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OLFACTORY LEARNING IN THE CRICKET *GRYLLUS BIMACULATUS*

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Summary

The olfactory learning capability of the cricket *Gryllus bimaculatus* was studied. Crickets were deprived of drinking water for 4 days and were individually trained to associate peppermint with water and vanilla with NaCl solution. Their odour preference was tested before and after training by allowing them to choose between peppermint or vanilla sources. The time spent visiting each source served as a measure of odour preference. Crickets exhibited an initial preference for vanilla over peppermint, but preference for the latter increased after only one training session. The olfactory memory formed by a single training session decayed with time but remained for at least 24 h. Memory formed by three training sessions was extremely robust, and did not decay significantly between 1 and 7 days after training. The preference formed was easily altered by reversal training in which vanilla was associated with water and peppermint with saline solution. This study shows that crickets have a highly developed olfactory learning capability characterized by fast acquisition, long retention and easy rewriting of memory.

Key words: learning, memory, olfaction, insect, cricket, *Gryllus bimaculatus*.

Introduction

The ability to recognize the odour or the taste of a food has selective advantage for animals since it helps them to select appropriate foods from a variety of choices. It is not surprising, therefore, that various species of animals, including insects, have developed the ability to learn factors related to olfaction and/or taste. Olfactory learning in insects has been studied most extensively in honeybees, *Apis mellifera*, using a classical conditioning paradigm in which reflexive extension of the proboscis in response to a sucrose reward was conditioned to odour stimulation (Kuwabara, 1957; Menzel, 1999). Odour learning by honeybees occurs after only one conditioning trial; the memory that is formed leads to the animals making highly reliable choices and it is retained for several days at least (Menzel et al., 1993). Olfactory learning capability has also been demonstrated in other insects, including fruit flies *Drosophila melanogaster* (Dudai, 1977; Tully and Quinn, 1985), house flies *Musca domestica* (Fukushi, 1979), cockroaches *Periplaneta americana* (Balderrama, 1980) and the parasitic wasp *Microplitis croceipes* (Lewis and Tumlinson, 1988).

Olfactory learning in insects has proved to be one of the best models for studying the cellular and molecular bases of learning and memory (Milner et al., 1998; Menzel, 1999). In fruit flies, *Drosophila melanogaster*, biochemical pathways underlying olfactory memory formation have been studied, and the essential role of the cyclic AMP cascade in the formation of olfactory memory has been demonstrated (Dabnau and Tully, 1998). In honeybees, neural pathways subserving olfactory memory formation have been examined electrophysiologically, pharmacologically and by local cooling of the brain, and the participation of the antennal lobe (the primary olfactory centre) and the mushroom body (a higher associative centre) in olfactory memory processing has been demonstrated (Erber et al., 1980; Menzel and Müller, 1996; Menzel, 1999). Some neurons in the honeybee brain exhibit activity changes associated with olfactory memory formation (Hammer, 1993; Mauelshagen, 1993). However, the neural mechanisms underlying olfactory memory processing remain largely unknown.

Crickets are one of the best animal models for studying neural mechanisms of behaviour since they provide the opportunity for electrophysiological measurements of neural activity in animals behaving normally (Böhm and Schildberger, 1992). The activities of brain neurons during phonotactic orientation (Böhm and Schildberger, 1992; Staudacher and Schildberger, 1998) and of neurons in the terminal abdominal ganglion during escape responses evoked by air currents (Hörner, 1992; Kohstall-Schnell and Gras, 1994) have been examined.

There have been many studies of the acoustic, tactile, visual and mating behaviour of crickets (Kutsch and Huber, 1989; Schildberger et al., 1989; Gnatzy and Hustert, 1989; Honegger and Campan, 1989; Matsumoto and Sakai, 2000a,b), but no attention has been directed to their olfactory behaviour.

Anatomical studies of the palps of *Gryllus bimaculatus* suggested that their sensilla act as olfactory and contact chemoreceptive receptors (Klein, 1981), and the use of contact chemoreceptive cues for mate recognition has been noted in some crickets (*Acheta domesticus* and *Gryllus integer*, Otte and Cade, 1976; *Teleogryllus commodus*, Rence and Loher, 1977). However, we have found no documentation of the olfactory capability of crickets. In the present study, we examined the performance of *Gryllus bimaculatus* in a discriminatory olfactory learning paradigm and found that they are able to associate a specific odour with a reward. Crickets may prove to be a useful model for studying the neural basis of olfactory learning.

Materials and methods

Insects

Adult male crickets, *Gryllus bimaculatus* DeGeer, reared in a plastic case (80 cm×45 cm×20 cm) on a 12 h:12 h light:dark photoperiod (photophase 08:00–20:00 h) at 27±2 °C, were fed a diet of insect food pellets and water *ad libitum*.

Four days before the start of the experiment, a group of 30 crickets was placed into a container (30 cm×20 cm×15 cm); they were fed insect food pellets, but they were deprived of fluid to enhance their motivation to search for water. On the day of the experiment, they were placed individually in 100 ml beakers. A sheet of paper was placed on the bottom of each beaker to provide a normal substratum for locomotion and to absorb excreta. Animals were used in experiments 1–2 weeks after the imaginal moult.

Experimental arrangement

The training paradigm was a modification of that used by Balderrama (1980) to study olfactory learning in cockroaches. The apparatus consisted of three chambers (Fig. 1), a 'training chamber' (15.5 cm×25 cm×7 cm) and two removable 'waiting chambers' (5 cm×7 cm×7 cm each), one of which was placed at the 'waiting position' and the other at the 'entrance position'. Between the waiting chamber at the entrance position and the training chamber, there was a sliding door that could be opened and closed manually. The cover of the training chamber was removable and had many small openings to provide ventilation.

On the floor of the training chamber were two circular holes (3 cm in diameter) that connected the chamber with two of six sources of odour (Fig. 1). Each odour source consisted of a cylindrical plastic container (4 cm in diameter × 4 cm in height) covered with a fine gauze net. The six containers were mounted on a holder (consisting of a plastic disc 20 cm in diameter and 3 mm in thick) with a rotating axle in its centre and fixed to the front wall of the training chamber. Two odour sources could be located simultaneously just below the holes at the 'offer position' by rotating the container holder.

Experimental procedure

The experiment consisted of two stages, a preference test and training. Before each stage, a filter paper (15 mm×15 mm) soaked with either peppermint (Kyoritsu-Shokuhin Co., Tokyo, Japan) or vanilla (Meijiya Co., Tokyo, Japan) artificial essence, diluted fivefold with water, was placed inside each container. For both the preference test and the training, three odour sources, one vanilla (–) and two peppermint (+), were used (Fig. 2). All three odour sources were located in each half of 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 the surroundings. The waiting chamber was then slid into the entrance position, and the door connecting it to the training chamber was opened (Fig. 2A). When the cricket entered the training chamber, the door was closed and the test started. Two minutes later, the container holder was rotated, and the relative positions of the vanilla and peppermint sources were interchanged (Fig. 2B). Thus, one change in position occurred during every preference test. 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 registered cumulatively with a stop watch. If the total time of a visit to either source was less than 20 s, the data were rejected. Every preference test lasted for 4 min.

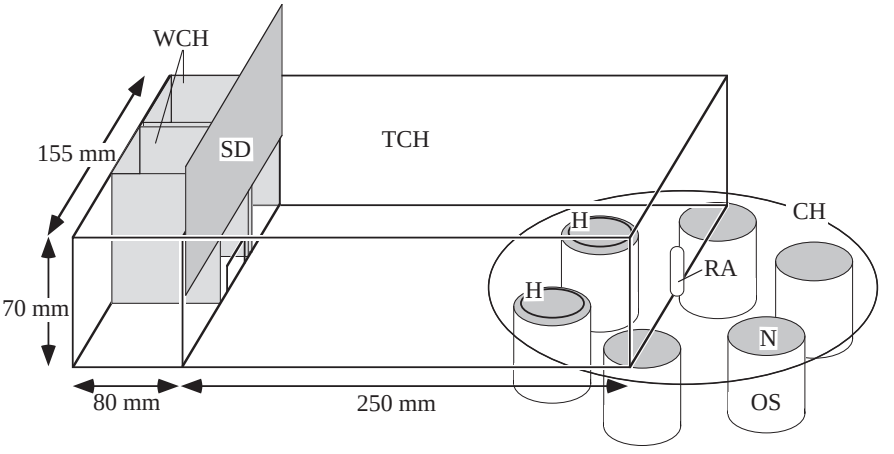


Fig. 1. A diagram of the experimental apparatus. WCH, waiting chambers; TCH, training chamber; CH, container holder; RA, rotating axle; OS, odour source; N, gauze net; SD, sliding door; H, holes connecting the chamber with two of the six odour sources.

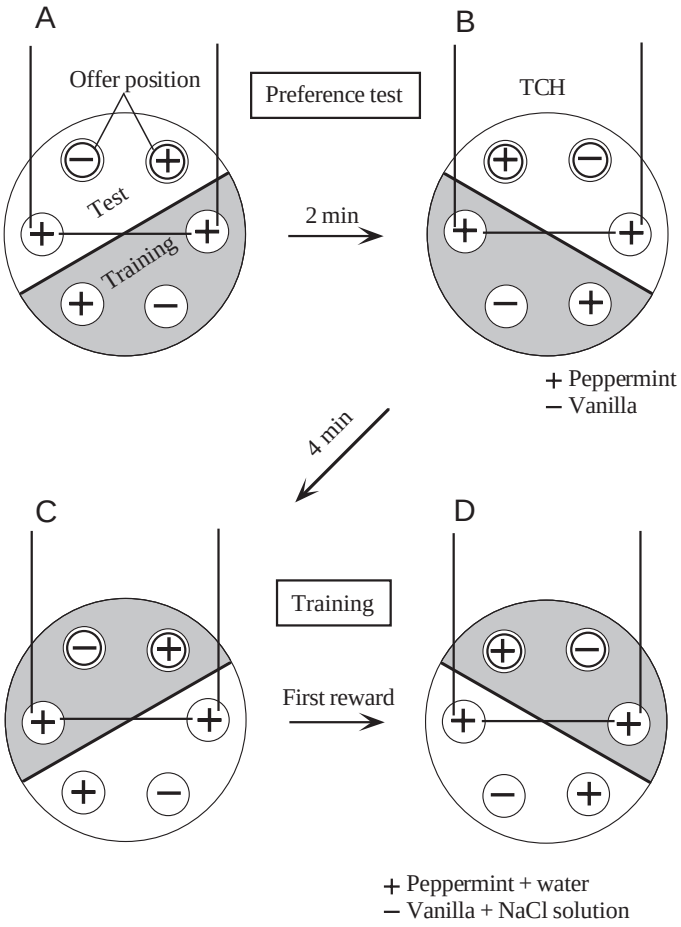


Fig. 2. Procedures for the preference test and training are shown as viewed from above the container holder. + represents the peppermint source, and – represents the vanilla source. The three odour sources in the white half of the holder are for the preference test. Relative positions during the test are shown in A and B. The three sources in the stippled 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 water, and the top net of the vanilla source contained saline solution.

Immediately after the end of a preference test, a training session was started. Because crickets had an innate preference for vanilla over peppermint (see Results), training was designed to associate peppermint with water and vanilla with a 20 % sodium chloride solution: a 30 µl drop of water was placed on the top net of the peppermint source, and a 30 µl drop of saline solution was placed on the top net of the vanilla source. A rewarded visit, i.e. a visit to an odour source with water, was limited by the observer to 20 s, and after this time the relative positions of the vanilla and peppermint sources were interchanged (Fig. 2C,D). Training was concluded after two rewarded visits or after 4 min had elapsed.

At the end of the training session, the sliding door was opened, the cricket was gently pushed into the waiting chamber using a Lucite plate (9 cm×12 cm) and the door was closed. The waiting chamber was removed, and the cricket was placed into a beaker. Daily testing and training began at 11:00 h, with a maximum duration of 4 h.

At various times after training, crickets underwent preference tests, which we refer to as ‘retention’ tests. The interval between the last training session and a ‘retention’ test is called the ‘retention’ interval. During the ‘retention’ interval, the cricket was placed in a beaker with insect food pellets and given a drop of water *via* a pipette once daily.

Data analysis

Relative odour preference was measured using the ‘peppermint preference index’ (%), the total time spent at the peppermint source divided by that spent at either the peppermint or the vanilla source, multiplied by 100, and by the ‘learning index’ (%), the preference index after training minus that before training. The Mann–Whitney *U*-test was used to compare the odour preference of crickets before and after training, and the χ^2-test was used to evaluate initial preference.

Results

General behaviour of crickets during the experiment

The typical behaviour of crickets during the experiment was as follows. During the preference test, the cricket walked about with one antenna in contact with the wall and approached one of the odour sources. The cricket then vigorously swung its antennae over the odour source and probed the gauze net covering the top of the source with its mouth. Almost all the crickets visited the vanilla and peppermint sources alternately and, thus, little difference was found in the number of visits to either source. However, the time spent in probing the top net with the mouth differed between the two sources. The crickets visited the odour sources many times for at least the first 2 min of the test but, thereafter, many crickets no longer approached the odour sources and remained close to the sliding door.

When a cricket visited the vanilla source during the training session and probed the saline solution with its mouth, it immediately retreated. However, touching the saline with the antennae or maxillary pulps did not induce retreat. When a cricket probed the water at the peppermint source, it drank the water and then licked the net intensively. Changing the positions of sources at this time induced a rapid orientation towards the new position of the peppermint source with intense movement of the antennae. Generally, the crickets completed two rewarded visits during each training session, but they seldom visited the vanilla source twice.

Initial preference

In the initial preference test, 104 of 117 crickets exhibited a peppermint preference index of less than 50 % (PT-0 of Figs 3–5), and the χ^2-test showed that they had a significantly greater preference for vanilla than for peppermint (P<0.001). This is thought to represent the innate preference of crickets, since they had never experienced a vanilla or peppermint odour before the test.

Effects of a single training session

Two hours after training (PT-1 of Fig. 3), crickets exhibited a significantly greater preference for peppermint than before training (PT-0, $P<0.0001$), indicating that one training session is sufficient to establish olfactory learning. When measured 6 h (PT-2), 12 h (PT-3) and 24 h (PT-4) after a single training session, the preference for peppermint was significantly less ($P<0.01$ for PT-2; $P<0.0001$ for PT-3; $P<0.001$ for PT-4) than that 2 h after training (PT-1). However, these peppermint preferences were still significantly greater ($P<0.0001$ for PT-2; $P<0.05$ for PT-3; $P<0.001$ for PT-4) than the initial preference (PT-0). Memory formed by a single training session is, therefore, retained for at least 24 h.

It can be argued that the decay of memory that occurs between 2 and 24 h and shown in Fig. 3 may be due to the extinction effect of the three intervening retention tests (PT-1–PT-3) in which odours were presented without water or saline. Alternatively, it may reflect natural memory decay with time. To distinguish between these possibilities, we performed the first retention test 24 h after a single training session in another group of crickets and compared the results (PT-1 in Fig. 4) with those of the fourth retention test given 24 h after a single training session (PT-4 in Fig. 3). The initial preference and the preference after 24 h of training did not differ significantly between the two groups of crickets. Moreover, statistical comparisons using the learning index, i.e. the difference in the peppermint preference index before and after training, also showed no significant difference between the two groups. We conclude that the extinction effect of the retention test is small, at least under the present experimental conditions.

Effects of three training sessions

The effects of three training sessions performed on three consecutive days are shown in Fig. 4. The peppermint preference increased with an increase in the number of training sessions: after the third training session (PT-3), the preference for peppermint was significantly greater than that after the first training session (PT-1, $P<0.0001$). It was notable that the preference for peppermint at 7 days after the third training session (PT-4) did not differ significantly from that 1 day after the third training session (PT-3), indicating that the memory did not decay significantly between 1 and 7 days after training.

Effects of reversal training

The effects of reversal training are shown in Fig. 5. After three training sessions to associate peppermint with water and vanilla with saline, crickets were retrained to associate vanilla with water and peppermint with saline. After the first (PT-1), second (PT-2) and third (PT-3) training sessions, the

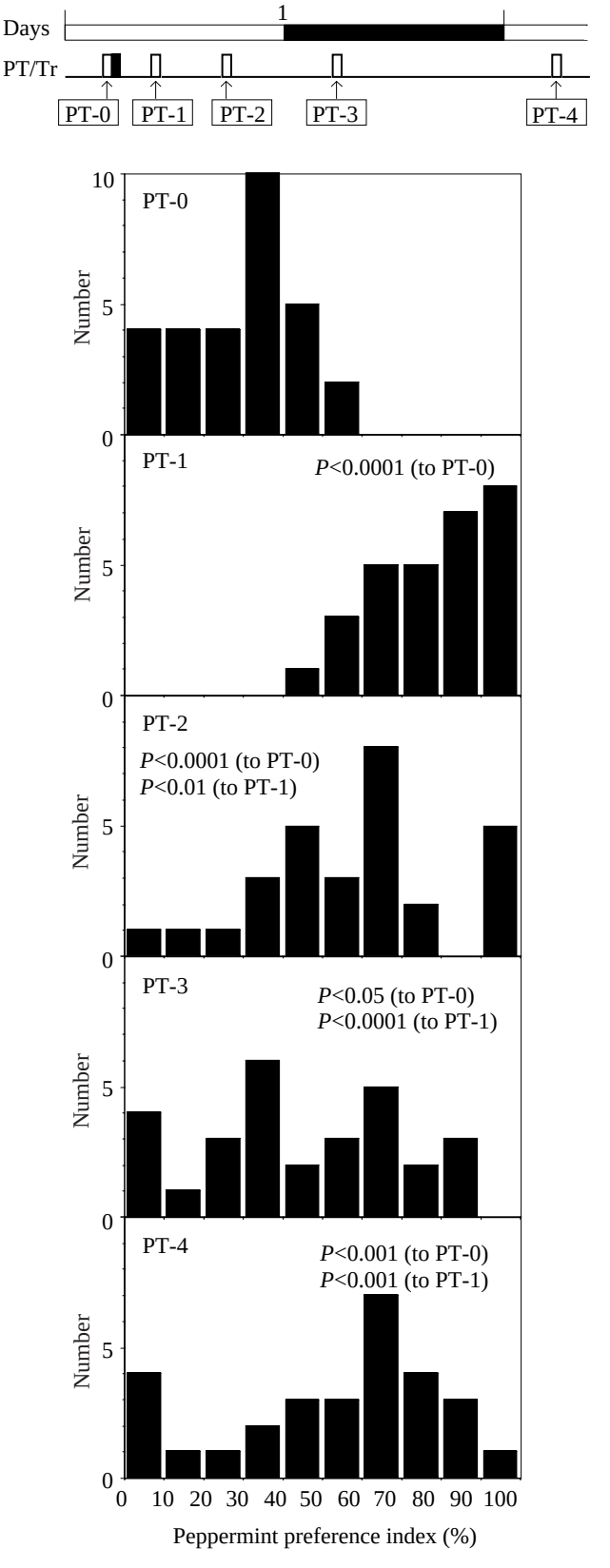
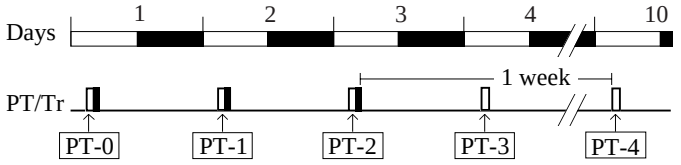
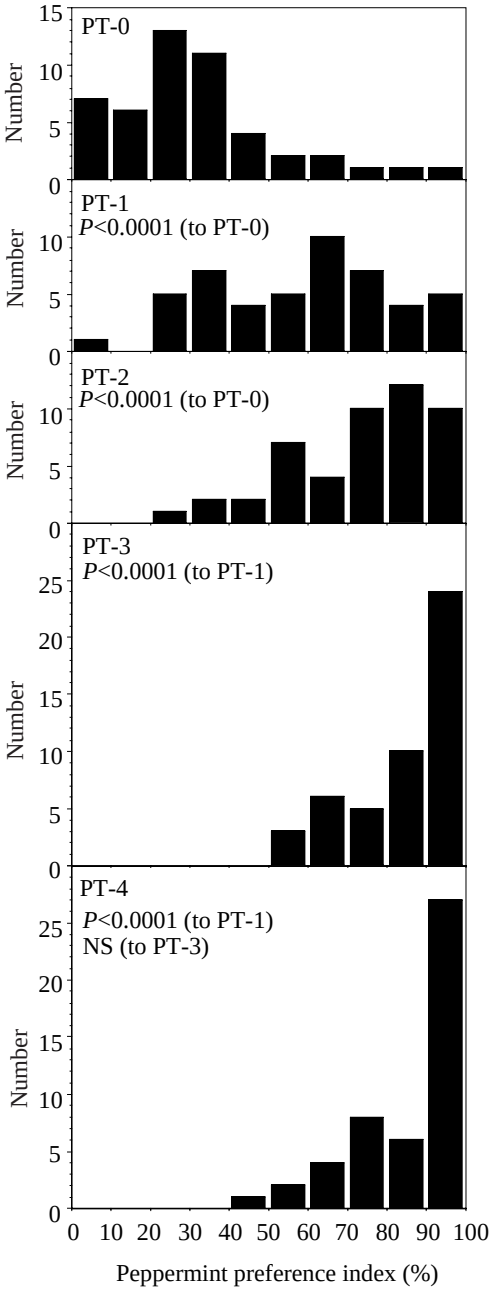


Fig. 3. The effects of a single training session. The diagram at the top shows the time schedule for the preference test (PT, white bar) and for training (Tr, black bar). The white and black parts of the time bar indicate photophase (12 h) and scotophase (12 h), respectively. The histograms show the distribution of the peppermint preference index (see Materials and methods) for each individual in a group of crickets (N=29) in tests made before (PT-0) and 2 h (PT-1), 6 h (PT-2), 12 h (PT-3) and 24 h (PT-4) after training. In each histogram, the results of a statistical comparison, using the Mann–Whitney U-test, are shown with the significance level (P).

preference for peppermint was significantly greater (P<0.0001) than that before training (PT-0), as in tests for which the results are shown in Fig. 4. After the first retraining session (PT-4), the preference for peppermint decreased significantly (compared with PT-3, P<0.0001). After the second (PT-5) and third retraining sessions (PT-6), the peppermint preference did not differ significantly from the initial preference (PT-0). Odour memory can, therefore, be easily rewritten by reversal training.





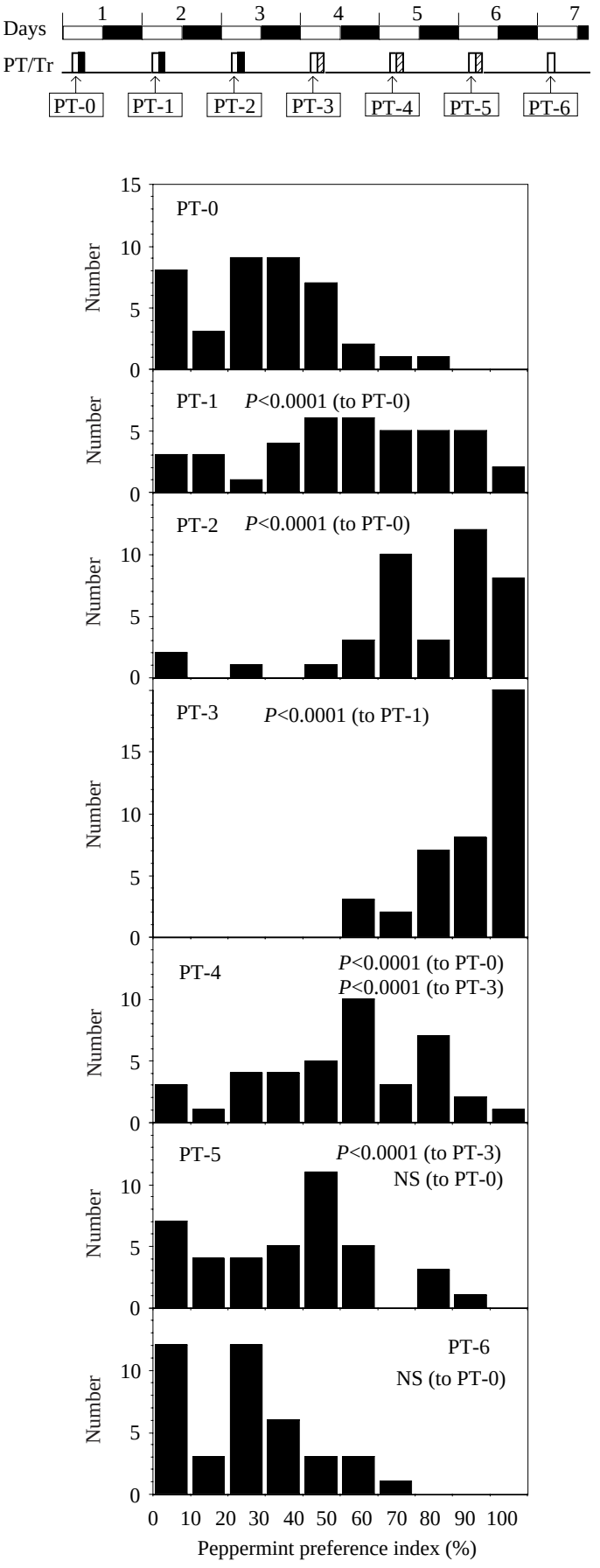
Discussion

Our observations show that crickets have a high capability for olfactory learning. The experimental design excluded the possibility that this learning could be accomplished using spatial, tactile or visual cues or their own odour marks. This study is, as far as we know, the first to document that crickets are capable of olfactory learning, although there have been reports of a learning capability in crickets in some paradigms, including shock-avoidance learning, in which the position of the leg is associated with an electric shock (Jaffe et al., 1992), Y-maze learning, in which crickets were trained to turn consistently to one side of a symmetrical Y-shaped maze using reinforcement with water (Jaffe et al., 1990), and visual learning of escape direction (Beugnon, 1986; Felicioni and Ugolini, 1991). Among orthopteran insects, the only other species for which olfactory learning capability has been reported is *Locusta migratoria*, in which nymphs deprived of protein have been shown to prefer an odour associated with food that contains protein (Simpson and White, 1990).

Consideration of the experiment paradigm

Since the performance of animals in learning experiments is strongly dependent on motivational status, we took the following precautions to ensure that the crickets were highly motivated to search for water and had a low motivation to search for a mate or for refuge. First, the crickets were deprived of drinking water for 4 days so that they would be highly motivated to search for water. Second, crickets at different levels of maturity may differ in their motivational status, so we used males 1–2 weeks after the imaginal moult. However, males become sexually mature within 1 week of the imaginal moult and begin to court females, especially in the subjective scotophase. For this reason, all experiments (except PT-2 and PT-3; Fig. 3) were performed during the photophase, when the motivation of the crickets to court was low. Third, care was taken not to stimulate or frighten the crickets before and during the experiment. Fourth, we did not use aversive substances such as quinine as a non-reward since such substances may cause illness and reduce the motivation of the crickets to search for water; instead, we used a high concentration of saline solution as a non-reward. Although crickets may require a small amount of salt in their diet, we never observed them drinking the saline solution during the experiment. This is

Fig. 4. The effects of three training sessions performed on three consecutive days. The upper diagram shows the time schedule. The histograms show the distribution of the peppermint preference index (see Materials and methods) for each individual in a group of crickets (N=48) in tests made before (PT-0) and 1 day after the first (PT-1), second (PT-2) and third (PT-3) training sessions and 7 days after the third training session (PT-4). In each histogram, the results of a statistical comparison, using the Mann–Whitney U-test, are shown with the significance level (P). NS, not significant.



probably because the insect food pellets given to them provided sufficient salt.

Olfactory learning capability of crickets

The results of our study may be summarized as follows. First, crickets exhibited an innate preference for vanilla over peppermint. It may be that the vanilla odour is similar to that of the fruits eaten by wild crickets, while the peppermint odour represents the odour of non-nutritious substances, so that this innate preference helps crickets to find suitable food in their natural surroundings. Peppermint has been shown to be repellent to *Drosophila melanogaster* (Thorpe, 1939). Second, a single training session was sufficient to alter this innate preference, and the altered performance was retained for at least 24 h, although significant decay of this memory occurred between 2 and 24 h after the training session. Third, a memory established by three training sessions did not decay significantly between 1 and 7 days after training. It is of considerable interest to determine how long this memory can be retained. Our preliminary observations suggest that a memory formed by nymphs over five training sessions can be maintained for at least 6 weeks (Y. Matsumoto and M. Mizunami, unpublished observations). Fourth, although olfactory memory formed by three training sessions is stable, as has been discussed above, it can easily be altered by a reversal trial. This is in contrast to the finding that no significant extinction effect was found in the case of the retention test, in which odours were presented without water or saline (Fig. 3). Crickets learn to associate an odour with the presence of a reward or non-reward much faster than they learn to associate it with the absence of a reward or non-reward.

These findings indicate that crickets possess a highly sophisticated system for processing olfactory memory, which is characterized by rapid acquisition, long retention and easy rewriting of retained events. Among insects, the only comparable process is the olfactory learning of honeybees (Menzel et al., 1993; Menzel, 1999). The excellent ability of crickets to process olfactory memory may reflect their omnivorous feeding habits, in that they select what is edible or inedible after testing various organic materials.

Perspective

Crickets are one of most suitable models for studying the neural mechanisms of behaviour because they are readily

Fig. 5. Effects of reversal training. The white and black parts of the upper diagram indicate the preference test and training, respectively. The hatched columns indicate reversal training, in which vanilla was associated with water and peppermint with saline solution. The histograms show the distribution of the peppermint preference index (see Materials and methods) for each individual in a group of crickets (N=40) in tests made before (PT-0) and 1 day after the first (PT-1), second (PT-2) and third (PT-3) training sessions and 1 day after the first (PT-4), second (PT-5) and third (PT-6) reversal training sessions. In each histogram, the results of a statistical comparison, using the Mann–Whitney U-test, are shown with the significance level (P).

accessible to detailed electrophysiological examination during natural behaviour (Böhm and Schildberger, 1992; Staudacher and Schildberger, 1998). Recently, we established a method of making extracellular recordings of the activities of brain neurons from freely behaving insects using thin wires (Mizunami et al., 1998; Okada et al., 1999) and a method of training crickets to associate odours with a reward or non-reward while making extracellular recordings of the activities of brain neurons (Y. Matsumoto and M. Mizunami, unpublished results). Crickets, therefore, may prove to be a pertinent model for studying neural mechanisms related to odour learning. The basic features of the olfactory learning of crickets described here provide a basis for electrophysiological studies.

Olfactory learning capabilities have been reported in at least six insect orders, the Hymenoptera (honeybees, Menzel, 1999; parasitic wasps, Lewis and Tumlinson, 1988), Diptera (fruit flies, Dudai, 1977; Tully and Quinn, 1985; house flies, Fukushi, 1979), Lepidoptera (moths, Fan et al., 1997), Coleoptera (Visser and Thiery, 1986), Blattaria (Balderrama, 1980) and Orthoptera (Simpson and White, 1990; this study). The feeding habits of these species encompass polyphagous herbivores (larvae of lepidopteran and hymenopteran species, Bernays, 1993), scavengers (fruit flies and house flies, see above), omnivores (crickets, this study; cockroaches, see above), fruit eaters (wasps *Vespula meculifrons*, Jander, 1998), nectar foragers (honeybees and moths, see above) and parasitoids (wasps, Lewis and Tumlinson, 1988; Turlings et al., 1993). Thus, it is likely that a capability for olfactory learning is more ubiquitous among insects than has been previously thought. This capability may have been established early in the evolution of hemimetabolous insects and passed on in many modern taxa. Further examination and a comparison of the neural and molecular mechanisms of olfactory learning in various species of insect may provide insights into the origin and evolution of brain systems subserving olfactory learning.

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