BEHAVIOURAL ANALYSIS OF OLFATORY CONDITIONING IN THE MOTH
SPODOPTERA LITTORALIS (BOISD.) (LEPIDOPTERA: NOCTUIDAE)

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Summary

We studied the associative learning capabilities for behaviourally relevant cues in the moth Spodoptera littoralis. The moths were trained to associate a conditioned stimulus (CS), geraniol odour, with an unconditioned stimulus (US), a sucrose solution. The occurrence of a proboscis extension reflex (PER) was tested. The PER performance during acquisition increased steadily with the number of training trials. Non-associative control procedures did not result in learning. PER conditioning was achieved when the CS was presented 1–3 s before the US. A wide range of inter-trial intervals was able to support conditioning. Males and females learned equally well. Moths could to some degree learn the CS–US association after a single trial. These results demonstrate that S. littoralis females and males have a good capability to associate an odour with a reward. The neural basis of olfactory coding in moths has been well studied; thus, the moth provides a powerful system in which to examine the neurobiology of olfactory learning.

Key words: associative learning, olfaction, proboscis extension reflex, inter-stimulus interval, inter-trial interval, acquisition function, learning, moth, Spodoptera littoralis.

Introduction

All organisms live in constantly changing environments. The availability of food sources varies with the seasons, and the abundance and types of predators or parasites change greatly over a lifetime. For an insect in such a dynamic habitat, the ability to predict positive and negative events is vital. The ability to learn to associate a certain cue with an abundant food source or with an approaching predator must thus be of great importance.

The analysis of learning in insects has been greatly advanced by the use of relatively simple protocols such as the proboscis extension reflex (PER) preparation. The PER preparation has been extensively used to study the learning ability of the honeybee Apis mellifera (Menzel and Müller, 1996). The PER of a naive, restrained honeybee is elicited when taste receptors on its antenna are stimulated with a sucrose solution (Kuwabara, 1957; Bitterman et al. 1983). The bee readily learns to extend its proboscis when presented with an odour. Experiments based on this procedure have yielded information regarding many aspects of memory and cellular mechanisms in the learning process (Menzel, 1990; Mauelshagen, 1993; Hammer and Menzel, 1995; for a review, see Menzel and Müller, 1996).

Moths have attracted much interest in investigations of chemical communication and olfactory function (Kanzaki and Shibuya, 1992; Hansson, 1995). Male moths use pheromones to track females over long distances (Baker, 1989), while female moths use odour cues to identify suitable oviposition sites (Renwick, 1989). Both sexes find food sources in the form of nectar-rich flowers partly by means of olfaction (Gabel et al. 1992). The olfactory system of the moth Spodoptera littoralis has been characterised in behavioural and neurophysiological experiments. Antennal receptor neurones and antennal lobe interneurones have been characterised both physiologically and morphologically (Anderson et al. 1993, 1995; Anton and Hansson, 1994; Ljungberg et al. 1993), and neurones responding to odours involved in several behavioural processes have been identified.

Like the honeybee, the moths Heliothis virescens and Helicoverpa armigera have been shown to be able to associate an odour with a reward in a PER preparation (Hartlieb, 1996). The moth thus provides a useful system in which to study the neural mechanisms underlying conditioned behaviour. An important step in such an analysis is to characterise the fundamental properties of conditioning (Menzel and Bitterman, 1983; Carew and Sahley, 1986). In the present study, we used the PER preparation to obtain the acquisition function, to examine the effect of inter-stimulus (ISI) and inter-trial intervals (ITI) and to investigate sex differences and non-associative learning in the moth Spodoptera littoralis.

Materials and methods

Insects

In total, 520 female and 30 male cotton leaf worm moths, Spodoptera littoralis (Boisd.), were used. Moths were obtained...
from a culture maintained at the Swedish University of Agricultural Sciences in Alnarp, Sweden. The moths were reared on a potato-based semi-synthetic diet (Hinks and Byers, 1976). Pupae were separated according to sex and put in emergence boxes (31 cm x 21 cm x 11 cm) in an air-conditioned room (23–25 °C, 70 % relative humidity, L:D 16 h:8 h). Immediately after emergence, the moths were given access to water for 6–8 h. They were subsequently transferred into other boxes (31 cm x 21 cm x 11 cm) without a water supply for 42±4 h. All experiments were conducted 2–3 days after emergence.

Analysis and procedure

The moths were brought to the laboratory 1–2 h before the start of the dark period. Each moth was introduced into the wide end of an Eppendorf tube from which the narrow end had been cut to allow the head and antennae to protrude. The animals were then kept in the light in the laboratory to adapt to the environment and to the change from light to dark. The experiments started 2 h into the dark period under red light (25 lx). During training, the moths were grouped as a squad containing 10–20 animals, depending on the design of the experiments (see below). The members of each squad were treated sequentially. The first animal was brought into the learning situation and given a trial, then the second animal, and so forth. The first animal was given its second trial only after the last animal in the squad had been given its first trial. During training, the head of the moth was placed 1 cm away from the outlet of a glass tube (i.d. 8 mm), so that the antennae were continuously flushed with a charcoal-filtered and moistened air stream flowing at 0.5 m s⁻¹. At the beginning of each conditioning trial, the moth received the airflow for 10 s to familiarize it with the mechanical stimulation. The conditioned stimulus (CS) was 100 μg of geraniol dissolved in paraffin oil and applied to a piece of filter paper (10 mm x 18 mm) in a Pasteur pipette. Geraniol was chosen because it is a flower odour related to the food sources of $S. \text{littoralis}$. A 3 s air pulse (5 ml s⁻¹) of the atmosphere in the Pasteur pipette containing the CS was injected into the continuously flowing air stream through an opening (i.d. 3 mm) in the glass tube by a stimulus controller. A wooden toothpick was moistened with 40 % sucrose solution and was used as an unconditioned stimulus (US). The moth’s antennae were touched briefly with the sucrose-moistened toothpick to elicit the proboscis extension reflex (PER). The moth was subsequently rewarded with sucrose solution from the toothpick for 2 s while it extended its proboscis. In the early experiments, a hypodermic syringe was used for applying the sucrose solution to the moths. However, the response levels of the moths rewarded from the toothpick and hypodermic syringe were similar and, since the toothpick is more easily handled, it was employed in subsequent experiments. During testing, all animals were tested twice: 15 and 120 min after training, the CS was presented to the moth without the reward. The testing periods, 15 and 120 min, corresponded to intermediate- and long-term memory consolidation, respectively (Erber and Menzel, 1980; Smith, 1991). The basic conditioning procedure was manipulated in several ways to obtain the acquisition function, to assess non-associative effects, to determine the optimal interval between the CS and the US (inter-stimulus interval, ISI) and the inter-trial interval (ITI) and to test for sex differences.

Experiment 1: acquisition function

Groups of female moths ($N=10$ each) received the CS–US pairing 1, 2, 4, 6, 8 or 10 times before being tested. A zero trial group received one trial with the CS alone as a control. The ISI was 1 s with forward conditioning (i.e. CS 1 s before US). The ITI was 5 min.

Experiment 2: non-associative control

Female moths ($N=10$) were trained 10 times using five different procedures. One group received a CS–US paired training. The ISI was 1 s and the ITI was 5 min. Four non-associative control procedures were also examined: (1) the CS and the US were each presented alone 10 times with 5 min intervals. The order of CS–US presentation was generated by random numbers (the CS–US unpaired); (2) neither odour nor sucrose solution was presented (air alone); (3) the CS was presented alone; and (4) the US was presented alone. The occurrence of a PER was observed during training and after training. In each CS–US training, the presence of a PER was observed to obtain an acquisition profile for individual moths.

Experiment 3: effect of inter-stimulus interval (ISI)

Each female moth ($N=10$) received one trial of forward or backward conditioning with different ISIs: +10, +5, +3, +1, 0, −3, −5 or −10 s, where ‘+’ indicates that the CS was presented before, and ‘−’ that it was presented after the onset of the US. Each PER elicited by the CS was recorded by an observer.

Experiment 4: effect of inter-trial interval (ITI)

Female moths ($N=10$) received 10 CS–US pairings with different ITIs; 1, 5 or 10 min. The ISI was kept constant at +1 s. During training, the animals of the 1 min group were treated individually. The first animal was brought into the training situation and given 10 trials, then the second animal, and so forth. In the 5 and 10 min ITI groups, the animals were grouped as a squad of 10 and 20, respectively. The members in each squad were trained sequentially (see Materials and methods).

Experiment 5: effect of sucrose solution or water as US

Female moths ($N=10$) received 10 pairings at an ISI of 1 s and an ITI of 5 min. The US was either 40 % sucrose or distilled water.

Experiment 6: effect of sex

One group of female ($N=10$) and one of male ($N=10$) moths received 10 pairings with an ISI of 1 s and an ITI of 5 min. The occurrence of a PER was observed during training and testing (experiment 2).
Data analysis

A conditioned response (CR) was scored if the moth displayed a PER (Fig. 1) during training and/or post-training. The observation time was 3 s between the onset of the CS and the US. When the moths were tested 15 and 120 min after training, the observation period was 30 s starting from the onset of the CS. Spontaneous PERs were noted when the moth extended its proboscis before the training and in the absence of a CS. The CR was recorded as 1 (responded to the CS) or 0 (failed to respond).

Mean percentages of CR probability were calculated on the basis of the response of the 10 moths in each replicate. The mean percentage of CR probability was calculated for statistical analysis. A repeated-measurement design with one-way analysis of variance (ANOVA) was used to determine the effects of ISI, ITI, sex, number of acquisition trials, type of rewards, non-associative procedures and test periods. Tukey’s honest significant difference (HSD) tests were employed for multiple comparisons.

Results

Overall, 23% of the 520 female and 20% of 30 male moths showed a spontaneous PER to the CS. During training, the animals that gave a spontaneous response were trained normally. A moth was excluded only when it failed to extend its proboscis after the antennae had been touched by the sucrose-moistened toothpick.

Experiment 1: acquisition function

Acquisition was characterised by a positive steady increase ($P<0.001$) with trial number (Fig. 2). For the 15 min test, Tukey’s HSD test revealed that the response level of 2- to 10-trial groups was significantly higher than that of the 0- to 1-trial group. Nevertheless, within the 2- to 10-trial groups, only the 10-trial group differed significantly from the 2- to 6-trial groups. When testing took place at 120 min compared with 15 min, the responses of the 2- to 6-trial groups decreased significantly ($P<0.05$), while the responses of the 8- and 10-trial groups showed no significant differences ($P>0.05$). There was no interaction effect between number of trials and testing period.

Fig. 1. The proboscis extension reflex (PER) in the moth *Spodoptera littoralis*. The PER of a restrained moth was elicited when taste receptors on the moth antennae were stimulated with a sucrose solution (see Materials and methods). The moth readily learned to extend its proboscis when signalled by an odour presentation. The photograph was taken by Mr P. Valeur.

Fig. 2. The acquisition function for *Spodoptera littoralis*. Seven groups of moths received 0, 1, 2, 4, 6, 8 or 10 learning trials, respectively. The moths were tested at 15 and 120 min after training. The same letter above the columns indicates that the values within the same post-training period are not significantly different when tested using Tukey’s HSD test.

Experiment 2: non-associative control

The response of the CS–US paired group was significantly
greater ($P<0.001$) than that of the CS–US unpaired, the CS alone, US alone and air alone groups (Fig. 3). During training, only the group receiving CS–US forward pairing with a 1 s CS–US interval showed an increased response with learning trials from 40.0 to 73.3 % ($P<0.001$). No significant learning was shown in the groups receiving the CS–US unpaired or non-associative training. A naive group showed a 3.3–10.0 % response to the CS ($P>0.05$). The CS alone group displayed a CR probability from 23.3 to 6.7 % (not significant) during a 1-to 10-trial experiment. The response of the US alone group varied between 10.0 and 33.3 % (not significant).

Experiment 3: effect of inter-stimulus interval (ISI)

Investigations of the ISI revealed an asymmetric and inverted U-shaped response profile (Fig. 4). Presenting the CS before the US resulted in a significant ($P<0.001$) increase in the response level for intervals ranging from +10 to +1 s. In tests conducted 15 min after training, ISIs of +1 and +3 s resulted in a higher response level than in the other ISI groups. The responses of the +3 and +5 s ISI groups were not significantly different. In the 120 min test, the ISI +1 s evoked a significantly greater response than the other ISIs except for the ISI +3 s. When the CS was presented simultaneously with the US (0 s) or after (–3 to –10 s) the US, no significant conditioning was observed. The response levels of ISI +3 s, ISI +5 s and ISI +10 s were reduced significantly ($P<0.05$) when testing took place at 120 min compared with 15 min. For the other ISIs, no difference was observed with post-training period.

Experiment 4: effect of inter-trial interval (ITI)

In the 15 min test, the response percentages of the 1, 5 and 10 min ITI groups were 46.7, 80.0 and 56.7 %, respectively. However, no significant difference was observed among the ITI groups. In the 120 min test, the corresponding responses were 33.3, 63.3 and 73.3 % (Fig. 5). The 5 and 10 min ITI groups displayed a significantly higher response level ($P<0.05$) than the 1 min group. When testing took place at 120 min compared with 15 min, the response of the 1 and 5 min ITIs decreased significantly ($P<0.05$) while that of the 10 min ITI group increased significantly ($P<0.05$).

Experiment 5: effect of sucrose solution or water as US

The mean response of a group rewarded with sucrose was significantly higher ($P<0.001$) than that of a water-rewarded group (Fig. 6). The responses of both sucrose- and water-rewarded groups did not differ within group between testing...
Experiment 6: effect of sex

During training, from the first to the fifth trial, the responses of male moths were 20.0–50.0 % and those of the females were 40.0–63.3 % (Fig. 7). Thus, the response level of males appeared to start at a lower level than that of females, but the difference was not statistically significant ($P > 0.05$). Both acquisition profiles approached an asymptotic value by the sixth trial. After 15 and 120 min, both the response levels of males were 53.3 % and those of females were 66.7 and 50 %, respectively. Furthermore, the interaction effects of sex and training, and those of sex and testing time, were not significantly different.

Discussion

The moth *Spodoptera littoralis* clearly displays an associative capability in olfactory conditioning. Increasing the number of training trials led to more stable memories in the moth (Fig. 2); the number of training trials was shown to influence the training process as well as the post-training period. At the 15 min post-training period, no differences were found among the groups receiving 2–10 training trials, indicating that even two trials were sufficient to saturate the memory of the moth for 15 min. However, when the groups were tested after 120 min, one- and two-trial learning experiences were no different from the spontaneous activity. Statistically significant learning occurred after four trials or...
more, with a saturation level at 10 trials. This indicates that more training induces a more stable associative memory in the moths. In honeybees, significant learning occurred after a single learning trial in tests with olfactory and visual (Menzel et al. 1993) conditioned stimuli. However, the memory was more stable after multiple training trials (Menzel, 1990). In dual-choice colour training, the acquisition curves of free-flying bees were characterised by an accelerated increase with trial number, and in olfactory conditioning by a steady increase with trial number. The moths thus display a slowly rising acquisition curve compared with that of honeybees in the dual-choice colour test. However, the moths show a similar acquisition curve to that observed during olfactory conditioning of the honeybee. These observations may be explained by the fact that honeybees use both visual and olfactory cues for nectar-searching and navigation, whereas moths rely almost entirely on olfactory cues for food-searching, mating and host-plant localization. Therefore, one might expect that moths would have a similar learning ability to honeybees during olfactory conditioning.

After a single CS–US pairing, most moths (70 %) displayed a conditioned response to the odour, while 23 % responded spontaneously. Associative learning was only achieved by forward conditioning with an optimum at 1–3 s ISI (Fig. 4). Compared with the results of similar experiments in the honeybee (Menzel and Bitterman, 1983), the moth performs as well as the honeybee at the corresponding ISI. This indicates that the CS–US interval is a sensitive parameter for establishing the CS–US association in both honeybees and moths (Menzel, 1983). Simultaneous and backward conditioning did not result in any excitatory associative learning in the moth. However, further experiments should be conducted to test whether inhibitory learning plays a role in simultaneous and backward conditioning. Interestingly, only the shortest ISI (1 s) was able to maintain the best retention score at the 120 min test. We therefore conclude that the CS–US association is optimal when the onset of the US followed the onset of the CS within 1–3 s.

No statistical differences were found among groups trained with different ITIs when tested at 15 min. When tested at 120 min, the 10 and 5 min ITIs did, however, result in a significantly higher proportion of the moths (73.3 and 63.3 %) responding to the CS compared with the 1 min ITI. In the honeybee, varying the ITI had no effect upon performance when tested up to 3 h after training. However, 24 h after training, a 10 min ITI resulted in a slightly greater proportion of the honeybees displaying a conditioned response (Sandoz et al. 1995). Thus, the conditioned PER was only influenced by the different ITIs during testing (Fig. 5). Rescorla (1988) and Gallistel (1990) (see also Gibbon et al. 1977) found that an association between the CS and US in a trial depended not only on the CS–US interval but also on the ratio between the CS–US interval and the ITI (the ISI/ITI ratio) such that the lower the ISI/ITI ratio, the better association proceeded. Thus, our result may reflect the fact that the ratio between the ISI and ITI was lower for the 10 and 5 min ITIs compared with the 1 min ITI and, consequently, the response level for the 10 and 5 min ITIs was higher than that for the 1 min ITI. The ISI/ITI ratios in the 10, 5 and 1 min ITI are 0.1, 0.2 and 1, respectively.

Although these experiments were designed to produce classical conditioning (i.e. a specific CS–US association) in any appetitive conditioning experiment, it is possible that, to some extent, operant conditioning (i.e. a US–PER association) may play a role as well (Konorski, 1967; Bitterman et al. 1983; Colwill and Rescorla, 1985). In our study, we cannot exclude the possibility of an operant component (US–PER) being involved in the proboscis extension preparation. Therefore, an omission and yoked control experiment (Bitterman et al. 1983; Pearce, 1997) will be conducted in a further study to investigate the extent to which the learned behaviour of the moths is mediated by a CS–US connection or by a US–PER association in the proboscis extension preparation.

Variation in behaviour occurs frequently between individuals. Sometimes, individual differences are properly dismissed as the variability that is inevitable in biological systems (Martin and Kraemer, 1987). Such differences were also present in our results. After single-trial training, the group of moths tested in the third experiment (Fig. 4) responded (70 %) to the CS significantly better (P<0.01) than a different group with the same treatment (46.6 %) in the first experiment (Fig. 2). These differences may be due to random variation between individual moths, but functional and evolutionary analyses suggest that much variation in behaviour between members of the same species of the same age and sex has also been maintained through evolution (Slater, 1981).

The moths tested in the present study had been deprived of food and water for 42±4 h before experiments began. This treatment was used to increase their motivation to search for nutrition (Robacher, 1991). When the sucrose solution was exchanged for distilled water as the US, the appetitive reflex was reduced from 73.3 to 23.3 %, which was equivalent to the spontaneous response (Fig. 6). Thus, no learning effect was found, indicating that distilled water was not an effective US (Ramaswamy, 1987) despite the fact that hygroreceptors are known to be present on both the antenna and the proboscis (Bernays and Simpson, 1982). Blowflies Phormia regina (Nelson, 1971) and honeybees (Menzel et al. 1993) have the ability to detect the quality of a food source, e.g. the quality is lower in water than in sucrose solution. In the honeybee, an identified neurone, the ventral unpaired median neurone number 1 of the maxillary neurones (VUMmx1), mediates the reinforcing properties of the sucrose reward in olfactory proboscis extension conditioning (Hammer, 1993). This suboesophageal ganglion neurone projects widely to areas that integrate olfactory information at three levels of the protocerebrum (antennal lobe, lip of mushroom body calyces and lateral protocerebrum). Since this neurone has access to information about the CS, it may be involved in second-order conditioning and blocking (Hammer and Menzel, 1995). In the light of our findings, it will be interesting to explore corresponding neural systems in the moth.

When the sexes were compared in S. littoralis, no significant
differences were observed (Fig. 7). However, in a different moth species, *H. virescens*, male PER learning was significantly less good than that of females (Hartlieb, 1996). It would be interesting to determine whether this species-specific difference reflects different behavioural requirements.

In the control experiments, we examined the effect of non-associative training (Fig. 3). Repeated non-associative procedures did not induce learning, indicating that the conditioned behaviour results solely from associative learning (Bitterman et al. 1983). Neither the CS nor the US alone can induce a conditioned PER (De Jong and Pham-Delegue, 1991). In the CS–US group, when the CS regularly precedes the US, the CS acquires excitatory value and evokes the conditioned response. This inhibitory effect has also been found in the honeybee (Menzel, 1983) and in the fly *Drosophila melanogaster* (Tully and Quinn, 1985). Further experiments are necessary to test such properties in moths and to address the question of whether the decrease in the response level to unpaired stimuli is due to a reduction of the predictive value of the CS or to a reduction of US strength.

The moth *Spodoptera littoralis* emerges as a useful system for investigating memory processes in insects. The moth can offer new approaches in studies of associative learning and olfactory recognition. The well-known olfactory system of the moth and the many identified, behaviourally active pheromones and host-plant-associated odours allow differences in memory processing for odours with different meaning to the moth to be investigated. In parallel, an examination of the neural elements responsible for the cellular chain of events occurring from the initial stimulation with the CS and the US to the actual formation of memory can be performed.

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