

Learning, odour preference and flower foraging in moths

John Paul Cunningham^{1,*}, Chris J. Moore², Myron P. Zalucki¹ and Stuart A. West³

¹Department of Zoology and Entomology, University of Queensland, St Lucia, Brisbane 4072, Australia,

²Department of Primary Industries, Yeerongpilly, Brisbane 4105, Australia and ³I.C.A.P.B., University of Edinburgh, Edinburgh EH9 3JT, UK

*Author for correspondence (e-mail: p.cunningham@uq.edu.au)

Accepted 30 September 2003

Summary

Floral volatiles play a major role in plant–insect communication. We examined the influence of two volatiles, phenylacetaldehyde and α -pinene, on the innate and learnt foraging behaviour of the moth *Helicoverpa armigera*. In dual-choice wind tunnel tests, adult moths flew upwind towards both volatiles, with a preference for phenylacetaldehyde. When exposure to either of these volatiles was paired with a feeding stimulus (sucrose), all moths preferred the learnt odour in the preference test. This change in preference was not seen when moths were exposed to the odour without a feeding stimulus. The learnt preference for the odour was reduced when moths were left unfed for 24 h before the preference test.

We tested whether moths could discriminate between flowers that differed in a single volatile component. Moths

were trained to feed on flowers that were odour-enhanced using either phenylacetaldehyde or α -pinene. Choice tests were then carried out in an outdoor flight cage, using flowers enhanced with either volatile. Moths showed a significant preference for the flower type on which they were trained. Moths that were conditioned on flowers that were not odour-enhanced showed no preference for either of the odour-enhanced flower types. The results imply that moths may be discriminating among odour profiles of individual flowers from the same species. We discuss this behaviour within the context of nectar foraging in moths and odour signalling by flowering plants.

Key words: Lepidoptera, phenylacetaldehyde, α -pinene, wind tunnel, volatiles, insect, preference, feeding.

Introduction

Nectar-feeding moths are attracted to the odours of their floral hosts (Heath et al., 1992; Zhu et al., 1993). These odours are a complex blend of individual volatile components, each with a different biosynthetic route to their production and a potentially different effect on insect behaviour (Pichersky and Gershenzon, 2002). The quest to understand the role these volatiles play in signalling between flowering plants and nectar-feeding insects is now well underway (Dudareva and Pichersky, 2000; Smith, 1993). Electrophysiological techniques can determine which volatiles present in a floral bouquet are ‘active’ (i.e. recognised at the level of the insect antennae; Bruce and Cork, 2001; Burguiere et al., 2001; Plepys et al., 2002; Raguso et al., 1996). Once active volatiles have been identified, their function in modifying insect behaviour (e.g. as attractants or deterrents) can be explored using behavioural studies in the laboratory and field (Bruce and Cork, 2001; Heath et al., 1992; Landolt et al., 1991; Plepys et al., 2002).

Pre-dating these techniques is a wealth of studies that focus on the ecological and evolutionary importance of insect learning in foraging behaviour (Marler and Terrace, 1984; Papaj and Lewis, 1993; Papaj and Prokopy, 1989). In Lepidoptera, learning has been shown to influence the nectar-

foraging behaviour of both butterflies (Lewis, 1989, 1993) and moths (Cunningham et al., 1998; Lewis, 1989; Weiss, 1997). Odour learning in feeding behaviour has been elegantly demonstrated using the insect proboscis extension reflex (PER), a technique pioneered by studies on bees (Menzel and Bitterman, 1983; Smith, 1993) and adapted for moths (Daly et al., 2001; Fan et al., 1997; Hartlieb, 1996). In PER studies, the insect is restrained and the feeding response to an odour stimulus can be measured before and after conditioning (Smith, 1993). In moths, proboscis extension in response to an odour stimulus is strongly influenced by associative learning (Daly et al., 2001; Fan et al., 1997; Hartlieb, 1996). However, the influence of learning on preferences for individual volatiles in free-flying moths remains to be demonstrated. If learning strongly influences a moth’s responses to floral volatiles during foraging, the study of responses in naive moths may tell us little about the extent to which certain odours attract moths in nature.

We investigated whether learning influences innate preferences for two volatiles, phenylacetaldehyde and α -pinene, in the nectar-feeding noctuid moth *Helicoverpa armigera*. These volatile compounds are common to many flowers that act as hosts for both nectar-feeding and egg-laying

Helicoverpa moths (Bruce and Cork, 2001; Burguiere et al., 2001). We studied preferences for odours using a dual-choice flight test within a wind tunnel. We tested (1) innate preferences, (2) preference immediately after a conditioning treatment and (3) preferences 24 h after conditioning. We used male moths to prevent interactions between oviposition and feeding behaviours from influencing the preferences for odours.

Our wind tunnel experiment used a simplistic environment with few natural stimuli and controlled air flow. Learning to distinguish odours in a wind tunnel may tell us little about the importance of learning individual odour components in nature. In a second study, we integrated learning of single odours into the context of flower-visiting behaviour in foraging moths. Here, we tested whether moths could distinguish between flowers of the same species that emitted odour blends that differed by a single volatile. To achieve this we artificially manipulated the odour profiles of tobacco flowers by adding selected volatiles. Moths were conditioned to a particular flower type, and flower preferences were tested against a natural odour background in an outdoor flight cage.

Materials and methods

Insect and plant culturing

Helicoverpa armigera Hübner moths were obtained as pupae from a laboratory culture reared at QDPI Toowoomba, Queensland, Australia. Larvae had been reared on a soyflour-based artificial diet for *Helicoverpa* spp., minimising any possible influence of experience of host plants at this stage (Jermy et al., 1968). Pupae were sexed and male moths were placed in a separate holding cage (200 mm×150 mm×150 mm) until eclosion. Newly emerged adult males were transferred to either sealed 120 mm-diameter plastic containers (Experiment 1) or to new holding cages (Experiment 2) two hours before sunset each day in order to obtain discrete age groups. Moths were deprived of food until used in experiments.

In Experiment 1a and 1b, adult moths were kept in a laboratory at 25°C under ambient light conditions. In Experiment 2, moths were transferred to new holding cages and placed outdoors, under shelter. To prevent the insects from dehydrating in Experiment 2, cages were sprayed lightly with water (using a hand-held sprayer) at noon each day.

Tobacco (*Nicotiana tabacum*) was cultivated from seed under glasshouse conditions. To maintain new floral growth, maturing fruits were removed, preventing seed production.

Volatiles

The odours phenylacetaldehyde and α -pinene (obtained from Sigma-Aldrich, Sydney, NSW, Australia) were used in the conditioning experiments. We used (racemic) α -pinene, which is a mixture of two (+ and -) enantiomers. Previous electroantennogram (EAG) studies have demonstrated that these compounds elicit a peripheral olfactory response in *H. armigera* (Bruce and Cork, 2001; Burguiere et al., 2001).

Experiment 1: Odour preference in conditioned and unconditioned moths

Dual-choice preference tests were carried out in a wind tunnel with a Perspex flight chamber measuring 1600 mm×650 mm×650 mm. Air was circulated through the flight chamber at 0.7 m s⁻¹ (as measured at the centre of the chamber) using a fan system. A clean airstream was maintained by passing the circulated air through an activated charcoal filter and a dust filter before allowing it to enter the chamber. A laminar airflow was obtained by directing air through a honeycomb of soda straws and then a fine stainless steel screen (1.25 mm aperture) prior to entering the chamber.

Adult *Helicoverpa* moths show a characteristic surge in activity commencing at sunset, which corresponds with location of feeding sites (Topper, 1987). Feeding activity subsides around 90 min later (Beerwinkle et al., 1993). In all experiments, trials commenced 15 min after sunset and were confined to a 90 min testing period. Moths were exposed to changing ambient light conditions associated with sunset in order to instigate and maintain a regular pattern of behaviour. Additional lighting (for observation) was supplied using a diffuse light source, with the light intensity in the flight chamber measuring less than 1 lux throughout the experiment. The temperature inside the wind tunnel during the experiment was 24.4±0.12°C (mean ± S.E.M.).

Three- and four-day-old moths that had been held in individual plastic containers (120 mm diameter) without access to food or water were used in experiments. One antenna of the moth was gently touched with a cotton wool bud that had been soaked in 25% (w/v) sucrose solution in order to test feeding responsiveness. Only moths that extended their proboscis once the cotton wool bud had made contact with the antenna were used in conditioning trials. Each moth was only used once.

Conditioning trials

Moths were randomly allocated to one of three treatment groups: (1) 'conditioned'; moths exposed to a volatile (phenylacetaldehyde or α -pinene) whilst feeding on sucrose solution; (2) 'exposed'; moths exposed to volatiles without allowing feeding; or (3) 'no exposure'; moths given no exposure to volatiles and left unfed. The groups were constructed in order to ascertain whether feeding was required to initiate any changes in preferences and whether any innate odour preferences existed. We did not look in detail at the precise nature of the pairing between the unconditioned stimulus (sucrose) and the conditioned stimulus (volatile) involved in odour conditioning. This has been covered by previous studies on *Helicoverpa* species using proboscis extension tests (Hartlieb, 1996; Hartlieb et al., 1999).

Treatment 1: conditioned

Odour sources (referred to as 'lures' hereafter) were created by inserting a 15 mm absorbent cotton wool plug to a depth of 25 mm below the wide end (5 mm diameter) of a 145 mm glass pipette. 2 μ l of either phenylacetaldehyde or α -pinene were

pipetted onto the cotton wool no more than 15 min before the start of each experiment. The narrow end of the pipette was inserted into a 40 mm×50 mm×50 mm block of floral foam (Smithers-Oasis Ltd, South Australia), positioning the odour source at a height of 145 mm above the floor of the wind tunnel. Feeding sites were constructed similarly by plugging the end of a glass pipette with a cotton wool wick soaked in 25% (w/v) sucrose solution. This second pipette was positioned such that the sucrose wick was situated 2 cm downwind of the lure. New feeding sites and lures were used in each experiment.

Conditioning trials commenced by placing an individual moth on the sucrose wick and allowing a 30 s feeding bout. Feeding was identified as contact of the extended proboscis with the sucrose wick. In this way, the moth fed approximately 2 cm downwind from the lure. After 30 s, the moth was removed with a wooden toothpick and placed 400 mm directly downwind from the lure/feeding site. Moths were then allowed to fly freely back to the feeding source. Upon contact with the sucrose wick, the moth was allowed to feed for a further 20 s and was then returned to the downwind starting position. This process was repeated until moths had been given a total of four feeding visits in the presence of the volatile; one initial 30 s feed and 3×20 s return feeds.

Treatment 2: exposed

Moths were exposed to either phenylacetaldehyde or α -pinene without being allowed to feed in order to test whether any differences in response between treatments may have occurred through exposure to the volatile, irrespective of feeding. Each insect was placed into a 50 mm×50 mm black mesh bag clipped (using a fold back clip) to a wooden skewer at a height of 130 mm. To expose the insect to the odour, the base of the skewer was inserted into floral foam immediately downwind from the odour source, holding the insect at the same height and position relative to the lures as insects used in the conditioning trials. To match the exposure time in this treatment group with that of the conditioning trials, each moth was placed in this downwind position for four bouts (1×30 s and 3×20 s). Intervals of 1 min were allowed between each exposure bout. During this interval, the moth was placed 30 cm upwind of the lure in the centre of the wind tunnel.

Treatment 3: no exposure

We used unfed male moths with no previous exposure to either volatile to determine the innate odour preferences. Adult moths were placed into individual sealed (120 mm diameter) plastic pots upon emergence and kept until testing at 3–4 days old. Preference for either α -pinene or phenylacetaldehyde was determined using the dual-choice testing procedure described below.

Preference testing

Each preference test comprised a dual-choice test using one α -pinene and one phenylacetaldehyde lure, the same procedure being employed for all three treatments. The lures were placed

300 mm apart at the upwind end of the wind tunnel. Smoke tests (titanium tetrachloride) showed that, at a wind speed of 0.7 m s^{-1} , these plumes remained separate within the wind tunnel. Two 200 mm×150 mm×150 mm Perspex wedges were placed at the downwind end of the wind tunnel, bringing odour plumes together at a distance of 800 mm from the lures and leaving a 200 mm gap through which the odours were directed into the rear 350 mm portion of the flight chamber.

Experiment 1a: preference testing (immediate)

Immediately following the conditioning treatment, the lure and feeding source were removed. The two odour lures were placed in position only when moths were in the 350 mm-long section at the downwind end of the wind tunnel, where both the plumes had merged. This method was used in preference to catching moths and placing them at the downwind end; disturbing moths in this way often instigated avoidance behaviours and erratic looping movements. In the absence of an odour plume, moths generally relocated to the downwind end of the wind tunnel, making it easy to position the lures. If a moth remained in the upwind end of the tunnel after a 3 min period it was caught and released downwind once the lures were in position. In the exposed and no exposure treatments, moths could be released directly into the downwind end of the wind tunnel.

Preference for a volatile was seen as a characteristic upwind flight pattern in the odour plume to within 100 mm of a lure. Once a lure had been approached, the odour source (lure type) was recorded and the test was terminated. If moths failed to approach either lure within a 5 min period, the test was terminated. The position of the feeding lure and odour source in the conditioning trials (centrally placed; 325 mm from either wall) differed from the position of either lure in the preference trials (200 mm from either the right- or left-hand side wall) so that learning the position of the feeding lure would not influence the choice of lure in the test. The position of each lure (nearest to the right- or left-hand wall of the chamber) was allocated randomly throughout the experiment to avoid positional biases. The volatile used in conditioning treatments was alternated throughout the experiment.

Experiment 1b: preference testing (24 h after conditioning)

Moths were conditioned to either odour source as in Experiment 1a. Once the conditioning treatment was completed, the moth was placed into a 120 mm-diameter airtight plastic container where it was held in the laboratory at 25°C under ambient light conditions for 24 h. The following night, preference tests were carried out as in Experiment 1a. Moths were released individually into the downwind end of the flight chamber and the lure approached was recorded.

Experiment 2: odour learning using odour-enhanced flowers

Tobacco flowers attract feeding adult *H. armigera* (Cunningham et al., 1998). We used standard volatiles-trapping techniques followed by GC-MS (gas chromatography-mass spectrometry) analysis to establish that

no phenylacetaldehyde or α -pinene was present among the volatile odour compounds of cut tobacco flowers. This is consistent with published data (Loughrin et al., 1990). Our choice of plant and test volatiles was directed in part by the desire to augment the natural flower odour with compounds that were normally absent.

Odour profiles of tobacco flowers were augmented by adding either phenylacetaldehyde or α -pinene into the base of the corolla tube. In this way, two types of flower were created, these flowers being identical in visual cues but differing in specific olfactory components detected by foraging moths. Tobacco flowers were picked one hour before dusk from plants reared in the glasshouse. Using a micropipette, 2 μ l of either phenylacetaldehyde or α -pinene were added into the inside base of the corolla tube. A third group of flowers, to which neither volatile was added, was prepared. The corolla tube was partially plugged using a small absorbent cotton wool plug that was lodged between the stamens at the lip of the corolla (Fig. 1A). The cotton wool plug was moistened with three drops (~0.1 ml) of 25% (w/v) sucrose solution administered from a pipette. This procedure provided sufficient sucrose solution for the duration of the conditioning experiments and prevented the insects from contacting either the floral nectar or added volatiles. Previous results have shown that moths that are fed from the top of the corolla will not attempt to enter the corolla tube in order to probe deeper (Cunningham et al., 1998).

To construct a standardised inflorescence, five flowers (from the same treatment group) were inserted, to the depth of the calyx, into a block of 'Oasis' floral foam (80 mm \times 60 mm \times 40 mm) such that a single flower protruded from each of five faces of the block (Fig. 1B).

Conditioning experiments

On any one night, moths were conditioned using a single treatment group of flowers: (1) odour enhanced using phenylacetaldehyde; (2) odour enhanced using α -pinene or (3) flowers with no added volatiles (non-enhanced). The standardised inflorescence was positioned at a height of 1 m on a bamboo cane in the centre of an outdoor flight cage (1.8 m \times 1.8 m \times 1.8 m).

To begin each conditioning trial, a single male was removed from the holding cage and encouraged to commence feeding using a cotton wool bud moistened with 25% (w/v) sucrose. Once proboscis extension was observed, the moth was placed onto the corolla lip of one of the tobacco flowers where it was allowed to feed on the sucrose wick for 30 s. The moth was then removed using a wooden toothpick and held at a distance of 200 mm from the flower head. Insects were allowed three return visits to the flowers, with 20 s feeding at each return. After the third return visit (insects having had a total of 90 s of feeding), the moth was caught and held in a plastic container. Moths were conditioned in this manner until feeding activity subsided (~90 min after the experiment commenced).

Preference testing

Preference testing of flower-conditioned moths was carried out

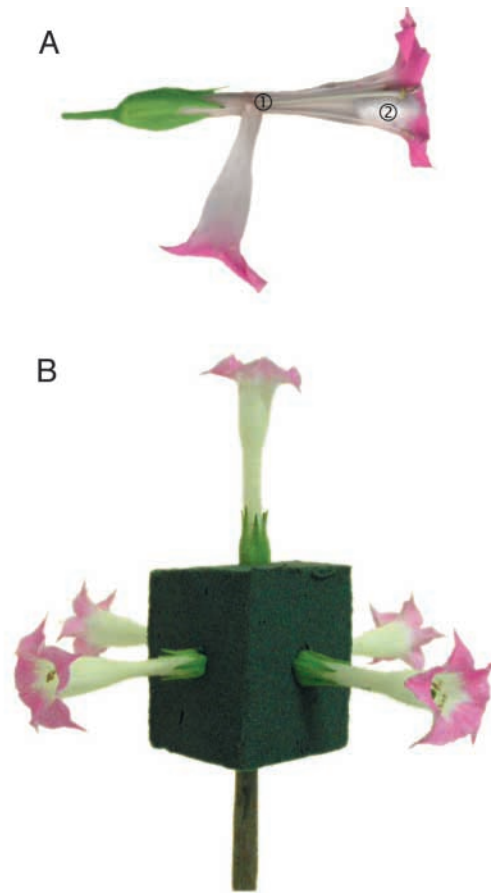


Fig. 1. Odour augmentation of tobacco flowers in Experiment 2. (A) Corolla tube partially removed to display the location of the additional single odour (1) and sucrose feeding site (2) in augmented flowers. (B) Standardised inflorescence used in conditioning trials and preference tests.

on the following night. Two standardised inflorescences were constructed as previously described; one inflorescence using odour-enhanced flowers augmented with phenylacetaldehyde, the other with flowers augmented with α -pinene. Flower corolla tubes were partially blocked with cotton wool, as in conditioning trials, but no sucrose solution was added to the cotton wool. Conditioned moths were released into the flight cage one hour before sunset. Fifteen minutes before sunset, the inflorescences were placed on 1 m canes at a distance of 1 m apart.

Moths that approached and landed on flowers were captured and the treatment group of the inflorescence visited was recorded. Once captured, moths were not re-released. The experiment was continued until all moths had been captured or until flight activity ceased, ~90 min later.

The experiment was repeated over 22 nights (seven trials for each odour-enhanced flower treatment and eight trials for the non-enhanced flower treatment). On each night of conditioning, the treatment group was assigned randomly. In preference tests, the position of the flower head within the cage was assigned randomly using a grid. Each insect was only used once.

Statistical analysis

Data were analysed using generalised linear modelling techniques (McCullagh and Nelder, 1989) in the GLIM statistical package (Crawley, 1993). Choice test outcomes were analysed as proportions, with the number of moths selecting a particular odour as the response variable and the total number of moths selecting either host as the binomial denominator. Binomially distributed error variances were assumed and a logit link function employed. In all cases, we initially fitted a maximal model to the data, with all explanatory variables and experimental treatments. We then used the process of stepwise deletion (see Crawley, 1993) to remove terms from the model until a minimal model was obtained. Hypothesis testing was carried out using a *G*-test on differences in deviance. Differences in treatments were assessed by testing whether grouping them caused a significant change in the deviance explained.

In Experiments 1a and 1b, due to the low number of moths tested each night (4–6 moths), data were pooled over the 27 nights of testing. Treatment order was randomised to prevent any biasing that may have resulted from the night of testing. In Experiment 2, night of testing was included in the analysis to avoid pseudo-replication.

Results

Experiment 1: odour preference in conditioned and unconditioned moths

In total, 160 adult male moths displayed upwind flight towards odour lures in the dual-choice preference tests carried out in the wind tunnel over 27 nights [mean (\pm S.E.M.) moths per night = 5.93 ± 0.32]. Of these moths, 80 were in the conditioned (odour + feeding) treatment group, 40 were in the no exposure (no odour + no feeding) group and 40 were in the exposed (odour + no feeding) group. Preferences for α -pinene and phenylacetaldehyde lures for all treatment groups are summarised in Table 1. Differences between treatment groups are presented in Table 2 as *G* values determined by GLIM (*G*-test). Preferences between treatment groups were significantly different ($G_{(6)}=82.5$, $P<0.001$). Position of the odour lures (left or right side of wind tunnel) did not influence odour plume choice ($G_{(1)}=0.20$, $P>0.05$).

The innate preferences of the adult male moths for either phenylacetaldehyde or α -pinene were determined by testing moths that had been given no experience of volatiles and no feeding experience before testing (no exposure treatment). These moths showed a significant preference for the phenylacetaldehyde lure ($G_{(1)}=7.312$, $P<0.01$).

All moths ($N=40$) tested on the same night as conditioning (Experiment 1a) flew to the lure emitting the volatile on which they had been conditioned. Feeding experience in the presence of a volatile therefore led to significant differences in odour choice ($G_{(1)}=55.45$, $P<0.001$). No differences were found between moths given no experience of volatiles or feeding (no exposure group) and moths exposed to volatiles for the same

Table 1. Results of Experiments 1a and 1b: number of moths selecting each lure for each treatment group

Treatment group	Number of moths tested	Lure selected		% selecting pinene
		Aldehyde	Pinene	
1a: aldehyde	20	20	0	0
1a: pinene	20	0	20	100
1b: aldehyde	20	17	3	15
1b: pinene	20	3	17	85
No exposure	40	26	14	35
Exposed aldehyde	20	12	8	40
Exposed pinene	20	15	5	25

The table displays the number of moths selecting each lure (phenylacetaldehyde and α -pinene) in dual-choice preference tests. The percentage of moths selecting α -pinene in each treatment group is also displayed (percentages selecting phenylacetaldehyde = 100–value for each treatment). Each treatment group represents a new set of moths (total 160 moths). Treatment groups: 1a, Experiment 1a (immediate test); 1b, Experiment 1b (24 h test); no exposure = moths given no exposure to either volatile and left unfed; exposed pinene/aldehyde = moths exposed to α -pinene or phenylacetaldehyde, respectively, without feeding. Pinene/aldehyde = moths in 'conditioned' group, using α -pinene or phenylacetaldehyde, respectively, as the conditioning stimulus. Statistical analysis of these data is presented in Table 2.

Table 2. Summary of results of hypothesis testing (*G*-test) to determine the significance of differences between treatments

Test	<i>G</i> _(d.f.)	<i>P</i>
All treatments	82.5 ₍₆₎	<0.001
Exposure vs no exposure	1.09 ₍₂₎	ns
aldehyde vs pinene	7.312 ₍₁₎	<0.01
1a: pinene vs aldehyde	55.45 ₍₁₎	<0.001
1a: pinene vs no exposure	35.97 ₍₁₎	<0.001
1a: aldehyde vs no exposure	14.35 ₍₁₎	<0.001
1b: pinene vs aldehyde	21.64 ₍₁₎	<0.001
1b: pinene vs no exposure	17.98 ₍₁₎	<0.001
1b: aldehyde vs no exposure	2.97 ₍₁₎	ns
1a pinene vs 1b pinene	4.402 ₍₁₎	<0.025
1a aldehyde vs 1b aldehyde	4.402 ₍₁₎	<0.025

See Table 1 and methods for explanation of treatment groups. ns, not significantly different ($P>0.05$).

amount of time as in conditioning trials but without pairing this with feeding (exposed group; $G_{(2)}=1.09$). Thus, changes in preference were attributed to classical conditioning; pairing of odour with feeding.

When moths were tested 24 h after conditioning (Experiment 1b), preference for the conditioned odour was significantly lower than in Experiment 1a moths ($G_{(1)}=4.40$, $P<0.025$). When the proportion of 'errors' (moths choosing the non-conditioned odour) per night was examined for Experiment 1b moths, night of testing was not found to be

significant ($G_{(6)}=5.288$); therefore, the decrease in preference in this group was not attributed to greater error on any one night. Moths conditioned on α -pinene showed a significantly higher preference for the α -pinene lure compared with moths conditioned on phenylacetaldehyde ($G_{(1)}=21.64$, $P<0.001$) and moths without associative conditioning (unconditioned moths and exposed moths; $G_{(1)}=17.98$, $P<0.001$). Preferences of Experiment 1b moths conditioned on phenylacetaldehyde were not significantly different from moths without associative conditioning ($G_{(1)}=2.97$, $P>0.05$).

Experiment 2: odour learning using odour-enhanced flowers

We carried out 22 trials using 111 adult male *H. armigera*. Moths trained on α -pinene- and phenylacetaldehyde-enhanced flowers (41 moths per treatment) were trained over 14 trials (seven trials for each flower type, 5.86 ± 0.48 moths per trial). Moths trained on non-enhanced flowers ($N=29$ moths) were trained over eight nights (3.63 ± 0.56 moths per trial). Treatment groups showed significant differences in preference for flowers ($G_{(2)}=19.5$, $P<0.001$).

Moths conditioned on the α -pinene-enhanced flowers showed a greater preference for these flowers when compared with moths trained on phenylacetaldehyde-enhanced flowers ($G_{(1)}=18.8$, $P<0.001$) and moths trained on non-enhanced flowers ($G_{(1)}=8.0$, $P<0.005$; Fig. 2). Moths trained on phenylacetaldehyde showed a preference for the flowers containing phenylacetaldehyde but this was not significantly different from the moths trained on non-enhanced flowers ($G_{(1)}=1.41$, $P>0.05$). Moths trained on non-enhanced flowers showed no difference in preference for either flower type ($G_{(1)}=0.07$, $P>0.05$).

Discussion

This study shows that the upwind flight of male *H. armigera* moths towards different odour sources is strongly influenced by previous odour experience. In wind tunnel dual-choice bioassays, moths that were fed in the presence of a single volatile showed a preference for that odour compared with a second volatile that they had not experienced. Moths with no experience of the volatiles did not differ in their relative preferences for either odour source from those exposed to volatiles without association with a food reward. The results demonstrate that associative conditioning influences preferences for host odours in foraging moths. Studies on the proboscis extension reflex (PER) in *H. armigera* have looked more closely at the nature of the pairing in this type of learning (Hartlieb, 1996).

Moths flew upwind to both odours in the absence of conditioning, which implies that an innate attraction to these odours exists. An innate preference for phenylacetaldehyde over α -pinene was demonstrated in this treatment group, which suggests that attraction to odours is hierarchical, with certain odours being more attractive than others. These innate preferences then become modified through experience. Strictly speaking, conditioning to the odours in this form is termed an

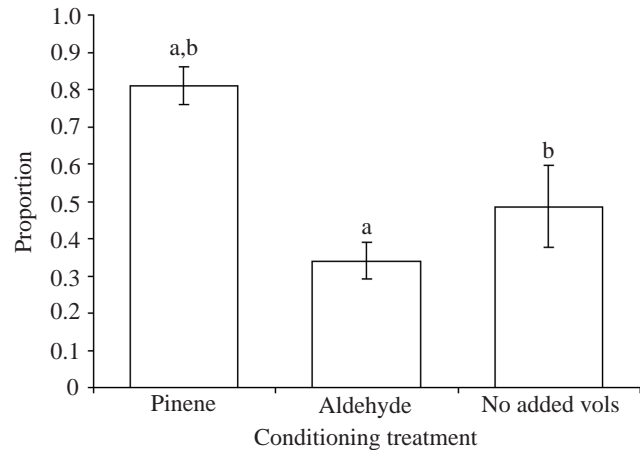


Fig. 2. The mean proportions of insects in Experiment 2 that selected tobacco flowers augmented with α -pinene in each treatment group. Moths had been conditioned on flowers augmented with either α -pinene (pinene), phenylacetaldehyde (aldehyde) or no extra volatiles. Corresponding proportions of insects selecting flowers augmented with phenylacetaldehyde are 1-mean proportions shown for each treatment. Common letters above bars denote significance of differences between treatments (G -test, GLIM): a, $P<0.001$; b, $P<0.005$. Values are means \pm S.E.M.

α -response, as a prior response to the conditioning stimulus (odour) already exists (Menzel et al., 1993).

Following a 24 h period without reinforcement, a strong preference for the odour on which the moths were conditioned the previous night was still evident. This suggests that foraging decisions that occur during one night of feeding influence behaviour on the following night. The fidelity to the learned odour after 24 h was lower than on the initial night of conditioning. A decline in the strength of the conditioned stimulus in eliciting a response with the absence of reinforcement is typical of classical conditioning (Papaj and Prokopy, 1989). Differences between the immediate test and the 24 h test may also be related to the changes associated with short- and long-term learning and memory (Menzel et al., 1993).

When the odour of tobacco flowers was enhanced with either phenylacetaldehyde or α -pinene, feeding experience again led to significant differences in flower visiting. Moths preferred to visit flowers enhanced with the same odour as the flowers on which they were trained. Moths could therefore discriminate between flowers that differed in a single volatile compound. We therefore show that the discrimination and learning of odours is not solely a product of a 'stimulus-deficient' wind tunnel environment, where only a single conditioning stimulus (odour) is present. Moths can detect differences in odours that may exist between flowers with many common visual and olfactory stimuli. These differences are learned associatively with feeding. Moths with experience of the non-enhanced flowers show no preference for either enhanced flower type. Here, differences

in preference may reflect natural variations in odour output of individual flowers.

The odour profile of flowers within a single species can vary in the presence, concentration and relative proportions of their constituents at different times of day (Baldwin et al., 1997; De Moraes et al., 2001; Heath et al., 1992; Shaver et al., 1997). Such variations in odour output have been linked to the attraction of pollinators and deterrence of pests (De Moraes et al., 2001; Heath et al., 1992). Other variables, such as insect damage (De Moraes et al., 2001; Kessler and Baldwin, 2001; McCall et al., 1994) and the onset of pollination (Schiestl and Ayasse, 2001), can lead to variations in odour among plants of the same species. Where such odour signals are consistent with changes in nectar rewards from flowers, recognising such correlations between odour and reward will have fitness benefits to foraging insects. Associative learning of these subtle differences in odour would be advantageous to the generalist forager.

In conclusion, we demonstrate that both innate and learned behaviours are playing important roles in attraction to the individual volatile components of a floral blend. Innate responses to odours predict the expected environment and will have a strong influence on floral choice in newly emerged adult insects. Learning shapes the insect's response to odours in its local environment, increasing the response to odours that have previously led to successful foraging. Thus, the role of odours in plant-insect communication cannot be determined by concentrating solely on the behavioural responses of naive moths. The 'attractiveness' of volatiles to moths in nature is likely to depend as much on ecological factors such as host abundance as on inherited odour preferences (Cunningham et al., 2001; West and Cunningham, 2002). Where learning has a strong influence on the preference for floral odours, the volatiles emitted from the most frequently visited rewarding host species will be those towards which the insect will be the most attracted. Clearly, response to odour is a dynamic system that is as dependent on an ever-changing environmental and behavioural context as it is on a highly evolved system of odour recognition and response.

Thanks to Dr Bronwen Cribb for comments and suggestions on the manuscript and to Kristy Willett for many long hours of research assistance. This project was supported by the Cotton Research & Development Corporation and the Leverhulme trust.

References

- Baldwin, I. T., Preston, C., Euler, M. and Gorham, D. (1997). Patterns and consequences of benzyl acetone floral emissions from *Nicotiana attenuata* plants. *J. Chem. Ecol.* **23**, 2327-2343.
- Beerwinkle, K. R., Shaver, T. N. and Lopez, J. D. (1993). Field observations of adult emergence and feeding behavior of *Helicoverpa zea* (Lepidoptera, Noctuidae) on dallisgrass ergot honeydew. *Environ. Entomol.* **22**, 554-558.
- Bruce, T. J. and Cork, A. (2001). Electrophysiological and behavioral responses of female *Helicoverpa armigera* to compounds identified in flowers of African marigold, *Tagetes erecta*. *J. Chem. Ecol.* **27**, 1119-1131.
- Burguiere, L., Marion-Poll, F. and Cork, A. (2001). Electrophysiological responses of female *Helicoverpa armigera* (Hubner) (Lepidoptera; Noctuidae) to synthetic host odours. *J. Insect Physiol.* **47**, 509-514.
- Crawley, M. J. (1993). *GLIM for Ecologists*. Oxford: Blackwell.
- Cunningham, J. P., West, S. A. and Wright, D. J. (1998). Learning in the nectar foraging behaviour of *Helicoverpa armigera*. *Ecol. Entomol.* **23**, 363-369.
- Cunningham, J. P., West, S. A. and Zalucki, M. P. (2001). Host selection in phytophagous insects: a new explanation for learning in adults. *Oikos* **95**, 537-543.
- Daly, K. C., Durtschi, M. L. and Smith, B. H. (2001). Olfactory-based discrimination learning in the moth, *Manduca sexta*. *J. Insect Physiol.* **47**, 375-384.
- De Moraes, C. M., Mescher, M. C. and Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel nonspecific females. *Nature* **410**, 577-580.
- Dudareva, N. and Pichersky, E. (2000). Biochemical and molecular genetic aspects of floral scents. *Plant Physiol.* **122**, 627-633.
- Fan, R. J., Anderson, P. and Hansson, B. S. (1997). Behavioural analysis of olfactory conditioning in the moth *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *J. Exp. Biol.* **200**, 2969-2976.
- Hartlieb, E. (1996). Olfactory conditioning in the moth *Heliothis virescens*. *Naturwissenschaften* **83**, 87-88.
- Hartlieb, E., Anderson, P. and Hansson, B. S. (1999). Appetitive learning of odours with different behavioural meaning in moths. *Physiol. Behav.* **67**, 671-677.
- Heath, R. R., Landolt, P. J., Dueben, B. and Lenczewski, B. (1992). Identification of floral compounds of night-blooming jessamine attractive to cabbage-looper moths. *Env. Entomol.* **21**, 854-859.
- Jermey, T., Hanson, F. E. and Dethier, V. G. (1968). Induction of specific food preference in lepidopterous larvae. *Entomol. Exp. Appl.* **11**, 211-230.
- Kessler, A. and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* **291**, 2141-2144.
- Landolt, P. J., Lenczewski, B. and Heath, R. R. (1991). Lure and toxicant system for the cabbage-looper (Lepidoptera, Noctuidae). *J. Econ. Entomol.* **84**, 1344-1347.
- Lewis, A. C. (1989). Flower visit consistency in *Pieris rapae*, the cabbage butterfly. *J. Anim. Ecol.* **58**, 1-13.
- Lewis, A. C. (1993). Learning and the evolution of resources: pollinators and flower morphology. In *Insect Learning: Ecological and Evolutionary Perspectives* (ed. A. C. Lewis), pp. 219-242. London: Chapman and Hall.
- Loughrin, J. H., Hamiltonkemp, T. R., Andersen, R. A. and Hildebrand, D. F. (1990). Headspace compounds from flowers of *Nicotiana tabacum* and related species. *J. Agric. Food Chem.* **38**, 455-460.
- Marler, P. and Terrace, H. S. (1984). *The Biology of Learning*. New York: Springer-Verlag.
- McCall, P. J., Turlings, T. C. J., Loughrin, J., Proveaux, A. T. and Tumlinson, J. H. (1994). Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L) seedlings. *J. Chem. Ecol.* **20**, 3039-3050.
- McCullagh, P. and Nelder, J. A. (1989). *Generalized Linear Models*. London: Chapman and Hall.
- Menzel, R. and Bitterman, M. E. (1983). Learning in honeybees in an unnatural situation. In *Neuroethology and Behavioral Physiology* (ed. H. Markl), pp. 206-215. New York: Springer-Verlag.
- Menzel, R., Greggers, U. and Hammer, M. (1993). Functional organisation of appetitive learning and memory in a generalist pollinator, the honey bee. In *Insect Learning: Ecological and Evolutionary Perspectives* (ed. A. C. Lewis), pp. 79-125. London: Chapman and Hall.
- Papaj, D. R. and Lewis, A. C. (1993). *Insect Learning: Ecological and Evolutionary Perspectives*. London: Chapman and Hall.
- Papaj, D. R. and Prokopy, R. J. (1989). Ecological and evolutionary aspects of learning in phytophagous insects. *Annu. Rev. Entomol.* **34**, 315-350.
- Pichersky, E. and Gershenzon, J. (2002). The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* **5**, 237-243.
- Plepys, D., Ibarra, F., Francke, W. and Lofstedt, C. (2002). Odour-

- mediated nectar foraging in the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae): behavioural and electrophysiological responses to floral volatiles. *Oikos* **99**, 75-82.
- Raguso, R. A., Light, D. M. and Pickersky, E.** (1996). Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. *J. Chem. Ecol.* **22**, 1735-1766.
- Schiestl, F. P. and Ayasse, M.** (2001). Post-pollination emission of a repellent compound in a sexually deceptive orchid: a new mechanism for maximising reproductive success? *Oecologia* **126**, 531-534.
- Shaver, T. N., Lingren, P. D. and Marshall, H. F.** (1997). Nighttime variation in volatile content of flowers of the night blooming plant *Gaura drummondii*. *J. Chem. Ecol.* **23**, 2673-2682.
- Smith, B. H.** (1993). Merging mechanism and adaptation: an ethological approach to learning and generalization. In *Insect Learning: Ecological and Evolutionary Perspectives* (ed. A. C. Lewis), pp. 126-157. London: Chapman and Hall.
- Topper, C. P.** (1987). Nocturnal behavior of adults of *Heliothis armigera* (Hubner) (Lepidoptera, Noctuidae) in the Sudan Gezira and pest control implications. *Bull. Entomol. Res.* **77**, 541-554.
- Weiss, M. R.** (1997). Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Anim. Behav.* **53**, 1043-1052.
- West, S. A. and Cunningham, J. P.** (2002). A general model for host plant selection in phytophagous insects. *J. Theor. Biol.* **214**, 499-513.
- Zhu, Y. C., Keaster, A. J. and Gerhardt, K. O.** (1993). Field observations on attractiveness of selected blooming plants to noctuid moths and electroantennogram responses of black cutworm (Lepidoptera, Noctuidae) moths to flower volatiles. *Environ. Entomol.* **22**, 162-166.