

Habituation of sexual response in male *Heliothis* moths

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Abstract. It has been generally hypothesized that habituation mediates the effects of pheromone-based disruption strategies used in the management of moth pests. The current study demonstrates that pheromone-mediated sexual response in the tobacco budworm moth, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), can in fact be modulated by conditions consistent with the production of habituation. An ethogram was used to measure response strength in a wind tunnel experiment where male moths were allowed to respond freely to one of two different blends of female pheromone in 16 trials over 4 days. Post-test measures were collected to investigate stimulus specificity and long-term effects. In conditions appropriate to the formation of habituation, habituation will develop and disrupt male sexual response to female sex pheromone. Males repeatedly exposed to plumes of synthetic pheromone blends display a habituated response lasting up to 96 h. Habituation rate and spontaneous recovery of response strength are greater with less intense stimuli. Additionally, males habituated to one blend express no habituation of sexual response when exposed to a different blend. This indicates a high degree of stimulus specificity, which could facilitate outbreeding, and that moths attend to the configuration of the pheromone blend, not simply to its elements.

Key words. Habituation, *Heliothis virescens*, pheromone, spontaneous recovery, stimulus specificity.

Introduction

As a consequence of *Heliothis virescens* evolving resistance to pesticides (Sparks, 1981) and their pernicious appetite for important agricultural plant species including corn, cotton, tobacco, soybean, tomato and wheat (King & Coleman, 1989), much research effort has focused on their behavioural control (Van Lenteren & Overholt, 1994). Behavioural control of male mating, known as mating disruption, takes advantage of the natural system whereby a female moth attracts males. By applying synthetically produced female sex pheromone into the surrounding atmosphere, nearly complete disruption of male mating effort can be achieved. A number of field and laboratory studies have clearly demonstrated the efficacy of mating disruption (Shorey *et al.*, 1967; Hendricks *et al.*, 1982).

Bartell (1982) outlined five different ways in which effective disruption may be achieved including: decreasing the signal-

to-noise ratio, production of false trails, manipulation of sensory input patterns (by superimposing one or many components upon the female signal), and by inhibiting or otherwise modifying the males response with antipheromones. The fifth, and by far the most promising, mediator of disruption of male sexual response is achieved by the manipulation of the peripheral and central nervous system via sensory adaptation and habituation (Kuenen & Baker, 1981). Both mechanisms are believed to be partially responsible for the observed disruption of male sexual response in laboratory and field experiments.

However, sensory adaptation and habituation are fundamentally different mechanisms expressed as a result of different environmental situations. Sensory adaptation is a short-term effect that, given adequate recovery time, completely dissipates (Domjan, 1993). Sensory adaptation to pheromones has been demonstrated in a number of moth species, including *Trichoplusia ni* (Ignoffo *et al.*, 1963; Shorey *et al.* 1964), *Epiphyas postvittana* (Bartell & Roelofs, 1973; Bartell & Lawrence, 1976c), *Anagasta kuehniella* (Traynier, 1970), *Agrotis segetum* and *Grapholita molesta* (Baker *et al.*, 1988), and *Choristoneura fumiferana* (Sanders, 1985).

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By contrast, pheromonal response recovery can be incomplete 4 days after intermittent pheromonal exposure in the laboratory setting (Figueredo & Baker, 1992), which suggests that habituation has occurred. Further evidence comes from Kuenen & Baker (1981) and Willis & Baker (1984), who found that pulsed pheromone delivery produced more response reduction than continuous pheromonal stimulation.

Whereas sensory adaptation is believed to reduce responsiveness by fatiguing the sensory system as a result of continuous exposure, habituation is considered a central non-associative learning process characterized by a progressive decrease in the activity of an unconditioned response that may occur with repeated presentations of an unconditioned stimulus (Domjan, 1993). Habituation has been documented in species ranging from humans to protozoans (Wood, 1970; Tomlinson, 1974; Jennings, 1976) and displays a surprisingly similar set of characteristics across many sensory modalities (for review see Thompson & Spencer, 1966; Groves & Thompson, 1970). Whereas there is a detailed description of habituation and the stimulus characteristics that produce it, little is known about its efficacy in pheromone-based mating disruption. Associative based olfactory learning has now been documented in three moth species, *H. virescens* (Hartleib, 1996), *S. littoralis* (Fan *et al.*, 1997) and *M. sexta* (Daly & Smith, 2000). Developing an olfactory-based non-associative learning paradigm using *H. virescens* could allow comparative studies.

The aim of this study was to develop a laboratory method to produce habituation in *H. virescens*. We thus developed a training and measurement system by which analysis of non-associative learning phenomena can be investigated. According to Thompson & Spencer (1966), to establish habituation it is not necessary to demonstrate all the different defining characteristics they list. They suggest, rather, that it may be attributed if a number of characteristics are explored with little or no deviation from the expected pattern. Five of the characteristics were thus investigated: the relationship between repeated brief exposures to pheromone blends and response strength; long-term changes in response strength; spontaneous recovery; stimulus intensity; and stimulus specificity.

Methods

Insects

Male *H. virescens* moths were obtained from Arizona Research Labs Division of Neurobiology (ARLDN, Tucson, AZ) as pupae, approximately one week prior to eclosion. Upon arrival, each pupa was isolated and individually housed in an environmental chamber that maintained a temperature of 28°C, a LD 16:8 h photocycle and 80% relative humidity. As adults, males had access to 10% sucrose solution via a cotton wick (Teal *et al.*, 1986). A minimum 2-day posteclosion period was allowed (M. Willis, personal communication) to ensure complete maturation of the male's olfactory system prior to experimentation.

Wind tunnel

The wind tunnel was constructed from clear Plexiglas sheets buttressed in a plywood frame. The dimensions of the wind tunnel were 1 m width × 2.2 m length × 0.85 m height. The side walls and top of the wind tunnel were formed of a single piece of Plexiglas arched in an inverted U shape. A black fabric with randomly placed solid blue fabric circles (10 cm diameter) was placed along the floor of the tunnel to provide random non-directional cues to indicate ground speed and direction of travel. An exhaust vent at the downwind end removed the exiting pheromone plume.

Wind speed in the tunnel was 0.6 m/s, measured using a hot wire anemometer. Titanium tetrachloride (liquid smoke) was used to gauge the turbulence of the wind tunnel. Results of this test indicated that there was a moderate amount of turbulence in the tunnel, causing the smoke plume to break into irregular puffs. These puffs widened from a diameter of ~2 cm at the source to ~15 cm at the downwind end.

Lighting was provided by an array of red photo-safe bulbs that were distributed evenly directly above and along the length of the wind tunnel with an additional soft-white bulb in the centre. Light levels in the tunnel, measured with a Sekonic brand Digipro X-1 light meter, ranged from <1–2.3 lux, with a mean of 1.8 lux.

Pheromone blends

Two pheromone blends were used. Both were obtained from ARLDN. We followed Vickers & Baker's (1992) details for blend ratios, purity, dosage and storage of pheromone. The first blend (2mix) contained the two necessary components for eliciting a sexual response from male *H. virescens*: (Z)-11-hexadecenal and (Z)-9-tetradecenal. The second blend (6mix) contained four additional components: (Z)-7-hexadecenal, hexadecenal, tetradecenal and (Z)-9-tetradecenal (Roelofs *et al.*, 1974).

Fifteen minutes prior to the start of the training procedure, 10 µL of pheromone/hexane solution (one female equivalent) was placed on a clean piece of white filter paper. The filter paper was held 20 cm above the floor by a stainless steel wire armature placed at the upwind end of the wind tunnel. A ¼-inch removable wire mesh shield was mounted on the end of the wire armature to restrict moths from direct contact with the pheromone source.

Habituation procedure

Only moths demonstrating controlled upwind flight were included in the study. Controlled flight was defined as the ability to fly to the upwind end of the wind tunnel in response to a light source. Males were then assigned an identification number. Their containers and testing cages were marked as such for the remainder of the experiment. At the beginning of each day, experimental and control males were placed in wire-mesh testing cages measuring 8 cm high and 6 cm in diameter.

Table 1. Ethogram scores and their behavioural definitions

Score	Behavioural description
0	Male is motionless with wings against body.
1	Male is fanning wings in low to medium amplitude.
2	Male walks upward within the cage while continuing to fan wings.
3	Male becomes airborne and is either orientated upwind or immediately orients upwind.
4	Male, while maintaining upwind orientation and forward position flies in broad arches, circles in a corkscrew fashion.
5	Male is casting from side to side through the pheromone plume while maintaining altitude and ground position.
6	Male makes forward progress while also moving left and right through the plume in a zigzagging fashion.
7	Male moves forward to within 10 cm of the source. There is a tendency to stop at this distance, or nearer.
8	Male touches or briefly lands on the pheromone source then immediately flies off.
9	Male lands on the source and begins to walk around on the pheromone source while vibrating wings with high amplitude strokes and flairs sexual apparatus.

These cages allowed excellent through-flow of air and odours. All cages with males inside were then placed in a plastic shoebox, which was then placed the wind tunnel to allow the males 1 h to acclimate.

Each experimental subject was exposed to one of the two pheromone blends in four habituation trials per day for four consecutive days (16 total trials). Males were flown in order, with one control male added to the end of each cycle of trials. This sequence produced a reasonably constant intertrial interval for each experimental subject lasting 15 min.

In sequence, each cage plus the male was placed into the pheromone plume at the downwind end of the wind tunnel. The open end of the container was closed briefly to insure that the subject had time to sense the pheromone and begin orientation. After 10 s, the cage was opened while still in the plume, so that the male could climb out. It was then allowed to behave freely within the pheromone plume. Each male was allowed 60 s to respond during every trial. If no response occurred in that time, the container and moth were placed back into the shoebox and 0 or 1 was scored (see below). Preliminary studies suggested that if the subject remained completely inactive or unresponsive for a minute or more, it usually remained that way indefinitely.

Subjects' response to the pheromone was scored on an ethogram (see Table 1) with a 10-point ordinal scale measuring the assumed progression of behavioural responses. Responses in the ethogram represent an ordinal sequence of progressive achievements from quiescence to location and landing on the source. This ethogram was developed emphasizing increments that were not only discrete and clearly observable, but were also progressive. Preliminary studies indicated that data collectors could be trained to use this scale with a high degree of agreement in as little as 30 min.

Experimental design

Figure 1 shows the general experimental design. Three basic treatments were used to investigate habituation. For each of these conditions, a total of 90 male *H. virescens* were used, 30 experimental and 60 controls. Experimental

subjects in all treatments were trained in three subgroups of 10 subjects (the maximum number that could be handled in a session).

For the 6MIX treatment condition (Fig. 1), experimental subjects were exposed, one at a time, to the 6mix and allowed to respond. Scores were recorded for each trial. After the habituation training trials were complete, each of the three subgroups, with the remaining controls, were post-tested for long-term habituation. Post-tests were performed at 48, 72 and 96 h for subgroups 1, 2 and 3, respectively. Subjects were again exposed to the pheromone plume, allowed to respond, and scored in the same manner as before for one trial.

In the 2–6MIX treatment condition, the same number of males were used and in the same manner as described in previous treatment condition with the two following variations. First, males were exposed to the 2mix, and then post-tested with the 6mix 24 h after training to assess stimulus specificity. In the 6–2MIX treatment condition the use of the pheromone blends was reversed, that is, subjects were trained with the 6mix and post-tested with the 2mix.

In all treatments, one control male was flown after all 10 experimental males had completed a trial. These controls were yoked to the treatment groups by age and time of trial, and used only once. They were exposed to the pheromone plume and scored in the same manner as the experimental males. They were used to test for within-days effects such as changes in pheromone emission rates and/or circadian rhythms (scotophase), and between-days effects such as ageing.

Data were collected from late February to mid-August. Climatic conditions in the Tucson area vary dramatically throughout the year and can have profound effects on moth behaviour (M. Willis, personal communication). From late February through to June conditions tend to be arid, whereas in mid and late summer Tucson experiences a humid monsoon season. During the monsoon season the males appeared to be somewhat more resistant to habituation. To account for climatic effects on pheromone response, data on water vapour pressure, a measure of the absolute humidity, were collected (post hoc) from a weather station approximately 5 miles north of the University of Arizona campus.

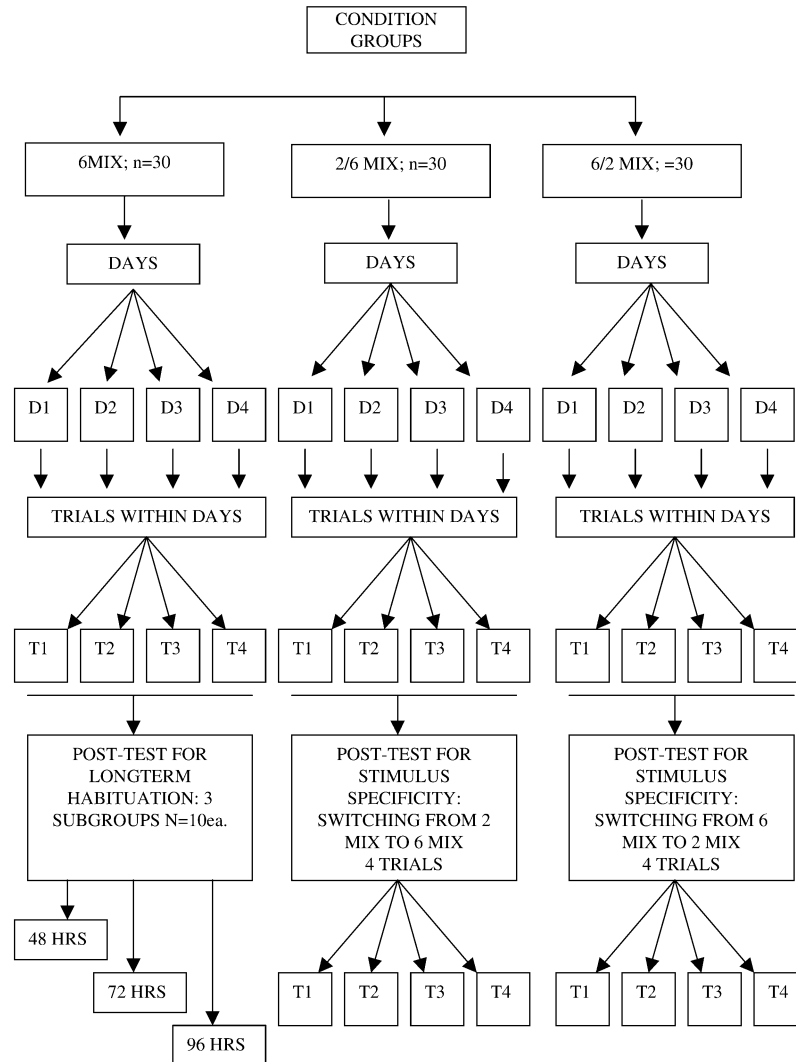


Fig. 1. Flowchart describing the experimental design. There were three different experimental conditions all of which received four habituation training trials on 4 consecutive days. The first group was habituated to the six-component blend then subdivided into three groups, which were post-tested in successive 24 h intervals. The second group was habituated to the two-component blend then received four post-test habituation trials on the fifth day with the six-component blend. The third group was first habituated to the six-component blend then post-tested in the same manner as the second group but with the two-component blend.

Statistical analysis

Significance tests and parameter estimations were produced using the General Linear Model (GLM) procedure with supporting regression analyses in a series of separate models (Cohen & Cohen, 1983; SAS, 1990). Additional analyses of a number of curvilinear functions were also tested. Data from all three treatment groups were pooled for analysis. Subsets of these pooled data were then analysed in separate models to test specific hypotheses.

Analysis 1: Control group. In this first model, we investigated the effects of ageing and time within the scotophase on performance as measured by the ethogram score. Age was a continuous variable ranging from 1 to 4

signifying which of the four sequential days that each control subject was used. Because the controls were matched by age, this variable tracked developmental effects. Log_Age was the natural logarithmic transformation of age. TIME was also a continuous variable, ranging from 1 to 4, distinguishing controls by which of the four trials, within days, that they were used. TIME explains variance due to the effects of scotophase and/or changes in pheromone emissions rates from the plume source within the training period.

Analysis 2: General characteristics of habituation. In this second analysis, all data excluding the post-test data (data collected after the 16th trial) were used to investigate the effect of repeated exposure to pheromone blends. VAPOUR was a continuous variable measuring the atmospheric water vapour

pressure, in millipascals, at the start of each training series. MIX was a binary variable, coded 0 and 1 for the groups receiving habituation training with the 2mix and the 6mix, respectively. MIX represents the main effect of stimulus intensity (i.e. differences in Y intercept). Subject identification (SID) was a class variable accounting for variance due to individual differences. LNT was the natural logarithmic function of the number of trials within a day. The linear form of trials (TRL) ranged from 1 to 4. Both the log and linear form of trials were treated as continuous variables and were used hierarchically to test for significance and fit. DAY was a class variable measuring the amount of spontaneous recovery between blocks of trials. The day effect was dummy coded D1, D2 and D3 for regression analysis. The MIX by LNT interaction tests for differences in rate of habituation as a function of stimulus intensity, while MIX by DAY tests for differences in the amount of spontaneous recovery between days as a function of stimulus intensity.

Analysis 3: Long-term effects. In this analysis, we investigated long-term retention of habituated response in two ways. For this, data from the 6MIX treatment condition were used, subdivided by post-test subgroups. The dependent variable, RECOV, was a measure of spontaneous recovery, created by taking the difference in ethogram score from the last trial of each day and the first trial of the comparison day. ITI compares spontaneous recovery between training days (coded 0) and post-test days (coded 1). To the degree that subjects maintained a habituated response, there should be no significant correlation between the ITI and the RECOV variables. Whereas ITI pooled all pretest data, the BLOCK variable did not. BLOCK was coded 1–4, distinguishing spontaneous recovery for each day, with the post-test day being coded as 4.

Analysis 4: Stimulus specificity. The most powerful test of stimulus specificity was to compare data from the first day to that of the post-test day. To the degree that habituation generalizes across the two different pheromone blends, the day 1 and post-test scores should show habituation-related differences. Alternatively, if there was no generalization between the two blends, post-test performance should not be different from day 1. Thus, POST was a variable differentiating all day 1 data (coded 0) from all of the post-test day (coded 1) for the 6–2MIX and 2–6MIX treatment conditions. POST tests for difference in intercept between day 1 and day 5 for each of the two groups. The GROUP variable differentiates the 2–6MIX and 6–2MIX treatment conditions (coded 0 and 1, respectively). The interaction of GROUP by LNT investigates possible pre-exposure effects of one odour upon responsiveness and rate of habituation to the other by comparing like pheromone blends in a between groups design.

Analysis 5: Interaction of POST by GROUP. Two models were created to look at pre-exposure effects that indicate the generalization of one blend to the other. Two variables were created to gain specific intercept and slope comparisons for POST by GROUP interactions. Again, a subset consisting of day 1 and post-test day data were used. In the first model 2MIX-POST represented a dummy coded variable that compared the day 1 data from the 2–6MIX

group and the post-test data from the 6–2MIX group, thereby producing a comparison of 2MIX data across the two treatment groups. This main effect tests for differences in response strength, while its interaction with LNT tests for differences in habituation rate. In the second model, 6MIX-POST compares the 6MIX data in the same way as described for the 2MIX data. It is important to recognize that the variables described above are cross-group comparisons but they allow the comparison of response patterns between naive subjects and subjects who have been pre-exposed to a different blend first.

Results

Effects of age and scotophase

There were no significant effects found of either age or time of day within blocks of trials, which means that as moths aged during testing they did not change the way in which they responded to the pheromone. Furthermore, within the range of the scotophase that we tested, there were no changes in moths' responsiveness to the pheromone. Finally, there were no significant behavioural effects attributable to changing pheromone levels within a day. These results are contrary to previous tests of the effect of age on pheromonal response (e.g. Figueredo & Baker, 1992) with the Oriental fruit moth; however, their analysis was over a greater number of days.

General characteristics of habituation

Table 2 indicates that vapour pressure (VAPOUR) had a small but significant effect on ethogram scores. The associated regression analysis indicated that with increasing vapour pressure the males were increasingly responsive to pheromones. Differences among the males (SID) explained 36% of the model variance.

There was a significant effect of trial (LNT) in the experimental groups accounting for 13% of the model variance (Fig. 2). In the standardized solution, LNTs produces a negative slope, indicating a decrease in response strength with successive trials. The linear function of trials was not significant when its variance component was estimated after LNT, whereas when the variance for the linear function of trial is estimated first, significant residual variance for the log LNT remains. Thus, by using LNT alone the same total variance is explained with one fewer variable.

DAY had a significant effect accounting for 10% of the model variance. The standardized solution indicates strong positive spontaneous recovery in ethogram scores between days. Figure 2 shows that the amount of spontaneous recovery between days was appreciable in all three treatment groups. The standardized solutions for the dummy coded comparisons of sequential days confirm this observation (Table 2).

Table 2. General linear model and supporting regression analysis explaining ethogram scores as functions of individual differences, odour intensity (mix), log and linear functions of trial number, and days. The dependant variable is the ethogram score. In the regression analysis D1-D3 represent levels of day and MD1-MD3 levels of mix by day

Predictor	NDF, DDF	R ² (sR ²)	F-Ratio	Probability
VAPOUR	1, 88	0.01	83.27**	0.0001
MIX	1, 88	0.05	13.43*	0.0004
SID	88, 261	0.36	22.65**	0.0001
LNT	1, 88	0.13	329.40**	0.0001
TRIAL	1, 88	0.00	1.88	NS
MIX*LNT	1, 88	0.01	16.26**	0.0001
LNT*SID	88	0.04	2.28**	0.0001
DAY	3, 261	0.10	104.02**	0.0001
LNT*DAY	3, 261	0.001	1.29	NS
MIX*DAY	3, 261	0.01	8.45**	0.0001
SID*DAY	261	0.08	1.79**	0.0001
Model	448, 980	0.87	10.51**	0.0001

Standardized Final Solution: ETHOGRAM=0.01*VAPOUR + -0.38*MIX + -1.85*LNT + 0.1.15*MIX*LNT + 0.88*D1 + 0.1.25*D2 + 0.1.41*D3 + -0.42*MD1 + -0.49*MD2 + -0.53*MD3
 * $P < 0.05$; ** $P < 0.0001$

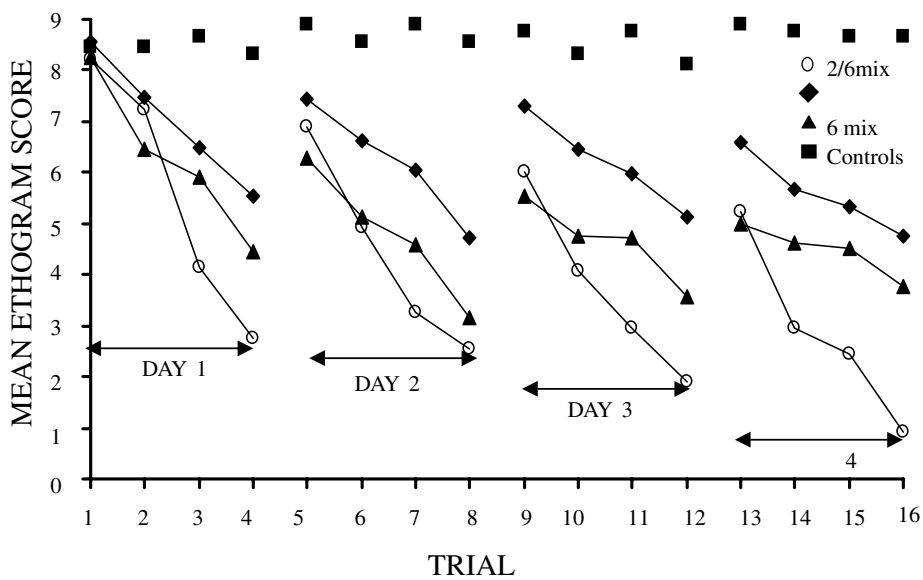


Fig. 2. Mean response strength for the three treatment groups and pooled controls (six- and two-component blends). Note that control (black squares: $n=9$ /per mean) response strength continues to be strong both within days and between days. Experimental groups show a consistent pattern of decreasing response strength within days and recovery between days. Also notice that the group receiving the 2MIX clearly habituates at a much higher rate with more spontaneous recovery.

MIX accounted for 5% of the model variance with a standardized estimate of -0.38 (Table 2). This significant negative effect indicates that the six-component blend produced a lower response strength than the two-component blend. This was not in accordance with our expectations. Figure 2, however, indicates that the reason for this dis-

crepancy is the generally weaker response strength of the 6MIX group; this may be due to seasonal differences in response patterns of the moths.

The MIX by LNT and MIX by DAY interactions tested the differences in slope between both of the 6mix groups (6MIX and 6-2MIX) vs. 2-6MIX treatment. The MIX by LNT

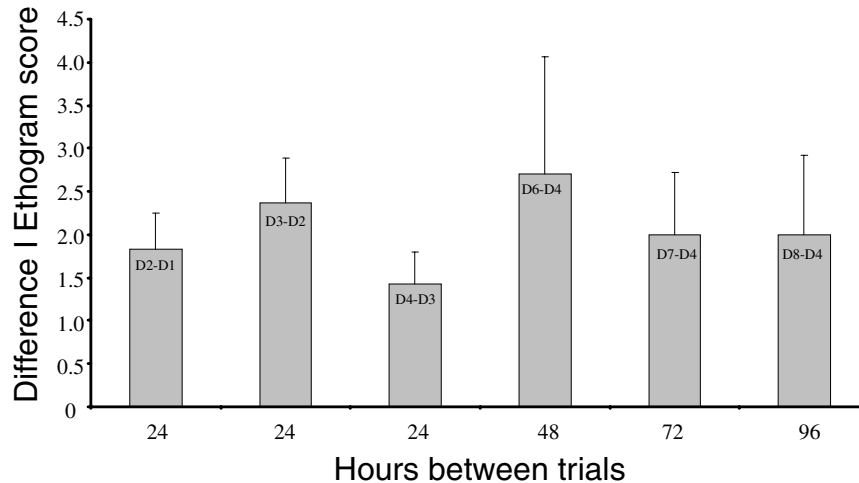


Fig. 3. Mean spontaneous recovery of response strength as measured by the difference in ethogram score between the last score of each day and the first score from the following day. The first three bars ($n=30/ea$) represent spontaneous recovery during habituation training. The second three bars ($n=10/ea$) measure spontaneous recovery from the last trial on the last day of training and the time at which each subgroup of 10 moths was post-tested (48, 72 and 96 h, respectively).

interaction accounted for a significant amount of the variance in scores, indicating that moths exposed to the 2mix habituated faster. MIX by DAY was also significant, with the standardized solution indicating that subjects receiving the 2mix experienced greater spontaneous recovery than those exposed to the 6mix.

Long-term retention

Three GLIMs were tested analysing long-term habituation effects for different post-test periods of increasing length. In all three subgroups ITI was found nonsignificant, indicating that the amount of spontaneous recovery between 24 and 48 h, 24 and 72 h, and 24 and 96 h did not increase. Similarly, the BLOCK variable was also found to be nonsignificant, indicating that there was no progressive change in spontaneous recovery across training and post-test. Figure 3 is a bar graph showing mean spontaneous recovery between days; notice that these scores are very similar. This means that while subjects continued to become more habituated with each block of trials (Table 2), they displayed the same amount of spontaneous recovery between days, indicating that there is a consistent retention of habituated response.

Stimulus specificity

Figure 4 displays mean response strength by trial for the 2-6MIX and 6-2MIX groups on day 1 and day 5. Each group has been subdivided to indicate training (PRE) or testing (POST). While there is increasing variation across trials, what is important here is that there is very little variation in the initial response across the PRE and POST phases, indicating very little generalization of learning. Table 3 displays the results of the GLIM investigating stimulus specificity. Note

that in the model the POST variable was not significant. This means that the Y intercept for day 1 and the post-test day are the same and indicates complete recovery of response strength when subjects are presented with the novel blend. GROUP and POST by GROUP variables, however, were significant, accounting for less than 1% and 4% of the total variance of ethogram scores, respectively. Regression analysis indicates that the 6-2MIX treatment had a slightly higher Y-intercept. This finding indicates that response strength in the 6-2MIX group was greater than to the 2-6MIX group. This contradicts findings in analysis 1 showing the 2MIX to produce a greater initial response strength.

Table 4a indicates that 2MIX-POST was not significant but its interaction with LNTs was. This effect was small, accounting for 2% of the variance with a change in slope of +0.04. The standardized solution indicates that subjects displayed a lower rate of habituation to the 2mix if they were pre-exposed and habituated to the 6mix. Table 4b indicates that, although the slope was the same for 6MIX-POST, the intercept increased as a function of pre-exposure to the 2MIX, although Fig. 2 indicates that this effect was small.

Discussion

A number of mechanisms have been proposed that might account for the reduction in male responses to female sex pheromones observed in mating disruption trials. Here we have shown that with successive trials there is a continued weakening of response strength (Fig. 2), which shows the classic characteristics of habituation. With respect to stimulus intensity, our results were mixed. We expected that because the six-component blend was capable of affecting a broader array of olfactory receptors that it would have elicited a

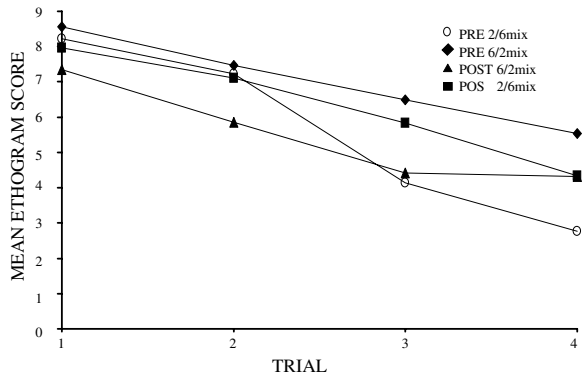


Fig. 4. Mean ethogram scores by trial from the 6–2mix ($n=30$) and 2–6mix ($n=30$) treatment conditions for day 1 (pre) and the day 5 (post).

stronger response in the moths. The lower Y intercept for the six-component blend in analysis 1 was therefore unexpected and may be attributable to seasonal differences, such as vapour pressure (a measure of absolute humidity). It may also be the case that *Heliothis* is not receptive to a number of the non-essential four components (Vetter & Baker, 1983). Our analysis points, in part, to seasonal effects but a more detailed study of these environmental effects is needed. Additionally, analysis 1 indicates that the moths habituated much faster to the two-component blend, and then showed greater spontaneous recovery. These significant interactions between odour blend and trial and between odour and day are in line with our expectations (Thompson & Spencer, 1966) and suggest that males are sensitive to at least one of the four additional odours present in the six-component blend.

We have also shown that there is a lack of recovery of the habituated sexual response in moths that persists for as long as 96 h after habituation has occurred, which is a substantial portion of the adult life span. This long-term effect would not be expected if the response decrement were solely due to sensory adaptation. Herein is the major advantage that habituation provides in and above the short-term effects of sensory adaptation, meaning males habituated to female

pheromone will continue to exhibit little or no response to calling females for many days. *Heliothis virescens* can relocate hundreds of kilometers in a single evening by riding upper-air currents (Fitt, 1989). Therefore, the long-term effects that habituation has upon male sexual response could conceivably have far-reaching regional effects if a male should leave a treated field.

One surprising finding of this study was the near complete lack of generalization of habituated response from one pheromone blend to the other, again suggesting that males are sensitive to at least one of the four additional components in the six-component blend. These results also strongly suggest that male moths attend, in part, to the configuration of the pheromone blend and not the individual elements per se. If it were the case that moths were attending specifically to the elements in the blend and not the blend configuration, then we would have expected that once habituated to the two primary components, there would have been little or no response recovery to the six-component blend because the additional four components are in and of themselves inadequate to elicit a sexual response. Furthermore, the fact that moths can be habituated to the six-component blend and show no generalization of that habituation when presented with the two components alone reinforces this conclusion. This finding suggests a general methodological approach to addressing theoretical disputes as to the biological relevance of secondary components found in gland extracts (Vickers *et al.*, 1991).

Typically, a blend similar in ratio to our two-component blend has been experimented with in agricultural applications for this species, although at higher emission rates. Our findings indicate that this two-component blend might be inadequate for the purposes of producing habituation in the field. This is because habituation of male sexual response to the two-component blend does not generalize to the natural female blend, at least at the concentrations used here. However, our understanding of the generalization gradient is still deficient; it may be that males can discriminate equally well much subtler differences in blend composition. Indeed, Smith (1983) reports that in *Lasioglossum zephyrum*, genotypic variation in pheromone blend provides enough specificity that males habituated to one female will generalize to other females only

Table 3. General linear model and supporting regression analysis modeling stimulus specificity effects. Note that there is no significant effect of POST, which compares the first day of the habituation trials and the post-test trials. The dependent variable is ethogram score.

Predictor	NDF, DDF	R ² (sR ²)	F-Ratio	Probability
SID	29	0.16	4.85**	0.0001
LNT	1, 29	0.28	237.05**	0.0001
GROUP	1 29	0.00	3.98**	0.0466
POST 1, 29	0.00	3.35	0.0680	
POST *GROUP	1, 29	0.04	33.53**	0.0001
Model	33, 438	0.48	12.68**	0.0001

Standardized Final Solution: ETHOGRAM = $-0.58 * LNT + 0.09 * GROUP + -11 * POST * GROUP$
 * $P < 0.05$; ** $P < 0.0001$.

Table 4. (a) General linear model and (b) supporting regression analysis modelling the significant interaction between POST by GROUP for the dependent variables activity and ethogram.

Predictor	NDF, DDF	R2(sR2)	F-Ratio	Probability
(a)				
SID	29, 199	0.20	2.96**	0.0001
LNT	1, 29	0.32	136.01**	0.0001
2MIX-POST	1, 29	0.00	0.00	NS
LNT*2MIX-POST	1, 29	0.02	9.50*	0.0023
Model	32, 199	0.54	7.23**	0.0001
Standardized Final Solution: ETHOGRAM = -0.66*LNT + 0.04*LNT*2MIX-POST				
(b)				
SID	29, 207	0.24	3.65*	0.0001
LNT	1, 29	0.27	117.45*	0.0001
6MIX-POST	1, 29	0.02	10.13*	0.0017
LNT*6MIX-POST	1, 29	0.00	0.74	NS
Model	32, 207	0.53	7.32*	0.0001
Standardized Final Solution: ETHOGRAM = -0.56*LNT + 0.17*6MIX-POST				

* $P < 0.05$; ** $P < 0.0001$

if they are related to the original female. Clearly, a more detailed study of stimulus specificity in *H. virescens*, using more closely related blends and higher concentrations, is called for.

There are some subtle pre-exposure effects, one of which can be brought to bear on the issue of stimulus generalization. We have shown that pre-exposure to the two-component blend reduced the responsiveness to the six-component blend, indicating potential stimulus generalization. However, the magnitude of this pre-exposure effect was small and could be due to group differences. Pre-exposure to the six-component blend, by contrast, reduced the rate of habituation that males expressed when they were trained with the two-component blend.

Our results have clearly implicated habituation in the modulation of male moth sexual response to female pheromone. However, do male moths experience similar conditions in the field? We attempted to create a simplified reproduction of the natural environment, controlling what are believed to be the relevant conditions that a male *H. virescens* might experience on a typical night. Wind speeds were low and subtly turbulent (non-laminar), creating a naturally discontinuous pheromone plume, lighting was typical of a dark night, and pheromone emissions, although not measured directly, were released at concentrations thought to closely resemble naturally occurring levels produced by a single calling female (Pope *et al.*, 1982; Vetter & Baker, 1983).

Given that there was little generalization between the two blends, it is reasonable to infer, as did Smith (1983), that habituation could facilitate outbreeding. By habituating to females that have already been encountered, a male may focus mating efforts on females that he has not encountered. Furthermore, if related females produce highly similar pheromonal signatures, males would be less likely to mate

with relatives of females that he had previously encountered, creating non-random mating preferences in the male based on prior experience. However, it cannot be concluded based on these data that habituation actually plays a role in outbreeding; a more focused analysis, investigating the discrimination of varying ratios of the complete pheromone blend would be necessary to substantiate this hypothesis.

The role of climate on habituation

The addition of an atmospheric measure was done post hoc to explain the differences in mean scores between the two groups receiving the six-component blend. Although the effects of absolute humidity were small, they did imply that atmospheric changes influence not only response strength of male behaviour but habituation rate as well. These results also suggested that our analysis of stimulus intensity could have been confounded by other related climactic conditions. There are at least two potential weaknesses with the VAPOUR measure as used in this study. First, it is only one of many possible climactic variables that may have affected moth behaviour. Second, because the weather station was 5 miles from where this study was carried out, there was no guarantee that the measure was perfectly accurate at predicting conditions within the laboratory. This problem was compounded by the fact that the study was carried out in a climate-controlled building. However, because the building is an open system with respect to the external environment, we do believe that there was a substantial correlation (although not a perfect one) between atmospheric conditions inside and outside the laboratory.

Nevertheless, the difference between the two groups receiving the six-component blend for training was clearly

visible. Further evidence came from a count of individuals that showed little or no habituation. Of the group flown in the winter, none of 30 males scored 8 or higher on the ethogram scale on the 16th trial. By contrast, five of 30 males flown in the summer scored 8 or higher. During the late summer when atmospheric conditions suddenly change, when there is abundant new plant life, and reproductive conditions are seemingly ideal, male moths appeared more responsive to the six-component blend. One could postulate that males that are more responsive and persistent during optimal times realize an adaptive advantage.

The role of individual differences

Individual differences had largest effect on male moth sexual response to female pheromone blends in this study. By far the most important point here is that some males exhibited no response reduction whatsoever across all 16 trials. That is these subjects exhibited the same high response, or displayed an increase in response strength, with successive trials. The fact that these individuals showed little or no habituation is perhaps surprising. Given more trials with longer exposure and shorter intervals between stimuli, perhaps these individuals would habituate. Perhaps too, the longer exposure that males would receive nightly in the field setting might produce habituation that was not detected in the laboratory. Nevertheless, the evidence that these individual moths did not habituate suggests the possibility that selective pressure favouring males who are slow to habituate could affect the gene pool.

Heliothis virescens evolves resistance to pesticides rapidly and laboratory evidence from selective breeding experiments (Collins & Cardé, 1989, 1990; Collins *et al.*, 1990) suggests that the pheromone-based sexual attracting system can be manipulated in *Pectinophora gossypiella*. Collins *et al.* (1990) have found that females can be selected to emit pheromone blends with dramatic increases in selected components but one still capable of eliciting male response. Collins & Cardé (1990) also showed that males can be selected to respond longer to nontypical pheromone blends. That males respond longer does not necessarily mean that they are slower to habituate, but based on our results, this possibility cannot be ruled out.

Acknowledgement

This work was supported in part by an award from NIH-NCRR (9 R01 RR14166-06) and contract from DARPA-CBS to Brian H. Smith.

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Accepted 8 March 2000