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# Pre-exposure Modulates Attraction to Sex Pheromone in a Moth

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## Abstract

In behavioural experiments we investigated the influence of previous short exposure to sex pheromone on subsequent response of male *Spodoptera littoralis* moths to sex pheromone. We found that pre-exposed males showed increased sensitivity to female sex pheromone after a single exposure to a pheromone plume compared to that found in naïve males. The increased responsiveness lasted for at least 27 h after the exposure, showing that it was not just a short-term sensitization of the males. Exposure to the odour source without upwind movement towards the source was enough to increase the responsiveness. Physical activation without exposure to odour did not affect responsiveness. The increase in responsiveness after exposure was higher when the males were pre-exposed to natural female pheromone gland extract than when they were exposed to a higher dose of the main component, even though both odour sources elicited similar upwind attraction in naïve males. Thus, the quality of the pheromone mixture to which males were exposed influenced the subsequent response.

Key words: *Spodoptera littoralis*, olfaction, Lepidoptera, mate finding, response threshold, odour detection

## Introduction

Odour cues are important for insects in many different aspects of their life, for example in finding a partner, food or suitable oviposition sites. The behaviour elicited by these cues have in many insects been shown to be modulated by experience. Classical conditioning studies have been performed mainly in bees that learn to associate food sources with odours (Menzel, 1993). In parasitic wasps, learning of odours in association with oviposition in their victims or during feeding has also been shown to be highly important (Vinson, 1984). Non-associative mechanisms, such as induction of preference, sensitization and habituation, can also influence behaviour through earlier experiences.

All of these examples are connected to food or oviposition site location. However, very little is known regarding the role of experience in sex-pheromone-dependent behaviour. The response to sex pheromones is considered to be innate and should not be modulated by experience. Mistakes while searching for a partner are very costly since they could drastically reduce the chances of obtaining a successful mating. Thus, a signal that is closely connected to reproduction is assumed to be stable and not affected by experience. Previous exposure to female-produced sex pheromone or to synthetic pheromone compounds has been shown to reduce male attraction to pheromone (Traynier, 1970; Bartell and Lawrence, 1973; Bartell and Roelofs, 1973; Figueredo and Baker, 1992; Daly and Figueredo, 2000). In vertebrates, pre-exposure to sexual attractants has been shown to affect subsequent responses to volatile sex pheromone compounds (Meredith, 1986; Fewell and Meredith, 2002; Moncho-Bogania *et al.*, 2002).

In Lepidoptera, males are attracted to a blend of compounds specifically emitted by females. Typically, the sex pheromone blend is a mixture of several compounds. Blend specificity is achieved by unique combinations or ratios of the compounds included in the female blend. In *Spodoptera littoralis* (Lepidoptera: Noctuidae), the main component of the sex pheromone was found to be (*Z*,*E*)-9,11-tetradecadienyl acetate—*ZE*-9,11–14:OAc (Nesbitt *et al.*, 1973; Campion *et al.*, 1980). In addition, several other potential pheromone compounds have been identified from the female gland. Field trials in Israel showed that a mixture of *ZE*-9,11–14:OAc and (*Z*,*E*)-9,12-tetradecadienyl acetate (99.5:0.5) was attractive to males (Dunkelblum *et al.*, 1982) and this blend has been used as an attractant in later field experiments (Kehat and Dunkelblum, 1993; Downham *et al.*, 1995).

In a preliminary study we found that males of *S. littoralis* showed an increased sensitivity to female gland extract after a brief exposure to female sex pheromone. These results were in contrast to earlier findings, where pre-exposure resulted in habituation to female sex pheromone compounds (Traynier, 1970; Bartell and Lawrence, 1973; Bartell and Roelofs, 1973; Figueredo and Baker, 1992; Daly and Figueredo, 2000).

In this study we have investigated the effect of pre-exposure to odours from female pheromone gland extract and synthetic sex pheromone compounds on the behaviour of virgin male *S. littoralis* to female sex pheromone in subsequent upwind attraction.

The reduced responsiveness found in former studies remained from a few hours to several days (Bartell and Lawrence, 1973; Figueredo and Baker, 1992; Daly and Figueredo, 2000). The effect of exposure may just be a short increase in sensitivity, i. e. a sensitization, or it may have a more long-term effect on behaviour. The duration of the effect may indicate the mechanism behind the increased sensitivity.

Therefore, we also attempted to determine if the experience had a short-term effect or a longer impact on behaviour.

The quality of the sex pheromone stimuli has been shown to have impact on learning in *S. littoralis* (Hartlieb *et al.*, 1999). Single components of the sex pheromone elicited different effects on behaviour than a female sex pheromone gland extract. The effects of pre-exposure have been shown to vary.

Thus, we investigated if the quality of the previously experienced pheromone affected the attraction behaviour.

In our preliminary study, the moths were both exposed to a sex pheromone source and physically activated and allowed to walk against the source. The different elements during the exposure may have different influence on the subsequent behaviour or may interact synergistically.

Therefore, we studied if detection of the pheromone was sufficient or if the physical activation and movement towards the odour source was also important to reinforce the odour attraction.

## Materials and methods

### Insects

Males of *S. littoralis* for the olfactometer experiments were obtained from a laboratory culture reared for many generations on an artificial diet (Hinks and Byers, 1976). The culture had been supplemented with moths collected from the wild yearly over the last 7 years. The males used in the wind tunnel experiments originated from field-collected insects. Eggs collected in Egypt were transported to Sweden where larvae were reared on cotton leaves until pupation. Both cultures were maintained at 25°C, 65% relative humidity and 16:8 h light:dark photoperiod. Male and female pupae were separated at the pupal stage and allowed to emerge in different climate chambers to exclude pre-exposure of males to female sex pheromone.

### Odour sources

In the experiments, both female pheromone gland extracts and synthetic pheromone compounds were used. For the extracts, the pheromone gland from 2–3-day-old females were dissected 2–3 h into the scotophase, which is the peak calling time for females (Dunkelblum *et al.*, 1987). After 2 h elution of the pheromone glands in hexane, the solvent was transferred to another vial and stored at –17°C until used in the experiments. Each female gland was found to contain about 18 ng of *ZE*-9,11–14:OAc. The analysis was performed on a Hewlett-Packard HP-6890 gas chromatograph equipped with a capillary HP-Innowax column (30 m × 0.25 mm ID) using a standard temperature programme (Jönsson and Anderson, 1999). *ZE*-9,11–14:OAc (96% pure) was diluted in hexane.

### Bioassays

#### *Olfactometer experiments*

Attraction of adult males of *S. littoralis* was tested in an open-arena walking olfactometer measuring 60 × 60 cm (Schlyter *et al.*, 1995). Charcoal filtered air was pushed through a baffle (40 × 5 cm) with spaced 2 mm holes generating a 0.5 m/s laminar airflow over the floor of the olfactometer. An exhaust at the other end sucked out the air from the olfactometer. Earlier studies have confirmed that *S. littoralis* behave normally to sex pheromone in the olfactometer (Hartlieb *et al.,* 1999). The experiments were carried out 2–4 h into the scotophase in red light, at 18–19°C and 40–60% relative humidity. Virgin males, 2–3 days old, were transferred individually to glass tubes (80 mm, i.d. 23 mm) with one end covered with a plastic net and transported to the experimental room just before the onset of the scotophase. Starting 2 h into the scotophase, males were introduced individually into the olfactometer at the centre of the odour plume 40 cm downwind from the odour source. Three different behavioural steps were scored: orientation; half way; and 2 cm from the source (see Figure 1 legend for description).

The odour solutions were applied on pieces of filter paper (5 × 10 mm), which after evaporation of the solvent were placed in the centre of the upwind end 5 mm above the floor of the olfactometer. The filter paper was replaced every 15 min. The different treatments within each experiment were tested in parallel. Thus, each treatment was tested on several days to minimize the effects of variations in conditions between days. After the entire experiment all males used in the experiments that day were tested against 1 female equivalent (fe) of gland extract. Males not activated within 2 min (8%) by the female extract were excluded from the data set.

In the first series of experiments, we investigated the male response to 1 fe of the female pheromone gland extract and to four doses—1, 10, 100 and 1000 ng—of the main sex pheromone component, *ZE*-9,11–14:OAc, diluted in 10 µl hexane.

In a second series of experiments, males were given a pre-treatment in the same olfactometer as used in the subsequent experiments just before the start of the scotophase. Males respond to sex pheromone and mate with females during both the photo- and scotophases in the laboratory (personal observation). After the pre-treatment, males were kept individually in glass tubes in the olfactometer room until tested, except for the males tested 27 h after exposure. These males were taken back to the rearing chamber and returned to the olfactometer room the next day, just before the onset of the scotophase.

Pre-treatments were as follows.

1. Males were exposed to 1 fe of female sex pheromone gland extract and allowed to walk up to, but not to contact, the pheromone source. The males were tested 3 h after the pre-exposure.
2. The same as above, except that the males were tested 27 h after pre-exposure.
3. As treatment 1, except that males were removed from the pheromone plume immediately after they were activated by the pheromone and started to orient to the source inside the glass tube.
4. As treatment 1, except that the males were pre-exposed to 1000 ng *ZE*-9,11–14:OAc.
5. Males were forced to walk 30 cm upwind in the olfactometer, but no odour source was applied at the upwind end.
6. Naïve males. These males were handled in the same way as the other treatments, but no pre-treatment in the olfactometer was made.

On average the pre-treatment time lasted ~10 s, but for treatment 3 it normally lasted only a few seconds.

The males were tested against 10 ng *ZE*-9,11–14:OAc, 3 or 27 h after the pre-treatment. This odour source was chosen for the experiments because an intermediate proportion of males were activated and walked up (Figure 1) to this dose and it is close to the natural amounts found in females.

### *Windtunnel experiments*

The windtunnel was a 2 × 1 × 1 m Plexiglas construction, through which cleaned air is pushed by a fan producing a 0.3 m/s laminar airflow. In the room with the windtunnel, the temperature and humidity could be controlled. The experiments were performed at 24–26°C, 60% relative humidity and at 1 Lux light intensity. Just before the start of the scotophase, 2–3-day-old virgin males were transferred to the windtunnel room. At the upwind end, an odour source, a standard rubber septum impregnated with 1 fe of female pheromone gland extract was applied after the solvent was allowed to evaporate. The rubber septum with the odour source was replaced every experimental day.

During the pre-treatment, males were exposed to a rubber septum impregnated with a high dose of female gland extract just after the start of the scotophase and allowed to start flying upwind towards the pheromone source. The flight was interrupted and the male placed in the glass vial again until tested. Three different pre-treatments, with a duration time of 10–30 s, were used, as follows.

A. Males pre-exposed to pheromone gland extract and tested again in the wind tunnel 3 h after pre-exposure.
B. As A, but the males were tested 27 h after pre-exposure.
C. Naïve moths, that were handled in the same way as the males in the previous treatments, but were not exposed to sex pheromone.

The males were tested against 1 fe, 3 or 27 h after pre-treatment. Starting 3 h into the scotophase, insects were released individually at the downwind end of the tunnel and the behaviour was recorded. Three different behaviours were scored: take-off; half way; and close (see Figure 3 legend for description)

### *Age-dependent response to female extract*

Naïve males, aged 1, 2, 3 and 4 days old, were tested in windtunnel experiments. The behavioural response to 1 fe of pheromone gland extract was recorded.

### *Statistics*

The numbers of males recorded for each behavioural step in the olfactometer and windtunnel experiments were compared using Ryan's test, a method of adjusted significance levels for proportions (Ryan, 1960).

## Results

### Olfactometer experiments

Most males (>90%) oriented, walked up and approached within 2 cm of the pheromone source when 1 fe of female gland extract and when the highest dose (1000 ng) of the main pheromone component was used as the odour source (Figure 1). For the 100 ng bait, 90% of the males oriented to, but only 63% approached within 2 cm of the pheromone source. Using 10 ng of the main component, 32% of the males oriented to and 26% reached within 2 cm of the source, whereas for 1 ng, 12% of the males oriented to and 8% came within 2 cm of the pheromone source.

In the experiment with different pre-treatments, the naïve males showed a similar response to 10 ng *ZE*–9,11–14:OAc as the males in the dose–response experiments (Figure 2). Three hours after pre-exposure to the female gland extract a higher percentage of the males was activated (84%) and approached within 2 cm of the pheromone source (82%). Similar response levels (85 and 80%) still remained when the males were tested 27 h after pre-exposure. When the males were exposed to 1 fe 3 h before the experiment, but

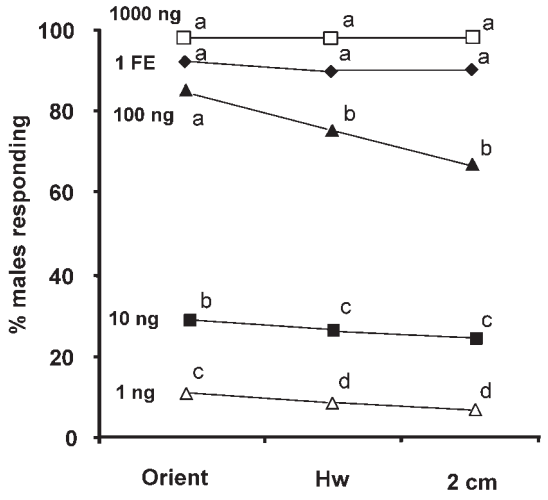


**Figure 1** Behavioural response of virgin 2–3-day-old male *Spodoptera littoralis* in an olfactometer to 1 fe of female sex pheromone gland extract and to four doses (1, 10, 100 and 1000 ng) of the main pheromone component, *ZE*-9,11–14:OAc. Three behavioural steps were recorded: 'orientation'—the male was activated by the odour plume and started to walk against the odour source; 'half way'—the male walked half way up to the odour source; '2 cm'—the male reached within 2 cm of the odour source. Different letters indicate values that are significantly different at the 5% level, using Ryan's test for multiple comparison of proportions (Ryan, 1960); $n = 30$ for female extract, 1 and 1000 ng; $n = 38$ for 100 ng; $n = 49$ for 10 ng.

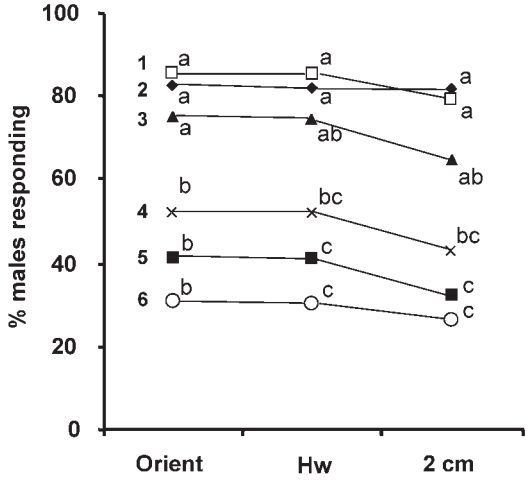


**Figure 2** Behavioural response of virgin 2–3-day-old male *Spodoptera littoralis* in an olfactometer to 10 ng *ZE*-9,11–14:OAc. For behavioural steps, see legend to Figure 1. Six different pre-treatments of the males were used, as follows. (1) Males exposed to 1 fe of female sex pheromone gland extract and allowed to walk up to, but not contact the pheromone source. The males were tested 3 h after the pre-exposure. (2) As (1), but the males were tested 27 h after pre-exposure. (3) As (1), except that males were removed from the pheromone plume immediately after they were activated by the pheromone. (4) As (1), except that the males were pre-exposed to 1000 ng *ZE*-9,11–14:OAc. (5) Males were forced to walk 30 cm upwind in the olfactometer, but no odour source was applied at the upwind end. (6) Naïve males. Different letters indicate values that are significantly different at the 5% level (Ryan, 1960); $n = 40$.

not allowed to walk up towards the pheromone source, 75% were activated and 65% walked up to within 2 cm of the source. When males were pre-exposed to 1000 ng of *ZE*-9,11–14:OAc, a significantly lower proportion (55 and 45%) of males responded than in the previous treatments. A higher proportion (not, however, significant) of the males pre-exposed to 1000 ng *ZE*-9,11–14:OAc responded 3 h later than the naïve males. Males that were forced to walk without exposure to pheromone did not differ from naïve males in their response to 10 ng of the main component.

### *Windtunnel experiments*

In response to 1 fe of gland extract, 44% of the naïve males took flight and 27% came within 10 cm of the pheromone source (Figure 3). Significantly more males took flight (71%) and approached within 10 cm of the source (53%) when they had been allowed to fly against a gland extract source 27 h earlier. Males tested 3 h after the experience showed an intermediate rate of response between the two former treatments.

### *Age effect*

No significant difference in the attraction to female gland extract was found among 1–4-day-old males (Figure 4).

## Discussion

A higher percentage of *S. littoralis* males that were pre-exposed to female sex pheromone responded to the pheromone on subsequent exposures when compared to naïve males. To reach similar response percentages in naïve males, as found in the pre-exposed, 10–100 times higher doses of pheromone compound were needed. A brief exposure that activated the males was sufficient to induce the change in responsiveness. In some cases the male only spent a few seconds within the pheromone plume until removed. In other studies examining the effect of prior exposure to pheromone on male moth attraction to pheromone sources, a higher response threshold to the female sex pheromone after exposure was found (Traynier, 1970; Bartell and Lawrence, 1973; Bartell and Roelofs, 1973; Figueredo and Baker, 1992; Daly and Figueredo, 2000). In these studies, males were pre-exposed statically or repeatedly to sex pheromone compounds and the males most likely became adapted to the pheromone compounds due to the exposure. Habituation was found also in the experiments where the pre-exposure time was down to 1 min (Bartell and Lawrence, 1973), which is a pre-exposure time that approaches the exposure times used in this study. However, the effect on subsequent behaviour was only studied for 1 h after pre-exposure. The aim in all these studies was primarily to investigate male response under situations similar to those when sex pheromones are used for insect control. The experiments performed in the present study come closer to a natural situation, where males experience shorter pulses of pheromone. In such a situation it seems unlikely that males

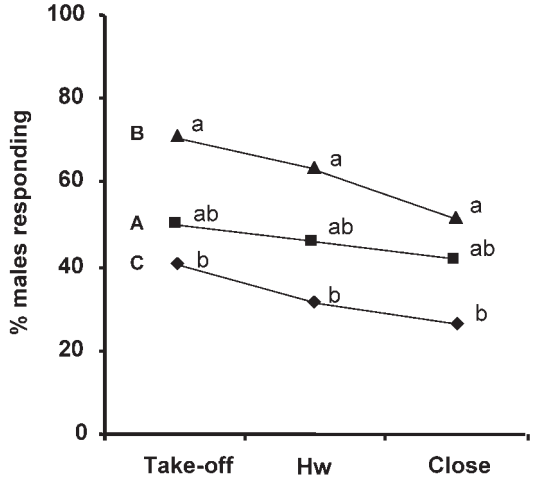


**Figure 3** Behavioural response of virgin 2–3-day-old male *Spodoptera littoralis* in the windtunnel to one fe of a female sex pheromone gland extract. Three different behaviours were scored: 'take-off'—the male was activated by the odour plume and started upwind flight against the odour source; 'half way'—the male flew half way up to the odour source; 'close'—the male flew up to within 10 cm of the odour source. Three different pre-exposure treatments were used, as follows. (A) Males pre-exposed and allowed to start flying towards a rubber septum impregnated with 1 fe of pheromone gland extract and tested again in the wind tunnel 3 h after pre-exposure. (B) As (A), but the males were tested 27 h after pre-exposure. (C) Naïve moths. Different letters indicate values that are significantly different at the 5% level (Ryan, 1960); $n = 50–60$.

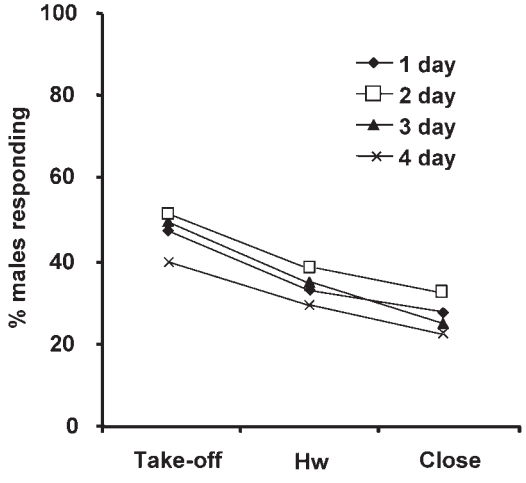


**Figure 4** Behavioural response of 1–4-day-old *Spodoptera littoralis* virgin males in the windtunnel. For behavioural steps see legend to Figure 3. No significant differences were found between the different ages (Ryan, 1960); $n = 40$.

should have a higher response threshold to female pheromones after an unsuccessful attempt to find a calling female. On the contrary, one would expect that not yet mated males that have experienced the presence of calling females should express similar motivation to respond to female sex pheromone as naïve males.

Sex pheromones in moths typically consist of several components mixed in unique combinations and ratios. The complete female blend is needed to get full attraction of males to females. Our results show that even though naïve males show a similar behavioural response to a single component and to the female blend, the two odour stimuli are perceived differently. Behavioural differences between males exposed to a single pheromone component and those exposed to female extract have also been found in other experiments with *S. littoralis*. In proboscis extension experiments, males were able to associate individual pheromone components with a sugar reward (Hartlieb *et al.*, 1999). However, when a female gland extract was used the male response to the reward was much lower and not significantly different from unconditioned control males. A general result from both the present study and that of Hartlieb *et al.* (Hartlieb *et al.*, 1999) is that the pheromone blend is perceived configurally, i. e. as a unique feature and not just as a sum of its elements.

Studies on mice and hamsters have reported that pre-exposure to sexual attractants affects subsequent responses to olfactory sex pheromone compounds (Meredith, 1986; Fewell and Meredith, 2002; Moncho-Bogania *et al.*, 2002). Male hamsters were exposed to female secretion connected to mating behaviour, that contained both volatile and non-volatile components. After exposure, males showed increased responses to the volatile components and were no longer dependent on the non-volatile components of the secretion for attraction and mating (Meredith, 1986; Fewell and Meredith, 2002). In mice, females exposed to male-derived compounds, non-volatile and volatile, were attracted to the volatile compounds that are neutral to naïve mice (Moncho-Bogania *et al.*, 2002). In both cases, increased responses to sex attractants were achieved after exposure to chemosensory stimuli without contact with other individuals and, thus, no mating experience.

The effect of pre-exposure to sex pheromone was not just a short-time sensitization of the males to sex pheromone. No differences in the behavioural response were found between the two tested intervals after pre-exposure. Male response to female sex pheromone has been shown to increase with increasing age (Gadenne *et al.*, 1993). We found no increase in naïve male responsiveness within the age range of 2–4 days used in the present study. The increase in responsiveness found for the experienced males cannot be explained by a higher motivation to respond to female sex pheromone depending on age. Thus, a longer or permanent change in male responsiveness to sex pheromone occurs.

A lower response of naïve males to 1 fe of female sex pheromone gland extract was found in the windtunnel experiments compared to that found in the walking olfactometer. This difference is partly explained by the fact that the males were exposed to lower amounts of the gland extract in the windtunnel. The extract was applied on a rubber septum that had a lower release rate than the filter paper that was used in the olfactometer experiments. The windtunnel and

olfactometer experiments were performed at different times. Another explanation could be variation in the general response to sex pheromone in the bioassays during different periods, as the olfactometer and windtunnel experiments were not performed in parallel. Such variation has been found in other systems studying responses to sex pheromone (Daly and Figueredo, 2000).

Our experiments showed that exposure to pheromone is crucial to induce a lower response threshold and that physical activation alone does not increase male responsiveness. We can only speculate about the mechanisms behind the observed behavioural changes after experience of the female sex pheromone. Males of *S. littoralis* given experience of the female sex pheromone were not offered a reward, i.e. a mating opportunity with a female, which excludes associative learning. The increase in responsiveness resembles a sensitization. However, the long duration of the increased responsiveness after exposure to female extract and the absence of increase in responsiveness after exposure to high doses of *ZE*-9,11–14:OAc contradicts this explanation. It is doubtful that memory formation through associative or non-associative mechanisms is at all involved in the phenomenon observed here.

The change in responsiveness could be explained by an increased sensitivity in peripheral pheromone receptors cells on the antennae and/or in neurons in the central nervous system (CNS). The experience of sex pheromone may trigger a long-lasting change in concentrations of hormones, neurotransmitters or biogenic amines in the brain. In hamsters, a minimal experience of sexual attractants was sufficient to activate strong Fos-expression in the medial preoptic area after stimulation with olfactory cues that was not found in naïve males (Fewell and Meredith, 2002). These changes suggest an experience-dependent synaptic modulation in this area of the brain. In the moth *Agrotis ipsilon*, increase in male responsiveness to female sex pheromone coincided with increasing juvenile hormone (JH) biosynthesis activity (Gadenne *et al.*, 1993). Juvenile hormone was found to modulate male responsiveness by acting on the sensitivity of olfactory interneurons in the antennal lobe (Anton and Gadenne, 1999). The action of JH was specific to sex pheromone processing neurons, as the sensitivity of interneurons responding to plant odours was not affected by increase in JH levels (Greiner *et al.*, 2002). Serotonin was suggested to be a possible mediator of JH action (Anton and Gadenne, 1999). The level of serotonin in the antennal lobes of the sphinx moth *Manduca sexta* was found to fluctuate and had the highest levels when the male was most active (Kloppenburg *et al.*, 1999). Furthermore, serotonin lowered response threshold and increased the magnitude of responses to stimulation with sex pheromone in projection neurons originating in the macroglomerular complex.

Serotonin and octopamine (OA) modulate male sensitivity to sex pheromone when injected into males of several moths species (Linn and Roelofs, 1984, 1986; Linn *et al.*, 1992). Injection of OA increased male behavioural responsiveness to sex pheromone and influenced the behaviour by modulating the activity of neurons located in the CNS (Linn, 1997). The activity of peripheral receptor neurons on moth antennae can also be increased by the action of OA (Pophof, 2000; Grosmaitre *et al.,* 2001). However, we have found no evidence for a change in the sensitivity of the peripheral sex pheromone receptors in *S. littoralis* after experience of the pheromone (M. Sjöholm, P. Anderson and B.S. Hansson, unpublished).

A brief experience of the female sex pheromone triggers a change, most likely in the CNS, that increases male responsiveness to female pheromone. In our study, the short experience triggered changes that lasted for at least 27 h and most probably longer. From an adaptive point of view, it is strange that the male needs experience of the female sex pheromone to show maximal responsiveness. Why does the naïve male not have this ability from the start? Ecological or physiological constraints can make it adaptive to not have full sensitivity to female sex pheromone until the females are indeed present. Further studies are needed to elucidate the mechanisms behind and the evolutionary significance of the lower behavioural threshold found after pre-exposure to sex pheromone.

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