

Investigation of a Long Range Male Sex Pheromone in the Millet Headminer Moth, *Heliocheilus albipunctella* de Joannis (Lepidoptera: Noctuidae: Heliiothinae).

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Abstract. The millet head miner moth, *Heliocheilus albipunctella*, is one of the most damaging pests of pearl millet in the Sahelian region. Previous unpublished research and circumstantial evidence suggested that both chemical and acoustic cues are involved in mate location in this species. Males display by generating a buzzing sound and exposing their genitalia, and receptive females fly directly to a male or male group to mate. The present investigation found good evidence of acoustical attraction of virgin females to males, but could find no evidence of chemical cues. Thus, analyses of solvent extracts of the genitalia of buzzing males and volatiles collected from buzzing males by gas chromatography-mass spectrometry failed to show any significant, male-produced volatiles. In particular, diethyl malonate, previously believed to be this species' male sex pheromone, was not found. No components in the extracts elicited significant responses from virgin female moths in linked gas chromatography-electroantennography analyses. In laboratory bioassays, virgin female moths were not attracted to extracts of male genitalia or to recordings of male buzzing. In contrast, female moths were strongly attracted to live male moths hidden from the females with or without an airflow to extract any chemicals emitted by the males. On the basis of these findings it would appear that there is little prospect of managing this pest by behavioural manipulation.

Key Words: *Heliocheilus albipunctella*, Noctuidae, millet, *Pennisetum glaucum*, mating behaviour, pheromone, lek.

Introduction.

The heliothine moth, *Heliocheilus albipunctella* De Joannis (syn. *Raghuva albipunctella*), damages pearl millet, *Pennisetum glaucum* L. throughout the Sahelian region of west Africa through the feeding activities of its larva, the millet head miner, inside developing panicles (Vercambre, 1978, Gahukar *et al.*, 1986, Nwanze and Sivakumar, 1990). Following the severe drought of the early 1970s, *H. albipunctella* has regularly inflicted serious crop losses in the Sahel (*ibid.*). In response, a significant research effort has been devoted to developing control measures for use against this pest, although success has been rather limited so far (reviewed in Nwanze and Youm, 1995).

Management by means of behavioural manipulation (Foster and Harris, 1997) would appear to be a promising option in the case of *H. albipunctella*. Circumstantial evidence from morphology and behavioural observation as well previous unpublished research, suggests that chemical communication is probably involved in the moth's mate location mechanism, as outlined below.

This species' mating behaviour differs substantially from the norm for noctuid moths, with the female approaching the male prior to mating (Matthews, 1987a,b, Green *et al.*, submitted). Males display during a 2-3 hour period following the onset of darkness, by fanning their wings whilst perched on low vegetation, sometimes doing so in multi-male groups which are considered to be leks (Green *et al.*, submitted). Percussive vibration of the structurally specialised forewings produces a distinctive buzzing sound, with pulse repetition rate of 135/sec at 27°C, and a predominantly audible sound spectrum (maximum power 1-8kHz) (Green *et al.*, submitted). Whilst the male is buzzing, the posterior abdominal segments are extended telescopically, exposing whorls of scales on the lateral and ventral posterior margins of abdominal segments 8 and 9, and the genitalic claspers (valvae) are everted and flexed periodically, thereby exposing a mass of filamentous scales associated with the genitalia (Plate 1). The exposure of these parts provides a very large surface area from which the dissemination of volatile substances could be promoted (see Birch *et al.*, 1990), particularly so since the abdomen tip is held just beneath the vibrating forewings in a region of air turbulence.

Nocturnal field observations of *H. albipunctella* mating behaviour were made in and around millet fields in Niger over four growing seasons (1996-99). In all observed matings, except those which took place on completely still nights, the female approached the buzzing male/s directly from downwind (Green *et al.*, submitted), consistent with the hypothesis that mate location is mediated by air-borne chemical cues. A further observation consistent with this hypothesis was that males were consistently observed to buzz very vigorously if perched adjacent to mating pairs in the field. This might be explained in terms of opportunistic adaptive signalling, since the scales associated with the male's genitalia remain exposed throughout copulation (Plate 2), and so buzzing males could gain an advantage by displaying close to a supplementary pheromone source. Attempted mating take-overs or rematings by such males were never observed.



Plate 1. Male *H. albipunctella* buzzing approximately 20cm above ground level on *Hibiscus sabdariffa* (Malvaceae), a common field weed. The wings here are at the extreme of the downstroke. Note the costal thickening and behind this the compliant panel or "blister". Abdominal segments 8, 9 and 10, which are covered with hair-like scales, are drawn largely inside segment 7 when the moth is not buzzing.



Plate 2. Male and female *H. albipunctella* in copula. Note the sizeable tuft of scales associated with the male genitalia which remains exposed throughout mating.

An unpublished, preliminary chemical analysis, quoted by Matthews (1987a), suggested that males do indeed release a volatile chemical. Diethyl malonate was identified as the single major volatile component in heptane extracts from the genitalic claspers of buzzing male *H. albipunctella*. Furthermore, diethyl malonate was found to produce an EAG response from female antennae, but not so in males.

Matthews (1987a) also reported that experiments conducted in Mali in 1986 using diethyl malonate failed to lure any adult *H. albipunctella*, but no further research on this aspect was conducted for a decade. Further field trials were eventually conducted in Niger during 1996 and 1997, testing various trap designs baited with diethyl malonate lures, formulated for slow release (0.37 mg/d at 27°C), or using caged live male moths. In both years hardly any *H. albipunctella* were captured by these means, despite a plentiful moth population at the field site in question. This prompted a re-assessment of the identity and role of the putative male pheromone, which is presented below.

Materials and Methods.

Collection of volatiles

Volatile substances from buzzing *H. albipunctella* males were collected by solvent extraction or air entrainment. For extraction, males were allowed to buzz for 15 minutes during the first hour of scotophase, then physically immobilised to allow rapid excision of the terminal abdominal segments with a scalpel blade. These segments were immersed in hexane (25 µl per individual) either as the complete genitalic complex or after further subdivision into genitalic claspers only and segments 7 and 8 without the claspers. After 5-10 min the hexane was removed with a syringe, the residue washed with a further aliquot of hexane and the combined extracts stored at 0°C prior to chemical analysis. The extraction procedure was performed in the laboratory at NRI using males from a laboratory culture, in the laboratory at ICRISAT Sahelian Centre using males obtained from a light trap and then kept under culture conditions during the following day, and in the field in Niger where buzzing males were freshly caught in a sweep net.

For air entrainment, one or two young (<3 days) adult males from the NRI laboratory culture were placed in glass chambers (10 x 3 cm) during the latter part of the photophase. A diaphragm air-pump (Capex Mk II, Charles Austen, UK) was used to draw air (1 l/min) into the chamber through an activated charcoal filter (20 x 2 cm, 6-18 mesh) and out through a collection filter containing Porapak Q (200 mg, 50-80 mesh; PhaseSep), held between plugs of silanised glass wool in a Pasteur pipette. Air was drawn over the insect for 1 hr before fitting the collection filter in order to remove surface contaminants. Buzzing was observed to take place during the early part of scotophase on those occasions when male behaviour was monitored, and volatile collection was continued throughout the scotophase. Trapped volatiles were removed from the Porapak with dichloromethane (Pesticide Grade; 3 x 0.5 ml).

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were carried out using a Thermoquest Finnigan-MAT Ion Trap Detector (ITD 700) operated in electron impact mode (230°C) coupled directly to a Carlo Erba Mega GC. The GC column was a fused silica capillary column (30 m x 0.25 mm i.d.) coated with either polar CPWax52CB (Carbowax equivalent; Chrompack UK) or non-polar CPSil5CB (methyl silicone; Chrompack, UK). Carrier gas was helium (0.5 kg/cm²) and injection was splitless (2 µl; 200°C). The oven temperature was held at 50°C for 2 min, then programmed at 6°C/min to 230°C.

Gas chromatography-electroantennography (GC-EAG)

GC-EAG analyses were done using a fused silica capillary column (30 m x 0.32 mm i.d.) coated with non-polar CPSil5CB (methyl silicone; Chrompack UK) and a flame ionisation detector (FID; 220°C). Carrier gas was helium (0.5 kg/cm²) and injection was splitless (2 µl; 200°C). The oven temperature was held at 50°C for 2 min, then programmed at 6°C/min to 250°C. The effluent from the GC column was split (approx 1:1) between the FID and a small glass vessel in the GC oven, as described by Cork *et al.* (1991). The contents of the glass vessel were expelled at intervals (3 sec every 17 sec) with nitrogen (200 ml/min) over a female *H. albipunctella* electroantennogram preparation. The latter was prepared from the intact moth by insertion of glass microelectrodes filled with saline into the distal and proximal ends of one antenna. The electrodes were connected via silver/silver chloride

electrodes to a AC/DC amplifier in DC mode (UN06, Syntech, Hilversum, The Netherlands), and both GC and EAG data were captured and processed with TurboChrom 4 software (Perkin Elmer-Nelson).

Laboratory bioassays

Laboratory bioassay experiments were carried out at NRI at 25°C using *H. albipunctella* moths from the NRI laboratory culture. Virgin female moths were released from a petri dish placed at the centre of a wire-framed, fabric netting cage (50 x 50 x 150 cm) lit by a dim red light during the scotophase. Movements of the moths were observed during the 15 min after release and the number of excursions into each end third of the cage recorded.

Behaviour of virgin females exposed to male genitalia extract

Fifteen minutes into scotophase, hexane extract (10µl; approx 0.2 male equivalent) of the complete male genitalic complex was placed onto a filter paper strip and positioned in an air stream (1m/sec at 10cm from a fan) directed into one end of the cage. Two females were released and their behaviour recorded over 15 min. In alternate trials an equal volume of pure hexane was used instead as a control.

Behaviour of virgin females exposed to recorded male buzzing.

The buzzing of a single male *H. albipunctella* was recorded on an audio cassette in the field in Niger using a Sony Walkman Professional (Green *et al.*, submitted). Audio speakers (Sony SRS-58) were positioned at each end of the bioassay cage, and the cassette played back from a Sony Walkman Professional through one speaker only. The active speaker was alternated between consecutive trials. Before the first trial, the sound intensity used for playback in all subsequent trials was set by matching it subjectively to that of a live buzzing male, i.e. approximately 50dB at 15cm dorsally (Green *et al.*, submitted). Single virgin females were released individually from a petri dish at the mid-point of the cage during the early part of scotophase (26-28°C) and her movements tracked for 15 minutes following her first excursion outside the petri dish. The time of each move between adjacent thirds of the cage was noted using a dictaphone, and hence the total amount of time each female spent inside each third of the cage was calculated.

Behaviour of virgin females exposed to live males buzzing in an extractive air stream.

For these experiments, a polythene funnel (mouth diameter 75mm) was positioned at floor level at each end of the bioassay cage, the mouth of the funnel facing inwards. Two live males (1-4 days post emergence) were placed inside one funnel 20 minutes before the end of photophase, and the funnel mouths were covered using black fabric netting secured by a rubber band. A gentle air stream (0.8l/min) was drawn through each funnel by means of tubes linking the funnels' narrow spouts to a pump, and exhausted into a separate room in order to remove any volatiles that the males might produce. This did not appear to inhibit the males from buzzing as at least one of the males buzzed continuously for >12 minutes during the onset of scotophase in 80% of trials that were staged. Whilst buzzing, males gripped the fabric netting and pointed their abdomens downwind into the funnel as they do in the field. Subsequent tests using an air current smoke tube (Draper CH216/25301) demonstrated the air flow was sufficient to draw in completely a smoke plume generated at 5cm beyond the funnel mouth. The control treatment for this bioassay was to follow the same procedure with two

males in the same funnel cage set up, but without the extractive airflow. Once continuous buzzing by at least one male was established the female was released and her movements within the cage were monitored as previously.

Other experiments

Beside the investigations outlined above, attempts were also made to observe the effect of interference in the moths' communication by surgically removing the antennae of virgin females and by making incisions in the sound generating region of the male forewing. However, these attempts were unsuccessful as the females suffered high post-operational mortality, and the males continued to generate a much reduced buzzing sound even after the complete removal of one costal thickening.

Results.

GC-MS analysis of male volatiles

GC-MS analyses were carried out on:

- nine hexane extracts of genitalia of one or two buzzing male *H. albipunctella* moths prepared in the laboratory at NRI;
- six samples of volatiles collected from two male *H. albipunctella* moths by air entrainment at NRI;
- five hexane extracts of genitalia of single buzzing male *H. albipunctella* moths prepared in the laboratory at ICRISAT;
- six hexane extracts of genitalia of single non-buzzing male *H. albipunctella* moths prepared in the laboratory at ICRISAT;
- two hexane extracts of genitalia of male *H. albipunctella* moths in copula prepared in the laboratory at ICRISAT;
- two hexane extracts of genitalia of single buzzing male *H. albipunctella* moths prepared in the field at ICRISAT.

Diethyl malonate was not detected in any of these samples. The synthetic material elutes at KI 1578 on the polar CPWax52CB GC column and KI 1043 on the non-polar CPSil5CB GC column and had characteristic ions at m/z 115, 133 and 161 in the mass spectrum. It was estimated that the level of detection was << 50 pg/male.

No other consistent, significant components of "interest" were detected, and there were no obvious differences between the various samples collected. Representative GC-MS traces are shown in Figs 1-3. Components observed were ≤ 10 ng/male and the most significant were identified from their mass spectra as traces of hydrocarbons or antioxidants from the solvents or phthalates or silicon compounds as impurities from materials used during collection and storage of the insects and/or samples.

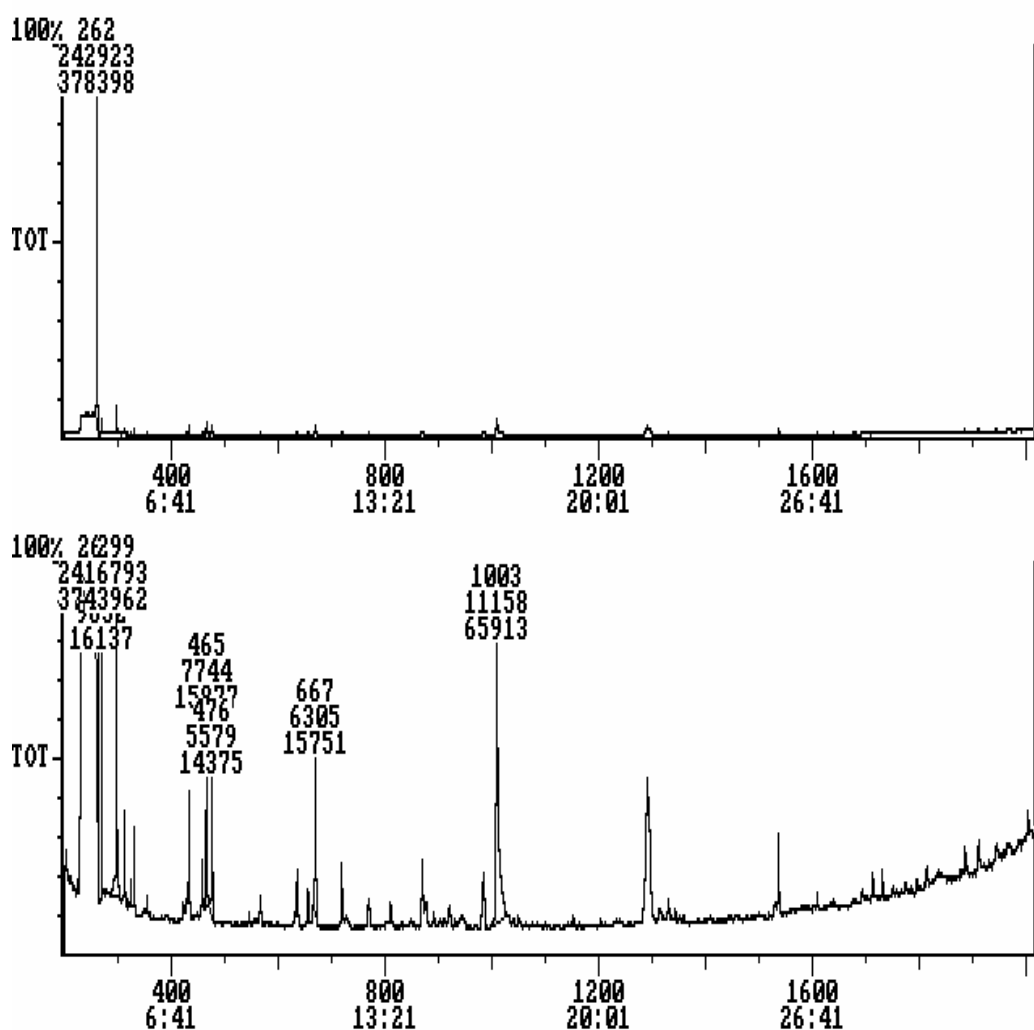


FIG. 1. GC-MS analysis (polar GC column) of hexane extract of genitalia of male *H. albipunctella* prepared at NRI (lower trace is amplified presentation of upper; component at m/z 262 is toluene in hexane).

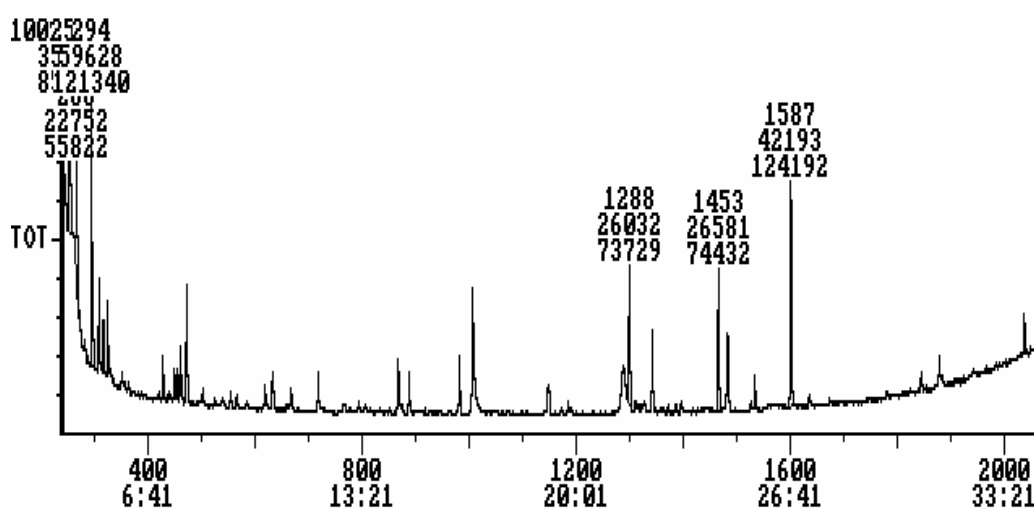


FIG. 2. GC-MS analysis (polar GC column) of volatiles collected from two male *H. albipunctella* moths at NRI.

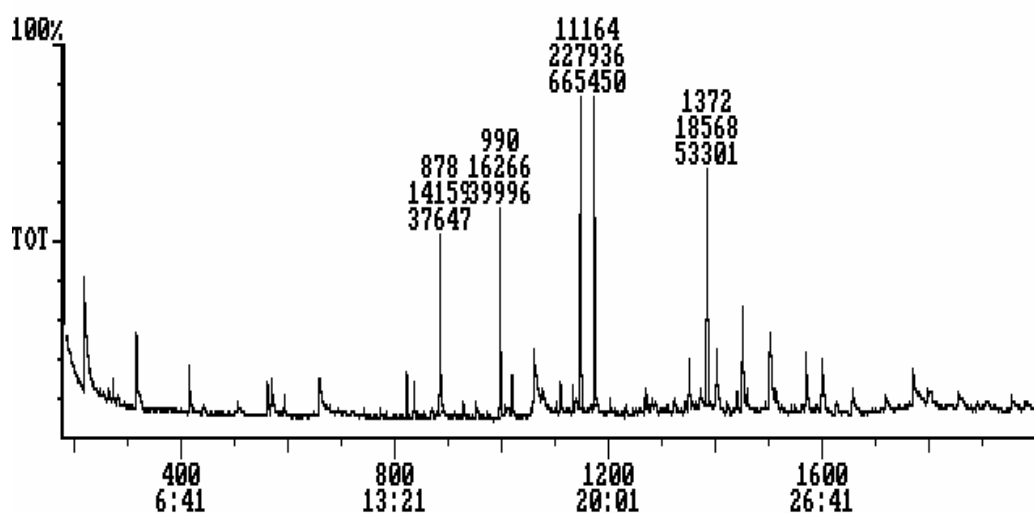


FIG. 3. GC-MS analysis (non-polar GC column) of hexane extract of genitalia of male *H. albipunctella* prepared at ICRISAT.

GC-EAG analysis of male volatiles

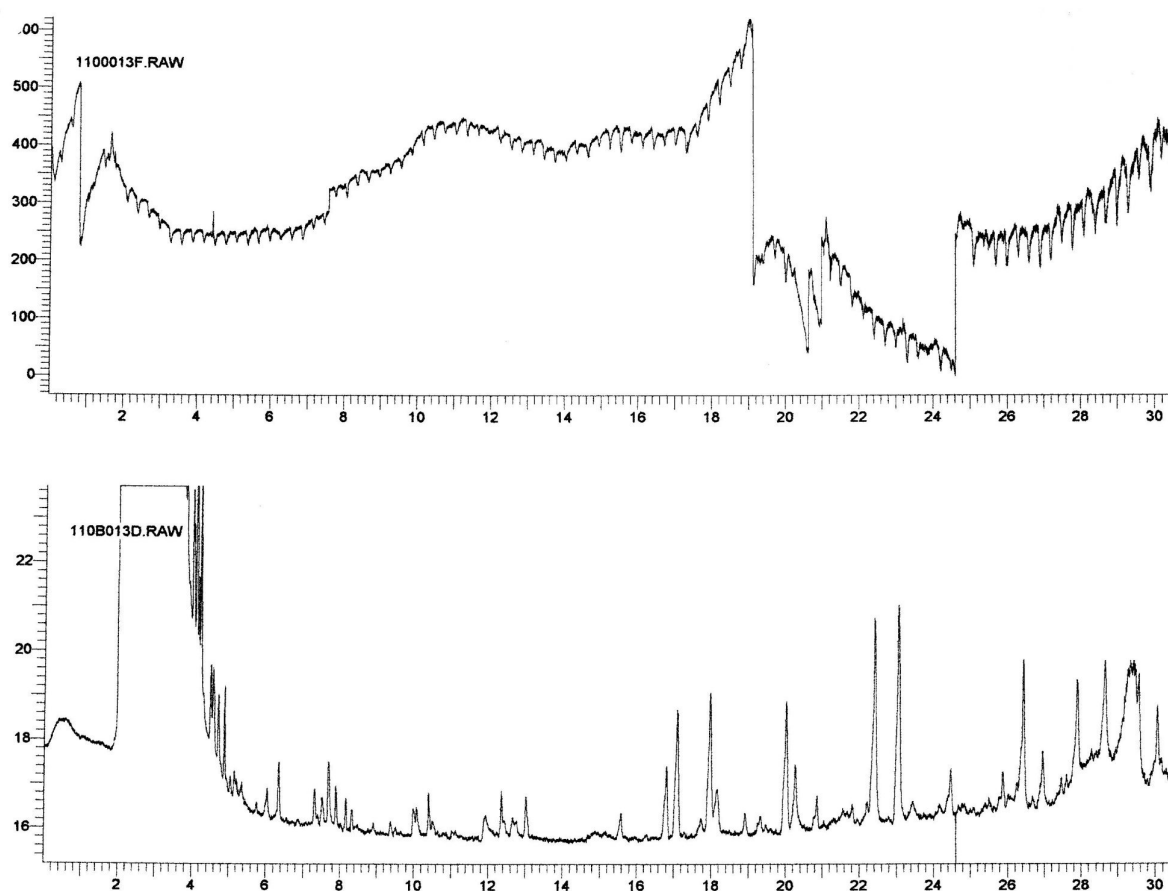


FIG. 4. GC-EAG analysis (non-polar GC column) of hexane extract of genitalia of male *H. albipunctella* prepared at ICRISAT against female *H. albipunctella* EAG preparation (upper GC, lower EAG).

Six samples of hexane extracts of genitalia of male *H. albipunctella* moths prepared at ICRISAT were analysed by GC-EAG against two different female *H. albipunctella* moths from the NRI culture. No significant (>10% increase in baseline response) and consistent EAG responses were recorded. A representative analysis is shown in Fig. 4.

Behavioural bioassays

Free flight was somewhat restricted within the bioassay cage, but most females remained active for much of the bioassay period, and movement around the cage was accomplished for the most part by a combination of walking, climbing and simultaneous wing fluttering. The female's first venture away from the release petri dish was typically a direct movement to one or other of the nearest side walls of the cage, followed by ascent to the cage wall/ceiling interface. Short free flights below the cage ceiling were not uncommon, but most of the subsequent movements around the cage were directed along the wall/ceiling interface, with periodic drops to the cage floor followed by re-ascent. In some cases however, females settled on the cage wall and remained stationary for considerable periods.

Virgin females exposed to male genitalia extract.

These trials were curtailed by a decline in the productivity of the laboratory culture of *H. albipunctella*. Whilst it would probably be unwise to draw any firm conclusions on the evidence of the current small sample size (6 bioassays with extract and 6 controls), there was no indication of female attraction upwind toward the extract of male genitalia (Table 1).

Table 1. Response of virgin female *H. albipunctella* moths to hexane extract of male genitalia (6 replicates treatment and control; mean \pm SE number of times female entered third of cage during 15 min; upwind is towards source)

Source	Upwind	Downwind
Male extract	2.67 \pm 0.95	3.33 \pm 1.12
Hexane control	2.83 \pm 0.83	1.83 \pm 0.79

Virgin females exposed to recorded male buzzing

Generally, females spent approximately half of the trial duration in the central third of the cage. On average, more time was spent in the third of the cage closest to the active speaker than in the third furthest away from it (Figure 5), but this difference was not statistically significant (paired sample t-test on log transformed data: $t=1.58$; $P=0.13$; $n=17$). Females did not exhibit any obvious directed movements toward the active speaker, in contrast to what is observed when a female approaches a buzzing male prior to mating (Green *et al.*, *submitted*). Matthews (1987a) previously also reported a failure to attract female *H. albipunctella* to playback of recorded male buzzing.

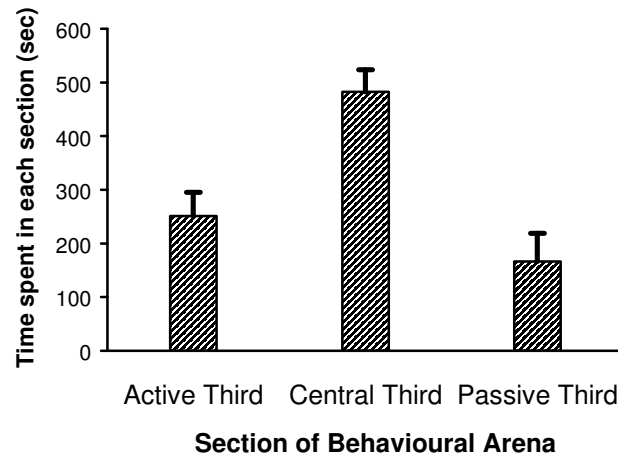


FIG. 5. Movement by virgin female *H. albipunctella* moths exposed to recorded male buzzing. (Playback of buzzing was from the "active third"; n=17; mean \pm SE).

Virgin females exposed to live buzzing males in an extractive air stream

In striking contrast to the two previous bioassay procedures, females showed strong attraction to buzzing males caged within an extractive air flow (Figure 6). This attraction, measured as the proportion of the trial duration that the female spent in the third of the cage nearest the males, was not significantly different ($P > 0.05$) from attraction to the males without the extractive air flow. The small observed reduction in attraction that was observed may have been a consequence of the slight hissing sound generated by the extractive air flow system, an extraneous acoustic factor which was absent in the control bioassays.

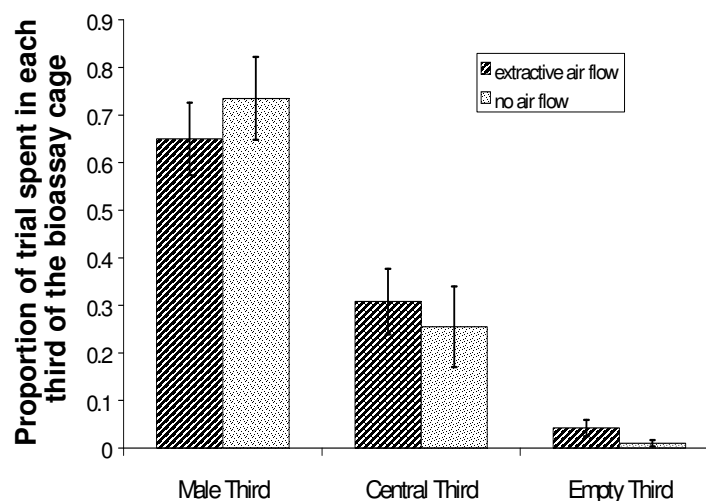


FIG. 6. Movement by virgin female *H. albipunctella* moths exposed to the buzzing of live males, held in an extractive airflow or without extractive. (Mean \pm SE; observation time 12 min; n=14 with extraction, n=8 without).

A further observation was made during these bioassays. In two separate control trials a virgin female was flying/climbing in the third of the cage containing the males, apparently attracted toward them. In each case, only one of the two males was buzzing, and then he stopped. Instantly the female landed on the cage wall, settled and then remained inactive for the remaining observation period (>20 minutes). During this period attempts were made to rouse the females using recorded buzzing, but these were unsuccessful. These replicates were not included in the final bioassay data set since in neither case did the continuous buzzing duration reach the qualifying time of 12 minutes.

Discussion.

The present study sought to elucidate the mechanism of mate location in the millet headminer moth, *H. albipunctella*. Previous studies and circumstantial evidence from morphology and field observation suggested that both chemical and acoustical stimuli would be involved in the attraction of females to males in this species, as suggested by Matthews (1987a). The present investigation found good evidence of acoustical attraction of females to live buzzing males, but no chemical or biological evidence for the existence of a long-range male pheromone, in contrast to the previous report (Matthews, 1987a).

Analytical studies

No significant quantities of any volatile substance were found in hexane extracts obtained either by extraction from buzzing males' genitalia, or by collection of volatiles from buzzing males. Specifically, no trace could be found of diethyl malonate (< 50 pg/male), the putative male pheromone which the previous unpublished study had found at approximately 1 µg/male) by the same extraction method used here, only using heptane instead of hexane (Matthews, 1987a). Furthermore, the present extracts did not elicit any consistent EAG response from virgin female *H. albipunctella* moths.

Behavioural bioassays

Initial behavioural bioassays were restricted by shortage of insects, but the results showed no indication of attraction of female *H. albipunctella* to hexane extracts of male genitalia. There was certainly nothing approaching the marked attraction of females to live males observed later.

In contrast, virgin females were strongly attracted to buzzing males held in an extractive air stream, but were not attracted to playback of recorded buzzing. These results indicate that the acoustic stimulus of buzzing is important in mate recognition and location, but that sound quality is a critical factor, and that the replayed recording was somehow deficient in this respect. Since *H. albipunctella* is a lek-forming species with a distinctive male display, pre-mating behaviour is probably subject to strong intersexual selection, and one might expect females to apply stringent discriminative criteria in mate selection and to reject poor quality signals (Höglund and Alatalo, 1995, Shelly *et al.*, 1997). However, since in the absence of any alternative mating signal females tested in the playback bioassay still were not attracted to recorded buzzing, it seems more likely that they failed to approach the active speaker because they did not recognise the recording as a signalling male conspecific. The tuning curve of the female moth's auditory system in relation to the sound spectrum of the buzz signal may be critical in this respect, but this was beyond the scope of the present study.

A less plausible explanation would be that the extractive air flow did not remove all traces of male volatiles in that bioassay, and that buzzing on its own (as in the playback bioassays) is not an effective mate attractant. However, smoke plume tests suggested that air flow through the funnel cage should have been adequate to draw away any odours, especially since the buzzing males in the funnel cage pointed their extended abdomens downwind into the funnel. The cessation of buzzing appeared to cause mate-seeking females to settle during certain control trials when there was no extractive air flow, and this suggests that male buzzing is an essential component in the mate-location mechanism. The failure of recorded buzzing to stimulate further activity in these females lends further support to the hypothesis that the playback sound quality was deficient in some respect.

Conclusions

Reassessing the circumstantial evidence for a male pheromone in the light of the above findings, it now seems likely that the female's pre-mating approach from downwind should be explained in terms of attraction to the acoustical signal alone. This is possible because the buzzing sound is perceptibly louder downwind, both as a consequence of the males' typical buzzing location and the buzzing posture. Firstly, buzzing males perch on the sheltered downwind side of the lower parts of millet plants and other low vegetation, and hence transmission of the acoustic signal upwind tends to be obstructed. Secondly, the buzzing male's body orientation is typically such that abdomen tip slopes diagonally downwards and points downwind, whilst sound radiation is greatest dorsally - effectively diagonally upwards in a downwind direction (Green *et al.*, submitted). Hence female attraction from downwind need not be a consequence of mate location by chemical means.

Despite the present findings, however, the abdominal morphology of male *H. albipunctella* still suggests that volatile substances should be involved in sexual communication in this species, unless the structures in question have become redundant quite recently in evolutionary time. It seems most likely now that chemical communication could occur only at close range, after the female has located the male by acoustic means. At the first arrival of the female, male buzzing intensifies and the genital claspers are everted and flexed repeatedly (Green *et al.*, submitted) and it may be that only at this stage are volatiles released. However, analyses of hexane extracts of genitalia of male *H. albipunctella* in copula did not show any obvious differences from those prepared at other times.

It remains unclear why the previous study should have found relatively large quantities of diethyl malonate in heptane extracts from male *H. albipunctella* genitalia made essentially in the same manner as in the present study. One possibility is that diethyl malonate is in fact the close-range pheromone hypothesised above, but it is very surprising that no trace of this substance was detected. Contamination of the previous samples is probably the most likely explanation, but in the previous work diethyl malonate was found in at least two separate extracts and the synthetic compound at levels > 100 ng at source off glass elicited an EAG response from female *H. albipunctella* moths. Whatever the explanation, it is unfortunate that the short, popular article by Matthews (1987a) resulted in the finding being detailed in a subsequent, high impact review article (Birch *et al.* 1990).

On the basis of the present findings it would appear that the prospects for managing this pest by means of behavioural manipulation are not good. The principal requirement would be the existence of a long-range sex pheromone, which could be mimicked to facilitate population monitoring, mating disruption or the trapping of mate-seeking females. The present study

has failed to demonstrate the existence of any volatiles from buzzing male *H. albipunctella* moths. In contrast, the acoustical stimuli from male buzzing seem to play the major role in mate attraction, and it appears that the quality of the acoustic signal is also critical. This would suggest that even if a male sex pheromone did exist then it would probably fail to disrupt mating. Support for this assertion comes from our experiences culturing *H. albipunctella*, since whilst problems have been experienced in maintaining cultures owing to larval mortality, obtaining matings in confined cages containing numerous individual moths has never proved a problem with this species.

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