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Full Length Research Paper

A sex attractant of the rough bollworm, *Earias huegeliana* (Gaede) (Lepidoptera: Noctuidae)

S. T. Lowor[1]*, A. P Del Socorro[2] and P. C Gregg[2]

[1]Cocoa Research Institute, Ghana, P. O. Box 8, Akim Tafo, Ghana
[2]Cotton Catchment Communities Cooperative Research Centre, Narrabri, NSW 2390 and School of Rural Science and Agriculture, University of New England, Armidale, NSW 2351, Australia.

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The sex attractant of the rough bollworm was identified using gas chromatography and mass spectrometry (GC-MS) from female gland and air collections. Identified compounds were formulated into blends and tested in the field for attractiveness to males. The Gas chromatograph-mass spectrometry (GC-MS) analysis revealed 4 compounds, (E,E)-10,12-hexadecadienal, (E,E)-10,12-hexadecadienol, (Z)-11-hexadecenal and (Z)-11-octadecenal in a ratio of 4:1:1:1 in the gland extracts. (E,E)-10, 12-hexadecadienol was not detected in the air collections. Field bioassay showed the 2 components, (E,E)-10,12-hexadecadienal and (Z)-11-hexadecenal to be essential for activity of the blend. This blend was highly attractive to males only. 2 trap designs, the funnel and delta traps were tested and the delta trap was the better of the 2. Male response to attractant baited traps was found to be in the second half of the night, between 0200 and 0500 h. This was found to be synchronised to female calling time. Use of the attractant blend developed as part of the integrated pest management system in cotton is discussed.

Key words: Rough bollworm, (E,E)-10,12-hexadecadienal, (E,E)-10,12-hexadecadienol, (Z)-11-hexadecenal, (Z)-11-octadecenal, pheromone traps, gas chromatograph-mass spectrometry, sex attractant.

INTRODUCTION

The rough bollworm (RBW), *Earias huegeliana* (Gaede) is endemic to Australia (Common, 1990) and considered an occasional pest of cotton in Australia (Pyke and Brown, 1996). Conventional insecticides sprayed to control the major pests of cotton such as Helicoverpa spp. usually controls adult rough bollworm populations in cotton. Insecticide control of *E. huegeliana* is difficult once the larvae burrow into the plant. Recent trends in the cotton industry indicate a reduction in insecticide use and the adoption of integrated pest management (IPM) approaches that rely on less chemical use and are more environmentally friendly. Also, it is likely that more Australian cotton will be grown in northern (tropical) areas in in future (Yeates, 2001). In these areas, irrigation water is more abundant, but there is also a greater diversity of Malvaceae (commonly known as bladder ketmia to which *Earias* spp. are specific) and knowledge of the distribution of other *Earias* species (Reed, 1994) suggests that the climate may be more favourable for *E. huegeliana* in such regions. Bladder ketmia, a key host of rough bollworm is less well controlled by glyphosate so may become more of a weed problem in roundup ready systems, hence more hosts for RBW. While transgenic (Bollgard II®) cotton may control RBW, the potential for resistance to this method in RBW is high because it is a Malvaceae specialist and at some times of the year it will be concentrated in cotton. Because of these factors, it is considered that rough bollworm might become economically more important in the future. Hence, there is a need to put in place improved control measures for this species. One of

*Corresponding author. E-mail: slowor2@yahoo.co.uk.

the options is the development of a good detection tool based on pheromones to monitor pest population and thus determine whether other control measures are necessary. Research on the sex attractants of the rough bollworm will further contribute to a better understanding of the ecology of this pest. A properly identified and formulated sex attractant blend for *E. huegeliana* would be a good monitoring tool if trap catches reflect field populations. It would also be another tool in research to shed some light on the distribution, phenology and host relationships and has a potential for use in mating disruption. This paper describes our attempts to study the pheromone system of *E. huegeliana,* including field bioassays to test the attractiveness of potential attractant components to males.

MATERIALS AND METHODS

Synthetic chemicals

(E,E)-10,12-hexadecadienal (E,E)-10,12:16Al, (E,E)-10,12-hexadecadienol (E,E)-10,12:16OH, (Z)-11-hexadecenal (Z)-11:16Al, (Z)-11-octadecenal (Z)-11:18Al and (E,Z)-10,12-hexadecadienal were obtained from plant research international B.V, Pherobank, Wageningen.

Insect rearing

Larvae of *E. huegeliana* were collected from Cecil Plains, Queensland on a malvaceous weed species, *Hibiscus trionum* (bladder ketmia). Larvae were carefully extracted from the bolls of the bladder ketmia using a scalpel and forceps. They were then transferred onto an artificial diet (Forrester et al. 1993) in 35ml plastic cups (Solo, P101M, Urbana, Illinois, USA). The rearing conditions in the insectary were 25 ± 1°C and 13:11 light-dark period with the scotophase during 1830 - 0530 h Australian eastern standard time (AEST). Emerging adults were sexed by gently squeezing the abodomen. Males had claspers and females protruding ovipositor when squeezed. They were held individually in 150 ml plastic containers and fed with 5% sucrose until ready for use in the experiments.

Pheromone collection and analyses

Air trapping

Trapping of volatiles was done using 3 - 6 day old unmated females and males. Volatiles were collected from 3 - 4 insects held in an all glass apparatus. Air was drawn into a flask (50 ml) through a filter of activated charcoal (10 cm x 2 cm; 10 **-** 18 mesh) by means of a pump (Capex L2C, Charles Austen Pump Ltd, Surrey, England) at a rate of 60 ml/min and the volatiles trapped on a 200 mg filter of super Q (80/100 mesh, Alltech associates Inc, U.S.A) held in place by salinised glasswool in a pasteur pipette. Air collection was done for 8 h. Trapped volatiles were eluted from the filter with 2 - 3 ml of hexane and concentrated to 500 µl under a gentle stream of nitrogen before analysis. For quantification purposes, (E,Z)-10,12-hexadecadienal was used as an internal standard.

Gland extracts

Gland extracts were done when females were calling, which was observed to be between 1530 and 1730 h AEST (that is, approximately 2 h before sunrise in the reverse-cycle experimental photoperiod). The extracts were prepared in batches, with each batch containing 3 or 4 excised abdominal tips of calling females soaked in 20 - 30 µl of hexane in an amber vial. Extracts were allowed to stand at room temperature for 10 min before transferring the supernatant into 1.5 ml amber sample vials with 100 µl limited volume inserts. Sample vials were stored in the freezer until analysis (about 24 h). Gas chromatographic-mass spectrometric (GC-MS) analyses were done using a Hewlett Packard 6890 series gas chromatograph and HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, U.S.A). The columns used on this GC were an AT 35 capillary column (30 m x 0.25 mm i.d x 0.25 µm) and a HP-5MS (5% phenyl methyl siloxane, 30 m x 0.25 mm i.d., 0.25 µm film thicknesses) fused capillary column. The carrier gas was ultrapure helium set at a flow rate of 1.0 m/s. The column temperature was programmed from 40°C (0.50 min hold) to 250°C at 20°C min^{-1}. Temperatures of the splitless injector and the GC-MS interface were set at 280 and 300°C, respectively. Total run time was 30 min. A mass spectrum was scanned from m/z 30 to 300 and acquired data collected and analysed on a Hewlett-Packard workstation using HP Chem station software. Mass spectra obtained were matched with spectra stored in the library of the HP Chem station software. Matches were then examined for molecular ions ($M^{\cdot+}$), M^{+} minus recognisable fragments and other fragment ions consistent with the structure proposed. These were then confirmed with spectra obtained from standard spectra run with retention times as well as co-injection with the identified compounds.

Wind tunnel bioassay

The wind tunnel used, temperature and light conditions of the experiment were as described by Del Socorro and Gregg (2001). Briefly, the tunnel was made of Plexiglas® with dimensions 260 x 60 x 60 cm and similar to that described by Cardé and Hagaman (1979). Airflow of 30 - 40 cm/s was maintained by means of a fan which pulled air through the tunnel using a 30 cm diameter exhaust tube leading to the outside of the building. The temperature of the air stream in the middle of the tunnel was maintained at 24 - 26°C throughout the experiments. A continuous red light source of intensity 1 **-** 1.5 Lux in the wind tunnel was provided by continuous red photographic safe lights (Encapsulite, Type R10, EncapSulite® International Inc., Rosenberg, Texas USA) suspended above the wind tunnel. To diffuse the light a plastic packaging material was placed between the tubes and the top of the wind tunnel. Observations were made in the second half of the scotophase when females are known to call. This experiment was designed to test the behaviour of males in the wind tunnel in the absence and presence of pheromones. Males were tested using an empty cage (blank), a 4:1 blend of (E,E)-10,12:16Al and (Z)-11:16Al (Blend B) and a 4:1:1 blend of (E,E)-10,12:16Al, (Z)-11:16Al and (Z)-11:18Al (Blend K). For each blend, a 13 mm diameter glass fibre filter paper disc (type A/E, Pall Corporation, Michigan, USA) was loaded with 4 µg of the mixture. A total of thirty 3 day old unmated males were used for each treatment. Individual males held in 150 ml meshed plastic cages were transferred to the wind tunnel room at least 7 h before the experiment to acclimatise. Either the empty cage or the pheromone blend was placed at the upwind end and each male was released from the cage at the downwind end of the tunnel. Males were allowed to respond for 5 min after one end of the cage was

Table 1. Coding for blends of *E. huegeliana* pheromone components used in trials.

	Blend / ratio					
Chemicals	**A**	**B**	**C**	**E**	**F**	**K**
(*E,E*)-10,12-hexadecadienal	4	4	4	4	-	4
(*E,E*)-10,12-hexadecadienol	1	-	-	1	-	1
(*Z*)-11-hexadecenal	1	1	-	-	1	1
(*Z*)-11-octadecenal	1	-	1	-	1	-

All blends contained butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10% of the total pheromone components. Blend K was based on ratios obtained from air samples, other blends were arbitrarily selected to test the effects of departures from this ratio.

opened and were scored for the following behaviours**,** (1) take off (2) upwind flight (3) downwind flight (4) approach to the source (5) contact with source (6) attempt at copulating/clasper extrusion. All observations were recorded using the observer (version 3.0) programme (Noldus information technology b.v, Costerweg 5, 6702 Wageningen, The Netherlands).

Field studies

A series of field experiments using delta traps (Phero Tech Inc, Delta, British Columbia, Canada V4G 1E9) were conducted to test the attractiveness of single components as well as various ratios and blends of potential pheromone components identified in *E. huegeliana* females. The different blends used in the field trials were coded as shown in Table 1. Trapping experiments were designed as latin squares with treatment (pheromone), trap position and day as the factors. The layout of traps varied between experiments, depending on the shape and size of the field. Where possible square layouts with equal inter-trap spacing were used, but sometimes the conditions of the field made this difficult. In all the experiments, traps were cleared and moved on one position daily. The major field trapping experiments were conducted at 2 sites, near Cecil Plains in the Darling Downs and near Mondure, Queensland (Qld).

Experiment 1 - comparison of blends A, B and C

The attractiveness of the full blend consisting of 4 components (A) was compared with that of 2 partial blends (B and C) and a blank control (CT). Field tests were carried out in February 2003 on flowering cotton at "Glen Shee" in Oakey, Qld. The experimental design was a 4 x 4 latin square with 3 different blends plus a control (CT), 4 rotation periods and 4 trap locations. Delta traps were located 100 m from each other and cleared each day before moved 1 position.

Experiment 2 - comparison of blends A, E and F

This experiment was a 3 x 3 latin square with 3 blends, 3 rotations and 3 trap locations aimed at further testing 2 other partial blends (E and F) compared with the full blend (A) using delta traps. The experiment was carried out in flowering cotton at "Glen Shee", Oakey, Qld in February 2003. During this experiment, observations of male response towards the sex attractant lures in the traps were done every night (for 3 nights) using night vision goggles (Litton Precision Products International, Rosebery, Sydney, Australia) and a torch fitted with red filter. Observations were done for 5 min every 2 h from 2000 to 0600 h. A male response to the pheromone was scored as "approach". An insect was said to have approached the trap when it flew in the characteristic zigzag manner and was about 5 cm from the mouth of the trap. Observations were recorded on a cassette tape and later transcribed. Numbers of male insects caught in the trap were counted at the end of each 1 hourly observation.

Experiment 3 – comparison of 2 types of traps using blend B

This experiment compared the suitability of the funnel traps for trapping *E. huegeliana* with the delta traps. The experimental design comprised 2 rows of traps, with the funnel and delta alternating within row. There were 3 rotation periods, 5 trap locations and 2 trap types. The experiment was conducted on a harvested wheat field containing many bladder ketmia (*Hibiscus trionum* L.) weeds at Mondure, Qld in January 2004. Traps were located 40 m from each other within rows and 100 m between rows and were cleared daily before rotation.

Statistical analyses

Statistical analyses of data were done using the R statistical package version 1.9.0 (R Development Core Team, 2004). Data were summarised using means and standard errors. Relationships between variables were determined using analysis of variance on log (x + 1) of the data followed by contrast to determine the least significant differences between means. Data on wind tunnel work was analysed using the generalised linear model (GLM) for logistic regression of binomial response variables.

RESULTS

Pheromone analysis and identification

Gas chromatographic traces showed the presence of 3 identifiable attractant compounds from the concentrated extracts of air collection from calling females and 4 compounds from the gland extracts (Figure 1). Compounds (*Z*)-11-hexadecenal (I), (E,E)-10,12-hexadecadienal (II) and (*Z*)-11-octadecenal (IV) were common to both the gland and air collections while compound (E,E)-10,12-hexadecadienol (III) was only found in the gland extract. Compounds were identified by comparison of the mass spectral data with standard spectra's and by co-injection with authentic standards.

Behavioural responses of conspecific males to synthetic attractant blends in a wind tunnel.

Figure 2 shows the various observations made on the

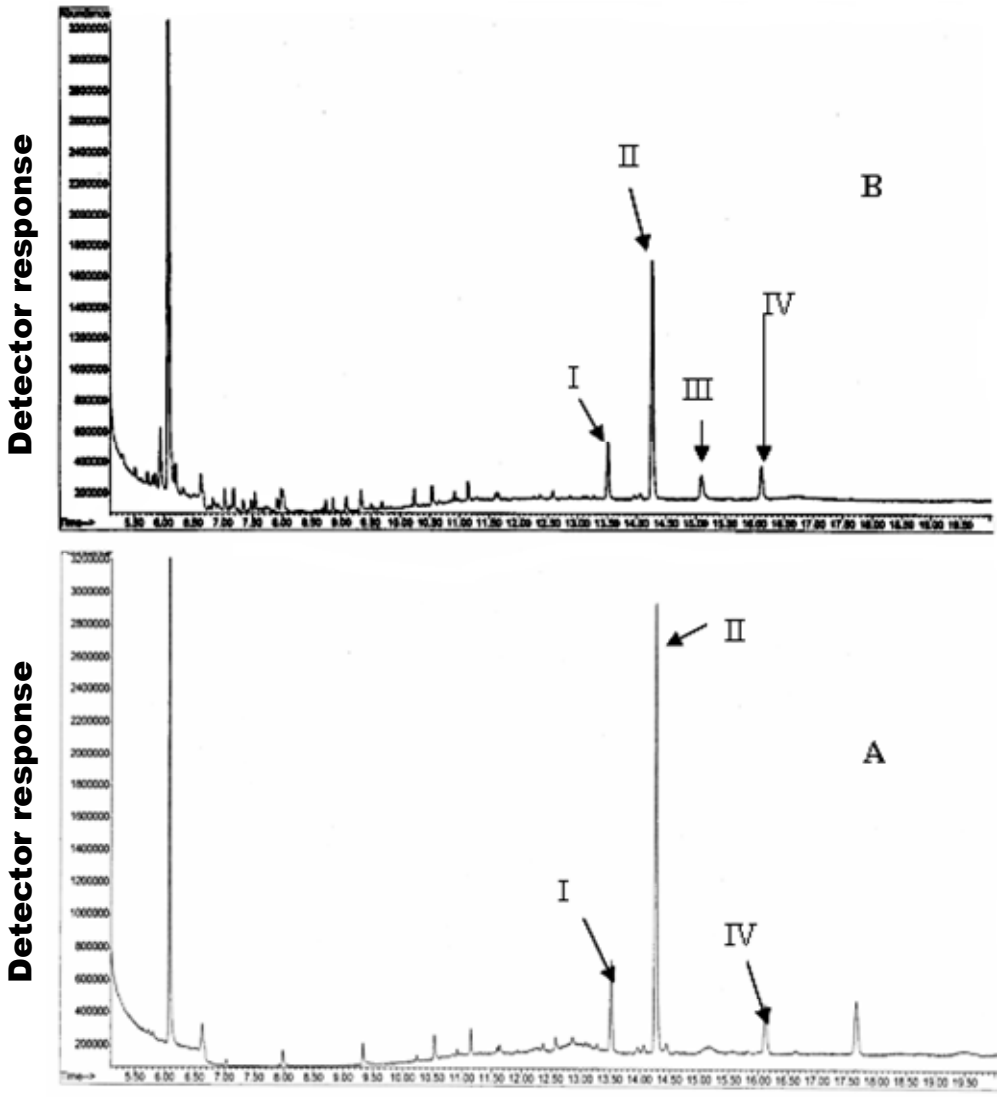

Figure 1. Gas chromatograms of samples from E. huegeliana female air collection (A) and female gland extract (B). I – (Z)-11-hexadecenal, II - (E,E)-10,12-hexadecadienal, III - (E,E)-10,12-hexadecadienol and IV - (Z)-11-octadecenal

male behaviour in the absence of a pheromone (blank or empty cage) and in the presence of 2 blends, B and K. In cases where males were presented with empty cages (blank, that is, no attractant source), 90% of them took off but only 50% flew upwind. Males usually took off and either spent some time upwind before coming to rest or moved downwind and there were no approaches or contact in the absence of pheromone source. On the other hand, male response behaviours such as approach, contact and copulatory attempt, were observed in males tested with blends B and K. The analysis of deviance from the GLM indicated that takeoff was not significantly different for the 3 treatments ($P = 0.108$). Many more male moths approached, made contact and attempted to copulate with the 2 attractant blends than the empty cages (blank). The analysis of deviance indicated highly significant differences in treatments for upwind flight, approach, contact and copulatory attempt ($P < 0.001$).

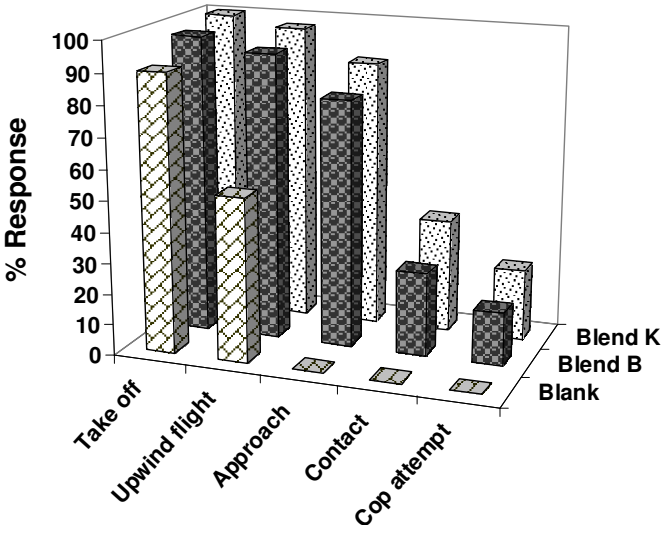

Figure 2. % of E. huegeliana males exhibiting different behaviours in the absence of a pheromone source (blank or empty cage) and in the presence of 2 blends, B (4:1 ratio of (E,E)-10,12:16Al and (Z)-11:16Al) and K (4:1:1 ratio of (E,E)-10,12:16Al, (Z)-11:16Al and (Z)-11:18Al). Males used were 4 days old.

Table 2. P-values showing the level of significance in the different treatments for *E. huegeliana* male response behaviours in a wind tunnel.

Behaviour	Comparison	P- Value
Approach	Blend B vs K	0.603
	Blend B vs blank	< 0.001
	Blend K vs blank	< 0.001
Contact	Blend B vs K	0.341
	Blend B vs blank	0.01
	Blend K vs blank	< 0.001
Copulatory attempt	Blend B vs K	0.446
	Blend B vs blank	0.053
	Blend K vs blank	0.006

Comparison of the mean response of upwind flight using contrast in R indicated no significant difference between blends B and K (P = 0.741) but highly significant differences between the 2 blends B and K and the blank (P < 0.001). Similar trends were observed for approach, contact and copulatory attempt. These results are summarised in Table 2. The results show no significant differences between blends B and K at any stage in upwind flight to source. Although copulatory attempts with blend B were not significantly different from those observed with the blank, there were significantly more attempts with blend K than with the blank.

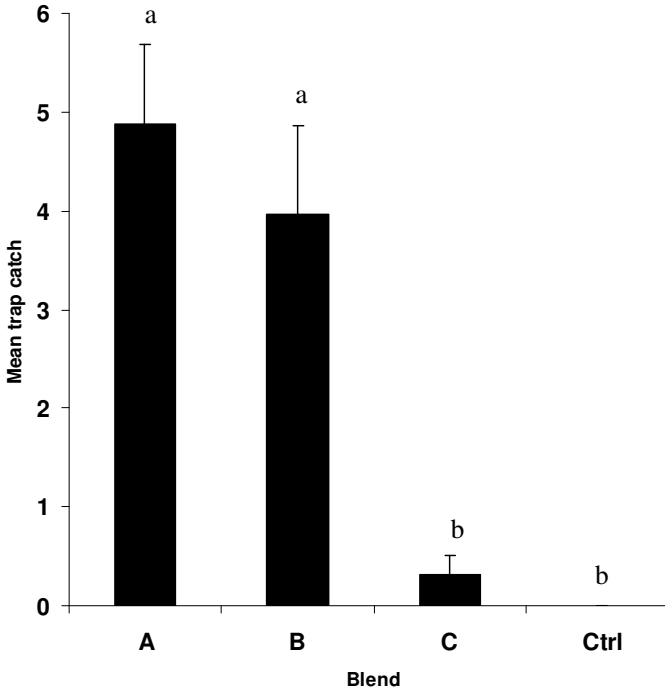

Figure 3. Experiment 1. Mean (± s .e) catches of E. huegeliana males in traps baited with blends A, B, C and CTRL in cotton "Glen Shee", O akey, Qld. Columns with common letter are not signifycantly different (P > 0.05).

Field bioassays

Experiment 1 – comparison of blends A, B and C

2 partial blends B and C were tested against the full blend A. The results indicated significant effects of blend (P < 0.001) and time factors (P < 0.001). Mean trap catches per night are shown in Figure 3. Comparison of the means showed no significant difference between blends A and B (P = 0.105) but highly significant differences (P < 0.001) between blends A and C, between blend A and control (CTRL), between blend B and control and between blends B and C. There were no significant differences between blend C and control. These results suggest that for any meaningful attraction of males to the pheromone to occur, both the major component (E,E)-10,12-hexadecadienal and a minor component (Z)-11-hexadecenal, should be present in the blend.

Experiment 2 - comparison of blends A, E and F

This experiment compared 2 other partial blends (E and F) with the full attractant blend (A). Only blend A caught moths during the 3 day period with mean trap catches of 21 moths per night whilst blends E and F did not catch

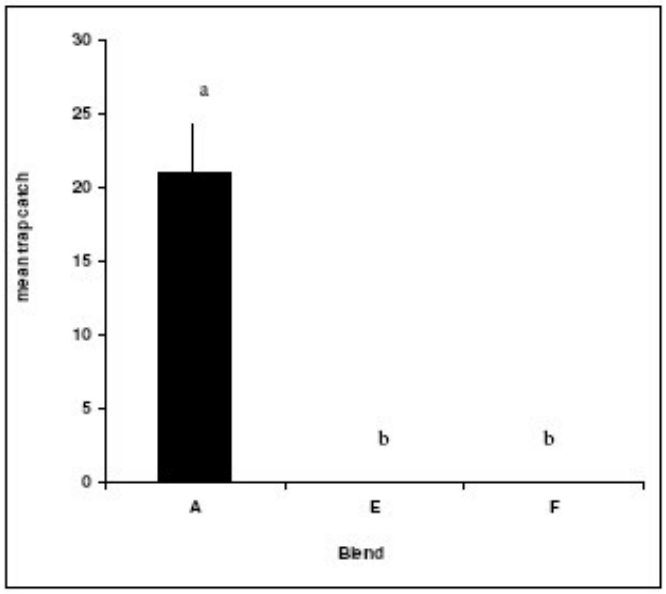

Figure 4. Experiment 1b. Mean (± s.e) delta trap catches of blends A, E and F in cotton, "Glen Shee", Oakey, Qld. Columns with common letters are not significantly different (P > 0.05).

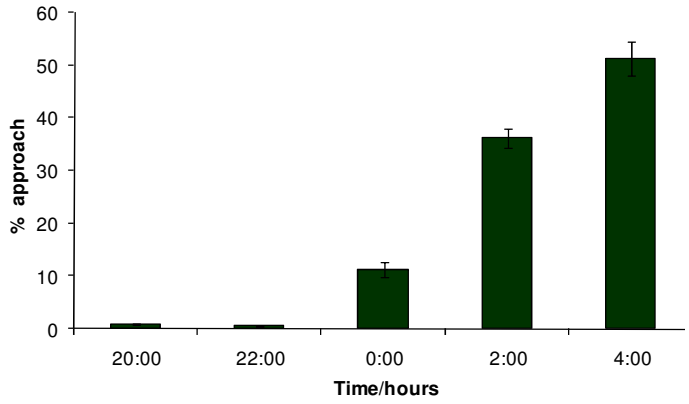

Figure 5. % (± s.e) of *E. huegeliana* males approaching the pheromone trap (baited with blend A) per night at different times of the night over the # day period in cotton, Glen Shee", Oakey, Qld.

any moths (Figure 4). Blend E was made up of a 4:1 mix of (E,E)-10,12-hexadecadienal and (E,E)-10,12-16OH, while blend F consisted of a 1:1 mix of (Z)-11: 16Al and (Z)-11:18Al.

The analysis of variance yielded highly significant effect of blend type (P < 0.001) but no significant effects of trap rotation, location and day. Again, these results suggest that (Z)-11: 16Al and (E,E)-10,12:16Al are essential for the formulated pheromone blend to attract males, as observed in experiment 1.

Table 3. Experiment 3. Mean (± s.e) catches of *E. huegeliana* per rotation interval in agrisense® and delta traps in bladder ketmia, Mondure. Qld.

	Agrisense® trap	Delta trap
Day 1	0.0 ± 0	0.4 ± 0.2
Day 2	0.0 ± 0	1.2 ± 0.6
Day 3	0.0 ± 0	0.4 ± 0.2
Day 4	0.0 ± 0	1.6 ± 0.8
Mean (± s.e)	0.0 ± 0	0.9 ± 0.3

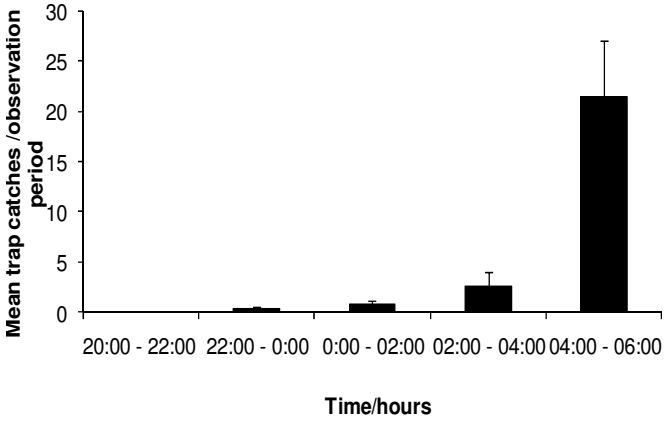

Figure 6. Mean (± s.e) catches of *E. huegeliana* males in delta traps baited with blend A at different times of the night in cotton, "Glen Shee", Oakey, Qld.

Male response to sex attractants in the field

E. huegeliana moths were observed to fly in the characteristic zigzag manner towards the full component blend (A) but not to the partial blends (E and F). On no occasion was a single insect seen flying around these partial blends, which did not catch any moths during this experiment (Figure 5). With blend A, moths were observed to approach the traps during the second half of the night (Figure 6). Less than 1% approached between 2000 - 2200 h, with approaches increasing from 11.2% at 2400 h to 51.3% at 0400-0600 h. This period was also observed in the laboratory to be the time period when the female exhibited calling behaviour. These results suggest that pheromone production in *E. huegeliana* females appeared to be synchronised with male response during the second half of the night. Even with synthetic lures that are emitting pheromone all the time, there is periodicity in response of the male moths. Peak periods of approaches at particular times of the night correlated with trap catches. Peak trap catches (68%) occurred between 0400-0600 h, the time when peak approaches were also observed (Figure 6).

Experiment 3 – comparison of 2 types of traps using blend B

The funnel trap was compared with the delta trap using blend B as the pheromone lure. Only the delta traps caught male rough bollworm moths while the funnel trap did not catch any moths during the 4 days of the trial (Table 3). GLM analysis showed no significant effect of day ($P = 0.100$) but a highly significant effect of the trap type ($P < 0.001$). The numbers of adult rough bollworms during this experiment were low. Nevertheless, the results clearly suggest that delta traps are likely to be more efficient traps for trapping *E. huegeliana* than funnel traps.

DISCUSSION

The sex attractant of female *E. huegeliana* has been identified as a mixture of the major component (E,E)-10,12-16Al and the minor components (Z)–11:16Al and (Z)-11:18Al in a ratio of 4:1:1, respectively. Most lepidopteran sex pheromone systems are multi-compo-nents and the relative composition may be critical to be effective attractants. Field trapping studies not only indica- ted that a 4:1 ratio of (E,E)-10,12-16Al and (Z)–11:16Al was effective in attracting male moths, but also, that these 2 compounds were essential for activity of the blend. The pheromone components identified in this species, (E,E)-10,12-16Al, (Z)–11:16Al and (Z)-11:18Al, are similar to the blend of the closely related species, *Earias vittella* (Fabricius). Similarities in morphology, pheromone components and time of release of phero-mones by females raise questions about their reproductive isolation and whether they are really different species. Electrophoretic or DNA studies to determine genetic differences might help resolve this. An experiment to determine the level of cross mating and hybridisation between males and females of the 2 species in the laboratory might also provide some useful information. In 2 other related species, *E. vittella* and *Earias insulana* where communication is via the use of these same chemical components, the addition of (E,Z)-10,12-16Al reduced catch drastically while the (Z,E)-10,12-16Al did not have any effect (Cork et al., 1988). Work remains to be done in this area with respect to *E. huegeliana*. The time of peak male response to the pheromone as indicated by peak catches in traps appeared to be synchronised with the time at which females were observed to be calling or releasing the pheromone in the laboratory. It was found to be restricted to the second half of the night, especially between 0400 and 0600 h AEST in the field.

Conclusion

The attractant lures developed in this study paves the way for the development of an effective pheromone management tool for *E. huegeliana*. However, for monitoring purposes, further data need to be collected to determine the correlation between trap catches and field infestations. Mating disruption applications might also be possible with this pest but a lot of data needs to be collected before any recommendations can be made. This technique has been used successfully in Pakistan (Critchley et al., 1987) for the closely related species *E. vittella*.

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