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Sex Pheromones of the Green Mirid, *Creontiades dilutus* (Stål) (Hemiptera: Miridae)

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Abstract: Whole body extracts and air collections from *Creontiades dilutus* males and females were analyzed to identify the sex pheromone components. The major component, hexyl hexanoate was found in whole body extracts and air collections from both sexes, while the minor component, (*E*)-2-hexenyl hexanoate, was only present in the female air collections. Field trapping experiments were conducted to determine the attractiveness of either of the single components and various binary blends to males. The optimum blend that consistently caught males in pheromone traps was a 5:1 ratio of hexyl hexanoate and (*E*)-2-hexenyl hexanoate. Trapping studies also showed that green mirids came to pheromone traps only between 18:00 and 06:00 h, suggesting that they might be nocturnal rather than diurnal insects as previously thought.

Key words: *Creontiades dilutus*, green mirid, sex pheromones, hexyl hexanoate, (*E*)-2-hexenyl hexanoate, pheromone traps

INTRODUCTION

The green mirid, *Creontiades dilutus* (Stål) has been identified as a pest of Australian cotton in the early 1970's and is also found in many other crops such as lucerne, potatoes, soybeans, mung beans, pigeon pea, stone fruits, sunflower and grapes (Hori and Miles, 1993 ; Malipatil and Cassis, 1997; Carver *et al*., 1991). Plant damage caused by mirids include pre-mature abortion or deformation of fruits, leaf wilt and disease transmission. In conventional or non-transgenic cotton, *C. dilutus* rarely reaches economic levels because the populations are usually suppressed by insecticides sprayed to control the key pests, *Helicoverpa* sp. In transgenic cotton, however, it is becoming a significant pest with the reduction in the use of broad spectrum insecticides and the use of more selective insecticides.

Current methods employed to monitor green mirid populations in Australian cotton include the beat sheet method, visual counts or suction sampling. Green mirids are small insects which easily take off from plants when disturbed and thus can be easily missed by these methods. The use of sex pheromones might be a more efficient method to monitor mirid populations in the field.

An earlier study demonstrated the attraction of *C. dilutus* males to conspecific females, presumably through sex pheromones. It was suggested that hexyl hexanoate, might be a component, but this compound was found in both males and females and it was not attractive to males in traps. This study describes further attempts to study the pheromone system of *C. dilutus*, including field bioassays to test the attractiveness of potential pheromone components to males in the field.

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MATERIALS AND METHODS

Insects

Nymphs of *C. dilutus* were collected in 2003 from a lucerne field in Armidale, NSW and were reared through to adults in the insectary at 25±1°C and 13:11 light: dark conditions. Five to ten mirids were held in 1 L plastic containers with meshed tops, provided with fresh beans daily as food. Adults were sexed immediately and males and females were kept in separate containers.

Pheromone Collection and Analysis

Pheromone collection was done by air collections and whole body extracts from both sexes. Volatiles were collected from 3-4 virgin females or males using an all glass apparatus. Air was drawn into a flask through a filter of activated charcoal (10×2 cm; 10-18 mesh) by means of a pump (Capex L2C, Charles Austen Pump Ltd, Surrey, England) at a rate of 60 mL min^{-1} and the volatiles trapped on a 100 mg filter of super Q (80/100 mesh; Alltech Associates Inc., USA) held in place by salinised glasswool in a pasteur pipette. Air collection was done for 15 h. Trapped volatiles were eluted from the filter with 2-3 mL of hexane and concentrated to 50 μL under a gentle stream of nitrogen before analysis. Volatiles were collected from 6-8 day-old mirids. Whole body extractions were done following the procedure of (Ho and Millar, 2002). Mirids were first immobilized in a freezer for 1-2 min. The immobilized insects were then put on a small piece of filter paper held between aluminium foils. The foil was then placed on another filter paper and insects squashed by applying gentle pressure. The external filter paper was discarded and the foil with the inner filter paper was then transferred using forceps into the collection chamber of the air collection apparatus. Air was sucked through the system at 60 mL min^{-1} for 15 h. Trapped volatiles were then eluted with hexane and concentrated to 50 μL under a gentle stream of nitrogen.

Gas Chromatographic-Mass Spectrometric (GC-MS) analysis were done using a Hewlett Packard 6890 series gas chromatograph and HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, USA). The columns used on this GC were an AT 35 capillary column (30×0.25 mm i.d x 0.25 μm) and a HP-5MS (5% Phenyl Methyl Siloxane, 30 m×0.25 mm i.d., 0.25 μm film thickness; J and W Scientific, Folsom, USA) fused capillary column. The carrier gas was ultrapure helium set at a flow rate of 0.8 m sec^{-1}. 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 analyzed 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 ($M^{\circ+}$), M^+ minus recognizable fragments and other fragment ions consistent with the structure proposed. These were then confirmed with spectra obtained from standard spectra run with retention times.

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 *C. dilutus* males and 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 spacings were used, but sometimes the conditions of the field made this difficult. In all the experiments, traps were cleared and rotated daily.

Table 1: Coding for blends of GM pheromone components used in field trials. All blends contained butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10% of the total pheromone components. Blend GM2 was based on ratios obtained from air samples, other blends were arbitrarily selected to test the effects of departures from this ratio

Blend	Hexyl hexanoate	(*E*)-2-hexenyl hexanoate
GM1	2	1
GM2	5	1
GM3	10	1
GM12	3	1
GM13	7	1
GMC	-	-

Experiment 1 -Comparison of Two-Component Blend and Single Components

This experiment aimed to test the two-component blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate in a 2:1 ratio, against each individual component. A 4×4 Latin square design with 4 treatments (GM1, GM5, GM6, GMC or control), 4 rotation periods and 4 trap locations was set up in soybeans (flowering stage) at Kurralinden, Cecil Plains, Qld in 2004. Traps were located 100 m within and between rows. Each lure was loaded with 2 mg of the blend or single component and 0.2 mg BHT as anti-oxidant. The lure for the control trap (GMC) was loaded with only BHT. The BHT (antioxidant) possibly prolongs the half-life of the active components.

Experiment 2-Optimization Studies of the Two-Component Blend

A series of experiments were done to determine the optimal ratio of the two-component blend. In one experiment, three ratios -2:1 (GM1), 5:1 (GM2) and 10:1 (GM3) were compared using a 3×3 Latin Square design with 3 rotations, 3 blends and 3 trap locations in lucerne (slashing stage) at Yarral, Narrabri, NSW. Delta traps were located 30 m apart within and between rows.

At another site in Jahlee, Mullaley, NSW, two 3×3 Latin Square experiments in mung beans (pod filled stage) and one in lucerne (one week post-slashing) compared 2:1 (GM2), 3:1 (GM12) and 7:1 (GM13) ratios. Traps were spaced at 40 m in the mung bean experiments and at 25 m intervals within and between rows in the lucerne experiment.

Experiment 3-Pheromone Trap Catches vs Time

This experiment aimed to determine what time the green mirids come to pheromone traps. Four funnel traps, (Entosol Australia Pty Ltd, Roselands, NSW, Australia) were set up on a lucerne field (Yarral, Narrabri, NSW) at 50 m-intervals. Traps were checked for any green mirids over 3 days at 18:00, 22:00, 06:00, 10:00 and 14:00 h.

Statistical Analysis

Statistical analyses of data were done using the R statistical package version 1.9.0 (R Development Core Team, 2004). Data were summarized 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 significant differences between means.

RESULTS

Pheromone Analysis and Identification

A total of 12 potential sex pheromone components were identified as shown in the GC-MS analysis of air collections and whole body extracts from both *C. dilutus* males and females (Fig. 1, Table 2). Hexyl hexanoate was the major component in both sexes and one minor component, (*E*)-2-hexenyl hexanoate was found in the female air collections only. Two other minor components, methyl salicylate and (*Z*)-3 hexenyl acetate, were also found from the air collections of both sexes. Compounds

Table 2: Calculated percent composition of air and whole body extracts of *C. dilutus* relative to hexyl hexanoate

		Whole body extract (n = 3)		Air (n = 3)	
Compound	Retention time (min)	Female	Male	Female	Male
3-Hexanone	7.655	0.00±0.0	0.00±0.0	1.05±0.5	5.89±0.6
2-Hexanone	7.751	0.00±0.0	0.00±0.0	1.42±0.7	4.97±0.4
Hexanol	9.455	4.16±2.4	3.16±1.5	0.00±0.0	0.00±0.0
(*Z*)-3 Hexenyl acetate	11.869	0.00±0.0	0.00±0.0	4.05±2.2	2.40±1.5
Octanal	11.932	0.32±0.2	0.18±0.1	6.30±3.8	24.46±4.2
Nonanal	13.714	0.51±0.3	0.24±0.2	10.43±4.2	40.85±0.6
Hexyl butyrate	15.020	0.91±0.5	0.79±0.7	0.00±0.0	0.00±0.0
Methyl salicylate	15.516	0.00±0.0	0.00±0.0	9.32±2.8	4.90±2.8
Pentyl hexanoate	16.524	3.44±2.0	0.99±0.8	0.00±0.0	0.00±0.0
Hexyl hexanoate	17.904	100	100	100	100
(*E*)-2- Hexenyl hexanoate	18.051	0.00±0.0	0.00±0.0	33.67±0.9	0.00±0.0
Heptyl hexanoate	19.254	2.37±1.4	2.21±1.3	0.00±0.0	0.00±0.0

Mean±SE

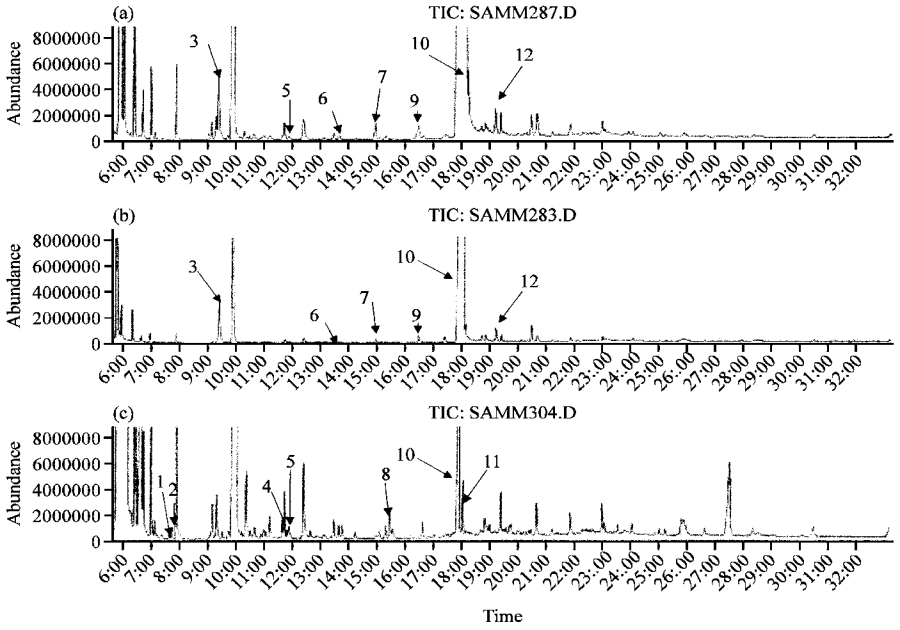

Fig. 1: Gas chromatographs of (a) male whole body extracts, (b) female whole body extracts and (c) female air collections of Creontiades dilutus. compound 1 = 3-hexanone, 2 = 2-hexanone, 3 = hexanol, 4 = (*Z*)-3-hexenyl acetate, 5 = Octanal, 6 = nonanal, 7 = hexyl butyrate, 8 = methyl salicylate, 9 = pentyl hexanoate, 10 = Hexyl hexanoate

were identified by comparison of the mass spectral data with standard spectra for hexyl hexanoate, (*E*)-2- hexyl hexanoate, methyl salicylate and (*Z*)-3 hexenyl acetate and by co-injection with authentic standards.

Field Bioassays

Experiment 1-Comparison of the Two Component-Blend and Single Components

Mean (±SE) catches per night of delta traps baited with the two-component blend (GM1), the single components hexyl hexanoate (GM5) and (*E*)-2-hexenyl hexanoate (GM6) and the blank or

Table 3: Mean catches per trap per night of *C. dilutus* males with the two-component blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate (GM1), hexyl hexanoate alone (GM5), (*E*)-2-hexenyl hexanoate alone (GM6) and control (GMC), at Kurralinden, Cecil Plains, Qld

Blend	Mean trap catch
GM1	3.9±1.0[a]
GM5	0.0±0[b]
GM6	0.0±0[b]
GMC	0.0±0[b]

Means followed by common letters are not significantly different (p>0.05)

Table 4: Mean catches per trap per night of *C. dilutus* males with 2:1 (GM1), 5:1 (GM2) and 10:1 (GM3) ratios of hexyl hexanoate and (*E*)-2-hexenyl hexanoate, in lucerne, Yarral, Narrabri, NSW

Blend	Mean trap catch
GM1	4.22±0.80[a]
GM2	7.22±1.44[b]
GM3	2.67±0.67[c]

Means followed by common letters are not significantly different (p>0.05)

Table 5: Mean catches per trap per night of *C. dilutus* males with 3:1 (GM12), 5:1 (GM2) and 7:1 (GM13) ratios of hexyl hexanoate and (*E*)-2-hexenyl hexanoate, in mung beans, site 1, Jahlee, Mullaley, NSW

Blend	Mean trap catch
GM2	7.8±1.6[a]
GM12	7.2±1.6[a]
GM13	6.2±0.8[a]

Means followed by common letters are not significantly different (p>0.05)

Table 6: Mean catches per trap per night of *C. dilutus* males with 3:1 (GM12), 5:1 (GM2) and 7:1 (GM13) ratios of hexyl hexanoate and (*E*)-2-hexenyl hexanoate, in mung beans, site 2, Jahlee, Mullaley, NSW

Blend	Mean trap catch
GM2	6.9±1.2[a]
GM12	6.4±1.0[a]
GM13	5.3±1.1[a]

Means followed by common letters are not significantly different (p>0.05)

control (GMC) is shown in Table 3. Male *C. dilutus* adults were caught in traps baited with the 2:1 blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate (GM1) at an average of 3.9 males per trap per night. The analysis of variance showed that effects of trap rotation and location ($p = 0.100$) and day factors ($p = 0.090$) were not statistically significant and the one highly significant factor influencing trap catch was the blend type ($p<0.001$). Comparison of the means using contrast in the R program indicated highly significant differences between blend GM1 and GM5, GM6 and GMC ($p<0.001$).

Experiment 2-Optimization Studies with the Two-Component Blend

In this experiment, three ratios of hexyl hexanoate to (*E*)-2-hexenyl hexanoate were compared in traps set up in a lucerne field. These ratios were 2:1 (GM1), 5:1 (GM2) and 10:1 (GM3). Mean male trap catches per night for blends GM1, GM2 and GM3 were 4.2, 7.2 and 2.7, respectively (Table 4). Analysis of variance yielded a significant effect of blend type ($p = 0.010$) and no significant effects of trap rotation, location and day ($p = 0.200$). Comparison of the means using contrast in the R program showed significant differences between all three ratios, with the 5:1 ratio (GM2) having the highest mean catch per trap.

Table 5 and 6 compare the trap catches of the two-component blend in 5:1 (GM2), 3:1 (GM12) and 7:1 (GM13) ratios done at two sites in mung beans. In both experiments, the analysis of variance showed no significant effects of blend or day ($p \geq 0.570$ and $p \geq 0.146$, respectively). Table 7 shows trap catches for a similar experiment carried out in a harvested lucerne site. The analysis of variance did not indicate any significant effect of blend type, row and position ($p \geq 0.140$). The effect of day was, however, significant ($p = 0.016$), indicating that some nights may not have been suitable for trapping, perhaps because of low temperatures.

Table 7: Mean trap catches of *C. dilutus* males per night with 3:1 (GM12), 5:1 (GM2) and 7:1 (GM13) ratios of hexyl hexanoate and (*E*)-2-hexenyl hexanoate, in lucerne, Jahlee, Mullaley, NSW

Blend	Mean trap catch
GM2	7.0±0.8[a]
GM12	5.2±1.0[a]
GM13	3.4±1.0[a]

Means followed by common letters are not significantly different (p>0.05)

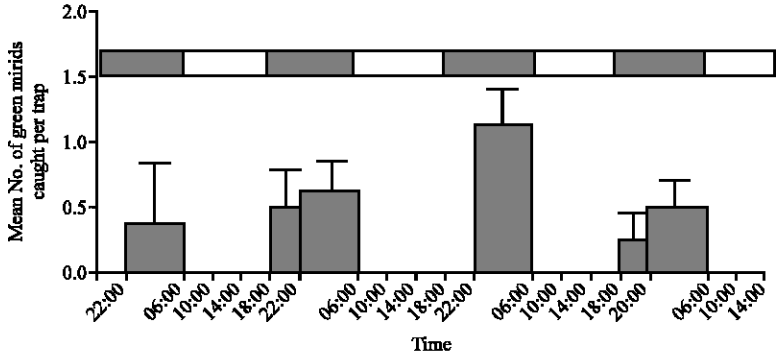

Fig. 2: Mean No. of green mirids per trap at various times. Black and white bar above graph represents night and day periods

Experiment 3-Pheromone Catches vs. Time

Although mirid numbers were low during this experiment, Fig. 2 shows that mirids came to the pheromone traps only between 1800 and 0600 h, with most of them coming between 22:00 and 06:00 h.

DISCUSSION

One way to identify potential sex pheromone components in insects is to determine differences between the chemical profiles of female and male extracts. The major component found in the whole body extracts and air collections from both *C. dilutus* males and females was hexyl hexanoate. No sex-specific compound was found in the whole body extracts, while in the air collections, (*E*)-2-hexenyl hexanoate was only found in the females.

Hexyl hexanoate and (*E*)-2-hexenyl hexanoate have also been reported in other heteropteran bugs. In addition to octyl butyrate, these two compounds have been identified as pheromones of the rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy) in Japan (Kakizaki and Sugie, 2001). A ratio of 10-100:1000:400-500 of octyl butyrate, hexyl hexanoate and (*E*)-2-hexenyl hexanoate was found to be attractive to males. (*E*)-2-hexenyl hexanoate and hexyl hexanoate have also been reported in secretions of *Blepharidopterus angulatus* (Fallen) (Miridae) (Knight *et al*., 1984) and *Homoeocerus unipunctatus* (Thunberg) (Coreidae) (Kitamura *et al*., 1984). Males of the bean bug *Riptortus clavatus* (Thunberg) produce male specific compounds (*E*)-2-hexenyl-(*Z*)-3-hexenoate, (*E*)-2-hexenyl-(*E*)-2-hexenoate, myristyl isobutyrate and (*E*)-2-hexenyl hexanoate, the latter being an alarm pheromone (Leal and Kadosawa, 1992) and a blend of the others as attractant pheromones attracting adults and second instar nymphs (Leal *et al*., 1995). In a similar species, *Riptortus serripes* (Fabricius) both males and females produced (*E*)-2-hexenyl hexanoate but its role as pheromone component is unknown (Aldrich *et al*., 1993).

Four compounds identified in the whole body extracts from both sexes but not in the air collections (hexan-1-ol, hexyl butyrate, pentyl hexanoate and heptyl hexanoate) could possibly be

alarm pheromones. Hexyl butyrate has been identified in both air and metathoracic gland collections of other Miridae like *Lygus hesperus* (Ho and Millar, 2002), *L. lineolaris* (Gueldner and Parrott, 1978) and *Phytocoris californicus* (Millar and Rice, 1998). While Zhang and Aldrich (2003b) reported this compound as a male produced anti-sex pheromone of *P. relativus*, another study (Zhang and Aldrich, 2004) indicates it, in addition to (*E*)-2-hexenyl butyrate in a ratio occurring in *L. lineolaris* and other mirids, acts as a kairomone attracting large numbers of scavenging Chloropid and Milichiid flies (Diptera). In *L. hesperus*, hexyl butyrate formed the major component of the metathoracic glands. Hexanol was present at 9.9% relative to the hexyl butyrate in both male and female air collections and 1.2 and 1.9% in the female and male glands, respectively. However, in squashed samples values were 400 and 419% for female and male air collections, while in glands these values were 429 and 140%, respectively. In *C. dilutus*, hexanol was not present in the air collections but about 4% was detected in the squashed whole body extracts. Ho and Millar (2002) detected the presence of heptanol in extracts of *L. hesperus* spiked with heptyl butyrate which formerly did not contain either compound. They concluded that extensive hydrolysis of the esters may have been the reason for the high level of hexanol in the squashed samples. It is possible that the presence of hexanol in the squashed extracts of both the female and male GM was the result of hydrolysis of the hexyl butyrate present. Pentyl hexanoate on the other hand, has only been cited as part of volatiles collected from apples, which act as an attractant for the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Zhang *et al*., 1999).

Other compounds identified in the female air collections included methyl salicylate and (*Z*)-3 hexenyl acetate. Methyl salicylate has been cited as an anti-aphrodisiac produced by male *Pieris napi* (Linnaeus) butterflies, synthesized and transferred at mating (Andersson *et al*., 2000). As a herbivore-induced plant volatile, it has been demonstrated as an attractant for the green lacewing, *Chrysopa nigricornis* (Burmeister) (James, 2003) and the western flower thrips, *Frankliniella occidentalis* (Pergande) (Chermenskaya *et al*., 2002). Its role in GM is not clear.

The source of pheromone secretions has been determined in other Miridae species. It is in the thorax for *P. relativus* (Millar *et al*., 1997), abdomen tip for *L. hesperus* (Graham, 1988), head and thorax for *C. verbasci* (Thistlewood *et al*., 1989) and is suggested to be in the metathoracic scent gland for *V. thapsis*, (McBrien and Millar, 1999) and *L. lineolaris* (Gueldner and Parrott, 1978). The location of pheromone glands in the *C. dilutus* has not been determined in this study.

Either hexyl hexanoate or (*E*)-2 hexenyl hexanoate alone did not catch any mirids in traps, suggesting that these two compounds are both required for the pheromone to work in *C. dilutus*. These results are similar to the situation in the rice leaf bug, *T. caelestialium*, where all the ten electroantennographic detector (EAD) active compounds on their own did not attract any males (Kakizaki and Sugie, 2001). In *C. dilutus*, the female-specific component, (*E*)-2-hexenyl hexanoate, did not attract any males in the absence of the major component, hexyl hexanoate, which is produced by both sexes. This situation where the attractive blend is a mix of a female component(s) and one produced by both sexes has also been reported in two other mirids, *P. relativus* and *P. californicus* (Millar *et al*., 1997; Millar and Rice, 1998). Another situation where both male and female extracts contain the same compounds but the pheromone is attractive to the males only has also been reported for the rice leaf bug, *T. caelestialium* (Kakizaki and Sugie, 2001) and *Phytocoris difficulis* (Zhang and Aldrich, 2003a).

Hemipteran pheromone systems, like those of other insects, are multi-component. A mixture of two female-specific components, butyl butyrate and (*E*)-2-butenyl butyrate in a ratio of 94:6 has been identified in the mullein bug, *C. verbasci*. In this species, pheromone release rates of 91 and 183 μL day^{-1} were found to be as attractive as five live virgin females (Smith *et al*., 1991; McBrien *et al*., 1994). The individual components on their own were found to be inactive. The pests of pistachio, *Phytocoris californicus* and *P. relativus*, on the other hand, use a 2:1 ratio of hexyl acetate, produced by both sexes, with the female-specific compounds (*E*)-2-octenyl acetate and (*E*)-2-octenyl

butyrate, respectively (Millar and Rice, 1998; Millar *et al*., 1997). While (*E*)-2-octenyl acetate did not inhibit *P. relativus* males, (*E*)-2-octenyl butyrate inhibited attraction of *P. californicus* males to traps (Millar and Rice, 1998).

Five different ratios of the major component, hexyl hexanoate to the minor component, (*E*)-2 hexenyl hexanoate (2:1, 3:1, 5:1, 7:1, 10:1) used in this study all caught green mirids. In all the optimization experiments, the 5:1 ratio caught the highest number of *C. dilutus* males in traps, indicating that this is the optimal ratio of the two-component blend.

Creontiades dilutus was previously thought to be diurnal as they are observed to be active during the day when crop scouting is usually done. Results from this study showed that no mirids were caught in the pheromone traps at day time. They came to the traps only between 18:00 and 06:00 h, suggesting that they might be nocturnal. In olfactometer studies, *C. dilutus* females were observed to be attracted to lucerne volatiles only under very low light (0.1-0.5 lux) between 18:00 h and midnight (Del Socorro and Gregg, unpubl.). The pheromone blend developed from this work has potential application as a monitoring tool for *C. dilutus* particularly in Australian cotton fields. The components are highly volatile, hence, further work will attempt to improve the longevity of pheromone lures in the field.

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