Sex pheromone of the jumping plant bug, *Halticus minutus*
Reuter (Hemiptera: Miridae)

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Abstract—The jumping plant bug, *Halticus minutus* Reuter, is a pest of sweet potatoes, soybeans and turfgrass in China. Here we present our analysis of the female-produced sex pheromone. These plant bugs release their sex pheromone, consisting hexyl acetate (HA), hexyl butyrate (HB) and (*E*)-4-oxo-2-hexenal (4-OHE), at night. These three components, tested individually, elicited strong antennal responses. Field tests indicated that a ternary blend of HA, HB and 4-OHE, at ratios of 1:16:10, effectively attracted male *H.minutus*. Our dose-response analysis showed optimal male capture at the trap dose of 13.5 mg/trap. Higher trap doses did not improve capture rates. Higher ratios of HB in the pheromone mix led to substantially reduced captures, from which we infer that HB may function as an alarm pheromone or defensive secretion. Pipette tips were used as long-term lures, and whose longevities were evaluated. Results showed effective duration of pipette tip lures were more than 5 weeks. Thus, we suggest that the pheromone-baited traps are economically viable tools for long-term monitoring and trapping of jumping plant bugs.

Key Words—jumping plant bug (*Halticus minutus*), sex pheromone, hexyl acetate, hexyl butyrate, (*E*)-4-oxo-2-hexenal

Introduction

The jumping plant bug, *Halticus minutus*, is a widespread pest in Asia (Amalin and Vasquez, 1993). Similar to its congeneric species *H. bractatus*, *H. minutus* is polyphagous, feeding on a wide recipe, including sweet potato, alfalfa, bean, barley, corn, oat, wheat, eggplant, tobacco, and cotton (Henry, 1983). In China, *H. minutus* is considered as an important pest in sweet potato and soybean (Tong and Wang, 1987). In recent years, *H. minutus* outbreaked in turfgrass in northern China, causing whitish spots on turfgrass leaves (Wang et al., 2005; Xu et al., 2010). In addition to direct feeding, *H. minutus*, as a leaf-sucking insect, indirectly harmed host plants through transmission of phytoplasmas (Mitchell, 2004). Nowadays, insecticide use is the sole pest control option available, which has caused problems including resistance development, pest resurgence and environmental pollution. Strategies exploiting the chemical communication of *H. minutus* could be an effective and environmentally-friendly management option against this pest.

For decades, insect sex pheromone has been demonstrated to be effective to monitor and control agriculture pests in an environmentally-friendly manner (Witzgall et al., 2010). Although the Miridae are the largest family of Heteroptera, including over 11,000 species (Cassis and Schuh, 2012), the studies and applications of mirid pheromone are still in infant. Up to now, sex pheromones of approximate 20 mirid species have been identified and verified, including *Adelphocoris* spp. (Zhang et al., 2015; 2016), *Apolygus* spp. (Yang et al., 2014, 2015), *Lygus* spp. (Byers et al., 2013; Fountain et al., 2014; Ho and Millar, 2002; Innocenzi et al., 2004, 2005), *Phytocoris* spp. (Millar and Rice, 1998; Millar et al., 1997; Zhang and

Aldrich, 2008), and species in other genus (Drijfhout and Groot, 2001; Kakizaki and Sugie, 2001; Lowor et al., 2009; Mahob et al., 2011; Smith et al., 1991; Sarfo et al., 2013; Yasuda et al., 2008;). Most identified communication compounds are saturated or unsaturated straight-chain esters and aldehyde.

As far as we know, there have been no reports published with respect to the chemical ecology of genus *Halticus*. The aim of our research is to identify potential sex pheromone components, to optimum their active ratios, and to further evaluate the activities of artificial lures in the field. This study could help us to decipher chemical communication of *Halticus* genus, and develop novel strategies to effectively monitor and control *H. minutus*.

Materials and Methods

Insects and Sample Collection

Nymphs of *Halticus minutus* were collected by sweep-netting from a lawn in Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, and subsequently cultured at 25 ± 2 °C, 50%~70% RH, and 12:12 (L:D) photoperiod in environmental growth chambers (Ningbo Jiangnan Instrument, Ningbo, China). The nymphs were reared on fresh turfgrass. Newly-emerged bugs were sorted by gender according to the ovipositor in female abdomen.

Emitted volatiles were collected using the aeration method described previously (Innocenzi et al., 2004). Briefly, volatiles from single virgin adult (~3-day-old) were drawn from a glass chamber (30 cm height $\times$ 3.5 cm diam) using a diaphragm pump (300 mL·min⁻¹) and adsorbed on a glass tube (0.5 cm diam) containing 50 mg of purified PorapakQ. Ten duplicates for both genders were conducted individually. All samples were collected for the first 2 hours of the scotophase. Then, adsorbed aeration volatiles were eluted from PorapakQ with 500 μ L of distilled dichloromethane. The extracts were stored at -20°C before GC-MS measurement.

GC-MS Analysis

Collections of volatiles emitted from *H. minutus* were analyzed with a gas chromatography-mass spectrometer (7890A-5975C, Agilent, Santa Clara, California) equipped with a DB-wax (30m $\times$ 0.25mm $\times$ 0.25 μ m, film thickness; Agilent) or DB-5 column

(30m×0.25mm×0.25µm, film thickness; Agilent). The carrier gas was helium in 1.0 mL·min⁻¹. The oven temperature was held at 50°C for 1 min and then programmed at 3°C·min⁻¹ to 120°C; then at 20°C·min⁻¹ to 250°C for 5 min. The MS operating conditions were as follows: transfer line temperature, 250°C; ion source temperature, 230°C; ionization energy, 70 eV; and scan range m/z. 50-400. Mass spectra were matched to those in the NIST14 database, and the identifications were confirmed by comparing retention indices and spectra with those of authentic standards. Retention indices were calculated by the retention times of target compound relative to those of n-alkanes. External standard curves, obtained from series concentration of each authentic compound, were used for quantitative analysis of pheromone compounds.**Electroantennography**

EAG apparatus (Syntech Ltd., Hilversum, Netherlands) was used in electroantennography assay to evaluate whether the identified compounds stimulate *H. minutus* using a previous method (Sun et al., 2014). In brief, the amputated antennae of *H. minutus* males (~3-days-old) with excision of tips were immediately attached to two electrodes of fork holder with electrically conductive gel (Syntech). Tested compounds were dissolved into liquid paraffin, and 10 µL of serial dilutions (10, 1, 0.1, 0.01 and 0.001 µg·µL⁻¹) were then added to a folded filter paper, which was inserted into a glass Pasteur pipette. An air stimulus controller CS-55 was used for air and chemical stimulant delivery to the antennae with a constant flow of 150 mL·min⁻¹ at 30 s intervals. Liquid paraffin was tested as a background and standard stimulus.

Chemicals

Hexyl acetate (98% purity) and hexyl butyrate (95% purity) were obtained from commercial sources (ABCR GmbH & CO. KG), (*E*)-4-Oxo-2-hexenal was synthesized as described by Moreira and Millar (2005). The purity of synthetic (*E*)-4-Oxo-2-hexenal was >95% by GC analysis. *n*-Alkanes were obtained from Sigma-Aldrich.

Lures and Traps

Lures used in field trials were prepared by polypropylene pipette tips (1 mL) with a 0.2 mm aperture (Fountain et al. 2014). Certain amounts of HA, HB and 4-OHE dissolved in sunflower oil (100 μ L; food-grade) were added to a cellulose acetate cigarette filter (14 $\times$ 6 mm), and then placed in a pipette tip (1mL, Fisher Scientific) with a 0.2 mm aperture. Tips were then sealed with a Teflon-lined crimp seal and wrapped in duct tape. Considering the poor flight ability of *H. minutus*, white delta traps with sticky floors (28 $\times$ 20 cm, Pherobio Technology Co. Ltd. Beijing) were set on the lawn directly.

Field Trapping

The field trials were carried out in turfgrass (*Festca arundinacea*) in Institute of Plant Protection (40°01'18.50"N, 116°16'44.26"E), Beijing. During our experiment period, the turfgrass was mowed every two weeks, and no pesticides were sprayed. Traps were randomly designed with at least 10 m between adjacent traps. Five duplicates were conducted for each treatment. We examined traps at 10:00 am every day, and replaced sticky inserts simultaneously. After each examination, traps were randomly rearranged to minimize positional effects. Mean daily trap captures were used as indicator for field activity. Trap catch data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's

honestly significant differences (HSD) test. A significance level of 0.05 was used for statistical analysis.

Field experiment 1 aimed to verify the existence of sex pheromone in *H. minutus* by using live females from July 15 to 22, 2014. Five virgin females caged in a glass vial (diam 2 cm; height 4.5 cm) with gauze were used as lures. During experiments, sunset usually occurred at ~19:00, and it get dark at ~20:00. To determine when *H. minutus* mated, treatments were conducted in three successive time periods: 5:00-12:00, 13:00-19:00 and 20:00-4:00(at night). The live bugs and their foods were refreshed at ~13:00. Field experiment 2 investigated the attractiveness of candidate components from August 11 to 18, 2014. Treatments were single HA, HB, 4-OHE, their binary and ternary blends, caged virgin females and control (100mg sunflower oil). Experiment 3 optimized the ratio by varying the amounts of one of HA, HB and 4-OHE each time from August 18 to September 8, 2014. Experiment 4 assessed the dose-attractiveness of HA, HB and 4-OHE at the ratio of 1:16:10 from August 8 to 15, 2015. Experiment 5 was carried out to verify the long-term attractiveness of artificial lures. Lures were prepared by adding 13.5 mg ternary blends of HA, HB and 4-OHE at the ratio of 1:16:10. Five traps with lures and five unbaited traps (as blank control) were set on the same lawn. Trap captures were recorded weekly from 11 August to 22 September, 2015.

Results

Preliminary Field Experiments

During all experiments, the mean captures of control traps baited with turfgrass in vials were less than 0.5 per replicate for both sexes. In experiment 1, we tested the mating peak of *H. minutus* in field. Fig.1 showed that significantly more *H. minutus* males were trapped by virgin females at night (Fig. 1. $F_{2,10}=526.6$, $P<0.001$), indicating that *H. minutus* copulate in nocturnal rather than diurnal. Captures of *H. minutus* females were no significant differences among all periods (Fig.1, $F_{2,10}=0.081$, $P=0.922$), and the mean number of captures was no more than 0.5 /trap/day.

Fig. 1. Captures of *Halticus minutus* in delta traps baited with virgin females during three periods of the day (July 15 to 22, 2014). Bars with the same letter are not significantly different ($p>0.05$).

Chemicals Identification

Volatiles were collected during scotophase because *H. minutus* females emitted sex pheromone for copulation at night. GC-MS analysis revealed that three major components were identified as HA, HB and 4-OHE in the volatiles of virgin *H. minutus* females (Table 1). The retention indices and the mass spectrums of these components were in accordance with those of commercial compounds. HB and 4-OHE were dominant in the volatiles of females, whereas HB was the most abundant component for males. In female volatiles, the ratio of HA, HB, and 4-OHE was analyzed as 4:100:40, and the amount of HB was estimated to be 2483 ± 987 ng per insect ($N=10$). Several trace compounds were also detected in adult volatiles, including hexanal, (*E*)-2-hexenal, hexanol, (*E*)-2-hexenyl acetate and pentyl butyrate (Table 1, Fig. S1).

Table 1. Analysis of volatiles collected from *Halticus minutus* adults (Mean $\pm$ SD)

Compounds	Retention indice ¹		Amounts (ng/bug) ²	
	DB-wax	DB-5	Female	Male
Hexanal	1069	803	45 $\pm$ 21	39 $\pm$ 18
(<i>E</i>)-2-Hexenal	1203	846	51 $\pm$ 32	46 $\pm$ 21
Hexyl acetate	1260	996	102 $\pm$ 38	59 $\pm$ 28
Pentyl butyrate	1310	1091	32 $\pm$ 13	ND ³
(<i>E</i>)-2-Hexenyl acetate	1335	1005	18 $\pm$ 7	11 $\pm$ 4
Hexanol	1352	871	53 $\pm$ 20	36 $\pm$ 15
Hexyl butyrate	1419	1190	2483 $\pm$ 787	2638 $\pm$ 825
(<i>E</i>)-4-Oxo-2-hexenal	1595	956	988 $\pm$ 355	226 $\pm$ 90
(<i>E</i>)-2-Hexenyl butyrate	1462	1201	ND ³	13 $\pm$ 6

¹Retention indices calculated according to the retention times of tested compounds and *n*-alkanes.

²The amounts of released volatiles were calculated by the standard compounds based on the peak

areas.

³ND = not detected.

EAG Analysis

During our previous EAG tests, only three major components ($10\ \mu\text{g}\cdot\mu\text{L}^{-1}$) elicited significant responses of antennae of *H. minutus* males (Unpublished data). Thus, we further tested the dose-response of the major compounds. Results showed that the EAG values of males increased significantly as the increases of HA, HB and 4-OHE by ten-fold each time. However, the dose-response of females was not obvious (Fig. 2), indicating that these compounds were the potential candidates of sexual communication components in *H. minutus*.

Fig. 2. Dose-dependent EAG response curves of both sexes to sex pheromone components.

Field Trials

While traps baited with single or binary of HA, HB and 4-OHE caught as low *H. minutus* males as control traps, the ternary blend significantly trapped more males than that of virgin

females (Fig.3, $F_{8,32}=126.4$, $P<0.001$). We conclude that HA, HB and 4-OHE should be essential components of *H. minutus* sex pheromone.

Fig. 3. Captures of *Halticus minutus* males in traps baited with hexyl acetate (HA), hexyl butyrate (HB), and (*E*)-4-oxo-2-hexenal (4-OHE), their binary blends and ternary blends and caged virgin females (August 11 to 18, 2014). Bars with the same letter are not significantly different ($p>0.05$).

To optimize the ratio of HA, HB and 4-OHE, lures were prepared with the variable amounts of one component and constant amounts of the other two. Amounts of each component used in the trials were based on field experiment 2. The results showed that captures of *H. minutus* males were heavily dependent on the ratio and dose of three components (Fig.4). When the amount of HA ranged from 0.075 to 0.15mg/tube, significantly more males were caught ($F_{4,16}=21.2$, $P<0.001$), and lures loaded 0.1 mg HA trapped the most bugs (Fig.4A, mean captures 4.23 ± 0.4 /trap/day). Amounts of HB affected the captures

significantly, and 1.6mg of HB addition was evaluated as the optimum (Fig.4B, mean captures 7.54 ± 0.69 /trap/day). Higher HB addition decreased the captures sharply, and when double dose of HB were added, the captures would be completely inhibited (Fig.4B, $F_{4,16}=53.3$, $P<0.001$). Similarly, lures loaded 1 mg 4-OHE trapped the most *H. minutus* males (Fig.4C, $F_{4,16}=106.1$, $P<0.001$), and extra 4-OHE reduced the number of captures (Fig.4C, mean captures 4.71 ± 0.43 /trap/day). As such, the optimum ratio of HA to HB to 4-OHE was determined to be 1:16:10.

Fig. 4. Captures of *Halticus minutus* males in traps baited with different ratio of HA, HB, and 4-OHE

(August 18 to September 8, 2014). A: from August 18 to 25; B: from August 25 to September 1; C:

from September 1 to 8. Bars with the same letter are not significantly different ($p>0.05$).

At optimum ratio, field experiment 4 was conducted to evaluate the dose-attractiveness with the total amounts ranged from 0.27 to 27mg per lure. Fig.5 showed that the attractiveness of lures significantly depended on the addition amounts of the tertiary blend of HA, HB and 4-OHE (Fig. 5, $F_{5,20}=280.6$, $P<0.001$). 13.5 mg of pheromone was tested as

optimum, because more addition could not increase the captures. Perhaps, high-dose of pheromone could extend the working life, which was determined by the capacity of the dispenser (Fountain et al. 2014). The long-term attractiveness of pipette tip lures were also evaluated, and Fig.6 showed that significantly more *H. minutus* males were caught in traps baited with lures than unbaited control during experiment period. Although the number of captures began to decrease at 6th weeks (Fig. 6, $F_{6,24}=5.63$, $P<0.01$), the effective duration of pipette tip lures were long-enough to use for monitoring or controlling.

Fig. 5. Dose-dependent captures of *Halticus minutus* males in traps baited with HA, HB, and 4-OHE at the ratio of 1:16:10 (September 8 to 15, 2014). Bars with the same letter are not significantly different ($p>0.05$).

Fig. 6. Captures of *Halticus minutus* males in traps baited with 13.5mg of HA, HB, and 4-OHE at the ratio of 1:16:10 (August 11 to September 22, 2015). Bars with the same letter are not significantly different ($p>0.05$).

Because delta traps were placed on the surface of turf directly, non-target insects were also caught on the viscous inserts, including spiders, ants, aphids, leafhoppers, flies and moths. However, the captures had no significant differences between treatments and control (unpublished data), suggesting these insects were caught by accident.

Discussion

To our knowledge, there have been no reports yet regarding the chemical ecology of genus *Halticus*. In our present study, three major components of *H. minutus* adults were identified as HA, HB and 4-OHE, which could elicit strong responses to male antennae. Their ternary blend was identified as sex pheromone of *H. minutus* due to trapping significantly more males in fields. HA, functioned as a sex pheromone component, had been detected in plant bugs, e.g. *Phytocoris* species (Millar and Rice, 1998; Millar et al., 1997; Zhang and Aldrich, 2003a, 2008). In addition to acting as sex pheromone, HA and other volatiles from *Phytocoris calli* Knight were believed to contribute to maintaining reproductive isolation of *Phytocoris* spp. (Zhang and Millar, 2008). HA was also determined as alarm pheromone (Blatt et al., 1998; Leal et al., 1994) and defensive compounds (Prudic et al., 2008) of species in family Coreidae.

HB and 4-OHE were also detected among various plant bugs, not only from Metathoracic Scent Glands (MSG) but also from emitted volatiles. They functioned as sex pheromone, defensive secretion, alarm pheromone or anti-sex pheromone. (Byers, 2006; Byers et al., 2013; Drijfhout et al., 2001; Fountain et al., 2014; Innocenzi et al., 2004, 2005; Oku and Yasuda, 2010; Wardle et al., 2003; Zhang and Aldrich, 2003b). Recently, HB and 4-OHE have been identified as key components of sex pheromone of *Lygus* spp. (Fountain et al. 2014), and estimated to play an important role in the reproductive isolation (Byers et al. 2013). One of our recent study showed that HB and 4-OHE composed the sex pheromone of *Adelphocoris* spp. (Zhang et al., 2015; Zhang et al., 2016). When *L. hesperus* and *L. lineolaris* were disturbed or attacked, they released more HB, 4-OHE and other volatiles as intraspecific alarm pheromone or as chemical weapons against predators (Byers, 2006; Wardle et al., 2003). In *Phytocoris difficilis* (Zhang and Aldrich, 2003b), *Lygocoris pabulinus* (Groot et al. 2001) and *Apolygus spinolae* (Yang et al., 2014, 2015), new-mated females released HB to prevent more copulations through interruption of sex communication, indicating that HB could modulate copulation rhythm. This cluster of study demonstrated that HB and other volatiles might play multiple roles in some Mirid species. In terms of the study

on *H. minutus*, we were thinking that sex pheromone compounds also functioned as alarm pheromone or defensive secretion, because we obviously smelled the specific odor of ester (such as HB) when we collected this insect by net-sweeping. Furthermore, this assumption was verified by further GC-MS analyses: more HA, HB and 4-OHE, especially HB, emitted from disturbed both sexes of *H. minutus* (unpublished data). The discrepancy, between analytic ratio from volatiles and optimized ratio in field test, might make sense as the sensitive insects were disturbed by slight vibration during collecting volatiles.

Rubber septa were usually used as pheromone dispenser for plant bugs (Millar et al. 1997; Zhang and Aldrich 2003a). Although sex pheromones of Mirid bugs would exhaust from septa only a few days (Innocenzi et al., 2004), septa were capable for preliminary screening of candidate components in short interval experiments (Yasuda et al., 2008, 2013). In recent years, more kinds of pheromone carriers were developed to improve the longevity and attractiveness of lures for plant bugs. Yasuda et al. (2013) developed high-density polyethylene tubes as pheromone dispensers of *Stenotus rubrovittatus*, the validity period was more than 28 days. And, they demonstrated that the ratio of esters and 4-OHE emitted from dispensers was similar to the blend initially loaded. Yang et al. (2014) used the same type of dispenser and caught significantly more *Apolygus spinolae* male in a relative long-term period. However, lower captures indicated that neither rubber septa nor high-density polyethylene tubes were suitable as the pheromone dispenser of *H. minutus*. A novel kind of pheromone lure for mirid, which could release ester and 4-OHE slowly, was developed by Fountain et al. (2014). We also employed pipette tips as long-term dispensers during field experiments. Lures loaded with HA, HB and 4-OHE (1mg, 16mg and 10mg, respectively) trapped significantly more *H. minutus* males in more than 5 weeks. During experiment period, there are no significant differences in the captures of blank control (were trapped by accident) (Fig. 6), suggesting populations of *H. minutus* did not decline. Meanwhile, we did not notice significant population declines when we collected bugs in another turfgrass which was about one kilometer distant from experiment site (Unpublished data). These results indicate the optimal longevity of lures should be ~5 weeks. Finally, we believe this kind of lures might be useful as long-term dispensers in control of plant bugs.

Our results, for the first time, revealed that the sex pheromone of *H. minutus* was composed of HA, HB and 4-OHE at ratio of 1:16:10. Traps with artificial lures caught significantly more males in 5 weeks period. Further studies should be conducted to determine the actual functions of HB and minor components. In addition, more efficient and convenient traps need to be designed and developed.

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