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Maja Derlink, Isabelle Abt, Romain Mabon, Charlotte Julian, Meta Virant-Doberlet, et al.. Mating behavior of *Psammotettix alienus* (Hemiptera: Cicadellidae). *Insect Science*, 2018, 25 (1), pp.148-160. 10.1111/1744-7917.12379 . hal-02628561

HAL Id: hal-02628561

<https://hal.inrae.fr/hal-02628561>

Submitted on 26 May 2020

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Running title: Behaviour of the leafhopper *P. alienus*

Title for authors: *M. Derlink et al.*

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Original article

Mating behaviour of *Psammotettix alienus* (Hemiptera: Cicadellidae)

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Abstract

The *Wheat dwarf virus*, the causal agent of the wheat dwarf disease, is transmitted by leafhoppers from the genus *Psammotettix* and currently the main protection strategy is based on the use of insecticide treatments. Sustainable management strategies for insect vectors should include methods that are targeted to disrupt reproductive behaviour and here we investigated the mating behaviour of *Psammotettix alineus* (Dahlbom 1850) in order to determine the role of vibrational signals in intra-specific communication and pair formation. Both genders spontaneously emit species- and sex-specific calling songs that consisted of

This is an Accepted Article that has been peer-reviewed and approved for publication in the Insect Science but has yet to undergo copy-editing and proof correction. Please cite this article as [doi: 10.1111/1744-7917.12379](https://doi.org/10.1111/1744-7917.12379).

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regularly repeated pulse trains and differ primarily in pulse train duration and pulse repetition time. Females preferred the conspecific male calling song. After a coordinated exchange of pulse trains, the male approached the stationary female. During the close range courtship and also immediately prior to copulatory attempts distinct male vibrational signals associated with wing flapping and wing vibrations were recorded from the substrate. In the presence of a receptive female, competing males emitted vibrational signals most likely aimed to interfere with male-female interaction. Mated females regained sexual receptivity after they laid eggs. Although results suggest that the viruliferous status of insects may have an effect on vibrational songs, our current results did not reveal a significant effect of virus on leafhopper performance in mating behaviour. However, the present study also suggests, that detailed understanding of plant-vector-virus interactions relevant for vector mating behaviour is essential for trying new approaches in developing future control practices against plant viruses transmitted by insect vectors.

Keywords: mating behavior, *Psammotettix alienus*, vibrational communication, Wheat dwarf virus

Introduction

Substrate-borne vibrations play an important role in mating behaviour of many insect pests from the order Hemiptera (e.g. Kanmiya, 2006; Čokl, 2008; Mazzoni *et al.*, 2009; Mazzoni *et al.*, 2010; Eben *et al.*, 2015). Leafhoppers (Hemiptera: Auchenorrhyncha: Cicadellidae) are, with more than 22 000 described species, one of the most speciose groups of phytophagous insects and are among the most important vectors of plant diseases (Weintraub & Beanland, 2006). Vibrational signals have been described in a great number of leafhopper species (e.g., Tishechkin, 2000; Čokl & Virant-Doberlet, 2003); however, taking into account the economic importance of leafhopper vectors and leafhopper diversity, it is surprising that a more detailed and comprehensive knowledge about the role of vibrational communication in leafhopper mating behaviour is limited to three species, namely *Graminella nigrifrons* (Heady & Nault, 1991; Hunt & Nault, 1991; Hunt & Morton, 2001), *Scaphoideus titanus*

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(Mazzoni *et al.*, 2009; Polajnar *et al.*, 2014) and *Aphrodes makarovi* (de Groot *et al.*, 2012; Kuhelj *et al.*, 2015).

In Cicadellidae, mate recognition and location are mediated exclusively by vibrational signals and the emerging general pattern of a leafhopper mating sequence is (a) that pair formation begins with the emission of a male advertisement call and (b) that after vibrational contact has been established, male searches for a replying stationary female (Heady & Nault, 1991; Hunt & Nault, 1991; Mazzoni *et al.*, 2009; de Groot *et al.*, 2012; Polajnar *et al.*, 2014). The leading role of the male in the pair formation is associated with the ‘fly/jump/walk-call’ strategy found in many Auchenorrhyncha (Hunt & Nault, 1991; De Luca & Cocroft, 2011; de Groot *et al.*, 2012; Polajnar *et al.*, 2014) and used by males to increase their signalling space in order to find the receptive females that are scattered unpredictably in the environment. The studies aimed at describing the role of vibrational signals in the context of species recognition showed (a) that vibrational signals are species- and sex-specific; (b) that signals emitted by males are more complex than those emitted by females; and (c) that partners exchange signals in a coordinated, stereotyped manner, in which a female reply follows vibrational signal emitted by a male. However, the results also revealed that leafhopper species differ greatly in the repertoire, structure and complexity of emitted signals, as well as in a duet structure (e.g. Tishechkin, 2000; Percy *et al.*, 2008; Mazzoni *et al.*, 2009; Derlink *et al.*, 2014).

Cereals, important crops in the world for both human and domestic animals, can be infected by different pathogens of which the *Wheat dwarf virus* (WDV, genus *Mastrevirus*, family *Geminiviridae* [ICTV report, 2012]) is the causal agent of dwarfing, mottling and yellowing symptoms in wheat, barley and oat. The wheat dwarf disease, described for the first time in the 1960s in a wheat (*Triticum aestivum* L.) field in the former Czechoslovak

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79 Socialist Republic (Vacke, 1961), is among the most important sanitary issues in cereals
80 production. WDV is transmitted from plant-to-plant by leafhoppers of the genus
81 *Psammotettix* (Deltocephalinae), holartic insects commonly found in cereal fields and in
82 grassland (Lindblad & Areno, 2002). Parameters linked to the WDV/*Psammotettix*
83 pathosystem are still poorly documented in the literature (reviewed in Abt & Jacquot, 2015).
84 Due to the lack of genetic resistance sources and the absence of anti-viral molecules,
85 currently the main protection strategy used against WDV is based on the area-wide use of
86 chemicals against the leafhoppers vectors.

87 The main objective of the present study was to provide the essential information on the
88 reproductive behaviour of *P. alienus* (Dahlbom 1850), which is considered the main WDV
89 vector (Zhang *et al.*, 2010). Although vibrational signals emitted by males of *P. striatus* (L.
90 1758), *P. confinis* (Dahlbom 1850), *P. narsikulovi* (Dlabola 1960), *P. pictipennis* (Kirchbaum
91 1868) and *P. poecilus* (Flor 1861) have been recorded (Tishechkin, 1999; Tishechkin, 2000),
92 the information on other aspects of vibrational communication associated with mating
93 behaviour in this genus is lacking. Genus *Psammotettix* is considered a taxonomically
94 challenging group and on the one hand, such knowledge is needed for a reliable delimitation
95 and identification of vectors, since behavioural characters are the most accurate ones to
96 delimit species (Schlick-Steiner *et al.*, 2010). On the other hand, a more detailed knowledge
97 on mating behaviour may also provide information on which the direction of more
98 environmentally friendly control practices can be developed (Weintraub & Beanland, 2006).
99 Due to increased awareness of harmful effects of pesticides on biodiversity and human
100 health, the exploitation of vibrational signals in pest management has in recent years received
101 an increased attention (Čokl & Millar, 2009; Eriksson *et al.*, 2012; Mankin *et al.*, 2013;

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Polajnar *et al.*, 2015; Polajnar *et al.*, 2016; Korinšek *et al.*, 2016). In the study we also included virus-free and viruliferous individuals in order to obtain some preliminary information whether viruliferous status of insects has an effect on the pre-copulatory mating performance.

Materials and Methods

Insects

Adult *P. alienus* leafhoppers were collected using a sweep net in June and September in years 2012 and 2013 in various cereal fields in North and South France and also from grassland and vineyards in West Slovenia in August 2014.

Based on the sanitary status of the gravid females collected in 2012 (determined by PCR methods [see Supplementary Material]), virus-free and viruliferous (WDV wheat strain [WDV-w] and WDV barley strain [WDV-b]) leafhopper populations were initiated using selected progenies. These leafhopper populations were reared in large plexi-glass cages (50 × 50 × 80 cm) in a temperature controlled chamber (24°C during the day/20°C during the night, 40% RH, 16/8, day/night period). Fresh potted plants were added to the cages every month. While virus-free and WDV-w leafhoppers were maintained on wheat cv. *Sunstar* (Posadas & Henry, 2002), WDV-b *Psammotettix* populations were maintained on barley cv. *Express* (Sadeghi *et al.*, 2000) according to the host specificity of the WDV strains described in the literature (reviewed in Abt & Jacquot, 2015).

Leafhoppers collected in the field in 2013 were maintained in the laboratory individually on a 2-leaf cereal plantlet covered by a micro-perforated cellophane bag in a temperature

controlled chamber as described above. For four weeks following the collection, the individual rearing systems were daily monitored for the presence of eggs and larvae. For each of the 330 gravid females collected in field, up to 10 larvae were transferred individually on young cereal plantlets and reared as described above in order to obtain virgin adults. The sanitary status of the 330 gravid females was determined by PCR method (see Supplementary Material).

Individuals collected in Slovenia in 2014 were maintained in individual rearing systems as described above until used in the experiments. The sanitary status of the insects was determined by PCR method.

Recording vibrational signals and behaviour

Experimental set-up All experiments were performed on wheat *cv. Sunstar* plantlets (approximate height 14 cm, 2-leaf stage, 1 leaf removed) at room temperature (20–25°C and at 40%–50% RH). Individual plantlet was planted in a plastic vial filled with vermiculite and placed upright into a jar filled with moist artificial substrate. The plantlet was positioned within a circular opening (10 cm diameter) of a custom made wooden tripod and the hole was covered with overlapping incised paper circles surrounding the plant thus creating a platform and separating the upper and lower part of the plantlet (de Groot *et al.*, 2012). The upper part was covered with a transparent plastic vial (height 9.5 cm, diameter 2.5 cm) to prevent leafhoppers from escaping.

Vibrational signals were registered from the plantlet 1 cm below the platform, with a laser vibrometer (PDV-100, Polytec, GmbH, Waldronn, Germany) and stored in a computer using a Sound Blaster 2 ZS sound card (Creative, Singapore) and Cool Edit pro 2.0 software

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(Syntrillium Software, Phoenix, USA) at the sampling rate 48 kHz and 16 bit resolution. In order to increase reflectance, a small piece of reflective tape was placed on the stem at the recording location 1 cm below the platform.

The set-up also enabled playback stimulation with the pre-recorded vibrational signals (see below). We applied vibrational stimuli to the plantlet stem approximately 3 cm below the platform via the conical tip of the 5-cm metal rod (4 mm in diameter) screwed firmly into the head of a vibration exciter (Minishaker type 4810, Brüel & Kjær, Nærum, Denmark). The vibration exciter was driven from the computer via the above mentioned sound card by Cool Edit Pro2 program. The amplitude of stimulation was adjusted to the level of naturally emitted vibrational signals registered at the point of recording.

Male and female calling songs

Calling signals were defined as signals that are emitted spontaneously by isolated individuals (de Groot *et al.*, 2012). We conducted seven overnight recording sessions (between 18:00 and 08:00 hours) of single individuals that included five males (three virus-free, one WDV-w viruliferous, one WDV-b viruliferous) and two females (both virus-free). A single leafhopper was placed to the plantlet above the platform and recorded vibrational signals were automatically stored in a computer every 30 min using Raven 1.4 software (Cornel Laboratory of Ornithology, Ithaca, USA). From the recordings, we determined the time periods when leafhoppers were signalling and measured the duration of calling bouts.

Subsequent experiments were carried out between 08:00 and 17:00 hours. Vibrational activity of single leafhoppers was recorded for 15 to 60 min. In order to induce signalling, females were stimulated with a pre-recorded *P. alienus* male call. From our library of

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recordings, we randomly chose a calling song of one virus-free male and the stimulatory sequence was 7.6 s long and included 6 pulse trains (pulse train duration, mean $\pm$ SD = 0.11 $\pm$ 0.01 s; pulse train repetition time = 1.51 $\pm$ 0.29 s). If a female did not start emitting vibrational signals, stimulation was repeated every 10 s for 20 min.

In the analyses of male calls, we included signals from 28 healthy males and from 7 and 12 WDV-w and WDV-b viruliferous leafhoppers, respectively. In the analyses of female calls we included signals from 18 virus-free leafhoppers and from 5 and 14 WDV-w and WDV-b viruliferous individuals, respectively. We measured the following parameters: pulse train duration, pulse repetition time within the train, pulse train repetition time, calling bout duration and dominant frequency.

Females used in mating experiments (see below) were after copulation kept separately on young wheat plantlets and after they had laid eggs their progenies were maintained as separate lineages. Vibrational signals of 12 F1 individuals (9 males and 3 females) from three virus-free lineages were recorded two months after the copulation as described above.

Female preferences

To test the preference of *P. alienus* females, 26 females (9 virus-free, 17 viruliferus [WDV-b]) were placed singly on a plantlet and stimulated with a sequence that included calling songs of virus free and viruliferus (WDV-b) *P. alienus*, calling songs of two *P. confinis* males and male advertisement call of the leafhopper *Gramphocraerus ventralis* (Table 1). Within the stimulation sequence, each song was repeated five times in a random order separated by 30 s silent intervals. All male songs included in the stimulatory sequence were taken from our

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library of recordings and had been recorded with the laser vibrometer mentioned above not more than 3 cm away from the calling male. Vibrational signals of *Psammotettix* males were registered on a wheat plantlet, while the male call of *G. ventralis* was recorded on the clover (*Trifolium pratense*). Each female was tested with the same stimulation sequence; however, for each trial the starting song was set randomly. Since female calling song can last for several minutes, the male calling song during which the female started signalling was scored as the preferred call. The playback was stopped at the end of the male song; however, the recordings continued until the end of the female calling bout. We measured the signalling latency (time from the beginning of the preferred calling song to the first emitted female pulse train) and the duration of the emitted female calling song.

Mating behaviour

Eighteen pairs of virgin individuals were used to describe mating behaviour and we included virus-free leafhoppers, as well as viruliferous individuals (Table 2). A male and a female were placed on a plantlet and their behaviour and emitted vibrational signals were monitored until the end of copulation. Care was taken to position the partners as far apart as possible; however, due to their small size (3–4 mm) and high mobility, the initial distance between the male and the female on the plantlet was variable. Vibrational signals emitted during the mating sequence were recorded as described above. For seven pairs leafhopper behaviour together with the emitted vibrational signals was simultaneously recorded with a 3CCD video camcorder (Canon DM XM2) fitted with a 4.2–84 mm zoom lens (Table 2).

In all experiments, we monitored the following parameters: male calling latency (time from the beginning of the experiment to the first pulse train emitted by male), female calling

latency (time from the beginning of the male calling song to the first pulse train emitted by the female), the number of emitted courtship signals, mating sequence duration (time from the emission of the first male or female pulse train to the beginning of copulation) and duration of copulation (Table S1). Whenever possible, we also determined the search duration (time from the onset of a duet to the location of the female) and close-range courtship duration (time between the location of the female and the beginning of copulation).

In addition, 49 females of *P. alienus* collected in the field in September 2013 that had laid eggs in the laboratory (i.e. they mated in the field) were after one month after egg deposition stimulated with a pre-recorded *P. alienus* song described above in order to determine whether they regained sexual receptivity.

Rivalry

To stage a rivalry situation, two males ($N = 25$ male pairs; 12 pairs of virus-free males, 5 pairs of a virus-free male and a WDV-b male, 3 pairs of a WDV-w male and WDV-b male, 5 pairs of WDV-b males) were placed on a wheat plantlet and we played them a pre-recorded female calling song. From our library of recordings, we randomly chose a 60 s long recording of a calling song of one virus-free female. The sequence contained 46 pulse trains (duration: mean $\pm$ SD = 0.56 ± 0.04 s; pulse train repetition time: 1.31 ± 0.07 s). The stimulatory sequence was played in a loop throughout the experiment; however, we randomly stopped the playback for 30 s intervals. Vibrational activity was recorded for 30 min.

Terminology and statistical analyses

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Pulse was defined as a unitary homogenous parcel of sound of finite duration (de Groot *et al.*, 2012), while a pulse train was characterized as a group of pulses with a characteristic sequence or form. We defined song as a bout of repeated pulse trains. Since pulse train repetition time was not always regular, the end of the bout was determined at the start of the inter-pulse train interval longer than 5 s. Vibrational signals recorded exclusively during the later close-range stages of the mating sequence were defined as courtship signals, while signals emitted immediately prior to copulation attempts were termed pre-copulatory signals.

Data are presented as means and standard deviations, together with the numbers of leafhoppers from which signals were obtained (N) and the numbers of signals analysed (n). Dominant frequency data are presented as medians together with ranges. For quantifying calling activity of single leafhoppers throughout the night, the cumulative calling time was used to represent the calling activity in each hour. We obtained the cumulative calling time for each individual by summing up the durations of all calling bouts (i.e. calling songs) in these periods.

The parameters of calling songs (pulse repetition time, pulse train duration, pulse train repetition time and dominant frequency) in each sex were compared between all viruliferous leafhoppers (WDV-w and WDV-b) and virus-free individuals (collected in the field and the progeny). Similarly, in each sex these parameters have been compared between virus-free individuals from the field and virus-free progeny reared in the laboratory. Data were first checked for normality with Shapiro-Wilk normality test and accordingly analysed either with nonparametric Wilcoxon rank sum test or parametric Student's t -Test. The proportions of all females responding to either conspecific or heterospecific male calls included in the stimulatory sequence used in female preference tests were compared with Test of equal of

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given proportions, while correlations between sanitary status of the females and their preferences for male calling songs were calculated using two-tailed Fisher's exact test. All statistical analyses were conducted using R version 3.2.1.

Results

General observations

Overnight recordings of single individuals revealed that both sexes emit vibrational signals spontaneously. The overall calling time during the night was longer in female (13020 s and 8522 s) than in males (2413 ± 2041 s), and was a result of longer pulse train durations (see below). Although calling activity varied between individuals, the emerging pattern suggests that while female calling activity is low in the period between midnight and 6 am, males are calling throughout the whole night (Fig. 1).

All recorded vibrational signals associated with the earlier stages of the mating sequence consisted of a series of single pulses and the main difference between the male calling song, female calling song and male rivalry song was in the pulse train duration and pulse repetition time (Table 3, Figs. 2, 5).

Male calling song

Male calling song is characterized by short pulse train duration, highly regular pulse repetition time within the train and less regular pulse train repetition time within the song (Fig. 2, Table 3). Pulse trains span broad frequency range and have clear harmonic structure

with dominant frequency between 130 and 1000 Hz. Duration of the registered male calling songs was variable (10.5 ± 5.9 s, $N = 70$, $n = 70$).

The analysed dominant frequencies, pulse train durations and pulse repetition time of the male calling song did not differ significantly between the virus-free and viruliferous males (Student's t -Test, $P > 0.05$), while viruliferous males emitted songs with significantly longer pulse train repetition time (Student's t -Test, $P = 0.04$).

Parameters of calling song emitted by field collected virus-free males and by virus-free males from a progeny produced in the laboratory were similar; however, in the latter, the pulse train repetition time was significantly longer (Student's t -Test, $P = 0.008$) (Table 3). However, eight of the nine F1 males tested belonged to the same lineage that appears to be characterized by long pulse train repetition time (Table S2).

Female calling song

In comparison with the male calling song, female calling song is characterized by longer pulse train duration, longer and less regular pulse repetition time within the train and more regular pulse train repetition time (Figs. 2, 3, Table 3). As in the male calling song, pulse trains span broad frequency range and also have similar dominant frequencies; however, without the harmonic structure. Duration of the registered female calling songs was highly variable (42.92 ± 43.95 s; $N = 41$, $n = 41$). The analysed parameters of female calling song did not differ significantly between the virus-free and viruliferous females (Student's t -Test, $P > 0.05$) and neither did between the female progeny and virus-free females (Student's t -Test, $P > 0.05$).

Female preferences

In the tested *P. alienus* females the emission of calling song was clearly associated with the conspecific male calling song (Test of equal or given proportions, $P < 0.001$) (Table 4). None of the females responded to *G. ventralis* male advertisement call; however, four *P. alienus* females started signalling during the playback of the *P. confinis* male calling song. In three of these females the preceding male calling song in the stimulatory sequence was the conspecific male song. Preferences for *P. alienus* males and *P. confinis* males in the virus-free and viruliferous females did not differ significantly (Fisher's exact test, $P = 0.591$) and we did not observe any correlation in preferences for the male calling songs emitted by the virus-free and viruliferous *P. alienus* males and virus-free and viruliferous females (Fisher's exact test, $P = 1$), however the number of the tested individuals was low (Table 4).

Mating behaviour

In general, observations did not reveal significant differences in mating behaviour between the virus-free and viruliferous leafhoppers (Table S1). On average, the complete mating sequence duration (excluding copulation) was 54 ± 26 min (minimum = 11 min, maximum = 101 min, $N = 15$). The main stages of the mating sequence in *P. alienus* are shown in the Supplementary movie and in Fig. S1. Broadly, the stages can be described as recognition, approach, courtship and pre-copula phase. In 17 out of 18 trials that finished with copulation, the first registered vibrational signal was a male calling song (male calling latency = 199 ± 255 s). On average, females emitted calling song 88 ± 164 s after the first emitted pulse train by male. The first vibrational interaction was a coordinated duet in which a male and a female regularly exchanged pulse trains in an M-F-M-F sequence (Fig. 3).

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After vibrational contact was established, the male started to approach the stationary female. On average, the approach stage lasted 24 ± 18 min (minimum = 3 min, maximum = 51 min, $N = 7$). During this stage vibrational interaction between a male and a female was less precisely coordinated and leafhoppers continued to emit calling songs when the partner stopped calling (Fig. 2b). Males were signalling only when they were not walking and often they only sporadically emitted single pulse trains.

When the male reached the female, he positioned himself behind or on the side of the female, facing in the same direction (Fig. S1A, B). On average, the close-range courtship lasted for 34 ± 17 minutes (minimum = 10 min, maximum = 64 min, $N = 7$). While during the courtship stage most females stopped calling, most males continued emitting the calling song; however, male calling activity was lower than during the earlier stages. Courtship stage was usually associated with a continuous emission of pulses emitted with irregular repetition time; however, we were not able to determine whether they were produced by a male or a female. During the close-range courtship males were rubbing and tapping the females with the front leg (Fig. S1C) and flapping their wings (Fig. S1D). The latter resulted in distinct courtship vibrational signals recorded in the substrate (Table 3, Fig. 4). Pulse trains produced by wing flapping span broad frequency range and within the main frequency band (100–1000 Hz) they have a clear harmonic structure with dominant frequency between 100 and 400 Hz. Courtship signals produced by wing flapping were registered in all mating sequences that concluded with copulation (minimum = 2; maximum = 25, $N = 16$).

Copulation attempts were associated with a distinct complex vibrational signal composed of a 1-3 s long pulse train (buzz), followed by high amplitude pulses produced by wing

flapping and vibrating one wing over the female (Fig. 4 and Fig. S1E). The average duration of copulation was 522 ± 88 s ($N = 11$).

Results of recordings of virus-free females that had mated in the field and have laid eggs in the laboratory showed that 26% of *P. alienus* females started signalling again when stimulated with the conspecific male calling song.

Rivalry behaviour

In seven out of 25 tested male pairs that were presented with a playback of the female calling song, males emitted another type of vibrational signal (Fig. 5). These signals were recorded in two pairs of virus-free males, two pairs of a virus-free male and a WDV-b male and three pairs that included a WDV-b male and a WDV-w male. Although males usually started to emit this signal in the interval between pulse trains in the female calling song, it always overlapped at least part of the pulse train emitted by the female (Fig. 5). Although the temporal and spectral parameters of this putative masking signal were variable, it is characterized by a long pulse train duration and long pulse repetition time (Table 3). Pulse train repetition time is not regular. Masking signal spans broad frequency range with dominant frequencies between 400 and 1200 Hz (Fig. 5).

In all trials in which the putative masking signals were recorded, they were preceded by the male calling song emitted by a live male. On average we recorded 23 (SD = 19, minimum = 2, maximum = 51) masking signals per trial. Due to the small size of *P. alienus* leafhoppers, it was not possible to determine which male emitted the recorded calling songs and masking signals.

Discussion

Results of the present study show that as in other Cicadellidae, mating behaviour in *P. alienus* is stereotyped and associated with the emission of species- and sex-specific vibrational signals. However, our results also revealed important differences with other leafhopper species so far studied in detail. The unique features of vibrational communication in *P. alienus* are: (i) that the pair formation is initiated by either gender; (ii) the same general, simple structure of male and female calling song and (iii) progression from a patterned to a loosely coordinated duet.

For leafhoppers, it is generally accepted that mating sequence is initiated by the emission of male calling signals (Claridge, 1985; Hunt & Nault, 1991; Čokl & Virant-Doberlet, 2003; Mazzoni *et al.*, 2009; de Groot *et al.*, 2012). However, one older study indicated that females also show low levels of spontaneous calling activity and can rarely also initiate a pair formation (Saxena & Kumar, 1984). Although in mating experiments, most duets were initiated by the male, taken together, the structure of the female calling song (see below) and high spontaneous female calling activity in overnight recordings suggest that in *P. alienus* females may be equally likely to trigger the exchange of vibrational signals. While apparently rare in leafhoppers, such behaviour seems to be more common in planthoppers (e.g. Virant-Doberlet & Žežlina, 2007; Mazzoni *et al.*, 2010). However, mating systems are shaped in part also by ecological aspects of the environment, including population density (Virant-Doberlet & Žežlina, 2007). Despite considerable economic importance of leafhoppers, we still know very little about their mating systems and reproductive strategies under field conditions and

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future studies should provide more insight into mechanisms that are involved in the evolution of different communication strategies in Auchenorrhyncha.

In contrast to males, mated leafhopper females stop signalling and, consequently, males, cannot locate them (Bailey & Nuhardiyati, 2005; Mazzoni *et al.*, 2009) and it is usually accepted that in leafhoppers, females, unlike males, most likely mate only once in their lifetime. Our results show that after they laid eggs mated *P. alienus* females regained their sexual receptivity. In the absence of studies of *P. alienus* mating behaviour under field conditions it is not clear whether this result reflects a natural situation. However, it has been observed that in the field leafhopper females can mate multiply (Hayashi & Kamimura, 2002) and that when ejaculate size is small, some females become responsive again after a post-mating refractory period (Bailey & Nuhardiyati, 2005).

Calling signals are long-range signals associated with the initial stage of mating behaviour when individuals advertise their presence and readiness to mate and should reveal the identity (species and gender) of the signaller and also provide information necessary to determine its location (Čokl & Virant-Doberlet, 2003). This is in agreement with our results that in *P. alienus* vibrational signals used in the recognition stage enabled a reliable identification of the partner. However, studies done so far show that in leafhoppers calling signals emitted by males are more complex than those emitted by females (Čokl & Virant-Doberlet, 2003; Percy *et al.*, 2008; Mazzoni *et al.*, 2009; de Groot *et al.*, 2012; Derlink *et al.*, 2014). In contrast, in *P. alienus* male and female calling songs do not differ in their complexity and in both genders calling songs are simple and formed by regularly emitted pulse trains. In particular, female calling song in this respect more closely resembles female vibrational signals in the hemipteran species in which females initiate vibrational exchange

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between partners (e.g. the planthopper *Hyalesthes obsoletus* (Mazzoni *et al.*, 2010) and the pentatomid bugs [Čokl, 2008]) than female replies described in other leafhopper species. Consequently, a duet structure in *P. alienus* also differs from the exchange of male and female vibrational signals described in other Cicadellidae (e. g. Heady *et al.*, 1986; Percy *et al.*, 2008; Mazzoni *et al.*, 2009; Derlink *et al.*, 2014).

Coordinated duet observed in the recognition stage to some extent resembles a duet structure described in the early stages of the mating sequence in the leafhopper *S. titanus* (Polajnar *et al.*, 2014) and the planthopper *H. obsoletus* (Mazzoni *et al.*, 2010). Additional studies are needed to determine whether in *P. alienus* such coordinated duet results from resetting the endogenous oscillator generating the song rhythm by the perceived pulse trains emitted by the partner or partners listen out for each other and reply (Polajnar *et al.*, 2014). During the approach stage, a continuous emission of female calling song may be important for successful and quick localization. For small plant-dwelling insects relying on vibrational communication, locating the partner is difficult (Virant-Doberlet *et al.*, 2006). The experimental environment used in the current study was not complex enough to test the localization ability of *P. alienus*; however, even for leafhoppers with the body size of around 3–4 mm, vibrational signals provide a reliable information needed to locate the female (Polajnar *et al.*, 2014). As in other leafhoppers (Mazzoni *et al.*, 2009; Kuhelj *et al.*, 2015), *P. alienus* males were alternating periods of signalling with periods of walking. Less coordinated duet observed during the approach stage may result from inability to coordinate the activity of muscles involved in signal production and walking, or from inability to detect female vibrational signals while walking, since leg movements likely stimulate the same leg mechanoreceptors that also detect vibrational signals.

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As in some other Auchenorrhyncha that emit relatively simple vibrational signals during the earlier stages of the mating sequence (e.g. Virant-Doberlet & Žežlina, 2007; Mazzoni *et al.*, 2009), in *P. alienus* more complexity has been introduced at the later stages of mating behaviour. In leafhoppers, production of vibrational signals is costly (Kuhelj *et al.*, 2015) and it has been hypothesized that the cost may be rationalized by emitting more complex and energetically more demanding signals after the recognition and location of the female have been completed (Polajnar *et al.*, 2014). Wing flapping and wing vibrations produced by males during the close-range stages have been observed in several leafhopper species (Shaw *et al.*, 1974; Shaw, 1976; Saxena & Kumar, 1984; Heady *et al.*, 1986; Hunt & Nault, 1991). Our results show that wing movements result in distinct and stereotyped vibrational signals; however, the role of these signals is not clear since wing flapping can also be associated with visual and/or chemical signals.

Our results suggest that in the presence of a receptive female, competing *P. alienus* males emit vibrational signals most likely to interfere with male-female communication. Such signals have been described in other leafhopper species (Mazzoni *et al.*, 2009; Kuhelj *et al.*, 2015). The data obtained in the current study by playback experiments are not sufficient to determine whether these putative masking signals are used to disrupt a coordinated male-female duet or to mask female calling song. Future studies should reveal whether this species coordinated duet is vulnerable to disruption and whether rival males also use satellite behaviour to approach the female duetting with another male.

Preliminary results obtained in the current study suggest that the viruliferous status of the leafhopper does not have a significant effect on leafhopper performance in mating behaviour; however, our results also showed that pulse train repetition time in male calling song was

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466 significantly longer in viruliferous than in the virus-free males. Although due to the low
467 numbers of tested individuals the current results are not conclusive, they show that it is
468 worthwhile testing in more detail whether reproductive behaviour in *P. alienus* is influenced
469 by the virus. Plant viruses can modify vector performance via indirect effects of virus
470 infection on plant nutritional composition (Fiebig *et al.*, 2004) or directly via altering vector
471 behaviour and physiology (Ingwell *et al.*, 2012; Moreno-Delafuente *et al.*, 2013; Wan *et al.*,
472 2015). Behaviour associated with mating (advertising, walking, rivalry interactions) is
473 energetically demanding (Kuhelj *et al.*, 2015) and better availability of nutrients in infected
474 plants may have an important role on performance (Morehouse *et al.*, 2010). Leafhoppers
475 keep their feeding stylets inserted into plant tissue while emitting vibrational signals (Kuhelj
476 *et al.*, 2015) and there is indirect evidence that *P. alienus* males may also be signalling while
477 feeding (Tholt *et al.*, 2015). Furthermore, virus infection also alters other parameters of the
478 host plant (phenotypic and physiological) (Scholthof *et al.*, 2011) that can affect plant
479 transmission properties that can in turn affect the ability of leafhoppers to communicate with
480 conspecifics. Future studies on virus-host plant-vector interactions should also take these
481 potential effects into account.

482 Developing new approaches in pest management that could help to reduce the use of
483 pesticides is one of the main challenges in sustainable agriculture. Until recently, exploitation
484 of vibrational signals used in intraspecific communication has been rarely considered in pest
485 management even from a theoretical viewpoint. However, recent work done on *S. titanus*
486 (Eriksson *et al.*, 2012; Polajnar *et al.*, 2016) and psyllid *Diaphorina citri* (Mankin *et al.*,
487 2013) highlights that, although currently due to the lack of obvious technical solutions, the

implementation of acoustic methods in pest management may seem limited, more directed efforts may provide new perspectives in management strategies.

Acknowledgments

The authors acknowledge Gabrijel Seljak for his help in leafhopper identifications during field survey in Western Slovenia. The presented work has been supported by the Institut National de la Recherche Agronomique (France), Montpellier SupAgro (France) and by funding from the Slovenian Research Agency (research programme P1-0255).

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Table 1 Temporal and spectral parameters of the male calling songs included in the stimulation sequence used to test preferences of *Psammotettix alienus* females.

Male calling song	Sequence duration (s)	Number of pulse trains	Pulse train duration (ms)	Pulse train repetition time (s)	Dominant frequency (Hz)
<i>P. alienus</i> , virus-free	10	12	76 ± 5	0.88 ± 0.34 s	586
<i>P. alienus</i> , WDV-b	19	12	68 ± 6	1.7 ± 1.1 s	375
<i>P. confinis</i> 1	19	41	40 ± 9	0.46 ± 0.21 s	703
<i>P. confinis</i> 2	10	25	40 ± 5	0.41 ± 0.16 s	773
<i>G. ventralis</i>	7	70	62 ± 21	0.10 ± 0.03 s.	280

Means ± standard deviations are shown while for dominant frequency medians, minimum and maximum measured values (in brackets) are given.

WDV-b: individuals viruliferous for the WDV barley strain.

Table 2 Sanitary status of
Psammotettix alienus
individuals included in
observations of mating behaviour.

Male status	Female status	<i>N</i>	<i>N_v</i>
virus-free	virus-free	7	3
virus-free	WDV-w	1	-
virus-free	WDV-b	1	1
WDV-w	virus-free	1	-
WDV-w	WDV-w	1	-
WDV-w	WDV-b	2	2
WDV-b	virus-free	1	1
WDV-b	WDV-w	1	-
WDV-b	WDV-b	3	-

N: number of pairs included in observations.

N_v: subset in which mating behaviour was also recorded on video.

WDV-w: individuals viruliferous for the WDV wheat strain.

WDV-b: individuals viruliferous for the WDV barley strain.

Table 3 Temporal and spectral properties of vibrational signals emitted by *Psammotettix alienus*.

	<i>N</i>	<i>n</i>	Pulse repetition time (ms)	<i>N</i>	<i>n</i>	Pulse train duration (s)	Dominant frequency (Hz)
Male calling song – virus free	9	94	6 ± 1	28	344	0.07 ± 0.01	492 (141–107)
Male calling song – WDV-w	5	51	7 ± 1	7	99	0.08 ± 0.01	409 (129– 603)
Male calling song – WDV-b	10	93	7 ± 1	12	134	0.08 ± 0.01	345 (129– 991)
Male calling song – progeny, virus free	9	92	6 ± 1	9	74	0.08 ± 0.01	609 (375 – 914)
Female calling song – virus free	8	75	20 ± 6	18	221	0.45 ± 0.06	363 (117– 914)
Female calling song – WDV-w	5	49	20 ± 4	5	52	0.46 ± 0.10	492 (492–539)
Female calling song – WDV-b	10	99	24 ± 3	14	156	0.42 ± 0.05	387 (234 – 722)
Female calling song – progeny, virus free	3	33	15 ± 5	3	140	0.48 ± 0.06	469 (375 – 511)
Courtship signal	8	28	300 ± 32	8	28	8.09 ± 1.47	140 (108–450)
Masking signal	7	516	13 ± 2	7	160	4.03 ± 2.09	574 (390 ± 1100)

Means ± standard deviations are shown while for dominant frequency medians, minimum and maximum measured values (in brackets) are given.

WDV-w: individuals viruliferous for the WDV wheat strain.

WDV-b: individuals viruliferous for the WDV barley strain.

A – Pulse train repetition time of the viruliferous males was statistically different than in the virus-free males (Student's t-Test, $P = 0.004$); B – Pulse train repetition time of the virus-free progeny males was statistically different from the field-collected virus-free males (Student's t-Test, $P = 0.004$).

Table 4 Responsiveness parameters of *Psammotettix alienus* females to stimulation with conspecific and heterospecific male calling songs,

Male calling song	Calling females (%)	Female calling song latency (s)	Female calling song
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	Virus-free	WDV-b	Virus-free	WDV-b	Virus-free
<i>P. alienus</i> , virus-free	15.4	38.5	5.23 ± 3.18	8.68 ± 4.07	41,3 ± 30,1
<i>P. alienus</i> , WDV-b	11.5	19.2	18.04 ± 13.72	10.86 ± 7,62	34,0 ± 34,6
<i>P. confinis</i> 1	7.7	7.7	7.8; 13.5	14.2; 6.8	92; 2
<i>P. confinis</i> 2	0	0	-	-	-
<i>G. ventralis</i>	0	0	-	-	-

Means ± standard deviations are shown. When N < 3, measured values are given.

Significantly higher number of females started calling during the presentation of the conspecific male calling songs (Test of equal or given proportions, $P < 0.001$).

Fig 1. Nightly calling activity of individual male and female *Psammotettix alienus*. Virus-free females (N=2): black circle and black diamond; Virus-free males (N=3): white squares; WDV-W infected male (N=1): asterisk; WDV-b infected male (N=1): cross.

Fig. 2 Male (A) and female (B) calling song of *Psammotettix alienus*. For each signal, the spectrogram is shown above the corresponding waveform. Insets show expanded individual female pulse trains. A male (M) – female (F) duet shown in (B) was registered during the approach stage.

Fig. 3 Coordinated male (M) – female (F) duet in *Psammotettix alienus* registered during the recognition stage.

Fig. 4 Representative courtship (a) and pre-copulatory (b) signals emitted by male *Psammotettix alienus*.

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724 Inset in (a) shows expanded section of the signal.

725
726 Fig. 5 Putative masking signals (MS) emitted by male *Psammotettix alienus*. (F) female pulse trains.
727 Inset shows expanded section of the signal.

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