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7
8 ***Original article***

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

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17
18 **Abstract**

19
20 The *Wheat dwarf virus*, the causal agent of the wheat dwarf disease, is transmitted by
21 leafhoppers from the genus *Psammotettix* and currently the main protection strategy is based
22 on the use of insecticide treatments. Sustainable management strategies for insect vectors
23 should include methods that are targeted to disrupt reproductive behaviour and here we
24 investigated the mating behaviour of *Psammotettix alineus* (Dahlbom 1850) in order to
25 determine the role of vibrational signals in intra-specific communication and pair formation.
26 Both genders spontaneously emit species- and sex-specific calling songs that consisted of

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

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

42 43 44 **Introduction**

45 Substrate-borne vibrations play an important role in mating behaviour of many insect pests
46 from the order Hemiptera (e.g. Kanmiya, 2006; Čokl, 2008; Mazzoni *et al.*, 2009; Mazzoni *et*
47 *al.*, 2010; Eben *et al.*, 2015). Leafhoppers (Hemiptera: Auchenorrhyncha: Cicadellidae) are,
48 with more than 22 000 described species, one of the most speciose groups of phytophagous
49 insects and are among the most important vectors of plant diseases (Weintraub & Beanland,
50 2006). Vibrational signals have been described in a great number of leafhopper species (e.g.,
51 Tishechkin, 2000; Čokl & Virant-Doberlet, 2003); however, taking into account the
52 economic importance of leafhopper vectors and leafhopper diversity, it is surprising that a
53 more detailed and comprehensive knowledge about the role of vibrational communication in
54 leafhopper mating behaviour is limited to three species, namely *Graminella nigrifrons*
55 (Heady & Nault, 1991; Hunt & Nault, 1991; Hunt & Morton, 2001), *Scaphoideus titanus*

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

58 In Cicadellidae, mate recognition and location are mediated exclusively by vibrational
59 signals and the emerging general pattern of a leafhopper mating sequence is (a) that pair
60 formation begins with the emission of a male advertisement call and (b) that after vibrational
61 contact has been established, male searches for a replying stationary female (Heady & Nault,
62 1991; Hunt & Nault, 1991; Mazzoni *et al.*, 2009; de Groot *et al.*, 2012; Polajnar *et al.*, 2014).
63 The leading role of the male in the pair formation is associated with the ‘fly/jump/walk-call’
64 strategy found in many Auchenorrhyncha (Hunt & Nault, 1991; De Luca & Cocroft, 2011; de
65 Groot *et al.*, 2012; Polajnar *et al.*, 2014) and used by males to increase their signalling space
66 in order to find the receptive females that are scattered unpredictably in the environment. The
67 studies aimed at describing the role of vibrational signals in the context of species recognition
68 showed (a) that vibrational signals are species- and sex-specific; (b) that signals emitted by
69 males are more complex than those emitted by females; and (c) that partners exchange signals
70 in a coordinated, stereotyped manner, in which a female reply follows vibrational signal
71 emitted by a male. However, the results also revealed that leafhopper species differ greatly in
72 the repertoire, structure and complexity of emitted signals, as well as in a duet structure (e.g.
73 Tishechkin, 2000; Percy *et al.*, 2008; Mazzoni *et al.*, 2009; Derlink *et al.*, 2014).

74 Cereals, important crops in the world for both human and domestic animals, can be
75 infected by different pathogens of which the *Wheat dwarf virus* (WDV, genus *Mastrevirus*,
76 family *Geminiviridae* [ICTV report, 2012]) is the causal agent of dwarfing, mottling and
77 yellowing symptoms in wheat, barley and oat. The wheat dwarf disease, described for the
78 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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102 Polajnar *et al.*, 2015; Polajnar *et al.*, 2016; Korinšek *et al.*, 2016). In the study we also
103 included virus-free and viruliferous individuals in order to obtain some preliminary
104 information whether viruliferous status of insects has an effect on the pre-copulatory mating
105 performance.

106

107 **Materials and Methods**

108

109 *Insects*

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

113 Based on the sanitary status of the gravid females collected in 2012 (determined by PCR
114 methods [see Supplementary Material]), virus-free and viruliferous (WDV wheat strain
115 [WDV-w] and WDV barley stain [WDV-b]) leafhopper populations were initiated using
116 selected progenies. These leafhopper populations were reared in large plexi-glass cages (50 ×
117 50 × 80 cm) in a temperature controlled chamber (24°C during the day/20°C during the night,
118 40% RH, 16/8, day/night period). Fresh potted plants were added to the cages every month.
119 While virus-free and WDV-w leafhoppers were maintained on wheat cv. *Sunstar* (Posadas &
120 Henry, 2002), WDV-b *Psammotettix* populations were maintained on barley cv. *Express*
121 (Sadeghi *et al.*, 2000) according to the host specificity of the WDV strains described in the
122 literature (reviewed in Abt & Jacquot, 2015).

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

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

131 Individuals collected in Slovenia in 2014 were maintained in individual rearing systems
132 as described above until used in the experiments. The sanitary status of the insects was
133 determined by PCR method.
134

135 *Recording vibrational signals and behaviour*

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

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

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

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

158

159 *Male and female calling songs*

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

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

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171 recordings, we randomly chose a calling song of one virus-free male and the stimulatory
172 sequence was 7.6 s long and included 6 pulse trains (pulse train duration, mean \pm SD = 0.11 \pm
173 0.01 s; pulse train repetition time = 1.51 \pm 0.29 s). If a female did not start emitting
174 vibrational signals, stimulation was repeated every 10 s for 20 min.

175 In the analyses of male calls, we included signals from 28 healthy males and from 7 and
176 12 WDV-w and WDV-b viruliferous leafhoppers, respectively. In the analyses of female calls
177 we included signals from 18 virus-free leafhoppers and from 5 and 14 WDV-w and WDV-b
178 viruliferous individuals, respectively. We measured the following parameters: pulse train
179 duration, pulse repetition time within the train, pulse train repetition time, calling bout
180 duration and dominant frequency.

181 Females used in mating experiments (see below) were after copulation kept separately on
182 young wheat plantlets and after they had laid eggs their progenies were maintained as
183 separate lineages. Vibrational signals of 12 F1 individuals (9 males and 3 females) from three
184 virus-free lineages were recorded two months after the copulation as described above.
185

186

187 *Female preferences*

188 To test the preference of *P. alienus* females, 26 females (9 virus-free, 17 viruliferous [WDV-
189 b]) were placed singly on a plantlet and stimulated with a sequence that included calling
190 songs of virus free and viruliferous (WDV-b) *P. alienus*, calling songs of two *P. confinis* males
191 and male advertisement call of the leafhopper *Gramphocraerus ventralis* (Table 1). Within
192 the stimulation sequence, each song was repeated five times in a random order separated by
193 30 s silent intervals. All male songs included in the stimulatory sequence were taken from our

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

204 205 *Mating behaviour*

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

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

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

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

227 228 *Rivalry*

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

237 238 *Terminology and statistical analyses*

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

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

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

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

265

266 **Results**

267

268 *General observations*

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

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

279

280 *Male calling song*

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

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

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

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

295 296 *Female calling song*

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

306

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307 *Female preferences*

308 In the tested *P. alienus* females the emission of calling song was clearly associated with
309 the conspecific male calling song (Test of equal or given proportions, $P < 0.001$) (Table 4).
310 None of the females responded to *G. ventralis* male advertisement call; however, four *P. alie*
311 *nus* females started signalling during the playback of the *P. confinis* male calling song. In three
312 of these females the preceding male calling song in the stimulatory sequence was the consp
313 ecific male song. Preferences for *P. alienus* males and *P. confinis* males in the virus-free and
314 viruliferous females did not differ significantly (Fisher's exact test, $P = 0.591$) and we did not
315 observe any correlation in preferences for the male calling songs emitted by the virus-free and
316 and viruliferous *P. alienus* males and virus-free and viruliferous females (Fisher's exact test, P
317 $= 1$), however the number of the tested individuals was low (Table 4).

318 319 *Mating behaviour*

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

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330 After vibrational contact was established, the male started to approach the stationary
331 female. On average, the approach stage lasted 24 ± 18 min (minimum = 3 min, maximum =
332 51 min, $N = 7$). During this stage vibrational interaction between a male and a female was
333 less precisely coordinated and leafhoppers continued to emit calling songs when the partner
334 stopped calling (Fig. 2b). Males were signalling only when they were not walking and often
335 they only sporadically emitted single pulse trains.

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

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

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

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

357

358 *Rivalry behaviour*

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

369 In all trials in which the putative masking signals were recorded, they were preceded by
370 the male calling song emitted by a live male. On average we recorded 23 (SD = 19, minimum
371 = 2, maximum = 51) masking signals per trial. Due to the small size of *P. alienus*
372 leafhoppers, it was not possible to determine which male emitted the recorded calling songs
373 and masking signals.

374

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375 **Discussion**

376

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

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

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

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

408 Calling signals are long-range signals associated with the initial stage of mating
409 behaviour when individuals advertise their presence and readiness to mate and should reveal
410 the identity (species and gender) of the signaller and also provide information necessary to
411 determine its location (Čokl & Virant-Doberlet, 2003). This is in agreement with our results
412 that in *P. alienus* vibrational signals used in the recognition stage enabled a reliable
413 identification of the partner. However, studies done so far show that in leafhoppers calling
414 signals emitted by males are more complex than those emitted by females (Čokl & Virant-
415 Doberlet, 2003; Percy *et al.*, 2008; Mazzoni *et al.*, 2009; de Groot *et al.*, 2012; Derlink *et al.*,
416 2014). In contrast, in *P. alienus* male and female calling songs do not differ in their
417 complexity and in both genders calling songs are simple and formed by regularly emitted
418 pulse trains. In particular, female calling song in this respect more closely resembles female
419 vibrational signals in the hemipteran species in which females initiate vibrational exchange

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

425 Coordinated duet observed in the recognition stage to some extent resembles a duet
426 structure described in the early stages of the mating sequence in the leafhopper *S. titanus*
427 (Polajnar *et al.*, 2014) and the planthopper *H. obsoletus* (Mazzoni *et al.*, 2010). Additional
428 studies are needed to determine whether in *P. alienus* such coordinated duet results from
429 resetting the endogenous oscillator generating the song rhythm by the perceived pulse trains
430 emitted by the partner or partners listen out for each other and reply (Polajnar *et al.*, 2014).
431 During the approach stage, a continuous emission of female calling song may be important
432 for successful and quick localization. For small plant-dwelling insects relying on vibrational
433 communication, locating the partner is difficult (Virant-Doberlet *et al.*, 2006). The
434 experimental environment used in the current study was not complex enough to test the
435 localization ability of *P. alienus*; however, even for leafhoppers with the body size of around
436 3–4 mm, vibrational signals provide a reliable information needed to locate the female
437 (Polajnar *et al.*, 2014). As in other leafhoppers (Mazzoni *et al.*, 2009; Kuhelj *et al.*, 2015), *P.*
438 *alienus* males were alternating periods of signalling with periods of walking. Less
439 coordinated duet observed during the approach stage may result from inability to coordinate
440 the activity of muscles involved in signal production and walking, or from inability to detect
441 female vibrational signals while walking, since leg movements likely stimulate the same leg
442 mechanoreceptors that also detect vibrational signals.

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443 As in some other Auchenorrhyncha that emit relatively simple vibrational signals during
444 the earlier stages of the mating sequence (e.g. Virant-Doberlet & Žežlina, 2007; Mazzoni *et*
445 *al.*, 2009), in *P. alienus* more complexity has been introduced at the later stages of mating
446 behaviour. In leafhoppers, production of vibrational signals is costly (Kuhelj *et al.*, 2015) and
447 it has been hypothesized that the cost may be rationalized by emitting more complex and
448 energetically more demanding signals after the recognition and location of the female have
449 been completed (Polajnar *et al.*, 2014). Wing flapping and wing vibrations produced by males
450 during the close-range stages have been observed in several leafhopper species (Shaw *et al.*,
451 1974; Shaw, 1976; Saxena & Kumar, 1984; Heady *et al.*, 1986; Hunt & Nault, 1991). Our
452 results show that wing movements result in distinct and stereotyped vibrational signals;
453 however, the role of these signals is not clear since wing flapping can also be associated with
454 visual and/or chemical signals.

455 Our results suggest that in the presence of a receptive female, competing *P. alienus*
456 males emit vibrational signals most likely to interfere with male-female communication. Such
457 signals have been described in other leafhopper species (Mazzoni *et al.*, 2009; Kuhelj *et al.*,
458 2015). The data obtained in the current study by playback experiments are not sufficient to
459 determine whether these putative masking signals are used to disrupt a coordinated male-
460 female duet or to mask female calling song. Future studies should reveal whether this species
461 coordinated duet is vulnerable to disruption and whether rival males also use satellite
462 behaviour to approach the female duetting with another male.

463 Preliminary results obtained in the current study suggest that the viruliferous status of the
464 leafhopper does not have a significant effect on leafhopper performance in mating behaviour;
465 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

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

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497

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630 **Table 1** Temporal and spectral parameters of the male calling songs included in the
631 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) | D |
|--------------------------------|-----------------------|------------------------|---------------------------|---------------------------------|-----|
| <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 |

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

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

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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.

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677 **Table 3** Temporal and spectral properties of vibrational signals emitted by *Psammotettix*678 *alienus*.

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| | <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 – 720) |
| 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 ± 111) |

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

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

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

685 A – Pulse train repetition time of the viruliferous males was statistically different than in the
686 virus-free males (Student`s t-Test, $P = 0.004$); B – Pulse train repetition time of the virus-free
687 progeny males was statistically different from the field-collected virus-free males (Student`s
688 t-Test, $P = 0.004$).

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

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| 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.

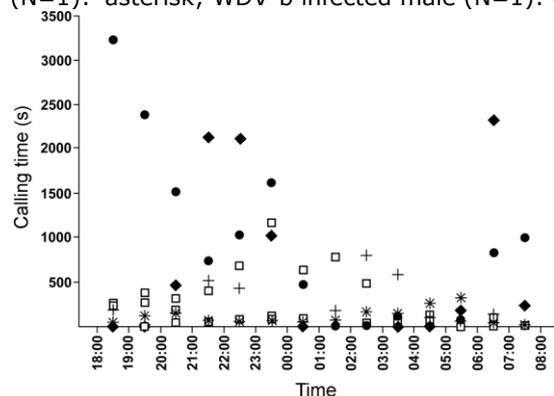
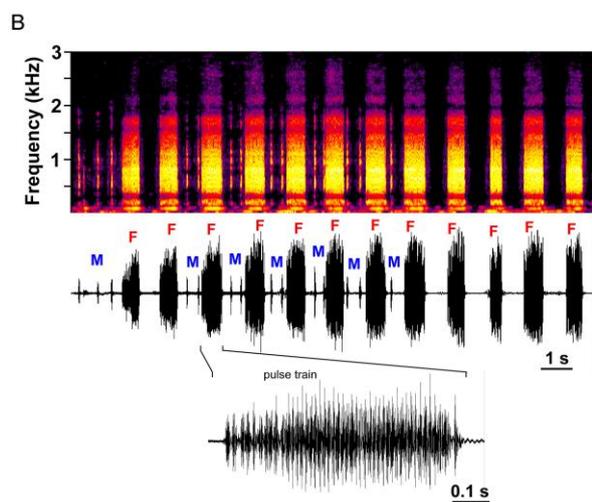
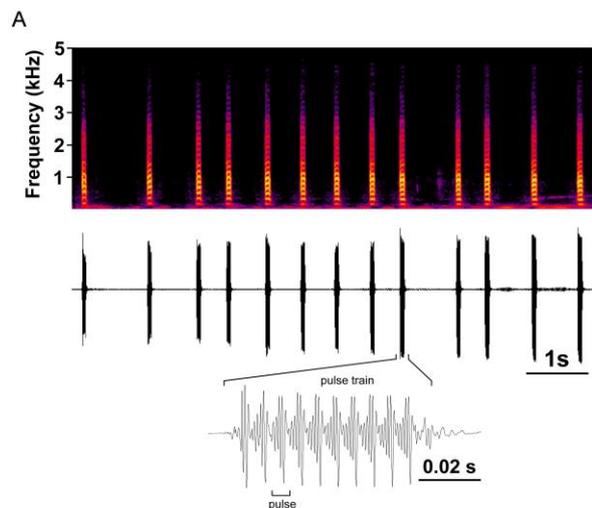


Fig. 2 Male (A) and female (B) calling song of *Psammotettix alienus*. For each signal, the spectrogram is shown above the corresponding waveform. Insets show expanded individual female pulse trains. A male (M) – female (F) duet shown in (B) was registered during the approach stage.

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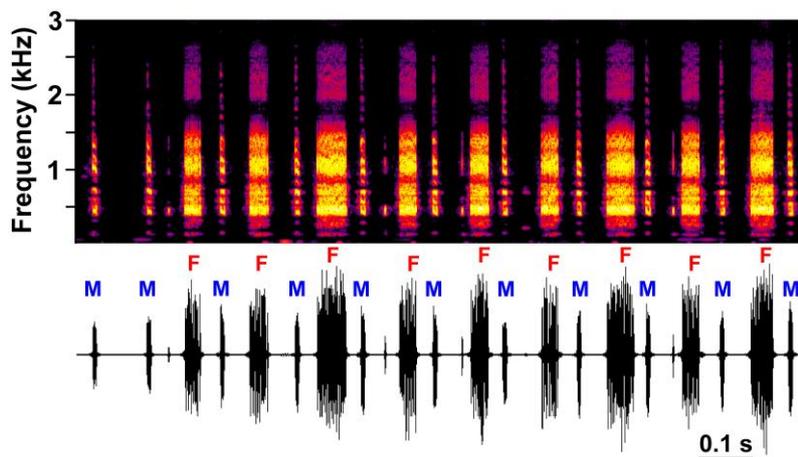


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Fig. 3 Coordinated male (M) – female (F) duet in *Psammotettix alienus* registered during the recognition stage.



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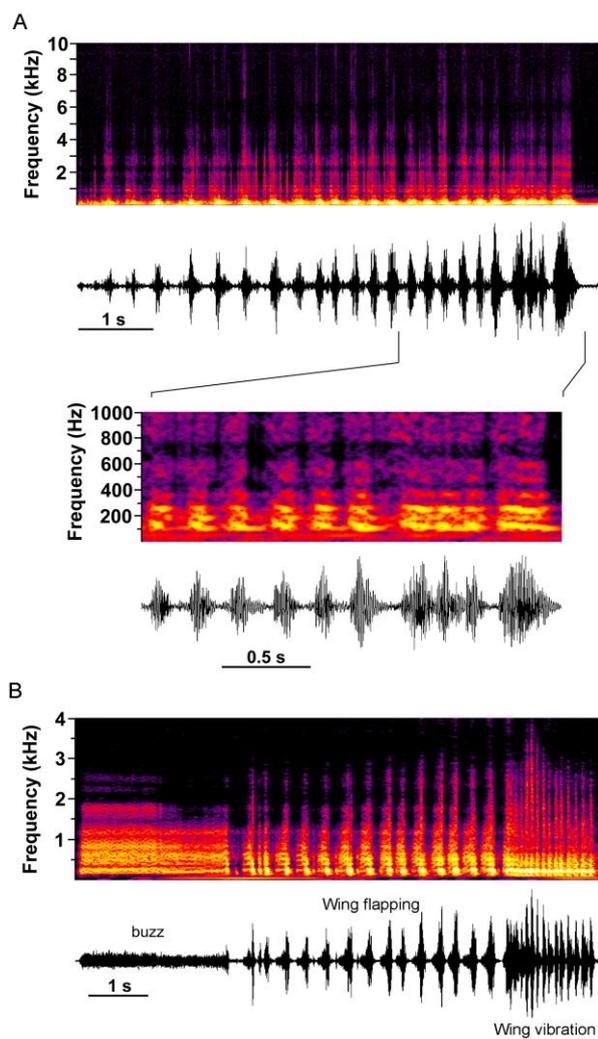
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Fig. 4 Representative courtship (a) and pre-copulatory (b) signals emitted by male *Psammotettix alienus*.

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

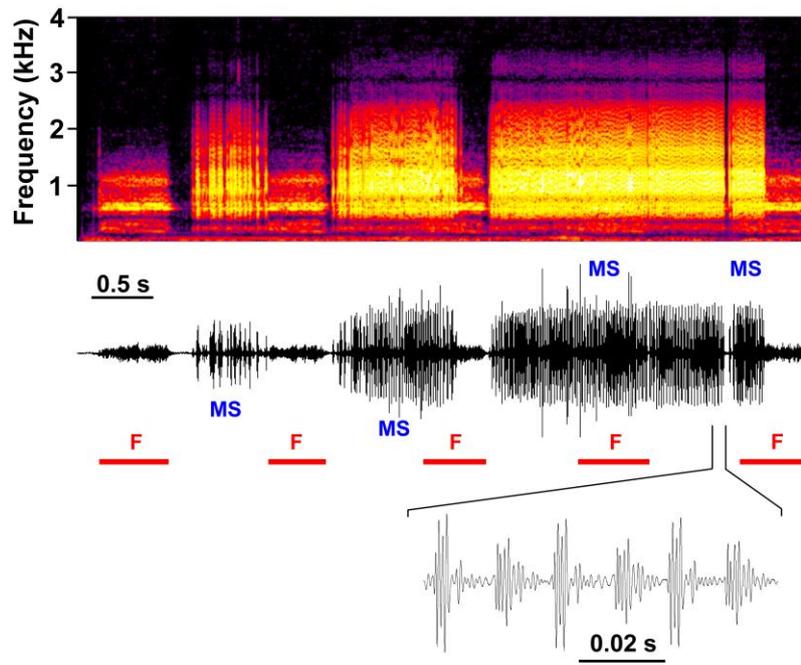


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726 Fig. 5 Putative masking signals (MS) emitted by male *Psammotettix alienus*. (F) female pulse trains.

727 Inset shows expanded section of the signal.

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