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The process of pair formation mediated by substrate-borne vibrations in a small insect

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ABSTRACT

The ability to identify and locate conspecifics depends on reliable transfer of information between emitter and receiver. For a majority of plant-dwelling insects communicating with substrate-borne vibrations, localization of a potential partner may be a difficult task due to their small body size and complex transmission properties of plants. In the present study, we used the leafhopper *Scaphoideus titanus* as a model to investigate duetting and mate searching associated with pair formation. Studying these insects on a natural substrate, we showed that the spatio-temporal structure of a vibrational duet and the perceived intensity of partner's signals influence the mating behaviour. Identification, localization and courtship stages were each characterized by a specific duet structure. In particular, the duet structure differed in synchronization between male and female pulses, which enables identification of the partner, while the switch between behavioural stages was associated with the male-perceived intensity of vibrational signals. This suggests that males obtain the information about their distance from the female and optimize their strategy accordingly. More broadly, our results show that even in insects smaller than 1 cm, vibrational signals provide reliable information needed to find a mating partner.

1. Introduction

Substrate-borne vibrational signalling is an ancient communication channel that is widely used by both invertebrates (Virant-Doberlet and Čokl, 2004; Cocroft and Rodriguez, 2005) and vertebrates (Hill, 2008). In insects alone, it is used by an estimated 195,000 species (Cocroft and Rodriguez, 2005), often exclusively, but has so far received much less attention than airborne sound communication.

The first step of mating sequences in sexually reproducing insects is pair formation that is achieved by identification and localization of a potential partner in the habitat (Alexander et al., 1997). Species-specific vibrational signals used in sexual communication enable identification of the emitter and provide directional information (e.g. Virant-Doberlet et al., 2006; Hill, 2008; Legendre et al., 2012; De Groot et al., 2012). In some insects that rely on vibrational communication, the searching for a mating partner has been described as "trial and error" (e.g. Gillham, 1992) while in others, individuals travelled a shorter path than they would during pure random search (Stewart and Sandberg, 2006; Legendre et al., 2012), suggesting that they extracted directional information from signals themselves.

Plants are the most common signalling substrate for invertebrates (Barth, 1998; Čokl and Virant-Doberlet, 2003; Cocroft and Rodriguez, 2005); however, they are complex structures and due to signal degradation and frequency filtering during transmission (Michelsen et al., 1982; Barth, 1998; Magal et al., 2000; Cocroft et al., 2006), signals may be distorted in the frequency and time domains (Michelsen et al., 1982; Miklas et al., 2001). Differences in amplitude and time of arrival of the vibrational signal to spatially separated vibration receptors in legs are the most obvious directional cues that insects may use (Virant-Doberlet et al., 2006). In insects, most vibration receptors are located in the legs (Čokl et al., 2006) and therefore the size (i.e. maximal leg span) of the insect is an essential factor for creating time or amplitude differences large enough to be directly used in orientation (Virant-Doberlet et al., 2006). Amplitude differences at distances as short as 2 cm are large enough to be detected in the nervous system of insects (Stritih et al., 2000; Čokl et al., 2006). On the other hand, the intensity gradient on plants may not be

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a reliable cue due to amplitude oscillations of vibrational signals during transmission (Michelsen et al., 1982; Čokl et al., 2007; Polajnar et al., 2012) and the role of amplitude in orientation behaviour is still under debate (Virant-Doberlet et al., 2006; Mazzoni et al., 2014). Furthermore, the majority of insects that rely on vibrational communication are smaller than 1 cm. In this case, deriving directional cues by directly comparing amplitude or time differences between sensory inputs may not be possible (Virant-Doberlet et al., 2006). Some small insects may instead be able to extract directional information from the mechanical response of the whole body (Cocroft et al., 2000), but solutions have been insufficiently studied.

In the present work, we describe pair formation and searching in a small plant-dwelling insect for which obtaining directional information may be difficult. We used the Nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae), which communicates with substrate-borne signals (Mazzoni et al., 2009a), as a model species. The body length of this leafhopper is around 5 mm, with a leg span that is probably too small to enable orientation by direct comparison of sensory inputs (Virant-Doberlet et al., 2006). Like other leafhoppers, *S. titanus* does not rely on chemical signals (Claridge, 1985; Mazzoni et al., 2009a), which allowed us to focus on vibrational cues alone.

In *S. titanus*, the male is searching for the female, and mating sequence is always initiated by the male emitting a calling signal (MCS) to which the stationary females respond with pulses emitted in gaps between the male pulses (Mazzoni et al., 2009a). A successful copulation is preceded by a male–female courtship duet (CrD), which can be disrupted by a rival male emitting a disturbance noise (DN) and taking over the duet. Duetting systems are common in arthropod communication (Claridge, 1985; Bailey, 2003; Virant-Doberlet and Čokl, 2004), often involving complex interactions where signalling is modified by the perception of the partner's reply (De Groot et al., 2012; Mazzoni et al., 2009a; Rodríguez et al., 2012). In such a system, replies by the stationary individual (usually the female) provide information needed for localization by the searching partner, but also by potential eavesdropping competitors (Bailey, 2003). Therefore, a male should optimize the process of gathering the necessary information from the female signals in order to reduce both the energetic costs and competition. To achieve this, a male should perform (1) accurate identification and (2) rapid localization, and should only begin with more complex and demanding courtship after these tasks have been accomplished. The same general principle has been recognized in numerous other animals before (Alexander et al., 1997), but the apparent monomodality of sexual communication in leafhoppers and the ability to accurately measure signals using laser vibrometry allow us to identify the cues that guide behaviour in these stages and trigger transitions between them. Understanding this process may then shed light on the problem of extracting information from vibrational signals by small insects.

In the present work, we therefore tested the following assumption: during pair formation, sexual behaviour progresses through different stages that are characterized and triggered by specific vibrational cues, favouring reliability of recognition and speed of localization before the onset of the most complex advertising stage of courtship. The process is facilitated by the ability of *S. titanus* males to use information in female signals to make directional decisions and detect female proximity despite their small body size.

2. Material and methods

2.1. Insects

Rearing of *S. titanus* from egg to adult followed the method described in Eriksson et al. (2011). All experiments were done with virgin and sexually mature males and females at least 8 days after their emergence (Mazzoni et al., 2009a). Each leafhopper was tested only once.

2.2. Experimental setup

We used grapevine cuttings with two different geometries as substrate. In each case the bottom of the stem was put in a glass vial filled with water to prevent withering and the vial was placed on an anti-vibration table (Astel s.a.s., Ivrea, Italy). In one case the cutting had two leaves (surface 6 cm × 10 cm) with petioles separated by a 10-cm long stem section (Fig. 1A), while in the other the cutting had three leaves with petioles separated by 5-cm long stem sections (Fig. 1B). For the purpose of analysis, the cuttings were divided into sections, each with a measuring point in the middle. For cuttings with two leaves, those were: (1) basal leaf, (2) basal petiole, (3) stem between the two leaves, (4) apical petiole and (5) apical leaf (Fig. 1A). For cuttings with three leaves, the section labelling was equivalent, but followed the position of male and female (Fig. 1B). The cuttings were replaced with equivalently shaped fresh ones as they wilted.

To prevent the insects from escaping, the setup was contained within a clear Plexiglass cylinder (50 cm × 30 cm). Mating behaviour was observed for 20 min or until the male reached the female, whichever came first. The experiments were performed at 23 ± 1 °C between 5 pm and 9 pm local time to obtain highest sexual activity from *S. titanus* (Mazzoni et al., 2009a), except in tests 1.2 and 1.4 where the temperature was 28 ± 1 °C.

Movement was recorded with a Canon MV1 miniDV camera (50 FPS). Vibrational signals were recorded with a laser vibrometer (Ometron VQ-500-D-V) and digitized with 48 kHz sample rate and 16-bit resolution, then stored directly onto a hard drive through a LANXI data acquisition device (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark). The laser beam was focused on a small piece of a reflective tape glued to each measuring point.

2.3. Data analysis and terminology

Spectral and temporal parameters of the recorded signals were analyzed with Pulse 14.0 (Brüel and Kjær) after applying Fast Fourier Transform (FFT) with window length of 400 samples and 66.7% overlap. The equipment was calibrated, which enabled direct measurements of the actual substrate velocity.

The terminology used for description of vibrational signals in *S. titanus* follows Mazzoni et al. (2009a). Vibrational signals not previously described were labelled according to their behavioural context.

2.4. Test 1. Signal parameters involved in pair formation

2.4.1. Test 1.1. Male–female synchrony in the course of mating behaviour

Pair formation was studied using a male and a female of *S. titanus* ($N = 20$ pairs), each placed on a different leaf of the same grapevine cutting with two leaves. Vibrational signals were registered from the measuring point on the lamina of the basal leaf with the male (Fig. 1A, point 1).

To analyze the synchrony of male–female pulses within vibrational duets, we measured pulse repetition time (=period) in male signal in presence and absence of female reply (i.e. female pulse), and female pulse latency (the interval between onsets of the male and the female pulse). Each of these parameters was analyzed throughout the whole male–female communication sequence, from the starting position when a male was on a different leaf than a female, through the male's searching phase to his arrival to the leaf with the female. To quantify the effect of female reply on

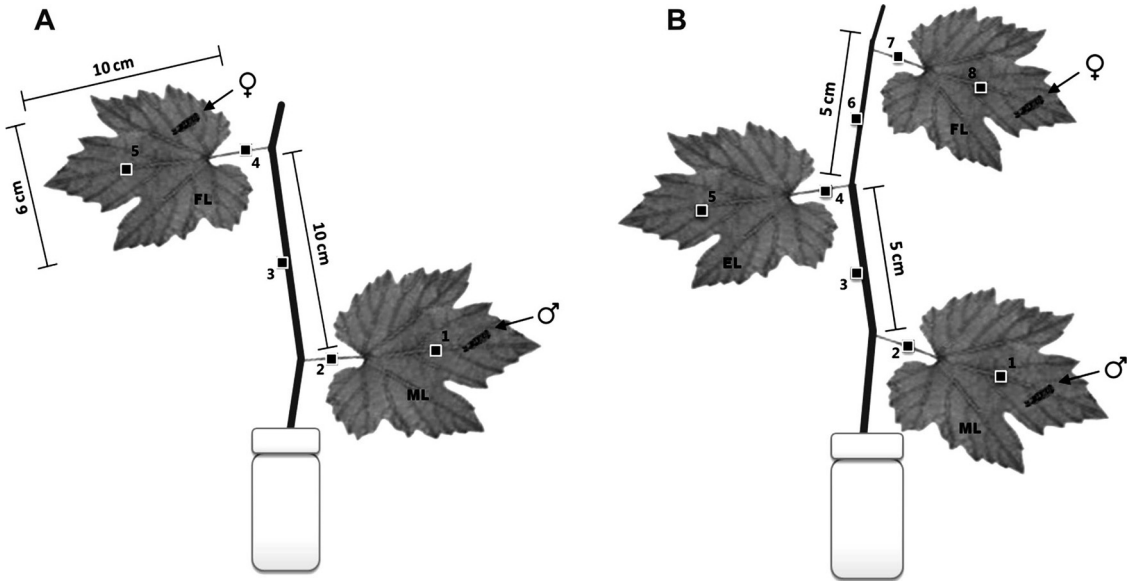

Fig. 1. Experimental setup of the two tests. In Test 1 (A), the laser was pointed towards a reflective tape on the male leaf lamina (ML, point 1). The other points are as follows: (2) male leaf petiole, (3) stem, (4) female leaf petiole, and (5) female leaf lamina. In Test 2.2 (B), 8 measuring points were distributed along the grapevine cutting: 6 on the leaves and 2 on the stem. The leaves were marked as male leaf (ML), female leaf (FL) or empty leaf (EL) according to the respective positions of male and female in each trial. In the example shown, the male was placed on the lower leaf, and the female on the upper one. The numbers therefore represent the following: (1) male leaf lamina, (2), male leaf petiole, (3) stem far from the female leaf, (4) empty leaf petiole, (5) empty leaf lamina, (6) stem near the female leaf, (7) female leaf petiole, and (8) female leaf lamina.

the period, we calculated male response phase (MRP) and female latency phase (FLP) (Greenfield, 1994). The MRP was equivalent to: $(((T' - T)/T) \times 360^\circ)$, where T and T' were the average pulse period in male signal in absence and in presence of female pulse, respectively. The FLP was equivalent to: $((\text{female pulse latency}/T) \times 360^\circ)$. The value of response phase delay was: $\alpha = \text{MRP/FLP}$. $\alpha = 1$ indicated a delay of an entire pulse period. A one tail paired t-test was used to compare the difference between T and T' in order to evaluate whether period increased during each behavioural stage. To determine whether female pulse latency and α values differed among the behavioural stages, we performed the Kruskal–Wallis test followed by the Steel-Dwass pairwise multiple comparison test, using statistics software KyPlot 5.0 (KyensLab Inc., Tokyo, Japan).

2.4.2. Test 1.2. Is courtship initiated by the male or by the female?

Test 1.1 clearly revealed a change of male signalling behaviour during the approach to a stationary female (see Section 3), detectable by the emission of a harmonic "buzz" that signifies progression from the location duet to the courtship duet (Mazzoni et al., 2009a).

Such a behavioural switch in a male may be elicited by a significant change in perception of the female response; however, the first question is whether the change of female signals is passive, i.e. a cue created by transmission properties of the substrate alone, or active. The latter would imply that the static female reacts to cues about distance in male signals and makes a change in some parameter of her reply that was previously overlooked in creating the signal classification described in Mazzoni et al. (2009a). To answer this question, we recorded female signals at the source and checked if the female emits a constant signal throughout the whole male mating approach or if there is an effective variation of any parameter in the female response that could elicit the male to progress from the Location to the Courtship signal.

We recorded mating behaviour of *S. titanus* pairs ($N = 18$) on a grapevine cutting with two leaves (Fig. 1A), the female being always placed on the apical leaf. Male positions during each trial were noted, and the vibratory emissions were recorded from the centre of the apical leaf in close vicinity (<5 cm) to the female (Fig. 1A, point 5). Up to 12 female replies were analyzed per male location per trial (total $n = 202$), and all the measurements from each plant section were pooled. The number of analyzed pulses was limited to prevent giving larger weight to trials where male search took longer. Sections were then compared using the Kruskal–Wallis test followed by the Steel-Dwass pairwise multiple comparison test.

2.4.3. Test 1.3 Courtship behaviour trigger

Test 1.2 showed that the female pulses remain relatively constant throughout the pre-copulative phase of the mating behaviour (see Section 3). This let us formulate a further hypothesis that males switch from location to courtship behaviour according to a cue in the female reply, related to their own perception of passive signal changes that indicate proximity of the female. We predicted that if the switch in behaviour normally occurs at a certain distance (or relative position on the plant, i.e. occurrence on the same leaf) from the female, we should detect a significant difference in any parameter's value between the measuring point where the change first occurred and all measuring points further away.

To test this hypothesis, a male and a female ($N = 30$) were put on separate leaves of the grapevine cutting with three leaves (Fig. 1B). The male and the female were randomly placed on either of the basal, middle or apical leaf, thus obtaining six different combinations, and leaves were labelled "female leaf", "empty leaf" and "male leaf" accordingly; therefore, males had to distinguish not only the leaf with the female from their starting position, but also from another, empty leaf.

Prior to the start of each trial, we used a minishaker (Type 4810; Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark) to vibrate the plant with playback of pre-recorded MCS in order to initiate mating behaviour. The female replied to the playback and, as a result of such duet, the male responded with rivalry (DN signal). The playback was then stopped to allow the male to establish a duet with the female. Location of the male during each phase of mating behaviour was determined from video recordings to synchronize the audio record with male movement.

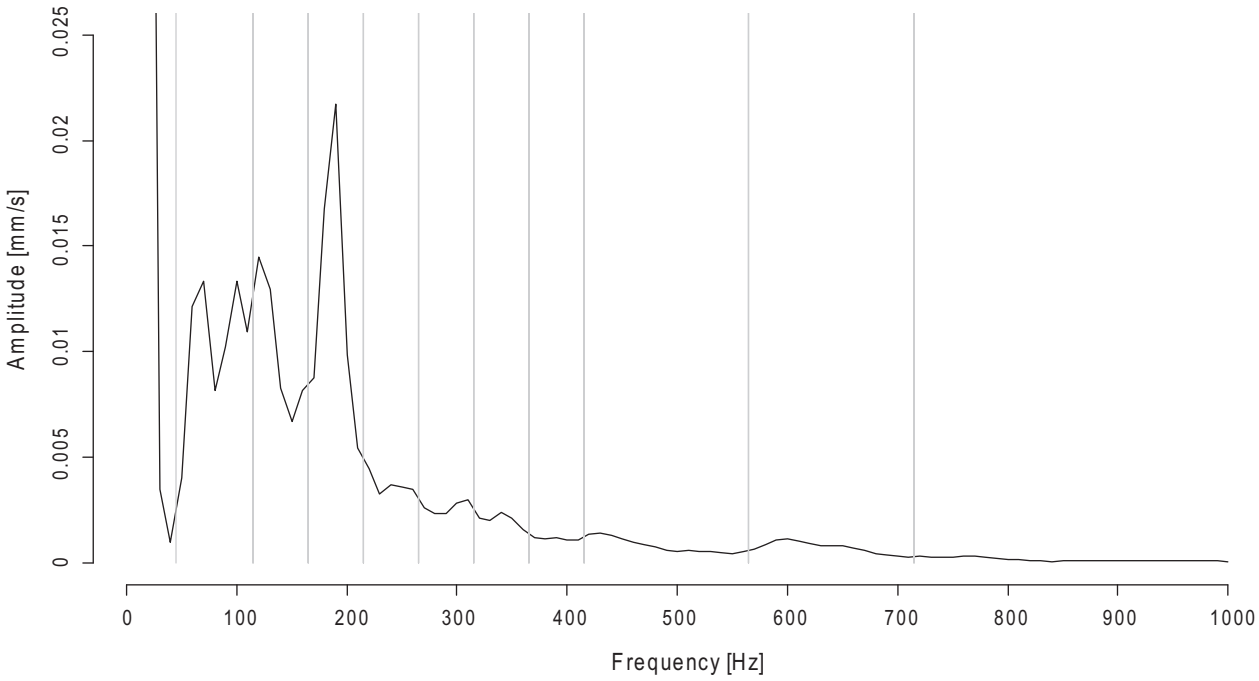

Fig. 2. Frequency spectrum of a "typical" female signal with frequency ranges used for analysis (delimited by grey lines). The lowest range 0–40 Hz comprises low-frequency external noise, and was excluded from analysis.

Since females remain stationary (Mazzoni et al., 2009a), the amplitude of their vibrational signals, measured as vibrational substrate velocity (μm/s) and its spectral components (Hz, frequency ranges), could be measured directly at 8 measuring points distributed on the grapevine cutting by moving the laser beam during male search (Fig. 1B). The goal was to record the signals at all the measuring points during each trial, so we did not necessarily follow the male's movement with the laser beam.

We calculated signal amplitude by averaging all the spectral components within the range 40–250 Hz where the majority of acoustic energy was concentrated (21 frequency bins at given FFT settings; Fig. 2). This measure, while absolute, should still be regarded as a proxy for the "true" amplitude because it is not yet certain which property of a broad-band signal the animals actually respond to. Additionally, we tested whether any particular frequency component of a signal, including low-amplitude ones, might also contribute to detection, by splitting the signal's frequency spectrum to 10 ranges with one dominant or subdominant spectral peak each, ranging up to 1 kHz (Fig. 2). Peak velocity value within each of those frequency ranges was averaged from three female pulses per measuring point per trial. To test for statistical differences of the female pulse amplitudes and frequency ranges between the measuring points, we performed the Kruskal–Wallis test followed by the Steel-Dwass multiple comparison test. Finally, with the help of the videos, we took note of the position on the plant (Fig. 1B) where the switch of male behaviour from location to courtship occurred. Thus, we associated the spectral analysis of the female pulse at each measuring point with the occurrence of behavioural switches on the plant.

2.4.4. Test 1.4. Confirmation of courtship behaviour trigger with playback

To further confirm the results of Test 1.3, we performed an additional playback test. We used playback to reduce variability caused by the male–female interaction, which enabled us to study transmission alone.

The cutting for this test was the same as for Test 1.2 (Fig. 1A). Again, the female was always placed on the apical leaf. A representative male calling signal (within the range of variability described in Mazzoni et al., 2009a) was played back to females ($N = 16$) using a static loudspeaker placed parallel to the empty basal leaf, at the distance of 3 cm. The amplitude of resulting vibrations near the female was adjusted beforehand to a level naturally experienced by females when listening to a male signalling from a different leaf, as determined from recordings made in Test 1.2. A loudspeaker was used for stimulation in order not to influence signal transmission near the point of excitation. Each female's responses were recorded by a laser vibrometer at all the recording points.

We examined response latency and two spectral parameters: peak amplitude, and frequency of the dominant spectral peak in each signal's frequency spectrum. Only relative peak amplitude in dB was considered because the absolute values were of less importance for the purpose of this test. Values were compared between plant sections with the Kruskal–Wallis test followed by the Steel-Dwass pairwise multiple comparison test.

2.5. Test 2. Directionality in S. titanus

To test the hypothesis that males make directional decisions on the basis of female reply, video recordings taken from Test 1.1 were also analyzed and the male's directional choices annotated. Three parameters were evaluated. First, we annotated whether the male started to walk towards the female (1), if yes, it was recorded as a right decision, if not, as a wrong decision. When males reversed the direction (2), it was annotated as a wrong decision if it turned away from the female and right if turned towards her. When males reached a fork between stem and leaf the right or wrong decisions at the branching point (3) were also annotated. To evaluate if males were able to make directional decisions other than by chance, the numbers of right and wrong decisions were compared in a one-tailed *t*-test for dependent samples for each parameter.

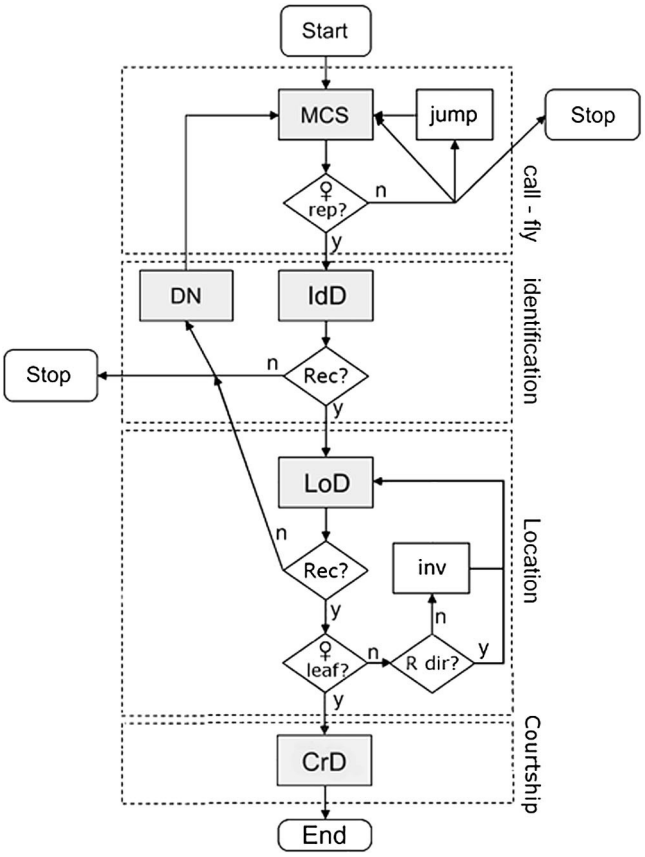

Fig. 3. Flow chart with the behavioural steps of male *Scaphoideus titanus* when searching for a female on a grapevine plant. MCS, male calling signal; DN, disturbance noise; IdD, identification duet; LoD, localization duet; CrD, courtship duet; ♀ rep, female reply; Rec, recognition; ♀ leaf, arrival at the female leaf; R dir, right decision; Inv, inversion of direction.

3. Results

3.1. Test 1. Pair formation in S. titanus

3.1.1. Test 1.1. Male–female synchrony in the course of mating behaviour

The main steps of the mating behaviour of *S. titanus* are summarized in Fig. 3. As described previously in Mazzoni et al. (2009a), in all trials ($N = 20$) males initiated vibrational communication with emission of a male calling signal (MCS). When females were not responding ($N = 4$), males either remained stationary or exhibited "call-fly" behaviour. When females responded ($N = 16$), most males ($N = 13$) emitted pulse trains with an irregular rhythm and with an increased pulse period (Table 1). The calculated male response phase delay (α) was 0.85 (Table 1), which indicates that female response resets the emission of male pulse for almost a complete pulse period. Such delayed exchange of male and female pulses was termed identification duet (IdD, Fig. 4A) and was observed only when a male and a female were placed on separate leaves. During IdD, males walked randomly on the leaf. Seven females also emitted short pulse trains ($m \pm SD = 3.03 \pm 1.28$ pulses, duration $= 0.89 \pm 0.61$ s, $n = 31$) in reply to the male signal. As a result, males either walked randomly and called again, or emitted disturbance signals (DN) (Mazzoni et al., 2009a).

Following IdD, males ($N = 13$) moved towards the petiole and walked to stem and towards the leaf hosting the female. In this stage female reply had a small but significant effect on the pulse period in male signal ($\alpha = 0.23$) (Table 1). This phase of male–female vibrational interaction was named localization duet (LoD; Fig. 4B)

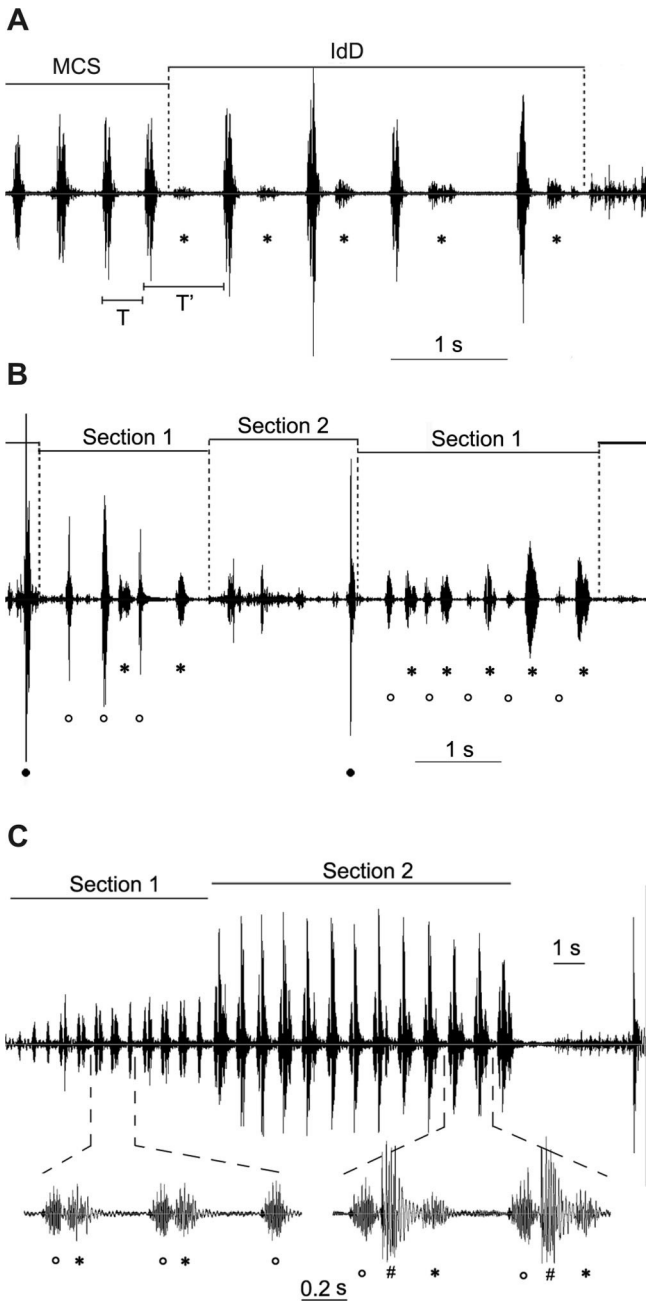

Fig. 4. Oscillogram of (A) identification duet (IdD), (B) localization duet (LoD) and (C) courtship duet (CrD). Recordings were made with the laser on the lower leaf hosting the male. (A) Male calling signal (MCS) that progresses into an identification duet (IdD), (B) two sections (S1 and S2) forming the localization duet with noise caused by the male walking following the duet. (C) S1 and S2 of a male courtship phrase that together with female pulses constitute CrD. Asterisks (*) indicate female pulses; open circles (○) male pulses; filled circles (●) the S2 single male pulse of section two; hashes (#) male pulse of type two (Mazzoni et al., 2009a). The male pulse period is T and T', in absence or presence of the female reply, respectively.

and was recorded from the beginning of the directional search until reaching the female leaf. LoD was composed of two sections repeated continuously. In section 1, males were stationary and emitted short series of pulses. In section 2, males walked for a few centimetres before stopping and often emitted a single strong pulse. Females were sometimes observed to emit multiple pulse trains ($n = 6$) (3.6 ± 1.39 pulses, duration $= 1.09 \pm 0.62$ s) after the last male pulse. The male behavioural response to the multiple female trains was either a directional search followed by another LoD ($N = 2$), or emission of disturbance signal and a restart of the

Table 1
α (male response phase delay) values from *Scaphoideus titanus* identification, localization (section 1) and courtship duet (section 1 and section 2).

	n	N	T/T' (s)	F latency (s)	α
Identification duet	10	7	$0.43 \pm 0.02/0.68 \pm 0.04$ *** ($t = 22.4$, df = 9)	0.30 ± 0.03 (25.5) b	0.85 ± 0.17 (34.7) b
Localization duet (section 1)	10	7	$0.44 \pm 0.01/0.50 \pm 0.07$ ** ($t = 3.61$, df = 9)	0.29 ± 0.01 (24.6) b	0.23 ± 0.27 (12.7) a
CrD duet (section 1)	10	7	$0.42 \pm 0.04/0.50 \pm 0.05$ *** ($t = 6.4$, df = 9)	0.23 ± 0.01 (9.1) a	0.37 ± 0.22 (19.7) a
CrD duet (section 2)	10	7	$0.62 \pm 0.06/0.69 \pm 0.08$ *** ($t = 6.9$, df = 9)	0.29 ± 0.01 (22.8) ab	0.26 ± 0.11 (14.9) a

MRP: male response phase; FLP: female latency phase; T, T': average pulse period in male signal in absence and in presence of female pulse, respectively; IdD: identification duet; LoD: location duet; CrD: courtship duet. Mean values ± SD of T, T', female latency α obtained from MRP and FLP of IdD and LoD recorded from males and females on different leaves as well as from CrD (S1 and S2) of males and females on the same leaf. The number of insect pairs tested for each signal (n) and the number of analyzed signals per pair (N) are shown. Different letters in the same column indicate significant difference after Kruskal–Wallis test (rank mean is given in brackets; F latency: $X^2 = 13.0$, df = 3, $P < 0.01$; α: $X^2 = 21.5$, df = 3, $P < 0.001$), followed by Steel-Dwass pairwise comparison test.
** $P < 0.01$ after one tailed paired t-test.
*** $P < 0.001$ after one tailed paired t-test.

communication with an IdD ($N = 4$), however, in the latter cases, the re-identification was limited to exchange of a few pulses between male and female – characterized by α value close to 1 – that immediately progressed into a LoD. The durations of IdD and LoD were similar (Table 2).

When the male arrived at the leaf hosting the female, courtship duet (CrD) was established ($N = 13$) (Fig. 4C). During CrD, males emitted pulses at a regular rhythm and female reply had again a small effect on the pulse period in the male signal. The phase delays during two sections of the CrD were similar to one determined for LoD and significantly lower than in IdD (Kruskal–Wallis test: $X^2 = 13.0$, df = 3, $P < 0.01$) (Table 1). The female pulse latency was constant throughout all stages of male–female vibrational interaction, with values significantly lower only in section 1 of CrD (Kruskal–Wallis: $X^2 = 21.5$, df = 3, $P < 0.001$).

3.1.2. Test 1.2. Is courtship initiated by the male or by the female?

Measurements from the apical leaf revealed no consistent changes in female reply during the course of the male approach. Out of the initial 18 pairs tested, $N = 18$ sets of measurements were obtained with the male calling from the basal leaf (initial male position), $N = 6$ from the basal petiole, $N = 10$ from the stem, $N = 12$ from the apical petiole, and $N = 10$ from the apical leaf. All the males switched to CrD after reaching the apical leaf lamina, at the distance of less than 10 cm from the female. A significant difference in the dominant frequency of female signals was measured only when males were calling from the basal petiole and the apical leaf (Kruskal–Wallis: $X^2 = 13.9$, df = 4, $P = 0.0078$). Conversely, female dominant peak amplitude was significantly higher when males were calling from the apical petiole than from the basal leaf (Kruskal–Wallis: $X^2 = 14.0$, df = 4, $P = 0.007$). Female response latency decreased from 210 ± 23 ms (male on the basal leaf, $n = 80$) to 182 ± 19 ms (male on the stem, $n = 22$), then remained stable, with 184 ± 26 ms ($n = 37$) when the male reached the apical leaf (Kruskal–Wallis: $X^2 = 44.2$, df = 4, $P < 0.001$), however, the Steel-Dwass test only showed significant difference between the basal leaf and the three sections closest to the female, i.e. stem, apical petiole, and apical leaf (Steel-Dwass, $P < 0.001$).

3.1.3. Test 1.3. Courtship behaviour trigger

Because female response latency changed only after the start of CrD in Test 1.1 and because we did not measure any changes in female reply consistent with behaviour in Test 1.2, we focused on spectral parameters and amplitude of female signals as perceived by the male.

Most energy of the female signal was concentrated in the chosen frequency range 40–250 Hz. Amplitude (expressed as average velocity of spectral bins within this range) of the female reply perceived by the male along the grapevine cutting is summarized in Fig. 5. There was no statistical difference in measured amplitude of female signals between male lamina, male petiole, empty lamina, empty petiole and stem, whereas the amplitude level of female pulse was significantly increased when perceived at the female leaf petiole and female leaf lamina (Kruskal–Wallis test: $X^2 = 400$, df = 6, $P < 0.001$; Fig. 5, Table 3). Twenty-five out of 27 courtship duets started on the section of the plant closest to the female, most commonly on the petiole. In the other two cases the courtship duet started on the stem, but it was always near the female leaf petiole.

No difference in dominant frequency was found between sections (Kruskal–Wallis test: $X^2 = 9.72$, df = 6, $P = 0.137$; Table 3). When peak frequencies within individual frequency ranges (Fig. 2) of the female reply were compared, up to three recording points differed significantly from the rest (Kruskal–Wallis test: df = 6, $P < 0.05$; Table 3), nevertheless, differences were never related to

Table 2
Descriptive statistics on duration of *Scaphoideus titanus* identification and localization duets.

	Duration (s)			
	IdD (single train)	LoD (section 1)	Identification (whole stage)	Localization (whole stage)
Mean ± SD	11.46 ± 7.86	3.74 ± 1.56	106.36 ± 70.98	83.45 ± 108.97
Max	32.96	6.92	236.96	393.11
Min	1.96	1.28	21.15	4.14
N	16	15	15	16

Mean ± SD, max and min values of duration are shown both for single trains and whole length of identification duet (IdD) and localization duet (LoD, section 1). N indicates the number of insect pairs analyzed.

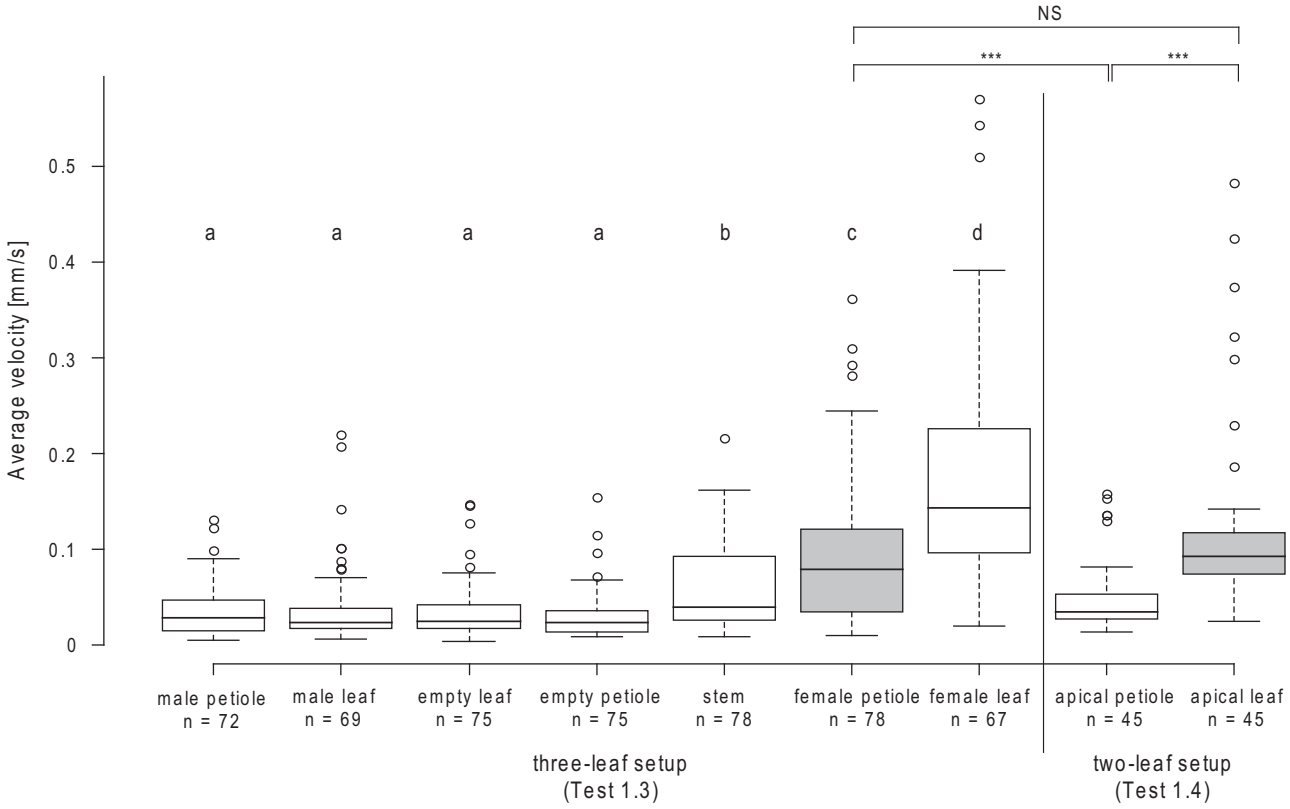

Fig. 5. Box-plots of average spectral velocity values in the range 40–250 Hz, measured from female signals on both kinds of experimental substrates. Different letters for values in the three-leaf setup (Fig. 1B, Test 2.2) indicate statistical significance after Kruskal–Wallis test followed by Steel-Dwass multiple comparison test. Section where the switch from male localization to courtship behaviour occurred is marked with grey (see also Fig. 4). Values that correspond to the behavioural switch match the values obtained on a two-leaf setup (Fig. 1A, Test 2.1), also corresponding to the behavioural switch, but at a different section (Mann–Whitney test with Bonferroni correction, see text for details).

Table 3
Spectral properties of female replies as perceived by the male at different sections on the cutting (Fig. 1B).

	Male petiole	Male leaf (lamina)	Empty petiole	Empty leaf (lamina)	Stem	Female petiole	Female leaf (lamina)
Dominant frequency (Hz)	105 ± 49	106 ± 44	95 ± 42	102 ± 42	107 ± 57	109 ± 52	122 ± 52
Avg. velocity within 40–250 Hz (μm/s)	3.5 ± 2.5	3.7 ± 3.9	3.3 ± 2.9	3.0 ± 2.5	5.6 ± 4.3	9.6 ± 7.6	17.1 ± 11.7
Peak frequencies within spectral ranges (Hz) (Fig. 5)							
40–100 Hz	71 ± 20	75 ± 20	68 ± 19	73 ± 19	69 ± 21	73 ± 20	80 ± 23
110–150 Hz	137 ± 15	133 ± 14	140 ± 16	139 ± 14	134 ± 16	138 ± 17	134 ± 14
160–200 Hz	184 ± 14	190 ± 17	182 ± 15	182 ± 15	183 ± 14	177 ± 10	181 ± 11
210–250 Hz	229 ± 14	235 ± 16	228 ± 13	236 ± 15	233 ± 14	232 ± 14	236 ± 16
260–300 Hz	283 ± 15	282 ± 15	288 ± 15	286 ± 15	282 ± 15	284 ± 16	283 ± 15
310–350 Hz	336 ± 16	333 ± 14	335 ± 16	334 ± 17	336 ± 15	334 ± 15	330 ± 13
360–400 Hz	387 ± 16	388 ± 17	387 ± 17	387 ± 16	387 ± 15	388 ± 16	385 ± 17
410–550 Hz	488 ± 50	473 ± 54	466 ± 49	451 ± 43	468 ± 49	475 ± 57	459 ± 48
560–700 Hz	614 ± 41	632 ± 41	627 ± 46	638 ± 40	619 ± 41	615 ± 43	610 ± 36
710–1000 Hz	815 ± 86	805 ± 83	814 ± 79	824 ± 81	824 ± 88	776 ± 76	804 ± 81
Peak velocities within spectral ranges (μm/s) (Fig. 5)							
40–100 Hz	8.8 ± 8.6	9.2 ± 9.4	8.7 ± 8.5	7.2 ± 6.0	16.2 ± 15.9	23.9 ± 18.1	33.3 ± 20.5
110–150 Hz	7.6 ± 11.0	6.2 ± 4.9	6.2 ± 6.5	6.7 ± 7.0	10.5 ± 9.0	19.2 ± 17.0	35.3 ± 32.9
160–200 Hz	5.1 ± 7.5	4.0 ± 2.9	4.3 ± 4.0	3.2 ± 3.5	7.5 ± 7.5	12.7 ± 11.8	27.4 ± 30.5
210–250 Hz	2.9 ± 5.1	2.0 ± 2.2	1.5 ± 1.6	1.4 ± 1.9	3.4 ± 4.2	4.2 ± 4.0	11.1 ± 12.5
260–300 Hz	1.5 ± 2.1	1.0 ± 1.1	0.9 ± 0.8	1.0 ± 1.1	1.6 ± 1.5	2.4 ± 2.8	6.8 ± 7.1
310–350 Hz	0.6 ± 0.6	0.7 ± 0.9	0.6 ± 0.6	0.5 ± 0.7	1.0 ± 1.3	1.4 ± 1.3	4.6 ± 4.8
360–400 Hz	0.5 ± 0.4	0.4 ± 0.3	0.4 ± 0.4	0.5 ± 0.9	0.9 ± 1.7	1.1 ± 1.2	3.0 ± 4.4
410–550 Hz	0.4 ± 0.5	0.7 ± 0.8	0.4 ± 0.6	0.4 ± 0.7	0.9 ± 1.7	1.0 ± 0.9	2.1 ± 2.6
560–700 Hz	0.3 ± 0.3	0.5 ± 0.6	0.3 ± 0.4	0.2 ± 0.3	0.5 ± 0.7	0.7 ± 0.8	1.2 ± 1.7
710–1000 Hz	0.2 ± 0.2	0.3 ± 0.3	0.2 ± 0.4	0.2 ± 0.2	0.4 ± 0.6	0.4 ± 0.5	0.6 ± 0.9
n	72	69	75	75	78	78	70

All values are mean ± SD.

the distance from the female. On the amplitude axis, peak amplitude within the following individual frequency ranges changed consistently with the distance from the female (i.e. significant difference between the female petiole and the stem section closest to the female leaf, sections further away with lower amplitude): 110–150 Hz, 160–200 Hz, 310–350 Hz, 360–400 Hz, and 410–550 Hz (Kruskal–Wallis test: df = 6, $P < 0.05$; Table 3).

3.1.4. Test 1.4. Confirmation of courtship behaviour trigger with playback

Measurements of female replies recorded from different points on the plant revealed changes caused by transmission along the substrate. The stimulus elicited replies by each female ($N = 16$), so we analyzed all the female signals (except the female pulse trains) from all the recording points in each trial (total $n = 643$). The baseline latency value, as measured on the apical leaf closest to the female (216 ± 38 ms, $n = 147$), did not increase significantly until the basal leaf at 232 ± 37 ms, $n = 147$ (Steel-Dwass, $P < 0.001$; Fig. 6A). A small, but statistically significant, difference occurred only between the apical petiole and the stem (Steel-Dwass, $P < 0.05$). Relative amplitude of the dominant peak decreased consistently, with all the differences between points significant (Kruskal–Wallis, $X^2 = 325.4$, df = 4, $P < 0.001$; Fig. 6B), except between the stem and the basal petiole, and between the basal petiole and the basal leaf (Steel-Dwass, P = NS). Dominant frequency differed significantly between recording points (Kruskal–Wallis, $X^2 = 99.6$, df = 4, $P < 0.001$), but no consistent changes related to the distance from the emitter was observed: frequencies at basal petiole, apical petiole and apical leaf were not statistically different, but all were higher than the frequency at the basal leaf, which was in turn higher than the frequency at the stem (Steel-Dwass, $P < 0.01$; Fig. 6C).

To validate the average substrate velocity at which the switch in behaviour was observed in Test 1.3, we combined behavioural data from Test 1.2 and recordings from Test 1.4, both obtained on a cutting with two leaves (Fig. 1A). For this purpose, the amplitude was expressed as the average velocity in the frequency range 40–250 Hz, the same as in Test 1.3, and compared between tests using the Mann–Whitney test with Bonferroni correction. Average velocity increased from 0.049 ± 0.037 ($n = 45$) at female petiole to 0.124 ± 0.101 ($n = 45$) at female leaf lamina (Mann–Whitney test, $W = 321$, $P < 0.001$) (Fig. 5). Average velocity at the female leaf lamina on the cutting with two leaves was not different from the average velocity at the female petiole on the cutting with three leaves (Mann–Whitney test, $W = 1416$, $P = 0.08$), while it was significantly higher than at the stem on the cutting with three leaves (Mann–Whitney test, $W = 781$, $P < 0.001$) (Fig. 5). Measured differences between plant sections for other analyzed parameters did not correspond to the behavioural switch in Test 1.2.

3.2. Test 2. Males are able to make directional decisions

The number of right or wrong directional decisions made by males moving towards a female after a female response is shown in Fig. 7. Significantly more decisions were towards the female (t-test: $t = 12.72$, df = 27, $P < 0.001$) and when reversing the direction, significantly more males made a correct rather than wrong directional decision (t-test: $t = 4.72$, df = 27, $P < 0.001$). On the other hand, no difference between correct and wrong directional decisions was observed at branching points (t-test: $t = 1.43$, df = 27, $P = 0.32$). A male that turned in the wrong direction made on average two (1.8 ± 1.4) additional moves in this direction before turning around.

4. Discussion

Pair formation in *S. titanus* starts with identification of the mating partner and continues with a localization stage until a final courtship stage before copulation. In general, signals should first inform the receiver about the sender's identity (who?), then the quality (what?) and the location (where?) (Pollack, 2000).

4.1. Male–female synchrony

Our results indicate that the first act of pair formation in *S. titanus* is male identification of a conspecific female through a strict synchronization with his own pulses. Female reply has to arrive within a specific time window, like in several other leafhopper species (Nuhardiyati and Bailey, 2005; Derlink et al., 2014), and should not overlap with the next male pulse, because overlapping would be mistaken by both partners for a disruptive signal emitted by a rival male (Mazzoni et al., 2009b); however, while it was previously thought that female pulses are emitted only in-between the male pulses (Mazzoni et al., 2009a), we also found in the present study that female pulses may be emitted as pulse trains, most often after the last male pulse. Such multiple replies occurred when the male was identifying or locating the female from distant plant parts, which may represent female adaptation for increasing detectability and/or traceability (i.e. serial redundancy). Male calling song pulses are emitted with regular rhythm, indicating that they are generated by an endogenous oscillator. Females do not reply to all male pulses (Mazzoni et al., 2009a), suggesting that they listen out for each male pulse and reply (or not) to it.

Resetting of the male endogenous oscillator by the central nervous system is comparable to signal interactions among chorusing males (Greenfield, 1994, 2005); however, *S. titanus* males do not form choruses, engaging instead in rival behaviour if a competing male is detected in the vicinity (Mazzoni et al., 2009b). The change in rhythm could in this case help the male to distinguish the female signal from conspecific male emissions in the first stage of mating behaviour, when recognition has not yet been achieved. Without a change in rhythm, two males emitting MCS out of phase might mistake each other for a conspecific female, while if a reply triggers prolongation of the pulse period, overlapping will necessarily ensue.

4.2. Directionality in S. titanus

The effect of female reply on male pulse period at later stages was small, suggesting that mate recognition is the main function of pulse period resetting in the calling phase. This observation indicates a complex and situation-dependent neuronal control of signal production in males. After identification has been achieved, accuracy of recognition became secondary and speed was the key, with male LoD signals three times shorter than IdD on average.

As noted before in this and other species of small plant-dwelling insects (Mazzoni et al., 2009a, 2010; Legendre et al., 2012), males interrupt walking bouts with calls after which directional decisions are made. Although plants constitute complex structures with branching points, leaves and stems, of woody and green tissues, males of *S. titanus* were able to make correct directional decisions when walking towards a stationary female, seemingly based on a continuous process of evaluating the perceived information. According to our results, *S. titanus* males are able to extract directional information from female reply during the search, since significantly more males walked towards the females; however, males made many mistakes at branching points. Males of the larger stink bug *Nezara viridula* (Heteroptera: Pentatomidae) (size 1 cm), which can orient reliably at branching points, stop and stretch their legs between branches, thus extending the leg span (Čokl et al., 1999). We never observed such behaviour in *S. titanus*. Instead, we showed that males are able to correct the direction after they had made a wrong decision, despite their short leg span. The correction process was rapid, with males making on average less than

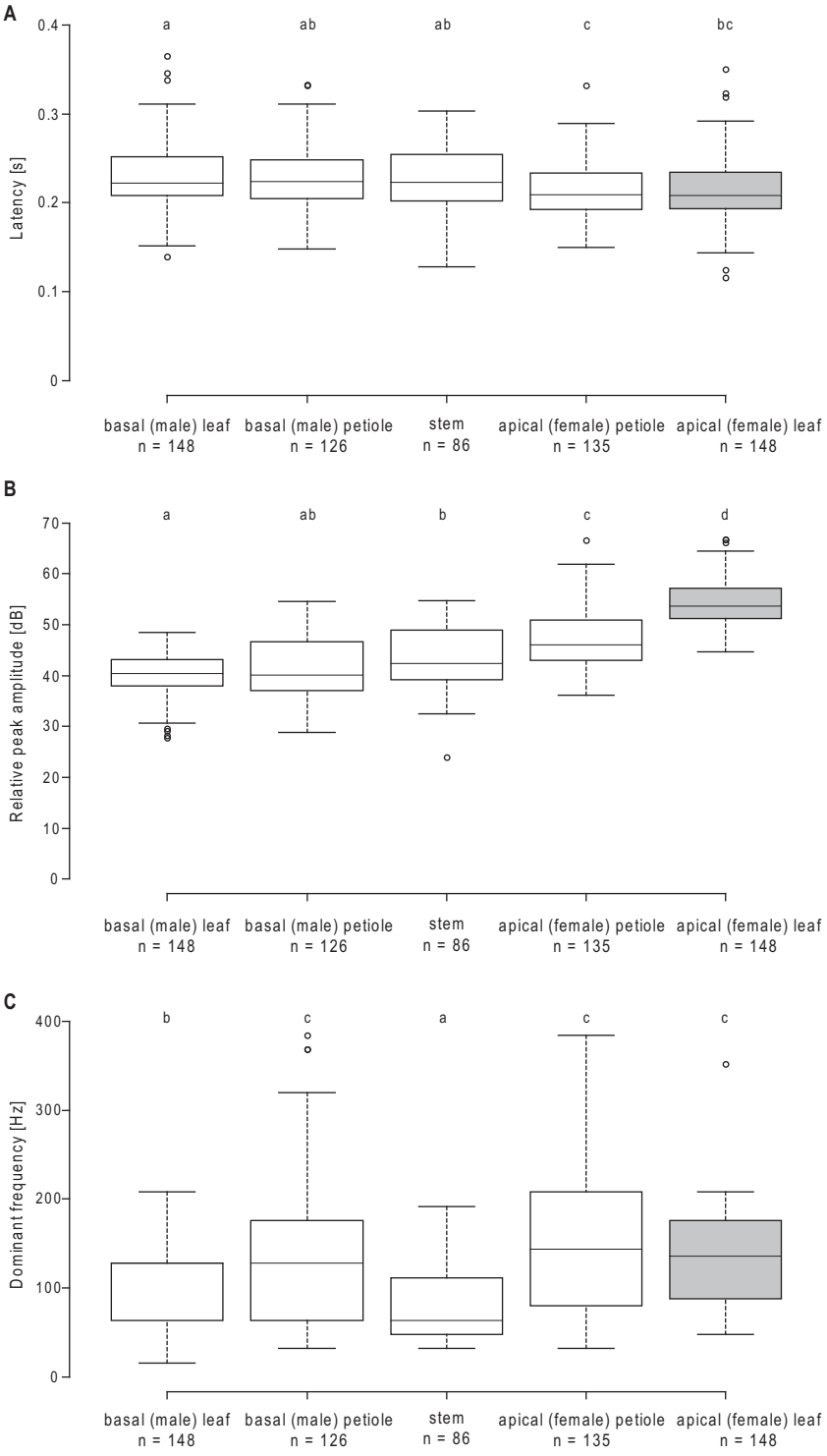

Fig. 6. Changes in female response caused by transmission along the substrate. (A) Latency, (B) relative amplitude of the dominant spectral peak in dB and (C) frequency of the dominant spectral peak. Different letters indicate statistical significances after Kruskal–Wallis test followed by Steel-Dwass multiple comparison test. Section where the switch from male localization to courtship behaviour occurred is marked with grey.

two moves away from the female before reversing their direction. This indicates a perception of a directional cue even in the orientation of such small insects, perhaps by comparing the relevant parameter of the female response between consecutive locations where they stop to signal. Decisions by a male were made as he walked after every identified female response, while in absence of female reply, he remained stationary. Such a search tactic would require short-term memory for comparison of signals between neighbouring locations, but not the capacity for direct stereo or multi-channel comparison, bearing more resemblance to triangulating search behaviour of some beetles and stoneflies on 2-D surfaces (Abbott and Stewart, 1993; Goulson et al., 1994) than to direct orientation of the more closely related Pentatomid bugs (Čokl et al., 1999).

In the leafhopper *Graminella nigrifrons*, searching is facilitated by positive phototaxis that, coupled with female preference for

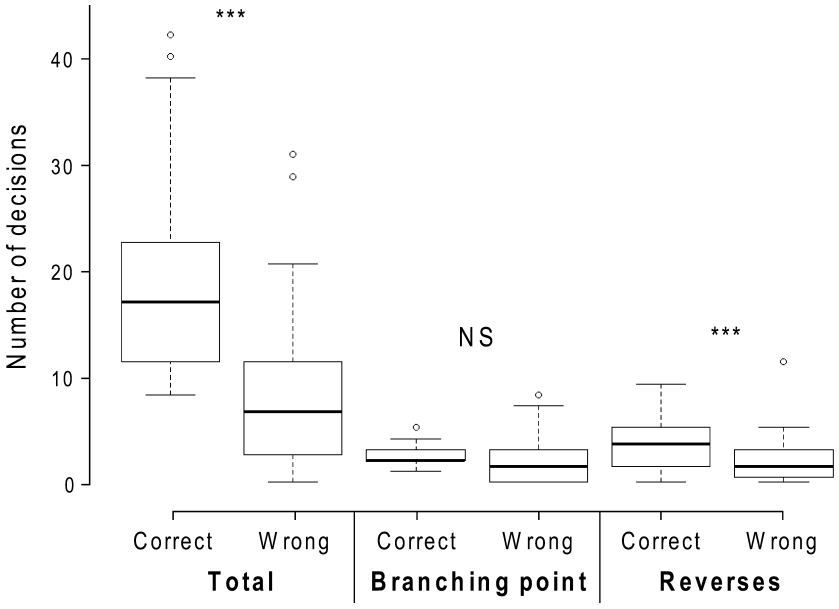

Fig. 7. Total number ($m \pm$ SD) of right (towards female) and wrong directional decisions made by males after receiving female response along a grapevine cutting (left). Directional decisions at branching points (middle). The number of males making a right or wrong decision after changing direction (right). Asterisks (***) indicate statistical significances after one-tail paired *t*-test ($P < 0.001$).

perching on the top of the plant, enables localization even without the need to extract exact directional information from vibrational signals (Hunt and Nault, 1991). We observed no such preference in *S. titanus*. Directional decisions at petiole-stem crossings appeared random and males found females perched on leaves below their starting location without difficulty, further confirming that vibrational signals alone provide the information about both identity and location in this species.

4.3. Courtship behaviour trigger

The change in behaviour as males progressed from identification to localization and ultimately to courtship suggests that a male is aware of whether the female is in close proximity or not. According to our results, female response is all-or-nothing and signal changes due to transmission through the plant act as a trigger for male courtship behaviour. Significantly higher female signal amplitude was detected on or near the female leaf compared to other sections of the plant. We demonstrated that the switch to courtship behaviour occurred when the average substrate velocity of female signals, as measured from calibrated frequency spectra of individual signals, increased to approximately 10 μm/s. Behavioural switch corresponded to the amplitude threshold, while exact geometry was not important. This is supported by previous results when pairs were placed on the same leaf at the start (Mazzoni et al., 2009a). In such a situation, males did not perform neither IdD nor LoD, and MCS immediately progressed into a courtship duet. It could be beneficial to restrain the emission of courtship signals, which are enriched with additional elements (two pulse types and the buzz, Mazzoni et al., 2009a) and thus likely more energetically demanding, to the stage when a female is already nearby. The complexity of the CrD contrasts with the relative simplicity of the LoD, which is formed by only one type of pulse and is shorter in duration than both IdD and CRS.

In the present study, none of the males expressed "call-fly" behaviour after the duet was established, which may be due to the small size of the experimental substrate. The "call-fly" behaviour is usually associated with a strategy to increase signalling space (Gwynne, 1987; Hunt and Nault, 1991) and the amplitude in our experiments was probably high enough for the males to perform a more localized search by walking, unlike in the case of inter-plant communication (Eriksson et al., 2011).

Previous work suggested that frequency-dependent attenuation may provide more reliable information about distance than total amplitude (Barth et al., 1988). We found several individual frequency components whose peak amplitude change reflected the change in the average velocity of the whole range 40–250 Hz. For this reason, they might have a role as a proximity cue, perhaps complementing the change in amplitude of the whole signal, which is the most likely trigger to switch localization stage into courtship; however, while we found significant changes on the frequency axis, no pattern consistent with behaviour or distance from the emitter was obvious, neither in the dominant frequency, nor the frequencies of individual subdominant peaks. Initially we also considered female response latency as a potentially relevant parameter, but while latency increase was consistent with distance, we found no abrupt significant change in this parameter between sections where the switch in behaviour occurred, so we did not consider it further. Hence, amplitude is the parameter we believe the males use, as opposed to response latency and frequency structure of a female reply.

Polajnar et al. (2012) previously demonstrated that due to resonance, amplitude is an unreliable cue for assessing distance from the emitter in species that use almost pure-tone signals. In broad-band signals, on the other hand, amplitude oscillations average out between frequency components and lead to monotonous attenuation with distance, as also observed in the present study. Unpredictable propagation of pure-tone signals because of resonance may therefore be an additional reason why the emission of a harmonic "buzz" as an element of male vibrational signals (Mazzoni et al., 2009a) is restricted to the final stage, emitted when the male is already close to the female. Another confounding factor is eccentricity of stem motion where perceived signal amplitude might depend on the angle relative to the emitter (McNett et al., 2006), but this is only noticeable very close to an emitter standing on a petiole.

While visual or chemical cues may also be involved in eliciting courtship behaviour at short distances, these seem to be

less likely possibilities. Our video recordings show that females were not visible from the petiole of the female leaf (i.e. where most courtship duets started). Until now there has been no evidence that chemical communication plays a role in reproductive behaviour of leafhoppers (Claridge, 1985), and adults rely exclusively on substrate-borne vibrations for intraspecific communication (Virant-Doberlet and Čokl, 2004; Mazzoni et al., 2009a). The antennae of *S. titanus* adults in particular have a reduced number of olfactory sensillae (Stacconi and Romani, 2012) and only the nymphs have been shown to use olfaction for recognition of the host plant (Mazzoni et al., 2009c).

To summarize, pair formation in leafhoppers is a dynamic process where the identification stage seems to be optimized for reliability and the localization stage for speed, while energetic demands may be rationalized by starting the costliest and most complex advertising stage only after the first two tasks have been accomplished. Secondly, leafhopper males are able to interpret relevant information contained in female signals' perceived amplitude and temporal synchrony with males' own signals, therefore utilizing not only female reply per se, but also transmission properties of the substrate to guide their behaviour.

It is the authors' belief that behaviour in our chosen model species may, because of its simplicity, provide further insights in the insect mating process, either through observation or through manipulation of signals (see also Mazzoni et al., 2009b; Eriksson et al., 2012). Building upon these basic insights, similarly optimized strategies for mate recognition and localization should also be searched for in other species of animals.

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