# Doctoral thesis
## University of Pisa
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# Mating disruption in *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) by vibrational signals



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# Abstract

Conventional pesticides have detrimental effects on the global health and a development of environmentally friendly alternatives to control agricultural pests is essential. Mating disruption is an example of such method, since it exploits the natural airborne pheromone plumes that females emit to attract males. When a synthetic pheromone is applied to a field, males are disorientated and mating is prevented in the treated area. However, not all insect species communicate with olfactory signals. It has been estimated that 150 000 species use vibrations to achieve mating and among them there are several pests and important vectors of plant diseases. To control such species, growers may need to apply large amounts of pesticides, which is both environmentally and economically costly.

The main goal of the present thesis was therefore to develop a vibrational mating disruption strategy. For this, the leafhopper *Scaphoideus titanus* was chosen as model species, since it uses vibrations both for mating and rivalry, along with being an economically important vector of the severe phytoplasma grapevine disease Flavescence doreé. Besides experiments concerning the proper mating disruption, laboratorial studies were made on signal transmission through grapevine tissues and on the ability of males and females to emit and receive substrate-borne signals. For the first time, it was shown that substrate-borne vibrational signals can allow communication between individuals despite lack of substrate continuity. This is an important contribution for an improved knowledge of the subject, but also to consider for control of insects that are distributed on closely adjacent plants like grapevine. Moreover, it was shown that males

are able to make directional decisions towards females and that there is an increased level of female signal intensity that triggers the male to initiate courtship. Pair formation in *S. titanus* starts with identification and proceeds with a location (search) stage before the final courtship. In the identification duets, male pulses were delayed after female reply, while they were fully synchronized during location and courtship duets. It is possible that mating disruption with vibrations is more successfully applied during the identification stage when external interferences could result in loss of important information that is needed to correctly identify the mating partner.

Finally, during the mating disruption experiments, a pre-recorded natural rivalry signal of *S. titanus* was used for disruption when transmitted via grapevine wires to plants, where it masked the communication between males and females. In both semi-field and field experiments, the number of mated females was significantly reduced in presence of disruptive signal while females were mated in the silent control plants. These results suggest that vibrational mating disruption may have an important impact on future integrated pest managements of agricultural productions. Moreover, it is possible that the method can be applied to control different vibrational communicating pests. Vibrating plants in greenhouses may be easier than in an open field due to the protected environment and presence of energetic source. Yet, although the results from this thesis have shown that the principle of the method is promising, a future goal will first be to optimize the energetic and economic expenses of the system.

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# Dedication

To Adriano and Gianfranco with all my heart

*La vita è bella!*
   Roberto Benigni

# Contents

# List of Publications

This thesis is based on the work contained in the following peer reviewed papers, referred to by Roman numerals in the text:

I. **Anna Eriksson**, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet, Valerio Mazzoni (2011) Inter-plant vibrational communication in a leafhopper insect. PLoS ONE 6(5):e19692

II. **Anna Eriksson**, Gianfranco Anfora, Andrea Lucchi, Francesco Lanzo, Meta Virant-Doberlet, Valerio Mazzoni (2012) Exploitation of insect vibrational signals reveals a new method of pest management. PLoS ONE 7(3):e32954

III. **Anna Eriksson**, Marco Valerio Rossi Stacconi, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet, Valerio Mazzoni. (2013) Signal parameters involved in pair formation mediated by substrate-borne vibrations (*Submitted)*

# 1. Introduction

## 1.1. Vibrational communication in insects

A signal may be classified as an information content that is encoded and transmitted by a sender to a receiver who recovers and decodes the message before acting accordingly (Hauser, 1996, Rendall et al., 2009). Insects mainly use visual (e.g. Hochkirch et al. 2006), chemical (e.g. Cross and Jackson, 2009) and mechanical (including acoustical) signals (Michelsen et al. 1982; Čokl and Virant-Doberlet, 2003; Bailey et al. 2006) for sending and receiving information among conspecifics. Many species use sounds or vibrations as primary communication channel for mating, rivalry or aggregation (Hill, 2008), while olfaction in such species may be a minor sense to for example orientate towards a host plant, as in the nymphs of the Nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) (Mazzoni et al., 2009c).

Substrate-borne vibrations have been estimated to be used by more than 150 000 insect species as primary communication channel and by another 45 000 species when together with other forms of mechanical signals (Cocroft and Rodriguez, 2005). In particular small insects belonging to the group Auchenorrhyncha (Hemiptera: Cicadomorpha and Fulgoromorpha) are known to communicate with vibrational signals (Claridge, 1985). Within Auchenorrhyncha there are so called planthoppers, leafhoppers, treehoppers, cicadas and spittlebugs. All of them are plant-feeders and some are vectors of severe plant diseases. For example, *Homalodisca coagulata* Say (Hemiptera: Cicadellidae) can transmit "Pierce's disease" to grapevine, almond and citrus plants (Redak et al., 2004) and *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) is a vector of the phytoplasma grapevine disease "Bois noir" (Lee et al 1998).

Within acoustics, it may be confusing to distinguish among all terminology. Vibration is defined as particle motion that either can be airborne, waterborne or substrate-borne and hence referred to as a song, sound or noise (Hill, 2008). However, typically, songs are distinguished from noises by having an harmonic tone with fundamental frequency and sounds are usually detectable by a human ear as airborne or waterborne acoustic waves. Vibrations may be referred to those waves that have frequencies which can be undetectable to humans unless specific sound recorders are used. Because of size constraints, few insect groups evolved communication with airborne sounds (Bennet-Clark, 1998), while substrate-borne vibrations are considered an efficient way to convey information (Hill, 2008). Substrate-borne vibrations may travel in a spiders' web, a plant, sand or on the ground where the signalling individuals feed or reproduce. A well researched example are plant tissues where many insects feed and reproduce and where the low frequencies suffer less from attenuation (Čokl and Virant-Doberlet, 2003).

The structure of vibrational signals is visually observed as pulses in oscillograms or spectrograms when being emitted at a certain frequency, with different amplitudes and temporal pattern (Čokl and Virant-Doberlet, 2003). Signals are produced through a selection of spectral and temporal properties and are influenced by both physical substrate properties and background disturbances (Rendall et al., 2009).

Vibrations may be either intentional or incidental and yet useful for the receiver (Hill, 2008). For example, parasitoids distinguish the vibrations produced by larvae and pupae (Meyhöfer et al., 1994) and predators locate preys by following the vibrations created from unintentional walking or feeding movements (Hill, 2008). When being transmitted intentionally as signals, they are used for conspecific communication to for example coordinate group living, protection, parental care and predator avoidance

(Virant-Doberlet and Čokl, 2004). During mating, pairs may exchange calls and replies with species-specific signals referred to as duets. During sexual communication, the signals may enable identification of the sender (species and sex), guidance of its location (e.g. Virant-Doberlet et al., 2006; Hill, 2008; Legendre et al., 2012; De Groot et al., 2012) or provide information about the quality of the partner, as during courtship (Bradbury and Vehrencamp, 2000). Some signals have developed for sexual advertisement during male-male competition (e.g. Ichikawa, 1982; Hill, 2008; Mazzoni et al., 2009b). Signals used by males during rivalry are sometimes referred to as agonistic vibrations (e.g. Fernandez-Montraveta and Schmitt, 1994; Hill, 2008) and may be stimulated by female responses in courtship duets or by other male calls (Ichikawa, 1982). The more males present, the higher percentage of the signals emitted are agonistic, as was shown in the wandering spider *Cupiennus getazi* (Schmitt et al., 1992) and in the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) (Ichikawa, 1982). In an experiment by Ichikawa (1982), agonistic signals in *N. lugens* were not produced when a single male was present but only when there was a group of individuals. In the planthopper *H. obsoletus*, males compete both with specific aggressive signals and violent body contact even in absence of females on the plant (V. Mazzoni in prep.). Within groups, a male may be inhibited from calling by other males as a result of dominance hierarchy. Moreover, in the group living leafhopper *Graminella nigrifrons* Forbes (Hemiptera: Cicadellidae), the male rivalry signals were not different from the songs produced for interactions with females (Hunt and Norton, 2001). Male-male competition in *G. nigrifrons* occurs when rival males alternate the other male signals with chorusing, which causes both inhibition and resetting, thus reduction of the call rate and delayed responses. The aim of chorusing is to adjust the timing of the calls to either overlap and alternate

during competition (Hunt and Norton, 2001) or synchronize during cooperation and group protection (Greenfield, 1994).

## 1.2. Signal production and reception

There are different mechanisms for producing sound or substrate-vibrations. Some vibrations are created when the particle motion from body movements reaches the substrate, others use percussion of one body part against another, or rely on the click mechanisms that results from contraction and relaxation of musculatures (Haskel, 1961).

Once the signal has been sent through the medium it will be decoded in the central nervous system of the receiver. The receptor organs for detection of vibrational signals are typically present in the tibial organs of the six legs (e.g. Rössler et al., 2006) but may also be situated on the fore wings (e.g. Prager and Larsen, 1981) or possibly in the antennae in case of airborne vibrations (House and Clardige, 1970; Jeram and Čokl, 1996; Romani et al., 2009; Rossi Stacconi and Romani, 2013). However, in insects, most vibration receptors are located in the legs (Čokl et al., 2006). For example the plant-dwelling bug *Nezara viridula* L. (Heteroptera: Pentatomidae) possesses a subgenual organ (SO) in each of the six legs that has been identified as the most sensitive organ to detect substrate-borne vibrations. Both the structure and sensitivity of SO to vibrations vary among different insect species (Hill, 2008). An insect may use amplitude (intensity) and time-of-arrival differences of signals for orientation. However, since variations in intensity may occur while the signal is travelling (McVean and Field, 1996) because of the complex filtering properties of plants (Michelsen et al. 1982), there is often no reliable intensity gradient on plants which could be used as a proper directional cue (Virant-Doberlet et al., 2006). The size (i.e. maximal leg span) of the insect is an essential factor for creating large enough differences (Virant-Doberlet

et al., 2006). Yet, in species with a leg span less than 1cm, such as most Auchenorrhyncha, there has not yet been evidenced that they are able to extract directional information from arrival of vibrational signals from plant parts where females are distant.

## 1.3. Transmission in plants

Plant leaves and stems are the most widespread substrates to send and receive vibrational signals (Cocroft and Rodriguez 2005). Reproductive isolation results from development on the same plant and when the insects shift host plant (Vrijer, 1992; Rodriguez et al., 2007). Spectral variables are more influenced by variation in plant substrates than temporal variables (Cocroft et al 2006), since plants are known to be frequency filters (Michelsen et al., 1982; Magal et al 2000). Frequency has been shown to differ between plants of the same species (Cocroft et al., 2006) and between native and non native plants (Polajnar et al 2013). Differences in signalling behaviour on native and non native plants were also reported in treehoppers (Hemiptera: Membracidae) that produced shorter and less signals on their non host plants (Sattman and Cocroft, 2003). Thus, the physical properties of plants have an important influence on both the structure of vibrational signals and on the mode of signal transmission (Čokl and Virant-Doberlet, 2003; Cocroft et al., 2006). In fact, plants constitute structurally complex substrates for transmission of vibratory signals due to the internal substrate differences within stems, branching points and leaves (Michelsen et al., 1982, Magal et al 2000) and these influence the ability of insects to accurately localize the direction of the signalling source (Virant-Doberlet et al., 2006). On a stem it may either move backwards or forwards, leaves contain both veins and laminae and at branching points there are several directions to choose between. Vibrations travel best as bending waves in

plant tissues, since these waves have low propagation velocity and a relatively low attenuation (i.e. less loss of energy during transmission) (Michelsen et al., 1982). The attenuation and frequency filtering vary according to the stiffness and radius of the substrate. For example, the transmission of vibrations is more efficient in leaf veins than in leaf laminae (Čokl et al., 2004) and the transmission is stronger in a leaf than in a stem closer to the source (Michelsen et al., 1982). Because the complexity of plants, insects may have adapted different behavioural strategies to acquire the directional information from a signalling source (Virant-Doberlet et al., 2006). For example, when two individuals are far distanced on different plant parts, some insects use call-fly strategies by alternating calling and flying (Hunt and Nault, 1991), or a combination of olfactory and visual cues before courtship at close distances (Čokl and Virant-Doberlet, 2003).

## 1.4. The Nearctic leafhopper *Scaphoideus titanus*

*S. titanus* (Fig. 1) (4-5 mm as adult) is an ampelophagous specialist and an economically important species, when being a vector of the severe phytoplasma grapevine disease Flavescence dorée (FD) (*Candidatus* phytoplasma vitis, 16Sr-V) (Schvester et al 1961; Boudon-Padieu, 2000; Bressan et al 2006). FD is marked as a quarantine disease in Europe.

**Figure 1.** *Scaphoideus titanus* adult (photo: P. Giannotti).

*S. titanus* was accidentally introduced to Europe in the 1950s from North America (Schvester et al 1961). So far from France it has been spread to Spain, Portugal, Switzerland, Italy, Austria, Slovenia, Hungary, Croatia and Serbia (Mazzoni et al. 2005; Der et al., 2006; Steffek et al., 2006). Flavescence dorée phytoplasma, is a quarantine organism included in the A2 EPPO list 11 (N°2000/297CE directive). In Italy, where both *S. titanus* and FD are present, there are mandatory chemical pesticide controls (Ministerial Decree nr 32442 from the 31st of May 2000) as well as removal of infected plants. Across Europe the chemical control of *S. titanus* is variable from one to three chemical treatments per season, according to the specific region. In Italy these consist of chitin depressors against nymphs and neurotoxic products against both adults and nymphs (Bosio et al., 2004).

S. titanus is monovoltine and overwinters as eggs laid the previous summer in two-year old grapevine canes (Vidano, 1964). Hatching occurs the following spring when there are five nymphal stages during approximately one month. In northern Italy nymphs are mainly found from May to June (few individuals may be observed also in late August) and

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adults from July to October (Lessio and Alma, 2004a). Most individuals remain in the area of the host plant and their flight activity in the evening and early morning hours (Lessio and Alma, 2004b). Early nymphal stages feed sap mostly from minor veins of the lower leaf page, while later stages (fourth and fifth instars), as well as adults, also feed on mid veins, green shoots and stems (Vidano, 1964). The acquisition of FD occurs from the third nymphal stage, although latter stages and adults are more effective vectors, since the latency period is 28-35 days (Schvester et al 1969).

*S. titanus* males are able to mate with three different females during eight hours while females mate once in a lifetime and remains stationary while the male searches (Mazzoni et al., 2009a). The sexual communication of *S. titanus* is mediated by substrate-borne vibrations (Lucchi et al., 2004; Mazzoni et al., 2009a). The production of vibrations is not yet known for *S. titanus*, although it is likely that it is a tymbal-like segment located dorsolaterally on the abdomen, such as the sound-producing organ in several Auchenorrhyncha (Ossiannilsson, 1949). The male leafhopper emits the more complex signals, which are composed of four elements (Mazzoni et al., 2009a): *pulses* (1) with a dominant frequency around 150 Hz and broadband characteristics, *noises* (2) and *rumbles* (3) that are short series of pulses emitted at 230 Hz or 570 Hz respectively and finally there is the *buzz* (4), which is a continuous sound with harmonic structure emitted at a frequency of 280 Hz. Males starts the communication by slowly moving the abdomen in a dorso-ventral way to emit a male calling signal (MCS) (Fig. 2a), constituted by a rumble, a pre-pulse of low amplitude and high frequency, followed by a series of pulses, referred to as male pulse 1 (MP1). MP1 are emitted with a rather regular repetition rate and with an increasing amplitude. Females respond with single pulses in-between the male pulses, which also are produced from dorso-ventral movements.
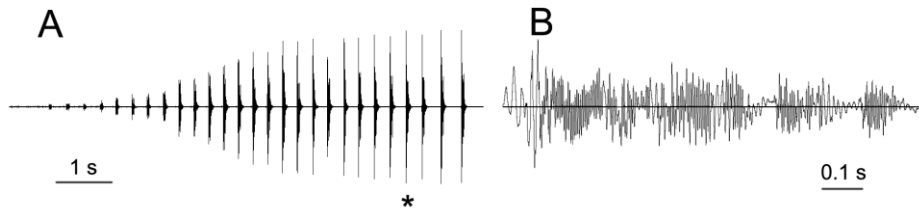
**Figure 2. Oscillogram** of **A.** Male calling signal (MCS) with female pulse indicated with an asterisk, and **B.** Disturbance noise (DN).

As the female has responded to the male and both insects are within the same leaf range, they may produce a courtship duet (CrD) before mating. In CrD, males emit a male courtship phrase (MCrP) (Fig 3).
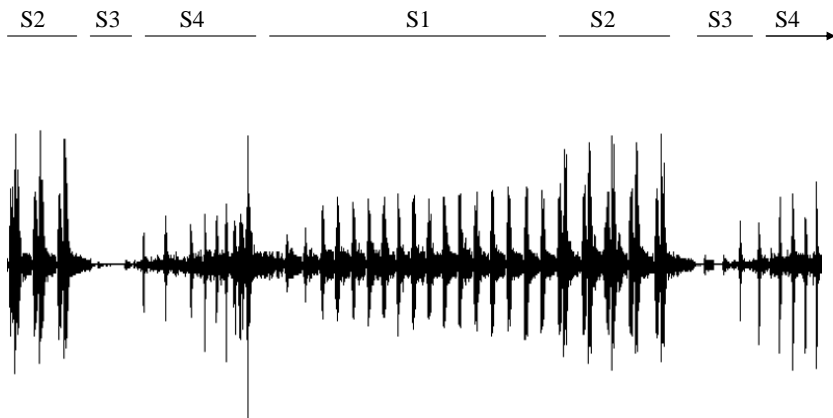


**Figure 3. Male courtship phrase (MCrP)**, consisting in section 1 - 4 of Male courtship phrase (MCrP) in duet with female (Mazzoni et al., 2009a).

Within MCrP, there are four sections S1-S4. In the first section, there is a train of MP1 with regular repetition time and with a buzz in-between, while in S2 there is a longer pulse repetition time and presence of a male pulse 2 (MP2) with higher amplitude than MP1. Section 3 consists of only a buzz

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section and S4 of short pulses associated with fast abdominal shakings. Rival males can interrupt CrD when emitting a disturbance noise (DN) (Fig. 2b). DN consists in a train of quickly repeated male disturbance pulses without presence of a pre-pulse. Disturbance pulses overlap with the female pulses with the immediate effect to interrupt the signalling of the courting male. Such masking of female signals was shown to substantially reduce the number of copulations in a laboratorial experiment (Mazzoni et al., 2009b).

The peak in male signalling activity is associated with twilight or early night and with a call-fly behaviour when males fly immediately after calling, even in absence of female response (Mazzoni et al., 2009a).

## 1.5. Mating disruption

Mating disruption (MD) is an environmentally safe management approach to control insect pests. Today MD aims to disrupt mating in insects that use species specific chemical signals, known as pheromones, for sexual communication (Witzgall et al. 2008). The hypothesized mechanisms of MD are to cause sensory adaptation at the antennal receptor level and at the central nervous system due to constant exposure of high concentration of pheromone, or a competition between natural and synthetic pheromone due to a uniform environment of synthetic pheromone (Cardé, 1990). The first attempts of deploying female sex pheromones to cause disorientation in males were made in the 1960s (Beroza, 1960; Wright, 1964) and since then the pheromone research has improved the pest management of several important crops worldwide (Witzgall et al. 2008). In the European grapevine production it is currently implemented on approximately 140,000 ha (Ioriatti et al 2011).

The success of MD depends on the dispensers' efficacy to release pheromones homogenously for the required period, weather factors such as

rain or winds and the density of dispensers and plants in the field (Ioriatti et al., 2004). Moreover, for efficient disruption, knowledge about the reproductive characteristics and the dispersal ecology of the species is crucial (Cardé, 1990).

An advantage with MD is the non-toxicity, which results in reduced or eliminated use of pesticides against a certain pest. Only if the pest population density exceeds the thresholds of MD, some pesticides can be used, either at the entire vineyard or at "hot spots" (Ioriatti et al 2011). Another advantage is that the species specificity makes it possible to both control and monitor the mating behaviour. However, the species specificity may also force growers to use pesticides against all other pests that are non-target to MD, which eliminates the environmentally friendly advantage. Such situations occur, for example, when vibrational communicating pests are present. Apart from the work published from the present thesis (Eriksson et al., 2012), the use of species specific vibrations for mating disruption has been considered only from a theoretical point of view, as when the DN signal in *S. titanus* was found (Mazzoni et al 2009a) and played back to insects in laboratory to disrupt mating duets (Polajnar and Cokl, 2008; Mazzoni et al 2009b) or when the jamming of the courtship by a rival male was studied (Miranda, 2006; Legendre et al 2012) and when pre-recorded vibrations were used in combination traps with pheromones (Čokl and Millar, 2009).

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## 2. Objectives

The main aim of this thesis was to develop a novel **mating disruption** strategy based on substrate-borne vibrations (chapter II), using *S. titanus* as a model pest species. Experiments were made in semi-field and with mature field-growing grapevine plants. The system was based on the natural rival signal from *S. titanus* (disturbance noise; DN), which was synthetically reproduced and transmitted from an electromagnetic tool (shaker), via vineyard supporting wires into grapevine tissues, with the aim to mask the natural communication between *S. titanus* males and females released on the grapevine plants.

Moreover, to enhance the efficacy of the method and to increase the general understandings on the mechanisms of substrate-borne vibrational communication, laboratorial studies were made on transmission properties of substrate-borne signals and on some undescribed aspects of the mating behaviour of *S. titanus*. The aim was first to investigate whether substrate continuity is essential for transmission of vibrational signals when male and female leafhopper were placed to **communicate from discontinuous substrates** (chapter I) and secondly, the role of the perceived **signal intensity of female reply for male orientation** and if *S. titanus* has **directionality** when searching for a mating partner situated on a distant leaf (chapter III).

# 3. Inter-plant vibrational communication in a leafhopper insect (Chapter I)

## 3.1. Introduction

Substrate-borne vibrational signaling is a widespread form of animal communication, not only in arthropods (Virant-Doberlet and Čokl, 2004; Cocroft and Rodriguez, 2005) but also among vertebrates (Hill, 2009; Caldwell et al., 2010). Although it has been recognized for centuries, its importance has long been overlooked (Virant-Doberlet and Čokl, 2004; Cocroft and Rodriguez, 2005; Hill, 2009). As with any communication channel, the effective communication range of vibrational signals depends on the amplitude of the emitted signals, on attenuation and degradation during propagation (Michelsen et al., 1982; Cocroft et al., 2006; Miklas et al., 2001) and on the sensitivity of the receiver's receptors (Endler, 1993). Depending on the size, the communication range of vibrational signals can extend up to eight meters (Michelsen et al., 1982; Čokl and Virant-Doberlet, 2003; Stewart and Zeigler, 1984; McVean and Field, 1996; Barth, 2002). At any rate, it is generally assumed to be limited to one plant or neighboring plants with interconnected roots or touching leaves (Cocroft and Rodriguez, 2005; Čokl and Virant-Doberlet, 2003; Ichikawa and Ishii, 1974; Hunt, 1993).

Until recently most studies on vibrational communication have been made within the range of few centimeters and have primarily focused on the species-specific vibrational repertoire (reviewed in (Čokl and Virant-Doberlet, 2003; Claridge, 1985). The ability of conspecifics to recognize and locate each other in the environment depends on the efficacy of their communication. In particular, species-specific signals used in sexual communication enable identification of the sender (species and sex) and provide information necessary to determine its location (Bradbury and

Vehrencamp, 1998; Gerhardt and Huber, 2002). In order to efficiently localize a conspecific partner, receivers should, in principle, determine not only a direction of the signal source, but also estimate its distance and adjust searching strategy accordingly. Currently there is no evidence of determination of source distance in plant-dwelling insects (Virant-Doberlet et al., 2006). However, it has been hypothesized that on plants, insects may be able to roughly estimate the distance by the extent of distortion and degradation due to differences in attenuation and filtering of different frequency components in the signal (Michelsen et al., 1982).

Signals that are perceived by insects as substrate-borne vibrations usually have a low intensity air-borne component (Čokl and Virant-Doberlet, 2003; Ossiannilsson, 1949; Percy et al., 2006) that potentially may be detected over few centimeters by antennal receptors (e.g. Kirchner, 1994) or even by vibration receptors in the legs (Shaw, 1994a). Antennal receptors suggested to be involved in perception of air-borne and substrate-borne vibrations have been described in *Oncopsis flavicollis* (Howse and Claridge, 1970; Claridge and Nixon, 1986), *Nezara viridula* (Jeram and Pabst, 1996), and *Hyalesthes obsoletus* (Romani et al., 2009). Therefore, we investigated whether continuity of the substrate is essential in the transmission of vibrational signals for successful communication between sexes.

As a model species we chose the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae), a major pest of grapevine, that transmits the phytoplasma responsible of the grapevine yellow disease "Flavescence dorée" in Europe (Schvester et al., 1963). The role of vibrational signals in intraspecific communication and pair formation of *S. titanus* on a single grapevine leaf has been described in detail. Pair formation begins with a spontaneous emission of a male calling signal (MCS) which in response to female reply may extend into a courtship phrase (MCrP). Females don't

emit vibrational signals spontaneously (Mazzoni et al., 2009a). In absence of female reply males may perform the "call-fly" behavior (Hunt and Nault, 1991), by alternating emissions of MCS with jumps from the plant (Mazzoni et al., 2009a).

We show here that discontinuity of substrate is not a barrier for communication in a vibrational communicating insect and that antennal receptors are not essential for detecting mating signals when partners are placed on discontinuous substrates. The results are discussed with regard to mate searching behavior associated with different levels of signal intensity.

## 3.2. Materials and Methods

### 3.2.1. Rearing of insects

*S. titanus* eggs originated from two-year-old grapevine (*Vitis vinifera*) canes collected from organic farms in Northern Italy (Povo, Trento, Italy). Egg hatching occurred in a climate chamber (24±1°C, 16L:8D photoperiod, 75% R.H.). Nymphs were removed daily into rearing boxes, consisting of plastic beakers (height 10 cm; 5 cm i.d.) with a moistened grapevine leaf laid on top of a 1-cm-layer of technical agar solution (0.8%) that was replaced twice a week. At emergence, adults were separated by sex and age (day of emergence), and kept in the rearing boxes. All experiments were made with virgin, sexually mature males and females at least 8 days old (Mazzoni et al., 2009a).

### 3.2.2. Terminology and recording of vibrational signals

In the current study we used terminology established by Mazzoni et al. (2009a). The experiments were performed in an enclosed room of the Entomology Section (Pisa University) at 23±1°C from June to August, between 5 pm and 9 pm which is the peak in sexual activity in *S. titanus*

[29]. The signals were recorded with a laser vibrometer (Ometron VQ-500-D-V, Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark) and digitized with 48 kHz sample rate and 16-bit resolution, then stored directly onto a hard drive through Plug.n.DAQ (Roga Instruments, Waldalgesheim, Germany). Signal spectral analysis was performed by means of Pulse 14 (Brüel and Kjær Sound & Vibration A/S). Recorded signals were analyzed with a FFT window length of 400 points. The leafhopper behavior was recorded with a Canon MV1 miniDV camera. The communication between males and females was observed for 20 minutes or until the male reached the female.

### 3.2.3. Test 1. Inter-plant communication

We placed a male and a female on leaves of two separate grapevine cuttings with one leaf (surface 6 x 10 cm) (see Figure 4). The gap width between the upper and lower leaf surface ranged from 0.5 cm to 7 cm. For each distance we recorded whether the female responded to the MCS emitted by male with the prompt emission of pulses. Then, we categorized and counted the male behavioral reactions to the female reply: (1) no reaction; (2) mating duet followed by call-fly; (3) mating duet with male search and location of the female.
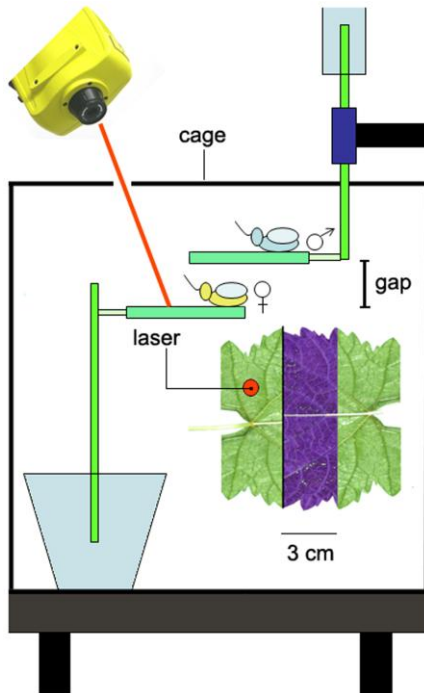
**Figure 4. A schematic drawing of experimental setup.** A male and a female were placed on leaves (surface 6 x 10 cm) of two separate grapevine cuttings. The bottom of the stem was put in a glass vial filled with water to prevent withering. One cutting was put on an anti-vibration table (Astel S.a.s., Ivrea, Italy). The second cutting was attached to a metal arm suspended from above – without any contact with the table - and positioned in parallel over half the surface of the lower leaf (as shown in the inset as viewed from above). The laser beam was focused on the lamina of the lower leaf with the female. To prevent the insects from escaping, recordings were made within a Plexiglas cylinder (50 x 30 cm), provided of two openings for the laser beam and the metal arm. Not drawn to the scale.

## 3.2.4. Test 2. Signal transmission

Transmission of MCS between grapevine leaves that were not connected by a common substrate was studied by playback of pre-recorded MCS. The spectral structure of *S. titanus* MCS is characterized by a series of several

prominent frequency peaks in the range between 80 and 300 Hz and maximum substrate vibration velocity above $10^{-2}$ mm/s. We recorded MCS at a close range on the grapevine leaf with a laser vibrometer as described above, from three different males. Since variability between spectral parameters among males was negligible we used a single randomly chosen MCS (composed of 27 pulses). Five pairs of leaves were tested from different cuttings, in the same experimental set up of figure 3, in absence of real insects and cage. The lower grapevine leaf was vibrated by a minishaker (Type 4810; Brüel and Kjær Sound & Vibration A/S) with a conical tip attached onto the leaf surface, 2 cm distant from the anterior border. The minishaker was driven from a computer via Adobe Audition 3.0 (Adobe Systems Incorporated). The amplitude of playback signal was adjusted to the natural emitted signal. The measurements were taken from the leaf lamina in two different randomly chosen points at least 2 cm distant from the border both of the lower and upper leaf by laser vibrometer. The gap between parallel leaf surfaces was 0.5, 1, 2, 3, 4, 5, 6, 7 and 11 cm. Spectral components and velocity of leaf vibration were analyzed along the distance by taking the average of nine randomly chosen recorded pulses from each distance and each leaf. To assess the velocity and frequency differences the Jonckheere test was performed (Siegel and Castellan, 1968).

### 3.2.5. Test 3. The role of antennae in perception of vibrational signals.

Females were put in a freezer (-25°C) for 30 seconds to cool them and prevent them from moving when placed under a stereomicroscope. Both antennae were cut off with microscissors. After ablation, females were kept separately in the rearing boxes for 24 hrs before they were used in experiments.

For the experiments, ten pairs consisting of intact males and of females whose antennae had been removed were first tested at close range on a single grapevine leaf to determine the female responsiveness after the ablation. In case of female response, they were subsequently tested on two leaves not connected via the common substrate and separated by a 5 cm gap as described above. The laser was focused on the leaf of the female.

## 3.3. Results

### 3.3.1. Test 1. Male-female inter-plant communication

We placed *S. titanus* male and female on different grapevine leaves separated by a gap of varying widths. In all trials males initiated communication behaviour with emission of MCS and females were observed to reply to male calls up to a 6-cm gap distance (Figure 5). As a result of female responses, most males established a duet with the female that ended either with female location or "call-fly" behaviour. Few males did not show any reaction to female responses. When mating duets were observed, they were composed of short series of male pulses alternated with one or more female pulses. Within the 5-cm gap distance, most females replied to male calls, although mate locations - achieved by the short jump from the upper leaf to the lower one with the female - were observed only at shorter distance. At 7-cm distance between leaves, none of the females responded to MCS.
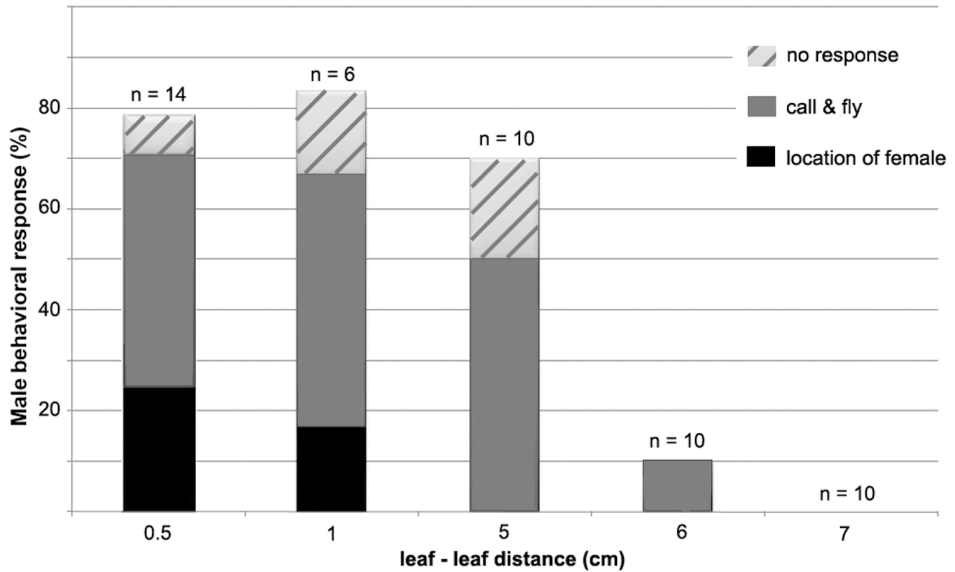
**Figure 5. Male-female communication in *Scaphoideus titanus* recorded on leaves without direct contact (Test 1).** Distances between upper and lower leaf were from 0.5 cm to 7 cm. The percentage of females that responded to the male calling signal (total column height) is divided according to the subsequent male behavioural response: mating duet, followed either by female location (black) or by call-fly (gray), and no male reaction (striped). n indicates the number of insect pairs tested.

## 3.3.2. Test 2. Signal transmission

We studied transmission of male vibrational signals between grapevine leaves that were separated by a gap of varying distance. In playback experiments (Figure 6), the mean substrate velocity progressively decreased with the distance (i.e. width of the gap) (Jonckheere test: $J_0$=5.93, P<0.001). In contrast, the dominant frequency increased ($J_0$=2.29, P=0.011). Compared with the signal recorded from the lower leaf, at 0.5 cm gap distance the decrease in vibration velocity was on average of 91.6±7.1% and at 11 cm gap distance the velocity was further reduced of

7.3±5.6%. Values of velocity measured between 0.5 – 1 cm were over 0.001 mm/s, whereas from 2 cm gap the mean velocity was constantly lower.



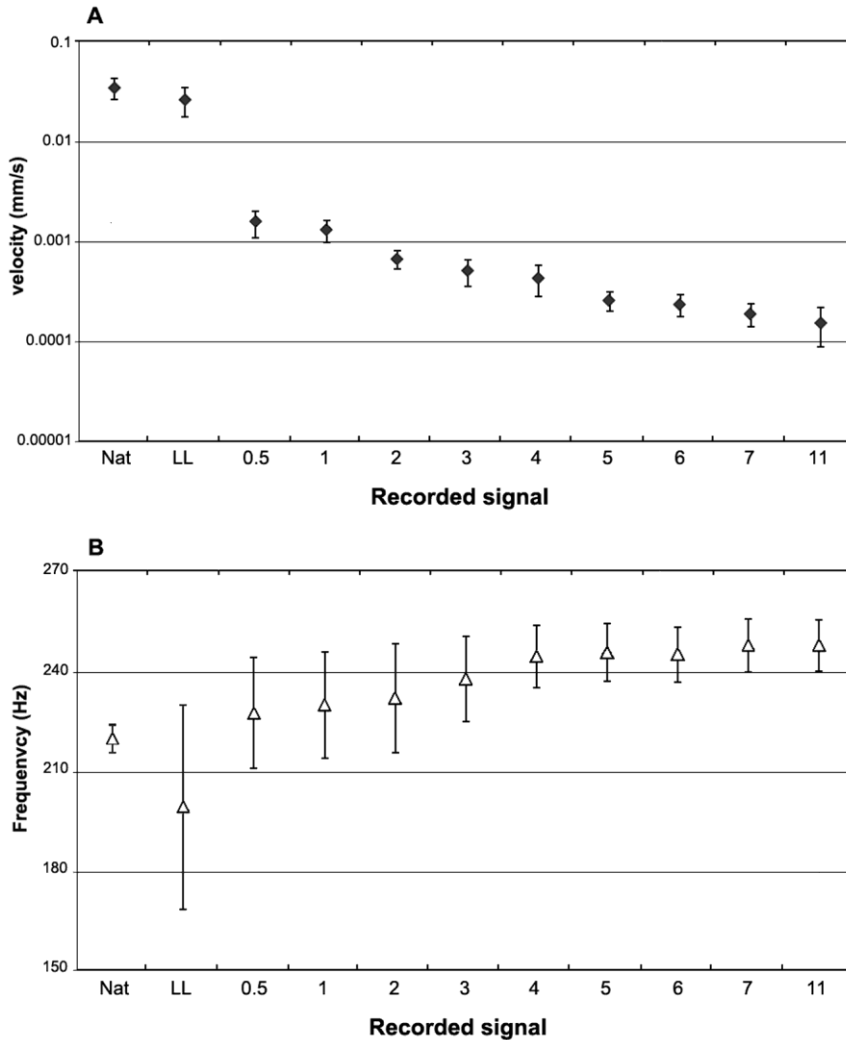**Figure 6. Signal properties measured on leaves with discontinuous substrate (Test 2).** Mean (±SE) values of maximum substrate vibration velocity (mm/s) (A, logarithmic scale) and frequency (Hz) (B) of pulses from MCS (Male calling signal) are shown. While substrate velocity progressively decreased (Jonckheere test: $J_0=5.93$, P<0.001) with the distance between leaves, the frequency increased

($J_0=2.29$, P=0.011). Nat: MCS emitted by natural male recorded on the same leaf; LL: MCS emitted by playback recorded on the same leaf; 0.5-11: MCS emitted on the lower leaf and recorded from the upper leaf with a progressive gap width of 0.5 – 11 cm..

### 3.3.3. Test 3. The role of antennae in perception of vibrational signals

When ten pairs of intact males and females with surgically removed antennae were tested on the same leaf, all females responded to the MCS. When pairs were tested on two leaves not connected via the common substrate and separated by 5 cm gap, seven out of ten females responded. This result is identical to test 1, when leaves were separated by 5 cm gap and females had intact antennae.

## 3.4. Discussion

Contrary to general belief, our findings demonstrate that the communication range of vibrational signals emitted by small insects is not limited to physically interconnected substrates. Production of low-frequency acoustic signals that are perceived by receivers as substrate-borne vibrations usually also results in emission of a low-intensity air-borne component (Michelsen et al., 1982; Ossiannilsson, 1949; Percy et al., 2006). Efficient radiation of acoustic sources in the air is possible only when emitter is bigger than 1/3 of the wavelength of the emitted sound (Markl, 1983; Bennet-Clark, 1998). For an insect of the size of *S. titanus* (4-5 mm), the optimal frequency of air-borne sound would be above 10 kHz. The effective air-borne range of low frequency vibrational signals with dominant frequencies in the range between 80-300 Hz emitted by *S. titanus* is short and we never heard air-borne sounds during their calling. Nevertheless, while communication at distances larger than a few cm is

mediated by vibrations of the substrate, at closer range the role of air-borne component cannot be excluded. At a range of a few cm, such signals may be detected by mechanosensory hairs (Keil, 1997) or the Johnston's organ in the antennae (Kirchner, 1994). Our results show that in *S. titanus* mechanoreceptors in the antennae are not involved in detection of air-borne component of vibrational signals. Heteropteran insects possess hairs that may be used for detecting air-particle displacement (Drašlar, 1973) however, a systematic survey of sensilla on the leafhopper body is lacking.

Our measurements showed that vibrations are transmitted from one leaf to another even when they were separated by a gap of 11 cm and that females responded to males up to a gap width of 6 cm. From our results it was not possible to determine explicitly whether the vibrational signals were detected as air-borne sound or as substrate vibrations induced in the leaf. However, some observations, indicate the latter as the more probable hypothesis. In some cases male and female leafhoppers were not positioned within the gap between leaves, but on external sides of leaf laminae. In such situation two leaves would represent severe obstacle to any low intensity air-borne sounds. On the other hand, it has previously been shown that leaf vibrations are transmitted through the air beyond the boundary layer of the leaf and that air particle displacement triggered by leaf vibrations has the same temporal pattern as substrate vibrations (Čokl et al., 2006). The fact that in our experimental set-up we used two partly overlapping leaves with relatively large surface may also explain why in other studies in which only the tips of the leaves were in close proximity, concluded that vibrational communication was limited to a continuous substrate. Situations in which leaves are separated by a gap but partly overlapping probably represent a more natural case for insects that communicate in a dense vegetation habitat.

The maximum intensity of vibrational signals on a leaf without any contact with the vibrated leaf, measured directly as velocity at gap distances at which females were still responding, was in the velocity range between $10^{-6}$ and $10^{-7}$ m/sec at dominant frequencies between 220-250 Hz. These values translate to displacement values between $10^{-9}$ and $10^{-10}$ m. The lowest neurobiologically determined velocity threshold values for subgenual organs in various insect groups are all in the range between $10^{-5}$ and $10^{-6}$ m/sec (Heteroptera: (Čokl et al., 2006; Čokl, 1983); Neuroptera: (Devetak et al., 1978); Orthopteroids: Shaw, 1994b; Čokl et al., 1995; Čokl and Virant-Doberlet, 1997). However, in all these insects conversion of velocity threshold values into displacement values results in threshold values below $10^{-9}$ m. In particular, in another hemipteran insect, the southern green stink bug *Nezara viridula*, threshold values of receptor cells in the subgenual organ follow the line of equal displacement (Čokl et al., 2006). This suggests that, although displacements induced in a leaf by vibrational signals emitted on another leaf nearby are low, they are not below the threshold values of the subgenual organ. In leafhoppers nothing is known about vibration receptors in the legs (Čokl and Virant-Doberlet, 2003). However, it is likely that leafhoppers possess subgenual organs on all six legs. In insects this is the most sensitive organ to detect substrate vibrations and it was described also in closely related insect groups such as froghoppers (Cercopidae) and bugs (Heteroptera) (Čokl et al., 2006; Debaisieux, 1938; Michel et al., 1983). Our measurements also revealed a significant increase in dominant frequency (from 200 to 250 Hz) when vibrational signals were transmitted through air from one leaf to another. It is interesting to note that resonant frequencies of sound-induced vibrations in bean leaves are in the frequency range between 190 and 290 Hz (Čokl et al., 2005). In the pentatomid bug *N. viridula*, for which bean is a preferred host plant, resonant frequencies correspond to best frequency sensitivity of

one of the two cells in the subgenual organ (Čokl, 1983). We argue that transmission of vibrational signals from one leaf to another via air may be a common phenomenon. High receptor sensitivity, together with potential tuning of plant resonant frequencies with spectral properties of vibrational signals may enable the insect to extend the communication range beyond the limit of one plant.

In addition, our results suggest that the intensity of the perceived vibrational signals may have crucial effects on the leafhopper behaviour. Mating duet followed by female location was observed only at the two shortest gaps, while call-fly behaviour prevailed at longer distances. Although the role of shifts in dominant frequency cannot be excluded, the observed differences are small (between 20 and 40Hz) in comparison with the 20 dB difference in intensity. When male and female were positioned on the same leaf at the beginning of our observations, MCS was immediately extended into a courtship phrase without the intermediate stage observed at other distances (Mazzoni et al., 2009a; Mazzoni et al., 2009b). It is conceivable that leafhoppers are able to compare the intensity of their own signals and perceived signals emitted by the duetting partner. Below a certain threshold the intensity may provide information that the female is not located on the same leaf as the male and that the male therefore needs to adjust the searching strategy accordingly. Since most studies on planthopper and leafhopper mating behaviour have been conducted in short range situations, the information about patterns of long-range communication is lacking.

The call-fly behaviour observed in males is usually associated with a strategy to increase effective signalling space (Hunt and Nault, 1991; Gwynne, 1987). However, when the position of the source of low intensity female reply is unpredictable for the courting male, call-fly strategy may enable a faster localization of the leaf hosting the female. In addition,

numerous changes of the position of the signalling male may reduce predation risk from eavesdropping predators like spiders (Virant-Doberlet et al., 2011).

In conclusion, we showed that the communication range of vibrational signals is not limited by substrate continuity and that in this situation antennal receptors are not essential in detection of vibrational mating signals. Moreover, our behavioural observations together with measurements of signal transmission between grapevine leaves suggest that behavioural responses of *S. titanus* may depend on the signal intensity.

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# 4. Exploitation of insect vibrational signals reveals a new method of pest management (Chapter II)

## 4.1. Introduction

For many insects, species-specific sex pheromones are essential in bringing together potential partners (Greenfield, 2002) and an early realization of the potential for exploiting chemical signals for pest control has led to a plethora of research and application through the last 50 years (Gaston et al., 1967; Witzgall et al., 2010; Čokl and Millar, 2009). Today disruption of chemical communication is an integral part of pest management in several important crops worldwide (Witzgall et al., 2010; Čokl and Millar, 2009; Ioriatti et al., 2008). However, numerous major insect pests do not rely on long-range chemical communication, most notably leafhoppers and planthoppers (Redak et al., 2004; Wientraub and Beanland, 2006; Janse and Obradovic, 2010) that comprise more than 30,000 species (Dietrich, 2004; Urban and Cryan, 2007). In these insects mate recognition and localization of the partner are mediated exclusively via substrate-borne vibrational signals (Čokl and Virant-Doberlet, 2003) and their populations are currently managed primarily by insecticide treatments. Surprisingly, although males use special species-specific disruptive vibrational signals to interfere with the courtship of rivals (Mirandax, 2006; Mazzoni et al., 2009a), mating interruption by induced vibrations has been rarely considered even from a theoretical viewpoint and there has been virtually no research on how to exploit this common insect communication channel (Cocroft and Rodriguez, 2005) as a tool for pest control (Čokl and Millar, 2009; Mazzoni et al., 2009b).

Here we present the first implementation of mating disruption based on substrate-borne vibrations. The leafhopper *Scaphoideus titanus*

Ball (Hemiptera: Cicadellidae), a vector of a lethal grapevine disease Flavescence dorée, was chosen as a model pest species. In Europe Flavescence dorée is a quarantine disease and compulsory measures to manage vector populations and prevent the spread of the disease include large-scale insecticide treatments (Bressan et al., 2006). In sexual communication of *S. titanus* a stable male-female vibrational duet is essential for successful localization of the female and, consequently, for copulation (Mazzoni et al., 2009a; Cocroft and Rodriguez, 2005; Eriksson et al., 2011). Because the initial step in pair formation of *S. titanus* is an emission of male calling signals (Mazzoni et al., 2009a; Eriksson et al., 2011), we first analyzed the velocity characteristics of these vibrational signals in semi-field conditions, by applying pre-recorded calls to one leaf of the grapevine plants that were later used for mating disruption tests. Next, we established whether disruptive vibrational signals can be applied to several grapevine plants simultaneously and whether under such circumstances these signals would mask male calls. Finally, we assessed copulation success of *S. titanus* in the presence of disruptive signals under simulated semi-field conditions (potted plants) and in a vineyard with mature, field growing grapevine plants. By testing transmission of male calling signals on different plant parts, we aimed to establish the sensitivity of mating signals in order to adjust the power of the mating disruption signals into effective species-specific masking signals (disturbance noise) (Mazzoni et al., 2009a). An electromagnetic shaker was used to vibrate the wire with disruptive signal that was transmitted as substrate vibrations to the plants in both potted and fully mature field grapevine plants.

## 4.2. Materials and Methods

### 4.2.1. Insects

We collected *S. titanus* eggs from organic farms in Villazzano (Trento, Italy) and from them reared the adults used in the semi-field and field trials as described previously (Mazzoni et al., 2009a-b; Eriksson et al., 2011). All experiments were made with sexually mature males and females that were at least 8 or exactly 10 days old, respectively (Mazzoni et al., 2009a).

## 4.2.2. Signal transmission through grapevine plants

A *S. titanus* male calling song (MCS) used in transmission study was recorded with a laser vibrometer (PDV-100, Polytech GmbH, Waldbronn, Germany) in the laboratory with male singing at 0.5 cm distance from the recording point. To verify the characteristics of this signal, we compared it to the signals recorded and described previously (Mazzoni et al., 2009a; Eriksson et al., 2011). The disruption signal was a pre-recorded natural disruptive signal (also termed disturbance noise, DN) (Mazzoni et al., 2009a) recorded from a rival *S. titanus* male with the above mentioned laser vibrometer during rivalry encounters on a single grapevine leaf (Mazzoni et al., 2009a-b). An exemplar with the best signal-to-noise ratio was chosen from a library of recordings at Fondazione Edmund Mach (Italy).

Measurements in a semi-field setting were made outdoors at Pisa University (Italy) in July 2010. Five potted grapevine plants were pruned to have similar morphological characters (height 70-75 cm, two main branches, eight fully developed leaves). A supporting metal wire that commonly is used in vineyards was tied to the main stem of the plants, while the wire ends were fixed to concrete poles. The plants were placed in a row at distances 180 cm (plant 1), 370 cm (plant 2), 560 cm (plant 3), 750 cm (plant 4) and 940 cm (plant 5) from a newly designed electromagnetic shaker (power = 1 W, CBC Europe Ltd., Milano, Italy), through which the disruptive vibrational signal was applied (DN source) (Figure S3A). The shaker was driven by a lap-top computer via audio software Adobe

Audition (version 3.0; Adobe Systems Inc.) and the amplitude of naturally emitted DN was amplified 20 times. MCS was applied to the lamina of the upper leaf via a conical rod attached to a mini-shaker (Type 4810; Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark), driven from a computer via Adobe Audition and the amplitude was adjusted to the level of naturally emitted calls (Eriksson et al., 2011). To study the signal transmission through the whole plant, small squares of reflective tape (n = 21) on which the laser beam was focused were placed on leaves (blades, veins and petioles) and along the stem (Figure 7).
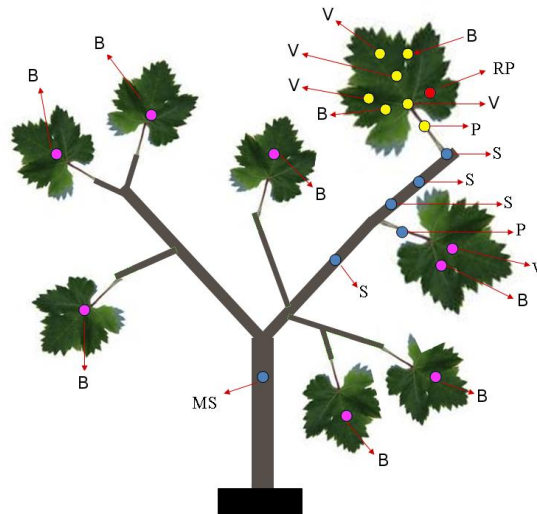


**Figure 7. Schematic drawing of the measuring points on the grapevine plants used in the transmission experiment.** Abbreviations: RP, reference point; B, blade; V, vein; P, petiole; S, stem; MS, main stem. Yellow and pink dots indicate the points used to analyze the signal intensity of the Vibrated Leaf and the Distant Leaves, respectively. RP is in red. Points of the stem (in blue) were not included in the analysis.

Vibrational signals were recorded with the above mentioned laser vibrometer and digitized with 48 kHz sample rate and 16-bit resolution,

then stored directly onto a hard drive through Plug.n.DAQ (Roga Instruments, Waldalgesheim, Germany). The intensity of recorded signals was measured directly as maximum substrate velocity (mm/s) by Pulse 14 (Brüel and Kjær Sound & Vibration A/S). Only the spectral component within the natural range of *S. titanus* vibrational signals (50-300 Hz) (Figure 8) was analyzed, using a FFT window length of 400 points.
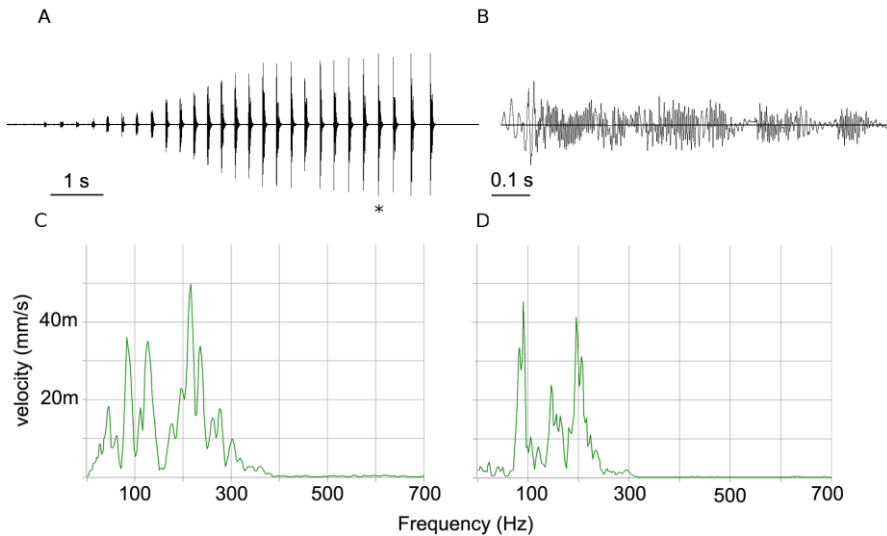


**Figure 8.** Oscillogram of a *Scaphoideus titanus* male calling song (MCS) (A) and of disturbance noise (DN) (B), both recorded on the same leaf, approximately 0.5 cm away from the male. Power spectra of a male pulse (indicated with the asterisk in A) of MCS and of the whole DN sequence are shown in (C) and (D), respectively. For mating disruption trials the amplitude of DN was amplified 20 times.

MCS and DN were played back three times respectively for each measuring point on every plant and the velocity was then taken for the three pulses with highest amplitude, thus obtaining an average velocity from 9 pulses per measuring point. An average across the three plants was calculated for

all points both from the vibrated leaf (VL: n = 7) and from all other leaves (distant leaves, DL: n = 8) (Figure 7). Points from the stem were excluded since *S. titanus* adults normally dwell on leaves. Our preliminary observations showed that the masking effect of DN on MCS was effective when the former was as high in intensity as the latter .

Field tests in a grape producing vineyard were conducted at Fondazione Edmund Mach (Italy) in July and August 2011. Mature rooted grapevine plants (height 1.5 m) grew in a row at distances 70 cm from each other with stems tied to a supporting metal wire. A MP3 driven electromagnetic shaker (EMS) used as source of disruptive signals (power = 1 W, CBC Europe Ltd., Milano, Italy) was attached to the wire and plants were chosen 100, 310, 520, 730 and 940 cm distant (Figure 9). Disruptive signals were recorded as described above from four leaves/plant (two points/leaf), randomly chosen among those enclosed in the net sleeves, used for the mating disruption test (see below).
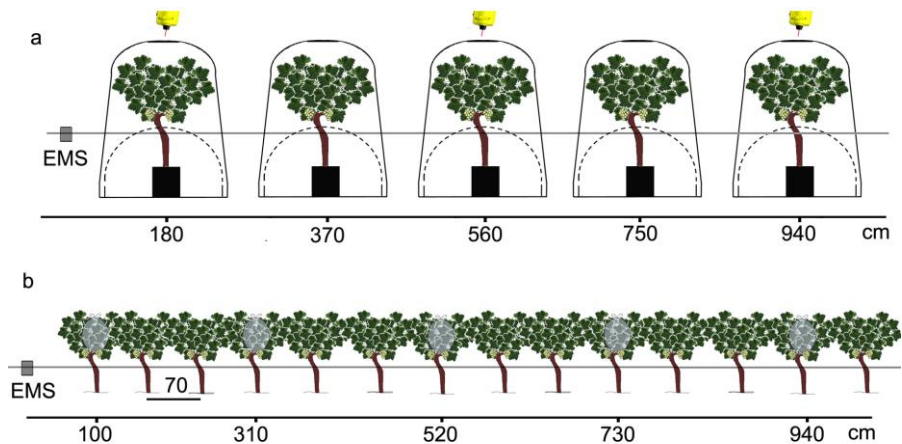


**Figure 9. Experimental set-up** of mating disruption in semi-field with potted plants surrounded by cages (a) and in a mature vineyard with shoots of the rooted plants enclosed in nylon netting sleeves (b). The disruptive signals (DN) were emitted from an electromagnetic shaker (EMS) attached to the supporting wire.

Recordings of vibrational signals were made with a laser vibrometer at 180 cm, 560 cm and 940 cm from EMS in semi-field and at all plants with sleeves in the vineyard. One insect pair was put in each cage/sleeve on grapevine plants at increasing distance from the source.

### 4.2.3. Mating disruption

Experiments with live *S. titanus* in the semi-field setting of five potted grapevine plants, as described above, were conducted outdoors at Pisa University in July and August 2010. In addition, two potted grapevine plants of similar size tied to a non-vibrated wire were used as controls. Each plant was isolated in a transparent polyester cage (75 x 75 x 115 cm) (Bugdorm 2400 Insect Rearing Tent, MegaView Science Co., Ltd., Taichung, Taiwan) with closable openings to release and collect the insects. As a control, one grapevine plant from a neighbouring row without disruptive vibrations was used.

In field experiments, a shoot from the middle part of each plant (with approximately 20 leaves) was isolated in a nylon-netting sleeve (30 x 70 cm) (Bugdorm Insect Rearing Sleeves) with closable openings to release and collect the insects (Figure 10).

**Figure 10. Mature vineyard** with the shoots of grapevine plants isolated by sleeves (photo: V. Mazzoni).

Since most mating activity in *S. titanus* occurs during twilight or during the night (Mazzoni et al., 2009a)*,* all trials were made between 5 pm and 10 am the following day when insects were recollected from the cages/sleeves. In each overnight trial one virgin male and female *S. titanus* were put on separated leaves of each grapevine plant. When a male or a female could not be found or when one individual was dead, the replicate was discarded. Collected females were placed individually in rearing containers without access to egg laying sites and dissected 10 days later. Difference between the number of mated and virgin females in the treated plants and control plants was assessed with a G test in contingency table, after Williams' correction. The G-test was followed by pair-wise comparisons between groups with Ryan's test for multiple comparisons of proportions (Ryan, 1960).

### 4.2.4. Definition of virgin and mated S. titanus females

In preliminary experiments 10 days old females (n=35) were placed together with males and observed until they copulated. Afterwards the females were kept for 10 days individually in rearing containers without suitable egg laying substrate. As a control, 20 days old virgin females (n=35) were used. Shortly before a dissection, a living female was put in the freezer for 40 seconds before she was put in ethanol (70 %) under the stereomicroscope. Virgin females had on average 1.3 ($\pm$ 1.6) eggs, while mated females of the same age dissected 10 days after copulation had significantly higher number of eggs (13.4 $\pm$ 3.7; n=35; one-tailed unpaired t-test: t = -17.8, P<0.001). The minimum number of eggs found in the mated females was 7, while the maximum number of eggs in the virgin females was 6. Accordingly, we defined all females with 0-6 eggs as virgin and the females with >10 eggs as mated. As a safety limit, two females with 7-9 eggs were discarded. The eggs found in the virgin females were probably unfertilized and without the potential for development, as was suggested in the closely related species *Homalodisca vitripennis* (Hemiptera: Cicadellidae) (Al-Wahaibi and Morse, 2009).

## 4.3. Results

### 4.3.1. Semi-field

In order to simulate a natural situation in a vineyard the potted grapevine plants were tied in a row to the grapevine supporting wire at various distances. On these plants the highest intensities of male calling signal were measured on the leaf which was vibrated with the pre-recorded calls (m = $1.45 \times 10^{-5} \pm 0.56 \times 10^{-5}$ mm/s), nevertheless, at almost all measuring points the recorded intensities were high enough to enable communication between

the male and female (Figure 11) (Eriksson et al., 2011). The mean substrate velocity measured from all other leaves was $2.19 \times 10^{-6} \pm 1.37$ mm/s.
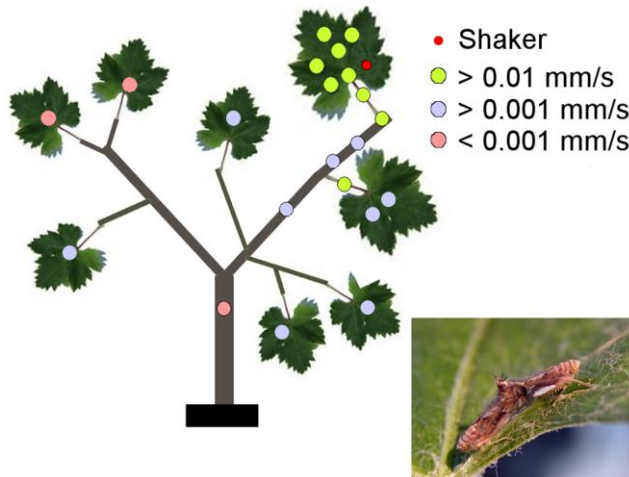


**Figure 11. Transmission of MCS through a grapevine plant.** The uppermost leaf of potted grapevine plants was vibrated with male calling signal (red dot, Shaker). The intensity of vibrational signals was measured at several points along the grapevine plants as substrate velocity at the dominant frequency (mm/s) and accordingly, three probability levels of successful mating communication were assigned to each point: »high«, velocity of mating signals > 0.01 mm/s, green circles; »median«, velocity of mating signals between 0.001 and 0.01 mm/s, blue circles; »low«, velocity of disruptive signals under 0.001 mm/s, pink circles. The latter is below the threshold level of signal detection of *S. titanus* (17). A mating pair of *S. titanus* is shown next to the grapevine plant (photo A. Lucchi).

The disruptive vibrational signals were applied to several potted plants simultaneously via the supporting wire up to 940 cm from the source of masking signals. An electromagnetic shaker was used to vibrate the wire with a pre-recorded *S. titanus* species-specific disruptive signal (disturbance noise) (Mazzoni et al., 2009a) and we determined the masking effect on male calling signal at several points along each plant. Although the ratio between the measured level of disruptive signal and male calling

signal decreased with increasing distance of the plant from the shaker, even at 940 cm from the source, disruptive vibrational signals still masked male calling signals at every measured point (Figures 11 and 12).
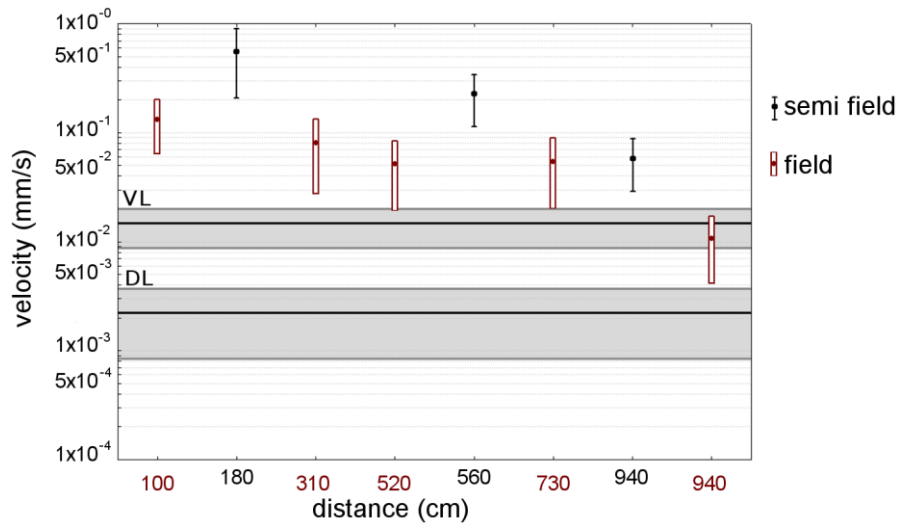


**Figure 12**. **Maximum (mean±SD) substrate velocity (mm/s, logarithmic scale) of disruptive signal (DN) recorded in the frequency range 50-300 Hz from potted plants in semi-field conditions and from rooted grapevine plants in field.** Semi-field and field recordings were made at three and five distances from a DN source, respectively (semi-field: black dots, distances 180 cm, 560 cm, 940 m; field: red dots, distances 100 cm, 310 cm, 520 cm, 730 cm, 940 cm). MCS played back into potted plants from a leaf showed highest substrate velocities within the same vibrated leaf (VL) range; a substantial decrease was found on all other leaves of the plant (distant leaves, DL). The transverse black lines represent the mean (±SD, gray areas) of maximum velocity of MCS of the VL or DL range.

Next, we assessed copulation success of *S. titanus* under simulated semi-field conditions as described above by comparing the number of eggs produced by females left with males overnight on vibrated and non-vibrated grapevine plants. In pairs that were placed on potted grapevine

plants vibrated with disruptive signals, significantly more females remained virgin when pairs were put on vibrated plants (Figure 13A; G=58.4, df=6, P<0.0001) and no significant difference in copulation success at different distances was found.

## 4.3.2. Mature vineyard

In a mature grape-producing vineyard, insect pairs were released overnight on plants positioned at similar distances as in semi-field trial. The last grapevine plant was positioned 940 cm away from the source of disruptive signals and the measured levels of disruptive signal were in the same intensity range as the naturally emitted *S. titanus* male calling signal (Figure 12). There was a significant difference in the number of virgin females between control and vibrated plants (Figure 13B; G=119.7, df=5, P<0.0001) but not between vibrated plants positioned at different distances.
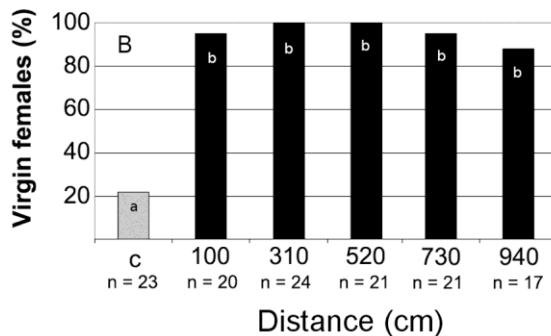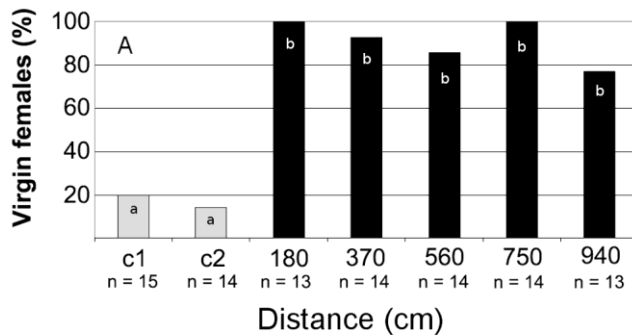
**Figure 13. Number of virgin and mated females found on vibrated and non-vibrated grapevine plants.** (A) Semi-field conditions with potted plants, (B) field trial in a vineyard. Black and gray bars show virgin females from plants at increasing distances from the source of disruptive signals and from control plants in the absence of these signals, respectively. Different letters indicate significant differences (P<0.0001) between treatments after G-test for contingency table (William's corrected) followed by a Ryan multiple comparison of proportions test. The number of replicates (n) at each distance from the source of disruptive signals and for controls (c) is given.

## 4.4. Discussion

Taken together, these results provide direct evidence that mating disruption based on playback of disruptive vibrational signals is an effective, environmentally friendly approach to manage insect pests. Although few females on vibrated plants placed at further distance from the source were mated, these matings could be explained by chance, as a result of call-fly behaviour (Hunt and Nault, 1991) when males accidentally landed on the same leaf close to the females and when other potential factors like short-range chemical or visual cues may enable partner recognition. Taking into account the intensity loss of mating vibrational signal measured on distant leaves, in the presence of disruptive signals mating communication between pairs placed on different leaves seems unlikely. However, such accidental location of the female would be even less likely in the open field, where the movements of the male would not be limited to only few leaves or to the same shoot.

As in mating disruption based on pheromones, management of insect pests by disruptive vibrational signals does not eliminate pests from the system but can keep populations below an acceptable economic damage threshold (Čokl and Millar, 2009). Since delays in mating result in reduced

female fecundity and fertility (Torres-Vila et al., 2002), long-term use of mating disruption can also decrease population levels of target pest species (Witzgall et al., 2010; Ioriatti et al., 2008). Future work should reveal whether disruptive vibrational signals also affect other behaviours of target pests, such as feeding and oviposition, as well as whether they have negative effects on beneficial fauna. Parasitoids (Laumann et al., 2007) and predators (Virant-Doberlet et al., 2011) use vibrational signals to locate their prey and masking signals could affect their localization ability. However, in our field trials spiders preying on *S. titanus* were a persistent problem and, potentially, visually-oriented predators like some spiders may be less affected.

Besides *S. titanus*, there are several other leafhopper and planthopper grapevine pests (Lenz et al., 2009; Costello, 2008; Mazzoni et al., 2010; Pavan and Picotti, 2009), including the vector of a lethal Pierce's disease (Redak et al., 2004), against which this new tool for insect pest control could be implemented. Although in the current study we used *S. titanus* species-specific disruptive signals, it may be possible to synthesize a disruptive signal suitable for managing several pests simultaneously. As vibrational signalling is widespread among insects (Cocroft and Rodriguez, 2005), mating disruption strategies for control of insect pests communicating via substrate-borne vibrational signals is likely to have wider application. Whiteflies are serious pests in greenhouses and vibrational signals are produced as part of their mating behaviour (Kanimiya, 2006). Vibrating large number of plants in the greenhouse may be easier than large scale field application for which additional work is needed to parameterise the effects of distance, and hence the spacing of vibrational sources, as well as potential interference of multiple sources of disruptive signals. Furthermore, it has been suggested that a monitoring trap, which combines pheromones and vibrational signals may provide a

solution for pests like stink bugs that rely on bimodal communication (Čokl and Millar, 2009). In short term, the main challenge for application of vibrational mating disruption may be to convince growers, as well as policy makers, that this is a viable alternative to conventional plant protection tactics and that vibrating wires could reduce or replace the use of chemical pesticides. Moreover, in combination with novel monitoring techniques and as a part of an integrated high-tech crop protection system (Clay, 2011), mating disruption based on substrate-borne vibrational signals can provide an efficient pest management with low environmental impact that in the near future could transform many farming systems.

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# 5. Signal parameters involved in pair formation mediated by substrate-borne vibrations (Chapter III)

## 5.1. Introduction

Animals induce vibrations in different substrates, both incidentally when walking and feeding or when producing signals aimed at conspecifics (Hill 2008). Substrate-borne 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). Likewise, it has been found in animals as large as elephants (O'Connel-Rodwell 2007) and as small as fruitflies (Mazzoni et al. 2013a; Fabre et al. 2012). While for example elephants communicate on soil as a signal transmission substrate, plants are the most common substrate for invertebrates (Barth 1998; Čokl and Virant-Doberlet 2003; Cocroft and Rodriguez 2005). However, plants 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 are distorted in frequency and time domain (Michelsen et al. 1982; Miklas et al. 2001). Species-specific vibrational signals used in sexual communication enable identification of the sender (species and sex) and provide information necessary to determine its location (e.g. Virant-Doberlet et al. 2006; Hill 2008; Legendre et al. 2012; De Groot et al. 2012). Intensity differences at distances as short as 2 to 4 cm are large enough to be detected in the nervous system of insects (Stritih et al. 2000; Čokl et al. 2006). However, due to intensity oscillations of vibrational signals during the transmission (Michelsen et al., 1982; Čokl et al. 2007; Polajnar et al. 2012), on plants there is often no reliable intensity gradient and the role of intensity in orientation behaviour is still under debate (Virant-Doberlet et al. 2006; Mazzoni et al. 2013b). Furthermore, the majority of insects relying on vibrational communication

is smaller than 1 cm and for them deriving directional cues by directly comparing amplitude or time differences on two sides of the body (left-right or front-back) may not be possible (Virant-Doberlet et al. 2006). However, alternatively, for small insects directional information may be available in the mechanical response of the body itself (Cocroft et al., 2000).

Differences in amplitude and time of arrival of 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 intensity differences large enough to be used in orientation (Virant-Doberlet et al. 2006).

In some insects relying on vibrational communication, the searching for a mating partner has been characterized as "trial and error" (e.g. Gillham 1992) while in others males travelled a shorter path than during pure random search (Legendre et al. 2012). The efficiency of recognizing and localizing a conspecific partner depends on the ability of the receiver to distinguish the signalling source from environmental noise and/or non-target species (Pollack, 2000).

In the Nearctic leafhopper, *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae), which communicates with substrate-borne signals (Mazzoni et al. 2009a), it was shown that a male and a female were able to establish a duet also when leaves were not physically interconnected (Eriksson et al. 2011). When *S. titanus* partners were communicating from two partly overlapping leaves separated by an air gap, the most commonly observed male reaction was "call-fly" behaviour (i.e. alternation of calling and flying (Hunt and Nault 1991)). In *S. titanus*, the male is searching for the female and mating sequence is always initiated by male emitting calling signal

(MCS) to which the stationary females respond with pulses in-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 rival males emitting a disturbance noise (DN).

In the present work we aimed to describe pair formation in small plant dwelling insects in which obtaining directional cues may be difficult. By using *Scaphoideus titanus* as model species we studied duetting and searching behaviour in a situation when signals were transmitted through tissues of a grapevine cutting over distances larger than 10-15 cm. The following three hypotheses were made: 1) during pair formation in *S. titanus* duet structure may change as mating behaviour is progressing from initial recognition stage to close-range courtship. We expected that during the initial recognition stage when male has to correctly identify the partner's species and sex , female reply may have a different effect on duet structure than during courtship phase. This was tested by comparing the synchronization of male and female pulse emissions within duets of different behavioural stages. Moreover, since it has been suggested that the intensity of perceived signals may affect leafhopper mating behaviour (Eriksson et al. 2011), we hypothesized 2) that males may adjust their behaviour according to the perceived intensity of the female reply and related spectral component of her reply. At last we hypothesized that 3) males are able to make directional decisions to locate the female despite the small body size of *S. titanus*.

## 5.2 Materials and methods

### 5.2.1. Insects

Rearing of *S. titanus* from egg to adult followed the method described previously (Eriksson et al. 2011). All experiments were done with virgin

and sexually mature males and females at least 8 days old (Mazzoni et al. 2009a).

## 5.2.2. Terminology and signal recording

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. The experiments were performed at $23 \pm 1°C$ between 5 pm and 9 pm to obtain highest sexual activity from *S. titanus* (Mazzoni et al. 2009a). 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 being stored directly onto a hard drive through LANXI data acquisition driver (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark). Spectral and temporal parameters of the recorded signals were analyzed with Pulse 14.0 (Brüel and Kjær) with a FFT window length of 400 samples and 66.7% of overlap.

## 5.2.3. Test 1. Pair formation and synchrony of male-female signals in *S. titanus*

To study pair formation a male and a female of *S. titanus* (n = 20 pairs) from start were placed each on a different leaf of the same grapevine cutting. The cutting had two leaves (surface 6 x 10 cm) with petioles separated by a 10-cm long stem (Fig. 1A). 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). The laser beam was focused on a small piece of a reflective tape (i.e. measuring point) placed on the lamina of the leaf with the male. To prevent the insects from escaping, all recordings were made within a Plexiglas cylinder (50 x 30

cm). Communication between a male and a female was observed for 20 minutes or until the male reached the female, whichever came first. Leafhopper behaviour, together with vibrational signals were recorded with a Canon MV1 miniDV camera (50 FPS).
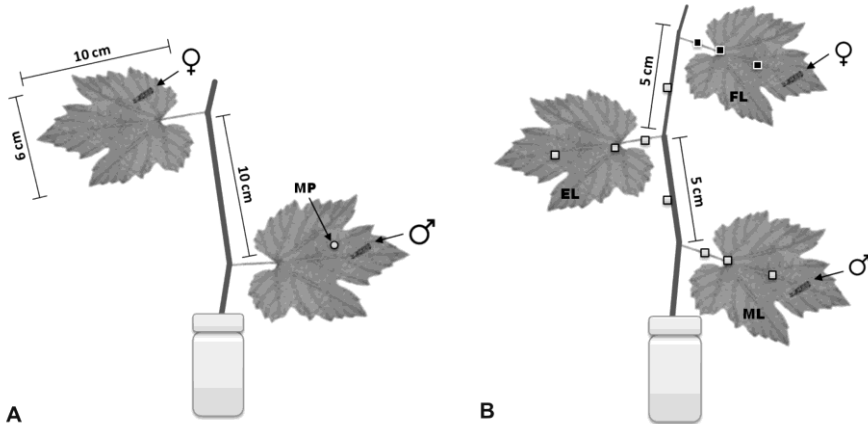


**Fig. 14. Experimental set up of the two tests.** In test 1 (**A**) the laser was pointed on a reflective tape (measuring point, MP) on the male starting leaf. In test 2 (**B**) 11 MP were distributed along the grapevine cutting, 9 on the leaves and 2 on the stem. The leaves were marked as male leaf (ML), female leaf (FL) or empty leaf (EL) according to the respective positions of male and female. Black squares indicate those MP where the intensity of the female signal was significantly higher (Kruskal-Wallis test followed by Steel-Dwass multiple comparison test) than the others (white squares).

To analyse 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 male and female pulse). Each parameter was analysed 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 location phase to his arriving on the leaf with the

female. The duration was measured for each male signal (i.e. pulse train) as well as for the entire behavioural stage. To quantify the effect of female reply on the period we calculated male response phase (MRP) and female latency phase (FLP) (Greenfield 1994). The MRP was equivalent to: $((T'-T)/T)\times360°)$, 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: $((\text{female pulse latency}/T)\times360°)$. The value of response phase delay was: $(\alpha = \text{MRP}/\text{FLP})$. $\alpha = 1$ 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 $\alpha$ values differed among the behavioural stages we performed Kruskal-Wallis test followed by Steel-Dwass pairwise multiple comparison test.

## 5.2.4. Test 2. Intensity and dominant frequency of female reply

To test the hypothesis that males may adjust their behaviour according to the perceived intensity of the female reply, a male and a female (n=30) were put on separate leaves of the same grapevine cutting and the level of vibrational substrate velocity (μm/s) from female reply was measured across measuring points of a grapevine cutting. Contemporarily, to test the role of the spectral component for male searching, we measured the dominant frequency (Hz) of the female signal on the measuring points. The cutting consisted of three leaves (surface 6 x 10 cm) with petioles and a 10-cm long stem (i.e. 5 cm stem between basal and middle leaf, 5 cm stem between middle and upper leaf) (Fig. 14B). A male and a female were randomly placed on either the basal, middle or upper leaf, thus obtaining six different combinations, and leaves were marked as "female leaf", "empty leaf" and "male leaf". Prior to the start, we used a minishaker (Type

4810; Brüel and Kjær Sound & Vibration A/S) to vibrate the plant with playback of pre- recorded MCS in order to stimulate mating behaviour. Female replied to the playback and, as a result of such duet, live male responded with rivalry (DN signal). Subsequently the playback was stopped to allow the male to establish a duet with the female. A laser vibrometer and a video camera (see above) were used to record male movements, mating behaviour and female signal intensity along the grapevine cutting. Recordings with laser vibrometer were made site per site on the three leaves at the centre of the lamina, base and petiole, as well as on the stem between basal and middle leaf or between middle and upper leaf (Fig. 14B). In total,11 measuring points were distributed for each trial. Since females remain stationary (Mazzoni et al., 2009a), the intensity of her vibrational signal could be measured directly at the measuring points by moving the laser beam during male search. To test for statistical differences of the female pulse intensities across the measuring points on the cutting we performed Kruskal-Wallis test followed by Steel-Dwass multiple comparison test. Furthermore, the measuring point of female intensity corresponding to the beginning of the courtship by males was determined from video recordings in combination with Pulse 14, in order to evaluate a possible threshold in signal intensity for courtship behaviour.

### 5.2.5. Test 3. Directionality in *S. titanus*

To test the hypothesis that males make directional decisions after receiving a female reply, video recordings were analyzed by annotating the male's directional choices. Three parameters were evaluated. First we annotated if the male walked towards female direction, if yes it was recorded as a correct decision, if not, as a wrong decision. When males made a change in direction it was annotated as a wrong decision, if it turned away from the

female and correct if turned towards her. When males reached a fork between stem and leaf the correct or wrong decisions at branching point were annotated. To evaluate if males were able to make directional decisions, the data from correct and wrong decisions were compared in a one-tailed t-test for dependent samples.

## 5.3 Results

### 5.3.1. Test 1. Behavioural stages and synchrony in male-female signals

The main steps of the mating behaviour of *S. titanus* are summarized in Fig. 15. As described previously in Mazzoni et al. (2009a), in all trials (n=20) males initiated vibrational communication with emission of a MCS. When females were not responding (n=4), males either remained stationary or expressed "call-fly" behaviour (Hunt and Nault, 1991).
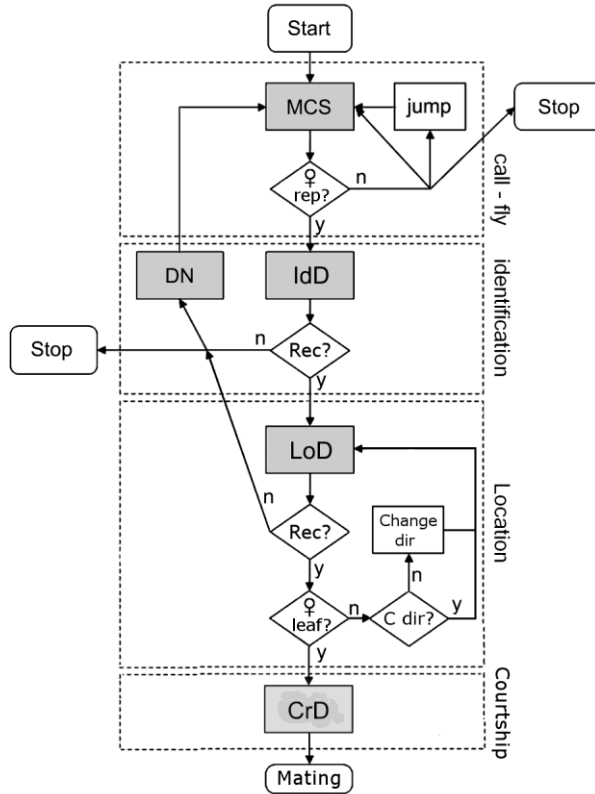
**Figure 15. Flow chart with the behavioural steps** of male *Scaphoideus titanus* when searching for a female on a grapevine plant. MCS = Male Calling Signal; DN = Disturbance Noise; IdD = Identification Duet; LoD = Location Duet; CrD = Courtship Duet; ♀ rep = Female reply; Rec = Recognition; ♀ leaf = arrival at the female leaf; C dir = correct decision; Change dir =change of direction.

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. 16A) and was observed only when male and female were placed on separate leaves.

**Table 1.** α (male response phase delay) values from *Scaphoideus titanus* Identification (IdD), Location (LoD) (Section 1) and Courtship Duet (Section 1 and Section 2).

| | **n** | **N** | **T / T' (s)** | **F latency (s)** | **α** |
|---|---|---|---|---|---|
| **IdD** | 10 | 7 | 0.43±0.02/0.68±0.04 *** (t = 22.4, df = 9) | 0.30±0.03 (25.5) b | 0.85±0.17 (34.7) b |
| **LoD (S1)** | 10 | 7 | 0.44±0.01/0.50±0.07 ** (t = 3.61, df = 9) | 0.29±0.01 (24.6) b | 0.23±0.27 (12.7) a |
| **CrD (S1)** | 10 | 7 | 0.42±0.04/0.50±0.05 *** (t = 6.4, df = 9) | 0.23±0.01 (9.1) a | 0.37±0.22 (19.7) a |
| **CrD (S2)** | 10 | 7 | 0.62±0.06/0.69±0.08 *** (t = 6.9, df = 9) | 0.29±0.01 (22.8) ab | 0.26±0.11 (14.9) a |

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 (in brackets is indicated the rank mean; 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. *** indicate p < 0.001 after one tailed paired t-test.

During IdD, males walked randomly on the leaf. Seven females emitted also short multiple pulse trains (m±SD = 3.03±1.28 pulses, N = 31, duration = 0.89±0.61 s) 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 small but significant effect on the pulse period in male signal (α=0.23) (Table 1). This phase of male-female vibrational interaction was named Location Duet (LoD; Fig. 16B) 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 few centimetres before stopping and, often emitting a single strong pulse. Females were observed to emit multiple pulse trains (n=6) (m = 3.6 ±1.39 pulses, duration 1.09 ± 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 communication with an IdD (n=4). However, in these cases the re-identification was limited to exchange of 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 to the leaf hosting the female a CrD was established (n=13) (Fig. 16C). 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: X² = 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 test: $X^2 = 21.5$, df $= 3$, $P < 0.001$).

**Table 2.** Descriptive statistics on duration of *Scaphoideus titanus* Identification and Location Duets.

| | IdD (single train) | LoD (Section 1) | Identification (whole stage) | Location (whole stage) |
|---|---|---|---|---|
| | | | **Duration (s)** | |
| **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 Location Duet (LoD, section 1). n indicates the number of insect pairs analysed.
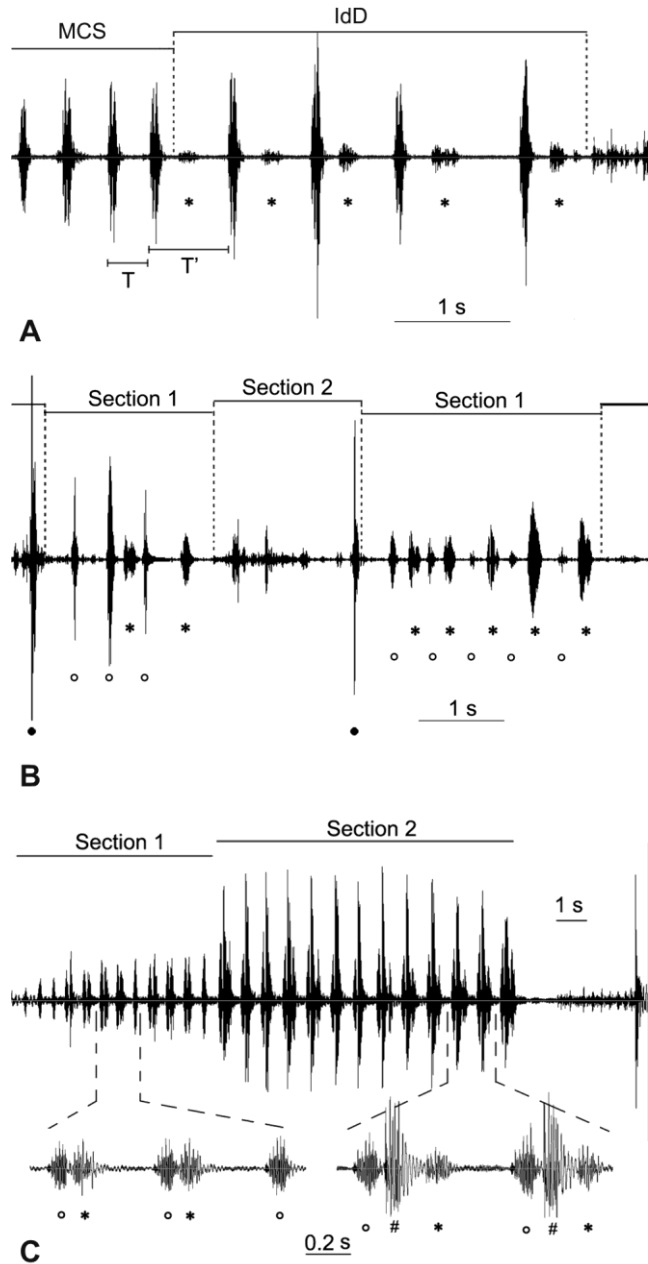
**Figure 16. Oscillogram** of (**A**) Identification Duet (IdD), (**B**) Location Duet (LoD) and (**C**) Courtship duet (CrD). Recordings were made with the laser on the lower leaf hosting the male. In (A) are shown: Male Calling Signal (MCS) that turns into

an Identification Duet (IdD). In (B) are shown the two sections (S1 and S2) forming the Location Duet with the vibrations due to male directional search. In (C) S1 and S2 of a male courtship phrase that together with female pulses constitute CrD. Asterisks (*) indicate female pulses; white dots (○) male pulses; black dots (●) 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 female's reply, respectively.

## 5.3.2. Test 2. Increased level of signal intensity associated with courtship behaviour

The intensity (measured directly as substrate velocity) of the female reply perceived by the male along the grapevine cutting is summarized in Figure 17. There was no statistical difference in intensity between male leaf, empty leaf and stem, whereas the intensity level of female pulse was significantly increased at the petiole, base and lamina female leaf (Kruskal-Wallis test: $X^2 = 160.57$, df = 10, p < 0.001; Fig. 14 and 17). Twenty-five out of 27 courtship duets started on the female leaf, most commonly on the petiole. Only in two cases the courtship duet started on the stem, but it was always nearby the female leaf petiole (Fig. 17).

No difference in dominant frequency was found between the leaves and stem parts when the spectral component of the female reply was measured (Kruskal-Wallis test: $X^2 = 6.67$, df = 10, p = 0.7565).

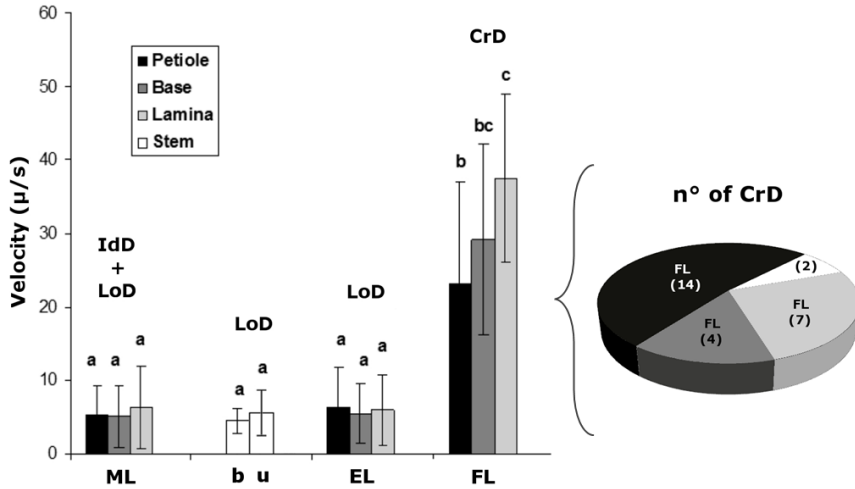**Figure 17. Mean (± SD) substrate velocity values of female responses detected from measuring points distributed along the grapevine cutting.** Different letters indicate statistical significances after Kruskal-Wallis test followed by Steel-Dwass multiple comparison test. ML = male leaf; FL = female leaf; EL = empty leaf; u = upper stem; b = basal stem. In the pie chart is indicated the number of courtship duets recorded on different parts of the female leaf.

### 5.3.3. Test 3. Males are able to make directional decisions

The number of males making correct or wrong directional decisions towards female after a female response is shown in Figure 18. Significantly more correct decisions were towards the female (t-test: t = 12.72, df = 27, P < 0.001) (Fig 18a) and when changing the direction, significantly more males made a correct than wrong directional decision (t-test: t = 4.72, df = 27, P < 0.001) (Fig 18b). A male that turned in the wrong direction made on average 1. 8 ± 1.4  wrong decisions before turning in correct decision. No difference between correct and wrong directional decisions was observed at branching points (t-test: t = 1.43, df = 27, P = 0.32) (Fig 18c).
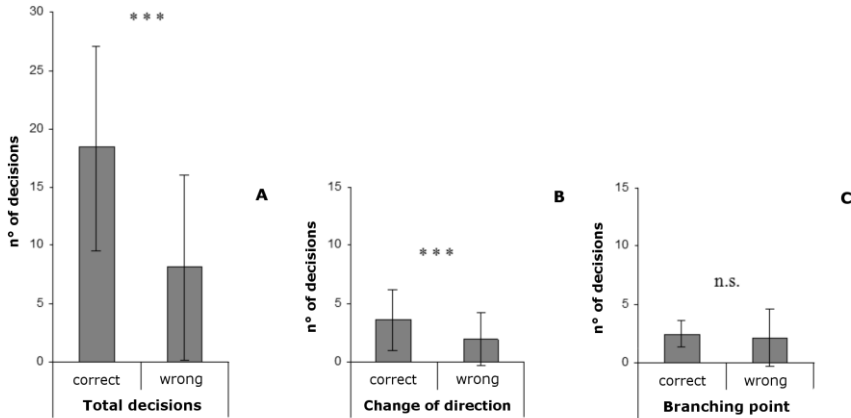
**Figure 18.** A) Total number (m ± SD) of correct (towards female) and wrong directional decisions made by males after receiving female response along a grapevine cutting. B) The number of males making correct or wrong decision after changing direction and C) directional decisions at branching points. Asterisks (***) indicate statistical significances after one-tail paired t-test (P < 0.001).

## 5.4 Discussion

Pair formation in *S. titanus* starts with identification of the mating partner and continues with a location stage until a final courtship before copulation. We have shown that the level of synchronization increased during the initial stages of pair formation as males proceeded from identification to location of females. During such searching process males were shown to make several directional decisions towards female position. There is a continuous process of interpreting the perceived information on different plant parts. 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, once the female had been identified. In general, in the beginning of a mating communication, signals should first tell the receiver

about the sender's identity (who?) before the quality (what?) and location (where?) (Pollack 2000). Our results indicate that during the search *S. titanus* males can extract directional information from female reply, since significantly more males walked towards the female. However, males made many mistakes at branching points. In contrast, in the larger stink bug *Nezara viridula* (size 1 cm), in which males can orient correctly at the branching point, males stopped and stretched their legs between branches thus extending the leg span (Čokl et al. 1999). We haven't observed such behaviour in *S. titanus*. Nevertheless, despite its' small leg span, we showed that males were able to correct the direction relatively quickly after they made a wrong decision (usually after 2 moves), which indicates a perception of a direction even in the orientation of such small insects. Decisions by a male were taken as he walked after every identified female response and he remained stationary in absence of female reply. The change in behaviour as males progressed from identification to location and finally courtship suggests that a male is aware of whether he is on a different or on the same leaf as the female. The significantly higher intensity of the female response was shown on the female leaf. It is therefore possible to suggest that males may use the abrupt intensity increase of 10 dB (Fig. 17) as a reliable cue to proceed from searching behaviour on the stem to courtship duetting on the petiole of the female leaf. While visual or chemical cues may also be involved in eliciting courtship behaviour at short distances, these seems to be less likely possibilities. Video recordings show that in our experiments females were not visible from the petiole of the female leaf (i.e. where most courtship duets started). Up to now there is no evidence that chemical communication plays a role in reproductive behaviour of leafhoppers and the antennae of *S. titanus* adults also have a reduced number of olfactory sensilla (Rossi Stacconi and Romani, 2012). Only the nymphs have been shown to use

olfaction for recognition of the host plant (Mazzoni et al., 2009c). Given that the between male and female varied randomly, it seems unlikely that a sudden switch of behaviour was caused by presence or release of odours. Since the adults rely exclusively on substrate-borne vibrations for intra specific communication (Mazzoni et al., 2009a), it is most likely that the male perceived the large difference in vibrational velocity of female signal on the female leaf and that the lower intensity on other plant parts provided the information that the is female not located on the same plant part as the male. Previous work found that frequency dependent attenuation gives more predictable information about distance than amplitude (Barth, 1988). In our study we found no frequency changes of the female reply across the plant. The trend of the dominant frequency was rather variable and without a specific degradation. Indeed, when the frequency on female leaf was compared to other plant parts there was such high variability that it could not be considered as a reliable cue for the male during location of the female.

In the bushcricket *Phaneroptera nana*, which uses air-borne sound communication, it was suggested that females might disregard low intensity male pulses because of the indication that the male is too far away (Tauber and Pener 2000). However, the intensity of vibrational signals is strongly dependent on the substrate in which the vibrations are transmitted and has a high variability in plants due to the complex transmission properties (Michelsen et al. 1982; Čokl et al. 2007). The perceived low intensity of female response in the early stage of mating sequence when partners are duetting from distant leaves could explain also the delayed emission of male pulses during the initial identification phase. Since the male pulse period increased for approximately an entire pulse period after a female reply, males may not be able to immediately and correctly identify the female (gender) or right species. This is supported by previous results when

pairs were from the start placed on the same leaf (Mazzoni et al. 2009a). In such a situation, males did not perform neither IdD nor LoD, and MCS immediately progressed into courtship duet.

Our results also show that male recognizes a conspecific female signal because it is coupled with his own pulse. In preliminary playback tests, males were subjected to pre-recorded female pulses in order to verify the effect of female response intensity on male behaviour. When males emitted MCS, female pulses were played back to the leaf at various intensity levels and all males remained stationary (A. Eriksson, pers. ob.). The reason for the absence of searching behaviour may be the wrong timing of female pulse, since female response was triggered manually. In addition, it explains why males, in response to alive females sometimes emitted a disturbance signal when identification of the female had not yet been achieved. Indeed, female reply has to appear in a specific time window and should not overlap with the next male pulse, since 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 multiple trains, especially, after the last male pulse. Such multiple replies occurred when the male was identifying or locating the female from distant plant parts, and it is possibly an evolution of the female to increase her traceability when receiving delayed male pulse periods. However, since males often replied with disturbance signals, additional studies are needed to determine the function of female multiple signals. In male calling songs, pulses are emitted with regular rhythm, indicating that they are generated by an endogenous oscillator. In contrast, 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. Taking into

account the longer pulse period in identification signals and the duration of the female reply, female pulses would overlap with the next male pulse in MCS. In this respect, the resetting of male endogenous oscillator is necessary to maintain a contact with the female. Furthermore, the change in rhythm could help the male to determine that the perceived reply is not a biotic noise in the environment. Such resetting of male endogenous oscillator to the basal level by central nervous system is comparable to the signal interactions among chorusing males (Greenfield 1994; 2005). Interestingly, although pulse period in male signal in the absence of female reply at different stages of male-female interaction does not change, the effect of female reply on pulse period at later stages was small. This observation suggests a complex neuronal control of signal production.

The "call-fly" behaviour observed in males is usually associated with a strategy to increase signalling space (Gwynne 1987; Hunt and Nault 1991) and therefore such strategy would be most adaptive when signals have a limited communication range. In *S. titanus*, "call-fly" behaviour may also represent a tactic to minimize the advantage of satellite males and/or predation by eavesdropping predators (Virant-Doberlet et al. 2011). Male-male competition can have an important influence on the structure of a duet and male searching behaviour (Bailey 2003). The location duet observed in the present study closely resembled a duet when the courting male was aware of the presence of a rival male on the same leaf (Mazzoni et al. 2009a, b). In *S. titanus* a rival male relying on silent approach to a duetting female (satellite behaviour) can move around while the courting male which is maintaining a duet with the female remains stationary for most of the courtship phrase and therefore simplifying and shortening the signal reduces the time needed for localization.

In conclusion, animals that communicate with substrate-borne vibrations could interpret a relevant part of the information provided by

received signals because of their perceived intensity and synchrony with their own signals. Decisions on direction are therefore made according to transmission properties of the substrate. Consequently, external interferences, either environmental or anthropogenic, may result in loss of information necessary either for identification or location and thus preventing the mating. An example of it was the use of synthetic rivalry signals applied to grapevine plants for mating disruption with vibrational signals (Eriksson et al. 2012). To interfere with mating communication it is therefore important to have knowledge about all stages and aspects of mating behaviour in order to target the more susceptible behavioural phases.

## 5.5 References

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# 6. Conclusions and future perspectives

The results of the present thesis revealed the following three main results. Mating disruption based on playback of disruptive vibrational signals is a promising approach to manage vibrational communicating insect pests in agricultural field situations (1). Furthermore, the communication range of substrate-borne vibrational signals is not limited to physically interconnected substrates (2) and males of *S. titanus* are able to extract directional information from a female reply despite their small body size and also associate courtship behaviour with a certain level of signal intensity (3).

When developing a successful pest control strategy it is important to study the biology of the pests and how they communicate with their conspecifics. The experiments made in this thesis were based on the results from previous laboratorial experiments in which *S. titanus* was shown to communicate with vibrations during mating or male rivalry (Lucchi et al., 2004; Mazzoni et al., 2009a) and when synthetically reproduced rivalry signals interrupted male and female communication on the same leaf (Mazzoni et al., 2009b). The results from the present thesis then revealed the first successful field data and important findings for an increased understanding of the general communication mechanisms by means of substrate vibrations. When *S. titanus* communicated from discontinuous substrates it was demonstrated for the first time that the transmission of vibrational signals from a small insect not is limited to the same substrate (plant). Such finding was crucial also for the development of a disruptive control method in the field. Not only did it become important to consider the single plant range, but also the neighbouring plants with closely adjacent leaves. In fact, more likely for most insects are situations in which leaves are separated by a gap but partly overlapping, as in a dense vegetation habitat like grapevine. When playback experiments were made

with a synthetic calling signal reproduced to one grapevine leaf and recorded from another not physically interconnected leaf, it was shown that such transmission was possible until an air gap of 11 cm. With alive insects, the communication between males and females was verified up to a gap width of 6 cm. It may be speculated whether the vibrational signals were detected as air-borne sound or as substrate vibrations induced in the leaf, although, it is likely that the latter is a more probable hypothesis. During the experiment, male and female leafhoppers were often positioned on the external sides of leaf laminae and two leaves should represent a too severe obstacle for low intensity air-borne sounds.

The transmission of vibrational signals from one leaf to another via air occurs probably in many other species than *S. titanus* and therefore it may be a finding of importance also for mating disruption based on vibrational signals from other insect pests. Avoiding mating among vibrational pests that theoretically can find each other despite being initially separated on different plants, implies that the intensity levels of the disruptive signal are higher than the signal intensities transmitted through the plant tissues by males and females. It was therefore crucial to investigate the approximate intensity levels that are used for the natural mating communication in *S. titanus*, in order to subsequently create a masking effect able to cover the communication along several neighbouring plants.

Accordingly, when males and females were studied on different leaves of the same plant the males initiated courtship when reaching the leaf hosting the female, according to a specific level of female signal intensity. On plant parts that were distant from the female position, there were lower intensity levels, although not significantly different among each other. Possibly, males may perceive the relatively lower intensity of the signal as an information about female identity or that she not is located on

the same plant part as the male. In fact, males first have to identify the female (as in right sex and species) before searching for her location (Virant-Doberlet et al., 2006). The identification stage in *S. titanus* was shown when males from a distant plant part delayed the latency time between pulse emissions after receiving a female reply. Such identification duets were perceived from both insects at relatively low intensity levels and were characterized by long duration and irregular pulse repetition rate, when compared to signals emitted during the location and courtship stages. A possible explanation to why recognition of female not was immediate in the initial stage of pair formation may be the variation in signal intensity when the two individuals were separated by a longer distance (McVean and Field, 1996), as a result of the complex filtering properties of plants (Michelsen et al. 1982). Only as the male had recognized the female, he started searching and alternated callings with difficult decisions about the direction of the female. The difficulty was shown at branching points between leaves and stem, where males not were able to make decisions on the female direction. Therefore, it may be explained why males and females frequently produced location duets, as these short duets, alternated with quick walking, helped the male to accurately choose direction along the different plant parts. In fact, it was shown from the total number of directional decisions, that a significant number of males made correct decisions towards the female. This finding is interesting, since *S. titanus* is an example of a small insect with a body size less than 1cm, which theoretically should imply difficulties in the comparison of intensity or time differences on two sides of the body (left-right or front-back) (Virant-Doberlet et al. 2006). In insects it is known that most vibration receptors are located in the legs (Čokl et al. 2006) and that it is the size (i.e. maximal leg span) that creates time or intensity differences large enough to be used in orientation (Virant-Doberlet et al. 2006). Yet, we showed that that *S.*

*titanus* are able to make correct directional decisions, once the female had been identified. In an applicative point of view, it may be suggested that the identification stage should be the most sensitive part of the mating behaviour and therefore the more easy target for mating disruption.

The mating disruption experiments showed that successful results could be obtained until an approximate distance of 10 m from the source of disturbance signal. The few mated females found within such distance on the vibrated plants could be due to the call-fly behaviour of *S. titanus*, as males are known to alternate calling and immediate flying to other plant parts (Mazzoni et al., 2009a). It is possible that males by chance landed at a short distance from the female, which permitted mating communication at a level of intensity that was higher than that transmitted from disruptive signal. Yet, accidental locations of the female would be even less likely in the open field, since the use of cages or netting sleeves limited the movements of the male to only few leaves or to the same shoot.

Before application of mating disruption with vibrations will be possible, there are still some studies necessary to do both in laboratory and in field. Among them, one goal should be to continue with *S. titanus* as model insect and determine which intensity levels of their rivalry signal are necessary for disrupt communication between males and females on the same leaf or plant. Further, it will be important to study the sexual activity of the males in different environmental conditions, as when the temperature or atmospheric pressure changes. Then, studies may be performed with other insect species and in other agricultural fields. For example, in greenhouses there is an energetic system already present and there is the advantage to protect plants from external environmental factors. Instead, for application to viticulture, the future goal should be to increase the distance efficacy to at least 50 m or 100 m, in order to cover a whole row of grapevine plants at a minimum cost. However, before such application is

possible, major technological improvements are necessary. Possibly, one could aim to have a control system with different time durations or use solar energy for management in open field conditions.

Vibrations have a potential to become as important as pheromones for the integrated management systems since more than 150 000 insect species have been estimated to use vibrations as primary communication channel (Cocroft and Rodriguez, 2005). Among these there are in particular some important leafhopper pests (Hemiptera: Cicadellidae) of grapevine, that as opposed to *S. titanus*, cause economically important direct damage when feeding on the grapevine leaves. For example *Empoasca vitis* (Göthe), *Zygina rhamni* (Ferrari) and especially *Jacobiasca lybica* (Bergevin & Zanon) could be successfully controlled since their mating behaviour is mediated by vibrational signals. Possibly, a shaker with multiple channels could be developed in order to control several different pests simultaneously. Moreover, there should be studies made on the possible collateral effects on other pests or beneficial insects.

Finally, consumers have an increasing concern about safe food production and growers deal with problems of resistance development to pesticides in several pests. However, as with all new application methods, it will be a challenge to convince growers and others that vibrations could reduce or replace the use of chemical pesticides.

## 6.1. References

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# 7. Appendix. Media Coverage

## 7.1. Journals

- **Anna Eriksson**, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet, Valerio Mazzoni (2011). Inter-plant vibrational communication in a leafhopper insect. PLoS ONE 6(5):e19692

http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0019692

- **Anna Eriksson**, Gianfranco Anfora, Andrea Lucchi, Francesco Lanzo, Meta Virant-Doberlet, Valerio Mazzoni (2012). Exploitation of insect vibrational signals reveals a new method of pest management. PLoS ONE 7(3):e32954

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- **Anna Eriksson**, Marco Valerio Rossi Stacconi, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet, Valerio Mazzoni. (2013). Signal parameters involved in pair formation mediated by substrate-borne vibrations (*Submitted)*

## 7.2. Abstracts from scientific meetings

- Valerio Mazzoni, **Anna Eriksson**, Gianfranco Anfora, Meta Virant-Doberlet, Andrea Lucchi (2010). Mating disruption by vibrational signals: theory and possible applications to Hemipteran pests. IOBC/WPRS Integrated protection of fruit crops. Pp. 48

- Valerio Mazzoni, Federica Trona, Claudio Ioriatti, Andrea Lucchi, **Anna Eriksson**, Gianfranco Anfora (2011). IOBC/WPRS Integrated protection and production in viticulture. Pp. 281-284

- **Anna Eriksson**, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet, Valerio Mazzoni (2011). International symposium on vibrational commuication in arthropods. Long range communication through substrate-borne vibrations in *Scaphoideus titanus* (Hemiptera Cicadellidae). Pp. 206

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determines mating behaviour in *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). IOBC/WPRS Bulletin 85: 151-155

- **Anna Eriksson**, Valerio Mazzoni, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet (2012). Exploitation of insect vibrational signals for pest management. 6th European conference on behavioural biology, 19-22 July 2012, Essen, Germany

- Valerio Mazzoni, **Anna Eriksson** (2013). Mating disruption with vibrational signals: results of 2012. Future IPM in Europe, 19-21 March 2013, Riva del Garda, Italy

- **Anna Eriksson**, Marco Valerio Rossi Stacconi, Andrea Lucchi, Gianfranco Anfora, Meta Virant-Doberlet, Valerio Mazzoni (2013). How vibrational signals can guide mating behaviour in *Scaphoideus titanus*. Future IPM in Europe, 19-21 March 2013, Riva del Garda, Italy

## 7.3. Popular scientific web sites

- Inter-plant communication in "Science News" 18/05/2011, http://www.sciencenews.org/view/generic/id/74425/title/News_in_Brief_Life

- Mating disruption with vibrations, in "IASMA notizie", April 2012, http://www.iasma.it/UploadDocs/9029_Iasma_spec21web.pdf

## 7.4. Others

- Inter-plant communication, in "Il Tirreno", 10/06/2011, http://iltirreno.gelocal.it/livorno/cronaca/2011/06/10/news/il-canto-d-amore-degli-insetti-4410401

- Inter-plant communication, in "Corriere della sera", 30/08/2011, http://archiviostorico.corriere.it/2011/agosto/30/Decifrano_segnali_sessuali_bloccano_insetto_co_9_110830050.shtml

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- Mating disruption with vibrations, in "Rural Times", 11/06/2012, http://theruraltimes.blogspot.it/2012/06/micro-vibrazioni-contro-gli-insetti.html

## 7.5. Book Chapter

- Valerio Mazzoni, **Anna Eriksson**, Gianfranco Anfora, Andrea Lucchi, Meta Virant-Doberlet. Active space and the role of amplitude in plant-borne vibrational communication. (*In Press)*

## 7.6. Publications outside PhD

- Ulf Nilsson, Linda-Marie Rännbäck, Peter Anderson, **Anna Eriksson**, Birgitta Rämert (2011). Comparison of nectar use and preference in the parasitoid *Trybliographa rapae* (Hymenoptera:

Figitidae) and it's host, the cabbage root fly, *Delia radicum* (Diptera: Anthomyiidae). Biocontrol Science and Technology 21:1117-1132

- Ulf Nilsson, **Anna Eriksson,** Birgitta Rämert, Peter Anderson (2012). Behavioural responses of male and female *Trybliographa rapae* (Hymenoptera: Figitidae) to volatiles from food plants, infested host plants and combined volatiles. Arthropod-plant interactions 6:251-258

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