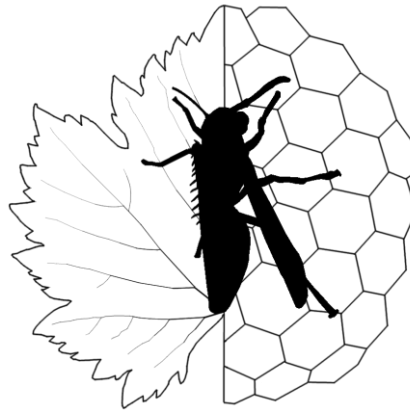


**DOTTORATO DI RICERCA IN
ETOLOGIA, ECOLOGIA,
ANTROPOLOGIA E BIOSISTEMATICA
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ECOLOGIA
(XXVIII CICLO)**

**Insect vibrational communication:
description, decoding, and manipulation**

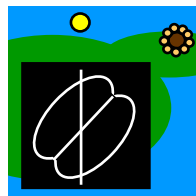
Tesi di

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description, decoding, and manipulation**

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Summary

Vibrations are extremely widespread and ancient among animals' communication modalities; nevertheless, their importance has been neglected for many years. During my PhD I wanted to increase the knowledge about the role of vibrational signals in insects. Therefore, I conducted behavioral bioassays and laser vibrometer recordings to describe and decipher vibrations produced by four species belonging to two different orders. The role of vibrational signals in intraspecific communication varies widely among different groups of insects. For this reason I chose to study two model groups, Hemiptera and Hymenoptera.

Hemiptera, in particular leafhoppers, rely almost exclusively on vibrations for intraspecific communication. Their reproductive strategy is based on the production of vibrational calling and courtship signals, which are necessary for identification and location of the mating partner. Similarly, male-male competition for mating is regulated by means of specific vibrational signals, which in many cases are used to interfere with an ongoing mating duet. The emission of specific disruptive noise gives the rival male a chance to access mating by replacing the calling male in the duet. Recent studies showed that disruptive signals can be played back into plants to effectively disrupt the mating behavior of the grapevine leafhopper, *Scaphoideus titanus*. These findings inspired my research, its aims and the experimental approach. First, I described and decoded the reproductive strategy and associated vibrational signals of two grapevine leafhoppers species, the green leafhopper, *Empoasca vitis* and the glassy-winged sharpshooter, *Homalodisca vitripennis*. Secondly, I used the acquired knowledge to select potential synthetic 'disruptive signals' and test their efficacy in disrupting the mating process of *E. vitis* in laboratory conditions.

Hymenoptera, on the other hand, such as paper wasps of the genus *Polistes*, use mainly semiochemicals to coordinate colony activities (e. g., to discriminate among individuals and their roles). However, the "mechanical switch hypothesis" suggested that vibrations produced by body oscillation movements of foundresses can bias larvae development towards a worker phenotype. That is, when a larva is subjected to low frequency vibrations it will develop into a worker. The social parasite - host system, *Polistes sulcifer* – *P. dominula*, was a very good model to investigate the potential caste determination function of body oscillation movements in paper wasps. *P. sulcifer*, the parasite, does not have a worker caste and its reproductive success rely exclusively on the brood cares provided by the host workers that emerge from usurped colonies. For this reason, I described and compared the vibrations transmitted to the nest by both species in usurped and not-usurped colonies. Moreover, the "mechanical switch hypothesis" predicts that vibrations manipulate larval development by modulating the "nutritional effect" (i.e. larvae that are fed more should develop into reproductive individuals and vice

versa). Therefore, I tested the *P. dominula* foundress ability to modulate the vibration emission in association or not with the feeding activity.

This research unveiled remarkable information in both model groups. Several original aspects in the leafhopper mating behavior have been discovered. Main peculiarities have been found in the daily activity and the potential role of visual stimuli in *E. vitis*, and in the complex structure of signals and male-male rivalry interactions in *H. vitripennis*. These results showed that multimodal communication (i.e. vision plus vibrations) and ecological adaptations still need to be studied in leafhoppers to fully understand how vibrational signals evolved and adapted to ecological constraints. From an applied point of view, we identified one disruptive signal that, in laboratory conditions, was highly effective in disrupting *E. vitis* mating process.

On the other hand, I described, for the first time in detail, the spectral properties of induced vibrations into a paper wasps nest produced by *P. dominula* and its social parasite *P. sulcifer*. By comparing the vibrations produced by *P. dominula*, in different larval nutritional conditions, and the parasite we found several significant differences. For example, the foundress varies the spectral and temporal properties when she is feeding the larvae; while the parasite produces vibrational events with some exaggerated features compared to the host (i.e. each event is composed of a higher number of pulses). Results have been discussed from an adaptive point of view considering the putative role of vibrations in leading larvae caste determination.

Overall, this thesis provides novel insights on the great variability of functions and adaptations of vibrational signals. The acquired knowledge can be used as a basis to perform further experiments on biological and applied aspects of biotremology.

Chapter 1

Introduction

1. Introduction

a. Vibrational communication in insects

The status of vibrational communication in the scientific community perfectly fits the observation about concealment of Dupin, the main character of the short story “The purloined letter” by Edgar Allan Poe (1844): “the physical oversight is precisely analogous with the moral inapprehension by which the intellect suffers to pass unnoticed those considerations which are too obtrusively and too palpably self evident.”. As a matter of fact, solid substrates, such as the ground or plant tissue, are overloaded with information transmitted as surface-borne vibrations and used by the majority of animals. Nevertheless, vibrations as a potential communication channel have been neglected for centuries and are still barely considered (Hill and Wessel 2016).

More than our incapability to perceive vibrations used by most animals, the human tendency to oversight what we think to be of minor significance precluded us to understand the importance of this communication modality (Markl 1983). In fact, already in the early 20th century scientists noticed animal behaviors that produce vibrational signals, but they never took into account vibrations as a reliable communication channel, just because they are hard to perceive, or difficult to measure even with sensitive instruments, or they undergo big physical and theoretical constraints (Hill 2009). In the late 40’s, Ossiannilsson suggested for the first time that signals transmitted through the substrate were used by leafhoppers for intraspecific communication (Ossiannilsson 1949). Even then, the proof of concept arrived only two decades later with the studies of Gogala and colleagues on cydnid bugs (Gogala et al. 1974) and of Ichikawa and colleagues on planthoppers (Ichikawa and Ishii 1974; Ichikawa et al. 1975; Ichikawa 1976). It was the first time vibrations were demonstrated to be crucial in intraspecific communication. Indeed, they suggested legs as the location of involved receptor organs.

Despite vibrational communication has been the more neglected communication modality by the scientific community up to the late years of the 20th century, today it is recognized as the most widespread and ancient (Cocroft and Rodriguez 2005). Likely, the communication of eukaryotic unicellular organisms already involved vibrational and chemical modalities (Hill and Wessel 2016). Therefore, it was potentially present in the ancestor of all living animals, and thus examples of species that use vibrations as informative cues or signals are present in mammals, such as elephants and moles, in fishes, amphibians, and reptiles (Hill 2009). Actually, they have been found to be used by every species in which it has been looked for vibrational communication. Recently, it has been shown that surface-borne vibrations are not only limited to animals, but they are used also in plant-insect interactions (Appel and Cocroft 2014). Nevertheless, the group in which vibrational communication has been

better studied and that well represent its ubiquitous is the first one in which it was described: arthropods, in particular insects (Cocroft and Rodriguez 2005).

Production and signal perception

Vibrational signals consist in mechanical waves that travel at the boundary of two media, one of them being the substrate the insect is living on (e.g. plant tissue, ground, water) (Michelsen 2014). Two types of waves are known to mainly encode signal's information: Rayleigh and bending waves (Hill 2009; Michelsen 2014). Both of them are a combination of longitudinal and transverse waves, and determine a displacement in substrate particles perpendicular to the direction of the wave transmission. However, bending waves occur in small diameter or thickness structures compared to the wavelength of the wave or the substrate itself (e.g. leaves and plant stems (Michelsen et al. 1982)); whereas, Rayleigh waves occur in massive substrates, such as the soil.

The rigid exoskeleton that covers arthropods enables them to easily produce surface-borne vibrations just by touching a solid surface. Thus, we can find several examples of insects that evolved a specific drumming behavior to encode information in the vibrations they produce. For instance, some termites drum their head against nest walls to alert the colony members of an approaching danger (Hertel et al. 2011). Some insects groups developed organs specialized in vibrations production, such as stridulatory organ in ants and tymbal-like organs in some Hemiptera (i.e. Cicadomorpha, Fulgoromorpha, and Heteropteroidea) (Cokl and Virant-Doberlet 2003; Wessel et al. 2014). The first consist in two external structures, a scraper or plectrum and a file or pars stridens, which are rubbed together. Instead, tymbal-like organs are similar to the cicada's tymbal but they lack of air-sacs, which serve as a resonant chamber to amplify air-borne sound. They consist in a very strong set of muscles that are anchored to a specialized integumental part (i.e. 'tymbal plates') on the first two abdominal tergites. The species-specific variability of the cuticular structure and the pattern of muscle contractions buckling the tymbal plate are responsible for the differentiation of signals among species. In addition, some insects can produce vibrations and transmit them to the below surface neither using specialized organs nor touching it, because the rapid movement of the abdomen is sufficient to elicit a displacement of the substrate (Hill 2009). During the years this behavior has been named in many different ways according with the species in which it was described, today it is commonly known as tremulation.

Some of these modalities produce vibrations with distinctive spectral features, and thus it is possible to make hypothesis on the emission modality knowing the spectrum of a signal and vice versa (Elias and Mason 2010). For instance, drumming signals have a broadband spectrum profile that means at the source all frequencies are detectable with almost the same amplitude; whereas, stridulatory signals have a clear harmonic structure. Vibrations produced by

tymbal-like organs are the most variable due to the different ways the insect can activate muscles and produce very narrow band signals with harmonics, pure tones, or high-pitch pulses (Wessel et al. 2014).

Insect mechanical receptors can be found on the external surface (i.e. campaniform sensilla, hair sensilla, or hairplates) or internal to the exoskeleton (i.e. scolopidial sensilla or multipolar/multidendritic sensilla) (Lakes-Harlan and Strauß 2014). To detect perpendicular surface displacements, insects rely mainly on scolopidial sensilla, which are arranged as units in small or large groups according to the insect order. In the latter case they are considered as complex sensory organs, such as the subgenual organs that are present in the proximal tibia or the Johnston's organ in the antennae. Once vibrational signals have been detected they are processed in the median ventral association center (mVAC), which is the neuropile area where also proprioceptive information are processed (Nishino et al. 2016).

b. Aim of the thesis

The study of vibrational communication, biotremology, is still in its childhood (Hill and Wessel 2016). Even if advances in technology to detect and measure animal vibrations is supporting and increasing the number of studies in this field, there are plenty of taxa to be studied and biological and applied questions to be addressed.

To deepen the knowledge of insects' vibrational communication, in the following thesis I took into examination two groups that are considered to be at the opposite sides according to the role of vibrations in their communication system. On the one hand, leafhoppers are known to rely almost exclusively on surface-borne vibrations (Claridge 1985; Cocroft and Rodriguez 2005). Recently, Horisk and Cocroft (2013) extended the definition of animal signals from just 'information' to a more general 'influence' of the behavior. Their stretched definition would include all animal signals examples: signals that encode a message for the receiver and signals that change the receiver behavior without giving it any information. Leafhoppers are a good model to investigate vibrational communication, since their communication incorporates examples of both kinds of signals. During the pair formation process partners base their decisions on the information they receive from the duet (Bailey 2003; Mazzoni et al. 2014; Polajnar et al. 2014). At the same time, unintended receivers can intercept the communication and manipulate other individuals behavior by means of 'masking' or 'disruptive' signals (Mazzoni et al. 2009b; Mazzoni et al. 2009a; Virant-Doberlet et al. 2014). Therefore, the first objective of this thesis was to increase knowledge about leafhoppers' mating and rivalry communication systems.

On the other hand, vibrations in paper wasps intracolony communication have been neglected for years (Jeanne 2009; Hunt and Richard 2013). Although, paper wasps have been one of the most important model to

study the evolution of social behavior (O'Donnell 1998; Jandt et al. 2014), only recently an intriguing hypothesis (i.e. the 'mechanical switch hypothesis' (Jeanne 2009)) suggested that larval development, and thus caste determination, can be manipulated by adults on the nest by means of vibrations. This hypothesis would locate vibrations produced by paper wasps among signals that 'influence' the behavior of the receiver. Indeed, the influence would be complete only at the end of the larva development, when the new adult emerges, and thus postponing the 'influence' effect. Nevertheless, the knowledge on vibrations features produced by paper wasps is still largely incomplete. For this reason, the second objective of my thesis was to give new insights on the features of wasps' vibrations that could be involved in wasps' caste determination.

c. Vibrations as the main channel of communication: Cicadellidae.

In the life cycle of leafhoppers (Hemiptera: Cicadellidae) the role of plants is extremely relevant, not only because they represent the only feeding resource, but also the main communication medium. In fact, cicadellid species rely mainly on surface-borne vibrations for pair formation and mating success (Claridge 1985; Cocroft and Rodriguez 2005).

As a general mating process, males actively search for a partner emitting calls. They use the "call-fly" strategy (i.e., alternate calling and jumping or flying to move through plants) to enlarge their signal active space (Mazzoni et al. 2014) and enhance the probability of finding a receptive female (Hunt and Nault 1991). On the contrary, usually females emit a vibrational response only when they detect a male call. A duet between partners is then established, and the repeated perception of the female signal triggers males to actively search for the female, which instead remains stationary (Saxena and Kumar 1984; Claridge 1985; Hunt and Nault 1991; Hunt et al. 1992; Mazzoni et al. 2009b; de Groot et al. 2012; Derlink et al. 2016). The duet is characterized by a predictable temporal association between both genders signals (Bailey 2003), which is crucial for the accomplishment of partner identification and its final location (Polajnar et al. 2014; Kuhelj et al. 2015; Kuhelj et al. 2016).

Male calls last from less than 1 s (e. g. in *Balclutha incisa*) to several seconds (e. g. 15 s in *Graminella nigrifons* and *Scaphoideus titanus*, 19 s in *Aphrodes makarovi*) and consist of several sections (Inoue 1982; Heady et al. 1986; Heady and Nault 1991; Gillham 1992; Nuhardiyati and Bailey 2005; Mazzoni et al. 2009b; de Groot et al. 2012). The number of sections is variable. For instance, the structure of *Alebra* males call is articulated in two subsequent sections: a burst followed by a sequence of repeated pulses (Gillham 1992). On the other hand, *A. makarovi* male call consists of five different sections (de Groot et al. 2012). Often a signal is specifically or more frequently produced in advanced stages of the pair formation process, when partners are close to each other (e.g., 'courtship signals') (Shaw et al. 1974; Saxena and Kumar 1984;

Heady et al. 1986; Nuhardiyati and Bailey 2005; Mazzoni et al. 2009b; Polajnar et al. 2014). Therefore, historically they have been named accordingly to the behavioral context in which they were performed. *Alebra* genus represent an interesting exception, as all four studied species have only one male signal, which is used in all behavioral contexts (Gillham 1992).

Completely different are female replies, which are simpler (i.e., lack of patterned sections) and interpose with one or more specific section of the male call (Inoue 1982; Heady et al. 1986; Heady and Nault 1991; Nuhardiyati and Bailey 2005; Mazzoni et al. 2009b; Kuhelj et al. 2015). In some species, such as *S. titanus* (Mazzoni et al. 2009b) and *A. makarovi* (Kuhelj et al. 2015; Kuhelj et al. 2016), the female reply overlaps part of the male call. However, the portion of the female reply that the male uses to gain directional information is the nonoverlapped one (Kuhelj et al. 2015). Beside directional information, female reply signals convey to males information about their reproductive availability. In fact, after the last moult they need a variable and species-specific period of time to become reproductively active and after mating they undergo a refractory period (Nuhardiyati and Bailey 2005; Mazzoni et al. 2009a).

Recently, the study on *Psammotettix alienus* mating strategy brought novelties to the stereotyped pattern known for leafhoppers in general. In fact, as far as we know, this is the only species in which both genders can initiate pair formation and have simple and similar signals (Derlink et al. 2016).

All communication systems must be look at as complex networks, in which the exchange of information is susceptible to be exploited by a unintended receiver, to its own advantage (Endler 1993). This is true also for vibrational interactions between individuals (Virant-Doberlet et al. 2014). In leafhoppers, when a rival male eavesdrops a conspecific signaling can use different strategies to disrupt courtship: alternation of male calls (Hunt and Morton 2001), production of rivalry signals aimed at masking the female reply (Mazzoni et al. 2009b; Mazzoni et al. 2009a; Derlink et al. 2016; Kuhelj et al. 2016), and silently approach the female while she is duetting with the first male (i.e., satellite behavior) (Mazzoni et al. 2009b). All these strategies enable the rival male to manipulate the behavior of the duetting male and increase the intruder's possibility to mate. The most studied strategy is the production of 'disruptive signals', which are supposed to disrupt the pair formation process occurring within the time window of the female reply and, therefore, masking it. The masking can be achieved covering part or all the female reply or confusing the male because it perceives stimulus from a spatially separated source from that of the female location (Hammond and Bailey 2003; Bailey et al. 2006; Mazzoni et al. 2009a). Rival interactions have been deeply investigated in the leafhopper *S. titanus*, in which two distinct signals are produced by rival males during an ongoing duet: the 'disturbance pulses' and the 'disturbance noise' (Mazzoni et al. 2009b). In particular, the 'disturbance noise' of *S. titanus* is hypothesized to mask the female signal (Mazzoni et al. 2009a), by overlapping the end of the female pulse.

Applied resources: vibrational mating disruption

Also humans can take advantage if we become able to manipulate insects' behavior. Many insects are threatening crops, either directly by feeding on them or indirectly as pathogens vectors (Oerke 2006; Sisterson and Stenger 2016). Today, to control pests population the use of integrated pests management (IPM) are encouraged (Epstein 2014). One of IPM strategies is to manipulate insect behavior to control birth. This can be achieved for instance with mating disruption techniques, which prevent mating partners from identifying and locating each other (Foster and Harris 1997). This method is successfully used in several agro-ecosystems (Cardé 1995; Gordon et al. 2005).

However, to date it is based on pheromones and so it is inapplicable to insects, like leafhoppers, that rely on substrate-borne vibrations for mating identification and location (Cokl and Virant-Doberlet 2003; Cocroft and Rodriguez 2005). Recently, several efforts have been done to develop a successful behavior manipulation method based on vibrations (Mankin 2012; Polajnar et al. 2015). Vibrational 'disruptive signals' are transmitted into the substrate through specific transducers with the aim of confounding and/or misleading individuals. The first successful application has been realized on the American grapevine leafhopper *Scaphoideus titanus* (Eriksson et al. 2012; Polajnar et al. 2016). The transmission of a species-specific 'disruptive signal' into grapevine tissues by means of electromagnetic shakers prevented 90% of mating in the tested pairs in semi-field conditions.

Several beneficial insects, such as parasitoids and predators, use vibrations as a communication channel (Cokl and Virant-Doberlet 2003), to preserve them it is crucial to make a 'disruptive signal' with a narrow frequency band, trying to occupy a little slice of the spectrum. Consequently, to assess the feasibility of a vibrational mating disruption method and develop it for a specific target species, it becomes crucial to know the signaling behavior of the target species. If a natural rivalry behavior aimed at masking conspecific signals is present, it can be selected to be used in the mating disruption approach (Mazzoni et al. 2009a; Eriksson et al. 2012).

Studied species: Empoasca vitis and Homalodisca vitripennis

The green leafhopper, *Empoasca vitis* Göthe (Hemiptera: Cicadellidae: Typhlocybinæ), is a polyphagous species with holarctic distribution (Alma 2002). It is an important grapevine pest in north Italy, Switzerland, Germany, France and Greece (Cerutti et al. 1991; Mazzoni et al. 2001; Alma 2002; Böll and Herrmann 2004), while in Asia it is reported as noxious for tea plants (Hazarika et al. 2009). Crop damages are associated to the phloem feeding activity that causes stress symptoms, such as vein browning and chlorosis of leaf margins under low-density conditions and leaf burn and phylloptosis at higher densities (Alma 2002; Böll and Herrmann 2004). *E. vitis* overwinters on

conifers and adults move to host crops in spring. There 2 to 4 generations, according to latitude, occur until fall, when they go back to overwintering sites (Alma 2002).

Recently, the insurgence of insecticide resistance in *E. vitis* (Girolami 2001), have urged the development of IPM methods. In the course of the years several approaches have been tested, such as the use of non-susceptible cultivars, and techniques of landscape management to favor the occurrence of predators and parasitoids (Decante and van Helden 2006; Pavan and Picotti 2009; Liu et al. 2015; Fornasiero et al. 2016). However, a truly effective solution is still missing.

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae: Cicadellinae), is a grapevine pest native to southeastern United States and northeastern Mexico (Triapitsyn and Phillips 2000), which invaded California in the late 1980s (Sorensen and Gill 1996; Stenger et al. 2010). GWSS is a polyphagous insect and a serious threat to agriculture due to its ability to transmit *Xylella fastidiosa* Wells et al., a xylem-limited bacterium that causes Pierce's disease in grapevines (Davis et al. 1978) and many other diseases in economically important woody crops. In California, GWSS reproduce from spring to fall producing at least two generations per year. During winter months, GWSS population densities decline sharply and are strictly associated with non-deciduous shrubs and trees. Several aspects of GWSS reproductive biology and behavior have been studied, including egg load and maturation dynamics (Sisterson 2008; Sisterson 2012; Sisterson 2014), reproductive maturity (Krugner 2010), oviposition behavior (Hummel et al. 2006), host preference for oviposition (Blua et al. 2001; Patt and Sétamou 2007; Krugner et al. 2009; Chen et al. 2010), and use of visual stimuli to recognize conspecifics (Mizell III et al. 2012).

Given the near-zero tolerance for GWSS in vineyards, long-term suppression of population densities will rely heavily on novel methods. While products (e.g., insecticides) are available to increase mortality of insect vectors of plant pathogens, research is needed to identify methods to reduce birth (Sisterson and Stenger 2016).

Exploitation of vibrational signals for disrupting mating of *E. vitis* and GWSS could prove to be a useful tool, but existing knowledge on their communication is completely missing (i.e., *E. vitis*) or insufficient (i.e., GWSS, Percy et al. 2008) to implement a management program for these pests.

d. Neglected signals: the case study of vibrational communication in social insects

Insect societies, as in all animals, rely on exchange of information, which is crucial to coordinate activities, differentiate individuals and their roles in the colony life (Wilson 1971). From half of the 20th century and up to date, intracolony communication have been considered to be driven mainly by chemical signals and cues (Wilson 1965; Richard and Hunt 2013). Several glands are specialized in the production of semiochemicals (Billen and Morgan 1998), which are used to signal to conspecifics food sources, danger for the colony, individual reproductive status and conditions (Richard and Hunt 2013). Among chemical messenger used by several social insect species as nestmate discrimination system are cuticular hydrocarbons (CHC), which have been extensively studied and proven to be the main responsible as recognition cues at least in paper wasps (Bruschini et al. 2011; Richard and Hunt 2013).

However, recently growing attention has been given to another communication modality, vibrations (Hunt and Richard 2013). The use of vibrational signals is reported for Hymenoptera and defined as widespread in Isoptera species (Cocroft and Rodriguez 2005). Even if it's not always easy to discriminate the mechanical components involved in communication (e. g., sound and surface-borne vibration), to date social insects are known to rely exclusively on vibrational one, with the only exception of honeybees (Kirchner 1997; Hunt and Richard 2013). In fact, *Apis mellifera* uses airflows generated by dancing bees to extrapolate directional information (Michelsen 2003). There are numerous functions that vibrational signals carry out in colony activities, from percussive alarm signals in termites (Hertel et al. 2011) to stridulatory recruitment signals in ants (Baroni-Urbani et al. 1988; Hölldobler and Roces 2000).

Indeed, among all social insect species, wasps of the subfamily Polistinae are considered to be a very good candidate for this kind of communication (Jeanne 2009; Hunt and Richard 2013), because (1) colony activities take place mainly on their nest that is made of paper material, known to properly convey superficial vibrations and (2) their body oscillatory movements, which are widespread in the group, potentially produce vibrations. The pattern of body oscillations vary according to the species and the context in which they are performed, from the shaking of the entire body to a drumming of just a part of it on the nest surface, such as the antennae or the abdomen. They are reported in at least three of the four genus and have been thoroughly studied in *Polistes* (Jeanne 2009). In this genus three distinct oscillatory behaviors have been described: 'lateral vibration' (LV), 'antennal drumming' (AD), and 'abdominal wagging' (AbW). LV consists in the wasp standing on the nest shaking the entire body horizontally to it, the shaking movement is so intense to produces an audible sound (Gamboa and Dew 1981). The other two movements involve only part of the body: AD is performed by the wasp hitting its antennae

on the cell rims (Pratte and Jeanne 1984), while AbW consists in the horizontal abdomen oscillation performed by foundress walking over cells, often rubbing the nest surface with the abdomen (Brillet et al. 1999; Brennan 2007). The meaning of all of them is not clear yet. The brood, in particular larvae, are considered to be the signal recipients, since all these behaviors are performed in strict connection with brood presence on the nest (i.e., larvae from the 3rd stage) and brood care, in particular feeding (Savoyard et al. 1998; Brillet et al. 1999; Cummings et al. 1999). Moreover, LV induced vibrations are supposed to signal the larvae to withdraw the liquid saliva they usually exchange in trophallaxis acts with adults, because they are going to be feed (Savoyard et al. 1998; Cummings et al. 1999).

In the last years, an intriguing hypothesis has been formulated regarding paper wasps vibrations. Contrary to advanced social insect species, primitively eusocial wasps are a good model to study the evolution and maintenance of social behavior, because caste determination is rather flexible and is believed to be affected by nutritional status (O'Donnell 1998; Jandt et al. 2014). Larvae that are fed more should grow faster and develop into reproductive individuals, gynes. But recently contradictory information suggested that the quantity of food received by the larvae is not sufficient to explain cast determination, mainly for two reasons: (1) faster larval development is correlated with smaller workers (Karsai and Hunt 2002; Kudô 2003) and (2) usually larval development time for workers increases during the colony life, while gynes have an intermediate time of development (Strassmann and Orgren 1983). Thus, evidences suggest the occurrence of an external modulation that Jeanne (2009) proposed to be carried out by vibrations. According to the “mechanical switch hypothesis”, vibrations modulate the biochemical pathways that direct the larval development towards a worker phenotype, and thus they would be at least in part responsible for cast determination (Jeanne 2009). Even if pieces of evidence have been reported for *P. biglumis* (Mignini and Lorenzi 2015), at the moment this hypothesis has been experimentally demonstrated only in one species, *P. fuscatus* (Suryanarayanan et al. 2011).

Social parasitism can lead to novel hints on the manipulation hypothesis

One of the key features of social insects success is the division of labor: few individuals concentrate on reproduction, while non-reproductive colony members spend their energies to rear the brood (Wilson 1971; Wilson 1990). The energetic investment of the worker caste is remarkable, since workers usually take the risk of foraging outside the nest and actively protect the brood inside of it. Likely the relevance and cost for parental care have benefit the independent evolution of several social parasite species in social insects, from ants (Hölldobler and Wilson 1990) to bees (Neumann et al. 2001; Hines and Cameron 2010) and wasps (Cervo 2006). In fact, social parasites exploit the

host workers to rear their own brood. The parasitic relation can be temporary (Mori et al. 2001), if the parasite is able to reproduce also in absence of the host, or obligate (Wilson 1971), if the parasite species have lost the ability to perform nest activities or to produce the worker caste. In the latter case the parasite brood is composed exclusively by reproductive individuals (Wilson 1971).

Obligate social parasite reproductive success depends totally on the host workers force (Cervo 2006). The strong selective pressure has driven an arms race between host and parasite species, which gave us the opportunity to study and understand many spectacular adaptations (Manna and Hauber 2016). For instance, social parasites are able to sneak in the colony life exploiting the individual recognition system of the host species. In this regard, the *Maculinea* butterflies case is remarkable. The larvae of this genus exploit both chemical and acoustic signals of the host species, *Myrmica* ants, to enter the nest, be accepted by the workers, and take the status of queen (Barbero et al. 2009).

In *Polistes*, three obligate social parasite species have been reported and deeply investigated (Cervo 2006). Besides morphological (Cervo 1994; Ortolani et al. 2010) and physiological (Cervo et al. 2004; Ortolani et al. 2008) adaptations to the parasitic life, it has been shown that they are able to exploit the chemical communication system of the host (Turillazzi et al. 2000; Dapporto et al. 2004; Lorenzi et al. 2004; Lorenzi 2006). If the “mechanical switch hypothesis” is true, social parasites of *Polistes* genus could exploit vibrations to manipulate the development of host larvae towards worker phenotype. One peculiar oscillatory behavior have been reported for *Polistes* social parasites (Cervo 1990), the female drums with the abdomen the nest surface and drumming is so intense to produce an audible sound. However, contrary to the ‘stroking’ behavior that is linked to the chemical mimicry (Dani et al. 1992), the *P. sulcifer* drumming has never studied in detail.

Studied species: Polistes dominula and its social parasite P. sulcifer

Among paper wasps, *P. dominula* is particularly common and has a worldwide distribution. It is native of Europe, but recently it has been reported as invasive species in North America (Cervo et al. 2000; Liebert et al. 2006). The colony cycle is typical of *Polistes* species (Reeve 1991). In spring colonies are founded by one or an association of multiple mated foundresses (‘founding phase’). When there are more than one foundress, a linear hierarchy is established soon after foundation by agonistic interactions and it is maintained by means of ritualized dominance behaviors (Pardi 1996). The individual with higher rank (i.e. alpha) usually monopolize reproduction on the nest (Pratte 1993) and acquires distinctive chemical profile and behaviors (Sledge et al. 2001a). In the temperate zone of the Northern Hemisphere, the ‘workers phase’ begins at the end of May, when the first workers emerge. These individuals are all females that help until the end of the season rearing additional brood, they do not develop ovaries unless a fertile foundress or abundant brood are missing on

the nest (Pardi 1996; Strassmann et al. 2004). Reproductive individuals, males and females reared by workers, emerge only later in the season, from the end of July to August. Mating occur outside of the colony at the end of summer (Beani 1996) and only mated females overwinter to start a new colony the following spring.

P. sulcifer is an obligate social parasite specialized on *P. dominula* (Cervo 2006). Contrary to the wide distribution of its host, *P. sulcifer* presence is patched and limited to the Mediterranean and the Caspian basin (Cervo 2006). However, in the areas of presence, the parasite pressure on the host populations is quite high (ranging from 20% to 50%) (Cervo and Turillazzi 1996). Prior the emergence of workers, *P. sulcifer* mated females usurp host nests fighting with *P. dominula* foundresses (Cervo and Turillazzi 1996; Ortolani et al. 2008). After the usurpation the parasite take the role of the higher ranked female by mimicking its chemical profile (Sledge et al. 2001b; Dapporto et al. 2004). Even if the host reproductive activity is not completely suppressed (Cini et al. 2014), the parasite lays its eggs on the nest and rely exclusively on the host workers force to rear its brood, and thus produce reproductive individuals (Cervo et al. 2004).

e. Thesis work plan

When a new world is discovered, the first necessary step is to describe it in detail. This consideration is valid also for new science field, such as biotremology. Therefore, in order to fulfil the objectives of my thesis I started to investigate two species for each group, two leafhoppers (*Empoasca vitis* and *Homalodisca vitripennis*) and two Polistinae wasps (*Polistes dominula* and its social parasite *P. sulcifer*), whose vibrational communication and signaling behavior have never been studied or it was poorly known. To increase knowledge about leafhoppers mating and rivalry communication systems I described in detail and then decoded the leafhoppers vibrational communication systems in different intraspecific interactions contexts, such as isolated individuals, male-female pairs and two males competing for one female (chapter 2, 3, and 4). Indeed, for *E. vitis*, which is also an important pest, I used the knowledge acquired in the description and decoding steps to understand if and how the pair formation process could be artificially disrupted by vibrational manipulation (chapter 3).

Moreover, to give new insights on the features of wasps' vibrations that could be involved in caste determination I recorded and compared the vibrations produced by *P. dominula* when larvae were in two different nutritional status (starved or not) to check if the foundress is able to modify its behavior and produce different vibrations. Furthermore, I compared the vibrations induced by the host and the parasite abdomen oscillation movements to investigate if they are species-specific and which temporal or spectral features differentiate them (chapter 5).

References

- Alma, A.** 2002. Auchenorrhyncha as pests on grapevine. *Denisia* **176**, 531–538.
- Appel, H.M., & Cocroft, R.B.** 2014. Plants respond to leaf vibrations caused by insect herbivore chewing. *Oecologia* **175**(4), 1257–1266.
- Bailey, W.J.** 2003. Insect duets: underlying mechanisms and their evolution. *Physiological Entomology* **28**, 157–174.
- Bailey, W., Macleay, C., & Gordon, T.** 2006. Acoustic mimicry and disruptive alternative calling tactics in an Australian bushcricket (Caedicia; Phaneropterinae; Tettigoniidae; Orthoptera): does mating influence male calling tactic? *Physiological Entomology*, **31**, 201–210.
- Barbero, F., Bonelli, S., Thomas, J., Balletto, E., & Schönrogge, K.** 2009. Acoustical mimicry in a predatory social parasite of ants. *The Journal of Experimental Biology*, **212**, 4084–4090.
- Baroni-Urbani, C., Buser, M.W., & Schilliger, E.** 1988. Substrate vibration during recruitment in ant social organization. *Insectes Sociaux* **35**, 241–250.
- Beani, L.** 1996. Lek-like courtship in paper wasps: “a prolonged, delicate, and troublesome affair.” In *Natural history and evolution of paper wasps* (Ed. by Turillazzi S. & West-Eberhard M.J.), pp 113–125. Oxford University Press, Oxford.
- Billen, J., & Morgan, E.D.** 1998. Pheromone communication in social insects: sources and secretions. In *Pheromone communication in social insects ants, wasps, bees, and termites* (Ed. by Vander Meer, R. K., Breed, M.D., Espelie, K.E., & Winston, M.L.) pp. 3–33. Westview Press, Boulder, Colorado.
- Blua, A.M.J., Redak, R.A., Morgan, D.J.W., & Costa, H.S.** 2001. Seasonal flight activity of two *Homalodisca* species (Homoptera: Cicadellidae) that spread *Xylella fastidiosa* in southern California. *Journal of Economic Entomology* **94**, 1506–1510.
- Böll, S., & Herrmann, J.V.** 2004. A long-term study on the population dynamics of the grape leafhopper (*Empoasca vitis*) and antagonistic mymarid species. *Journal of Pest Science* **77**, 33–42.
- Brennan, B.J.** 2007. Abdominal wagging in the social paper wasp *Polistes dominulus*: behavior and substrate vibrations. *Ethology* **113**, 692–702.
- Brillet, C., Semenov Tian-Chansky, S., & Le Conte, Y.** 1999. Abdominal waggings and variation of their rate of occurrence in the social wasp, *Polistes dominulus* Christ. I. quantitative analysis. *Journal of Insect Behavior* **12**, 665–686.
- Bruschini, C., Cervo, R., Cini, A., Pieraccini, G., Pontieri, L., Signorotti, L., Turillazzi, S.** 2011. Cuticular hydrocarbons rather than peptides are responsible for nestmate recognition in *Polistes dominulus*. *Chemical Senses* **36**, 715–723.

- Cardé, R.T.** 1995. Control of moth pests by mating disruption: successes and constraints. *Annual Review of Entomology* **40**, 559–585.
- Cerutti, F., Baumgärtner, J., & Delucchi, V.** 1991. The dynamics of grape leafhopper *Empoasca vitis* Göthe populations in southern Switzerland and the implications for habitat management. *Biocontrol Science and Technology* **1**, 177–194.
- Cervo, R.** 1990. *Il parassitismo sociale nei Polistes*. Università di Firenze, Firenze.
- Cervo, R.** 1994. Morphological adaptations to the parasitic life in *Polistes Sulcifer* and *P. Atrimandibularis* (Hymenoptera-Vespidae). *Ethology, Ecology & Evolution*, 61–66.
- Cervo, R.** 2006. Polistes wasps and their social parasites : an overview. *Annales Zoologici Fennici* **43**, 531–549.
- Cervo, R., & Turillazzi, S.** 1996. Host nest preference and nest choice in the cuckoo paper wasp *Polistes sulcifer* (Hymenoptera: Vespidae). *Journal of Insect Behavior* **9**, 297–306.
- Cervo, R., Zacchi, F., & Turillazzi, S.** 2000. *Polistes dominulus* (Hymenoptera, Vespidae) invading North America: some hypotheses for its rapid spread. *Insectes Sociaux* **47**, 155–157.
- Cervo, R., Macinai, V., Dechigi, F., & Turillazzi, S.** 2004 Fast growth of immature brood in a social parasite wasp: a convergent evolution between avian and insect cuckoos. *American Naturalist* **164**, 814–820.
- Chen, W., Leopold, R.A., & Boetel, M.A.** 2010. Host plant effects on development and reproduction of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Homoptera: Cicadellidae). *Environmental Entomology* **39**, 1545–53.
- Cini, A., Nieri, R., Dapporto, L., Monnin, T., & Cervo, R.** 2014. Almost royal: Incomplete suppression of host worker ovarian development by a social parasite wasp. *Behavioral Ecology and Sociobiology* **68**(3), 467–475.
- Claridge, M.F.** 1985. Acoustic behavior of leafhoppers and planthoppers: species problems and speciation. In *The leafhoppers and planthoppers*. (Ed. by Nault, L.R. & Rodriguez, J.G.), pp 103–125. Wiley & Sons, Inc, New York.
- Cocroft, R.B., & Rodriguez, R.L.** 2005. The behavioral ecology of insect vibrational communication. *Bioscience* **55**, 323–334.
- Cokl, A., & Virant-Doberlet, M.** 2003. Communication with substrate-borne signals in small plant-dwelling insects. *Annual Review of Entomology* **48**, 29–50.
- Cummings, D., Gamboa, G., & Harding, B.** 1999 Lateral vibrations by social wasps signal larvae to withhold salivary secretions (*Polistes fuscatus*, Hymenoptera: Vespidae). *Journal of Insect Behavior* **12**, 465–473.
- Dani, F.R., Cervo, R., & Turillazzi, S.** 1992. Abdomen stroking behaviour and its possible functions in *Polistes dominulus* (Christ) (Hymenoptera,

- Vespidae). *Behavioral Processes* **28**, 51–58.
- Dapporto, L., Cervo, R., Sledge, M.F., & Turillazzi, S.** 2004. Rank integration in dominance hierarchies of host colonies by the paper wasp social parasite *Polistes sulcifer* (Hymenoptera, Vespidae). *Journal of Insect Physiology* **50**, 217–223.
- Davis, M.J., Purcell, A.H., & Thomson, S.V.** 1978. Pierce's disease of grapevines: isolation of the causal bacterium. *Science* **199**, 75–7.
- de Groot, M., Derlink, M., Pavlovčič, P., Prešern, J., Čokl, A., & Virant-Doberlet, M.** 2012. Duetting behaviour in the leafhopper *Aphrodes makarovi* (Hemiptera: Cicadellidae). *Journal of Insect Behavior* **25**:419–440.
- Decante, D., & van Helden, M.** 2006. Population ecology of *Empoasca vitis* (Göthe) and *Scaphoideus titanus* (Ball) in Bordeaux vineyards: influence of migration and landscape. *Crop Protection* **25**, 696–704.
- Derlink, M., Abt, I., Mabon, R., Julian, C., & Virant-doberlet, M.** 2016. Mating behaviour of *Psammotettix alienus* (Hemiptera : Cicadellidae). *Insect Science*. <http://doi.org/10.1111/1744-7917.12379>.
- Elias, D.O., & Mason, A.C.** 2010. Signaling in variable environments: substrate-borne signaling mechanisms and communication behavior in spiders. In *The use of vibrations in communication: Properties, mechanisms and function across Taxa* (Ed. by O'Connell-Rodwell, C.E.), pp 25–46. Transworld, Kerala.
- Endler, J.A.** 1993. Some general comments on the evolution and design of animal communication systems. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **340**, 215–225.
- Epstein, L.** 2014. Fifty years since silent spring. *Annual Review Phytopathology* **52**, 377–402.
- Eriksson, A., Anfora, G., Lucchi, A., Lanzo, F., Virant-Doberlet, M., & Mazzoni, V.** 2012. Exploitation of insect vibrational signals reveals a new method of pest management. *PloS One*, **7**(3), e32954.
- Fornasiero, D., Pavan, F., Pozzebon, A., Picotti, P., & Duso, C.** 2016. Plant resistance relative infestation level and sensitivity of grapevine cultivars to the leafhopper *Empoasca vitis* (Hemiptera : Cicadellidae). *Journal of Economic Entomology*, **109**, 1–10.
- Foster, S. P., & Harris, M. O.** 1997. Behavioral manipulation methods for insect pest-management. *Annual Review of Entomology*, **42**(1), 123–146.
- Gamboa, G.J., & Dew, H.E.** 1981 Intracolony communication by body oscillations in the paper wasp, *Polistes metricus*. *Insectes Sociaux* **28**, 13–26.
- Gillham, M.C.** 1992. Variation in acoustic signals within and among leafhopper species of the genus *Alebra* (Hornoptera, Cicadellidae). *Biological Journal of the Linnean Society* **45**, 1–15.
- Girolami, V.** 2001. Organophosphate resistance in grape leafhoppers and IPM strategies. *Redia* **84**, A1–A17.

- Gogala, M., Čokl, A., Drašlar, K., & Blažević, A.** 1974. Substrate-borne sound communication in cydnidae (Heteroptera). *Journal of Comparative Physiology* **94**, 25–31.
- Gordon, D., Zahavi, T., Anshelevich, L., Harel, M., Ovadia, S., Dunkelblum, E., & Harari, A. R.** 2005. Mating disruption of *Lobesia botrana* (Lepidoptera: Tortricidae): effect of pheromone formulations and concentrations. *Journal of Economic Entomology*, **98**(1), 135–142.
- Hammond, T. & Bailey, W.** 2003. Eavesdropping and defensive auditory masking in an Australian bushcricket, *Caedicia* (Phaneropterinae: Tettigoniidae: Orthoptera). *Behavior* **140**, 79–95.
- Hazarika, L.K., Bhuyan, M., & Hazarika, B.N.** 2009. Insect pests of tea and their management. *Annual Review of Entomology* **54**, 267–284.
- Heady, S.E., Nault, L.R., Shambaugh, G.F., & Fairchild, L.** 1986. Acoustic and mating behavior of *Dalbulus* leafhoppers (Homoptera: Cicadellidae). *Annals of the Entomological Society of America* **79**, 727–736.
- Heady, S.E., & Nault, L.R.** 1991. Acoustic signals of *Graminella nigrifons* (Homoptera: Cicadellidae). *The Great Lakes Entomologist* **24**, 9–16.
- Hertel, H., Hanspach, A., & Plarre, R.** 2011. Differences in alarm responses in drywood and subterranean termites (Isoptera: Kalotermitidae and Rhinotermitidae) to physical stimuli. *Journal of Insect Behavior* **24**, 106–115.
- Hill, P.S.M.** 2009. How do animals use substrate-borne vibrations as an information source? *Naturwissenschaften* **96**, 1355–71.
- Hill, P.S.M., & Wessel, A.** 2016. Biotremology. *Current Biology* **26**, R187–R191. doi: 10.1016/j.cub.2016.01.054
- Hines, H.M., & Cameron, S.A.** 2010. The phylogenetic position of the bumble bee inquiline *Bombus inexpectatus* and implications for the evolution of social parasitism. *Insectes Sociaux* **57**, 379–383.
- Hölldobler, B., & Wilson, E.O.** 1990. *The Ants*. Harvard University Press, Cambridge.
- Hölldobler, B., & Roces, F.** 2000. The behavioral ecology of stridulatory communication in leafcutting ants. In *Model systems in behavioral ecology: integrating empirical, theoretical and conceptual approaches* (Ed. by Dugatkin, L.), pp 92–109. Princeton University Press, Princeton, New Jersey.
- Horisk, C., & Cocroft, R.B.** 2013. Animal signals: always influence, sometimes information. In *Animal communication theory: information and influence* (Ed. by Stegmann, U.), pp 259–278. Cambridge University Press.
- Hummel, N.A., Zalom, F.G., Toscano, N.C., Peng, C.Y.S., & Burman, P.** 2006. Seasonal patterns of female *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae) reproductive physiology in Riverside, California. *Environmental Entomology* **35**, 901–906.
- Hunt, J.H., & Richard, F.J.** 2013. Intracolony vibroacoustic communication in

- social insects. *Insectes Sociaux*, 403–417.
- Hunt, R.E., & Nault, L.R.** 1991. Roles of interplant movement, acoustic communication, and phototaxis in mate-location behavior of the leafhopper *Graminella nigrifrons*. *Behavioral Ecology and Sociobiology* **28**, 315–320
- Hunt, R.E., Fox, J.P., & Haynes, K.F.** 1992. Behavioral response of *Graminella nigrifrons* (Homoptera: Cicadellidae) to experimentally manipulated vibrational signals. *Journal of Insect Behavior* **5**, 1–13.
- Hunt, R.E., & Morton, T.L.** 2001. Regulation of chorusing in the vibrational communication system of the leafhopper *Graminella nigrifrons*. *American Zoologist* **41**, 1222–1228.
- Ichikawa, T.** 1976. Mutual communication by substrate vibrations in the mating behavior of planthoppers (Homoptera: Delphacidae). *Applied Entomology and Zoology* **11**(1), 8-21.
- Ichikawa, T., & Ishii, S.** 1974. Mating signal of the brown planthopper, *Nilaparvata lugens* Stål (Homoptera: Delphacidae): vibration of the substrate. *Applied Entomology and Zoology* **9**(3), 196-198.
- Ichikawa, T., Sakuma, M., & Ishii, S.** 1975. Substrate vibrations: mating signal of three species of planthoppers which attack the rice plant (Homoptera: Delphacidae). *Applied Entomology and Zoology* **10**(3), 162-171.
- Inoue, H.** 1982. Species-specific calling sounds as a reproductive isolating mechanism in *Nephotettix* spp. (Homoptera: Cicadellidae). *Applied Entomology and Zoology* **17**, 253–262.
- Jandt, J.M., Tibbetts, E.A., & Toth, A.L.** 2014. Polistes paper wasps: a model genus for the study of social dominance hierarchies. *Insectes Sociaux* **61**, 11–27.
- Jeanne, R.L.** 2009. Vibrational signals in social wasps: a role in caste determination? In *Organization of insect societies: from genome to sociocomplexity* (Ed. by Gadau, J., Fewell, J. & Wilson, E.O.), pp. 241–263. Harvard University Press, Cambridge.
- Karsai, I., & Hunt, J.H.** 2002. Food quantity affects traits of offspring in the paper wasp *Polistes metricus* (Hymenoptera: Vespidae). *Population Ecology* **31**, 99–106.
- Kirchner, W.H.** 1997. Acoustical communication in social insects. In *Orientation and Communication in Arthropods* (Ed. by Lehrer M.), pp 273–300. Birkhäuser Basel, Basel.
- Krugner, R.** 2010. Differential reproductive maturity between geographically separated populations of *Homalodisca vitripennis* (Germar) in California. *Crop Protection* **29**, 1521–1528.
- Krugner, R., Groves, R.L., Johnson, M.W., Arnel, P. Hagler, J.R., Morse, J.G., & Flores, A.P.** 2009. Seasonal population dynamics of *Homalodisca vitripennis* (Homoptera: Cicadellidae) in sweet orange trees maintained under continuous deficit irrigation. *Journal of Economic*

Entomology **102**, 960–973.

- Kudô, K.** 2003. Growth rate and body weight of foundress-reared offspring in a paper wasp, *Polistes chinensis* (Hymenoptera, Vespidae): no influence of food quantity on the first offspring. *Insectes Sociaux* **50**, 77–81.
- Kuhelj, A., de Groot, M., Blejec, A., & Virant-Doberlet, M.** 2015. The effect of timing of female vibrational reply on male signalling and searching behaviour in the leafhopper *Aphrodes makarovi*. *PLoS One* **10**:1–15.
- Kuhelj, A., de Groot, M., Blejec, A., Virant-Doberlet, M.** 2016. Sender-receiver dynamics in leafhopper vibrational duetting. *Animal Behavior* **114**, 139–146.
- Lakes-Harlan, R., & Strauß, J.** 2014. Functional morphology and evolutionary diversity of vibration receptors in insects. In *Studying vibrational communication* (Ed. by Cocroft, R.B., Gogala, M., Hill, P.S.M., Wessel, A.), pp 277–302. Springer Berlin Heidelberg.
- Liebert, A.E., Gamboa, G.J., Stamp, N.E., Curtis, T.R., Monnet, K.M., Turillazzi, S., & Starks, P.T.** 2006. Genetics, behavior and ecology of a paper wasp invasion: *Polistes dominulus* in North America. *Annales Zoologici Fennici* **43**, 595–624.
- Liu, S., Li, Z., Sui, Y., Schaefer, D. A., Alele, P. O., Chen, J., & Yang, X.** 2015. Spider foraging strategies dominate pest suppression in organic tea plantations. *BioControl*, **60**(6), 839–847.
- Lorenzi, M.C.** 2006. The result of an arms race: the chemical strategies of *Polistes* social parasites. *Annales Zoologici Fennici* **43**, 550–563.
- Lorenzi, M.C., Cervo, R., Zacchi, F., Turillazzi, S., & Bagnères, A.G.** 2004. Dynamics of chemical mimicry in the social parasite wasp *Polistes semenowi* (Hymenoptera: Vespidae). *Parasitology* **129**, 643–651.
- Mankin, R.W.** 2012. Applications of acoustics in insect pest management. *CAB Reviews* **7**, 1–7.
- Manna, T.J., & Hauber, M.E.** 2016. Recognition, speciation, and conservation: recent progress in brood parasitism research among social insects. *Current Opinion in Behavioral Sciences* **12**, 1–5.
- Markl, H.** 1983. Vibrational communication. In *Neuroethology and Behavioral Physiology* (Ed. by Huber F., & Markl H.), pp 332–353. Springer-Verlag, Berlin Heidelberg.
- Mazzoni, V., Cosci, F., Lucchi, A., & Santini, L.** 2001. Occurrence of leafhopper (Auchenorrhyncha, Cicadellidae) in three vineyards of the Pisa district. In *Integrated Control in Viticulture IOBC wprs Bulletin*, pp 267–271.
- Mazzoni, V., Lucchi, A., Cokl, A., Presern, J., & Virant-Doberlet, M.** 2009a. Disruption of the reproductive behaviour of *Scaphoideus titanus* by playback of vibrational signals. *Entomologia Experimentalis et Applicata*, **133**(2), 174–185.
- Mazzoni, V., Presern, J., Lucchi, A., & Virant-Doberlet, M.** 2009b. Reproductive strategy of the Nearctic leafhopper *Scaphoideus titanus* Ball

- (Hemiptera: Cicadellidae). *Bulletin of Entomological Research*, **99**(4), 401–13.
- Mazzoni, V., Eriksson, A., Anfora, G., Lucchi, A., & Virant-Doberlet, M.** 2014. Active space and the role of amplitude in plant-borne vibrational communication. In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp. 349–374. Springer, Berlin Heidelberg.
- Michelsen, A.** 2003. Signals and flexibility in the dance communication of honeybees. Karl von Frisch lecture. *Journal of Comparative Physiology A Neuroethology, Sensory, Neuronal, and Behavioral Physiology* **189**, 165–174.
- Michelsen, A.** 2014. Physical aspects of vibrational communication. In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp. 199–213. Springer, Berlin Heidelberg.
- Michelsen, A., Fink, F., Gogala, M., & Traue, D.** 1982. Plants as transmission channels for insect vibrational songs. *Behavioral Ecology and Sociobiology* **11**, 269–281.
- Mignini, M., & Lorenzi, M.C.** 2015. Vibratory signals predict rank and offspring caste ratio in a social insect. *Behavioral Ecology and Sociobiology* **69**, 1739–1748.
- Mizell III, R.F., Andersen, P.C., Brodbeck, B.V., Hunter, W.B.** 2012. Congener response reduces risks from bottom-up and top-down forces: behavioral parsimony by a Xylophage. *American Entomologist* **58**, 106–115.
- Mori, A., Grasso, D.A., Visicchio, R., & Le Moli, F.** 2001. Comparison of reproductive strategies and raiding behaviour in facultative and obligatory slave-making ants: the case of *Formica sanguinea* and *Polyergus rufescens*. *Insectes Sociaux* **48**, 302–314.
- Neumann, P., Radloff, S.E., Moritz, R.F., Hepburn, H.R., & Reece, S.L.** 2001. Social parasitism by honeybee workers (*Apis mellifera capensis* Escholtz): host finding and resistance of hybrid host colonies. *Behavioral Ecology* **12**, 419–428.
- Nishino, H., Mukai, H., & Takanashi, T.** 2016. Chordotonal organs in hemipteran insects: unique peripheral structures but conserved central organization revealed by comparative neuroanatomy. *Cell Tissue Research* 1–24.
- Nuhardiyati, M., & Bailey, W.** 2005. Calling and duetting behavior in the leafhopper *Balclutha incisa* (Hemiptera: Cicadellidae: Deltocephalinae): opportunity for female choice? *Journal of Insect Behavior* **18**, 259–280.
- O'Donnell, S.** 1998. Reproductive caste determination in eusocial wasps (Hymenoptera : Vespidae). *Annual Review of Entomology* **43**, 323–346.
- Oerke, E.C.** 2006. Crop losses to pests. *Journal of Agricultural Sciences* **144**, 01, 31.
- Ortolani, I., Turillazzi, S., & Cervo, R.** 2008. Spring usurpation restlessness:

a wasp social parasite adapts its seasonal activity to the host cycle. *Ethology* **114**, 782–788.

- Ortolani, I., Zechini, L., Turillazzi, S., & Cervo, R.** 2010. Recognition of a paper wasp social parasite by its host: evidence for a visual signal reducing host aggressiveness. *Animal Behavior* **80**, 683–688.
- Ossiannilsson, F.** 1949, Insect drummers. a study on the morphology and function of the sound-producing organ of Swedish Homoptera Auchenorrhyncha with notes on their sound production. *Opuscula Entomologica: Supplementum*. **X**, 146.
- Pardi, L.** 1996. *Polistes*: analysis of a society. In *Natural history and evolution of paper-wasps* (Ed. by Turillazzi S, & West-Eberhard MJ), pp 1–17. Oxford University Press, Oxford.
- Patt, J.M., & Sétamou, M.** 2007. Olfactory and visual stimuli affecting host plant detection in *Homalodisca coagulata* (Hemiptera: Cicadellidae). *Environmental Entomology* **36**, 142–150.
- Pavan, F., & Picotti, P.** 2009. Influence of grapevine cultivars on the leafhopper *Empoasca vitis* and its egg parasitoids. *BioControl* **54**, 55–63.
- Percy, D.M., Boyd, E.A., & Hoddle, M.S.** 2008. Observations of acoustic signaling in three sharpshooters : *Homalodisca vitripennis*, *Homalodisca liturata*, and *Graphocephala atropunctata* (Hemiptera: Cicadellidae). *Annals of the Entomological Society of America* **101**, 253–259.
- Polajnar, J., Eriksson, A., Rossi Stacconi, M. V., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2014. The process of pair formation mediated by substrate-borne vibrations in a small insect. *Behavioural Processes*, **107**, 68–78.
- Polajnar, J., Eriksson, A., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2015. Manipulating behaviour with substrate-borne vibrations - Potential for insect pest control. *Pest Management Science* **71**, 15–23.
- Polajnar, J., Eriksson, A., Virant-Doberlet, M., & Mazzoni, V.** 2016. Mating disruption of a grapevine pest using mechanical vibrations: from laboratory to the field. *Journal of Pest Science* **89**, 1–13.
- Pratte, M.** 1993. Experimental Changes of Hierarchical Rank in *Polistes dominulus* Christ Foundresses. *Ethology* **95**, 97–104.
- Pratte, M., & Jeanne, R.L.** 1984. Antennal drumming behavior in *Polistes* wasps (Hymenoptera: Vespidae). *Ethology* **66**, 177–188.
- Reeve, H.K.** 1991. *Polistes*. In *The Social Biology of Wasps* (Ed. by Ross K.G. & Matthews R.H.), pp 99–148 . Cornell University Press, Ithaca, NY.
- Richard, F.J., & Hunt, J.H.** 2013. Intracolony chemical communication in social insects *Insectes Soiauxc* **60**, 275–291.
- Savoyard, J.L., Gamboa, G.J., Cummings, D.L.D., & Foster, R.L.** 1998. The communicative meaning of body oscillations in the social wasp, *Polistes fuscatus* (Hymenoptera, Vespidae). *Insectes Soiauxc* **45**, 215–230.
- Saxena, K.N., & Kumar, H.** 1984. Acoustic communication in the sexual

- behaviour of the leafhopper, *Amrasca devastans*. *Physiological Entomology* **9**, 77–86.
- Shaw, K.C., Vargo, A., & Carlson, O.V.** 1974. Sounds and associated behavior of some species of *Empoasca* (Homoptera: Cicadellidae). *Journal of the Kansas Entomological Society*, 284–307.
- Sisterson, M.S.** 2008. Effects of insect-vector preference for healthy or infected plants on pathogen spread: insights from a model. *Journal of Economic Entomology* **101**, 1–8.
- Sisterson, M.S.** 2012. Host selection by *Homalodisca vitripennis*: the interplay between feeding, egg maturation, egg load, and oviposition. *Arthropod-Plant Interactions* **6**, 351–360.
- Sisterson, M.S.** 2014. Evaluation of a method to quantify glassy-winged sharpshooter (Hemiptera : Cicadellidae) egg maturation during a feeding assay. *Journal of Economic Entomology* **107**, 206–214.
- Sisterson, M.S., & Stenger, D.C.** 2016 Disentangling effects of vector birth rate, mortality rate, and abundance on spread of plant pathogens. *Journal of Economic Entomology* **109**, 487–501.
- Sledge, M.F., Boscaro, F., & Turillazzi, S.** 2001a. Cuticular hydrocarbons and reproductive status in the social wasp *Polistes dominulus*. *Behavioral Ecology and Sociobiology* **49**, 401–409.
- Sledge, M.F., Dani, F.R., Cervo, R., Dapporto, L., & Turillazzi, S.** 2001b. Recognition of social parasites as nest-mates: adoption of colony-specific host cuticular odours by the paper wasp parasite *Polistes sulcifer*. *Proceedings Biological Sciences of the Royal Society* **268**, 2253–2260.
- Sorensen, J.T., & Gill, R.J.** 1996. A range extension of *Homalodisca coagulata* (Say) (Hemiptera: Clypeorrhyncha: Cicadellidae) to southern California. *Pan-Pacific Entomologist* **72**, 160–161.
- Stenger, D.C., Sisterson, M.S., & French, R.** 2010 Population genetics of *Homalodisca vitripennis* reovirus validates timing and limited introduction to California of its invasive insect host, the glassy-winged sharpshooter. *Virology* **407**, 53–59.
- Strassmann, J.E., & Orgren, F.M.C.** 1983. Nest architecture and brood development times in the paper wasp, *Polistes exclamans* (Hymenoptera: Vespidae). *Psyche (New York)* **90**, 237–248.
- Strassmann, J.E., Fortunato, A., Cervo, R., Turillazzi, S., Damon, J.M., & Queller, D.C.** 2004. The cost of queen loss in the social wasp *Polistes dominulus* (Hymenoptera: Vespidae). *Journal of the Kansas Entomological Society* **77**, 343–355.
- Suryanarayanan, S., Hermanson, J.C., & Jeanne, R.L.** 2011. A mechanical signal biases caste development in a social wasp. *Current Biology* **21**, 231–5.
- Triapitsyn, S.V., & Phillips, P.A.** 2000. First record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae) with notes on the distribution of the

host. *Florida Entomologist* **83**, 200–203.

- Turillazzi, S., Sledge, M.F., Dani, F.R., Cervo, R., Massolo, A., & Fondeli, L.** 2000. Social hackers: integration in the host chemical recognition system by a paper wasp social parasite. *Naturwissenschaften* **87**, 172–176.
- Virant-Doberlet, M., Mazzoni, V., de Groot, M., Polajnar, J., Lucchi, A., Symondson, W.O.C., & Čokl, A.** 2014. Vibrational communication networks: eavesdropping and biotic noise. In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp 93–123. Springer, Berlin Heidelberg.
- Wessel A, Mühlethaler R, Hartung V, Kuštor, V., & Gogala, M.** 2014. The tymbal: evolution of a complex vibration-producing organ in the *Tymbalia* (Hemiptera excl. Sternorrhyncha). In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp 395–444. Springer, Berlin Heidelberg.
- Wilson, E.O.** 1965. Chemical Communication in the social insects. *Science* **149**, 1064–1071.
- Wilson, E.O.** 1971. *The Insect Societies*. Oxford University Press, Cambridge.
- Wilson, E.O.** 1990. *Success and dominance in ecosystems: the case of the social insects*. Ecology Institute, Oldendorf / Luhe, Germany.

Chapter 2

The reproductive strategy and the vibrational duet of the leafhopper *Empoasca vitis* Göthe

*The results of this study have been submitted for publication.
The present version of the manuscript has been modified after peer
review revision.*

2. The reproductive strategy and the vibrational duet of the leafhopper *Empoasca vitis* Göthe

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Running title: The mating behavior of *Empoasca vitis*

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Abstract

The recent description of a new vibrational mating disruption method to control the leafhopper *Scaphoideus titanus* Ball opened questions about its possible application to other leafhopper pests. Since the prerequisite for the method's successful application is a deep knowledge of the species mating behavior and the exact role of associated signals, we conducted behavioral assays on the green leafhopper *Empoasca vitis* Göthe, a pest of grapevine and other crops in Europe and Asia. Laser vibrometer recordings of single and paired individuals (male and female) during a 24-hour period enabled us to detect and describe two male and one female signal. The pair formation starts when the female replies to a male call and a duet is established, then it continues through two different behavioral stages: Location and Courtship. The proper courtship begins only when the male locates the female. The latter is characterized by a significant change in temporal parameters that regards both the signals and the duet structure. Although, the male calling activity and the female replying rate were the same during the 24 hours, a lower number of matings was recorded during the night. We discuss the possible role of vision and of the species ecology as factors of reproductive success and mating strategy. Our conclusion is that the mechanical mating disruption technique seems feasible for future application to this species.

Key words: leafhopper, mating behavior, pest, vibrational communication, daily activity.

Introduction

The green leafhopper, *Empoasca vitis* Göthe (Hemiptera: Cicadellidae: typhlocybinae), is a polyphagous species with holarctic distribution (Alma, 2002). Despite being polyphagous, *E. vitis* is an important grapevine pest in north Italy, Switzerland, Germany, France and Greece (Cerutti et al., 1991; Mazzoni et al., 2001; Alma, 2002; Böll & Herrmann, 2004), while in Asia it is reported as noxious for tea plants (Hazarika et al., 2009). Damage is due to the phloem feeding activity that causes stress symptoms, such as vein browning and chlorosis of leaf margins under low-density conditions and leaf burn and phylloptosis at higher densities (Alma, 2002; Böll & Herrmann, 2004).

In recent years, several integrated pest management (IPM) approaches have been tested to control *E. vitis* population density, such as the use of non-susceptible cultivars, and techniques of landscape management to favor the occurrence of predators and parasitoids (Decante & van Helden, 2006; Pavan & Picotti, 2009; Fornasiero et al., 2015; Liu et al., 2015). However, none of them have been applied extensively to the field and population control of *E. vitis* is still achieved mainly with pesticides. Another sustainable approach would be to use behavioral manipulation methods such as a mating disruption technique that prevents mating partners from identifying and locating each other (Foster & Harris, 1997). Mating communication of leafhoppers rely on substrate-borne vibrations (Cocroft & Rodriguez, 2005). In particular, we know that vibrational signals are essential in mate recognition and partner location (Cokl & Virant-Doberlet, 2003) and for this reason they would represent a convenient target for a mechanical mating disruption technique (Polajnar et al., 2015; Polajnar et al., 2016a, b).

The latter is achievable when the precise characterization of the male and female vibrational signals and their association with specific behaviors during the process of pair formation are known (Mazzoni et al., 2009a; Eriksson et al., 2012; Polajnar et al., 2014). In general, leafhopper males call first. They use the “call-fly” strategy to enlarge their signal active space (Mazzoni et al., 2014) and enhance the probability of finding a receptive female (Hunt & Nault, 1991), who in turn replies to a male call with a simpler vibrational signal. A duet between partners is then established, and the perception of the female signal triggers males to actively search for the female, who remains stationary (Saxena & Kumar, 1984; Claridge, 1985; Hunt & Nault, 1991; Hunt et al., 1992; Mazzoni et al., 2009b; de Groot et al., 2012; Derlink et al., 2016). Predictable temporal association between the male call and the female reply characterizes a proper duet (Bailey, 2003), and are crucial for the accomplishment of partner identification and its final location (Polajnar et al., 2014; Kuhelj et al., 2015a). More specifically, not much is known about *Empoasca* species and the typhlocybinae in general. Shaw et al. (1974) recorded the vibrational signals of 7 American species of the genus *Empoasca*. All reported male signals involved in mating consisted of a repetition of tonelike

pulses grouped in phrases, while female signals were described as “rumbling”. However, specific studies on *E. vitis* mating behavior are still missing. Not only the vibrational communication, but also information such as the age of sexual maturity and daily sexual activity are still unknown. In other leafhoppers, for example, males are reproductively active at earlier age than females and are able to mate multiple times (Mazzoni et al., 2009b). Instead females have a post-mating refractory period during which they are not responsive to male calls (Bailey & Nuhardiyati, 2005; Mazzoni et al., 2009b; Derlink et al., 2016). Among Auchenorrhyncha, a few studies investigated the exact time of the day in which mating occur. Usually, the reproductive activity is considered to be restricted to a narrow time window specific to the species. For instance, in *S. titanus* matings are concentrated during late afternoon/evening (Mazzoni et al., 2009b), while in *Metcalfa pruinosa* during the night (Virant-Doberlet & Žežlina, 2007). Therefore, the main goal of this work is to describe the reproductive strategy and characterize all vibrational signals associated with the mating behavior produced by males and females of *E. vitis*. This knowledge will be the basis for which to plan the next research specifically aimed at investigating the feasibility of vibrational mating disruption technique to control pests.

Materials and methods

Insects rearing

Adults and nymphs of *E. vitis* were manually collected in the vineyard of the Fondazione Edmund Mach, San Michele all’Adige (Trento, Italy) during summer and spring of 2013 (for test 1), and spring 2014 (for test 2). Immediately after collection the animals were placed inside a net cage (Bugdorm-6620, 60x60x120 cm) and provided with grapevine plants in a greenhouse in S. Michele all’Adige, at 25±2 °C, 80±5% relative humidity, and 16:8 (L:D) photoperiod, with the scotophase starting at 5:00 AM local time. Individuals of second or further generation were used for experiments. Twice a week vine leaves in the cage were checked for newly hatched individuals and they were moved to rearing boxes that consisted of plastic beakers (height 10 cm; 5 cm interior diameter) with a moistened grapevine leaf laid on top of a layer (1 cm) of technical agar solution (0,8%) that was replaced every 3 days. The rearing boxes were checked daily and new adults were separated by sex and date of emergence.

Recordings vibrational signals and behavior

All recordings were made in a laboratory room of the Fondazione E. Mach (S. Michele all’Adige, Italy), on an anti-vibration table (Astel s.a.s., Ivrea,

Italy) at temperature $22 \pm 1^\circ\text{C}$. A laser vibrometer (Ometron VQ-500-D-V, Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark), focused on a reflecting-sticker, was used to detect the vibrational signals from the leaf lamina. The signals were digitized with 48 kHz sample rate and 16-bit depth and stored onto a hard drive of a laptop (HP, EliteBook 8460 p) using Adobe® Audition™ version 1.0 (© 1992-2003, Adobe Systems Incorporated) and the inbuilt audio drive (Audio Definition Device, SRS Premium Sound).

Test 1 - Characterization of vibrational signals and mating behavior

To study the daily pattern of male calling and mating activity, we divided the day into three main periods: morning, from dawn to 3:00 pm, afternoon, from 3:00 pm to sunset, and night, from sunset to dawn. Sunrise and sunset times changed during the experimental period (from June to September) and, therefore, they were defined according to the exact day. No artificial light was used during the recording of insect behavior, so in the night period recordings were done in darkness, while the room was illuminated by natural sunlight coming through the windows during daytime. Insects were placed on a fresh grapevine leaf (surface area: $54.66 \pm 0.40 \text{ cm}^2$) inserted into a vial with water and let acclimatize for three minutes. All tested individuals were seven to 20 days old counting from eclosion. The position in which insects were placed on the leaf and, for pair trials, the order in which they were introduced to the arena, were randomized during trials. For pair trials, the male and female were always placed on different halves of the leaf. The space between the stem of the leaf and the opening of the vial was closed using parafilm, to prevent the animals going into the water and to keep the leaf stable. To thwart leafhoppers from escaping, the vial with the leaf was placed into a plastic cube (20x20x20 cm) with a hole on the top for the laser beam.

To explore which individuals of *E. vitis* produce substrate-borne vibrations and the role of these signals in the mating behavior, three different conditions were simulated for each period of time: single female (total n=80) (test 1.1), single male (total n=156) (test 1.2), and pairs, one male and one female (total n=159) (test 1.3). Individuals' behavior was recorded starting from their positioning on the leaf for 15 minutes or, in pair trials, until copula occurred if it happened before the 15-minute mark. In addition, to assess if vibrational signals were produced during or after copulation, 13 pairs that successfully mated were recorded throughout the copula duration until 30 minutes after it.

Test 2 - Playback experiment

A playback experiment was conducted to assess if substrate-borne vibrations alone can elicit a female response and trigger a duet, and if the

female replying activity varies according to female age and reproductive status (e.g. virgin or mated). An electromagnetic mini-shaker (Type 4810; Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark), controlled by a computer and Adobe® Audition™, was added to the setup of experiment 1. A conical rod attached to the mini-shaker was in contact with the lower lamina of the leaf. The contact was ensured by a small amount of blue-wax on the tip of the rod (Surgident Periphery Wax, Australia). We tested 83 virgin females sorted by age (i.e. days after moult: group 1= from 2 to 6 days n=20, group 2 = from 7 to 10 n=24, group 3 = from 11 to 13 n=16, group 4 = from 14 to 20 n=23) and 15 mated females (from 8 to 20 days from moult and tested 24 to 48 hours after mating). In each trial, a female was placed on the leaf and left to acclimatize for three minutes, then stimulated with two different playbacks separated by a gap of 30 s of silence. Each playback consisted of a sequence of 6 male calls recorded from an individual that successfully mated in test 1. Females were scored as sexually active if they replied to at least one male call. The order in which they were played was randomized between trials.

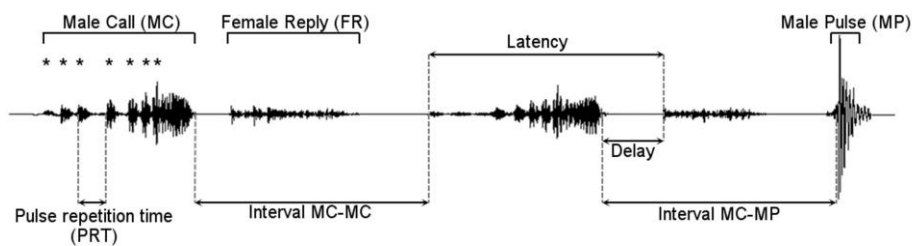


Figure 1 Oscillogram of all *E. vitis* mating signals and measured temporal parameters. Asterisks indicate pulses composing the first section of the Male Call.

Signal characterization and analysis

Spectral analysis was performed with Raven 1.2.1 (The Cornell Lab of Ornithology, Ithaca, NY) using Fast Fourier Transform (FFT) type Blackman, window length of 512 samples and 75% of overlap. The following parameters were measured when applicable for each signal and/or section of it: duration, pulse repetition time (PRT, measured as the distance between the onsets of two consecutive pulses), interval between signals/sections, as the time between two consecutive signals or sections, fundamental frequency (Ff), and dominant frequency (Df). To describe the duet temporal pattern we measured the latency and the delay of the female signal respectively from the onset and from the end of the immediately preceding male signal. All temporal parameters are indicated in figure 1.

Recordings of two signals from 30 single males and four signals of each type from 30 pairs that successfully mated were used to describe the vibrational repertoire of *E. vitis*. Statistics were conducted using KyPlot version 2.0 beta 15 (1997-2001 Koichi Yoshioka) and Statistica version 13 (© Dell Inc., Tulsa, OK). Since the number of pulses composing section 1 of the male signal (see Results) varied from 6 to 22, to compare the PRT at the beginning and at the end of the section we performed the Friedman test (nonparametric repeated measures ANOVA) with two replications followed by pairwise multiple comparisons (Siegel & Castellan, 1988). In particular, we selected three pulses from the first half and three from the second half of the male call, always including the first pulse and the last one. To determine whether the spectral and temporal parameters of male and female signals varied during the pair formation process, a Wilcoxon T test for paired data followed by Bonferroni correction was used to compare each parameter in the two behavioral phases (see Results). In addition, a Principal Component Analysis (PCA) was performed on the multivariate signals recorded on 30 pairs that successfully mated in order to visualize the results of the mating process and assess the contribution of the different signal parameters.

Behavioral analysis

In all tests we measured, when applicable, the following parameters: the calling/replying activity as the number of individuals, male or female respectively, who emitted at least one signal during the trial; the latency to the first emitted signal (= calling latency); the “call-fly” activity as the number of individuals who alternated flying or jumping with signal emission for at least once during the trial; the number of pairs that successfully mated; the duration (s) of the pair formation process as the time between the establishment of the duet and the copula; the replying rate of female as the rate between the total number of female replies and male calls during the trial. For the 13 pairs that were recorded during and after copulation, we also measured the length of copulation and the latency to the first vibrational signal emission after the mating end. For the playback experiment we considered an active female as one which replied to at least one call during the trial, and we measured the female replying activity as the number of active individuals. To compare the calling activity, the “call-fly” activity, and the number of mating during the three day periods, we performed a G-test in a contingency table (2x3) followed by a Ryan multiple comparisons for proportions (Ryan, 1960). The same test was used to compare the female reply among females of different ages. The Kruskal-Wallis test was performed to compare the female replying rate in different periods of the day. The Mann-Whitney U-test was used to compare the male calling latency between single and pair trials and the female replying rate between pairs that successfully mated and pairs that did not.

Results

Vibrational signals

Females emitted only one type of signal, the Female Reply (FR), while males emitted two types of signals: the Male Call (MC) and the Male Pulse (MP). All *E. vitis* vibrational signal emissions were associated with a slightly visible tremulatory-like movement associated with dorso-ventral abdominal oscillation, except for MP, which was observed in association with a peculiar body movement: the forepart of the body (e. g. head and thorax) was quickly swayed up and down. However, due to the small size of the insect (adults are about 2 mm long), it was not clear to us if this signal was produced by body contact with the leaf or not. None of the *E. vitis* signals was recorded during copulation (n = 13).

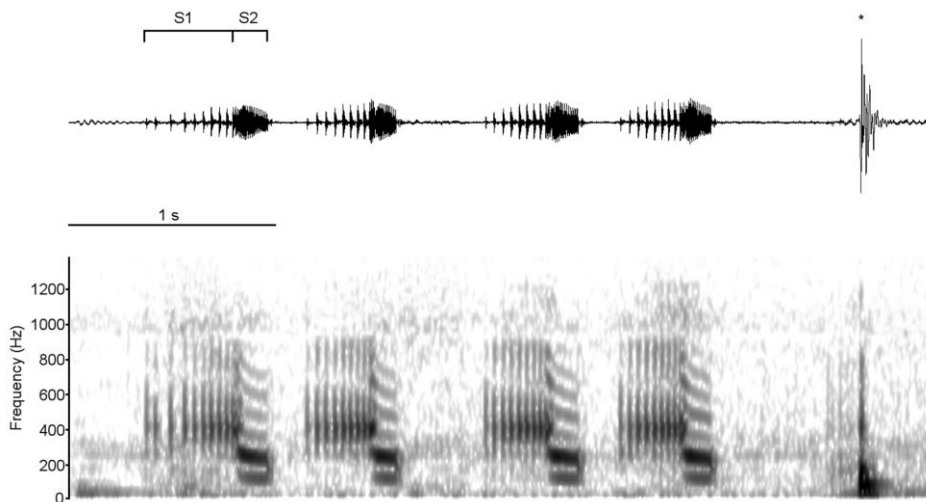


Figure 2 Oscillogram (above) and spectrogram (below) of spontaneous *E. vitis* male vibrational signals. Four male calling signals (MC) composed of two sections (S1 and S2) followed by a male pulse (MP). The asterisk indicates the MP.

Male signals. The MC (Table 1, 2; Fig. 2 - 5) was emitted by males in both single and pair tests. Its structure can be divided in two sections: section 1 (S1), consisting of a series of pulses at relatively high Df (about 400 Hz), and section 2 (S2), with clear harmonic structure (Ff about 125 Hz) and most energy usually associated with the second harmonic (Table 1). The PRT was constant among the first three pulses composing S1, while it was shorter between the last three pulses (Friedman Test followed by Bonferroni-Dunn multiple comparisons, $\chi^2 = 227.18$, $df = 5$, $p < 0.001$) (Fig. 4). S1 is longer than S2,

the latter about ¼ of the MC. The MP is the shortest signal of *E. vitis*. It consists of a single broadband pulse with relatively low Df (about 160 Hz). The MP emission was often alternated to MC and separated from it by a short silence gap (e. g., MC-MP interval, Table 1-2). Sometimes we recorded MP isolated or even sequences of two to three consecutive MPs. MP was produced by all duetting males (n=114) and only by 25% of active single males (n=117).

Female signal. The FR (Table 1, 2; Fig. 3) has a simpler structure if compared to MC, in that it consists of a single broadband unit with Df in between the Df of S1 and S2 of the MC (mean \pm SD: 266.65 \pm 116.09 Hz). The length of FR is about half of MC length. The FR can either overlap part of the MC S2 or not (see below).

Table 1 Spectral and temporal parameters of *E. vitis* single male signals.

	Signal / section	Parameter	N	n	Mean \pm SD
Spectral	S1	Df (Hz)	30	2	396.454 \pm 97.115
	S2	Df (Hz)	30	2	253.372 \pm 72.719
		Ff (Hz)	30	2	124.173 \pm 10.520
	MP	Df (Hz)	12	2	161.513 \pm 58.554
Temporal	S1	Length (S)	30	2	0.386 \pm 0.119
	S1	N pulses	30	2	9.700 \pm 2.157
	S2	PRT (S)	30	2	0.039 \pm 0.007
		Length (S)	30	2	0.105 \pm 0.023
	MP	Length (S)	12	2	0.026 \pm 0.008
	MC-MC	Interval (S)	30	2	25.818 \pm 58.232
	MC-MP	Interval (S)	12	2	0.316 \pm 0.071

S1 = first section of male call (MC), S2 = second section of MC, MP = male pulse, Df = dominant frequency, Ff = fundamental frequency, N pulses = number of pulses, PRT = pulse repetition time, N = number of individuals analyzed, n = number of signals analyzed for each individual.

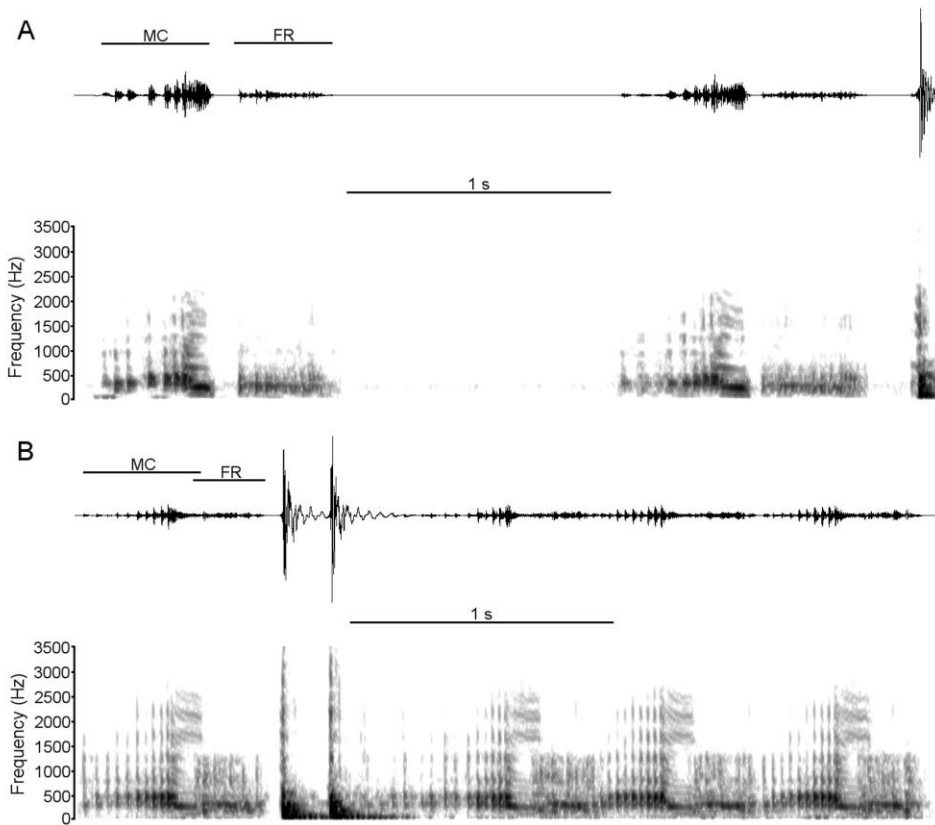


Figure 3 Oscillogram (above) and spectrogram (below) of a *E. vitis* duet in two following stages of the pair formation process: Location (A) and Courtship (B). The first male call is indicated with MC, the first female reply with FR.

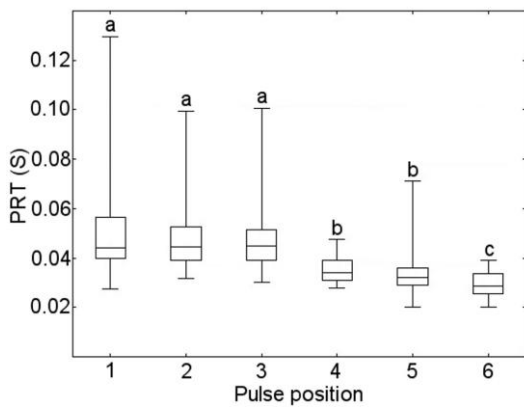


Figure 4 Comparison of PRT of the first and last three pulses composing S1 of MC. Different letters show significant difference between PRT after Friedman Test followed by Bonferroni-Dunn multiple comparisons. A total of 30 individuals were analyzed and two male calls for each of them were included in the analysis.

Table 2 Spectral and temporal parameters of *E. vitis* male and female signals in the two phases of the mating behavior.

Signal / section	Parameter	Location		Courtship		Wilcoxon test	
		Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	T	p-value
S1	Df (Hz)	376.959 \pm 69.180	413.447 \pm 86.004	-991	< 0.001		
S2	Df (Hz)	272.035 \pm 60.136	249.063 \pm 61.983	462	0.039		
	Ff (Hz)	126.806 \pm 11.039	125.369 \pm 9.302	159	0.416		
MP	Df (Hz)	156.482 \pm 62.432	165.093 \pm 84.364	-51	0.849		
FR	Df (Hz)	272.758 \pm 154.506	260.548 \pm 56.870	-400	0.133		
S1	Length (S)	0.299 \pm 0.160	0.325 \pm 0.116	-463	0.089		
	N pulses	7.783 \pm 2.308	9.950 \pm 2.664	-1321	< 0.001		
	PRT (S)	0.037 \pm 0.010	0.032 \pm 0.006	1122	< 0.001		
S2	Length (S)	0.083 \pm 0.019	0.119 \pm 0.027	-1739	< 0.001		
MP	Length (S)	0.035 \pm 0.011	0.038 \pm 0.014	-711	0.008		
MC-MC	Interval (S)	0.916 \pm 0.756	0.134 \pm 0.075	1818	< 0.001		
MC-MP	Interval (S)	0.471 \pm 0.240	0.287 \pm 0.80	1594	< 0.001		
	Length (S)	0.206 \pm 0.097	0.160 \pm 0.055	854	0.002		
FR	Latency (S)	0.433 \pm 0.175	0.410 \pm 0.127	278	0.307		
	Delay (S)	0.052 \pm 0.044	-0.034 \pm 0.026	1830	< 0.001		

For each pair (N = 30), two signals of each type and phase of the mating behavior were analyzed. S1 = first section of male call (MC), S2 = second section of MC, MP = male pulse, FR = female reply, Df = dominant frequency, Ff = fundamental frequency, N pulses = number of pulses, PRT = pulse repetition time. Statistical significant differences after Bonferroni correction are boldfaced.

Description and daily rhythm of the mating behavior

Females never emitted FR spontaneously in single trials. In contrast, most single males (74%, N = 116/156) emitted MC with a widely variable calling latency (mean \pm SD: 162 \pm 228 s). A similar percentage of males (72%, N = 115/159) emitted MC during pair tests with very similar calling latency (129 \pm 186 s) (Mann-Whitney test, U=6392, P=0.55). In general, in the absence of FR, the male continued emitting signals (15.83 \pm 18.80 MC) for the entire recording time, sometimes changing position on the leaf, either walking or flying (e. g. “call-fly” strategy). The “call-fly” strategy was displayed more

often in single trials than in pair trials (G test after Williams correction: $df=1$, $G=17.33$, $P<0.001$) (Table 3).

Table 3 Behaviors and signals of *E. vitis* males and females in single male and pair trials.

Time of day	Trial	N	Type of behaviour			
			Male calling	Call-fly	Duet	Mate
Night	single males	63	33	9	-	-
	pairs	63	17	0	19	7
Morning	single males	42	25	4	-	-
	pairs	53	5	2	10	24
Afternoon	single males	51	33	11	-	-
	pairs	43	4	2	9	15
Total		156	91	24	-	-
		159	26	4	38	46

N= number of trials, male calling = without female reply; call-fly = the male alternated flying or jumping with calling at least once; duet = male and female alternated MC and FR; mate = the pair accomplished copula. In “Type of behavior”, each column is exclusive.

When a receptive female was present on the leaf, 50% of the time she replied after 1-4 MC (median = 2, maximum = 188) (N=81). In one pair trial a female produced one FR a few seconds before the MC. Location of the female by the male always was accomplished in the presence of FR emission, and the mating success was associated with a higher female reply rate, which was lower in pairs that did not mate (Mann-Whitney test, $N_{mated} = 44$ vs. $N_{not\ mated} = 37$, $U = 1112.5$, $P = 0.02$): 0.78 ± 0.19 (mean \pm SD) and 0.63 ± 0.35 in mated and unmated pairs, respectively.

For convenience, we divided the whole pair formation process into two main phases: (1) Location, where the male and female establish a duet, then the male alternates MC emissions with walking until he locates the stationary female; (2) Courtship, the male in close proximity to the female (at approximately one body length from her) keeps duetting with her and attempts copulation.

During Location the male’s path to the female was not always straightforward and turns occurred repeatedly before reaching the Courtship position. At this stage males emitted few MC (mean \pm SD: 11.10 ± 4.62 , $n = 46$) before rapidly spinning around to join their genitalia with those of the females. This sequence was repeated until mating. The pair formation process from the

first duet to copula took on average 335 ± 189 s (mean \pm SD) ($n = 46$), while individuals stayed in copula for 841 ± 303 s (mean \pm SD) ($n = 13$). The latency of male calling after copula was 1322 ± 273 s (mean \pm SD) ($n=8$), while in only five trials males did not call within 30 minutes from the end of the mating.

We did not find significant differences between morning, afternoon, and night, neither in the daily calling activity of males (G-test with Williams correction, $N_{\text{morning}} = 95$, $N_{\text{afternoon}} = 94$, $N_{\text{night}} = 126$, $df = 2$, $G = 3.51$, $P = 0.17$) nor in “call-fly” activity (G-test with Williams correction, $df = 2$, $G = 2.64$, $P = 0.27$) (Table 3). Among pairs that established a duet, the number of matings was significantly lower during the night than during the rest of the day (morning and afternoon) (G-test with Williams correction, $df = 2$, $G = 12.16$, $P < 0.01$). Within trials in which the male called at least once, the number of females that replied (e. g., duetting and mated pairs) was significantly higher during day time (e.g. morning and afternoon) compared to the night (G-test with Williams correction, $N_{\text{morning}} = 41$, $N_{\text{afternoon}} = 30$, $N_{\text{night}} = 43$, $df = 2$, $G = 6.08$, $P < 0.05$) (Table 3); however, the female reply rate did not significantly differ between night and day (Kruskal-Wallis test, $N_{\text{morning}} = 34$, $N_{\text{afternoon}} = 24$, $N_{\text{night}} = 27$, $\text{Chi}^2 = 1.71$, $df = 2$, $P = 0.42$).

Changes of signals parameters in the pair formation process

The first two Principal Components, PC1 and PC2 respectively, accounted for the 45.53% of variance. The PCA biplot (PC1xPC2) showed that major contributors to the transition from Location to Courtship were temporal parameters (Fig. 6). The scoreplot indicated that Courtship and Location were clearly separated in the Y direction (i.e. PC2 = 20.56%). In terms of loadings, PC2 showed a positive contribution of the length of the MC second section, which was significantly shorter in Location than Courtship (Wilcoxon test: $T = -1739$, $p < 0.001$) (Tab. 4). Conversely, other parameters showing a negative contribution to PC2 were significantly longer in Location than in Courtship (Table 2): the interval between consecutive MC ($T = 1818$; $p < 0.001$), the interval between MC and MP ($T = 1594$; $p < 0.001$), the FR length ($T = 854$; $p = 0.002$) and its delay ($T = 1830$; $p < 0.001$) (Tab. 4). In particular, the negative value of FR delay in Courtship indicated that the MC and the FR partially overlapped. The overlapping time was variable (mean \pm SD: 0.03 ± 0.02 s) and covered about 22% of FR and 28% of S2 of MC. It is worth noting that the two phases were not separated along the X-axis (i.e. PC1 = 24.97%). In terms of loadings, PC1 showed a strong contribution of MC first section, PRT, and FR latency, parameters that can be associated with inter-individual variability.

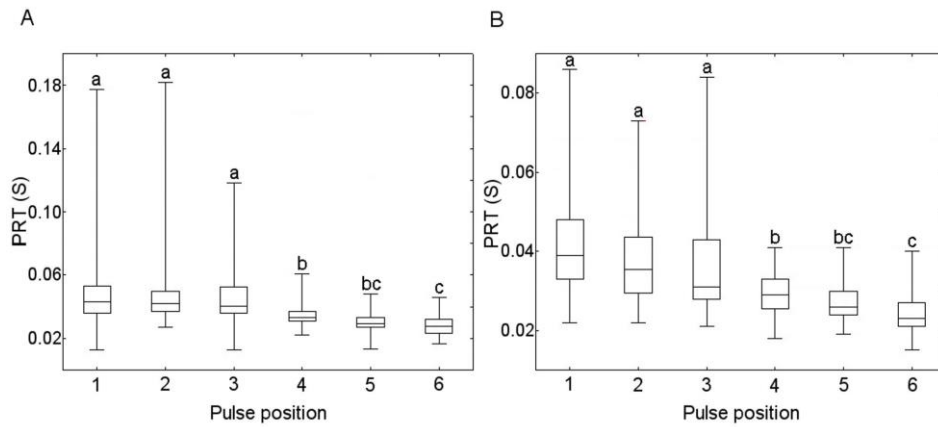


Figure 5 Comparison of PRT of the first and last three pulses composing S1 of MC in Location and Courtship. Different letters show significant difference between PRT after Friedman Test followed by Bonferroni-Dunn multiple comparisons (Location: $\chi^2=112.47$, $Df=5$, $p<0.001$; Courtship: $\chi^2=107.50$, $Df=5$, $p<0.001$). A total of 30 individuals were analyzed and two male calls for each male and phase of the mating process were included in the analysis.

Female reply to playback stimulation

The stimulation with MC playbacks elicited some females to emit FR and to establish a duet as observed in trials with real males. The probability that a female would reply to playback stimulation was dependent on their age and reproductive status: neither females prior to 7 days from eclosion, nor mated, emitted FR in response to MC stimulation. By contrast, most females replied to the playback after 7 days from eclosion, without significant differences among age-class groups (G test: $G = 1.58$, $df = 2$, $P = 0.45$): 71%, 88%, and 78% for group 2 (7-10 days), 3 (11-13), and 4 (14-20), respectively.

Table 4 Principal components coordinates of the measured parameters (factor loadings), based on correlations.

Parameter	Component	
	First	Second
Df S1	-0.287	0.281
Df S2	-0.323	-0.277
Ff S2	-0.589	-0.198
Df MP	0.040	0.081
Df FR	-0.200	-0.039
Length S1	0.917	0.043
Length S2	0.363	0.726
N pulses	0.563	0.422
PRT	0.843	-0.373
int MC-MC	0.087	-0.794
Length MP	0.528	0.123
int MC-MP	0.148	-0.628
Length FR	0.252	-0.528
Latency FR	0.894	-0.162
Delay FR	-0.015	-0.830

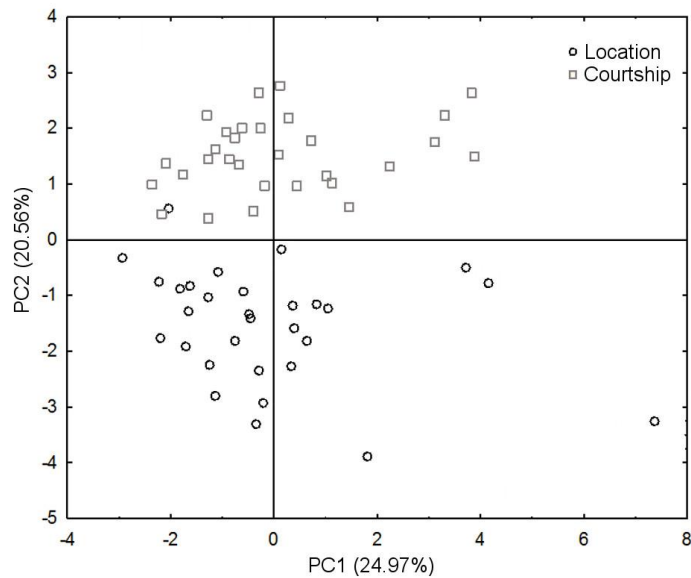


Figure 6 Score plot of the two main principal components (PC1 and PC2 respectively) obtained in the principal component analysis.

Discussion

Our study is the first complete description of the mating behavior and associated vibrational signals of the green leafhopper, *E. vitis*. We found that substrate-borne vibrations are essential to ensure mating in this species, because males search for a mating partner only after they have perceived a vibrational female response (FR) to their calls. In general, the reproductive strategy of *E. vitis* resembles the pattern known for other leafhopper species: males are more active than females, they are the only sex to emit spontaneous calling signals and then to search for the potential partner, which is stationary during pair formation (Saxena & Kumar, 1984; Claridge, 1985; Hunt & Nault, 1991; Cokl & Virant-Doberlet, 2003; Mazzoni et al., 2009b; de Groot et al., 2012). On the other hand, we found elements that characterize this species which are not common in other leafhoppers: (1) the use of mainly one type of male calling signal throughout the pair formation process, (2) the increase of the duet rhythm from Location to Courtship and (3) the constant calling activity during the 24 hours.

In closely related typhlocibinae species, different signals have been found in association with different behavioral contexts. For example, in *Amrasca devastans* (Distant) two signals were described, ‘croaking’ and ‘pattering’ that are emitted by the male just prior to copula (Saxena & Kumar, 1984). Shaw et al. (1974) reported two common sounds and one specific courtship sound involved in the pair formation process for seven *Empoasca* species. On the contrary, in *E. vitis* the MC is used for both calling and duetting. The use of one main signal in different behavioral contexts has been described in four typhlocybininae species of the genus *Alebra*, where the structure of the male call resembles *E. vitis* MC: two distinct sections, one made of a pulse sequence and the other with harmonic structure; although, in *Alebra* the section order is reversed, with the pulsed part at the end of the signal (Gillham, 1992). Indeed, we found a second *E. vitis* signal that we called Male Pulse (MP) and that could be considered a signal more strictly related to the male-female interaction than MC, since it was emitted frequently during duetting and more rarely from single males. The use of broadband single pulses is typical also of other leafhoppers, such as the courtship signals of deltocephalinae (Heady et al., 1986; Nuhardiyati & Bailey, 2005; Mazzoni et al., 2009b). In *S. titanus*, for example, the male initiated emission of specific courtship signals only after female localization was accomplished; whereas, different signals and behaviors were adopted during the previous location phase, while he was searching for the female’s leaf on a grapevine shoot with more leaves (Mazzoni et al., 2014; Polajnar et al., 2014). On the contrary, in our experiments the MP was produced as soon as the male engaged in a duet with a female. We do not know whether the relative proximity (e.g. same leaf) of the two partners facilitated mate identification and localization so that the courtship, meant as a behavior that aims at increasing the female acceptance, started as soon as the female replied.

In the case of *S. titanus* it has been hypothesized that the perception of the female pulse and, in particular, of its amplitude was the trigger to switch from Location to Courtship behavior. In fact, *S. titanus* males that were placed on the same female leaf immediately emitted courtship signals at the first duet (Mazzoni et al., 2009b). At the moment we cannot exclude that locating the pair in a more complex environment, such as a grapevine plant, could lead to more separated pair formation stages and also a delayed involvement of MP in the *E. vitis* duet.

Besides increasing knowledge of signal structure and features, only a few studies also investigated leafhopper duet structure (Derlink et al., 2014; Kuhelj et al., 2015a; Kuhelj et al., 2016). In general, the temporal parameters defining the duet are considered to be species-specific and remain more or less constant during the pair formation process (Bailey, 2003). However, there are some exceptions, such as in the leafhopper *Dalbulus* spp., where the male and female alternation rate increases when partners get close to each other (Heady et al., 1986). Similarly, in *E. vitis* the transition from Location to Courtship is characterized by temporal parameters changes which lead to a significant rhythm increase of the duet. In particular, the length increase of the MC second section and the shortening of the distances between MC-MC, MC-MP, and MC-FR were the most relevant variations. Eventually, this determines a peculiar trait within the leafhoppers' duet: in advanced stages of pair formation, the FR partially overlaps the MC. A female vibrational reply before the end of male signal has been observed in few species: in the planthopper *Javesella* spp. (De Vrijer, 1983), in the leafhoppers *Graminella nigrifrons* Forbes (Hunt et al., 1992) and *Aphrodes makarovi* Zachvatkin (de Groot et al., 2012), in the treehopper *Enchenopa binotata* Say (Rodríguez & Coccoft, 2006), in the psyllids *Schedotrioza* spp. and *Trioza* spp. (Percy et al., 2006), and in stoneflies (Stewart & Sandberg, 2005). In *A. makarovi*, where females always overlap the male call, it has been demonstrated that the overlapping reply ensures the female will respond in the narrow time window critical for species recognition (Kuhelj et al., 2015a). However, since in *E. vitis*, the interval between the end of the MC and the onset of the FR changes from positive to negative values during the pair formation process, the time window explanation must be modified for our model. An alternative hypothesis has been proposed for the bushcrickets *Caedicia* spp., in which case the tendency to begin replying before the conclusion of the male call may be related to high motivation of the females (Bailey & Hammond, 2004). The latter explanation seems more suitable to discuss our results. It has been demonstrated in leafhoppers that longer female replies provide better information for localization, thus ensuring shorter location time (de Groot et al., 2011; Kuhelj et al., 2016). Our results suggest that a higher number of FR perceived by the male while duetting with the female (e.g., female reply rate) facilitates mating success, at least in the 15 minutes of trials. One possibility is that at the beginning of the pair formation process, in Location, when the highest priority is to be localized, the delayed FR ensures

the male will be able to use the whole FR to acquire spatial information. However, during Courtship, when the male has already reached the female, the female shows her acceptance to mate to the male. This would mean that FR possibly encodes information on female availability to mate, as suggested by the female reply to playback. On the one hand, females younger than one week do not reply to MC because they are probably not reproductively mature, as has been indicated in other leafhoppers (Mazzoni et al., 2009b; Krugner, 2010); on the other hand, the lack of response by mated females to playback could be due to a temporary refractory period (Bailey & Nuhardiyati, 2005; Mazzoni et al., 2009b) with mating activity that could be restored later as seen for other leafhoppers (Derlink et al., 2016). However, we did not find significant differences in female reply between female groups over seven days from eclosion, which suggests that most of the females achieved reproductive maturity at the same time (after 7 days from eclosion) and age seems not to affect females' attitude to mate. In contrast with the female refractory period, males can start calling a few minutes after copulation, clearly indicating that they can mate multiple times and they do not have a refractory period.

Such a difference between genders may depend on the different reproductive costs that are asymmetrically distributed within genders. In leafhopper females investment is higher than males, and as a consequence they are choosier, while males spend more energy in searching and courting a potential partner (Trivers, 1972; Alexander et al., 1997). Vibrational signaling is energetically costly and the cost for the individual is related to the number of signals emitted (Kuhelj et al., 2015b). It is a common strategy to save energy and avoid possible predation in the first stages of the pair formation process, while expending all efforts in the last part (Polajnar et al., 2014). From this point of view, it is possible that in *E. vitis*, as in other leafhoppers (de Groot et al., 2012), females reply after several MC, the number of which is highly variable, to evaluate males from their call. Once the duet is established, the male avoids predation and loss of energy at the first stage (Location), but in an advanced pair formation stage (Courtship), he spends much more energy since the probability of mating, once the female has been located, is higher.

Males and females of *Dalbulus* spp. mate at any time of day or night (Heady et al., 1986) and in *Psammotettix alienus* (Dahlbom) calls have been recorded also at night (Derlink et al., 2016). However, in the other Auchenorrhyncha species, where activity throughout 24 hours has been studied extensively, matings are restricted to a narrow time window in which both males and females are active: during late afternoon/evening, in the case of *S. titanus* (Mazzoni et al., 2009b), or during the night, in the case of *M. pruinosa* Say (Virant-Doberlet & Žežlina, 2007). In *E. vitis*, males showed no differences in their calling activity and "call-fly" behavior throughout the day and this contrasts with the lower number of matings during the night. In Cicadellidae, vibrational signals are considered to be an exclusive communication channel and little is known about other sensory modes. Some studies have shown that

chemical and visual stimuli (Saxena & Kumar, 1984; Mazzoni et al., 2009b; Rossi Stacconi et al., 2014) can play a role in leafhoppers ecology and behavior. Colored sticky traps are commonly used to monitor their population dynamics (Cerutti et al., 1991; Lessio & Alma, 2004), but there is only one study on *A. devastans* that suggested a possible role of vision in mating behavior (Saxena & Kumar, 1984). As well as in *E. vitis*, the calling activity of *A. devastans* was independent from the presence of light but the number of matings was lower in the dark (Kumar & Saxena, 1986). Our results suggest that visual stimuli can actually be involved, at least at short distance in mating behavior, and can facilitate reproductive success. In fact, although many pairs established vibrational duets during the night (and the female reply rate was not different from the day time), the number of pairs able to mate within the given time was lower compared to daytime. It is likely that by giving additional time (the duration of our trials was 15 minutes), they would have ended up mating, nevertheless our observations indicate that mating is more difficult to accomplish in the dark. The question is why calling occurs at night. The longer time required to mate entails increased risks of predation (Virant-Doberlet et al., 2011) and energy consumption (Kuhelj et al., 2015b), therefore it would be safer to stop calling activity during the night when the chance to mate is reduced. A possible response to this question comes from the species ecology. It is known that ecological aspects of the environment, such as population density, can shape mating systems (Virant-Doberlet & Žežlina, 2007). *E. vitis*, as well as many other typhlocybinae, is polivoltine and can produce a large number of offspring (Alma, 2002). For this reason, it can cause relevant direct damage to crops because of high population density and related feeding activity (the action threshold is set at 2 nymphs per leaf, Fornasiero et al. 2015); on the contrary, other leafhopper subfamilies are often noxious only when they serve as vectors of phytopathogen agents. Polivoltinism and short life cycle make generations overlap in the field, especially in summer. Therefore, we should consider the occurrence of relatively high population densities on the vegetation, that together with a low availability of females (they must be at least seven days old and unlike males once mated they have a refractory period) implies higher competition for mating among males. This factor, which indeed must still be studied in detail, could make it convenient for partners to call even during the night to increase the chance of mating. If so, this strategy should be common in high density species in which, however, individuals do not live in groups. Unfortunately in the literature there is not much information on the relation between calling activity and population ecology of a species. For instance *M. pruinosa*, a planthopper that is active only during the night (Virant-Doberlet & Žežlina, 2007), lives in high density environments, but in aggregations. More appropriate is the similarity with the planthopper *Hyalesthes obsoletus* Signoret (Fulgoromorpha: Cixiidae), which is univoltine, but its ecology is more similar to *E. vitis*: adults lives in relatively high populations but without forming dense aggregations (Bressan et al., 2007) and it is active all day (Mazzoni et al.,

2010). Of course, more studies on species with similar ecology, in general, and on other tephroclybinae, in particular, are needed to assess this hypothesis.

To conclude, since the whole pair formation process of *E. vitis* is mediated by vibrational communication, while an involvement of other sensory cues to accomplish mating seems limited at the short range distance, we consider the application of mechanical mating disruption feasible. In particular, we think that a method aimed at masking vibrational signals of the *E. vitis* duet should be effective in preventing or interrupting mating duets between *E. vitis* males and females as well as in *S. titanus* (Polajnar et al., 2015; Polajnar et al., 2016a, b). Future research must be focused on the selection of the most suitable disruptive signals and on laboratory and field mating disruption experiments.

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Disclosure

The authors declare that there are no conflicts of interest, including specific financial interests and relationships and affiliations (other than those affiliations listed in the title page of the manuscript) relevant to the subject of this manuscript.

References

- Alexander, R. D., Marshall, D. C., & Cooley, J. R.** 1997. Evolutionary perspectives on insect mating. In *The evolution of mating systems in insects and arachnids* (Ed. by J. C. Choe & B. J. Crespi), pp. 4–31. Cambridge University Press, Cambridge.
- Alma, A.** 2002. Auchenorrhyncha as pests on grapevine. *Denisia* **176**(176), 531–538.
- Bailey, W. J.** 2003. Insect duets: underlying mechanisms and their evolution. *Physiological Entomology* **28**(3), 157–174.
- Bailey, W. J., & Hammond, T.** 2004. Female reply strategies in a duetting Australian bushcricket, *Caedicia* sp. (Phaneropterinae: Tettigoniidae: Orthoptera). *The Journal of Experimental Biology* **207**(5), 803–811.
- Bailey, W. J., & Nuhardiyati, M.** 2005. Copulation, the dynamics of sperm transfer and female refractoriness in the leafhopper *Balclutha incisa* (Hemiptera: Cicadellidae: Deltocephalinae). *Physiological Entomology* **30**(4), 343–352.

- Böll, S., & Herrmann, J. V.** 2004. A long-term study on the population dynamics of the grape leafhopper (*Empoasca vitis*) and antagonistic mymarid species. *Journal of Pest Science* **77**(1), 33–42.
- Bressan, A., Turata, R., Maixner, M., Spiazzi, S., Boudon-Padieu, E., & Girolami, V.** 2007. Vector activity of *Hyalesthes obsoletus* living on nettles and transmitting a stolbur phytoplasma to grapevines: a case study. *Annals of Applied Biology* **150**(3), 331–339.
- Cerutti, F., Baumgärtner, J., & Delucchi, V.** 1991. The dynamics of grape leafhopper *Empoasca vitis* Göthe populations in southern Switzerland and the implications for habitat management. *Biocontrol Science and Technology* **1**(3), 177–194.
- Claridge, M.** 1985. Acoustic Signals in the Homoptera: behavior, taxonomy, and evolution. *Annual Review of Entomology* **30**(1), 297–317.
- Cocroft, R. B., & Rodriguez, R. L.** 2005. The behavioral ecology of insect vibrational communication. *BioScience* **55**(4), 323–334.
- Čokl, A., & Virant-Doberlet, M.** 2003. Communication with substrate-borne signals in small plant-dwelling insects. *Annual Review of Entomology* **48**, 29–50.
- de Groot, M., Čokl, A., & Virant-Doberlet, M.** 2011. Search behaviour of two hemipteran species using vibrational communication. *Central European Journal of Biology* **6**(5), 756–769.
- de Groot, M., Derlink, M., Pavlovčič, P., Prešern, J., Čokl, A., & Virant-Doberlet, M.** 2012. Duetting behaviour in the leafhopper *Aphrodes makarovi* (Homoptera: Cicadellidae). *Journal of Insect Behavior* **25**(5), 419–440.
- De Vrijer, P. W. F.** 1983. Variability in calling signals of the planthopper *Javesella pellucida* (F.) (Homoptera: Delphacidae) in relation to temperature, and consequences for species recognition during distant communication. *Netherlands Journal of Zoology* **34**(3), 388–406.
- Decante, D., & Van Helden, M.** 2006. Population ecology of *Empoasca vitis* (Göthe) and *Scaphoideus titanus* (Ball) in Bordeaux vineyards: influence of migration and landscape. *Crop Protection* **25**(7), 696–704.
- Derlink, M., Abt, I., Mabon, R., Julian, C., & Virant-Doberlet, M.** 2016. Mating behaviour of *Psammotettix alienus* (Homoptera: Cicadellidae). *Insect Science* **00**, 1–13.
- Derlink, M., Pavlovčič, P., Stewart, A.J.A., & Virant-Doberlet, M.** 2014. Mate recognition in duetting species: the role of male and female vibrational signals. *Animal behaviour* **90**, 181–193.
- Eriksson, A., Anfora, G., Lucchi, A., Lanzo, F., Virant-Doberlet, M., & Mazzoni, V.** 2012. Exploitation of insect vibrational signals reveals a new method of pest management. *PloS One* **7**(3), e32954.
- Fornasiero, D., Pavan, F., Pozzebon, A., Picotti, P., & Duso, C.** 2015. Plant resistance relative infestation level and sensitivity of grapevine cultivars

- to the leafhopper *Empoasca vitis* (Hemiptera: Cicadellidae). *Journal of Economic Entomology* **109**, 1–10.
- Foster, S. P., & Harris, M. O.** 1997. Behavioral manipulation methods for insect pest-management. *Annual Review of Entomology* **42**(1), 123–146.
- Gillham, M. C.** 1992. Variation in acoustic signals within and among leafhopper species of the genus *Alebra* (Homoptera, Cicadellidae). *Biological Journal of the Linnean Society* **45**, 1–15.
- Hazarika, L. K., Bhuyan, M., & Hazarika, B. N.** 2009. Insect pests of tea and their management. *Annual Review of Entomology* **54**, 267–284.
- Heady, S. E., Nault, L. R., Shambaugh, G. F., & Fairchild, L.** 1986. Acoustic and mating behavior of *Dalbulus* leafhoppers (Homoptera: Cicadellidae). *Annals of the Entomological Society of America* **79**, 727–736.
- Hunt, R. E., Fox, J. P., & Haynes, K. F.** 1992. Behavioral response of *Graminella nigrifrons* (Homoptera: Cicadellidae) to experimentally manipulated vibrational signals. *Journal of Insect Behavior* **5**(1), 1–13.
- Hunt, R. E., & Nault, L. R.** 1991. Roles of interplant movement, acoustic communication, and phototaxis in mate-location behavior of the leafhopper *Graminella nigrifrons*. *Behavioral Ecology and Sociobiology* **28**, 315–320.
- Krugner, R.** 2010. Differential reproductive maturity between geographically separated populations of *Homalodisca vitripennis* (Germar) in California. *Crop Protection* **29**(12), 1521–1528.
- Kuhelj, A., de Groot, M., Blejec, A., & Virant-Doberlet, M.** 2015a. The effect of timing of female vibrational reply on male signalling and searching behaviour in the leafhopper *Aphrodes makarovi*. *PLoS ONE* **10**(10), 1–15.
- Kuhelj, A., de Groot, M., Blejec, A., & Virant-Doberlet, M.** 2016. Sender-receiver dynamics in leafhopper vibrational duetting. *Animal behavior* **114**, 139–146.
- Kuhelj, A., de Groot, M., Pajk, F., Simčič, T., & Virant-Doberlet, M.** 2015b. Energetic cost of vibrational signalling in a leafhopper. *Behavioral Ecology and Sociobiology* **69**(5), 815–828.
- Kumar, H., & Saxena, K. N.** 1986. Certain environmental factors influencing the acoustic communication in the sexual behaviour of the leafhopper *Amrasca devastans* (Distant) (Homoptera: Cicadellidae). *Applied Entomology and Zoology* **21**(1), 55–62.
- Lessio, F., & Alma, A.** 2004. Dispersal patterns and chromatic response of *Scaphoideus titanus* Ball (Homoptera: Cicadellidae), vector of the phytoplasma agent of grapevine flavescence dorée. *Agricultural and Forest Entomology* **6**(2), 121–127.
- Liu, S., Li, Z., Sui, Y., Schaefer, D. A., Alele, P. O., Chen, J., & Yang, X.** 2015. Spider foraging strategies dominate pest suppression in organic tea plantations. *BioControl* **60**(6), 839–847.

- Mazzoni, V., Cosci, F., Lucchi, A., & Santini, L.** 2001. Occurance of leafhopper (Auchenorrhyncha, Cicadellidae) in three vineyards of the Pisa district. *Integrated Control in Viticulture IOBC wprs Bulletin* **24**, 267–271.
- Mazzoni, V., Eriksson, A., Anfora, G., Lucchi, A., & Virant-Doberlet, M.** 2014. Active space and the role of amplitude in plant-borne vibrational communication. In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp. 349–374. Springer, New York.
- Mazzoni, V., Lucchi, A., Cokl, A., Presern, J., & Virant-Doberlet, M.** 2009a. Disruption of the reproductive behaviour of *Scaphoideus titanus* by playback of vibrational signals. *Entomologia Experimentalis et Applicata* **133**(2), 174–185.
- Mazzoni, V., Lucchi, A., Ioriatti, C., Virant-Doberlet, M., & Anfora, G.** 2010. Mating behavior of *Hyalesthes obsoletus* (Hemiptera: Cixiidae). *Annals of the Entomological Society of America* **103**(5), 813–822.
- Mazzoni, V., Presern, J., Lucchi, A., & Virant-Doberlet, M.** 2009b. Reproductive strategy of the nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). *Bulletin of Entomological Research* **99**(4), 401–13.
- Nuhardiyati, M., & Bailey, W.** 2005. Calling and duetting behavior in the leafhopper *Balclutha incisa* (Hemiptera: Cicadellidae: Deltocephalinae): opportunity for female choice? *Journal of Insect Behavior* **18**(2), 259–280.
- Pavan, F., & Picotti, P.** 2009. Influence of grapevine cultivars on the leafhopper *Empoasca vitis* and its egg parasitoids. *BioControl* **54**(1), 55–63.
- Percy, D. M., Taylor, G. S., & Kennedy, M.** 2006. Psyllid communication: acoustic diversity, mate recognition and phylogenetic signal. *Invertebrate Systematics* **20**(4), 431–445.
- Polajnar, J., Eriksson, A., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2015. Manipulating behaviour with substrate-borne vibrations – potential for insect pest control. *Pest Management Science* **71**(1), 15-23.
- Polajnar, J., Eriksson, A., Rossi Stacconi, M. V., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2014. The process of pair formation mediated by substrate-borne vibrations in a small insect. *Behavioural Processes* **107**, 68–78.
- Polajnar, J., Eriksson, A., Virant-doberlet, M., Lucchi, A., & Mazzoni, V.** 2016a. Developing a bioacoustic method for mating disruption of a leafhopper pest in grapevine. In *Advances in Insect Control and Resistance Management* (Ed. by A. R. Horowitz & I. Ishaaya), pp. 165–190. Springer, New York.

- Polajnar, J., Eriksson, A., Virant-doberlet, M., Lucchi, A., & Mazzoni, V.** 2016b. Mating disruption of a grapevine pest using mechanical vibrations: from laboratory to the field. *Journal of Pest Science* **89**(4), 1-13.
- Rodríguez, R. L., & Cocroft, R. B.** 2006. Divergence in female duetting signals in the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Ethology* **112**(12), 1231–1238.
- Rossi Stacconi, M. V., Hansson, B. S., Rybak, J., & Romani, R.** 2014. Comparative neuroanatomy of the antennal lobes of 2 homopteran species. *Chemical Senses* **39**(4), 283–294.
- Ryan, T. H.** 1960. Significance tests for multiple comparison of proportions, variances, and other statistics. *Psychological Bulletin* **57**(4), 318–328.
- Saxena, K. N., & Kumar, H.** 1984. Acoustic communication in the sexual behaviour of the leafhopper, *Amrasca devastans*. *Physiological Entomology* **9**, 77–86.
- Shaw, K. C., Vargo, A., & Carlson, O. V.** 1974. Sounds and associated behavior of some species of *Empoasca* (Homoptera: Cicadellidae). *Journal of the Kansas Entomological Society*, 284–307.
- Siegel, S., & Castellan, N.** 1988. *Non parametric statistics for the behavioral sciences* (2nd editio). McGraw-Hil, New York.
- Stewart, K. W., & Sandberg, J. B.** 2005. Vibratory communication and mate searching behaviour in stoneflies. In *Insect sounds and communication: physiology, behaviour, ecology, and evolution* (Ed. by S. Drosopoulos & M. F. Claridge), pp. 179–186. CRC Press, Boca Raton.
- Trivers, R. L. L.** 1972. Parental investment and sexual selection. In *Sexual Selection and the Descent of Man* (Ed. by B. Campbell), pp. 136-179. Aldine, Chicago.
- Virant-Doberlet, M., King, R. A., Polajnar, J., & Symondson, W.O.C.** 2011. Molecular diagnostics reveal spiders that exploit prey vibrational signals used in sexual communication. *Molecular Ecology* **20**(10), 2204–2216.
- Virant-Doberlet, M., & Žežlina, I.** 2007. Vibrational communication of *Metcalfa pruinosa* (Hemiptera: Fulgoroidea: Flatidae). *Annals of the Entomological Society of America* **100**(1), 73–82.

Chapter 3

Vibrational mating disruption in *Empoasca vitis*: natural and artificial strategies.

3. Vibrational mating disruption in *Empoasca vitis*: natural and artificial strategies.

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Abstract

The green leafhopper, *Empoasca vitis* Göthe, is a polyphagous pest of grapevine and tea plantations. To date population density is controlled mainly by insecticidal applications; therefore, the development of a healthier method, such as vibrational mating disruption, would be beneficial for the environment and humans. In this study, we assessed two main topics: if a ‘disruptive signal’ involved in rival interaction aimed at preventing mating naturally occurs in this species and if it or other vibrational signals can be used to artificially disrupt mating by playing them back to a leaf. With behavioral trials of two males and two males and one female, we described male-male rival interactions and recorded a species-specific disruptive signal, which consists in a single pulse which overlaps the competitor Male Call. The *E. vitis* disruptive signal interferes with the locating ability of the rival male, thus giving the mating opportunity to the disrupting male. Laboratory playback disruption trials revealed that the pair formation process was affected by artificial disturbance noises that were based on the following features: the *E. vitis* disruptive signal, *Scaphoideus titanus* disturbance noise, and a pure tone (250 Hz). Among these, the most efficient noise to prevent mating was the pure tone. To simultaneously disrupt the mating of *E. vitis* and *S. titanus*, the possibility to use a playback made of the *S. titanus* disturbance noise combined together with the pure tone is discussed.

Key words: biotremology, vibrational communication, leafhopper, rivalry, disruptive signals.

Introduction

The basic concept of mating disruption was ideated 40 years ago with the use of sex attractant pheromones to interfere with insect communication and reduce pest population. Ever since both basic research and chemical industry worked, often together, at developing this method that, however, is still far from its full application potential (Miller and Gut 2015). The idea of developing a vibrational mating disruption method is much more recent, but nevertheless due to the numerous pests using substrate-borne vibrations to communicate, the interest in its applicability is growing fast (Hofstetter et al. 2014; Polajnar et al. 2015; Lujó et al. 2016; Polajnar et al. 2016a; Polajnar et al. 2016b). Currently, two possible approaches to the vibrational mating disruption have been explored. The first is the use of a natural disturbance noise emitted by rival males to mask and interrupt the pair formation process. This was tested and applied to the grapevine leafhopper, *Scaphoideus titanus*, (Mazzoni et al. 2009b) by looping the disturbance signal as playback transmitted into host plants to disrupt mating (Mazzoni et al. 2009a; Eriksson et al. 2012). A second and more recent approach consists of mimicking the female reply signal to interfere with their localization by males. This was tested on the psyllid, *Diaphorina citri* (Lujó et al. 2016) and it is based on a system that must detect male calls and respond with reliable synthetic replies (Mankin et al. 2013).

Another pest species that could be a target of vibrational mating disruption is the green leafhopper, *Empoasca vitis* Göthe (Hemiptera: Cicadellidae: Typhlocybinae). This is an important pest in Europe, where damages are reported on grapevine in north Italy, Switzerland, Germany, and France (Cerutti et al. 1991; Mazzoni et al. 2001; Alma 2002; Böll and Herrmann 2004), and on tea plantations in Asia (Hazarika et al. 2009). The phloem feeding activity of immature and adult individuals directly causes stress symptoms in plants. Evident symptoms of low density populations are leaf veins browning and chlorosis of margins, while at higher densities leaf burn and phylloptosis occur (Alma 2002; Böll and Herrmann 2004). Despite the fact that several IPM strategies have been tested to control *E. vitis* population density, such as the use of non-susceptible cultivars, and techniques of landscape management to favor the occurrence of predators and parasitoids (Decante & van Helden, 2006; Pavan & Picotti, 2009; Liu et al., 2015; Fornasiero et al., 2016) to date, insecticides are still commonly used. Developing a vibrational mating disruption method to control *E. vitis* population level would be a beneficial alternative and will help to reduce chemical treatments.

As in other leafhoppers, the pair formation process in *E. vitis* is mediated by substrate-borne vibrational signals (Nieri and Mazzoni, under review, see Chapter 2). The male alternates the emission of Male Calls (MC) and jumping (i.e. ‘call-fly strategy’, Hunt and Nault 1991) waiting for the reply of a receptive female. When a female replies to the male, a duet is established and ensures the location of the female by the male and the mating success (Nieri

and Mazzoni, under review). During the duet, male and female alternate MC and female reply (FR) and at this stage a second male signal, the Male Pulse (MP), is produced by the male between the FR and the following MC. Even if vibrations emitted by *E. vitis* consist of broadband and harmonic signals, in both MC and FR, most of the energy is concentrated around 250 Hz. Starting from this knowledge, we investigated the *E. vitis* male rivalry behavior and the occurrence of related vibrational signals able to interfere with the pair formation process. Secondly, we evaluated the feasibility of a vibrational mating disruption approach using artificial playbacks.

Materials and methods

Insect rearing

All tested *E. vitis* individuals were reared in a greenhouse of the Fondazione E. Mach (S. Michele all'Adige, Italy), at 25 ± 2 °C, $80 \pm 5\%$ relative humidity and 16:8 (L:D) photoperiod, with the scotophase starting at 5:00 AM local time. Virgin adults of *E. vitis* were obtained by collecting late instar nymphs from massive rearing net cages (Bugdorm-6620, 60x60x120 cm), provided with grapevine plants, and moved to rearing boxes that consisted of plastic beakers (height 10 cm; 5 cm i.d.) with a moistened grapevine leaf laid on top of a layer (1 cm) of technical agar solution (0.8%) that was replaced every 3 days. The rearing boxes were checked daily and new adults were separated by sex and date of emergence. Individuals were tested at least 7 or 10 days after molt, respectively for males and females.

Recording vibrational signals and behavior

All recordings were made in an enclosed room of the Fondazione E. Mach (S. Michele all'Adige, Italy), on an anti-vibration table (Astel s.a.s., Ivrea, Italy) at a temperature of 22 ± 1 °C. To detect the vibrational signals from the leaf lamina, one or two laser vibrometers (Ometron VQ-500-D-V, Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark; PDV 100, Polytec, Germany), were used according to the test. To maximize the signal-to-noise ratio, the laser beam was focused on a reflecting sticker. The signals were digitized with a 48 kHz sample rate, 16-bit depth, and stored directly onto a hard drive through a multichannel LAN-XI data acquisition hardware (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark).

Trial 1 Male rivalry

To identify potential rivalry signals, virgin males were tested in two contexts: pair of two males ($n = 18$) and trio, i.e. two males and one female ($n =$

30). The stem of a single fresh cut grapevine leaf was inserted into a plastic tube (2 mL). To enable the observer to follow the insects movement on both sides of the leaf, the tube was inserted into a circular arena (diameter: 9 cm, height: 3 cm) through a hole on the lower half of one arena's face (Fig. 1). The leaf (surface about: 8x8 cm; petiole about: 2 cm) did not touch the arena walls. Two holes (diameter: 8 mm) on two opposite sides of the arena were used to insert *E. vitis* individuals. Trials started when all the insects inside the arena settled on the leaf surface and ended after 15 min, even if one male eventually mated with the female earlier. To identify which male was producing substrate-borne vibrations during the recording time, two lasers were used simultaneously. Preliminary to the trials, the leaf surface was regularly covered with reflective stickers separated from each other by 5-6 mm, so that during the trials each laser beam was focused on the sticker closest (maximum 2-3 mm distant) to each male. In this way the amplitude recorded by means of each laser was higher for the closest male and we were able to distinguish which male was producing vibrations at each time.

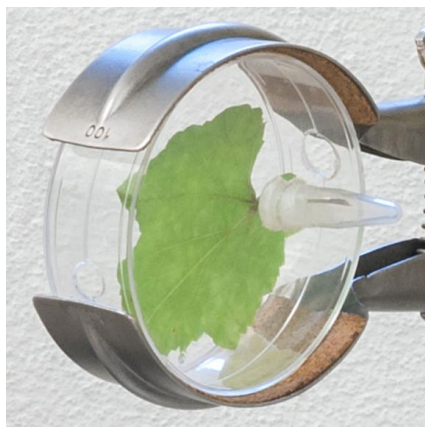


Figure 1 – Circular arena used in trial 1.

Trial 2 Mating disruption

Laboratory mating disruption trials were conducted to evaluate the efficacy of three candidate disturbance noises (DN) ($n = 20$) to disrupt *E. vitis* mating: an intraspecific signal (DN_{intra}), an interspecific signal (DN_{inter}), and a continuous pure frequency tone of 250 Hz (DN_{pure}). DN_{intra} consisted of a sequence of the disruptive signal recorded in trial 1 (see Results) with repetition time 0.4 s to ensure it would have overlapped any potential MC, since the duration of MC on average is 0.5 s (Nieri and Mazzoni, under review). DN_{inter} was the disturbance noise successfully used to disrupt *S. titanus* (Mazzoni et al. 2009a; Eriksson et al. 2012; Polajnar et al. 2014). The DN_{pure} frequency was chosen to match the dominant frequency of *E. vitis* male and female signals. As negative control, trials were conducted in absence of a disturbance playback (n

= 24) and as positive control we used a playback composed of white noise with a flat spectrum over 0 to 1000 Hz (n = 20). The white noise and the pure tone playbacks were synthesized using Adobe® Audition™ version 3.0 (© 1992-2003, Adobe Systems Incorporated). Pairs, one male and one female, were placed on a single fresh cut grapevine leaf inserted into a vial with water. An electrodynamic mini-shaker (Type 4810, Brüel & Kjær, Inc., Norcross, GA), driven by an mp3 reader (Irradio Style MP3/MP4 player, Melchioni S.p.A., Milano, Italy), was placed in contact with the lower lamina of the leaf through the conical tip of a 6-cm metal rod by using a small amount of blue wax (Surgident Periphery Wax, Australia). One laser vibrometer, focused on the leaf lamina, was used to record and monitor the vibrational emissions of insects and the amplitude of playback stimulations, which was adjusted to the level of the highest recorded natural leafhopper male signals as registered at the point of recording. To prevent insects from escaping, both the leaf and the mini-shaker were placed into a plastic cube (20x20x20 cm) with a hole on the top for the laser beam. Individuals were allowed to acclimatize on the leaf for 3 min, then females were stimulated with male calls following the protocol for female stimulation of Nieri and Mazzoni (under review). The FR to the playback stimulated the male to call and establish a duet with the female. The playback, either DN or white noise, was turned on after a male-female vibrational duet was established and the male started searching; pairs were exposed to the DN playback for 15 minutes.

To assess whether individuals were able to promptly restore the mating duet after the playback suspension, pairs were monitored for additional 3 minutes after the DN ended.

Parameters and data analysis

Known *E. vitis* vibrational signals were named according to Nieri and Mazzoni (under review). Newly described signals were named according to their behavioral context. A ‘pulse’ was defined as a physically unitary or homogeneous sound, composed of a brief succession of sine waves (Alexander 1967) and a ‘disruptive calling’ was defined as male signaling over a duet (Bailey et al. 2006). Spectral analysis was performed with Raven Pro 1.4 (The Cornell Lab of Ornithology, Ithaca, NY) using Fast Fourier Transform (FFT) type Blackman, window length of 512 samples and 75% of overlap. From each trials in which rivalry occurred, three Male Pulse (MP), Female Reply (FR), and disruptive signal (see Results) (total n=45 for each signal type) were selected and the following parameters were measured: duration, dominant frequency (df), and latency from the onset of the preceding Male Call (MC) and the second section of MC (S2).

The following behavioral parameters were measured when applicable: the male calling probability, as the number of individuals who emitted at least

one MC during the trial; the female activity as the number of females that replied at least once to a MC on the total of calling males; the latency (in seconds) to the first emitted MC, to the female location by the males, and mating; the number of males that located the female; the number of mating attempts; the number of pairs that successfully mated; the number of male-male pairs that exhibited a rival behavior; the number of MC prior the first FR. We measured the proportion of MC that were overlapped by a disruptive signal, and the proportion of overlapped and non-overlapped signals that elicited a female response. We also took note of which male first called, searched, located and mated with the female. The presence and parameters of signals that occurred during playback stimulation were not measured for the positive control, because the frequency structure of the white noise disabled us to clearly see *E. vitis* signals.

Statistical analysis

All statistics were conducted using KyPlot version 2.0 beta 15 (1997-2001 Koichi Yoshioka). To describe the disruptive signal, we compared temporal and spectral parameters of male signals (male pulse, MP and disruptive pulse, DP; see Results) performing a Mann-Whitney U-test. DP, MP, and FR latencies from the onset of the preceding MC were compared using a Kruskal-Wallis test followed by a Steel-Dwass multiple comparison test. The proportion of female reply to MC and disrupted MC was compared using a Wilcoxon signed rank test for paired data. To estimate the efficacy of DN playback in disrupting mating we performed a G-test in contingency table followed by a Ryan multiple comparison (Ryan, 1960) on the number of mating success, male and female activity, and successfully located females.

Table 1 Behavior of *E. vitis* males in pair (two males) and trio (two males and one female) trials.

Number of active males	Behavior	Pair	Trio
0		5	8
1	Calling	5	1
	Duet	-	4 (2)
	Rivalry	0	0
2	Calling	8	2
	Duet	-	0
	Rivalry	0	15 (14)
Tot		18	30

Active males indicate the number of males that emitted at least one MC during the trial. The number of pairs that successfully mated is reported in brackets.

Results

Male rivalry trial 1

When two males were on the leaf without a female, in 13 out of 18 (72%) trials at least one male was active and emitted male calls (MC) and 44% (8 out of 18) of times both males emitted MC (Table 1). In the latter case, a male alternated calling and walking on the leaf but without any apparent interaction with the other one, both physical and vibrational. In trials with two males and one female, when both males called (57%, 17 out of 30), the female replied in 15 trials out of 17 and most of the time to both males (14 out of 15, 93%). Considering trials in which one or both males called (19 out of 30), in 15 of them (79%) a male-male rival interaction was recorded in terms of emission of a specific signal, the Disruptive Pulse (DP). The latency of rivalry behavior from the male-female duet establishment was 91.84 ± 152.37 s (mean \pm SD). In 53% of trials in which rivalry occurred (8 out of 15), rivalry began after one of the two males started walking to search for the female. A rivalry behavior was established always after at least one male-female duet (i.e., one MC followed by one FR); however, most of the time (60%, 9/15) the first DP emitted was in conjunction with a MC followed by FR. After the establishment of a rivalry behavior, the following DP were produced by rival males both in conjunction with MC either in presence (100%, 15/15) and in absence (80%, 12/15) of a female reply. The total number of MC overlapped by DP was variable between trials, from a 3% minimum to 45% maximum (21 ± 14 %). The proportion of FR elicited by non-disrupted MC was higher than by disrupted MC (Wilcoxon signed rank test, $n=15$, $T=101$, $P<0.01$) (Fig. 2).

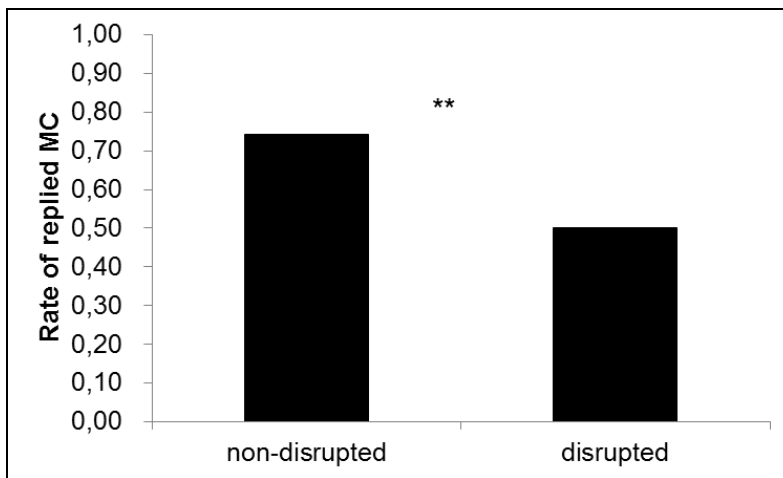


Figure 2 Proportion of FR elicited by disrupted and non-disrupted MC. ** $P<0.01$ after Wilcoxon signed rank test.

Even if rivalry occurred, most of the time a male eventually mated with the female (93%, 14/15). The male that was observed mating with the female was the first male that started calling, or that established a duet, or reached the female. The rival male called again a few seconds after mating occurred (mean \pm SD: 2.65 ± 2.22 ; $n=14$). In two trials, in which both males were close to the female when she mated with one of them, after the copula began the rival male attempted to mate with the mating couple, rapidly spinning around to join its genitalia with those of the females. In both cases the couple did not stop mating and the rival male walked away after one and two attempts respectively.

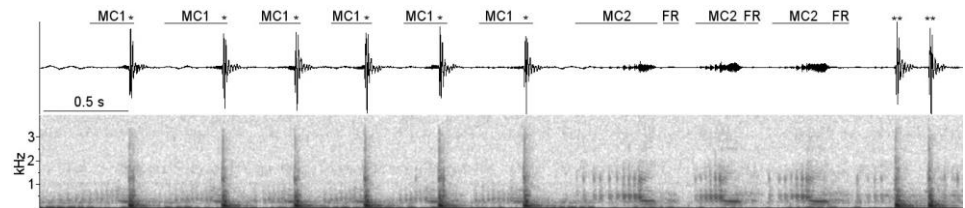


Figure 3 Oscillogram (above) and spectrogram (below) of MC overlapped by DP and no-overlapped MC. MC are produced by two different males, MC1 and MC2 respectively. * indicate DP position, ** indicate MP. In this particular case, FR followed exclusively no-overlapped MC (MC2).

DP was a short (0.03 ± 0.01 s) and low frequency (125.98 ± 61.89 Hz) single pulse (Fig. 3). The production of DP was associated with a vertical movement of the body that resembles the movement of *E. vitis* emitting MP: the forepart of the body (e. g. head and thorax) was quickly swayed up and down. We did not measure any significant difference between DP and MP neither with *df* (Mann-Whitney U-test: $n=45$, $U=821$, $P=0.12$) nor duration ($n=45$, $U=897.5$, $P=0.35$). The DP latency from the onset of MC was lower than MP and FR latency respectively (Kruskal-Wallis test, $\chi^2=48.08$, $Df=2$, $P<0.001$) (Fig. 4). Usually, DP overlapped the second section (S2) of MC (latency from the beginning of S2; mean \pm SD: 0.07 ± 0.03 s).

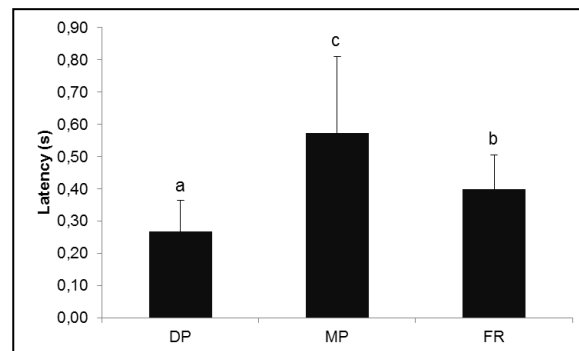


Figure 4 Mean and SD of DP, MP, and FR latencies from the onset of the MC. Different letters indicate significant difference after Kruskal-Wallis test followed by Steel-Dwass multiple comparison test.

Table 2 DN effect on the pair formation process of *E. vitis*

	Cont -		Cont +		DNinter		DNpure		DNintra	
	N	mean \pm SD	N	mean \pm SD	N	mean \pm SD	N	mean \pm SD	N	mean \pm SD
MC latency (S)			19	129.67 \pm 166.27	18	270.08 \pm 243.21	20	200.46 \pm 186.59		
MC prior FR			17	9.24 \pm 11.15	a 15	3.87 \pm 3.16	ab 17	3.53 \pm 3.74	b	
Location latency (S)	21	286.44 \pm 208.05 a (3)	(3)	(449.95 \pm 350.23)	7	512.14 \pm 265.34 b (1)	(799.29)		11	505.79 \pm 274.97 ab
Mating latency (S)	21	423.57 \pm 266.09 a (2)	(2)	(592.39 \pm 380.42)	5	434.34 \pm 168.07 a			8	884.27 \pm 330.76 b
Mating attempt	21	3.90 \pm 4.01 (2)	(2)	(0.50 \pm 0.71)	7	2.86 \pm 1.95			9	3.33 \pm 2.65

Different letters in the same row indicate significant difference after Kruskal-Wallis test followed by Steel-Dwass multiple comparison test. The value in brackets were not used for statistical analysis.

Table 3 Delayed effect of DN on the behavior of *E. vitis*

	Control		DNinter		DNpure		DNintra	
	N	mean \pm SD	N	mean \pm SD	N	mean \pm SD	N	mean \pm SD
MC latency (S)	18	4.83 \pm 7.50	11	15.44 \pm 41.68	15	9.55 \pm 23.31	19	3.16 \pm 2.93
MC prior FR	17	2.12 \pm 2.18	10	1.50 \pm 0.71	15	3.60 \pm 6.21	18	1.66 \pm 1.78
Location latency (S)	11	121.24 \pm 32.53 (2)	(2)	(120.95 \pm 67.75)	7	96.45 \pm 58.72	10	87.26 \pm 58.26
Mating latency (S)	6	131.34 \pm 25.12 (1)	(1)	80.01	(3)	96.34 \pm 51.93 (4)	(4)	110.83 \pm 61.23
Mating attempt	8	1.63 \pm 1.19 (3)	(3)	(1.33 \pm 1.53)	5	2.4 \pm 2.07	6	1.67 \pm 1.86

The value in brackets were not used for statistical analysis.

Mating disruption trial 2

All tested DN affected the pair formation process of *E. vitis* during the vibrational stimulation and immediately after it.

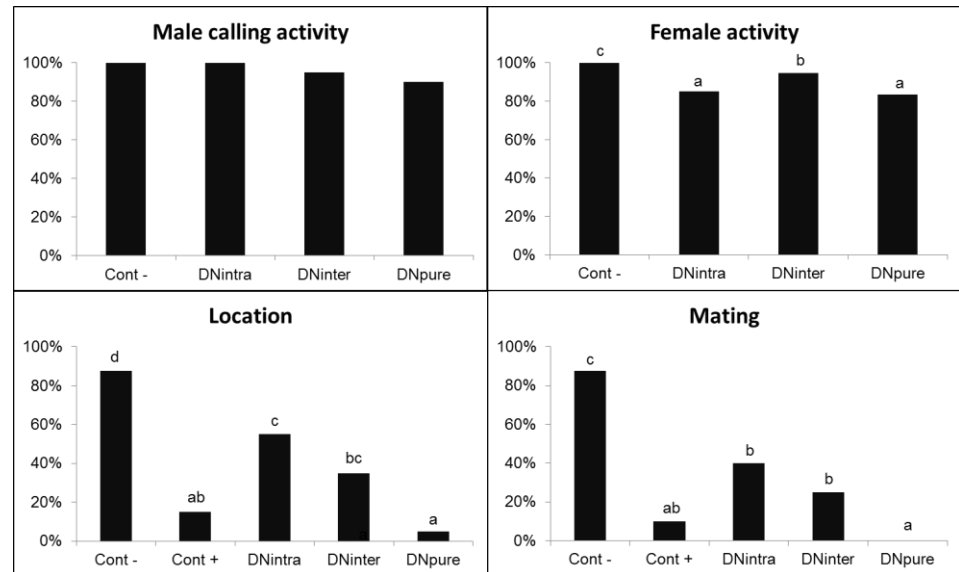


Figure 5 Effect of artificial playbacks on different stages of *E. vitis* pair formation process. (a) male calling activity, (b) duet activity, (c) female location, and (d) accomplished mating. Different letters above columns indicate significant difference after G-test followed by Ryan multiple comparisons.

DN effect on the pair formation process

Both males and females continued emitting vibrational signals during 15 minutes of artificial playback stimulation (Fig. 5). Even if there was a small number of males (during DNinter and DNpure) that ceased to signal, the reduction was not significant (G-test, $G=2.83$, $P=0.42$). The rate of females that replied to MC was significantly lower in all treatments, but in particular during DNintra and DNpure (G-test, $G=31.01$, $Df=3$, $P<0.001$). Comparing the three DN, there was no significant difference in MC latency (Kruskall-Wallis test, $Df=2$, $\chi^2=4.66$, $P=0.10$). However, females replied after a higher number of MC in the presence of the DNinter (Kruskall-Wallis test, $Df=2$, $\chi^2=6.35$, $P=0.04$) (Table 2). The number of males that successfully located the female were significantly lower when a DN was playing; the most efficient playback in reducing location was DNpure, while during DNintra more than half of the males located the female (G-test, $G=43.43$, $P<0.001$). Comparing trials in which the male located the female, during DNinter the location latency was

significantly longer (Kruskal-Wallis test, $Df=2$, $\chi^2=7.70$, $P=0.02$) (Table 2). Also the number of pairs that successfully accomplished mating was significantly reduced by all DN. In the presence of DNpure none of the pairs mated, while there was a significant reduction with the other treatments (G-test, $G=52.16$, $P<0.001$) (Fig. 5). Considering pairs that successfully mated, the mating latency was particularly high in presence of DNintra, but not significantly different in the other treatments compared to the control (Kruskal-Wallis test, $Df=2$, $\chi^2=9.39$, $P<0.01$). The number of mating attempts was not different comparing DNinter, DNintra, and negative control (Kruskal-Wallis test, $Df=2$, $\chi^2=0.04$, $P=0.98$).

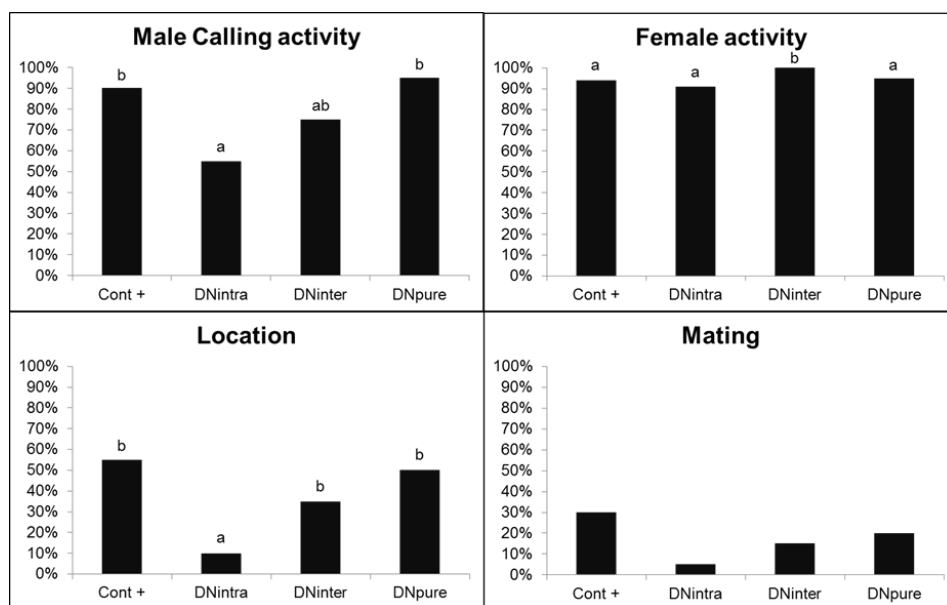


Figure 6 Delayed effect of artificial playback on *E. vitis* pair formation stages. (a) male calling activity, (b) duet activity, (c) female location, and (d) accomplished mating. Different letters above columns indicate significant difference after G-test followed by Ryan multiple comparisons.

Delayed effect of the DN

When the DN ended, usually the male started calling again, but the number of males that called was significantly lower after DNintra (G-test, $G=11.44$, $P<0.01$) (Fig. 6; Table 3). The number of females that replied to the male call, and therefore of the duet that were re-established, was significantly higher only after DNinter (G-test, $G=13.13$, $Df=3$, $P<0.01$). In contrast, the number of males that located the female was not different between DNinter, DNpure, and the control, while it was significantly lower after DNintra (G-test, $G=14.61$, $P<0.01$) and there was not a significant difference in the number of

accomplished mating (G-test, $G=4.75$, $P=0.20$). However, there were no significant differences between treatments considering the MC latency (Kruskal-Wallis test, $Df=3$, $\chi^2=1.46$, $P=0.70$), the number of calls after which the female replied (Kruskal-Wallis test, $Df=3$, $\chi^2=2.60$, $P=0.46$), the location latency (Kruskal-Wallis test, $Df=2$, $\chi^2=1.22$, $P=0.54$), and the number of mating attempts made by the male who located the mating partner (Kruskal-Wallis test, $Df=2$, $\chi^2=0.80$, $P=0.66$) (Table 3). The mating latency, reported in Table 3, was not compared due to the small number of pairs that mated in the given time.

Discussion

Results of this study showed that *E. vitis* naturally presents a rival behavior primarily expressed by the emission of a disruptive signal, the DP. Even if naturally occurring rivalry in most cases did not prevent mating, it was possible to disrupt the mating process by playing back an artificial DN into the leaf tissue.

Vibrational communication must be considered a complex network, in which the exchange of information is susceptible to being exploited by a receiver, which was not directly involved in the communication, to its own advantage (Virant-Doberlet et al. 2014). In leafhoppers, which rely on substrate-borne vibrations to identify and locate the mating partner, when a rival male eavesdrops a conspecific signaling can use different strategies to disrupt courtship and increase its possibility to mate: alternation of male calls (Hunt and Morton 2001), production of rivalry signals aimed at masking the female reply (Mazzoni et al. 2009a; Mazzoni et al. 2009b; Derlink et al. 2016; Kuhelj et al. 2016), and silently approach the female while she is duetting with the first male (i.e., satellite behavior) (Mazzoni et al. 2009b). The most studied strategy is the production of ‘disruptive signals’, which are supposed to disrupt the pair formation process occurring within the time window of the female reply and therefore masking it. The masking can be achieved by covering part or all the female reply or by confusing the male because it perceives stimulus from a spatially separated source from that of the female location (Hammond and Bailey 2003; Bailey et al. 2006; Mazzoni et al. 2009a). Rival interactions have been deeply investigated in the leafhopper *S. titanus*, in which two distinct signals are produced by rival males during an ongoing duet: the disturbance pulses and the disturbance noise (Mazzoni et al. 2009b). In particular, the ‘disturbance noise’ of *S. titanus* is hypothesized to mask the female signal (Mazzoni et al. 2009a), by overlapping the end of the female pulse. In contrast, the rival male-male interaction of *E. vitis* appears to us simpler, as it is for the reproductive behavior in general (Nieri and Mazzoni, under review). We found only one type of ‘disruptive signal’, which is equal to one of the two male signals used to interact with the female (i.e., the MP). The only difference is that

DP is emitted from a rival male at the same time of a competitor's MC. Moreover, the *E. vitis* DP anticipates the FR emission, and since DP is a short signal, it does not overlap the FR. The whole DP is included in the S2 of MC, and it has been demonstrated, at least in one leafhopper species, that males are not able to detect and use signal perceived while calling to locate a source (Kuhelj et al. 2015). Therefore, it is unlikely that DP is able to effectively mask the FR. It seems that DP function is to mask part of the competitor MC to decrease the FR occurrence. In fact, the proportion of elicited FR is lower when DP overlapped S2. The ratio of FR on MC is crucial to accomplish mating in *E. vitis* (Nieri and Mazzoni, under review), thus the lower number of FR would make more difficult for the calling male to locate the female and eventually mate. Females instead of males were already demonstrated to be the receiver of disruptive signals in two treehoppers species (Miranda 2006; Legendre et al. 2012). In particular, in *Tylopelta gibbera*, the rival overlapping signal reduced the directional information available to the competitor male (Legendre et al. 2012); our hypothesis is that *E. vitis* DP has the same function. The relative high number of mating despite rivalry in our trial was probably due to the small size of the leaf that reduced the efficacy of a strategy that aimed to interfere with the locating ability of the competitor male. This is the first evidence of such strategy in a leafhopper species and would be certainly worthy of further investigation. However, it goes beyond our aim to find a natural masking signal to be used in mating disruption. Future experiments are required to definitely assess how DP interfere with pair formation.

Similarly to what was observed in trials with two males, during which the two males ignored each other while calling, artificial noises never inhibited males and after a few minutes from the onset of the playback they started calling again. This was probably because they already entered the location stage and their motivation was high enough to maintain their calling activity. In contrast, females seem to react very differently to DN, since the number of them who kept replying to calling males reduced in all treatments. In leafhoppers, females are stationary during the pair formation process (Saxena and Kumar 1984; Claridge 1985; Hunt and Nault 1991; Hunt et al. 1992; Mazzoni et al. 2009b; de Groot et al. 2012; Derlink et al. 2016) and need to tune their response with the male signal (Kuhelj et al. 2015). Maybe the DN obstructed the female perception of the MC, reducing its ability to reply. On the other hand, males do not stop signaling because they do not need a vibrational trigger to start signaling (Nieri and Mazzoni, under review). Interestingly, females react differently according to the DN that was played back. In fact, in the presence of an interspecific signal, such as the *S. titanus* DN, the female remained silent for a longer time, but then the number of females that reply to MC was higher compared to other DN. Indeed, after playback suspension, the number of females that replied to MC was the highest if they were previously disrupted with *S. titanus* DN. Probably because the interference with the female receptor system is different according to the DN spectral features. In contrast, after

suspension of the DN playback, males were more reluctant to signal, as suggested by the reduced male calling activity (DN_{intra} and DN_{inter}) and successful female location (DN_{intra}). The possibility to temporarily suspend the DN would benefit the energetic cost of the transducers and thus the field applicability of a vibrational mating disruption method (Polajnar et al. 2016a). However, when observed the calling activity was restored a few seconds after the playback suspension, such as in *S. titanus* (Mazzoni et al. 2009a). Therefore, to ensure the efficacy of the method a continuous DN is recommended.

The results of the mating disruption trial suggest that the DN_{inter} empowered at 250 Hz could be a good disturbance noise candidate, to be tested in the field. The DN of *S. titanus* has already been successfully applied to disrupt mating of *S. titanus* in semi-field trials (Eriksson et al. 2012; Polajnar et al. 2016a) and our results showed that it is able to reduce the number of successful location, mating, and also to increase the location time in *E. vitis*. Leafhoppers species often are sympatric in vineyards and insecticides are used to control more species with the same application, thus a vibrational mating disruption method able to affect two species simultaneously would be an extremely useful tool. This would be feasible by incorporating a 250 Hz band in the *S. titanus* DN. Such a signal would interrupt or rather would not allow the establishment of a mating duet and therefore it would prevent pair formation operating as a 'masking signal' (Mazzoni et al. 2009b; Mazzoni et al. 2009a).

Thinking at the field applicability, we must consider that *E. vitis* is a polyphagous species, unlike *S. titanus*, and overwintering adults arrive in the vineyard in late spring when they are probably already mated (Böll and Herrmann 2004). So a mating disruption method will not be able to reduce the first generation, but could be effective on the successive ones (i.e., second and third). Nevertheless, this could contribute to maintaining a low population density and thus the leaf damage which is due only to the feeding activity. To date the vibrational mating disruption method has been developed to be applied in the vineyard, where vibrations are transmitted through existing trellises to the plants (Eriksson et al. 2012; Polajnar et al. 2016a). The applicability to different crops besides grapevine, which are hosts of *E. vitis*, such as kiwi, tea plants, and apple orchards, needs to be investigated and can increase the applicability of a vibrational mating disruption method and also to other polyphagous pests.

In conclusion, this study showed that a vibrational mating disruption method can be developed even in the absence of an effective species-specific natural masking signal. Moreover, it gives useful insights on the parameters that a potential disruptive noise needs to be effective in the long term. In the future, this acquired knowledge will be used to assess the applicability of the method in semi-field and field conditions, to control *E. vitis* and *S. titanus* simultaneously.

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References

- Alexander, R.D.** 1967 Acoustical communication in arthropods. *Annual Review of Entomology* **12**, 495–526.
- Alma, A.** 2002 Auchenorrhyncha as pests on grapevine. *Denisia* **176**, 531–538.
- Bailey, W., Macleay, C., & Gordon, T.** 2006 Acoustic mimicry and disruptive alternative calling tactics in an Australian bushcricket (Caedicia; Phaneropterinae; Tettigoniidae; Orthoptera): does mating influence male calling tactic? *Physiological Entomology* **31**, 201–210.
- Böll, S., & Herrmann, J.V.** 2004 A long-term study on the population dynamics of the grape leafhopper (*Empoasca vitis*) and antagonistic mymarid species. *Journal of Pest Science* **77**, 33–42.
- Cerutti, F., Baumgärtner, J., & Delucchi, V.** 1991 The dynamics of grape leafhopper *Empoasca vitis* Göthe populations in southern Switzerland and the implications for habitat management. *Biocontrol Science and Technology* **1**, 177–194.
- Claridge, M.** 1985 Acoustic Signals in the Homoptera: Behavior, Taxonomy, and Evolution. *Annual Review of Entomology* **30**, 297–317.
- de Groot, M., Derlink, M., Pavlovčič, P., Prešern, J., Čokl, A., & Virant-Doberlet, M.** 2012 Duetting behaviour in the leafhopper *Aphrodes makarovi* (Hemiptera: Cicadellidae). *Journal of Insect Behavior* **25**, 419–440.
- Decante, D., & van Helden, M.** 2006 Population ecology of *Empoasca vitis* (Göthe) and *Scaphoideus titanus* (Ball) in Bordeaux vineyards: influence of migration and landscape. *Crop Protection* **25**, 696–704.
- Derlink, M., Abt, I., Mabon, R., Julian, C., & Virant-doberlet, M.** 2016 Mating behaviour of *Psammotettix alienus* (Hemiptera: Cicadellidae). *Insect Science*. doi: 10.1111/1744-7917.12379.
- Eriksson, A., Anfora, G., Lucchi, A., Lanzo, F., Virant-Doberlet, M., & Mazzoni, V.** 2012 Exploitation of insect vibrational signals reveals a new method of pest management. *PLoS One* **7**, e32954.
- Fornasiero, D., Pavan, F., Pozzebon, A., Picotti, P. & Duso, C.** 2016 Plant Resistance relative infestation level and sensitivity of grapevine cultivars to the leafhopper *Empoasca vitis* (Hemiptera: Cicadellidae). *Journal of Economic Entomology* **109**, 1–10.
- Hammond, T. & Bailey, W.** 2003 Eavesdropping and defensive auditory

- masking in an Australian bushcricket, *Caedicia* (Phaneropterinae: Tettigonidae: Orthoptera). *Behavior* **140**, 79–95.
- Hazarika, L.K., Bhuyan, M., & Hazarika, B.N.** 2009. Insect pests of tea and their management. *Annual Review of Entomology* **54**, 267–284.
- Hofstetter, R. W., Dunn, D. D., Mcguire, R., & Potter, K. A.** 2014. Using acoustic technology to reduce bark beetle reproduction. *Pest Management Science*, **70**(1), 24–27.
- Hunt, R.E., & Nault, L.R.** 1991. Roles of interplant movement, acoustic communication, and phototaxis in mate-location behavior of the leafhopper *Graminella nigrifrons*. *Behavioral Ecology and Sociobiology* **28**, 315–320.
- Hunt, R.E., Fox, J.P., & Haynes, K.F.** 1992. Behavioral response of *Graminella nigrifrons* (Homoptera: Cicadellidae) to experimentally manipulated vibrational signals. *Journal of Insect Behavior* **5**, 1–13.
- Hunt, R.E., Morton, T.L.** 2001. Regulation of chorusing in the vibrational communication system of the leafhopper *Graminella nigrifrons*. *American Zoologist* **41**, 1222–1228.
- Kuhelj, A., De Groot, M., Blejec, A., & Virant-Doberlet, M.** 2015. The effect of timing of female vibrational reply on male signalling and searching behaviour in the leafhopper *Aphrodes makarovi*. *PLoS ONE*, **10**(10), 1–15.
- Kuhelj, A., de Groot, M., Blejec, A., Virant-Doberlet, M.** 2016. Sender-receiver dynamics in leafhopper vibrational duetting. *Animal Behavior* **114**, 139–146.
- Legendre, F., Marting, P.R., & Cocroft, R.B.** 2012. Competitive masking of vibrational signals during mate searching in a treehopper. *Animal Behavior*, **83**, 361–368.
- Liu, S., Li, Z., Sui, Y., Schaefer, D. A., Alele, P. O., Chen, J., & Yang, X.** 2015. Spider foraging strategies dominate pest suppression in organic tea plantations. *BioControl*, **60**(6), 839–847.
- Lujo, S., Hartman, E., Norton, K., Pregmon, E.A., Rohde, B.B., & Mankin, R.W.** 2016. Disrupting mating behavior of *Diaphorina citri* (Liviidae). *Journal of Economic Entomology*, **0**, 1-7. doi: 10.1093/jee/tow202.
- Mankin, R., Rohde, B., & Mcneill, S.** 2013. *Diaphorina citri* (Hemiptera: Liviidae) Responses to microcontroller-buzzer communication signals of potential use in vibration traps. *Florida Entomologist*, **96**, 1546–1555.
- Mazzoni, V., Cosci, F., Lucchi, A., & Santini, L.** 2001. Occurance of leafhopper (Auchenorrhyncha, Cicadellidae) in three vineyards of the Pisa district. In *Integrated Control in Viticulture IOBC wprs Bulletin*, pp 267–271.

- Mazzoni, V., Lucchi, A., Cokl, A., Presern, J., & Virant-Doberlet, M.** 2009a. Disruption of the reproductive behaviour of *Scaphoideus titanus* by playback of vibrational signals. *Entomologia Experimentalis et Applicata*, **133**(2), 174–185.
- Mazzoni, V., Presern, J., Lucchi, A., & Virant-Doberlet, M.** 2009b. Reproductive strategy of the Nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). *Bulletin of Entomological Research*, **99**(4), 401–13.
- Miller, J.R., & Gut, L.J.** 2015. Mating disruption for the 21st century: matching technology with mechanism. *Environmental Entomology* **44**, 427–453.
- Miranda, X.** 2006. Substrate-borne signal repertoire and courtship jamming by adults of *Ennya chrysurus* (Hemiptera : Membracidae). *Annals of the Entomological Society of America*, **99**, 374–386.
- Nieri, R., & Mazzoni, V.** (under review) The reproductive strategy and the vibrational duet of the leafhopper *Empoasca vitis* Göthe.
- Pavan, F., & Picotti, P.** 2009. Influence of grapevine cultivars on the leafhopper *Empoasca vitis* and its egg parasitoids. *BioControl* **54**, 55–63.
- Polajnar, J., Eriksson, A., Rossi Stacconi, M. V., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2014. The process of pair formation mediated by substrate-borne vibrations in a small insect. *Behavioural Processes*, **107**, 68–78.
- Polajnar, J., Eriksson, A., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2015. Manipulating behaviour with substrate-borne vibrations - Potential for insect pest control. *Pest Management Science* **71**, 15–23.
- Polajnar, J., Eriksson, A., Virant-Doberlet, M., Lucchi, A., & Mazzoni, V.** 2016a. Developing a bioacoustic method for mating disruption of a leafhopper pest in grapevine. In *Advances in Insect Control and Resistance Management* (Ed. by A.R. Horowitz & I. Ishaaya), pp 165–190. Springer, New York.
- Polajnar, J., Eriksson, A., Virant-Doberlet, M., & Mazzoni, V.** 2016b. Mating disruption of a grapevine pest using mechanical vibrations: from laboratory to the field. *Journal of Pest Science* **89**, 1–13.
- Ryan, T.H.** 1960. Significance tests for multiple comparison of proportions, variances, and other statistics. *Psychological Bulletin*, **57**, 318–328.
- Saxena, K.N., & Kumar, H.** 1984. Acoustic communication in the sexual behaviour of the leafhopper, *Amrasca devastans*. *Physiological Entomology*, **9**, 77–86.
- Virant-Doberlet, M., Mazzoni, V., de Groot, M., Polajnar, J., Lucchi, A., Symondson, W.O.C., & Čokl, A.** 2014. Vibrational communication networks: eavesdropping and biotic noise. In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp 93–123. Springer, Berlin Heidelberg.

Chapter 4

Mating behavior and vibrational mimicry in the glassy-winged sharpshooter, *Homalodisca vitripennis*

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4. Mating behavior and vibrational mimicry in the glassy-winged sharpshooter, *Homalodisca vitripennis*

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Abstract

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, is an important vector of *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevine. GWSS control relies mainly on insecticides; therefore, an alternative method, such as vibrational mating disruption, is required. However, knowledge of GWSS intraspecific communication is necessary to evaluate applicability of such methods. Mating behavior and associated vibrational signals were described in different social contexts: individuals, pairs, and one female with two competing males. Behavioral analysis showed that GWSS mating communication involved the emission of three male and two female signals, with specific roles in two distinct phases of mating behavior, identification and courtship. Mating success depended on vibrational duets between genders, which were temporarily interrupted in the presence of male rivalry. Male rivalry behavior involved the emission of three distinct rivalry signals. Two rivalry signals resemble female signals and were associated with replacement of the female in the duet by the rival male. The third rivalry signal was emitted by competing males. Data suggested that rival males used mimicry and hostile signals to interrupt the ongoing duet and gain access to a female. In the future, knowledge acquired from this study will be essential to develop a mechanical mating disruption method for GWSS control.

Key words: vibrational communication, mating disruption, rivalry, leafhopper, *Xylella fastidiosa*.

Key message

- *Homalodisca vitripennis* (GWSS), an important vector of *Xylella fastidiosa* that causes Pierce's disease of grapevine, has little known about its mating behaviors.
- Male and female mating communication and vibrational signals involved in the pair formation process were described.
- GWSS mating involved two main stages with characteristic male and female vibrational calls during each stage.
- In presence of multiple males, a unique male rival interaction occurred: male rivalry calls mimicked female signals interrupting the mating duet.

Introduction

Vibrational communication is widespread within insects, where 92% of species have been estimated to use substrate vibrations (Cocroft and Rodriguez 2005). In most Hemiptera, pair formation generally consists of distinct mating communication phases (i.e., mate identification, localization, and courtship) that are essential for accomplishing mating occurring mainly via exchange of vibrational signals (Alexander et al. 1997; Čokl and Virant-Doberlet 2003; Polajnar et al. 2014). In the family Cicadellidae, typically, a male emits vibrational advertisement signals through the plant until establishment of identification duet with a female. Signals are species-specific in temporal and spectral characteristics and essential for proper mate identification (Bailey 2003). After the initial duet, the male localizes the stationary female on the plant (Hunt and Nault 1991; Mazzoni et al. 2009b; de Groot et al. 2012), approaches her, and emits species-specific courtship signals (Polajnar et al. 2014).

In the presence of male competition, the apparently straightforward mate selection process can be delayed or interrupted by emission of interference signals by the rivals attempting to disrupt an ongoing duet between a male and female (Booij 1982; Heady et al. 1986; Nuhardiyati and Bailey 2005; Bailey et al. 2006; Miranda 2006; Mazzoni et al. 2009b). For example, natural mating disruption via emission of male rivalry signals occurs in *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) (Mazzoni et al. 2009b). Rival males emit masking signals to stop an ongoing male-female duet (Mazzoni et al. 2009a; Mazzoni et al. 2009b). Exploitation of the rivalry signals as a novel pest management strategy has been investigated for *S. titanus* by transmitting a synthetic male "disturbance noise" through wires of vineyard trellis (Eriksson et al. 2012; Polajnar et al. 2016). In field trials, mating of male-female pairs was suppressed by about 90%. These promising results opened the floodgates for studying the feasibility of this method to disrupt mating of other grape pests,

such as the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germer) (Hemiptera: Cicadellidae).

GWSS is a grapevine pest native to southeastern United States and northeastern Mexico (Triapitsyn and Phillips 2000) that invaded California in the late 1980s (Sorensen and Gill 1996; Stenger et al. 2010). GWSS is a polyphagous insect and a serious threat to agriculture due to its ability to transmit *Xylella fastidiosa* Wells et al., a xylem-limited bacterium that causes Pierce's disease in grapevines (Davis et al. 1978) and many other diseases in economically important crops. On grapevines in California, GWSS reproduce from spring to fall producing at least two generations per year. During winter months, GWSS population densities decline sharply and are strictly associated with non-deciduous shrubs and trees. In laboratory conditions, highest fecundity and longevity observed for a single GWSS female were 967 eggs and 296 days, respectively (Krugner 2010). Given the near-zero tolerance for GWSS in vineyards, long-term suppression of population densities will rely heavily on novel methods.

Population size is a result of the combined action of births, deaths, immigration, and emigration. While products (e.g., insecticides) are available to increase mortality of insect vectors of plant pathogens, research is needed to identify methods to reduce birth (Sisterson and Stenger 2016). Several aspects of GWSS reproductive biology and behavior have been studied, including egg load and maturation dynamics (Sisterson 2008; Sisterson 2012; Sisterson 2014), reproductive maturity (Krugner 2010), oviposition behavior (Hummel et al. 2006), and host preference for oviposition (Blua et al. 2001; Krugner et al. 2009; Chen et al. 2010). Exploitation of vibrational signals for disrupting mating of GWSS could prove to be a useful tool, but existing knowledge on GWSS communication is insufficient to implement a management program for this pest in California. Although, a GWSS male signal was described, male-female duets were never recorded (Percy et al. 2008). Therefore, the objective of this study was to identify and characterize GWSS vibrational signals and provide insights for understanding mate selection behaviors of sharpshooters.

Materials and Methods

Insects and plants

Adult GWSS were collected in spring 2014 from citrus orchards in Bakersfield and Ojai, California, USA and transported to a laboratory at the USDA-ARS San Joaquin Valley Agricultural Sciences Center in Parlier, California. Insects were reared in mesh cages (Bug Dorm-2®, BioQuip Products, Rancho Dominguez, CA) containing four plant species: cowpea (*Vigna unguiculata* L. Walp. cv. 'Blackeye'), okra (*Abelmoschus esculentus* (L.) Moench) (both from Vermont Bean Seed Co., Randolph, WI), basil

(*Ocimum basilicum* L. cv. 'Genovese'), and sunflower (*Helianthus annuus* L. cv. 'American Giant Hybrid') (both from Ferry-Morse Seed Co., Fulton, KY). Late-instar (4th and 5th) GWSS nymphs obtained from colonies were isolated by gender in cages to obtain virgin adult individuals. After molting to the adult stage, females were transferred to an individual mesh-screen tube cage (10 cm diameter \times 40 cm height) containing a cowpea plant. Reproductive maturity in about 150 individually caged females was determined by deposition of non-fertilized eggs. Male insects used in the experiments were of the same age as reproductively active females (Krugner 2010). After female reproductive maturity and virginity were confirmed (deposition of non-fertilized eggs, without embryo development), test insects were used in the recording experiments. Each individual was tested only once.

Experimental setup

Experiments were conducted in an arena that provided a uniform background and both reduced airborne noise and observer interference. The arena was a transparent experimental cage (60 \times 60 \times 80 height cm) made of 1-cm thick acrylic walls, centered inside a chamber formed by 86 \times 86 \times 98 cm high blackout fabric and sound isolating walls. The arena and chamber were placed on an active vibration isolation table (Model 20-561, Technical Manufacturing Corporation, Peabody, MA). Insect behaviors were monitored via video surveillance (Panasonic Lumix GH4). Vibrational signals produced by individuals were recorded using a laser Doppler vibrometer (NLV-2500, Polytec, Inc., Irvine, CA) and digitized with Adobe Audition® C26 (Adobe Systems, Inc., San Jose, CA) at a 44.1 kHz sample rate and 32 bits resolution. The laser vibrometer focused on a small piece of reflective tape glued to the stem of a potted okra plant placed in the center of the arena. All plants were about 30 cm in height, with two apical leaves (approximate surface 7 \times 7 cm).

Mating behaviour and signal characterization

Trials were conducted between 0800 and 1900 h at room temperature. Before testing, insects were allowed 15 min to acclimatize to ambient conditions in 130-ml plastic vials placed within the chamber. After the acclimatization, insects were released on the plant. Three types of trials were performed using 1) individual, 2) one male and one female, or 3) one female and two males. In trials with an individual, a male ($n = 21$) or female ($n = 26$) was placed alone on a host plant to identify spontaneous calling. In trials with a male-female pair ($n = 33$), the order of male and female introduction was randomized. In trials with three individuals, two males were placed on the plant before adding a female ($n = 30$). Trials consisted of 90 min observations, except for trials with an individual female that was 45 min, due to preliminary trials where female signaling rate was higher than males.

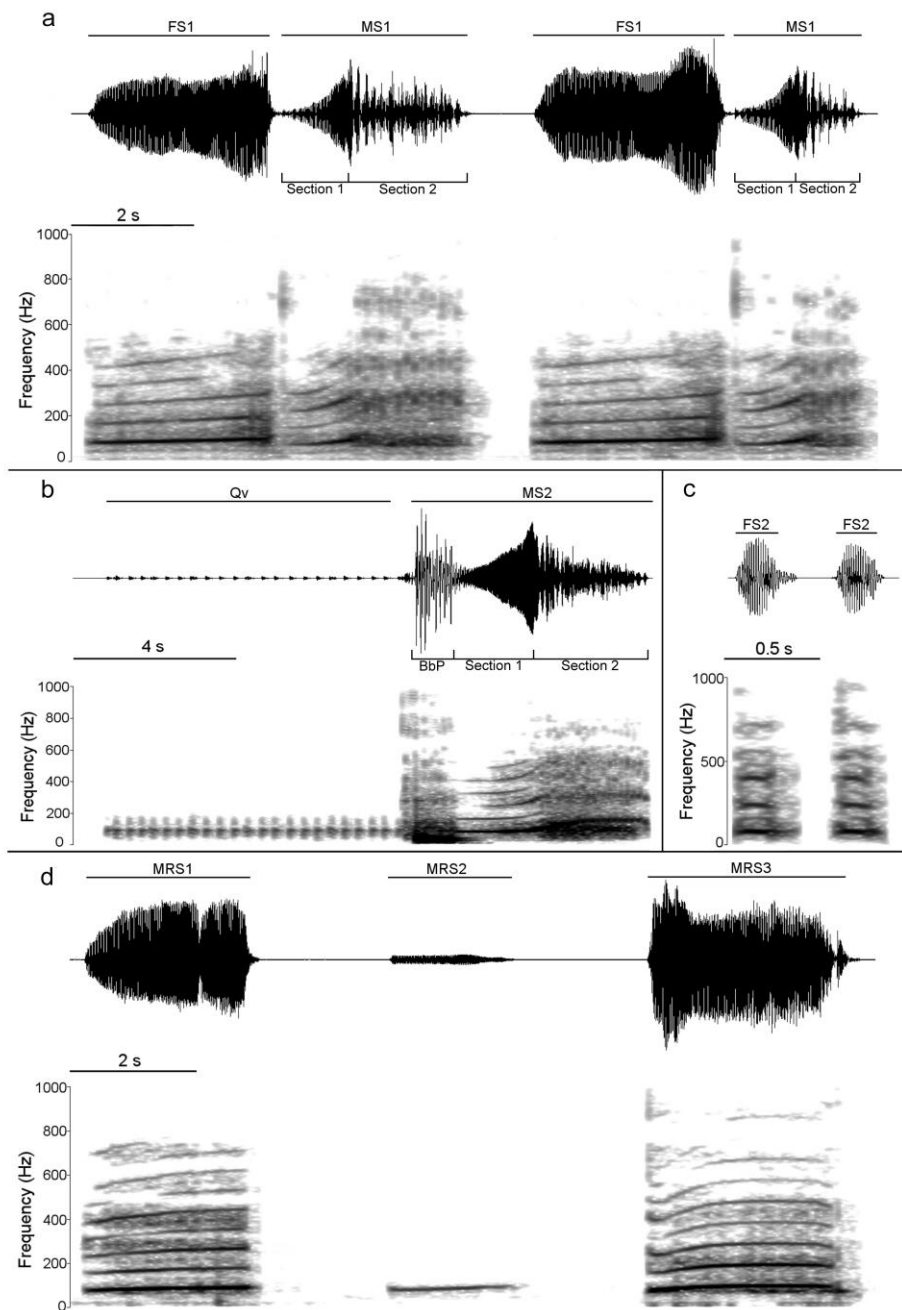


Figure 1 Oscillogram (above) and spectrogram (below) of GWSS vibrational signals. In (a) the Identification duet formed by two FS1 and two MS1 alternated; (b) is MS2 preceded by Qv; (c) two consecutive FS2; (d) three different MRS: MRS1, MRS2, and MRS3--from left to right.

Terminology and signal characterization

Vibrational signals were identified and named using a combination of signal structure and behavioral context. ‘Calling signals’ were defined as signals emitted spontaneously to trigger a reply from the opposite sex, ‘pulse’ was defined as a physically unitary or homogeneous sound, composed of a brief succession of sine waves (Alexander 1967), ‘pulse train’ was defined as a succession of repetitive and temporally well-distinct group of pulses (e. g. Mazzoni et al. 2009b), and ‘quivering’ was a rhythmic pulse-like vibration associated to abdominal quivering (Fabre et al. 2012). A signal, or part of it, was defined as ‘fragmented’ when its emission was not continuous but characterized by regularly repeated interruptions.

Spectral and temporal parameters of signals were analyzed with Raven Pro 1.5 (The Cornell Lab of Ornithology, Ithaca, NY) using Fast Fourier Transform (FFT) type Hann with window length of 8192 samples and 80% overlap. The following parameters, when applicable, were measured for each signal: duration, pulse (or fragment) repetition time (measured as the distance between the onset of two consecutive elements), percentage of male signal length made up of Section 2 (fragmented part, see below), fundamental frequency (ff), and relative amplitude (measured as root mean squared (RMS) (Charif et al. 2010). Because signal amplitude is relative to the distance between emitter and measuring location on the plant (laser beam), RMS was measured only from stationary individuals in single and pair trials. To describe frequency dynamics of a harmonic signal, the ff was measured at the beginning (b), middle (m), and end (e) of the signal. To determine the ff rate of increase/decrease within a signal, modulation rate (MR) was calculated as follows:

$$MR_{xy} = \frac{ff_y - ff_x}{t_{xy}}$$

where x and y indicate the section (0.1 sec) of the signal in which the ff was measured, t was the time (in seconds) between sampling points x and y. Recordings of single males (n = 5) and females (n = 10) that emitted signals while placed alone on the plant, pairs that mated (n = 12), and trios that displayed rivalry behavior (n = 17) were used to characterize vibrational signals of GWSS. A total of 40 signals per type (at most five samples per individual) were used for statistical analyses (either Wilcoxon test for paired data or Kruskal-Wallis test followed by multiple comparisons). To compare RMS of male and female signals, Friedman test with five replications (Kyplot, Koichi Yoshioka, 1997-2001 vers. 2.0 beta 15) was performed followed by pairwise multiple comparisons (New 1994).

To determine whether spectral and temporal features of male and female signals varied between steps of the mate selection behavior, statistical analysis was conducted across the two identified behavioral phases (see

Results). A stepwise discriminant analysis was used to determine whether signals could be distinguished based on temporal (duration) and spectral (ffb, MRbm, and MRme) profiles.

Analysis of behavioral parameters

For insects tested individually on plants (trial 1), the following parameters were recorded: time from beginning of trial to emission of the first vibrational signal (call latency), number of individuals that emitted at least one signal during trials (signaling activity), and number of signals emitted during the trial. Since the number of males that emitted signals when placed alone on the plant was low, statistical analyses were not performed to compare male and female call latency and number of signals emitted. Parameters recorded for insects tested in pairs (trial 2) were as follows: latency to first duet (first reply to a signal regardless of gender), duration of identification duet (see Results), and latency to mating (time between first duet and copula).

For analysis of the temporal sequence of signal exchange between individuals, a first-order Markovian behavioral transition matrix for the pair formation process was created for each individual using data from all insect pairs ($n = 12$) that successfully mated. Data were pooled in the analysis assuming non-significant differences among individuals. Transition probabilities were calculated from the observed frequency of a transition between two events (either a signal emission or a behavior) divided by the total number of occurrences of the first of the two events (Haccou and Meelis 1992). Male signals used in the analysis were MS1, MS2, and Qv; female signals were FS1 and FS2 (see Results for descriptions). Selected behaviors used in the analysis were identification duet, movement (i.e., walking), mating attempt, and copula. To eliminate loops while generating the matrices, only one repetition of single or coupled signals (e.g., repetitive duets, alternation of Qv and MS2) was considered. Expected values were calculated using the iterative proportional fitting method of Goodman (1968), then G-test (Williams' corrected) was performed to determine the significance, after Bonferroni method, of the overall table and of transitions by collapsing the table in a 2×2 matrix (Zar 1999).

Because of unreliability in distinguishing the source of signals in a trio (trial 3), behavioral analyses based on Markovian transitional matrices were not performed for trios. However, rivalry signals ($n = 40$ per each type, see Results) were randomly selected to determine which signal/behavior preceded and followed it. In particular, four types of signals (MS1, MS2, Qv, and Male Rival Signals) and two behavioral states (walking and stationary) were recorded. To determine which transitions were most commonly associated with emission of rivalry signals, G-test (William's corrected), followed by Ryan's multiple comparison test for proportions was conducted (Ryan 1960). In addition, the number of assays where male-male exchange of vibrational signals occurred

was recorded, as well as length of the identification duet (see Results) and latencies for first duet, first rivalry signal, and mating. Non-parametric test for unpaired data was used to compare latency to copula between pairs alone on the plant and pairs in the presence of a second male engaged in rivalry. G-test (Williams' corrected) was used to determine whether male rivalry behaviors affected the probability of accomplishing mating.

Results

Signal characterization

A total of two female signals (FS1 and FS2), three male signals (MS1, MS2, and Qv), and three male rivalry signals (MRS 1-3) were identified (Figures 1-2, Table 1). Signal emission was associated with dorso-ventral abdominal oscillation. In the initial portion of MS2 there was a rapid flicking of all wings with the remainder of the signal the same as MS1. The pair formation process was separated in two main phases: identification (Phase 1) and courtship (Phase 2). The identification phase was characterized by stationary individuals that exchanged FS1 and MS1. During courtship, males alternated signal emissions (while stationary) with walking to approach the female. The female remained stationary on the plant at all times and replied with FS1 or FS2.

Female signals

FS1 was the most common female signal with a clear harmonic structure and an increasing ff (Figure 1a; 2a,b,d). The ff of FS1 during the identification phase (FS1_1) had a constant positive slope increase ($0 < MR_{bm} = MR_{me}$; Wilcoxon: $n = 40$, $T = -188$, $P = 0.21$), whereas in the courtship phase (FS1_2) it had a significantly sharper increasing slope during the second half of the signal ($0 < MR_{bm} < MR_{me}$; $n = 40$, $T = -398$, $P < 0.01$) (Table 1).

FS1_1 was significantly longer than FS1_2 and FS2 (Figure 1c; 2a,b,d) (Kruskal-Wallis, $n = 40$, $Df = 9$, $\chi^2 = 196.94$, $P < 0.001$). Similarly, FS1_1 had higher amplitude than FS1_2 and both had higher amplitude than FS2 (Friedman, $n = 30$, $Df = 2$, $\chi^2 = 43.1$, $P < 0.001$). The starting frequency of FS2 was lower than both FS1 signals ($n = 40$, $Df = 9$, $\chi^2 = 157.66$, $P < 0.001$). The ff of FS2 decreased constantly during signal emission ($0 > MR_{bm} = MR_{me}$; $n = 40$, $T = -236$, $P = 0.10$). FS2 was repeated in sequences (up to 13 consecutive repeats) with variable pulse repetition time (median, 0.48 s; range, 0.13-4 s).

Table 1 Frequency modulation rate of GWSS signals.

	FS1_1	FS1_2	FS2	MS1_1	MS1_2	MS2	MRS1	MRS2	MRS3
MR _{bm}	9.6 (3.7; 17.7)	6.8 (-7.4; 17.9)	0.0 (-72.7; 5.9)	7.4 (0.0; 53.7)	7.9 (0.0; 85.3)	5.9 (0.0; 26.8)	12.9 (2.8; 22.0)	5.2 (-6.6; 12.8)	10.3 (1.7; 28.0)
MR _{me}	10.3 (-1.5; 36.8)	11.4 (0.0; 38.9)	0.0 (-41.7; 22.2)	32.7 (18.2; 75.3)	36.0 (13.0; 154.3)	33.3 (15.0; 79.6)	4.9 (0.0; 12.6)	3.6 (0.0; 11.5)	-1.7 (-27.7; 8.8)
	Ns	<0.01	Ns	<0.001	<0.001	<0.001	<0.001	Ns	<0.001

Median (minimum; maximum) of modulation rate (MR) calculated for the first (MR_{bm}) and second (MR_{me}) halves of 40 GWSS signals. Significant differences between MR_{bm} and MR_{me} within each signal are indicated (Wilcoxon test for paired data, P values ≤ 0.05).

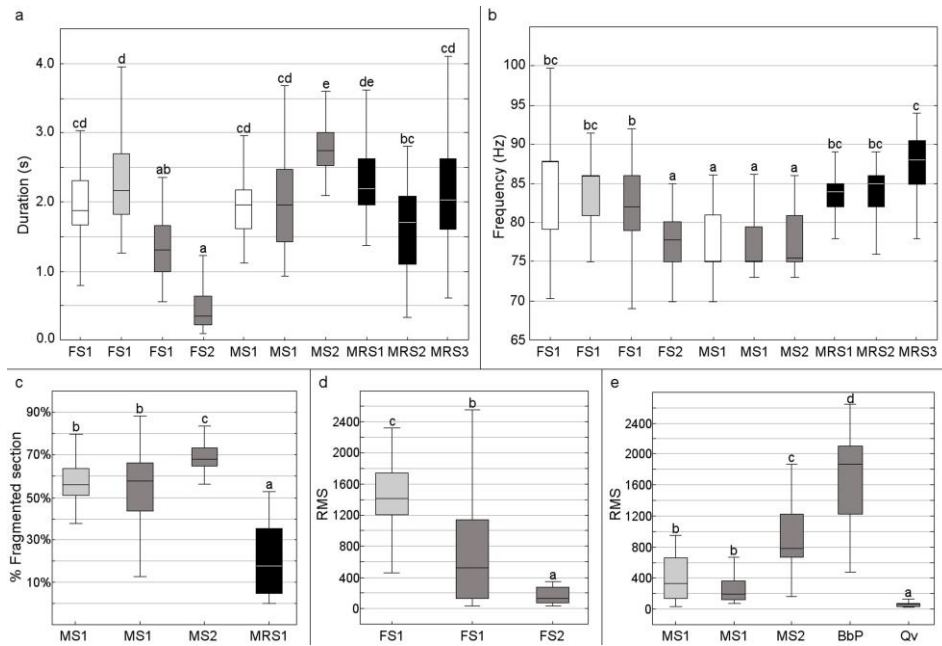


Figure 2 Box-plot of temporal and spectral parameters of GWSS vibrational signals. In (b), the frequency is the fundamental at the beginning of each signal. White bars refer to signals in single trials, light grey bars from signals of Phase 1 (identification), dark grey bars from Phase 2 (courtship), black bars from rivalry contest. Different letters indicate significant difference among signals after Kruskal-Wallis test followed by multiple comparisons test (a, b, c; n = 40 signals per type) or Friedman test followed by non-parametric multiple comparison (d and e; n = 30 signals per type).

Male courtship signals

MS1 (Figure 1a; 2a,b,c,e) was characterized by two distinct sections. The first was a continuous emission characterized by a significant increasing slope ($MR_{me} > MR_{bm} > 0$) both in identification (MS1_1; Wilcoxon: $n = 40$, $T = -806$, $P < 0.001$) and in courtship (MS1_2; $T = -792$, $P < 0.001$), before the onset of the second section, which was fragmented at a constant frequency.

Similar to MS1, MS2 (Figure 1b; 2a,b,c,e) encompassed the two MS1 sections, being characterized by a significant increasing slope ($MR_{me} > MR_{bm} > 0$; $n = 40$, $T = -814$, $P < 0.001$). The main difference was an additional strong broadband pulse (BbP) that anticipated the first section, corresponding to the rapid wing flicking. In addition, MS2 duration and percentage of the fragmented section were significantly higher than in both MS1 (duration: Kruskal-Wallis, $n = 40$, $Df = 9$, $\chi^2 = 196.94$, $P < 0.001$; percentage of fragmented section: $n = 40$, $Df = 9$, $\chi^2 = 95.04$, $P < 0.001$). The amplitude of MS2, without considering the BbP, was higher compared to MS1 and the BbP amplitude was the highest compared to all male courtship signals (RMS: Friedman, $n = 30$, $Df = 4$, $\chi^2 = 116.02$, $P < 0.0001$).

Qv (Figure 1b; 2e) was a train of low amplitude pulses with variable duration (0.5 to 240 sec) and regular pulse repetition time (mean \pm SD: 0.23 ± 0.03 sec). In two cases, sudden rhythm acceleration was observed with pulses that fused into a continuous signal (max. 1.7s) with harmonic structure and constant ff of about 75 Hz.

Table 2 Standardized canonical discriminant function coefficients.

	Function 1	Function 2
Basal Frequency	-0.178	0.739
MR_{bm}	-0.017	0.724
MR_{me}	0.845	-0.366
Signal duration	0.607	0.577

Male rivalry signals

Three types of MRS signals were identified (Figure 1d; 2a,b,c,e). MRS1 had clear harmonic structure with ff that significantly increased during signal emission, but unlike the other male rivalry signals the first half increased more than the second half ($MR_{bm} > MR_{me} > 0$; Wilcoxon: $n = 40$, $T = 751$, $P < 0.001$). In 80% of samples analyzed, the last part of MRS1 was fragmented, as in MS1 and MS2. However, this section was significantly shorter than in MS1_1 (median, 56%; range, 8-95%) and MS1_2 (median, 58%; range, 13-88%) (Kruskal Wallis: $n = 40$, $Df = 9$, $\chi^2 = 95.04$, $P < 0.001$). In addition,

the fragment repetition time of MRS1 (median, 0.10 s; range, 0.08-0.12 s) was significantly longer than in MS1 (MS1_1: median, 0.08 s; range, 0.06-0.11 s; MS1_2: median, 0.08 s; range, 0.06-0.12 s) and MS2 (median, 0.09 s; range, 0.08-0.10 s) ($Df = 3$, $\chi^2=18.97$, $P < 0.001$). MRS2 was characterized by a constant increase of ff ($MR_{bm} = MR_{me} > 0$; $T = 162$, $P = 0.27$). In 20% of samples analyzed, a short MRS2 fragmentation was observed; although, repetition rate was hardly measurable due to low intensity of the signal. Finally, MRS3 was variable in duration, not fragmented, and with a peculiar ff trend that initially increased, and then decreased from about half-length of the signal ($MR_{bm} > 0 > MR_{me}$; $T = 820$, $P < 0.001$). MRS temporal and spectral features are more similar to female than male signals: the duration of MRS1 and MRS3 was not significantly different from FS1, while the MRS2 duration was significantly shorter and comparable to FS2 ($\chi^2 = 196.94$, $Df = 9$, $P < 0.001$). The starting frequencies of all MRS and FS1 signals were similar ($Df = 9$, $\chi^2 = 196.94$, $P < 0.001$).

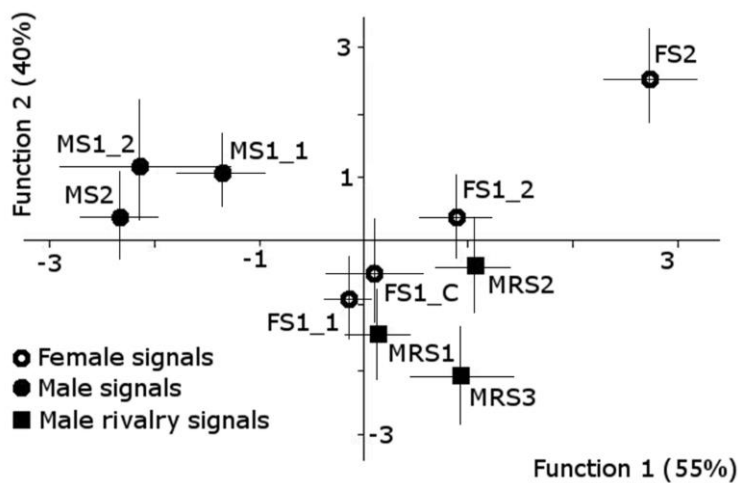


Figure 3 Combined-groups plot showing functions 1 and 2 derived from the discriminant function analysis of signal duration, starting frequency, and modulation rates (MR_{bm} and MR_{me}). Functions 1 and 2 explain 55% and 40% of variance, respectively, separating MS1 and MS2 from FS2, FS1, and MRS. Only centroids (calculated as averages (\pm SD) of canonical variables) are shown. Discrimination between FS1 and MRS is low, in particular between MRS1 and FS1_1/FS1_C (Call) and between MRS2 and FS1_2.

Discriminant Analysis

Discriminant analysis revealed that temporal and spectral parameters have a role in determining signal specificity; although, the accuracy of

discrimination was not high (50.8% of the signals were correctly classified). The first two discriminant functions explained 95.8% of the variance (function 1 = 54.6%, canonical correlation = 0.85, Wilks' lambda = 0.083, Chi² = 869, P < 0.001; function 2 = 41.2%, canonical correlation = 0.81, Wilks' lambda = 0.294, Chi² = 427, P < 0.001). Standardized canonical discriminant function coefficients are reported in Table 2. The plot of first vs. second roots of the discriminant analysis (Figure 3) showed that male signals used during identification and courtship (MS1, MS2) can be easily distinguished from female signals, while uncertainty occurs when trying to distinguish between FS1 and male rivalry signals. In particular, uncertainty occurs when trying to distinguish FS1_1 from MRS1 and FS1_2 from MRS2. On the contrary, FS2 and MRS3 were well discriminated (accuracy > 60%) (Table 3).

Table 3 Classification results from discriminant analysis.

Original Type	Predicted Signal Type									
	FS1_Call	FS1_1	FS1_2	FS2	MS1_1	MS1_2	MS2	MRS1	MRS2	MRS3
FS1_Call	27.5	17.5	7.5	2.5	5.0	5.0	5.0	2.5	25.0	2.5
FS1_1	25.0	40.0	10.0	0.0	2.5	0.0	0.0	17.5	2.5	2.5
FS1_2	20.0	7.5	47.0	0.0	2.5	0.0	0.0	5.0	18.0	0.0
FS2	0.0	0.0	12.5	87.5	0.0	0.0	0.0	0.0	0.0	0.0
MS1_1	0.0	5.0	5.0	0.0	60.0	17.5	12.5	0.0	0.0	0.0
MS1_2	0.0	0.0	7.5	0.0	25.0	35.0	27.5	2.5	0.0	0.0
MS2	7.5	7.5	2.5	0.0	2.5	20.0	62.5	0.0	0.0	0.0
MRS_1	0.0	20.0	5.0	0.0	0.0	0.0	7.5	45.0	15.0	20.0
MRS_2	2.5	15.0	27.5	5.0	0.0	0.0	0.0	7.5	35.0	7.5
MRS_3	0.0	0.0	2.5	0.0	0.0	0.0	0.0	15.0	20.0	62.5

Male and female signals were assigned to a certain type of signal based on temporal (duration) and spectral features (starting frequency, MR_{bm}, and MR_{me}) of the signal. Percentages in bold font represent signals that were correctly assigned to the signal type, whereas percentages in gray cells represent incorrect signal assignments (higher than 20%).

Behavioral Analysis

Trial 1. Individuals. When placed alone on plants, 77% (20/26) of females emitted FS1 and 24% (5/21) of males emitted MS1. Females spontaneously emitted calls (median, 5, range, 1-143) after a few minutes (median, 383.50 s; range, 28-2444 s). Males emitted two signals (median) per individual (range, 1-8), after 30 min (median) (range, 204-2295 s).

Trial 2. Pairs. When placed together, 64% (21/33) of pairs initiated a duet. Of the duets, females were the first individual to emit a signal in 71% (15/21) of the trials. Among pairs that engaged in duets, 57% (12/21) mated in the given time. Latency (median, 507 s; range, 72-2645 s) and length (median,

28 s; range, 10-342 s) of identification duet were variable (n = 21). While during identification the female:male response ratio was close to 1:1 (median, 1; range, 0.33-2.25), the number of female replies to male signals in courtship was much lower (1:4) (median, 0.25; range, 0-0.63) (n = 12). Finally, when a male arrived at a short distance (two to three body lengths) from the female, FS2 was emitted. Latency to mating ranged from 625 to 3572 s (median, 2482.50 s).

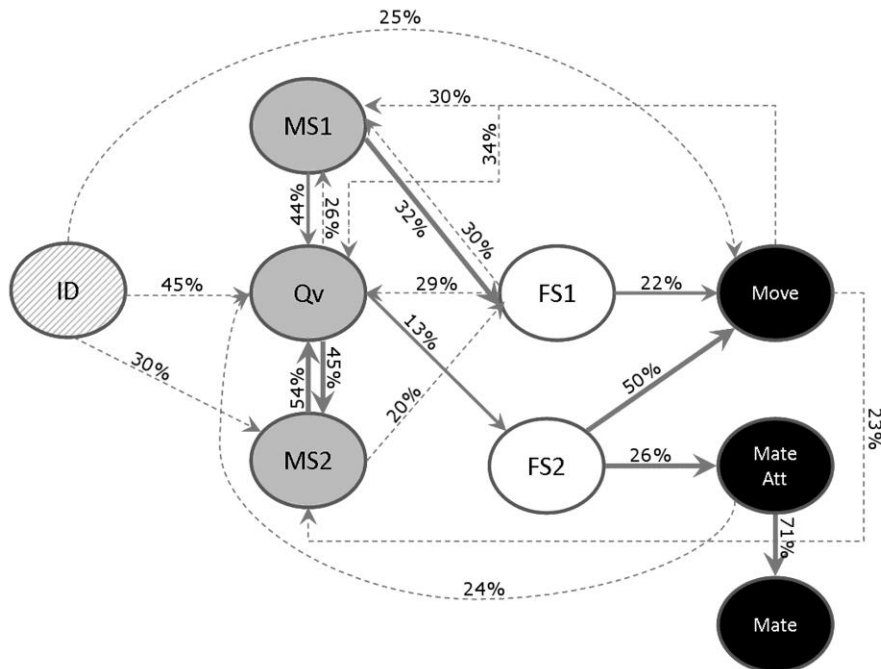


Figure 4 Ethogram describing transitions probabilities between signals and behaviors that constitute the process of pair formation in glassy-winged sharpshooter starting from the Identification Duet (ID). Male (MS1, MS2, and Qv) and female (FS1 and FS2) signals are shown in gray and white circles, respectively. Select behaviors were male movement (i.e., walking) (Move), mating attempt (Mate att), and mating (Mate). Dashed lines are non-significant transitions ($P > 0.05$); whereas, solid lines represent significant transitions ($P < 0.05$ = normal line and $P < 0.01$ = bold line). The percentage of observed transitions is indicated over each line. Non-significant transitions with percentages less than 15% were not included in the ethogram

Behavioral analysis based on the Markovian transition matrix (Figure 4) revealed that males were equally as likely to start courtship with MS2, Qv, or walking. In courtship, males alternated emission of MS1 and MS2, interspaced by Qv. In particular, emission of MS1 appears to be linked to emission of FS1, which in turn elicited establishment of longer duets or male movement. In contrast, emission of Qv elicited emission of FS2, which was the signal that

preceded male mating attempts. However, in three cases (Supplement table 1) the mating attempt was preceded by emission of FS1, MS2 or Qv; in one trial the male located the female and mated without any detectable female signal emission during the courtship phase. During identification and courtship phases, a female could still reject the male that located her. The female non-receptive behavior was displayed by lifting the posterior part of the abdomen (at about a 45° angle from the plant stem) and stretching the hind legs outward.

Trial 3. Trios. In 77% of the two male/one female trials (22/30), a male-female duet was established after 402 s (median) (range, 67-4643 s) into the recording period (latency to identification duet). In 90% of these trials (19/21), vibrational male-male interactions (rivalry) were detected after 250 s (median) (range, 2-3424 s) from duet establishment. Rivalry initiated after emission of male rivalry signals (MRS) during an ongoing duet between the female and the other male. Rivalry primarily occurred during the courtship phase (n = 15) but with some cases in the identification phase (n = 3).

Emission of MRS triggered duets between males. From the analysis of signal sequences before and after emission of any MRS (Figure 5), interactions with higher probability to occur were: 1) male identification/courtship signals to MRS1 and MRS2, 2) MRS (MRS2 and MRS3) to MRS2 and MRS3, and 3) male movement on the plant followed by MRS3. As soon as rivalry behaviors occurred, the rival male emitted MRS1 and/or MRS2. At that point, the female ceased to signal and was replaced in the duet by MRS1 and/or MRS2 of the rival male. While MRS1 primarily was followed by other male signals, MRS2 led to MS and male movements. In particular after MRS2, male-male duets elicited walking behavior in the first male that moved him closer to the rival male. When the two males were relatively close (less than two body distances), MRS3 was emitted often in a repeated series. At this stage, MRS emissions were elicited also from the first male, while MS emissions were temporarily interrupted. Emission of MRS3 was associated with a typical body movement often performed by individuals in tandem: both males lowered the posterior part of the abdomen forming an arc during the emission. At this stage, males often attempted to mount with the closer individual (either male or female).

During rival contests, females did not emit vibrational signals. Only after a male resumed emission of MS (either MS1 or MS2) a new duet with the female could be re-established. Copula occurred in 44% (n = 18) of trials where males emitted MRS. Similar to pair trials, mating attempts were preceded by emission of FS2 (six out of eight trials), though in two cases mating occurred without any detectable signal emission by females. Comparing trials in which a duet was established, the number of pairs that mated in presence or absence of rivalry contests was not significantly different ($G = 1.2$, $P = 0.27$). The time spent to achieve copula, when rivalry occurred, varied from 970 to 5362 s (median, 3546 s; n = 8) and was not significantly longer than in absence of

rivalry (range, 625-3572 s; median, 2482.5 s; n = 12; Mann-Whitney: U = 27, P = 0.11).

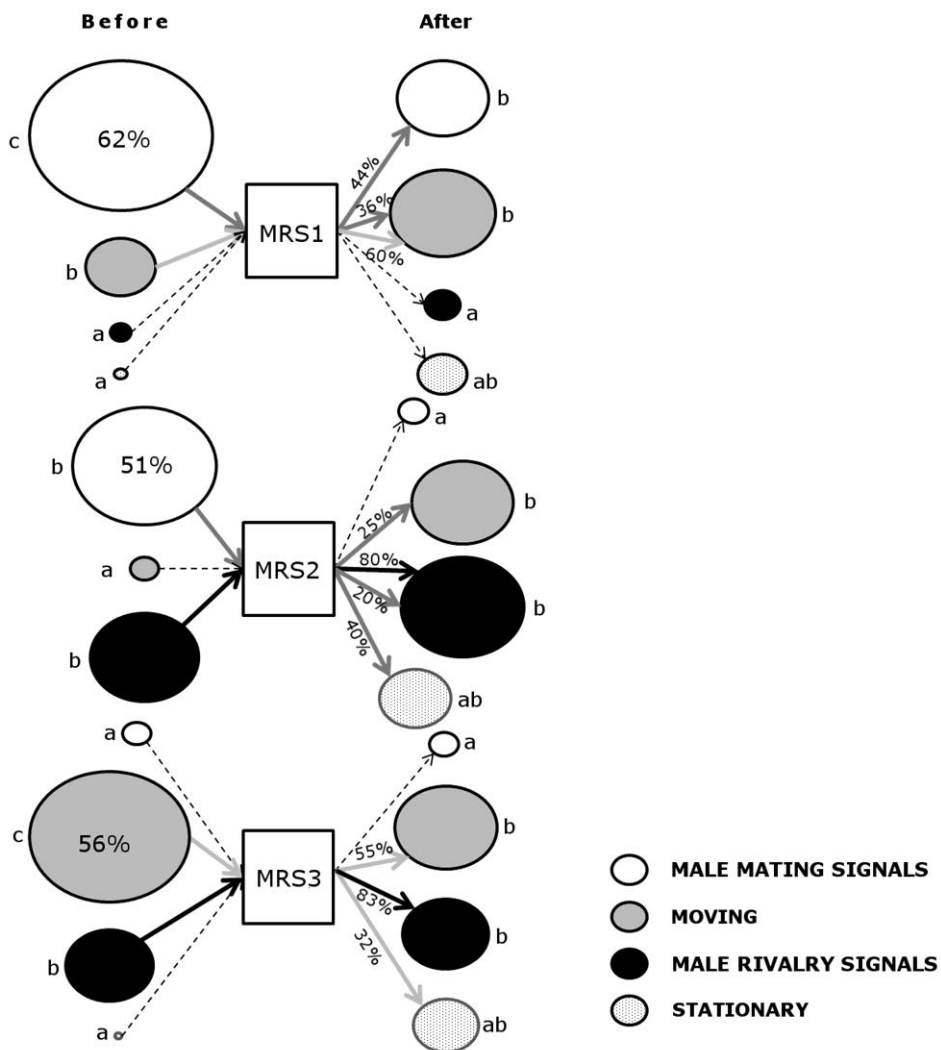


Figure 5 Interaction of the three types of male rivalry signals (MRS1, MRS2, MRS3) with preceding and following male behaviors (movements and signaling). Different letters within individual ethograms indicate significant differences ($P < 0.05$) on the percentages of observed transitions either preceding (“before”) or following (“after”) any MRS. Circle sizes are proportional to the observed frequency of the transition. Dashed lines indicate transitions $< 10\%$. Percentages on the arrows on the “after” side indicate how the main before behavior followed after the emission of MRS (split values $< 20\%$ are not reported)

Discussion

This study provided a comprehensive description of substrate-borne signals produced by GWSS and novel insights for the general mate selection behavior of leafhoppers. Similar to other species in Auchenorrhyncha (Čokl & Virant-Doberlet, 2003), GWSS vibrational signals were necessary to establish interaction between sexes, dependent on the behavioral context, and were essential to accomplish mating. However, GWSS mating behaviors and associated signals differ in many aspects from the stereotyped scheme known for other leafhoppers (Saxena and Kumar 1984; Hunt and Nault 1991; Mazzoni et al. 2009b; de Groot et al. 2012).

First, when placed individually on plants both male and female GWSS spontaneously emitted calling signals, but in contrast with other leafhopper species females were more likely to signal than males. Therefore, duets in GWSS appear to be initiated more commonly by the female, which led communication in the initial phase of the mate selection behavior. Percy et al. (2008) reported a few spontaneous GWSS male signals (similar to MS2 described here) and absence of female signaling activity, which presumably occurred due to reproductive immaturity of tested females. Spontaneous signaling from both genders is well known to occur in planthoppers (Ichikawa and Ishii 1974; Booiij 1982; Virant-doberlet and Žežlina 2007; Mazzoni et al. 2010), but not in leafhoppers where usually males initiate the pair formation process (Claridge 1985; Heady et al. 1986; Hunt et al. 1992; Čokl and Virant-Doberlet 2003; Mazzoni et al. 2009b; de Groot et al. 2012). Second, there was a rapid inversion of female leading/male replying roles resulting in males leading subsequent duets to locate the female.

In general, energetically costly signals are emitted later in the process during courtship (Polajnar et al. 2014), while during identification of potential partners the energetic demand is rationalized (Kuhelj et al. 2015). However, GWSS females emitted the longest signals, presumably the more energetically demanding, during the first phase of the mating process: identification; FS1 and FS2 used in the second phase, courtship, were shorter and sporadically emitted. After identification, GWSS males emitted a larger number of courtship-specific signals (MS2 and Qv) presumably to elicit female acceptance, which was ultimately expressed by emission of FS2. As an illustration, there was a large reduction in relative female signaling activity, as determined by the female:male signaling ratio, between identification (1:1) and courtship (1:4) phases. Observations from rearing conditions similar to conditions described in this study showed that GWSS preoviposition period was variable and ranged from nine to 285 days after adult molt (Krugner 2010). Therefore, one hypothesis to be tested is that a GWSS female spends more energy to provide the male(s) with cues about availability of a potential mate, but once identified by the male the remainder of the mate selection process relies heavily on male efforts. A similar behavioral pattern was observed in the planthopper, *Hyalesthes obsoletus*

Signoret (Hemiptera: Cixiidae) (Mazzoni et al. 2010), with females starting the duet. After an exchange of *H. obsoletus* male pulses and female pulse trains, there was an inversion of roles resulting in females reducing their signaling activity. Such behavioral similarities could be a consequence of ecological convergence due to an aggregated population structure, which is typical of both species.

GWSS males were able to locate females even during relatively long periods of an absence of female signals. In one case, mating occurred without female signaling during courtship. When potential mates were relatively close to each other, it is possible that other cues (e. g. visual or chemical) could be used by males to find a mate. Further studies with varying initial distances and more complex plant architectures should be performed to fully understand the roles of GWSS signals. In addition, it cannot be excluded that GWSS mate selection behaviors on an architecturally more complex host plant could be further separated into additional phases, as observed in the leafhopper *S. titanus* where males initiated emission of courtship signals only after arriving on the same leaf of the female (Mazzoni et al. 2014; Polajnar et al. 2014).

Male-male interactions and emission of rivalry signals are common in Auchenorrhyncha; usually the role is to disturb or mask an ongoing male-female duet (Booij 1982; Heady et al. 1986; Nuhardiyati and Bailey 2005; Miranda 2006; Mazzoni et al. 2009a; Mazzoni et al. 2009b). Compared to the aggressive male interactions occurring in leafhoppers and planthoppers (Mazzoni et al. 2009a; Mazzoni et al. 2009b), GWSS rivalry behavior was more similar to rivalry behaviors of the treehopper, *Ennya chrisura* Fairmaire (Hemiptera: Membracidae) (Miranda 2006). In both species, the rival signal is emitted by both males when in close proximity to each other, often including non-aggressive physical contact and sometimes mounting. Miranda (2006) hypothesized that the siren signal emitted by males could be indication that the male is in contact with another male, but also used for courtship interference. In GWSS, a ritual competition between males was performed, made of visual (e.g., the bent body) and vibrational signals. Emission of MRS by the rival male interrupted previously ongoing duets. Therefore, results presented here seem to support the hypothesis of Miranda (2006).

GWSS male rivalry behavior contains unique elements that include a repertoire of three distinct rivalry signals with different temporal and spectral features as well as subsequent functions. Discriminant and behavioral analyses suggest that MRS1 and MRS2 are FS1 mimics of identification and courtship, respectively. Hence, MRS1 would serve to mimic a female signal to 1) disrupt the ongoing male-female duet and 2) establish a new duet between males. Similarly, MRS2 elicited walking, leading males to meet on the plant. Finally, the close contact male-male competition was performed accompanied by emission of MRS3. To our knowledge, this behavior is not known to occur in leafhoppers or other insects that use acoustic mimics. In general, intraspecific sexual mimicry is found in species in which a large number of males compete

for few females, as an alternative tactic to disrupt the rival and thus increase own chances to mate (Greenfield 2002; Bailey et al. 2006).

Examples of acoustic female mimic occurs within Cicada and stoneflies; in both examples, the male mimicking the female is the one who initiated the duet (Luo and Wei 2015; Boumans and Johnsen 2015). In contrast, mimicry in GWSS is performed by the second (rival) male. A similar case was described in bushcrickets, genus *Caedicia*, where males stimulated with a playback of male-female duet emitted female-like clicks (Bailey et al. 2006). The authors concluded that males use this tactic to distract the other male and have a chance to mate. In our experiments, the rival (second male) was able to interrupt the duet, thus supporting the ‘male distraction hypothesis’, in that mimicry is usually performed to increase opportunities for the alternative male to acquire a mate (Forsyth and Alcock 1990; Field and Keller 1993). However, the lack of significant differences in mating accomplishments in presence and absence of rivalry behavior indicates that females accept either of the rival males. This means that this type of male rivalry behavior might give a selective advantage for female choice, which is common in several bird species that use vocal mimicry (Dalziell et al. 2015). Although additional evidence is needed to demonstrate that, it is possible that rivalry behaviors in GWSS establish male hierarchy.

In conclusion, GWSS communication is characterized by emission of vibrational signals with specific roles in all stages of the mate selection behavior. Given the relatively large repertoire of GWSS signals and length of communication leading to copula, results suggest that further studies are warranted to identify disruptive tactics using artificial playback.

Compliance with ethical standards

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Author contribution statement

RN, VM, and RK conceived and designed research. RN conducted experiments. RN, VM and SG analyzed data. All authors wrote and approved the manuscript.

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References

- Alexander, R.D.** 1967. Acoustical communication in arthropods. *Annual Review of Entomology*, **12**, 495–526.
- Alexander, R.D., Marshall, D.C., & Cooley, J.R.** 1997. Evolutionary perspectives on insect mating. In *The evolution of mating systems in insects and arachnids* (Ed. by J.C. Choe & B.J. Crespi), pp 4–31. Cambridge University Press.
- Bailey, W., Macleay, C., & Gordon, T.** 2006. Acoustic mimicry and disruptive alternative calling tactics in an Australian bushcricket (Caedicia; Phaneropterinae; Tettigoniidae; Orthoptera): does mating influence male calling tactic? *Physiological Entomology*, **31**, 201–210.
- Bailey, W.J.** 2003. Insect duets: underlying mechanisms and their evolution. *Physiological Entomology* **28**, 157–174.
- Blua, A.M.J., Redak, R.A., Morgan, D.J.W., & Costa, H.S.** 2001. Seasonal flight activity of two *Homalodisca* species (Homoptera: Cicadellidae) that spread *Xylella fastidiosa* in Southern California. *Journal of Economic Entomology* **94**, 1506–1510.
- Booij, C.J.H.** 1982. Biosystematics of the Muellierianella complex (Homoptera, Delphacidae), interspecific and geographic variation in acoustic behaviour. *Zeitschrift für Tierpsychologie*, **58**, 31–52.
- Boumans, L., & Johnsen, A.** 2015. Stonefly duets: vibrational sexual mimicry can explain complex patterns. *Journal of Ethology*, **33**, 87–107.
- Charif, R.A., Waack, A.M., & Strickman, L.M.** 2010. *Raven Pro 1.4 User's Manual*. Cornell Lab. Ithaca, NY
- Chen, W., Leopold, R.A., & Boetel, M.A.** 2010. Host plant effects on development and reproduction of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Homoptera: Cicadellidae). *Environmental Entomology* **39**, 1545–53.
- Claridge, M.F.** 1985. Acoustic behavior of leafhoppers and Planthoppers: Species Problems and Speciation. In *The Leafhoppers and Planthoppers*. (Ed. by Nault, L.R. & Rodriguez, J.G.), pp 103–125. Wiley & Sons, Inc, New York.
- Cocroft, R.B., & Rodriguez, R.L.** 2005. The behavioral ecology of insect vibrational communication. *Bioscience* **55**, 323–334.
- Čokl, A., & Virant-Doberlet, M.** 2003. Communication with substrate-borne signals in small plant-dwelling insects. *Annual Review of Entomology* **48**, 29–50.
- Dalziell, A.H., Welbergen, J.A., Igic, B., & Magrath, R.D.** 2015. Avian vocal mimicry: a unified conceptual framework. *Biological Reviews*, **90**, 643–668.

- Davis, M.J., Purcell, A.H., & Thomson, S.V.** 1978. Pierce's disease of grapevines: isolation of the causal bacterium. *Science* **199**, 75–7.
- de Groot, M., Derlink, M., Pavlovčič, P., Prešern, J., Čokl, A., & Virant-Doberlet, M.** 2012. Duetting behaviour in the leafhopper *Aphrodes makarovi* (Hemiptera: Cicadellidae). *Journal of Insect Behavior* **25**:419–440.
- Eriksson, A., Anfora, G., Lucchi, A., Lanzo, F., Virant-Doberlet, M., & Mazzoni, V.** 2012. Exploitation of insect vibrational signals reveals a new method of pest management. *PloS One*, **7**(3), e32954.
- Fabre C.C.G., Hedwig B., Conduit G., Lawrence P.A., Goodwin S.F., Casal J.** 2012. Substrate-borne vibratory communication during courtship in *Drosophila melanogaster*. *Current Biology* **22**, 2180-2185.
- Field, S.A., & Keller, M.A.** 1993. Alternative mating tactics and female mimicry as post-copulatory mate-guarding behaviour in the parasitic wasp *Cotesia rubecula*. *Animal Behaviour* **46**:1183–1189.
- Forsyth, A., & Alcock, J.** 1990. Female mimicry and resource defense polygyny by males of a tropical rove beetle, *Leistotrophus versicolor* (Coleoptera : Staphylinidae). *Behavioral Ecology and Sociobiology*, **26**, 325–330.
- Goodman, L.A.** 1968. The analysis of cross-classified data: independence, quasi-Independence, and interactions in contingency tables with or without missing entries. *Journal of the American Statistical Association*, **63**, 1091–1131.
- Greenfield, M.D.** 2002. *Signalers and receivers mechanisms and evolution of arthropod communication*. Oxford University Press, New York
- Haccou, P., & Meelis, E.** 1992. *Statistical analysis of behavioural data: An approach based on time-structured models*. Oxford University Press, Oxford.
- Heady, S.E., Nault, L.R., Shambaugh, G.F., & Fairchild, L.** 1986. Acoustic and mating behavior of *Dalbulus* leafhoppers (Homoptera: Cicadellidae). *Annals of the Entomological Society of America*, **79**, 727–736.
- Hummel, N.A., Zalom, F.G., Toscano, N.C., Peng, C.Y.S., & Burman, P.** 2006. Seasonal patterns of female *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae) reproductive physiology in Riverside, California. *Environmental Entomology* **35**, 901–906.
- Hunt, R.E., & Nault, L.R.** 1991. Roles of interplant movement, acoustic communication, and phototaxis in mate-location behavior of the leafhopper *Graminella nigrifrons*. *Behavioral Ecology and Sociobiology* **28**, 315–320.
- Hunt, R.E., Fox, J.P., & Haynes, K.F.** 1992. Behavioral response of *Graminella nigrifrons* (Homoptera: Cicadellidae) to experimentally manipulated vibrational signals. *Journal of Insect Behavior* **5**, 1–13.
- Ichikawa, T., & Ishii, S.** 1974. Mating signal of the brown planthopper, *Nilaparvata lugens* Stal (Homoptera: Delphacidae): vibration of the

- substrate. *Applied Entomology and Zoology*, **9**, 196–198.
- Krugner, R.** 2010. Differential reproductive maturity between geographically separated populations of *Homalodisca vitripennis* (Germar) in California. *Crop Protection* **29**, 1521–1528.
- Krugner, R., Groves, R.L., Johnson, M.W., Arnel, P. Hagler, J.R., Morse, J.G., & Flores, A.P.** 2009. Seasonal population dynamics of *Homalodisca vitripennis* (Hemiptera : Cicadellidae) in sweet orange trees maintained under continuous deficit irrigation. *Journal of Economic Entomology* **102**, 960–973.
- Kuhelj, A., de Groot, M., Pajk, F., Simčič, T., & Virant-Doberlet, M.** 2015. Energetic cost of vibrational signalling in a leafhopper. *Behavioral Ecology and Sociobiology*, **69**(5), 815–828.
- Luo, C., & Wei, C.** 2015. Intraspecific sexual mimicry for finding females in a cicada: males produce “female sounds” to gain reproductive benefit. *Animal Behaviour* **102**, 69–76.
- Mazzoni, V., Lucchi, A., Cokl, A., Presern, J., & Virant-Doberlet, M.** 2009a. Disruption of the reproductive behaviour of *Scaphoideus titanus* by playback of vibrational signals. *Entomologia Experimentalis et Applicata*, **133**(2), 174–185.
- Mazzoni, V., Presern, J., Lucchi, A., & Virant-Doberlet, M.** 2009b. Reproductive strategy of the Nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). *Bulletin of Entomological Research*, **99**(4), 401–13.
- Mazzoni, V., Lucchi, A., Ioriatti, C., Virant-Doberlet, M., & Anfora, G.** 2010. Mating behavior of *Hyalesthes obsoletus* (Hemiptera: Cixiidae). *Annals of the Entomological Society of America*, **103**(5), 813–822.
- Mazzoni, V., Eriksson, A., Anfora, G., Lucchi, A., & Virant-Doberlet, M.** 2014. Active space and the role of amplitude in plant-borne vibrational communication. In *Studying vibrational communication* (Ed. by R. Cocroft, M. Gogala, P.S.M. Hill, & A. Wessel), pp. 349–374. Springer, Berlin Heidelberg.
- Miranda, X.** 2006. Substrate-borne signal repertoire and courtship jamming by adults of *Ennya chrysur* (Hemiptera: Membracidae). *Annals of the Entomological Society of America*, **99**, 374–386.
- New, M.** 1994. Statistics for the behavioural sciences. *Behavioral Research and Therapy*, **32**, 591.
- Nuhardiyati, M., & Bailey, W.** 2005. Calling and duetting behavior in the leafhopper *Balclutha incisa* (Hemiptera: Cicadellidae: Deltocephalinae): opportunity for female choice? *Journal of Insect Behavior* **18**, 259–280.
- Percy, D.M., Boyd, E.A., & Hoddle, M.S.** 2008. Observations of acoustic signaling in three sharpshooters: *Homalodisca vitripennis*, *Homalodisca liturata*, and *Graphocephala atropunctata* (Hemiptera: Cicadellidae). *Annals of the Entomological Society of America* **101**, 253–259.
- Polajnar, J., Eriksson, A., Rossi Stacconi, M. V., Lucchi, A., Anfora, G.,**

- Virant-Doberlet, M., & Mazzoni, V.** 2014. The process of pair formation mediated by substrate-borne vibrations in a small insect. *Behavioural Processes*, **107**, 68–78.
- Ryan, T. H.** 1960. Significance tests for multiple comparison of proportions, variances, and other statistics. *Psychological Bulletin*, **57**(4), 318–328.
- Saxena, K. N., & Kumar, H.** 1984. Acoustic communication in the sexual behaviour of the leafhopper, *Amrasca devastans*. *Physiological Entomology*, **9**, 77–86.
- Sisterson, M.S.** 2008. Effects of insect-vector preference for healthy or infected plants on pathogen spread: insights from a model. *Journal of Economic Entomology* **101**, 1–8.
- Sisterson, M.S.** 2012. Host selection by *Homalodisca vitripennis*: the interplay between feeding, egg maturation, egg load, and oviposition. *Arthropod-Plant Interactions* **6**, 351–360.
- Sisterson, M.S.** 2014. Evaluation of a method to quantify glassy-winged sharpshooter (Hemiptera: Cicadellidae) egg maturation during a feeding assay. *Journal of Economic Entomology* **107**, 206–214.
- Sisterson, M.S., & Stenger, D.C.** 2016 Disentangling effects of vector birth rate, mortality rate, and abundance on spread of plant pathogens. *Journal of Economic Entomology* **109**, 487–501.
- Sorensen, J.T., & Gill, R.J.** 1996. A range extension of *Homalodisca coagulata* (Say) (Hemiptera: Clypeorrhyncha: Cicadellidae) to southern California. *Pan-Pacific Entomologist* **72**, 160–161.
- Stenger, D.C., Sisterson, M.S., & French, R.** 2010 Population genetics of *Homalodisca vitripennis* reovirus validates timing and limited introduction to California of its invasive insect host, the glassy-winged sharpshooter. *Virology* **407**, 53–59.
- Triapitsyn, S.V., & Phillips, P.A.** 2000. First record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae) with notes on the distribution of the host. *Florida Entomologist* **83**, 200–203.
- Virant-doberlet, M., & Žežlina, I.** 2007. Vibrational communication of *Metcalfa pruinosa* (Hemiptera: Fulgoroidea: Flatidae). *Annals of the Entomological Society of America*, **100**(1), 73–82.
- Zar J.H.** 1999. *Biostatistical analysis*. Pearson Education India, New Delhi.

Supplementary material

Supplement table 1 Markovian transition matrix. Number of transitions observed between signals and behaviors that constitute the process of pair formation in glassy-winged sharpshooter, starting from the Identification Duet (Phase 1).

	MS1	MS2	FS1	FS2	Qv	Move	M At	Mate	Total
Phase 1	0	6	0	0	9	5	0	0	20
MS1	20	15	51	1	71	3	0	0	161
MS2	18	11	33	11	90	2	1	0	166
FS1	38	7	15	0	37	28	1	0	126
FS2	1	3	0	0	8	25	13	0	50
Qv	62	108	22	32	0	14	1	0	239
Move	22	17	3	5	25	0	1	0	73
M At	0	0	0	1	4	0	0	12	17
	161	167	124	50	244	77	17	12	852

Select signals: MS1 = male signal 1, MS2 = male signal 2, Qv = quivering, FS1 = female signal 1, and FS2 = female signal 2. Select behaviors: Move = male movement (i.e., walking), M At = mating attempt, and Mate = mating.

Chapter 5

Abdominal drumming produces exaggerated host's vibrations in a social parasite wasp

5. Abdominal drumming produces exaggerated host's vibrations in a social parasite wasp

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Abstract

Caste determination is an intriguing issue in the study of sociality, particularly in primitive eusocial species, such as *Polistes* paper wasps in which the difference between workers and reproductive individuals is limited. Recently, it has been suggested that the vibrations induced in the nest by oscillation movements of the foundresses modulate nourishment effect on pre-imaginal caste determination (“mechanical switch hypothesis”). The obligate social parasite *Polistes sulcifer* lacks the worker caste, it does not provide any brood care, and it relies exclusively on the host workers force to rear its own brood. Interestingly, after having usurped a host colony, *P. sulcifer* performs a peculiar and intense abdomen oscillatory movement (Abdominal drumming, AbD) which resembles the Abdominal Wagging (AbW) performed by the host foundresses. Here we (a) characterized and compared the AbW and the AbD induced vibrations and (b) evaluated whether the AbW can be modulated by the emitter. Thanks to a laser vibrometer we recorded the vibrations produced by the host while feeding or not larvae on both usurped and not-usurped colonies. On usurped colonies, we also recorded the parasite vibrations induced by its peculiar oscillatory movement. Results show that both species produce surface-borne vibrations consisting in groups of repeated broad-band pulses. The host is able to modulate the dominant frequency and the rate of pulses when feeding larvae, respectively decreasing and increasing the two parameters. The vibrations produced by AbD of the parasite can be clearly discriminated by the AbW vibrations mainly for the higher number and duration of the pulses composing each event. We hypothesized that the host foundress might modulate its behavior when feeding larvae to better influence the nourishment effect and that the AbD of the parasite might represent an exaggeration of the host AbW aimed to ensure the host larvae development into workers, and thus the presence of adults which will provide cares to its own brood.

Key words: paper wasp, *Polistes*, abdominal wagging, caste determination, laser vibrometer, biotremology.

Introduction

Insect societies rely on activities coordination, individuals and roles differentiation among colony members (Wilson 1971). Such a complex coordination has been shown to be mainly related to semiochemicals, and chemical communications has indeed been considered almost the exclusive communication channel governing insect social life for a long time (Richard & Hunt 2013). Recently, however, vibrational signals have been shown to play crucial roles within insect societies communication. Indeed, in some species vibration production modalities and signals can replace or reinforce chemical communication (Cocroft & Rodriguez 2005; Hunt & Richard 2013), such as drumming alarm signals in termites (Hertel et al. 2011) and stridulatory recruitment signals in ants (Hölldobler & Roces 2000).

Among all social insects, social wasps of the genus *Polistes* are very good candidates for this kind of communication, as (1) colony activities take place mainly on their nest which is made of paper material, known to properly convey superficial vibrations and (2) the body oscillatory movements, which are widespread in the genus, potentially produce vibrations (Hunt & Richard 2013).

Body oscillations in *Polistes* paper wasps can be divided in three distinct oscillatory behaviors: ‘lateral vibration’ (LV), ‘antennal drumming’ (AD), and ‘abdominal wagging’ (AbW). LV consists in the wasp standing on the nest shaking the entire body horizontally to it. The shaking movement is intense and produces an audible sound (Gamboa & Dew 1981). The other two movements involve only part of the body: AD is performed by the wasp hitting its antennae on the cell rims (Pratte & Jeanne 1984), while AbW consists in the horizontal abdomen oscillation performed by foundress walking over cells, often rubbing the nest surface with the abdomen (Brillet et al. 1999; Brennan 2007). The AbW, which is the only oscillation movement known to occur in the common European species, *P. dominula*, is the only one whereof the surface-borne vibrations produced have been recorded by an accelerometer and analyzed (Brennan 2007). However, Brennan description considers only AbW performed while the performing individual was feeding larvae, but some AbW occur when the foundress is inspecting cells content without being actively involved in feeding (Brillet et al. 1999). Indeed, all three oscillatory movements are considered to be signals directed to the brood, because they are strictly related to the presence of larvae in the nest and they are mainly performed while the wasp is feeding them (Savoyard et al. 1998; Brillet et al. 1999; Cummings et al. 1999). However, the function of these putative signals is not clearly defined for all of them and in all the species in which they have been observed. For instance in *P. fuscatus*, LV signals to larvae to withdraw their saliva, which is usually released to adults, because they are going to be feed (Savoyard et al. 1998; Cummings et al. 1999). But recently, a more intriguing hypothesis involving low frequency vibrations and caste determination has been proposed (Jeanne 2009). Contrary to advanced social insect species, in primitively

eusocial wasps such as *Polistes* paper wasps, caste determination is rather flexible, because they lack a fixed morphological difference and females are categorized in queen, workers, and gynes mainly on their behavior and occurrence according to the different phases of colony development (Reeve 1991). The alpha is the highest rank foundress that monopolize the reproduction on the nest (Queller et al. 2000), workers are the first emerging offspring of the alpha, and gynes are females emerging late in the summer that mate, overwinter, and become the foundresses the following year. The overwinter ability of gynes is likely due to the well-developed fat-bodies, which are lacking in workers (Strassmann et al. 1984; Toth et al. 2009). Even if the physiological mechanisms which bias individual development into a worker or gynes phenotype are not completely solved, they are supposed to occur in a pre-imaginal phase and to be likely related to the nutritional status of larvae (i.e. well-fed female larvae develop into gynes) (O'Donnell 1998; Hunt & Amdam 2005; Hunt et al. 2007). The “mechanical switch hypothesis” suggests that low frequency vibrations transferred to the nest by oscillatory movements would act as a modulator of nourishment affecting the biogenic amine levels and eventually results in a differentiated gene expression, affecting larval growing rates and development of caste-specific traits (Jeanne 2009). In particular, the foundress produces vibrations which are transmitted to the larvae by the comb, resulting in a manipulation of larvae development into workers. To date, this hypothesis has been demonstrated in *P. fuscatus* through mechanical manipulation, in which the transmission of AD similar frequencies transduced by a piezoelectric device to the nest determined the emergence of individuals with low fat-bodies quantities (i.e. a worker phenotype) (Suryanarayanan et al. 2011). Given that AbW in *P. dominula* transmits vibrations to the nest (Brennan 2007) and recently field evidences show a relation between caste ratio and AbW occurrence in *P. biglumis* (Mignini & Lorenzi 2015), it has been suggested that also AbW vibrations can be involved in caste determination in species other than *P. fuscatus* (Mignini & Lorenzi 2015).

Obligate social parasites of genus *Polistes* represent a good opportunity to experimentally test the “mechanical switch hypothesis”. In fact, as obligate social parasite species do not have a worker caste and their reproductive success depends on the parental behavior performed by workers of the host species towards their brood (Cervo 2006), they would benefit from exploiting the vibrational mechanism involved in the host caste determination to drive the development of host larvae towards worker phenotype and/or that of their own larvae towards reproductive phenotype. *Polistes* social parasite successfully exploit the host communication systems to increase their fitness (Cervo 2006; Lorenzi 2006). For instance, they are very able in mimicking the behavior and cuticular hydrocarbons chemical profile of the alpha foundress to integrate in the host colony (Turillazzi et al. 2000; Sledge et al. 2001; Dapporto et al. 2004; Lorenzi 2006). Thus, it would not be surprising that the parasite females exploit

the host caste determination mechanism to drive phenotypic plasticity of usurped colony larva at its advantage.

Since *P. dominula* is known to produce vibrations only by AbW (Brillet et al. 1999; Brennan 2007) and *P. sulcifer* is an obligate social parasite specialized on *P. dominula* (Cervo 2006), the host – parasite system *P. dominula* – *P. sulcifer* seems to be a good model to study vibrations' role on *Polistes* nest. Noteworthy, *P. sulcifer* females perform a movement of the abdomen perpendicular to the nest, which is so intense to produce an audible sound (Cervo 1990; Cervo 2006). Likely this behavior is responsible for vibrations at the same manner that host foundress AbW does. However, the behavior itself and the features of the putative produced vibrations remain unknown.

To investigate the role of vibrations in the parasite – host system, which can lead to interesting insights on the role of vibrations in caste determination in *Polistes*, we first recorded and compared the vibrations produced by the two species on usurped and not-usurped nests. Secondary, we investigated if the host species modulate its vibrations according to the contexts: feeding and while inspecting cells. In fact, if vibrations interfere with biochemical pathways associated with larval nutrition, we expect them to be enhanced while larvae are fed.

Materials and methods

Host and parasite colony cycle

P. dominula has a typical *Polistes* colony cycle (Reeve 1991). Colonies are founded in spring by one or more mated foundresses; in the latter case, soon after foundation, a linear hierarchy is established by agonistic interactions and it is maintained by means of ritualized dominance behaviors (Pardi 1996). One foundress becomes the alpha female and lays most of the eggs (more than 90%, Queller et al. 2000) while the other females become subordinates and perform all the colonial works. At the end of May, the worker-phase starts with the emergence of the first workers. Workers help until the end of the season rearing additional brood. Reproductive individuals, males and females, emerge only later in the season, from the end of July. Mating occurs outside of the colony at the end of summer (Beani 1996) and only mated females overwinter to start a new colony the following spring. Contrary to the wide distribution of *P. dominula* (Cervo et al. 2000), *P. sulcifer* distribution is limited to the Mediterranean and the Caspian basin (Cervo 2006). Mated parasite females fight with *P. dominula* foundresses to usurp their nest prior the emergence of workers (Cervo and Turillazzi 1996; Ortolani et al. 2008). After the usurpation the parasite takes the role of the higher ranked female by mimicking its chemical profile (Sledge et al. 2001; Dapporto et al. 2004). Even if the host reproductive activity is not completely suppressed (Cini et al. 2014), the

parasite lays its eggs in the nest and rely exclusively on the host workers force to rear its brood (Cervo et al. 2004).

Animal collection, laboratory rearing and usurpation trials

Colonies of *P. dominula* were collected during spring 2014 (n = 11) and 2015 (n = 14) in various Italian sites for recording respectively the host vibration and the parasite vibration after colony parasite usurpation (see below). At the collection, colonies had single (16 out of 25) or multiple (9 out of 25) foundresses, 45.56 ± 16.80 (mean \pm SD) cells which were full with eggs, larvae and pupae. All colonies were transferred in laboratory where they have been maintained in plastic cages (15 x 25 x 15 cm) for the entire experimental period. Water, sugar and *Galleria melonella* larvae or fly maggots were provided *ad libitum* to each colony. All cages were kept at 25 ± 2 °C and 16:8 L:D photoperiod. When multiple foundresses were present, each individual was marked with different colors and the dominance hierarchy was established. *P. sulcifer* females were collected in 2015 at the end of the overwintering phase (May) in central Italy. Once in laboratory, they were kept under overwintering conditions (7 °C) until activation. Parasites were activated at room temperature for 7 days, following Ortolani et al. (2008) protocol. After activation, at the end of May-beginning of June (when the usurpations occur in the wild), a host colony to usurp was offered to each parasite by introducing a parasite inside a host colony cage and leaving the parasite free to approach and enter the host colony (Cini et al. 2011). Successfully parasitized colonies (n = 11) were maintained in laboratory condition as previously described for the host ones.

Recording trials

Laboratory observations of 15 minutes per colony were conducted at a temperature range of 25 – 30 °C and in the central hours of the day (11:00 – 16:00), when wasps are more active on the colony. Individuals' behaviors and vibrations were recorded simultaneously by means of a video camera (HDC-TM700, Panasonic Corporation of North America) and a laser vibrometer respectively (Ometron VQ-500-D-V, Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark; PDV 100, Polytec, Germany). Prior to mount the nest in the cage, a reflecting sticker was attached on its back surface and used to focus and maximize the sensitivity of the laser beam. The substrate-borne vibrations were digitized with 48 kHz sample rate, 16-bit depth, and stored directly onto a hard drive through a multichannel LAN-XI data acquisition device (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark). All trials were carried out during the second half of May and the first of June of both years, which corresponds to the period of the host colony cycle when the first workers are ready to emerge or just emerged.

All usurped colonies were recorded once between three and 18 days after usurpation. On the other hand, each not-usurped colony was recorded twice: during a ‘default’ condition and after starving the larvae present on the nest (i.e., ‘feeding’ condition). To starve larvae we isolated all adults from the nest for 72 h, after this period we let the alpha come back to the nest and we immediately offered her a *G. melonella* larva. The recording trials after the larvae starving period were done while the alpha was feeding the larvae. Only AbW produced by α were taken into account for description and analysis.

At the recording time, there were 1.13 ± 0.74 (mean \pm SD) and 1.18 ± 0.40 host foundresses on usurped and not-usurped nests respectively. On usurped colonies there were 2.43 ± 1.50 workers, while no workers were present in not-usurped colonies.

Terminology and data analysis

All newly described vibrations were named as the behavior that produced them. We define an ‘event’ as the substrate-borne vibrations recorded with the laser vibrometer while the wasp was oscillating or beating the abdomen on the nest surface (Brennan 2007). A ‘pulse’ was defined as a physically unitary or homogeneous sound, composed of a brief succession of sine waves (Alexander 1967). The oscillatory movement of the abdomen performed by *P. sulcifer* on the nest surface was named Abdominal Drumming (AbD).

Analyzed individuals were chosen among all trials as following: the α of *P. dominula* colony that performed at least one AbW event in the ‘default’ condition and one in the feeding condition (n=5), the parasite of usurped colonies that performed at least one AbD (n=7), and the α of *P. dominula* usurped colonies that produced at least one AbW (n=6). Spectral and temporal parameters of recorded vibrations were analyzed with Raven Pro 1.4 (The Cornell Lab of Ornithology, Ithaca, NY) using Fast Fourier Transform (FFT) type Hann with window length of 256 samples and 50% overlap. To characterize spectral and temporal properties of vibrations produced by AbW and AbD for *P. dominula* and *P. sulcifer* respectively the following parameters were measured for each event and pulse composing it (see Results): duration, dominant frequency (domfreq), number of pulses, and rate of pulses in the event, as the ratio between number of pulses and the event duration.

To compare if and how AbW performed by the same foundress varied between ‘default’ and ‘feeding’ conditions, we randomly selected 3 to 8 replications for each individual (total n = 35 for each condition) and a Wilcoxon signed rank test for paired data was performed. To compare AbW performed in usurped and not-usurped colonies a Friedman test with three replications per individual was performed.

A discriminant analysis was used to determine whether vibrational events could be distinguished between species and conditions (= ‘default’, ‘feeding’, and ‘usurped’) based on temporal (duration, number of pulses) and

spectral (domfreq of events and single pulses) parameters. To standardize the number of replications, three events for individual were chosen randomly to run the discriminant analysis (total n = 69).

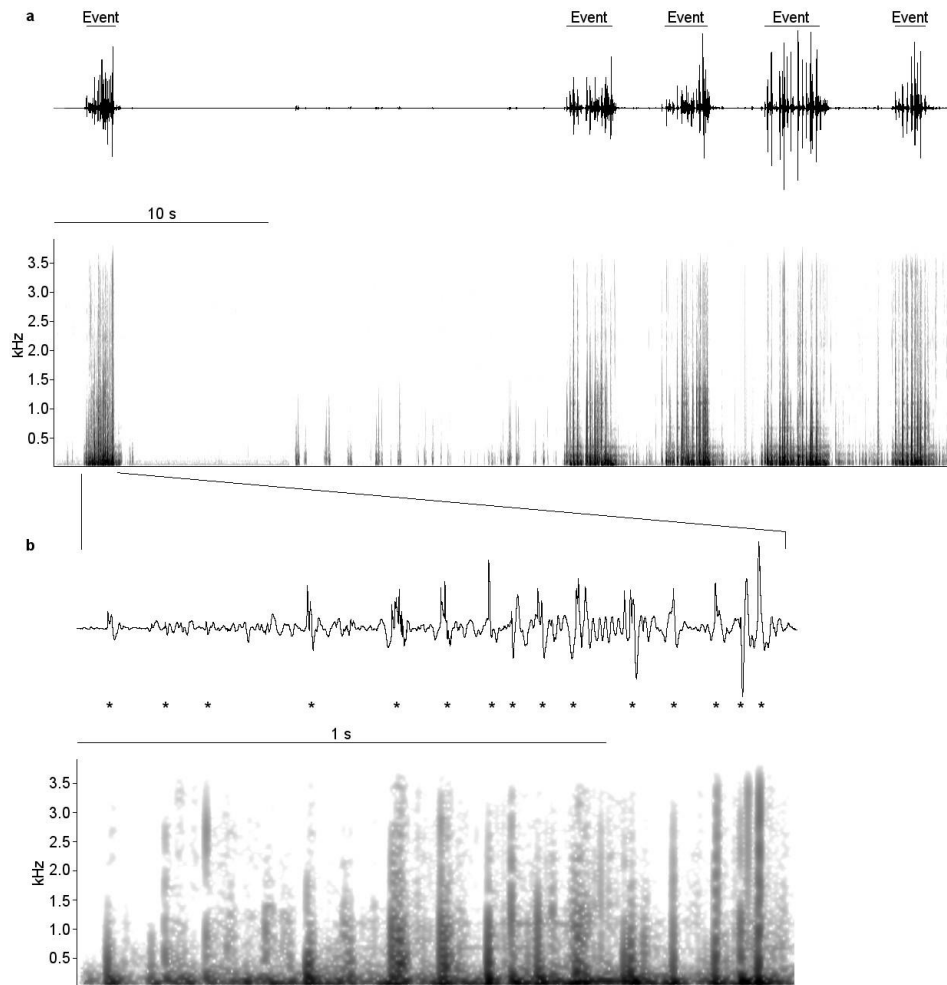


Figure 1 Oscillogram (above) and spectrogram (below) of five subsequent events of AbW performed by a *P. dominula* foundress on the nest in default conditions. In **b** the detail of an event, asterisks indicate the pulses composing the AbW.

Results

Concurrent analysis of the video and laser vibrometer recordings revealed that each abdominal wagging behavior performed by the *P. dominula* foundress on the nest produced a vibrational event detectable by the laser

vibrometer and distinguishable by its oscillogram and spectrogram, that was called AbW (Fig.1). Also the parasite produced distinguishable vibrational events when it beats the abdomen on the nest surface; we defined these vibrations Abdominal Drumming (AbD) (Fig. 2). Both vibrational events, AbW and AbD, consisted of a repetition of broad-band pulses.

Table 1 Spectral and temporal parameters of AbW and AbD.

Type	<i>P. dominula</i>			<i>P. sulcifer</i>	
	AbW_D	AbW_F	AbW_U	AbD	
N	98	72	97	160	
Event	Duration	0.61 ± 0.42	0.48 ± 0.31	0.48 ± 0.34	1.51 ± 1.05
	domfreq	48.70 ± 26.90	44.00 ± 23.68	39.92 ± 19.06	45.80 ± 26.03
	n pulses	7.17 ± 4.41	6.64 ± 3.40	6.70 ± 3.79	22.80 ± 15.60
	Pulses rate	13.72 ± 6.48	15.66 ± 5.48	16.04 ± 5.61	15.69 ± 3.81
Pulse	Duration	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	domfreq	49.34 ± 19.19	44.66 ± 16.42	38.93 ± 16.14	51.31 ± 20.19

AbW_D = AbW in not usurped colonies in ‘default’ condition; AbW_F = AbW in not usurped colonies in ‘feeding’ condition; AbW_U = AbW in usurped colonies in feeding condition. All values are given as mean ± SD. N = number of events analyzed; duration is given in seconds; domfreq = dominant frequency is given in Hz; pulse rate = pulses per second.

Host substrate-borne vibrations (Table 1-3; Fig. 1-2)

Usually AbW events lasted less than one second in all conditions and the domfreq was below 100 Hz (Table 1). Each event was composed on average of less than 10 pulses separated by a gap of silence, the number of pulses per second was variable from a minimum of 4 in ‘default’ to a maximum of 38 in ‘feeding’ context (Table 1).

The AbW varied depending on the context in which it was performed (Table 2). Therefore, we named AbW_D the AbW performed by foundresses in ‘default’ condition and AbW_F the ones performed by foundresses while feeding the larvae. Comparing AbW produced by foundresses in the two conditions (N = 5), AbW_F events had lower domfreq (Wilcoxon signed rank test, T = 220, P = 0.03). The length (Wilcoxon signed rank test, T = 228, P = 0.06) and the number of pulses (Wilcoxon signed rank test, T = 61, P = 0.62) composing the AbW were not significantly different in the two conditions, even if we observed a tendency of AbW_F to be shorter compared to AbW_D. However, there was a significant difference in the rate of pulses, which was higher in AbW_F (Wilcoxon signed rank test, T = -510, P < 0.001). Regarding

pulses features, they reflected what has been observed for the total event: significantly lower domfreq was observed for AbW_F (Wilcoxon signed rank test, $T = 425$, $P < 0.001$), while there was no difference in pulses duration (Wilcoxon signed rank test, $T = 58$, $P = 0.63$).

Table 2 Comparison of *P. dominula* AbW in ‘default’ (AbW_D) and ‘feeding’ (AbW_F) contexts after Wilcoxon signed rank test for paired data (number of colonies = 5; number of events = 35).

		AbW_D	AbW_F	T	P-value
Event	Duration	0.73 ± 0.45	0.52 ± 0.39	228	0.06
	domfreq	50.29 ± 26.07	42.06 ± 25.47	220	0.03
	n pulses	8.11 ± 4.93	7.14 ± 4.21	61	0.62
	Pulses rate	12.00 ± 3.40	15.99 ± 6.09	-510	< 0.001
Pulse	Duration	0.03 ± 0.01	0.03 ± 0.01	58	0.63
	domfreq	53.66 ± 21.58	41.25 ± 13.79	425	< 0.001

All values are given as mean ± SD. Abbreviations as Table 1. In bold, parameters which were significantly different.

All AbW of foundresses on usurped colonies (AbW_U) were performed while the wasp was feeding the larvae. Parameters of all recorded AbW_U are reported in Table 1. Comparing AbW_U with AbW performed by foundresses in not-usurped colonies in the same context (i.e. ‘feeding’, AbW_F) most of the parameters were not significantly different (see Table 3). However, a significant difference was observed in the single pulse duration, which was higher in AbW_U compared to AbW_F (Friedman test, $df = 1$, $\text{Chi}^2 = 6.94$, $P < 0.01$).

Table 3 Comparison of *P. dominula* AbW in ‘feeding’ contexts performed by foundresses on usurped (AbW_U) and not usurped colonies (AbW_F) after Friedman test with three replications (number of colonies = 5; degree of freedom = 1).

		AbW_F	AbW_U	Chi^2	P
Event	Duration	0.46 ± 0.33	0.51 ± 0.35	0.01	0.92
	domfreq	38.40 ± 17.94	36.27 ± 16.52	0.22	0.63
	n pulses	7.07 ± 5.12	6.20 ± 3.51	0.35	0.55
	Pulses rate	17.22 ± 6.91	14.54 ± 6.43	1.15	0.28
Pulse	Duration	0.03 ± 0.01	0.05 ± 0.01	6.94	< 0.01
	domfreq	39.77 ± 12.97	36.05 ± 12.60	3.20	0.07

All values are given as mean ± SD. Abbreviations as Table 1. In bold, parameters which were significantly different.

Parasite substrate-borne vibrations (Table 1; Fig. 2)

On average the AbD produced by parasites lasts more than one second (mean \pm SD: 1.51 ± 1.05 s) and consisted of a 22.80 ± 15.60 pulses (Table 1). On average, pulses were repeated at the rate of 15.69 ± 3.81 pulses per second, but the rate was particularly variable ranging from a minimum of 6 to a maximum of 49. Most of the energy produced by AbD was concentrated below 100 Hz, in particular at 51.31 ± 20.19 Hz for the single pulses and at 45.80 ± 26.03 Hz considering the whole event (Table 1).

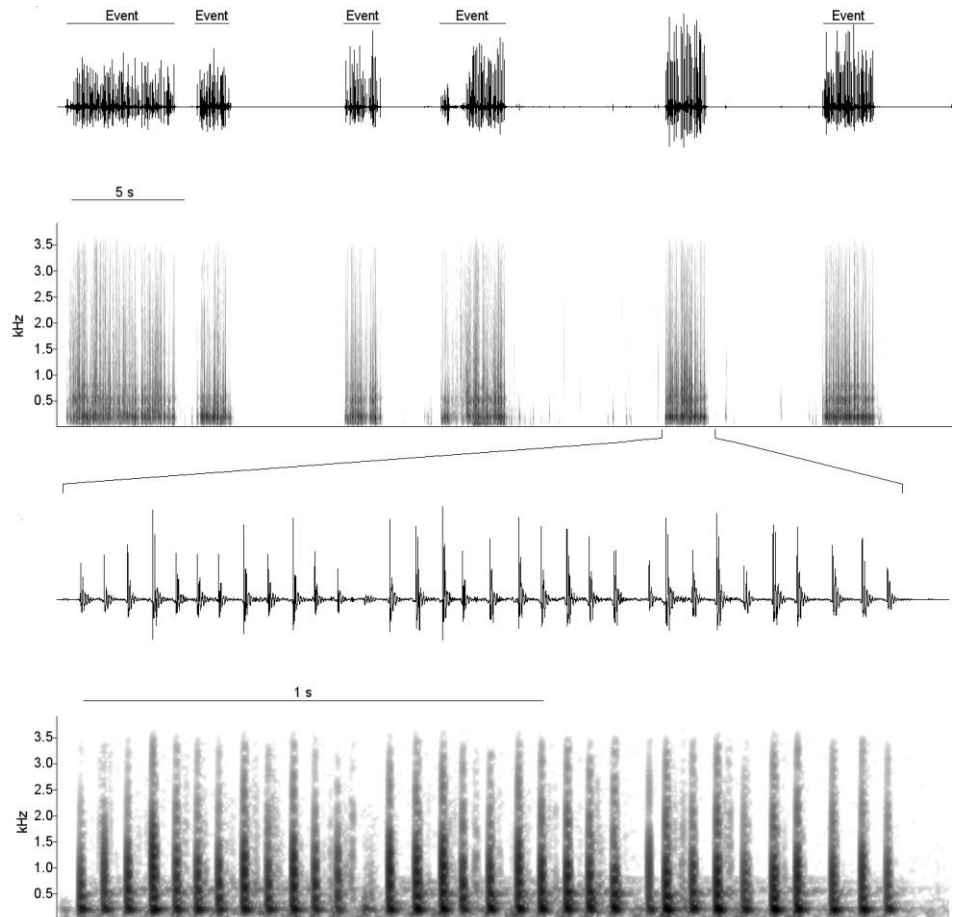


Figure 2 Oscillogram (above) and spectrogram (below) of six events of AbD produced by a *P. sulcifer* female on a usurped nest.

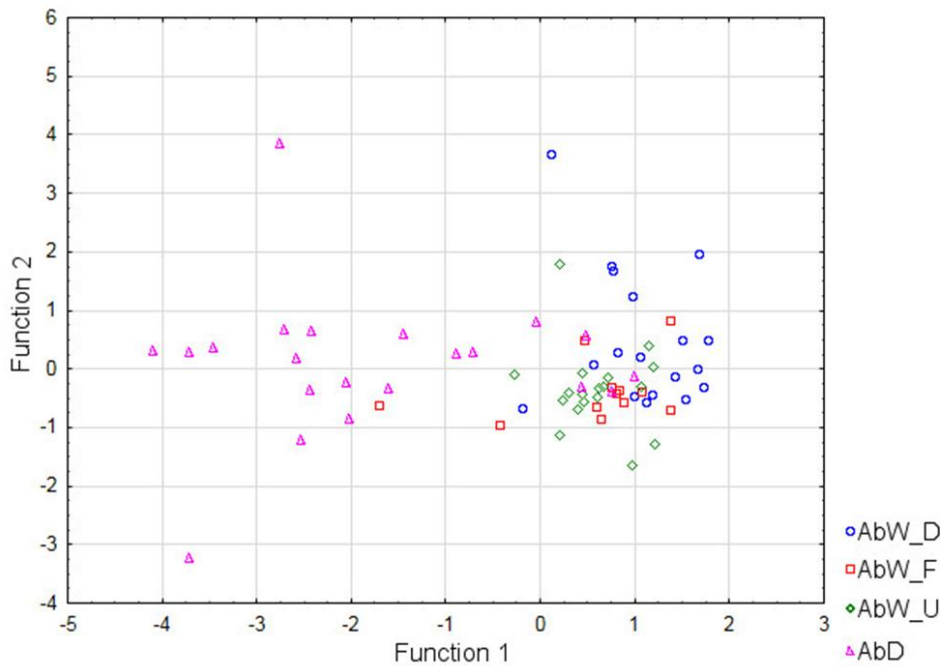


Figure 3 Combined-groups plot showing functions 1 and 2 derived from the discriminant function analysis of duration, frequency of the event and of pulses, and number of pulses. Functions 1 explained 88.2% of variance, function 2 accounted for 7.8% of variance. AbW stands for Abdominal Wagging performed by foundresses in different conditions: AbW_D in ‘default’, AbW_F in feeding, and AbW_U in usurped colonies. AbD stands for Abdominal Drumming produced by *P. sulcifer*.

Comparison between host and parasite vibrations (Table 4-5, Fig. 3)

The discriminant analysis revealed that the number of pulses and their duration had the main role in differentiating AbD and AbW performed in different conditions (Wilks’ Lambda = 0.34, $F_{(15,168)} = 5.42$, $P < 0.00001$). The significance values for each parameter are reported in Table 5. The total discriminant accuracy was 67%, but it increased to 76% when differentiating AbD from all the other AbW (Table 4). The lowest accuracy was observed when discriminating AbW in feeding conditions (42%). The first discriminant function explained the 88.2% of variance, while the second function only accounted for 7.8%. Both functions were mainly based on the number of pulses (standardized coefficients: -1.48 and -1.29 respectively). The second function was in part based also on the event duration and the domfreq of pulses (standardized coefficients: 1.45 and 1.17 respectively); however, the discriminant analysis was not significant for these parameters (Table 5). The

plot of the first function vs. the second function of the discriminant analysis (Fig. 3) showed that AbD can be easily distinguished from AbW. More uncertainty occurs when trying to distinguish AbW in the three conditions: ‘default’, ‘feeding’, and usurped colonies.

Table 4 Matrix of classification after discriminant analysis and percentage of discrimination accuracy.

	AbW_D	AbW_F	AbW_U	AbD	Percentage
AbW_D	12	1	5	0	67%
AbW_F	2	5	4	1	42%
AbW_U	3	2	13	0	72%
AbD	2	0	3	16	76%
Total	19	8	25	17	67%

AbW = vibrations produced by Abdominal Wagging in different contexts: AbW_D = in ‘default’ condition, AbW_F = AbW in ‘feeding’ condition, AbW_U = in usurped colonies; AbD = vibrations produced by Abdominal Drumming.

Table 5 Value of significance for each parameter after discriminant analysis

Parameter	Wilks' Lambda	P-value
Event duration	0.3676	0.1602
Event domfreq	0.3635	0.2145
n pulses	0.4523	0.0005
Pulse duration	0.4253	0.0027
Pulse domfreq	0.3656	0.1854

Significant parameters are in bold. Abbreviations as Table 1.

Discussion

This study provided a comprehensive description of the vibrations produced by two oscillation movement, AbW and AbD respectively produced by *P. dominula* and *P. sulcifer*. Moreover, we demonstrated that AbW vibrations produced by the same foundress are different if performed while it is directly involved in feeding the larvae or not and that, even if AbD events had similar structure to AbW (i.e., each event composed of a sequence of broad-band pulses), they were well differentiated according to the number of pulses and their duration.

The previous description of vibrations produced by AbW reported frequencies centered around 904 Hz (Brennan 2007). In contrast, our results showed that most of the energy is concentrated at much lower values of about 50 Hz. This difference is probably due to the sensitivity of the instrument used for recording and by the characteristics of the instrument itself. Contrary to the accelerometer used by Brennan, the laser vibrometer enables to completely avoid any load on the surface on which vibrations are transmitted, therefore to minimize interferences with surface-borne vibrations involved in insect communication (Michelsen & Larsen 1978; Michelsen et al. 1982; Hill & Wessel 2016). Since AbW produce vibrations with a broad-band spectrum, it is possible that only the higher components were detected by previous studies. Moreover, in both adults and immature insects, low frequencies have been demonstrated to be able to determine physiology changes, for instance the content of juvenile hormone in honeybee workers (Schneider et al. 2004), larvae growth in *Tribolium castaneum* (Hirashima et al. 1993), and fat bodies quantity in *P. fuscatus* (Suryanarayanan et al. 2011). Therefore, taking into account the “mechanical switch hypothesis”, even if *Polistes* oscillatory movements produce broad-band pulses, it is more likely that low frequency components can play a role in caste determination, triggering a cascade of events that starts from varying the quantity of biogenic amines and ends with modified gene expression, through different hormones release.

The fact that foundresses vary the parameters of their AbW, decreasing the domfreq and increasing pulse rate, while feeding larvae could supports the “mechanical switch hypothesis”. In fact, it predicts that vibrations role is necessary to modulate the effect of larval nourishment level (Jeanne 2009), which can only partially explain the phenotypic differences among workers and gynes (O’Donnell 1998; Hunt & Amdam 2005; Hunt et al. 2007; Jeanne 2009). Considering *Polistes* oscillating behaviors as modulators of nutrition, we hypothesized that the foundress modulates AbW when it is directly involved in feeding to maximize the mechanical effect in the exact moment in which nutrition occurs. The validity of this hypothesis should be tested in the future through dedicated experiments in order to experimentally demonstrate the effect of vibrations with different rate of pulses and domfreq.

Considering the production modality (drumming of the abdomen on the nest surface) and the wide broad-band spectrum, both AbW and AbD should be considered percussive signals (Elias & Mason 2010). One of the characteristic of this kind of vibrations is that the frequency profile is the result of the substrata properties (Pierce 1989). Therefore, it is possible that the paper nest structure itself emphasizes low frequencies. Future studies on the paper material and structure of the nest would be required to understand how much they affect the vibrations produced by oscillatory movements towards similar spectrum features. In this regard, the result of the discriminant analysis seems to support the hypothesis of homogeneity of spectral components. In fact, the variability of frequency features among different contexts and the two species is not sufficient

to distinguish them. On the contrary the number of pulses and their duration accounted for the major differences. It is known from spiders, in which percussive signals have been deeply investigated, that timing is the more relevant features of this kind of signals and is strictly related to the energetic costs (Elias & Mason 2010). In particular, the rate of drumming is energetically demanding (Kotiaho et al. 1998). Since the duration is not relevant in discriminating between AbW and AbD, the higher number of pulses produced by *P. sulcifer* suggests that the parasite is able to drum the abdomen on the nest surface at higher rate compared to the host, as it happens at a lighter rate when *P. dominula* is feeding larvae. This suggests that (1) the parasite spends more energy in producing these vibrations and (2) AbD can be an exaggeration of the host's AbW. Supernormal manipulation has been reported and demonstrated for several host – social parasite systems. Usually the immature brood of the parasite exaggerate begging signals to attract more care from the host adults, such as *Atemeles pubicollis* in nests of the ant *Formica polyctena* (Hölldobler & Wilson 1990) or cuckoo chicks in host nests (Redondo 1993). We suggest an inversion of the supernormal manipulation as it has been demonstrated for other animals: if vibrations modulate the nourishment effect in *P. dominula* caste determination, it is possible that AbD itself is an exaggeration of the host behavior (i.e. AbW) made by the adult parasite to manipulate the host's brood. In fact, the parasite relies exclusively on the rearing abilities of the host workers that will emerge after the nest usurpation to rear its own brood (Cervo 2006). Therefore, the possibility to manipulate their future behavior as adult workers would be surely beneficial. Why the exaggeration is not greater to entail a higher discrimination between AbW and AbD? One possibility is that there is a physical constraint to perform events with higher number of pulses, for example due to the energetic cost of moving the abdomen. A second possibility, which does not exclude the first one, is that the parasite must not stretch too much the features of the host behavior to avoid to be recognized as intruder and reject from the colony. In fact, a model developed on the game theory shows that exaggerated signals in social parasite are evolutionary supported only at low rate of parasitism or if they remain below a definite threshold to disable the host to recognize the intruder and thus reject it (Holen et al. 2001). In our system the parasite pressure is quite high, ranging from 20% to 50% (Cervo & Turillazzi 1996), thus remain under the threshold would be extremely important. However, the final proof will be to assess the role of these vibrations in *P. dominula* in future experiments, for instance testing different playbacks effect on larval development. Our results suggest that, at constant frequency components, higher number of pulses and rate should be more likely to drive larvae development towards workers phenotype, such as lower fat bodies quantity.

In conclusion, we demonstrated that *P. sulcifer* as other *Polistes* species produce surface-borne vibrations, but performing a distinctive drumming behavior within *Polistes*, AbD. The characteristics of the parasite vibrations

suggest that it is a case of exaggerated behavior performed by a social parasite to manipulate the host behavior and give useful suggestions on the parameters more likely to play a role in the “mechanical switch hypothesis”.

References

- Alexander, R.D.** 1967 Acoustical communication in arthropods. *Annual Review of Entomology* **12**, 495–526.
- Beani, L.** 1996. Lek-like courtship in paper wasps: “a prolonged, delicate, and troublesome affair.” In *Natural History and Evolution of Paper Wasps* (Ed. by Turillazzi S. & West-Eberhard M.J.), pp 113–125. Oxford University Press, Oxford.
- Brennan, B.J.**, 2007. Abdominal wagging in the social paper wasp *Polistes dominulus*: behavior and substrate vibrations. *Ethology*, **113**(7), 692–702.
- Brillet, C., Semenoff Tian-Chansky, S. & Le Conte, Y.**, 1999. Abdominal waggings and variation of their rate of occurrence in the social wasp, *Polistes dominulus* Christ. I. quantitative analysis. *Journal of Insect Behavior*, **12**(5), 665–686.
- Cervo, R.**, 1990. *Il parassitismo sociale nei Polistes*. Università di Firenze.
- Cervo, R.**, 2006. *Polistes* wasps and their social parasites: an overview. *Annales Zoologici Fennici*, **43**, 531–549.
- Cervo, R. & Turillazzi, S.**, 1996. Host nest preference and nest choice in the cuckoo paper wasp *Polistes sulcifer* (Hymenoptera: Vespidae). *Journal of Insect Behavior*, **9**(2), 297–306.
- Cervo, R., Zacchi, F., & Turillazzi, S.** 2000. *Polistes dominulus* (Hymenoptera, Vespidae) invading North America: some hypotheses for its rapid spread. *Insectes Sociaux* **47**, 155–157.
- Cervo, R., Macinai, V., Dechigi, F., & Turillazzi, S.** 2004 Fast growth of immature brood in a social parasite wasp: a convergent evolution between avian and insect cuckoos. *American Naturalist* **164**, 814–820.
- Cini, A., Bruschini, C., Poggi, L., & Cervo, R.** 2011. Fight or fool? Physical strength, instead of sensory deception, matters in host nest invasion by a wasp social parasite. *Animal behavior*, **81**(6), 1139–1145.
- Cini, A., Nieri, R., Dapporto, L., Monnin, T., & Cervo, R.** 2014. Almost royal: Incomplete suppression of host worker ovarian development by a social parasite wasp. *Behavioral Ecology and Sociobiology* **68**(3), 467–475.
- Cocroft, R.B. & Rodriguez, R.L.**, 2005. The behavioral ecology of insect vibrational communication. *BioScience*, **55**(4), 323–334.
- Cummings, D., Gamboa, G. & Harding, B.**, 1999. Lateral vibrations by social wasps signal larvae to withhold salivary secretions (*Polistes fuscatus*, Hymenoptera: Vespidae). *Journal of insect behavior*, **12**(4), 465–473.
- Dapporto, L., Cervo, R., Sledge, M.F., & Turillazzi, S.** 2004. Rank

- integration in dominance hierarchies of host colonies by the paper wasp social parasite *Polistes sulcifer* (Hymenoptera, Vespidae). *Journal of Insect Physiology* **50**, 217–223.
- Elias, D.O. & Mason, A.C.**, 2010. Signaling in variable environments: substrate-borne signaling mechanisms and communication behavior in spiders. In *The use of vibrations in communication: Properties, mechanisms and function across Taxa* (Ed. by C. E. O’Connell-Rodwell), pp. 25–46. Kerala: Transworld.
- Gamboa, G.J. & Dew, H.E.**, 1981. Intracolony communication by body oscillations in the paper wasp, *Polistes metricus*. *Insectes Sociaux*, **28**(1), 13–26.
- Hertel, H., Hanspach, A. & Plarre, R.**, 2011. Differences in alarm responses in drywood and subterranean termites (Isoptera: Kalotermitidae and Rhinotermitidae) to physical stimuli. *Journal of Insect Behavior*, **24**(2), 106–115.
- Hill, P.S.M. & Wessel, A.**, 2016. Biotremology. *Current Biology*, **26**(5), R187–R191.
- Hirashima, A., Nagano, T. & Eto, M.**, 1993. Stress-induced changes in the biogenic-amine levels and larval growth of *Tribolium castaneum* Herbst. *Bioscience Biotechnology and Biochemistry*, **57**(12), 2085–2089.
- Holen, Ø.H., Saetre, G.P., Slagsvold, T., & Stenseth, N.C.** 2001. Parasites and supernormal manipulation. *Proceedings. Biological sciences / The Royal Society*, **268**(1485), 2551–2558.
- Hölldobler, B., & Wilson, E.O.** 1990. *The Ants*. Harvard University Press, Cambridge.
- Hölldobler, B. & Roces, F.**, 2000. The behavioral ecology of stridulatory communication in leafcutting ants. In *Model systems in behavioral ecology: integrating empirical, theoretical and conceptual approaches* (Ed. by L. Dugatkin). Princeton, New Jersey: Princeton University Press, pp. 92–109.
- Hunt, J.H. & Amdam, G. V.**, 2005. Bivoltinism as an antecedent to eusociality in the paper wasp genus *Polistes*. *Science*, **308**, 264–267.
- Hunt, J.H., Kensinger, B.J., Kossuth, J.A., Henshaw, M.T., Norberg, K., Wolschin, F., & Amdam, G.V.** 2007. A diapause pathway underlies the gyne phenotype in *Polistes* wasps, revealing an evolutionary route to caste-containing insect societies. *Proceedings of the National Academy of Sciences of the United States of America*, **104**(35), 14020–5.
- Hunt, J.H. & Richard, F.J.**, 2013. Intracolony vibroacoustic communication in social insects. *Insectes Sociaux*, 403–417.
- Jeanne, R.L.** 2009. Vibrational signals in social wasps: a role in caste determination? In *Organization of insect societies: from genome to sociocomplexity* (Ed. by Gadau, J., Fewell J. & Wilson E.O.), pp. 241–263. Harvard University Press, Cambridge.
- Kotiaho, J.S., Alatalo, R.V., Mappes, J., Nielsen, M.G., Parri, S., & Rivero,**

- A. 1998. Energetic costs of size and sexual signalling in a wolf spider. *Proceedings of the Royal Society B: Biological Sciences*, **265**(1411), 2203–2209.
- Lorenzi, M.C.**, 2006. The result of an arms race: the chemical strategies of *Polistes* social parasites. *Annales Zoologici Fennici*, **43**(5/6), 550–563.
- Michelsen, A. & Larsen, O.N.**, 1978. Biophysics of the ensiferan ear - I. Tympanal vibrations in bushcrickets (Tettigoniidae) studied with laser vibrometry. *Journal of Comparative Physiology* **123**(3), 193–203.
- Michelsen, A., Fink, F., Gogala, M., & Traue, D.** 1982. Plants as transmission channels for insect vibrational songs. *Behavioral Ecology and Sociobiology* **11**, 269–281.
- Mignini, M. & Lorenzi, M.C.**, 2015. Vibratory signals predict rank and offspring caste ratio in a social insect. *Behavioral Ecology and Sociobiology*, **69**(10), 1739–1748.
- O'Donnell, S.**, 1998. Reproductive caste determination in eusocial wasps (Hymenoptera : Vespidae). *Annual review of entomology*, **43**, 323–346.
- Ortolani, I., Turillazzi, S., & Cervo, R.** 2008. Spring usurpation restlessness: a wasp social parasite adapts its seasonal activity to the host cycle. *Ethology* **114**, 782–788.
- Pardi, L.** 1996. *Polistes*: analysis of a society. In *Natural history and evolution of paper-wasps* (Ed. S. Turillazzi & M. West-Eberhard), pp. 1-17. Oxford University Press, Oxford.
- Pierce, A.D.**, 1989. *Acoustics: an introduction to its physical principles and applications*, Woodbury, NY: Acoustical Society of America.
- Pratte, M. & Jeanne, R.L.**, 1984. Antennal drumming behavior in *Polistes* wasps (Hymenoptera: Vespidae). *Ethology*, **66**, 177–188.
- Queller, D. C., Zacchi, F., Cervo, R., Turillazzi, S., Henshaw, M.T., Santorelli, L.A., & Strassmann, J.E.** 2000. Unrelated helpers in a social insect. *Nature*, **405**(6788), 784–787.
- Redondo, T.**, 1993. Exploitation of host mechanism for parental care by avian brood parasites. *Etología*, **3**, 235–297.
- Reeve, H.K.**, 1991. *Polistes*. In *The Social Biology of Wasps* (Ed. by K. G. Ross & R. H. Matthews), pp. 99–148. Ithaca, NY: Cornell University Press.
- Richard, F.J., & Hunt, J.H.** 2013. Intracolony chemical communication in social insects. *Insectes Sociaux*, **60**, 275-291.
- Savoyard, J.L., Gamboa, G.J., Cummings, D.L.D., & Foster, R.L.** 1998. The communicative meaning of body oscillations in the social wasp, *Polistes fuscatus* (Hymenoptera, Vespidae). *Insectes Soiauxc* **45**, 215–230.
- Schneider, S.S., Lewis, L.A. & Huang, Z.Y.**, 2004. The vibration signal and juvenile hormone titers in worker honeybees, *Apis mellifera*. *Ethology*, **110**(12), 977–985.
- Sledge, M.F., Dani, F.R., Cervo, R., Dapporto, L., & Turillazzi, S.** 2001. Recognition of social parasites as nest-mates: adoption of colony-specific

- host cuticular odours by the paper wasp parasite *Polistes sulcifer*. *Proceedings Biological Sciences of the Royal Society* **268**, 2253–2260.
- Strassmann, J.E., Lee, R., Rojas, R., & Baust, J.G.** 1984. Caste and sex differences in cold-hardiness in the social wasps, *Polistes annularis* and *P. exclamans* (Hymenoptera: Vespidae). *Insectes Sociaux*, **31**(3), 291–301.
- Suryanarayanan, S., Hermanson, J.C. & Jeanne, R.L.**, 2011. A mechanical signal biases caste development in a social wasp. *Current biology*, **21**(3), 231–5.
- Toth, A.L., Bilof, K., Henshaw, M., Hunt, J.H., & Robinson, G.E.** 2009. Lipid stores, ovary development, and brain gene expression in *Polistes metricus* females. *Insectes Sociaux*, **56**(1), 77–84.
- Turillazzi, S., Sledge, M.F., Dani, F.R., Cervo, R., Massolo, A., & Fondeli, L.** 2000. Social hackers: integration in the host chemical recognition system by a paper wasp social parasite. *Naturwissenschaften* **87**, 172–176.
- Wilson, E.O.** 1971. *The Insect Societies*. Oxford University Press, Cambridge.

Chapter 6

Conclusions and discussion

6. Conclusions and discussion

This thesis was developed in a new born and continuously improving environment, Biotremology, a new science that was introduced into the scientific community only in March 2016 by a primer published on the Journal Current Biology (Hill & Wessel 2016).

One of the main reasons why vibrational communication was not investigated in depth in the past was the scarce availability of sensitive instruments to record and transmit back to the substrate vibrational signals. Instruments like laser vibrometers and electromagnetic mini-shakers were not available until a couple of decades ago together with the possibility to use software that could handle gigabytes of data. Likely due to these technical constraints the role of vibrations in animal communication has been neglected for years and it's still in its childhood. This is true not only for insects, such as social wasps, that communicate mainly with semiochemicals, but also for leafhoppers. In fact, despite Cicadellidae are considered a model group to investigate vibrational communication, because of the crucial role in reproduction of vibrational signals, for most subfamilies, including Cicadellinae and Typhlocibinae object of the present study, the already existing knowledge was virtually absent.

Therefore, we were not surprised to unveil numerous original features while making this study (chapter 2, 3, and 4). The broad variability of signals that we found can be partially explained by a generally high selection pressure on mating signals. Furthermore, bending waves behave differently according to the substrate in which they are transmitted, thus vibrational signals produced by insects often tune with the substrate of their host plant. For this reason, the coevolution of the insect and the plant is considered to be involved in the speciation of some treehopper species (McNett & Coccoft 2008). In addition, we suggested that ecological habits of the studied species could have played a crucial role in shaping the evolution of the different reproductive strategy process we described in *Empoasca vitis* and *Homalodisca vitripennis*. The two species live in very different social contexts, for instance *H. vitripennis* aggregates and spend most of its time feeding on the same branch, while *E. vitis* is extremely active throughout the 24 hours and rely on the "call-fly" strategy. Thus, it is possible that the high number of signals and the complex structure of stages in the pair formation process of *H. vitripennis*, in contrast with the simpler acoustic repertoire and mating strategy of *E. vitis*, could have been shaped by its ecological habits. The same concept can be applied to the rivalry strategies that reflect this complex versus simple structure in the two species. In fact, the disruption of the duet made by rival males of *E. vitis* is extremely simpler than the articulated female mimic strategy of *H. vitripennis*. However, our hypothesis cannot be generalized yet, because of the low number of species that have been studied in detail and that makes it difficult to assess with certainty the role of ecological constraints in shaping the evolution of such

strategies. For instance, the female mimic strategy that we described in *H. vitripennis* has never been reported for any insect species, neither in acoustic or vibrational communication. Therefore, until more leafhopper species are investigated from a behavioral and phylogenetic point of view our hypotheses cannot be confirmed.

Another remarkable contribution of this thesis on leafhoppers communication systems is the potential role of stimuli other than vibrations in their reproductive process. In fact, their relevance has been neglected in the past, because vibrations have a clear dominant role. However, both the low number of matings in the dark of *E. vitis* and the peculiar bended body position of rival males of *H. vitripennis* indicate that visual cues can play a relevant role in their reproductive strategy. Therefore, to avoid the mistake that has been done in neglecting vibrations as putative signals in many animal taxa, future studies should consider the occurrence of multimodal communication also in groups in which so far investigation was limited to vibrational signals.

On the other hand, the lack of attention for vibrations led scientists to consider for more than 50 years fruit flies' (*Drosophila* spp.) mating communication exclusively mediated by airborne sounds. Only recently (Mazzoni et al. 2013), it was demonstrated that vibrational signals can play a major role in their reproduction, probably even more important than the airborne component. Similarly, in the colony life of *Polistes* a low number of studies were conducted with a proper biotremology approach, because chemical communication is dominant. In fact, most of the previous studies investigated the body oscillation movements that produce vibrations, but not the actual vibrations generated and transmitted to the nest. The study reported in this thesis is the first one describing the actual frequency composition of Abdominal Wagging and Abdominal Drumming (chapter 5), reporting also the very low frequencies that were overestimated by the previous study on Abdominal Wagging (Brennan 2007). Whether mechanical stimulations are involved in the modulation of the larvae development or in the interaction between adults and brood, putative functions of these signals can be tested only knowing the exact temporal and spectral structure of the vibrations transmitted into the nest. Therefore, our results are crucial notions to develop playbacks that will be tested in future manipulation experiments to definitely assess the function of body oscillation movements.

Moreover, the notions of the study on *Polistes* should be taken into account to develop an aspect of biotremology that, to the best of my knowledge, has not been studied yet. Besides a behavioral influence, which can be detected by observations of the phenotype or the behavior of individuals, the molecular mechanisms connecting vibrations to gene expression are still largely unexplored. These proximate aspects of biology have been poorly investigated just in some model species (such as red flour beetle larvae (Hirashima et al. 1993) and mice (Rubin et al. 2007)), but paper wasps have proven to be a very good model to better investigate the molecular mechanisms. In fact, in this

model system gene expression involved in caste determination is known and the relation mechanical stimuli – gene expression would be crucial to sustain the colony structure proper of a social insect society (Standage et al. 2016). Thus, the effect of vibrations should be evident and can rely on previous notions about which genes are expected to be involved.

Overall, technology was essential to achieve the main aims of this thesis that were the description of vibrations produced in two very different groups and to highlight the ecological role of vibrations in insects' interactions. In my opinion, technology will be crucial also in the future of biotremology, for instance to enlarge the applicability of behavioral interference techniques to the field conditions. Our study clearly indicates that *E. vitis* is susceptible to mating disruption in laboratory conditions (chapter 3). We selected and tested different 'disturbance signals' and finally we found the most effective (250 Hz pure tone) that could be used for practical purposes of pest management. However, the field applicability will be possible only once some technological constraints will be solved (e.g. energy and dissipation issues) (Polajnar et al. 2015).

To conclude, background studies as this thesis are needed in order to interfere with the behavior or physiology of insects, whether the tested hypothesis are about biological or applied issues. I'm confident that in the near future the synergy of basic knowledge here provided and technology improvement will enable us to deepen biological aspects, such as the molecular basis under wasps' caste determination, and applied resources, such as the development of vibrational methods for pest management.

References

- Brennan, B.J.**, 2007. Abdominal wagging in the social paper wasp *Polistes dominulus*: behavior and substrate vibrations. *Ethology*, **113**(7), 692–702.
- Hill, P.S.M. & Wessel, A.**, 2016. Biotremology. *Current Biology*, **26**(5), R187–R191.
- Hirashima, A., Nagano, T. & Eto, M.**, 1993. Stress-induced changes in the biogenic-amine levels and larval growth of *Tribolium castaneum* Herbst. *Bioscience Biotechnology and Biochemistry*, **57**(12), 2085–2089.
- Mazzoni, V., Anfora, G. & Virant-Doberlet, M.**, 2013. Substrate vibrations during courtship in three *Drosophila* species. *PloS one*, **8**(11), e80708.
- McNett, G.D. & Cocroft, R.B.**, 2008. Host shifts favor vibrational signal divergence in *Enchenopa binotata* treehoppers. *Behavioral Ecology*, **19**(3), 650–656.
- Polajnar, J., Eriksson, A., Lucchi, A., Anfora, G., Virant-Doberlet, M., & Mazzoni, V.** 2015. Manipulating behaviour with substrate-borne vibrations - Potential for insect pest control. *Pest Management Science* **71**, 15–23.
- Rubin, C.T., Capilla, E., Luu, Y.K., Busa, B., Crawford, H., Nolan, D.J., Mittal, V., Rosen, C.J., Pessin, J.E., & Judex, S.** 2007. Adipogenesis is

inhibited by brief, daily exposure to high-frequency, extremely low-magnitude mechanical signals. *Proceedings of the National Academy of Sciences of the United States of America*, **104**(45), 17879–84.

Standage, D.S., Berens, A., Glastad, K., Severin, A.J., Brendel, V.P., & Toth, A.L. 2016. Genome, transcriptome and methylome sequencing of a primitively eusocial wasp reveal a greatly reduced DNA methylation system in a social insect. *Molecular ecology*, **25**(8), 1769–84.

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"I am part of all that I've met"
(*Ulysses* by A. Tennyson)

Grazie

Rachele