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Novel approaches to study vibrational signaling of insects

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Abstract of the dissertation

Vibrational communication is one of the oldest modes of communication that represents an important component of animal behavior, yet it is the least explored among the different modalities. The study of substrate-borne vibrations has contributed to the understanding of multiple intra and interspecific interactions in insects. However, despite the increasing research in the field of biotremology, the exchange of vibrational signals in complex communication networks and in ecological contexts represents an understudied dimension of research. Therefore, the aim of the present work is to expand the study of vibrational signaling behavior of insects into more ecological systems, simulating natural situations in which they live and therefore provide more reliable information about their behavior. Different methodologies were applied according to different research questions. The study consisted of continuous recordings with a laser vibrometer of insect communication throughout a specific behavior, in a multiple individuals' scenario and in natural conditions. Laboratory trials were conducted to reproduce and study the parental care behavior in burying beetles in relation to their stridulations. Comparing the stridulating behavior of *Nicrophorus vespilloides* between pre- and post-hatching care revealed higher signaling activity in post-hatching and the occurrence of different patterns of signals, which implies that the signals are likely involved in attracting the larvae toward the carrion and/or in coordinating their provisioning. These results raise questions about the exact function of the stridulations in the biparental care behavior of *Nicrophorus* beetles. Moving to semi-field conditions,

individuals of the same sex of the spittlebug *Philaenus spumarius* have been recorded throughout their adult stage season. We found a higher and delayed vibrational signaling activity of females compared to males and complex intrasexual interactions consisting of signal overlapping in the case of females and signal alternating, partial or complete overlapping in the case of males. Our study has permitted to describe interactions that could mediate cooperative or competitive intrasexual behaviors in this species. Further reasearch is therefore needed to determine the functions of the reported intrasexual interactions. Finally, field recording trials in an organic vineyard showed that low vibrational signaling activity of an insect community was significantly associated with conditions of high temperature and wind velocity. Furthermore, the field recording methodology enabled the temporal monitoring of the agricultural pest *Halyomorpha halys* and the grapevine leafhopper *Scaphoideus titanus.* These findings confirm the validity of our method in assessing vibrational signaling in the vineyard and open the possibility to the use of biotremology techniques to detect the presence of insect pests in an orchard as a tool of monitoring. In this way, further research is needed to optimize the methodology to implement it in comparing vibrational signaling in vineyards with different management systems and in different types of orchards. Despite the challenges to use vibrational sensitive equipment in such ecological systems where interferences from noise, studied insects and the environment were encountered, the use of biotremology techniques has been proven feasible. Vibrational signals are better manifested when insects are studied in their natural habitats, interesting results can be obtained, and further questions would be asked for the ultimate understanding of this modality of animal communication. Overall, this thesis provides novel approaches to record and study vibrational signals of insects, which can be used as a basis to perform further experiments in the field of biotremology.

Chapter 1

General introduction

1. Animal communication

Communication is the process of information transfer from a sender to a receiver through signaling, so that the receiver extracts information from the signal and ultimately acts based on the received message (Markl, 1983, Stevens, 2013). Animals depend on their sensory system, that processes information present in their surrounding environment, to achieve an effective communication and therefore make important decisions for their reproduction and survival (Dominoni et al., 2020). Communication occurs in a network environment where it mediates important behaviors such as, conspecific recognition (Yorzinski, 2017), sexual advertisement (Greenfield, 2002), prey location (McGregor, 2005), parental care (Whittingham and Dunn, 2001), competition (West-Eberhard, 1984), group foraging (Fellowes et al., 2005), and defense against predators (Schmidt, 1998).

A signal is therefore any act that influences the behavior of organisms that receive it, and which evolved specifically because of its effect (Stevens, 2013, Markl, 1983, Hebets and Papaj, 2005). Signals could be of different types and often, multiple modalities can be involved in intra- and interspecific communication. For instance, bees use both ultraviolet visual (Kevan et al., 2001) and electromagnetic (Clarke et al., 2013) signals to learn the location of flowers which contain a high amount of nectar. Snakes use vibrational stimuli to localize their preys (Young, 2003). Wild African elephants can produce vibrations in the substrate as they vocalize (O'Connell-Rodwell et al., 2006) and they can discriminate between familiar and unfamiliar seismic alarm signals (O'Connell-Rodwell et al., 2007). Bats use ultrasonic frequencies to picture their world with sonar (Denzinger and Schnitzler, 2013).

In arthropods three basic communication categories exist: chemical, visual and mechanical (by sound and substrate vibrations) (Greenfield, 2002). The signals are received through different sensory systems (smell, sight, hearing) and specific receptors (Hill, 2009, Hill and Wessel, 2016). While chemical signals are often continuously emitted throughout the activity period of an individual, visual and mechanical signals are transmitted in the form of temporally discrete amounts of energy, which may represent an important signal feature or even different signals (Greenfield, 2002). Communication can be multimodal, which means that different categories can work together in association with a certain behavior in order to improve the efficacy of information transfer in different sensory environments (Hebets and Papaj, 2005). For example, stinkbugs can use visual, tactile, chemical, and vibrational signals for mating communication (Borges et al., 2017, Čokl et al., 2017). In particular, the simultaneous use of sound and substrate vibrations is one of the most common types of multimodal communication.

2. Acoustic and substrate vibrational communication in Arthropods

Airborne sound and substrate-borne vibrations are both produced by specific structures or organs in the signaler's body (Caldwell, 2014). Both communication modalities propagate as mechanical waves and can be perceived by the same sensory receptors (scolopidia) (Greenfield, 2002). Compared to chemical signals, both sound and substrate vibrations travel fast but are transient, and are constrained by noise in different ways. The major difference between substrate vibrations and sounds is that they propagate trough different pathways and waves types (Bradbury and Vehrencamp, 2011, Cremer and Heckl, 2013). Airborne sound travels as compressional (pressure) waves longitudinally through air or water, or as transverse waves in solids. While substrate-borne vibrations travel mostly as Rayleigh waves (through the ground) and bending waves (in plants) (Markl, 1983, Michelsen et al., 1982, Hill, 2009). However, an airborne signal can be accompanied by substrate-borne vibrations since they could be produced simultaneously when the signaler is in contact with a substrate (Caldwell, 2014).

The type of substrate, through which vibrational signals are transmitted, can affect the quality of information animals perceive (Mazzoni et al., 2014, Virant-Doberlet et al., 2006). Solid substrates are often more structurally complex, supporting a greater diversity of wave types, and spatially heterogeneous than air or water (Markl, 1983, Mortimer, 2017). Therefore, sound travels long distances through homogeneous media where the speed of sound is fairly constant, whereas the propagation velocity of substrate-borne waves varies widely and vibrations travel generally shorter distances before losing their detectability (Cocroft and Rodríguez, 2005). This may imply an effect on the efficacy and evolution of vibrational communication (Virant-Doberlet et al., 2006).

Vibrational communication can be either an exclusive modality or one channel in a multimodal signaling strategy that involves chemical or acoustic modalities (e.g., Hill and Shadley, 1997, Čokl et al., 2019b, Gordon and Uetz, 2011). For instance, insects may use pheromones for long-range attraction then switch to vibrational signals, once on the same plant, for a short-range communication (Čokl et al., 2019a). The use of multimodal communication allows them to overcome the fact that substrate vibrations do not travel long distances without damping off (Michelsen et al., 1982), to minimize the effect of noise interfering in a single modality (Wilson et al., 2013) and to send more complex information using different signals by different channels (Hebets et al., 2016). However, the strategy of involving more than one channel might increase the risk of eavesdropping by antagonists such as parasitoids and predators (Roberts et al., 2007).

3. Mechanisms of production and perception of substrate vibrations

Arthropods have evolved various mechanisms for producing substrate-borne vibrations. They can emit vibrations by “percussion”, which means by drumming their appendages, head, or abdomen against the substrate on which they stand, or by “tremulation”, which means by vibrating body parts thanks to contractions of muscle placed between thorax and abdomen. Vibrations can also be produced by “stridulation” which is achieved by rubbing exoskeletal structures against each other, which usually produce simultaneously substrate borne and airborne components, or by “tymbals buckling” where the tymbals are thin exoskeleton membranes homologous of those present in cicadas (Hill, 2008, Drosopoulos and Claridge, 2005, Virant-Doberlet and Cokl, 2004). Vibrations of biotic and abiotic origin are perceived by means of receptors, the scolopidia, specialized in detecting particle motions perpendicular to the direction of propagation (Hill, 2009). These sense organs are internal mechanoreceptors called chordotonal organs (groups of scolopidia), which are located in the legs and other body parts, and enable an accurate localization of the substrate vibration source in a two dimensions space (Virant-Doberlet et al., 2006). Chordotonal organs play the role of both a filter and a transducer of signals that are processed in the central nervous system (Field and Matheson, 1998, Hill, 2008).

Vibrational information can be produced intentionally to take part in a communication strategy as signals, or incidentally as cues

(Bradbury and Vehrencamp, 2011). Unintended receivers could detect vibrational cues generated when specific actions are performed, for example, by insect movements (Devetak et al., 2007) or feeding (Guedes and Yack, 2016), but also when specific signals are sent to other individuals like in the mating communication when rival males can eavesdrop the ongoing communication between a female and another male to their own advantage (i.e.,Mazzoni et al., 2009b). Some species, in fact, may perceive signals of their same-sex neighbors and assess their motivation of aggressiveness or competitiveness over mate, food or space (Greenfield, 2002). Consequently, these vibrations can provide cues of location, identity, or even qualitative aspects of an individual, which might be exploited by both intended and unintended receivers (Virant-Doberlet et al., 2011, Guedes and Yack, 2016, Meyhöfer et al., 1994). Examples of unintended and interspecific receivers are parasitoids and predators which may detect vibrations of their hosts and preys (Djemai et al., 2004, Laumann et al., 2007, Devetak and Arnett, 2015). On the other hand, the same ability to eavesdrop vibrations allows many insects to detect vibrational cues of their predators and therefore avoid them (Virant-Doberlet et al., 2014, Oberst, 2017).

In addition to the characteristics of transmitted signals and the type of substrate through which they travel, the quality of vibrational communication depends also on the background noise (Cocroft and Rodríguez, 2005, Čokl and Virant-Doberlet, 2003, Hill, 2008, Hill et al., 2019a). Noise is an ubiquitous perturbation feature that decreases the reliability of information provided in the environment, by preventing a receiver from detecting or accurately deciphering the information encoded in a signal which would, otherwise be perceived clearly (Brumm and Slabbekoorn, 2005). Noise may originate from biotic (conspecific or heterospecific signals or cues) or abiotic sources (such as wind or rain, or anthropogenic noise caused by human activity) and may act by interfering with signal transmission and detection (Forrest, 1994). It can limit the ability of animals to communicate by preventing mate attraction, prey's detection, and escaping from predation (De Groot et al., 2011, Dias et al., 2021, Wu and Elias, 2014). On the long term, noise might result in converting animal habitats or ultimately restructuring their communities (Slabbekoorn and Halfwerk, 2009, Tishechkin, 2007, Tishechkin, 2013, Dominoni et al., 2020, Kight and Swaddle, 2011). Consequently, by influencing behavior, fitness, and physiological functions of animal perception, noise can be a major driver of animal evolution (Virant-Doberlet et al., 2014, Dominoni et al., 2020). As an adaptation to overcome the effect of abiotic noise, some animals may adopt strategies such as shifting signal intensity and/or frequency to increase the signal to noise ratio (Brumm and Slabbekoorn, 2005, Nemeth et al., 2013), increasing their signaling activity when noisy conditions have stopped (Velilla et al., 2020), or exploiting temporal gaps of silence for communication (Gordon et al., 2017, Mazzoni et al., 2009a, McNett et al., 2010).

4. Biotremology

Biotremology is a new discipline of animal communication which deals with the production, perception and transmission of vibrations through a substrate (Hill and Wessel, 2016). The latter can be of any type: soil, plants, a spider's web or a honeybee's honeycomb (Hill, 2009). Vibrational communication is an ancient and widespread modality of information exchange that occurs in a diversity of species (Hill, 2008, Cocroft and Rodríguez, 2005). It is a major element of insect behavior covering a wide range of contexts, such as mating and related behavior (Mazzoni et al., 2009b, Polajnar et al., 2014), maternal care (Mukai et al., 2012), group foraging (Cocroft, 2005), prey-predator and host-parasitoid interactions (Virant-Doberlet et al., 2019), defense mechanisms (Manrique and Schilman, 2000), mutualism behavior (Travassos and Pierce, 2000) and food quality assessment (Inta et al., 2007). However, behaviors occurring within same-sex groups are still virtually unexplored. Many organisms share communication channels, generating complex signaling networks. In this regard, intrasexual interactions have been mainly investigated in bioassays that involve mating pairs or trios (e.g.,Mazzoni et al., 2009a, Kuhelj and Virant-Doberlet, 2017). The studies involving multiple individuals are usually those conducted on social and eusocial insects (but see Bedoya et al., 2020), although they did not focus on the communication associated to individuals but rather to the whole nest (Hill et al., 2019b).

Biotremology studies have provided major new insights into both the function and evolution of substrate-borne vibrational signals (Cocroft et al., 2014) and has helped clarifying and extending some taxonomic boundaries in insects (Henry et al., 1993, Tishechkin, 2005, Claridge and de Vrijer, 1994). More insect taxa are still to be adequately studied in order to answer further questions regarding the role of vibrations such as in Coleoptera or Thysanoptera (Čokl and Virant-Doberlet, 2003). Studies on the effect of vibrations generated by anthropogenic activities on animal behavior have demonstrated the ecological consequences on their fitness (Caorsi et al., 2019, Phillips et al., 2020, Velilla et al., 2021) and suggested mitigation measures for conservation strategies (Dominoni et al., 2020).

On another hand, environmentally friendly techniques, based on substrate vibrations, are currently being developed to manipulate insect behaviors by the emission of artificial signals able to interfere with a target pest vibrational signals (Eriksson et al., 2012, Mazzoni et al., 2009b, Polajnar et al., 2016, Gordon and Krugner, 2019, Avosani et al., 2020, Polajnar et al., 2019). Such promising technologies are developed as control strategies of insect pests and disease vectors, and can represent an innovative approach alternative or complementary to the application of chemical substances, in the context of integrated pest management strategies and biological control (Polajnar et al., 2015). The use of substrate vibrations for behavioral manipulation is not only limited to crop pests, but is also applied to disrupt the reproduction of wood borer pests (Aflitto and Hofstetter, 2014, Hofstetter et al., 2019).

An important constraint of biotremology regards the study of insects in their natural environment. Field investigations and trials are not yet very common, due to many technical issues that made this approach hard to perform in the past. The study of the vibrational landscape (vibroscape) associated to a certain environment would open new scenarios in the exploration of the natural vibratory world (Šturm et al., 2019), and would constitute a major step forward in studying vibrational communication in a complex ecological context. Biotic and abiotic environmental factors can concur to shape a vibroscape that can be characteristic of an ecosystem, natural or anthropic. Changing abiotic factors notably affects not only signaler's behavior, but also receiver responses to the signals (Jocson et al., 2019, Mhatre et al., 2016, Ritchie et al., 2001, Ahmed et al., 2016, McNett et al., 2010, Tishechkin, 2013). At this stage, the effects of abiotic environmental factors have been mainly studied by testing them separately and in controlled conditions. The role of temperature, wind, relative humidity and other climatic parameters need to be studied in the field for a better comprehension of their importance in determining the signaling behavior of insect species.

5. Thesis aim

Several approaches have been developed to study different aspects of vibrational communication of insects either in the laboratory or directly in the field. Trials in controlled conditions have represented a classic approach that is still widely used in biotremology, and that consists of recording vibrational signals (and associated behaviors) of target species on their host plant, or enclosed in net cages or constructed arenas. Recordings are usually carried out in a sound-proof chamber and on an anti-vibrational table to minimize the background noise, which is required for highly sensitive recording devices such as laser doppler vibrometer. Such set-up allows precise standardized comparisons among individuals, populations, and species. However, as already mentioned, this approach eliminates the effect of external factors in that the studied individuals are taken out of communication networks where they usually live and in which they have evolved under the influence of other conspecific and heterospecific organisms. Furthermore, experiments in controlled conditions may exclude the effect of environmental factors (photoperiod, solar intensity, temperature, wind, air pressure and relative humidity) on the signaling behavior.

The aim of my thesis was to expand the study of vibrational signaling behavior of insects into realistic or real ecological systems, simulating a specific behavior, social interactions, and natural conditions, and using different methodologies to answer different questions that cannot be answered using the classic laboratory approach. A major challenge for these studies was to design experiments that accurately simulated the natural situations.

6. Thesis outline

This thesis consists of 5 chapters. Following this general introduction there are three original research chapters, and the thesis concludes with a general discussion and conclusion chapter. Each chapter focuses on a different approach depending on the aim of research. I covered three situations to answer specific questions and shed the light on others. In chapter 2, parental care behavior, occurring underground, was reproduced in the laboratory using the burying beetle, *Nicrophorus vespilloides* as a model. Stridulatory signals produced by the beetles were recorded throughout the parental care process in order to investigate whether they play a possible role in this behavior. Chapter 3 introduces a semi-field approach to investigate the intrasexual behavior of insects based on vibrational signals. The context of high population density was simulated by including multiple individuals of the same sex. Vibrational signals were studied throughout the day and the adult stage of the insect model *Philaenus spumarius* in order to investigate the intrasexual behavior based on vibrational signaling. In Chapter 4 the effect of abiotic factors on insect vibrational signaling was studied in natural conditions. We investigated the daily vibrational signaling of a natural insect community, by analyzing the vibroscape in a vineyard and then we discussed the use of the recording approach as a monitoring tool of insect pests and biodiversity in agroecosystems.

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Chapter 2

Stridulatory signal differences between pre- and post-hatching care in the burying beetle *Nicrophorus vespilloides*

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Abstract

Acoustic signaling is common in Coleoptera as it mediates various behaviors such as mating, defense, aggregation, and parental care. Burying beetles of the genus *Nicrophorus* (Silphidae: Coleoptera) are model organisms in evolutionary and behavioral ecology by their elaborate biparental care in the rearing of their offspring. They reproduce on small vertebrate carcasses and typically cooperate in feeding and defending their young. During the intensive period of parental care, burying beetles produce sound by stridulating. However, the specific functions of the stridulatory signals are not yet known. The goal of this work was to gather a first understanding of how the parents' stridulations are involved in the biparental care behavior. Using *N. vespilloides* as a model species, we investigated the signals produced by the beetles and addressed the question of whether their stridulating behavior varied between pre- and post-hatching care. We found an increase of stridulatory signaling during post-hatching which clearly showed that it plays a vital role in parental care, especially after hatching has occurred. We formulated hypotheses that should be tested in future studies on the possible functions of the stridulations throughout the parental care process.

Keywords: biparental care, biotremology, *Nicrophorus,* stridulation.

1. Introduction

Communication is the process of information exchange using different sensory stimuli (e.g., chemicals, sound, light, and substrate vibrations) (Bradbury and Vehrencamp, 2011, Stevens, 2013) and the immense diversity and multitude of signals that organisms use for communication has contributed to our understanding of the underlying mechanisms, function, and evolution of multiple signals and signal complexity (Candolin, 2003, Hebets and Papaj, 2005, Higham and Hebets, 2013, Johnstone, 1996). Communication plays a vital part in the evolution of any form of cooperative behavior, in particular in complex social systems but also in early family life (Bradbury and Vehrencamp, 2011).

Parents and offspring can use different communication means to interact with each other (Costa, 2006). Although, airborne and substrate-borne signals are crucial in insect communication (Greenfield, 2016, Cocroft and Rodríguez, 2005) and can be involved in parent-offspring and inter-offspring interactions (Cocroft and Hamel, 2010), studies of parental care as a part of family life in insects have so far mainly focused on chemical communication (Nehring and Steiger, 2018, Steiger and Stökl, 2017).

Burying beetles of the genus *Nicrophorus* (Silphidae) exhibit elaborate biparental care in the rearing of their offspring, which has made them model organisms in evolutionary and behavioral ecology (Creighton et al., 2015, Engel et al., 2016, Jarrett et al., 2017, Head et al., 2014, Paquet and Smiseth, 2017, Parker et al., 2015, Rozen et al., 2008, Steiger, 2015, Trumbo, 2017, Vogel et al., 2017). Based on their rather complex family life and the interactions between partners as well as their offspring, a sophisticated recognition and communication process has evolved in these species (Steiger, 2015). The "social complexity hypothesis" states that communication systems should be more complex in groups with complex social systems, which is not limited to true sociality but to any context in which the animals face a complex array of interactions during which communication is necessary (Freeberg et al., 2012). However, even though Darwin (1871) already mentioned the stridulatory organ in *Nicrophorus*, the vast majority of studies that targeted their communication to date have focused on olfactory signals (Engel et al., 2016, Müller et al., 2003, Smiseth et al., 2010, Steiger, 2015).

Burying beetles provide important ecological benefits to the ecosystem by facilitating decomposition processes and soil nutrient cycling (Rozen et al., 2008, Scott, 1998). *Nicrophorus* species bury a small vertebrate carcass by repeatedly moving under it, digging up the soil and then rolling the carcass into a ball to prepare it as a food source for their offspring. The couple protects the carrion against detrimental microbes with secretions (Duarte et al., 2018, Rozen et al., 2008, Shukla et al., 2018) and the female lays single eggs in the soil surrounding the carcass. Upon hatching, larvae move towards the buried carcass and usually aggregate in or near the top of it. During the development of the larvae through three instars, parents exhibit uni- or biparental care, feeding and defending the larvae until their development is almost complete. When the carcass is mostly consumed, the larvae build separate pupal chambers in the surrounding soil. Pupation usually occurs several days later, and adults emerge a few weeks after the carcass was buried (e.g., 30 days in *Nicrophorus vespilloides*). Burying beetles are known to divide the tasks during brood care, with females doing more of the feeding of the larvae, whereas males do most of the guarding against intruders (Fetherston et al., 1990, Fetherston et al., 1994, Smiseth and Moore, 2004). Burying beetles are more efficient due to this task specialization and one parent cannot fully compensate for the loss of the other although males for example will increase their feeding rate substantially and will stay longer with the brood if the female is removed (Fetherston et al., 1990, Rauter and Moore, 2004).

Several coleopteran groups are characterized by concurrent evolution of parental care and acoustic communication, such as in Passalidae or Scarabaeinae (Drosopoulos and Claridge, 2006, Costa, 2006, Schuster, 1983). In the case of *Nicrophorus*, from mating and throughout their parental care, both parents produce audible sound using their stridulatory organs that consist of a plectrum (located on the ventral side of the elytra), which is moved across the pars stridens (located on the fourth and fifth abdominal segment) (Darwin, 1871, Freeberg et al., 2012, Hall et al., 2013, Niemitz, 1972, Pukowski, 1933). However, since stridulating organs produce airborne and substrate-borne components simultaneously (Hill, 2009) it is likely that vibrational signals have a role in communication. Despite sound perception with pressure-sensitive receptors has been reported for some Coleoptera (Greenfield, 2016), sound and substrate vibration receptors in *Nicrophorus* have not been thoroughly investigated. Studies on stridulations produced by *Nicrophorus* species have been limited so far to sound characterization and stridulatory structures description (Hall et al., 2013), while their function in family life remains largely unknown.

In order to gather a first understanding of the role that stridulations play during biparental care in burying beetles, we investigated the signals produced by these beetles and whether the stridulating behavior varied between pre- and post-hatching care. We used *N. vespilloides* as a model species whose larval reliance on the post-hatching care is intermediate between the highly dependent species, *N. orbicollis* in which larvae do not survive in the absence of parental care, and the independent species, *N. pustulatus* in which larvae are nutritionally independent of their parents (Capodeanu-Nägler et al., 2016).

2. Material and Methods

2.1 Rearing and maintenance of beetles

Experimental *N. vespilloides* beetles used in the study were descendants of individuals collected from carrion-baited pitfall traps in a forest near Bayreuth, Germany (49°55′18.192′′N, 11°34′19.9488′′E, WGS84) and were maintained in temperature-controlled chambers at 20 °C on a 16:8 h light: dark cycle. Once they had emerged as adults, groups of up to 5 beetles of the same sex and family were kept in small plastic containers (10 × 10 cm and 6 cm high) filled with moist peat. To ensure optimal outbreeding we used the program Kinshipper (CAT, Bayreuth, Germany) to calculate optimal mating pairs. Beetles were fed freshly cut larvae of either darkling beetles (*Zophobas morio*) or whole fly larvae (*Lucilia sericata*) *ad libitum* twice a week. At the time of experiments, beetles were virgin and 30-40 days old.

2.2 Signal recording and analysis

Pairs of *N. vespilloides* (N=4) were separately placed in a 19,5x19,5 cm plastic box filled with a thin layer of moistened peat substrate and covered with an antireflection glass. Trials were carried out in the dark and the behavior was recorded using an infrared camera (Somikon DV-883.IR, PEARL, Buggingen, Germany). Recording sessions (12 days) started when reproduction was induced by providing each mating pair with a 20 g (± 2.5 g) thawed mouse carcass (Frostfutter.de—B.A.F Group GmbH, Germany) and lasted until the larvae had dispersed for pupation.

Stridulations were recorded by pointing a Laser-Doppler Vibrometer (Polytec PDV-100, PolytecGmbH) through a hole in the glass cover (Ø ca. 5 mm), perpendicularly on a reflective tape (Ø ca. 1 cm) attached to one side of the box base, and was set up at 40 cm distance from the box. Signals were digitized using the software Raven pro 1.4 (The Cornell Lab of Ornithology, 151 Ithaca, NY), at a sampling rate of 44.1 kHz and a depth resolution of 16 bit, through a multichannel calibrated data acquisition device (TASCAM Celesonic US-20x20 interface), and were stored onto a computer as date and time-coded 10-minute files using a 24-bit soundcard.

We selected recordings from the first 3 days after the start of the experiment, as pre-hatching care, and then starting from day 5, as post-hatching care. We selected day 5 because *N. vespilloides* eggs hatch not earlier than 64-68 hours after the parents are given a carcass. For each care period we analyzed signals of the 1st, 8th, 16th, 24th hour of each day using the software Raven Pro 1.5 (The Cornell Lab of Ornithology, 151 Ithaca, NY) applying Fast Fourier Transform, type Hann, with a window length of 512 samples, frequency resolution of 8 Hz and 75% overlap. A signal was defined as a train of chirps (continuous sound characterized by a fundamental frequency and a harmonic structure) and stridulating activity was calculated as the time (in minutes) the beetles spent signaling per hour.

2.3 Statistical analysis

Statistical analyses were conducted in R version 4.0.2 (R Core Team, 2018), run in the R studio interface (RStudio Team 2020). Plots were made using R packages: "ggpubr" and "ggplot2" (Kassambara, 2020, Wickham et al., 2015). The stridulating activity, the number of signals and the average duration of signals were compared between pre- and post-hatching care using a Mann Whitney two sample test. Spectrograms were generated in R package "Seewave" (Sueur et al., 2020) with Hanning windows of 1024 samples with 75% overlap and application of 1000–4000 bandpass Butterworth frequency filter.

3. Results

3.1 Stridulating activity

The stridulating activity (range: 0.6-55 min) was significantly higher in post-hatching compared to pre-hatching care (W = 48, *p*-value <0.001, Figure 1). This increase in the beetles stridulating activity in post-hatching was associated with both higher number and longer signals (W = 66.5, *p*-value <0.001, W = 149, *p*-value <0.001 number of signals and average duration of signals respectively, Figure 2A, B).

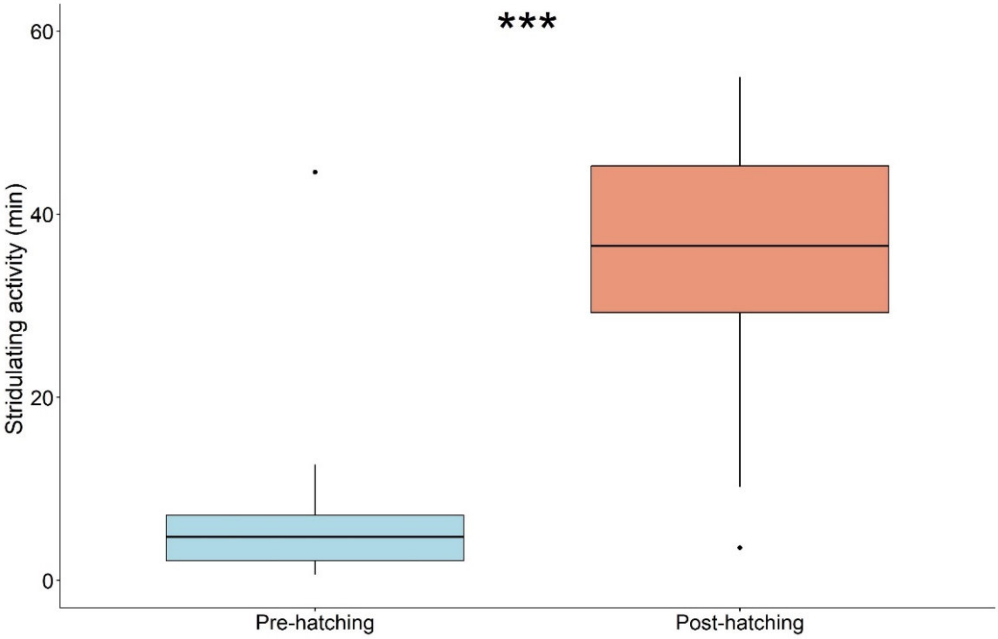

Figure 1: Variation of the stridulating activity of *N. vespilloides* between pre- (n=39) and post-hatching care (n=27). Asterisks indicate significant differences according to Mann–Whitney two-sample test (*p*-value<0.001).

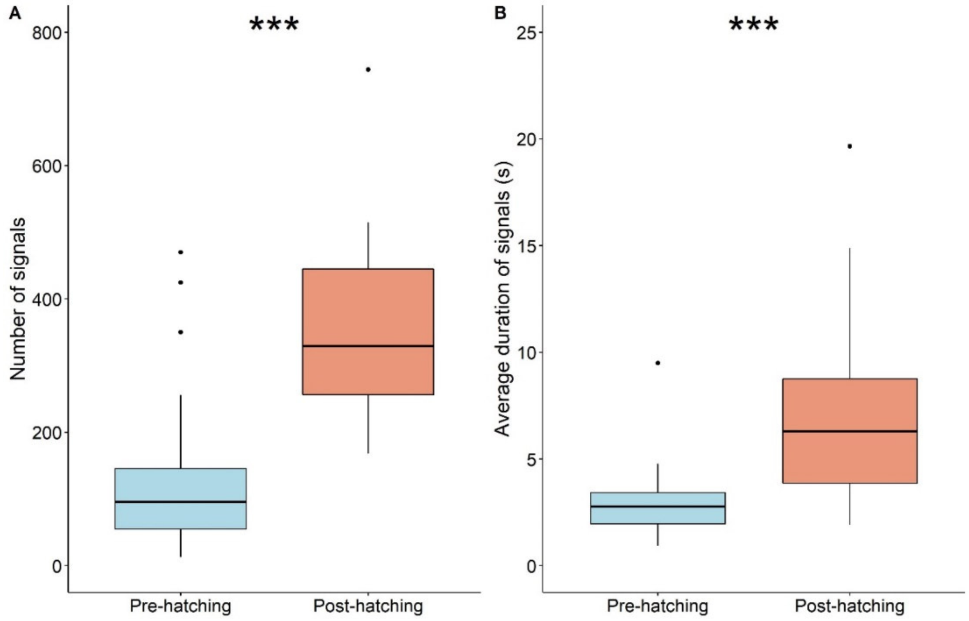

Figure 2: Variation of the number of signals (**A**) and average duration of signals (**B**) produced by *N. vespilloides* between pre- (n=39) and post-hatching care (n=27). Asterisks indicate significant differences according to Mann–Whitney two-sample test (*p*-value<0.001).

3.2 Stridulatory signals

Both parents started to stridulate as soon as they were in contact with the carcass. Stridulations were emitted during carcass preparation and burial, during copulation, and during post-hatching care. All the recorded signals consisted of a series of separated chirps, emitted either isolated or clustered in trains. A variable number of trains of disyllabic chirps were produced by the forward and backward movements of the stridulatory organ. Furthermore, each chirp unit was composed of a fundamental frequency component, which in most cases also corresponded to the dominant frequency, and several harmonics.

In pre-hatching care, two patterns of stridulatory signals were detected (Figure 3A, B) and signals of both parents often overlapped when simultaneously stridulating (Figure 3C). In post-hatching care one pattern of signals was detected (Figure 3D).

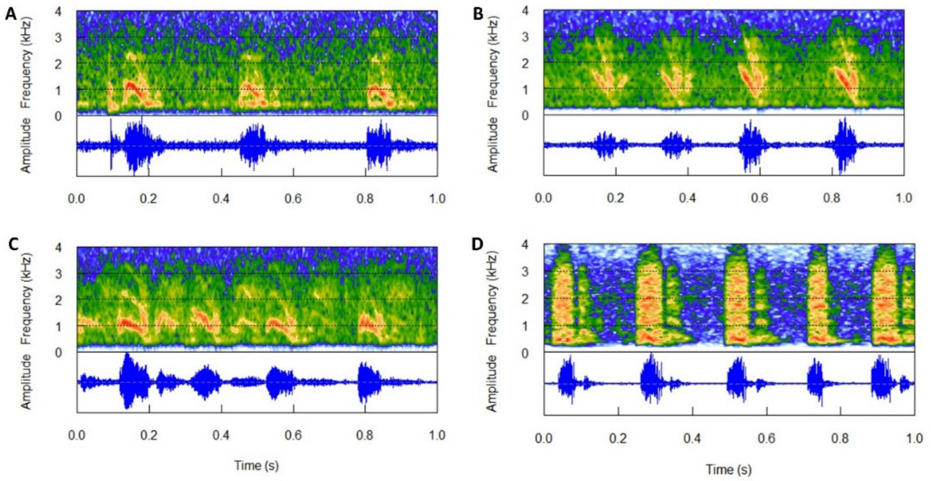

Figure 3: Examples of spectrograms (above) and oscillograms (below) of *N. vespilloides* stridulatory signals recorded during pre-hatching (**A**, **B** and **C**) and post-hatching care (**D**).

4. Discussion

Our results clearly show that stridulations produced by the burying beetle, *N. vespilloides*, change from pre- to post-hatching care and seem to play a relevant role in brood care. Our hypothesis is that *N. vespilloides* use the stridulatory signals to "call" their offspring and coordinate their care. Previous studies, in fact, have shown that the inhibition of stridulations in *Nicrophorus* affects reproduction and offspring survival (Huerta et al., 1992). Marking by elytral clipping also significantly reduced brood size in *N. americanus,* probably because stridulatory signals were significantly altered (Hall et al., 2015). This effect of stridulatory organs alteration in Nicrophorinae has been suggested to be critically linked to the origin of parental care (Cai et al., 2014), since the behavior is present only in the subfamily Nicrophorinae while it is absent in Silphinae, although the latter are also carrion-feeding as larvae and adults and do not possess stridulatory organs. The high stridulating activity during post-hatching care could mean that the stridulations might be used in parent-offspring communication or in parents' coordination of brood care. The increase in the number of signals can be explained by the fact that parents might have to coordinate their larval feeding, patrolling and cadaver manipulation in order to ensure providing an adequate care to their young. In our trials, signals were continuously produced by one of the parents while feeding the larvae, which explains the higher duration of signals when the larvae were present. We assume that the signals are used upon hatching, to direct larvae towards the carcass as well as to coordinate their feeding when they are gathered inside the carcass. Especially since young larvae are often found deep within the carcass, they might be helped by "calling" them to the feeding cavity (Leech, 1934). It will not be surprising if stridulatory signals may eventually turn out to be related to both behaviors or to another behavior such as defending their territory.

If the signals are used to "call" the larvae towards the feeding site, as already suggested by Niemitz and Krampe (1972), we would expect a peak of stridulations immediately after hatching. To test this hypothesis, further research should focus on the stridulating activity during the exact time of hatching. Bioassays using silenced parents would further confirm this hypothesis by evaluating whether the absence of signals would affect the time larvae spend to reach the feeding site. While if the signals are solely used to coordinate brood provisioning, we would expect higher signaling when all the larvae are on the feeding site. Since *N. vespilloides* can exhibit facultative biparental care (Capodeanu-Nägler et al., 2016), manipulation of parental presence would also provide more information about the intended receivers of the signals.

Stridulations of *N. vespilloides* could also have functional implications in intersexual interactions to facilitate the cooperative carrion processing and brood care, the reason why beetles stridulate in pre-hatching as well. Vibrational signaling can mediate interactions between parents and with offspring to achieve group member cohesion in some insects (Costa, 2006, Cocroft, 2001). However, because silenced beetles can still reproduce and provide brood care (Conrad, unpublished data), the parents' behavior needs to be further investigated and described in relation to their stridulating activity. The stridulatory signals could also be complementing the use of chemical substances. Both vibrational and acoustic signals can replace or reinforce chemical communication in social insects (Cocroft and Rodríguez, 2005, Hunt and Richard, 2013), such as drumming alarm signals in termites (Hertel et al.,

2011) and stridulatory recruitment signals in ants (Hölldobler and Roces, 2019).

The occurrence of different signal patterns suggests possible differences between the stridulatory signals produced by the beetles. We hypothesize that beetles modulate their stridulatory signals depending on the behavioral context and on the presence of the larvae. A study on other *Nicrophorus* species reported interspecific differences encoded in the temporal characteristics of their stridulatory signals (Hall et al., 2013). The temporal and frequency features of the signals need to be characterized and compared among the potential types of signals that beetles produce throughout the reproduction and brood care.

The recording approach used in this study can be applied for further experiments to record and analyze stridulatory signals of *N. vespilloides* and other *Nicrophorus* species. The dependence of larvae on the biparental care can be variable in this genus (Capodeanu-Nägler et al., 2016) and according to the social complexity hypothesis we could expect species-specific signal repertoires. Consequently, further studies might also give us an opportunity to connect the evolution of family life with the evolution of complex communication systems.

Overall, this study provides the first evidence that stridulatory signals play an important role in the social communication of burying beetle *Nicrophorus*, that seems to be particularly important after hatching has occurred.

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Chapter 3

Intrasexual vibrational behavior of *Philaenus spumarius* in semi-field conditions

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Abstract

Insects that communicate by vibrational signals live in a complex interactive network of communication. Most studies on insect intrasexual behavior, based on plant-borne vibrational signals, have targeted few individuals. Despite their importance, behaviors that occur within groups were often overlooked. The study of multiple individuals, when insects occur in high density could simulate the environment in which they live and provide more reliable information on their behavior. In semi-field conditions, we investigated the intrasexual behavior of the meadow spittlebug, *Philaenus spumarius*. Vibrational signals exchanged among individuals of the same sex were recorded throughout their adult stage, from late spring to early autumn, and during the day, from the morning to the evening using a laser vibrometer. Males were less active than females throughout the season and their interactions were less frequent compared to females. Intrasexual interactions were characterized by signal overlapping in both unisex groups, in addition to signal alternating only in the case of males. In conclusion, the study of signaling behavior in intrasexual groups contributed to a better understanding of *P. spumarius* social behavior. We discuss the hypothesis of a possible competitive behavior between males and cooperative behavior between females.

Keywords: Vibrational signals, Meadow spittlebug, intrasexual behavior, intrasexual interactions

1. Introduction

Vibrational communication is an important and prevalent component in animal communication (Hill, 2008). In nature, insects that communicate by vibrational signals live in a complex interactive environment, where they perceive signals from conspecifics, other species, and the surrounding environment (Čokl and Virant-Doberlet, 2003, Virant-Doberlet et al., 2014). Insects are therefore able to distinguish qualitative and quantitative characteristics of inter and intraspecific substrate-borne vibrations and react accordingly (Castellanos and Barbosa, 2006, Evans et al., 2009). In fact, the exchange of vibrational signals occur in different situations and can have several functions such as attraction and localization of a mate (Mazzoni et al., 2010, Mazzoni et al., 2009b), competition over a mate (Mazzoni et al., 2009b), coordination with conspecifics (Cocroft, 2005), and prey detection or predators avoidance (Virant-Doberlet et al., 2019).

Studying vibrational signals of insects in different contexts has provided relevant understanding of their behavior, ecology, and evolution. However, few studies have dealt with intrasexual interactions (e.g., Bedoya et al., 2020) and those that exist concern social and eusocial insects (Hill et al., 2019). Studies of the vibrational behavior of insects, mostly focus on the mating behavior (Virant-Doberlet and Cokl, 2004) and signals associated to pair formation, with bioassays that involve a pair (more often, a male and a female), sometimes in the presence of a third individual (e.g.,Mazzoni et al., 2009a, Kuhelj and Virant-Doberlet, 2017). Therefore, by targeting few individuals, behaviors that occur within groups are excluded even if for many species (especially of Hemipterans) the presence of groups on the same host plant is the rule and not the exception (Addesso et al., 2012, Biedermann, 2003, Kusmayadi et al., 1990, Park et al., 2006, Pérez-Rodríguez et al., 2017). For this reason, the simulation of the environment in which insects live, by involving multiple individuals, could provide more reliable knowledge of their interactions that could be useful if applied to practical aspects that involve decision making for conservation or pest control.

In this research, we aimed to investigate the vibrational behavior in a context of intrasexual groups in order to reveal the types of intrasexual signals and interactions. We chose, as a model species, the meadow spittlebug *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae). This insect is the major vector of *Xylella fastidiosa* subspecies pauca, the causal agent of the Olive Quick Decline Syndrome, a severe vascular disease that is leading to serious economic losses in olive production in Southern Italy (Cornara et al., 2018, Saponari et al., 2014) and poses severe risks to several other European countries (Schneider et al., 2020). The mating behavior of *P. spumarius* is mediated by vibrational signals and the role of intraspecific vibrational signals has already been described (Avosani et al., 2020). Since the calling behavior of females depends on their sexual maturation (Avosani et al., 2021), their responsiveness to mating signals is delayed and increases through the season. Adults of *P. spumarius* can occur at high densities at the same and on nearby host plants (Bodino et al., 2019, Bodino et al., 2020, Mangan and Wutz, 1983, Cornara et al., 2018, Weaver and King, 1954), suggesting that their vibrational environment includes multiple individuals simultaneously signaling.

To study the vibrational signaling behavior of intrasexual groups of *P. spumarius,* we first investigated the signaling activity of males and females separately (in order to prevent mating) throughout the adult stage and during the day. Second, we characterized the types of signals emitted by both unisex groups and evaluated the effect of type of signals and number of signaling individuals on the number of emitted signals. Finally, we investigated the types of interactions that occurred among same-sex individuals signaling simultaneously. The adopted approach provided insights about the intrasexual behavior of *P. spumarius,* as it might be manifested in nature.

2. Materials and methods

2.1. Insect collection and rearing

Second to fifth instar nymphs of *P. spumarius* were collected on their host plants from meadows in the Trentino region in Northeastern Italy, from April to May 2018. Collected nymphs were transferred into mesh cages (Bugdorm-6620, 60x60x120 cm^3, MegaView Science Co., Ltd., Taiwan) using a brush. Cages were supplied with *Vicia faba*, *Trifolium repens*, *Rumex* spp and *Helianthus annus* plants, and were maintained under controlled conditions (25±2 °C, L16:D8, 75±5% RH) in a glasshouse at Fondazione Edmund Mach (San Michele all'Adige, Trentino, Italy). After adult molting, insects were sexed and reared separately to prevent mating, according to conditions described in Avosani et al. (2020).

2.2. Signal recording

In each recording session, males ($n = 10$) and females ($n = 10$) were randomly collected from the rearing cages using a mouth aspirator and separately released into two mesh cages (Bugdorm-6620, 60x60x120 cm^3, MegaView Science Co., Ltd., Xitun Dist., Taiwan), placed outdoor in a shaded area and always oriented in the same direction. Each cage contained a 1-year-old potted grapevine plant (*Vitis vinifera* L. cv. Pinot noir grafted on Kobber 5BB) which was grown under greenhouse-controlled conditions (24±1°C, L16:D8, 75±5% RH) and were not subjected to pesticide treatments. Cages were separated by a distance of 0.5m in order to prevent any possible transmission of vibrations from one cage to another. At the end of each recording session insects were put back in their rearing cages. Recordings were conducted outdoors to simulate field conditions.

Vibrational signals emitted by males and females were simultaneously recorded using two laser Doppler vibrometers (Ometron VQ-500-D-V Ltd, U.K., and PDV 100, Polytec, Inc. Dexter, MI, U.S.A.). Each laser was pointed at a reflective sticker attached to the stem (diameter 1 cm) of the grapevine plants. Since *P. spumarius* tended to move towards the green and tender apical shoots of the plants (personal observation), the sticker was placed approximately 10 cm below the apical shoot. Recordings were digitized with the software Pulse 21 (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark) at a 44.1 kHz sample rate and 16-bit depth resolution through a data acquisition device (LAN XI type 3050-B-040, Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark), then stored onto a hard drive of a computer (HP, EliteBook 8460 p). To accommodate the processing of signals to manageable proportions, a "recording" was acquired as a 10 min segment.

2.3. Analyzed parameters

To evaluate the insect signaling throughout the season, trials covered most of the insect adult stage, which was divided into two parts: "early" season (from 14th June to 31st of July 2018), and "late" season (from 1st of August to 28th of September 2018). Early and late seasons corresponded to the periods associated with the absence and presence of ovarioles in females, respectively (Avosani et al., 2021). The signaling during nighttime was not evaluated, given that pilot recordings suggested that *P. spumarius* has a negligeable signaling activity during night (data not shown). The "recording session" consisted of three different periods of the day: morning (from 06:30 to 11:00), afternoon (from 11:30 to 16:00) and evening (from 16:30 to 21:00). Each recording session was replicated 12 times throughout the season, resulting in a total of 162 hours for each sex (3 recording sessions × 4.5 h × 12 replicates).

In order to characterize the types of signals emitted by the tested males and females, using a random number generator we randomly chose three recordings from each recording session, where insect vibrational signals occurred. In total, 74 recordings of females and 95 of males were used for data analysis. Analysis of signal spectrograms was performed with the software Raven Pro 1.4 (The Cornell Lab of

Ornithology, 151 Ithaca, NY, USA) using Fast Fourier Transform type Hann, a frequency resolution of 8 Hz with 75% overlap and window length of 512 samples.

To investigate whether the co-presence of same-sex individuals on the same plant elicited signaling of other individuals and influenced the type of emitted signals, we assessed whenever possible: the types of signals and number of signaling individuals. Vibrational signals were characterized according to (Avosani et al., 2020) as follows: female calling signal (FCS), female rejection signal (FRjS), male calling signal (MCS), male courtship signal (MCrS), and male-male signal (MMS). These signals can be composed of two elements, namely pulses (homogenous units of sound of specific duration (Broughton, 1963)) and chirps (continuous sound characterized by a fundamental frequency and a clear harmonic structure (Avosani et al., 2020)). To assess if signaling of two or more individuals tended to alternate or overlap, the type of interaction between signaling individuals was evaluated. In this regard, signals were ranked as "overlapped" when emitted at the same time by different individuals (the start and the end of the signals coincide by 0.05 – 0.1s), "partially overlapped" when there was not a perfect overlap between the start and the end of the signals, and "alternated" when emitted with a delay of 0.5 – 1 s.

2.4. Statistical analyses

Statistical analyses were performed with the software R version 4.0.2 (R Core Team, 2018) run in the R studio interface (RStudio Team,

2020) . Plots and graphic design were done using R packages: “ggpubr”, “ggplot2” (Kassambara, 2020, Wickham et al., 2015).

2.4.1. Vibrational signaling throughout the season and during the day

To explore the variation of signaling of both males and females, the signaling duration was calculated as the time that individuals spent signaling per recording session. The signaling duration of males and females was compared using the Wilcoxon rank-sum test. To evaluate the effect of date and period of the day on the signaling duration, we fitted a generalized least squares (gls) model for each sex using the function “gls” from the “nlme” package (Pinheiro et al., 2007). The signaling duration was used as the response variable, while the date was transformed into numbers and used as a numeric variable and the period of the day (morning, afternoon, and evening) was used as a categorical factor. Date for females and period for males were used as variance covariates. In the case of males, the model showed a significant effect of period, therefore we calculated pairwise comparisons among periods of the day using the R function “lsmeans” from the “lsmeans” package (Lenth and Lenth, 2018).

2.4.2. Types of signals throughout the season

To compare the types of emitted signals in the season, the proportion of each type of signal per recording session was compared between “early” and “late” season using a Mann–Whitney two-sample test for each sex.

2.4.3. Signaling according to the number of individuals and type of signals

To determine the effect of the number of signaling individuals and type of signals on the number of emitted signals, we conducted a Permanova test using the function "adonis" from the "vegan" package (Oksanen et al., 2013). When the effect of a variable was significant, we applied a post hoc test: Mann-Whitney pairwise test, with Bonferroni correction. Whenever a variable did not meet the assumptions of the test, no further post hoc were applied.

2.4.4. Types of interactions

Finally, we performed a descriptive analysis of the type of interactions among signaling individuals for each sex (i.e., overlapped or alternated signals).

3. Results

3.1. Vibrational signaling throughout the season and during the day.

In our experiment, the signaling duration of males (mean ± SD: 20.51±23.2 min) was significantly lower than that of females (mean ± SD: 60.17±69.09 min) (Wilcoxon signed-rank test: W = 437, *p*-value = 0.005).

The signaling of both males and females differed within the season (F= 11.337, *p*-value= 0.002, F= 32.856, *p*-value <0.001, males and females respectively) (Table 1: for model estimates). While males started to emit signals from the 15th of June, females rarely produced signals before the 24th of July 2018 (Figure 1A). The signaling of females significantly increased as the season progressed. A similar trend was observed in males although with much lower increasing rate (Figure 1A). The period of the day had a significant effect on the signaling duration of males (F= 4.728, *p*-value= 0.016) (Table 1, Table S1). The signaling duration in the evening was significantly longer than in the afternoon (*p*-value=0.0342), while no differences in the males' signaling duration were observed between morning and evening (*p*-value=0.201) or morning and afternoon (*p*-value=0.4412) (Figure 1B). The period of the day did not affect the females' signaling duration (F= 0.243, *p*-value= 0.785) (Table 1, Table S1), even if the signaling activity tended to be higher in the evening, as shown by the median values (Figure 1C).

Table 1: Estimated regression coefficients, standard error, z ratio and *p*-value from the gls model testing the effect of date and period of the day on the number of signals. The intercept corresponds to the period afternoon.

Sex	Parameter	Estimate	Std. Error	T-value	*p*-value
Males	Intercept	1.087	3.174	0.342	0.734
	Date	0.174	0.052	3.368	0.001
	Period Evening	24.967	8.656	2.884	0.007
	Period Morning	7.078	5.626	1.258	0.217
Females	Intercept	-1.271	1.099	-1.157	0.256
	Date	1.041	0.191	5.427	<0.001
	Period Evening	-2.010	6.349	-0.316	0.753
	Period Morning	-1.522	2.305	-0.660	0.514

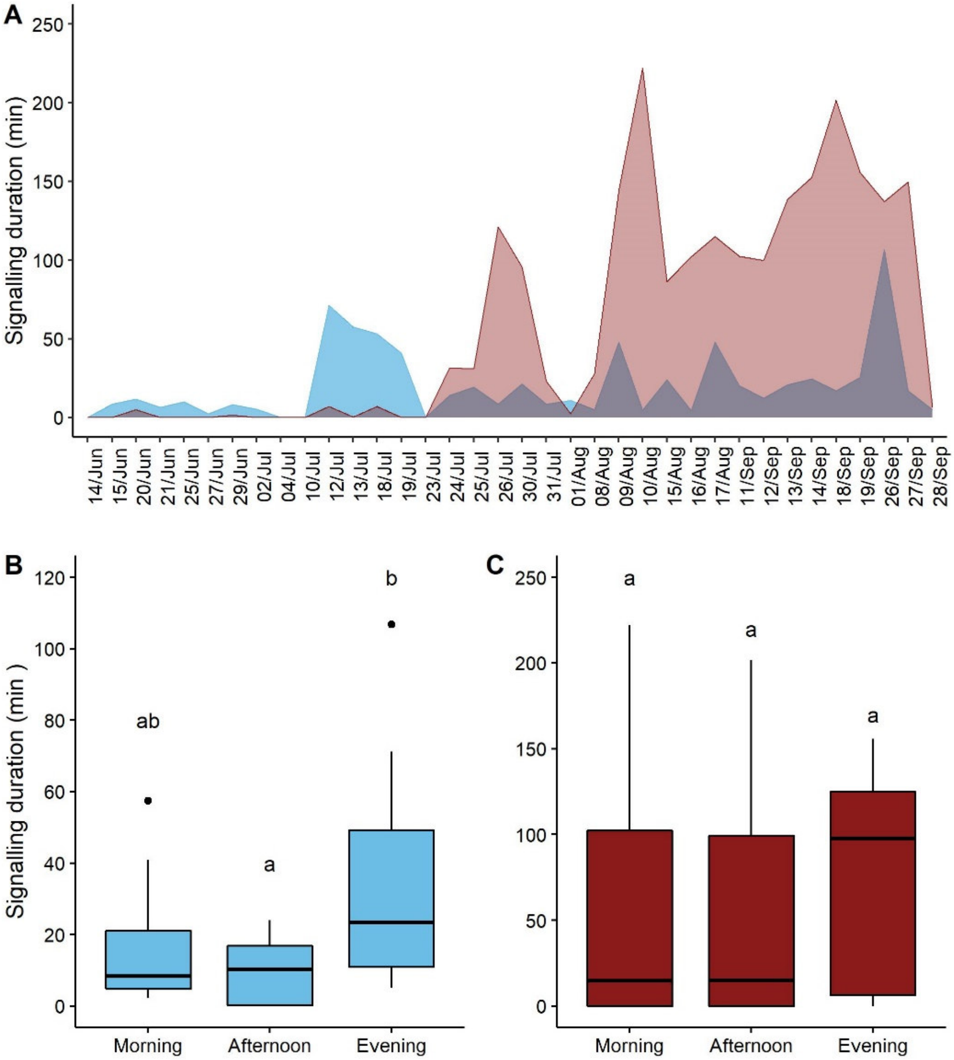

Figure 4. Vibrational signaling of males (blue) and females (red) of *P. spumarius* throughout the season and during the day: (**A**) Signaling duration per recording session of males and females; (**B**) Signaling duration of males per period of the day; (**C**) Signaling duration of females

per period of the day. Different letters (a, b) indicate significant pairwise differences (*p*-value< 0.05).

3.2. Types of signals in “early” and “late” season

All types of previously described male signals the MCS, the MMS and the MCrS (Avosani et al., 2020)) were recorded during our trials. Nonetheless, males also emitted short sequences of chirps (2-3) or pulses (6-7) without a clear temporal pattern. The proportion of MMS was higher in the late season than in the early season (Mann-Whitney pairwise test, U = 10, *p*-value = 0.0312), while no significant differences in the number of chirps, MCS and MCrS were observed (Mann-Whitney pairwise test, U = 18, *p*-value = 0.281, U = 183.5, *p*-value = 0.066 and U = 15, *p*-value = 0.609 respectively) (Figure 2A).

All types of female signals were detected, except for FRsS (female response signal). Female signals (i.e., the FCS and the FRjS) are composed of chirps, of which repetition time and duration depend on the type of the signal (Avosani et al., 2020). In our trials, females produced sequences of 2–3 chirps, which differed from the FCS and the FRjS for their temporal features (time between chirps and duration of the signal sequence). The proportion of chirps was higher in the early season than in the late season (Mann-Whitney pairwise test, U = 116, *p*-value = 0.022), while the proportion of FCS was significantly lower in the early than in the late season (Mann-Whitney pairwise test, U = 32, *p*-value = 0.007). The proportion of emitted FRjS was similar between the early and late seasons (Mann-Whitney pairwise test, U = 43.5, *p*-value = 0.4347) (Figure 2B).

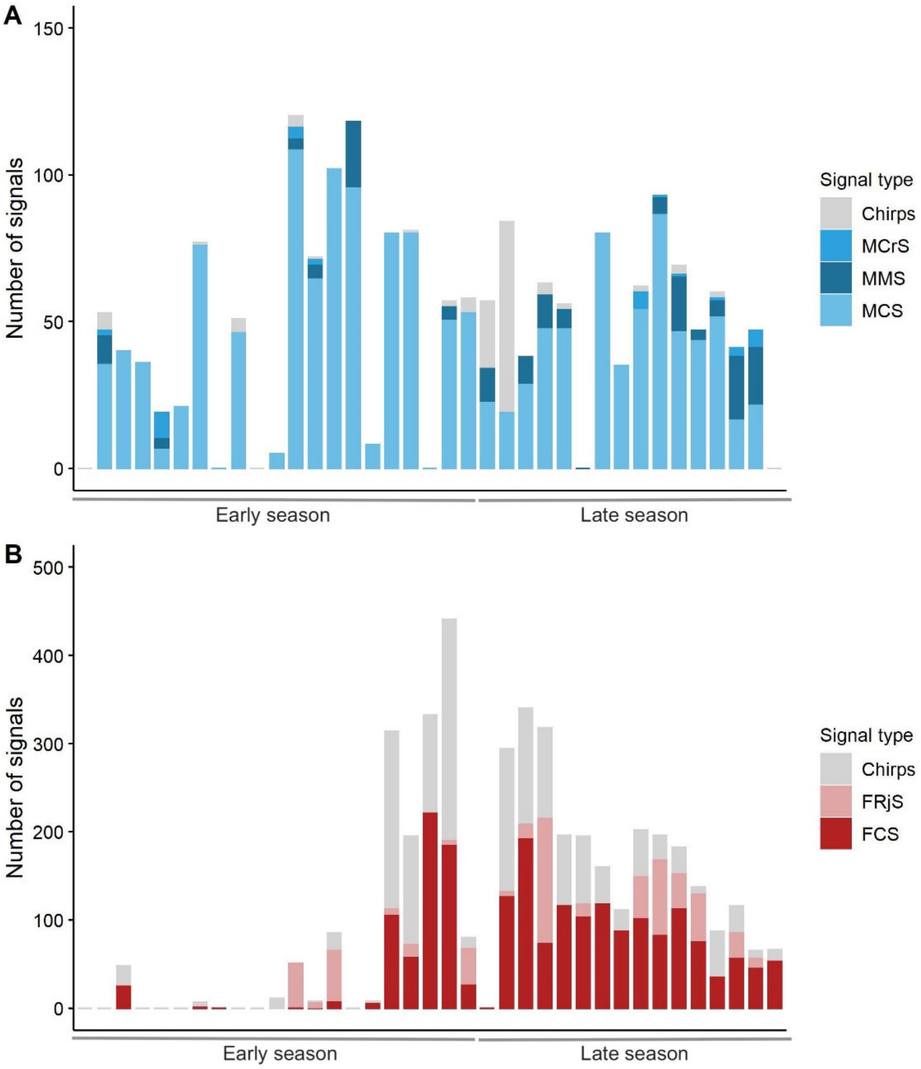

Figure 5. Number of signals per types emitted by males (A) and females (B) throughout the season. "Early" season: from 14th June to 31st of July 2018. "Late" season: from 1st of August to 28th of September 2018.

3.3. Signaling according to the number of individuals and type of signals

The number of signals was significantly different between the type of signals emitted by males (Permanova test, F=24.969, *p*-value=0.001), as the number of MCS was higher than the number of MCrS (*p*<0.001), MMS (*p*-value<0.001) and isolated chirps (*p*<0.001) (Figure 3A, see Table S2 for the other combinations). The effect of number of individuals on number of signals emitted was not considered (see Table S3 for complete results of the test), given the heterogeneity of dispersion found among the groups (permutation test for homogeneity of dispersion, F=3.777, *p*-value= 0.023, Table S4). Furthermore, the interaction between type of signals and number of signaling individuals was not significant (F=1.178, *p*-value=0.287).

The number of signals emitted by females was significantly different between the type of signals (Permanova test, F=3.161, *p*-value= 0.016) and the interaction between the type of signals and the number of signaling individuals was significant (Permanova test, F=3.617, *p*-value= 0.001). The number of signaling individuals had no significant effect (Permanova test, F=1.231, *p*-value=0.280). Overall, females emitted fewer chirps than FCS and FRjS (Figure 3B). The type of signals depended on the number of individuals simultaneously signaling. In the case of one signaling female, the number of chirps was higher than the number of FRjS (*p*-value=0.013), while no statistical difference was detected for the other combinations (*p*-value >0.05). When two females were signaling, the number of signals was not statistically different among the types of signals (*p*-value>0.05). When three individuals were signaling, females produced significantly more FCS than chirps (*p*-value=0.005) while no statistical difference was detected among the other combinations (*p*-value >0.05) (Figure 3B, Table S5).

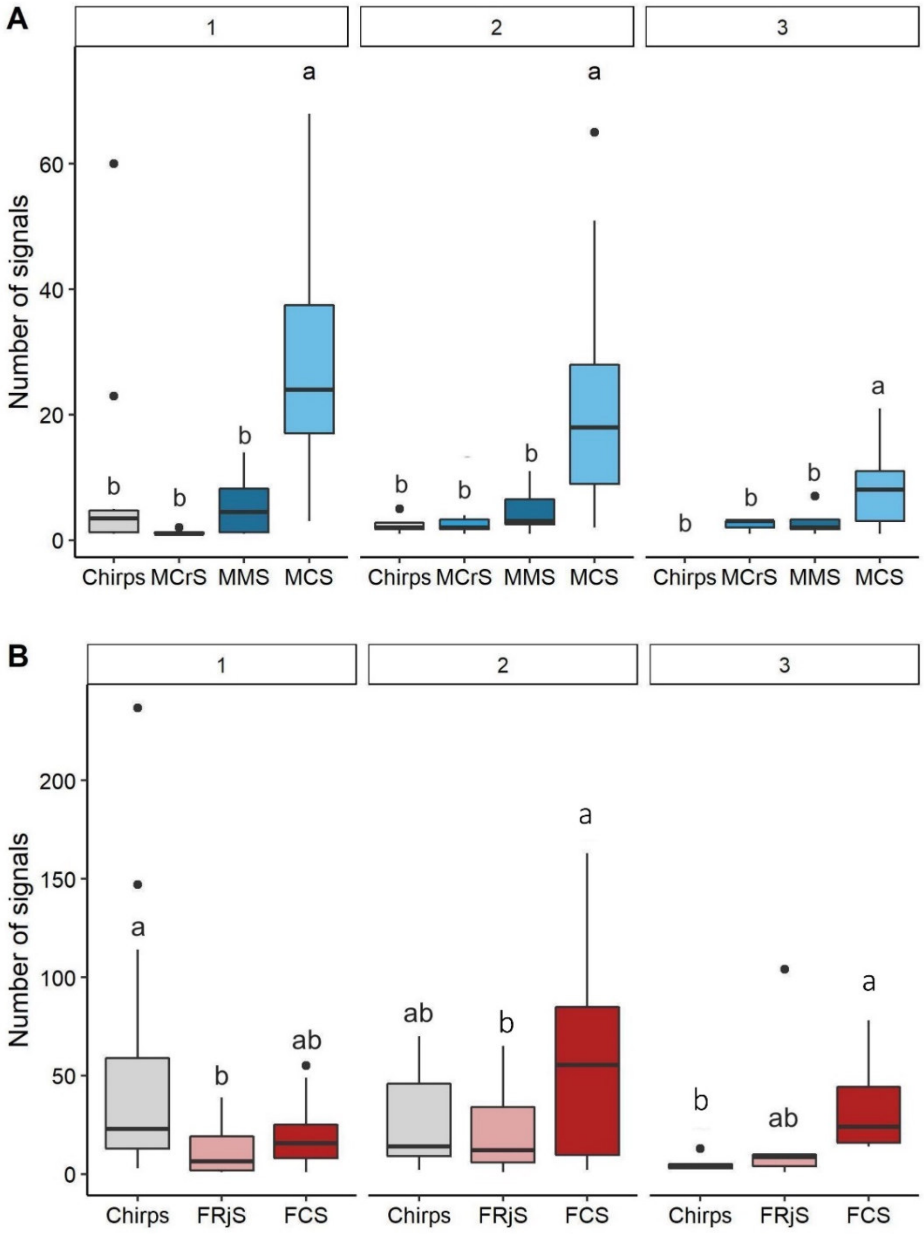

Figure 6: Number of signals emitted by males (A) and females (B) according to the type of signals and the number of signaling individuals (1, 2 and 3). Different letters (a,b) indicate significant pairwise differences

(p-value< 0.05) between types of signals for each number of signaling individuals.

3.4. Type of interactions

Male interactions consisted mostly of overlapped signals (62.63%), but also on partially overlapped (20.62%) and alternated signals (16.75%) of same or different types. Females tended almost exclusively to overlap their signals (96.5%).

4. Discussion

This study investigated intrasexual communication and associated types of vibrational signals occurring in non-social insect groups. Under semi-field conditions (e.g., natural daylight and temperatures), the signaling behavior of *P. spumarius* females was recorded from the 24th of July 2018, earlier than a previous study in laboratory conditions that reported the occurrence of female mating signals from August, in correspondence with the maturation of their reproductive apparatus (Avosani et al., 2021). Females produced intersexual vibrational signals, when tested alone, in the presence of another male or when subjected to a playback only when they were sexually mature. Even if female signaling was mainly concentrated in the second part of the summer, their overall activity was significantly higher than that of males. Although the male signaling also increased during the summer, this trend was much stronger in females, who were basically silent during the first half of the season. The male signaling activity varied during the day, being higher in the evening and lower in the afternoon, while females tended to produce signals regardless of the time of the day. Unlike leafhoppers (Mazzoni et al., 2009b, Nieri et al., 2017), *P. spumarius* females are the calling gender. The increased interest in finding a mate might explain why female signaling occur during the entire day. Since *P. spumarius* can reach high population densities in the field (Cornara et al., 2018), females likely dilute their signaling throughout the day to enhance their possibility to find a valuable partner, even if this strategy can be energetically costly (Kuhelj et al., 2015).

Differently from Avosani et al. (2020) where chirps were reported as short elements that compose the calling signals of both males and females of *P. spumarius*, chirps were detected in our trials also as isolated signals without a clear temporal pattern. Females produced fewer isolated chirps as the season progressed. Only in the later summer, when females had reached full sexual maturation, they did emit calling signals. Males, on the other hand, emitted isolated chirps without any seasonal trend, even if less frequently than females. Given that isolated chirps were produced by both sexes, the role of these signals remained unclear. One hypothesis is that chirps may be used to assess the presence of nearby conspecifics regardless of their sex. Although males and females could not directly interact, specific mating signals such as the female calling signal and the male courtship signal were recorded in our trials. As expected, the female response signal (which is emitted in tight synchrony within the male courtship to establish a duet (Avosani et al., 2020)) was not produced by females. On the other hand, although the male courtship is usually emitted by males in response to female calling signals (Avosani et al., 2020), it was produced by males in absence of females during our trials. The same behavior was also observed in the absence of other males and in response to playbacks of male signals (Avosani et al., 2021). It is possible that the male calling signal triggered other males to produce courtship signals, due to similarities with the female calling (i.e., they are both composed of chirps). However, a more probable hypothesis is that the courtship signal (and with some degree the female calling signal as well), could be used by the spittlebugs to assess the behavior of their (same sex) neighbors. In fact, insects may adjust their signals to the same level (cooperative interaction) or modify the spectral and temporal features of their signal to decrease their rival fitness (competitive interaction) (Greenfield, 1994a, West-Eberhard, 1984). In some insect species, collective signaling behaviors allow them to regulate local population densities and assess competitiveness of neighbors over food, mate or space (Greenfield, 2002, Wynne-Edwards, 1962). When two individuals approach one another or come in contact, an aggressive behavior can also be manifested (Greenfield, 2002). In our study, *P. spumarius* females and males expressed aggression by emitting female rejection signals and male-male signal, respectively.

Males of *P. spumarius* produced overall more male calling signals, independently from the number of signaling individuals. Contrary to our expectations, the male signaling activity was lower when more individuals were simultaneously signaling, suggesting that they may avoid interacting with their signaling neighbors. The situation was different in the case of females. When females emitted signals alone, they produced more chirps, while they emitted more female calling signals when interacting with other females. The presence of signaling females may trigger the activity of others, creating a cooperation that may increase their chances of finding/attracting a suitable mate (Greenfield, 2002). Moreover, the cooperation expressed as a possible signaling chorus would enlarge the female active space (the three dimensional area over which a signal can be detected by a potential receiver) on the same plant, overcoming the energetic cost of signaling throughout the day (Greenfield, 1994b, Mazzoni et al., 2014). Playback bioassays of the different types of signals, using same-sex groups, would confirm or reject these hypotheses.

Aside from the type of emitted signals, our study demonstrated that signals emitted by individuals of the same sex during the same time window were alternated or overlapping. Alternated signals in the case of males may impair the signaling of others (Hunt and Morton, 2001), particularly, as mentioned before, that male activity was reduced when more individuals were signaling. Furthermore, alternated signals may also refer to competition between males over space on the same plant. When males are clustered in space, they strongly compete over their territory (West-Eberhard, 1984). On the other hand, the overlap of signals that occurred in both intrasexual groups may underlie a cooperative behavior driving adults of *P. spumarius* to enhance their feeding by aggregation on the same part of the plant. *Philaenus spumarius* is a xylem-feeder that requires a great amount of energy to overcome the high tension in the xylem mainstream of the plant (Malone et al., 1999). The potential aggregation of adults on the same plant might be a strategy to overcome the xylem tension that results in less energy expenditure (Cornara et al., 2018). A similar strategy is used by the group-living treehopper *Calloconophora pinguis*, in which nymphs overlap their vibrational signaling to recruit further members to a new feeding source (Cocroft, 2005). Moreover, this behavior may also improve the survival of the spittlebug by protecting them from predators. Nymphs of *P. spumarius* aggregate on the same plant and share the same spittle mass, which provides them with a shelter and protects them from natural enemies (Wise et al., 2006, McEvoy, 1986). Similarly, adults could prevent being localized by natural enemies by synchronizing their signals (Greenfield, 1994b).

5. Conclusions

Investigating the intrasexual behavior of *P. spumarius*, based on vibrational signals, allowed us to discern substantial behavioral differences between males and females and revealed that the type of intrasexual interactions that occur among simultaneously signaling males consisted of overlapping and alternated signals, while they consisted of only overlapping signals in the case of females. Besides providing ethological insights, similar information may support the development of behavioral manipulation techniques based on substrate vibrations. By exploiting the competitive or cooperative behavior to target individuals of the same sex (Nieri et al., 2021). Further research based on vibrational signal playbacks is needed to determine the exact function of these intrasexual interactions in the case of *P. spumarius*.

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Supplementary Materials

Table S1: Signaling activity per period of the day as Mean ± standard deviation (SD).

Sex	**Period of the day**	**Mean ± SD**	**n**
Males	Morning	16.75±17.10	12
	Afternoon	9.86±8.75	12
	Evening	34.93±31.27	12
Females	Morning	53.32±74.41	12
	Afternoon	53.12±72.33	12
	Evening	74.07±64.04	12

Table S2. Complete results of the Mann-Whitney pairwise test, with Bonferroni correction, to test differences in the type of signals for both sexes.

Sex	**Contrast**	***p*-value**
Males	MCrS- Chirps	1.00
	MCS- Chirps	2.0e-05
	MMS - Chirps	1.00
	MCS- MCrS	4.1e-07
	MMS- MCrS	0.11

	MMS- MCS	1.1e-08
Females	FCS- Chirps	1.0000
	FRjS- Chirps	0.0734
	FRjS- FCS	0.0065

Table S3. Complete results of the Permanova test of the number of signals for both sexes.

Sex	**Parameter**	**Df**	**SumOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
Males	Signal	3	8.50	2.84	24.97	0.34	0.001
	Individuals	2	1.19	0.60	5.26	0.05	0.002
	Signal: Individuals	5	0.67	0.13	1.18	0.03	0.287
	Residuals	128	14.54	0.11		0.58	
	Total	138	24.91			1.00	
Females	Signal	2	1.01	0.51	3.16	0.04	0.016
	Individuals	2	0.40	0.20	1.23	0.02	0.280
	Signal: Individuals	4	2.33	0.58	3.62	0.10	0.001
	Residuals	129	20.79	0.16		0.85	
	Total	137	24.54			1.00	

Table S4. Complete results of the analysis of multivariate homogeneity of group dispersions for both sexes as an assumption for the Permanova test. In the case of males, the heterogeneity of dispersion was significant for the number of individuals. Therefore, the low *p*-value in the

Permanova test was not considered and no further pairwise test was conducted for this factor.

Sex	Parameter		Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
Males	Type of signals	Groups	3	0.08	0.03	0.72	999	0.569
		Residuals	135	5.15	0.04			
	Number of individuals	Groups	2	0.13	0.07	3.78	999	0.023
		Residuals	136	2.42	0.02			
Females	Type of signals	Groups	2	0.04	0.02	0.54	999	0.577
		Residuals	135	4.74	0.03			
	Number of individuals	Groups	2	0.04	0.02	0.55	999	0.566
		Residuals	135	5.40	0.04			

Table S5. Complete results of the Mann-Whitney pairwise test, with Bonferroni correction, to test differences in the type of signals in each number of signaling individuals in the case of females.

Number of individuals	Contrast	*p*-value
1	FCS- Chirps	0.172
	FRjS- Chirps	0.013
	FRjS- FCS	0.278
2	FCS- Chirps	0.119
	FRjS- Chirps	1.000
	FRjS- FCS	0.072

3	FCS- Chirps	0.005
	FRjS- Chirps	1.000
	FRjS- FCS	0.082

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Chapter 4

Vibroscape study of a vineyard

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Abstract

Environmental conditions are crucial factors that influence communication systems and affect animal behavior. Research in the field of biotremology has improved our understanding of insect behavior, ecology, and evolution. However, the interactions between vibrational signaling and the environmental factors are less studied, mainly because of technical issues faced in field trials. We therefore developed and tested an approach to investigate the effect of abiotic factors on insect vibrational signaling and evaluated its implementation as a monitoring tool of insect vibrational signals, using a vineyard as an agroecosystem model. Our results show a significant decrease of insect signaling activity during unsuitable conditions of high temperature and wind velocity. We also determined the daily signaling pattern of the two vineyard pests *Scaphoideus titanus* and *Halyomorpha halys* and the spatial occurrence of their signals in the vineyard. We conclude that biotremology techniques could be profitably used to evaluate not only quantitative information but also to monitor the biodiversity associated to insect vibrational signaling. The method implemented in this study could be applied in particular to compare the quality of cultivated areas at different management systems.

Keywords: vibroscape, biotremology, vibrational signaling, vineyard.

1. Introduction

Animals that use sounds or substrate vibrations to communicate live in complex ecosystems where sending and receiving information is crucial for their reproduction and survival (Dominoni et al., 2020). Environmental conditions, presence of noise, and interactions with their surroundings may directly or indirectly influence their signaling behavior (Halfwerk et al., 2011, Römer et al., 2010, Trillo et al., 2016). Therefore, it is essential to achieve a comprehensive understanding of the relationship between animal behavior and the environment where they live in order to determine the influence of environmental conditions on their vibrational communication.

The study of soundscape ecology has contributed to develop the discipline of eco-acoustics that investigates the relationship between the sound and the environment (Sueur and Farina, 2015). This discipline studies the sounds characterizing diverse ecosystems and relies on non-invasive methods to assess, for example, the impact of human activities or derived effects (i.e., climate change) on biodiversity (Linke et al., 2018, Desjonquères et al., 2020, Krause and Farina, 2016). On the other hand, in biotremology, which studies the production, perception, and transmission of mechanical vibrations through a substrate (Hill and Wessel, 2016), eliminating the background noise from the recordings is necessary to facilitate the identification and characterization of substrate borne vibrations. However, noise filtering is challenging because of its overlap with insect vibrations. Beside this, the vibroscape study, which consists of recording biological, geophysical, and anthropogenic vibrations deriving from a given landscape, requires a conspicuous and heavy equipment (i.e., laser vibrometer and associated devices that require handling by more than one person), a constant need of power supply and the presence of personnel on the spot during long recording sessions (Šturm et al., 2019). These technical issues have, in the past, made biotremology trials confined to laboratory soundproof chambers and reduced the possibility to perform recordings outdoor and, consequently, to measure the effect of the environment on the insect vibrational behavior. Although in the last few years some studies have started to investigate the vibroscape in natural contexts (Šturm et al., 2019, Tishechkin, 2013), there is still much to do to explore the potential of biotremology in the study of community ecology, especially in association to anthropic and abiotic factors.

In nature, the assemblage of multiple individuals and species of insects, signaling together, often results in time and space signaling variability (Šturm et al., 2019). One driver of it resides in abiotic factors, such as wind and temperature, which can impact insect signaling (Gasc et al., 2018, McNett et al., 2010). Agroecosystems in particular, compared to other ecosystems, are usually characterized by relatively low biodiversity and dominated by one or few specialized species (e.g., pests) (Sisterson et al., 2020, Samways, 2005) with the occurrence of many generalists. Because of this simplification, a vibroscape investigation conducted on a crop would be less complicated than in a natural environment and could provide relevant information about the presence, abundance, and phenology of certain species typical of that crop.

In this study, we first investigated how the vibrational signaling activity of an insect community in a vineyard changes according to abiotic factors and time during a summer day. We also evaluated whether the approach adopted to record vibrational signals could be implemented as a monitoring tool of key pest species occurring in the vineyard. To accomplish these aims, we chose an organic vineyard as a model agroecosystem that represents a habitat which includes many insects that primarily communicate by substrate-borne vibrations. Among these, two widespread species occurring in most European vineyards are the leafhoppers (Hemiptera, Cicadellidae) *Scaphoideus titanus* and *Empoasca vitis* (Mazzoni et al., 2009, Nieri and Mazzoni, 2018). The monitoring of these two major grapevine pests is based on traditional techniques, such as direct observation and the use of sticky traps (Decante and van Helden, 2006). Such techniques are not specific and may capture many non-target species also including beneficial insects; in this way, the detection of vibrational signals would be a more ecosystem friendly monitoring alternative. Furthermore, many other insects, such as the Brown Marmorated Stinkbug (BMSB), *Halyomorpha halys*, can also occur in the vineyards as occasional generalist pests (Hamilton et al., 2018). The BMSB vibrational communication has been already described (Polajnar et al., 2016) and since it is very common in the investigated area, a vibroscape analysis would provide us with prominent information about the presence of this species in a vineyard, about its signaling activity throughout the day and in association to abiotic factors.

In this regard, to examine how the vibrational signaling strategy might be affected by temporal and abiotic variables, we investigated the effect of time during the day, position of recording (i.e., middle or at the border of the vineyard), and environmental factors on the signaling activity of insects composing the vineyard vibroscape in the period of July-August.

2. Materials and methods

2.1 Recording and analyses of vibrational signals

2.1.1 Recording of vibrational signals in the vineyard

Trials were conducted from the beginning of July to the end of August 2019, in an organic vineyard of 0.28 ha in the campus of Fondazione Edmund Mach (San Michele all 'Adige, Trentino, Italy; 46.18953 N, 11.13625 E, WGS84). The vineyard was limited by a wood (*Carpinus*, *Alnus*, *Crataegus*, *Quercus* the main trees) on the east, a road on the west and vineyards on north and south sides. Recording sites were chosen randomly in the vineyard, the distance between neighboring points ranged between 6 -18 m (see Figure S1 in supplementary material). To record the insect signaling throughout the day, two continuous sessions were conducted: S1, from 7:00 to 14:00 and S2, from 15:00 to 21:00. Each session was replicated 13 times (26 sessions in total), resulting in a total duration of 190 hours. Data of hourly climate measurements were obtained from the automated weather station website of Fondazione Edmund Mach in San Michele all' Adige (http://meteo.iasma.it/meteo/index.php).

2.1.2 Recording of vibrational signals of *S. titanus* in semi-field conditions

The goal of this experiment was to assess the *S. titanus* daily vibrational signaling when the population density is known (semi-field condition) and to compare it with that measured when the population density is unknown (field condition). Because females do not emit signals spontaneously but only when elicited to respond by male calling songs and also because the presence of active females can trigger males rivalry behaviors (Mazzoni et al., 2009), we decided to exclude females from the cages and study only the signaling behavior of males. The latter were collected, in August 2020, from an infested vineyard near the investigated area and immediately transferred into mesh rearing cages (Bugdorm-6620, 60x60x120 cm, MegaView Science Co., Ltd., Xitun Dist., Taiwan) that were supplied with one year old grapevine plants (*Vitis vinifera* L. cv. Pinot noir grafted on Kobber 5BB). Five minutes before initiating recordings, 6 males were randomly collected from the rearing cages and placed on another grapevine plant inside another mesh cage that was placed outdoor. Similar to the vineyard experiment, trials were performed in two recording sessions (S1: from 7:00 to 14:00 and S2: 15:00 to 21:00). Each session was replicated 6 times (12 sessions in total), resulting in total duration 84 hours of recording.

2.2 Signal recording and analysis

Vibrational signals were recorded using a laser doppler vibrometer (Ometron VQ-500-D-V Ltd, U.K.). The laser beam was focused onto a reflective sticker, attached to a green stem of the grapevine plant (diameter ca. 1 cm) in both the vineyard and the semi-field experiments. Signals were digitized using the software BK Connect (Brüel and Kjær Sound & Vibration A/S, Nærum, 104 Denmark) at a sampling rate of 44.1 kHz and a depth resolution of 16 bit, through a multichannel calibrated data acquisition device (LAN XI type 3050-B-040, Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark), and stored to a computer hard drive (HP, EliteBook 8460p). To accommodate the processing of signals to manageable proportions, each recording was acquired as a 10-minute file. Spectrogram analysis was performed with the software Raven Pro 1.5 (The Cornell Lab of Ornithology, 151 Ithaca, NY) using Fast Fourier Transform type Hann, with a window length of 512 samples, a frequency resolution of 8 Hz and 75% overlap. Each recording was visually and orally screened to characterize the vibrotypes (i.e., different types of vibrational signals according to distinct temporal and spectral characteristics). Among 30 relatively different vibrotypes, vibrational signals of *S. titanus* (Mazzoni et al., 2009) and *H. halys* were identified (Polajnar et al., 2016).

2.3 Statistical analyses

2.3.1 Signaling variation in the vineyard

Statistical analyses was performed with R version 4.0.2 (R Core Team, 2018) run in the R Studio interface (RStudio Team, 2020). Plots and graphic design were done using R packages: “ggplot2” and “cowplot” (Wickham, 2016, Wickham and Wilke, 2019).

The signaling activity (SA) was calculated as the time (in minutes) insects spent signaling per hour. A Tweedie generalized mixed model

(GLMM), with a log link function, was applied using the function "glmmTMB" from the package "glmmTMB" (Brooks et al., 2017). Covariates consisted of "time" (hours during the day), "number of vibrotypes" (different types of signals), and "position" of recording in the vineyard (middle and border) as fixed factors. In addition, temperature (°C), wind velocity (m/s), relative humidity (%), and air pressure (hPa) were used as covariates representing environmental conditions. To account for the dependency among observations that were acquired during the same recording session and on the same site in the vineyard, "session" was used as a random factor. The variance inflation factors were calculated and relative humidity was dropped because of its highly significant negative correlation with temperature (Zuur et al., 2010). After developing the full model (Equation 1), model assumptions and spatial dependency were verified (Zuur and Ieno, 2016). Akaike Information Criterion (AIC) was used for model selection, with further optimization of the covariates by backward selection (see Table S1 in Supplementary material).

$SA_{ij} \sim \text{Tweedie}(\mu, \sigma^2, \varrho)$

$E(SA_{ij}) = \mu_{ij}$

$\log(\mu_{ij}) = \text{Time}_{ij} + \text{Number of vibrotypes}_{ij} + \text{Temperature}_{ij} + \text{Wind velocity}_{ij} + \text{fPosition}_{ij} + \text{Session}_{i}$

$\text{Session}_{i} \sim N(0, \sigma^2)$

(Equation 1)

Where SA_{ij} is the j^{th} recording in $Session_i$ ($i = 1$ to 26), and $Session_i$ is the random intercept, which is assumed to be normally distributed with mean 0 and variance σ^2.

2.3.2 Signaling of *H. halys* and *S. titanus* in field and semi-field conditions

To show the daily activity of *H. halys* and to compare those of *S. titanus* in the vineyard and in semi-field conditions, the SA was calculated as previously described.

3. Results

3.1 Vibrotypes and abiotic factors changes during the day

The morning (07:00-14:00) and evening (18:00-21:00) of the recording period were characterized by low temperature and low wind velocity, with the occurrence of many vibrotypes (range, 0-13) (Figure 1). While the afternoon (14:00-18:00) was characterized by high temperature and high wind velocity, with few vibrotypes (range, 0-7) (Figure 1).

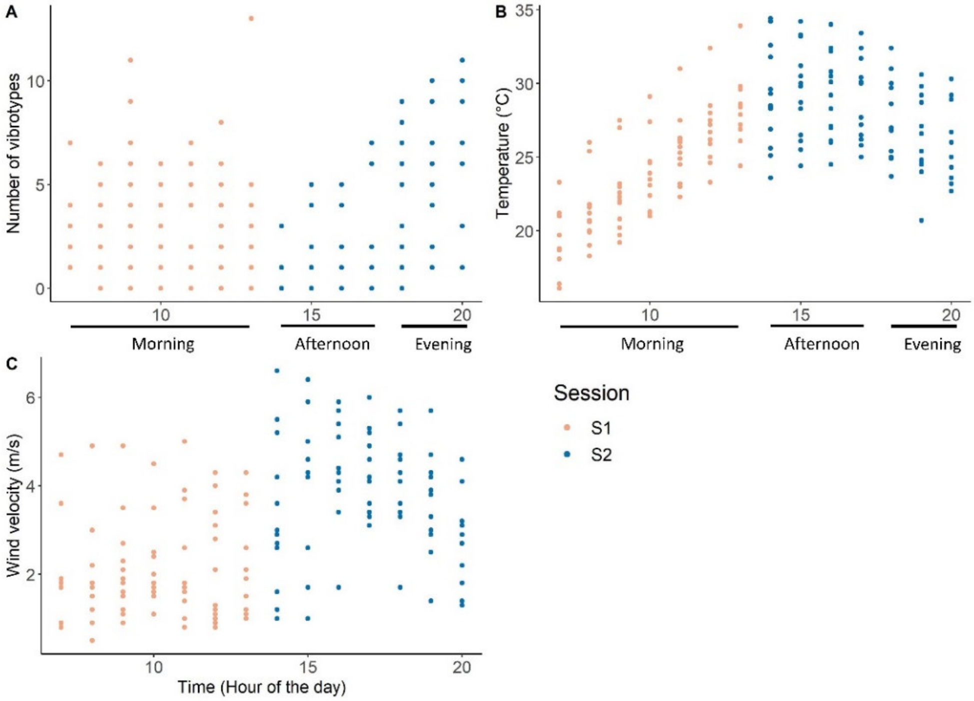

Figure 7. Variation of the covariates during the day: (**A**) Number of vibrotypes; **(B)** Temperature and (**C**) Wind velocity. S1: 7:00 to 14:00 (morning) and S2: 15:00 to 21:00 (afternoon and evening).

3.2 Signaling variation in the vineyard

The insect SA was highly variable throughout the day (0-54 min/hour). The SA was significantly influenced by time, number of vibrotypes, temperature, and wind velocity (Table 1). Fixed effects alone (marginal R^2 =0.42) and together with the random factor (conditional R^2= 0.66) had a high explanatory power in the model.

Table 2. Estimated regression parameters, standard errors, z ratio, and *p*-values from the Tweedie GLMM testing the effect of number of

vibrotypes, time, temperature, wind velocity and position. Session was used as a random factor and the estimated value for $\sigma_{Session}$ is 0.973. The intercept corresponds to position: border.

	Estimate	Std. error	z value	p-value
Intercept	0.734	0.315	2.331	0.020
Time	0.624	0.165	3.779	< 0.001
Number of vibrotypes	0.801	0.109	7.353	< 0.001
Temperature	-0.510	0.138	-3.702	< 0.001
Wind velocity	-0.515	0.138	-3.723	< 0.001
Position: middle	-0.612	0.427	-1.433	0.152

Most signaling occurred in the morning and in the evening, with a significant decrease in the afternoon (Figure 2A). The signaling significantly increased with the number of vibrotypes (Figure 2B). In total, 30 vibrotypes were detected with a maximum of 13 vibrotypes per hour (13:00-14:00). High SA was recorded in mild conditions of temperature (Figure 2C; 20-30°C) and wind velocity (Figure 2D; <4 m/s), while it strongly decreased at higher temperature (2D; >30°C) and wind velocity (Figure 2D, >4m/s).

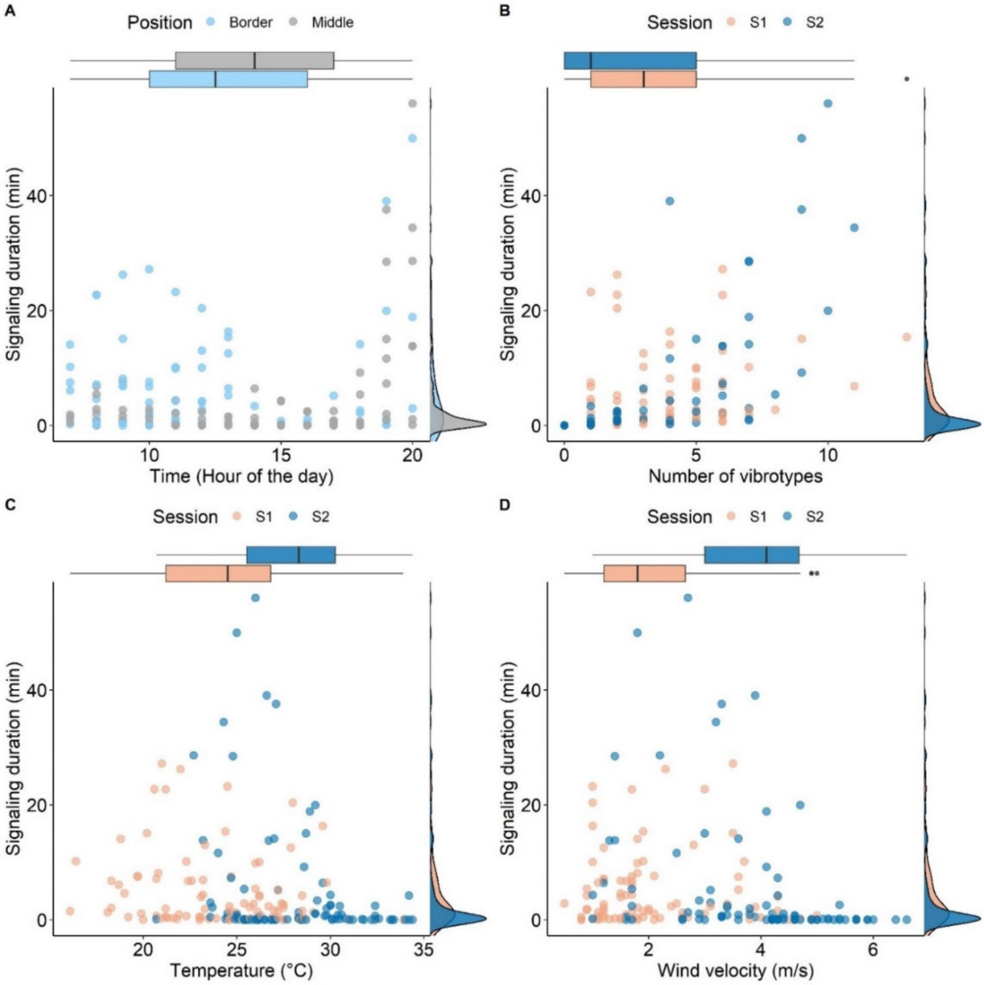

Figure 8. Scatterplots showing the effect of time of the day (**A**), the number of vibrotypes (**B**), temperature (**C**), and wind velocity (**D**) on the signaling activity. Boxplots above indicate the variation of the covariates between positions (**A**) and sessions (**B**, **C** and **D**). Density plots (on the right) indicate the frequency of signaling activity in position (**A**) and sessions (**B**, **C**, **D**). S1: 7:00 to 14:00 (morning) and S2: 15:00 to 21:00 (afternoon and evening).

3.3 Signaling of *H. halys* and *S. titanus* in the vineyard

The signaling of *H. halys* mostly occurred in the morning and evening, while very low SA was recorded in the afternoon (Figure 3). Whereas SA of *S. titanus* was concentrated in the evening, and it was the main driver of the increase of overall signaling activity at that period of the day. SA of *H. halys* and *S. titanus* according to position in the vineyard are illustrated in Figure S2, in Supplementary material.

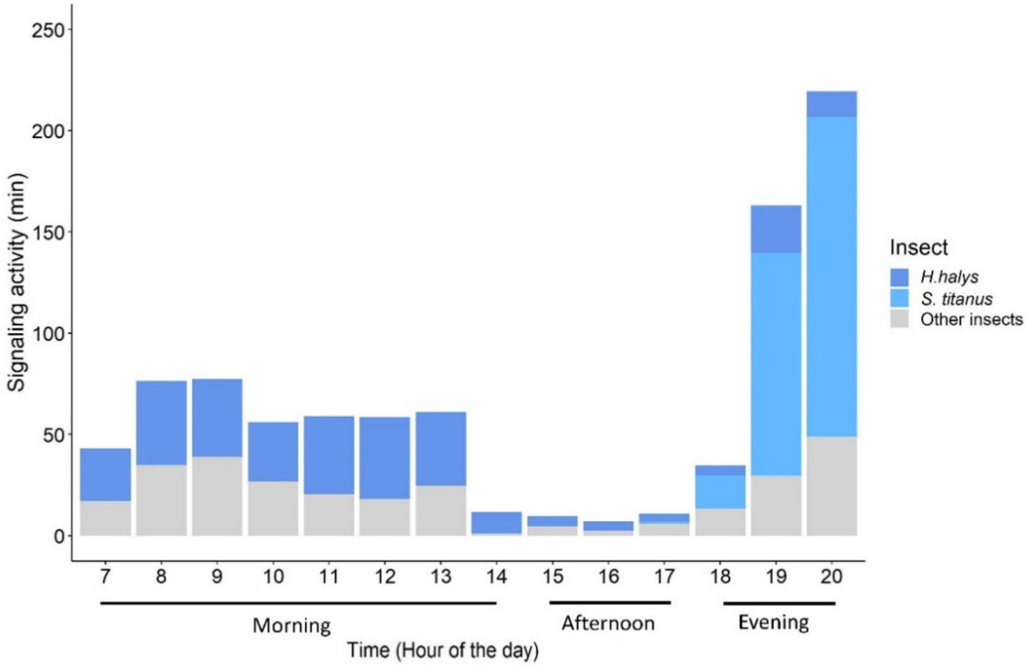

Figure 9: Signaling activity of *H. halys*, *S. titanus* and other, not identified, insects in the vineyard during the day.

3.4 Signaling of *S. titanus* in field and semi-field conditions

The daily pattern of *S. titanus* SA was similar in both field and semi-field trials, confirming that insects were mainly active in the evening (Figure 4). However, in semi-field conditions vibrational signals of *S. titanus* were also recorded earlier in the day (from 11:00 to 13:00) and the peak of activity started from 17:00 while it started from 18:00 in the field conditions.

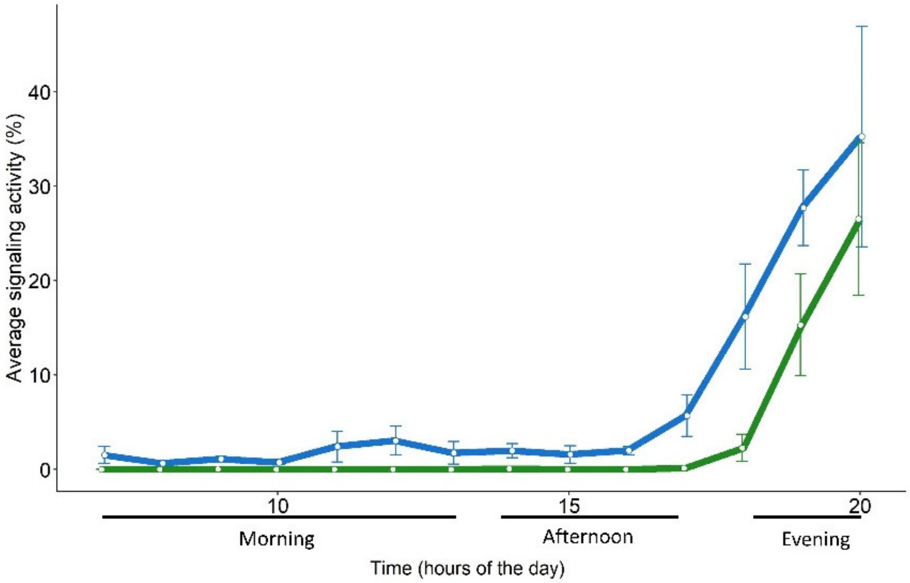

Figure 4. Daily pattern of the signaling activity of *S. titanus* in the vineyard (in green) and in semi-field conditions (in blue) presented as the average time insect spent signaling per hour. Standard error of the mean is shown with error bars.

4. Discussion

4.1 Effect of abiotic factors on vibrational signaling

The present study investigated the daily vibrational signaling of insects in field conditions and provided novel information of the effect of environmental conditions on their signaling activity. Although the total measured signaling activity resulted from vibrational signals of several different insect species, a common pattern was obtained representing a general trend of insect activity during a summer day. As expected, the signaling activity increased with the number of vibrotypes, which however are not necessarily associable to different species but also to different signals of the same species. We can assume that when more insects are signaling, the signaling activity is higher but in the absence of a library of signals we cannot make precise assumptions about biodiversity.

Results showed that insect signaling in the vineyard changed also according to time, temperature, and wind. In fact, most signaling occurred in the morning and evening when temperature and wind velocity were low, with the exception of the early morning (7:00-08:00), which could be explained by the very low temperature (16-20°C). Cold conditions slow down the insect biological processes and for this reason many species can start calling only when a certain temperature is reached (Kingsolver, 2009). On the other hand, we consistently found low signaling activity in the afternoon when temperature and wind velocity reached the highest values. Our analysis indicates a significant effect of these two factors which was also found in a previous study of the influence of these two parameters (even though their effect was investigated separately) on the mating behavior of *Nilaparvata lugens* (Hemiptera: Delphacidae) in controlled conditions (Ahmed et al., 2016). A similar daily pattern was also reported in field recordings of *Enchenopa binotata* (Hemiptera: Membraciade), where the daily vibrational signaling activity was associated to wind velocity (McNett et al., 2010). Vibrational signaling of insects at different time windows can be a strategy to increase their communication efficiency. This strategy might be adopted to escape the masking of their signals by interferences from biotic (interspecific signals) or abiotic noise (wind and rain) (McNett et al., 2010, Tishechkin, 2013), or to avoid being detected by parasitoids and predators (Virant-Doberlet et al., 2019, Vélez and Brockmann, 2006).

Our results showed a significant effect of temperature as well. In fact, temperature can affect the characteristics of vibrational signals as it can increase the signal frequency and pulse rate in some species of spiders, bees, flies and planthoppers (Brandt et al., 2018, Brandt et al., 2020, Conrad et al., 2017, Ritchie et al., 2001, De Vrijer, 1984, Shimizu and Barth, 1996). For instance, temperature induces differences in mating signals and therefore affects mate preferences (Brandt et al., 2018, Jocson et al., 2019, Symes et al., 2017). The performance of the muscles involved in producing vibrations may depend on the thoracic temperature, which in turn depends on the ambient thermal conditions. The high temperature might increase the muscle contraction rate (Greenfield, 2002), which would result in high energy expenditure (Kuhelj et al., 2015). In our study, insects have likely reduced their vibrational signaling at higher temperature as a strategy to save energy. Unfavorable conditions of temperature and relative humidity have been shown to reduce and inhibit the sexual vibrational communication and behavior of the leafhopper *Amrasca devastans* (Hemiptera, Cicadellidae) (Kumar and Saxena, 1986). Although, relative humidity was not included in our analysis, it cannot be excluded that its effect is likewise important.

Leafhoppers, as dominant species in the vineyard, prefer microclimate of moderate temperature and relative humidity as they usually settle on the underside of leaves (Vidano, 1959).

On the other hand, it was not surprising that wind has also affected insect vibrational signaling since it constitutes a major source of noise disturbing arthropods that communicate by substrate-borne vibrations (Cocroft and Rodríguez, 2005, Cocroft et al., 2014, Virant-Doberlet et al., 2014, Tishechkin, 2007). Even at the peak time of their activity, insects may reduce their signaling when exposed to wind gusts (Velilla et al., 2020). As a result, they may adjust the timing of their signaling according to wind velocity fluctuations or to the level of perceived noise (derived by the wind). Moreover, in conditions where strong wind occurs constantly during the day, insects may move to areas that are protected from the wind (Tishechkin, 2007, Tishechkin, 2013). Temperature and wind represented unfavorable conditions for insect signaling in the vineyard during a summer day. Thus, these two abiotic factors should be considered when recording and evaluating vibrational signaling of insects in the field. In future studies it would be interesting to compare the signaling activity of the same insect community between areas characterized by different weather conditions. It would be fruitful to investigate whether wind and temperature could represent a selection pressure on the timing of insect vibrational signaling.

4.2 Signaling of *H. halys* and *S. titanus* during the day

Besides information about insect activity across abiotic factors, we determined the daily pattern of the insect species *H. halys* and *S. titanus*. While the signaling pattern of *H. halys* more or less reflected the general daily signaling trend in the vineyard, *S. titanus* was mainly active in the evening. Vibrational signals of *E. vitis* were not detected in our recordings. One possible reason is that, since the active space of *E. vitis* is restricted to a leaf area and the intensity of their vibrational signals is very low (R. Nieri 2021, pers. comm.), the signals could have been attenuated before reaching the plant stem because of heterogeneity of the plant parts (Mazzoni et al., 2014) or they could have been masked by the high background noise. Therefore, because the active space of insects cannot be predicted, acquiring signals at different points on the same plant would be an optimization of the approach, used in this study, in order to use it not only to study temporal changes in the vibroscape but also to characterize the vibrational community composing it. The development of a vineyard reference library of vibrational signals is therefore crucial to associate vibrotypes to insect species.

4.3 Signaling of *S. titanus* in field and semi-field conditions

Recordings in semi-field conditions revealed a very similar signaling pattern of *S. titanus* that was obtained in the vineyard (which consisted of signaling mainly in the evening). The semi-field recordings represented a positive control to the signaling activity of *S. titanus* obtained in the vineyard, validating the approach used to record vibrational signals in this field study. These results are also consistent with previous research conducted in controlled conditions, which showed that the sexual activity of *S. titanus,* mediated by vibrational signals, was associated with the twilight (Mazzoni et al., 2009). The flight activity of *S. titanus* depends on the photoperiod (Lessio et al., 2011) and increases with low temperature (Chuche and Thiéry, 2014), which supports the occurrence of the peak of their activity in the evening. Furthermore, adults of *S. titanus* exhibit an aggregative spatial distribution (Bosco et al., 1997) and their movement is mainly restricted to within the vineyard (Lessio et al., 2014), which explains detecting their signals mainly in the middle part of the vineyard.

In semi-field conditions, signals of *S. titanus* were recorded in the morning and one hour earlier in the evening in contrast to field conditions. One possible explanation could be that the sensitivity of our recording method depends on the insect population level. In semi-field conditions a higher number of individuals were consistently present on the same plant compared to the field conditions. Another reason could be that in semi-field conditions the presence of several individuals in proximity have triggered their interaction through vibrational signaling. Therefore, trials on different population levels and density should follow in order to test whether they might influence the sensitivity of the recording method.

5. Conclusions and perspectives

The approach used in the present study can be regarded as the basis of a promising tool to monitor the presence and the signaling activity of vibrational signaling insects. In particular for leafhoppers, of which monitoring techniques in the vineyard are generally invasive (e.g., sticky traps, sweeping nets), and not highly specific, affecting non-targets such as pollinators and natural enemies. Further optimizations of the method are needed: for instance, enlarging the matrix of sampling points at plant and vineyard level, in order to detect insects that are characterized by a very narrow active space such as *E. vitis*. Because moving from a plant to another during the recording sessions was challenging in our study, the use of light equipment such as accelerometers, would facilitate recording in multiple spots during shorter periods with a priory planned timing, number, and position of recording. A better option would be using fixed vibrational sensors deployed in a regular grid to record vibrational signals simultaneously and continuously, which could also enable evaluating the seasonal signaling variation and the vibroscape composition, once a large library of vibrational signals is developed. A matrix of sensors may also point out the transition of insects through the agroecosystem such as the case of *H. halys* in the vineyard. Since vibrational signals are species and sex specific (Čokl and Virant-Doberlet, 2003, Cocroft et al., 2014, Cocroft et al., 2006, Virant-Doberlet et al., 2006), and automated vibrational sensors have already been developed in biotremology (Korinšek et al., 2019), their use in agroecosystems would represent a highly specific alternative to remotely detect and monitor insects without interfering with their behavior. For specific insect species the set-up of sensors would be used to determine their daily signaling activity and target their monitoring at the peak time of their activity, for example in the evening in the case of *S. titanus*.

Furthermore, integrating a real time monitoring system with vibrational pest control would optimize the schedule of interventions according to periods of actual occurrence and sexual activity of target insects or insect life stages. In turn it would monitor the dynamics of the controlled pest and consequently evaluate the efficacy of the pest control strategy. As demonstrated by the present study, insect signaling activity depends on abiotic factors, which represent another aspect that regards the development of a vibrational mating disruption strategy (Polajnar et al., 2016). By including weather forecasting data, the devices that emit the disturbance noise would be actioned according to environmental factors such as wind and temperature which would give an important contribution in terms of energy management. These multiple attributes highlight the need of the design and implementation of vibrational sensor networks for long-term and large-scale monitoring projects. Similar to ecoacoustics where acoustic diversity indices have been correlated with traditional diversity indices (Gage et al. 2001) and used as a method of biodiversity assessment (Sueur et al. 2008b, 2012) we think that biotremology could be applied for studies to monitor and compare the biodiversity in agroecosystems.

To conclude, the present study provided some evidence and perspectives on how biotremology could be used in agriculture to monitor the presence and phenology of certain pests that use vibrational signals for intra-specific communication. This study is only a first step in the direction of a larger use of biotremology techniques for new aims. Both biotremology and bioacoustics techniques could be complementarily implemented and together with chemical ecology, they would form a multidisciplinary approach that can achieve an accurate description of a given ecosystem.

Acknowledgments

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Supplementary material

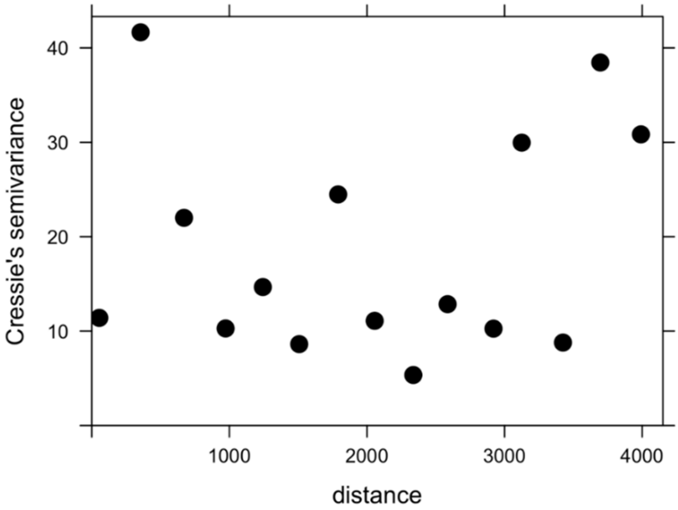

Figure S1: Semi-variogram for the residuals from the final model (Equation 1).

Table S1: Akaike information criterion (AIC) for the candidate models.

Model formulation	AIC
SA ~ Temperature * Position * Wind velocity + Number of vibrotypes + Air pressure + Time	742.71
SA ~ Temperature * Position * Wind velocity + Number of vibrotypes + Time	741.37
SA ~ Temperature + Position + Wind velocity + Number of vibrotypes + Air pressure + Time	741.32
SA ~ Temperature + Position + Wind velocity + Number of vibrotypes + Time	739.95

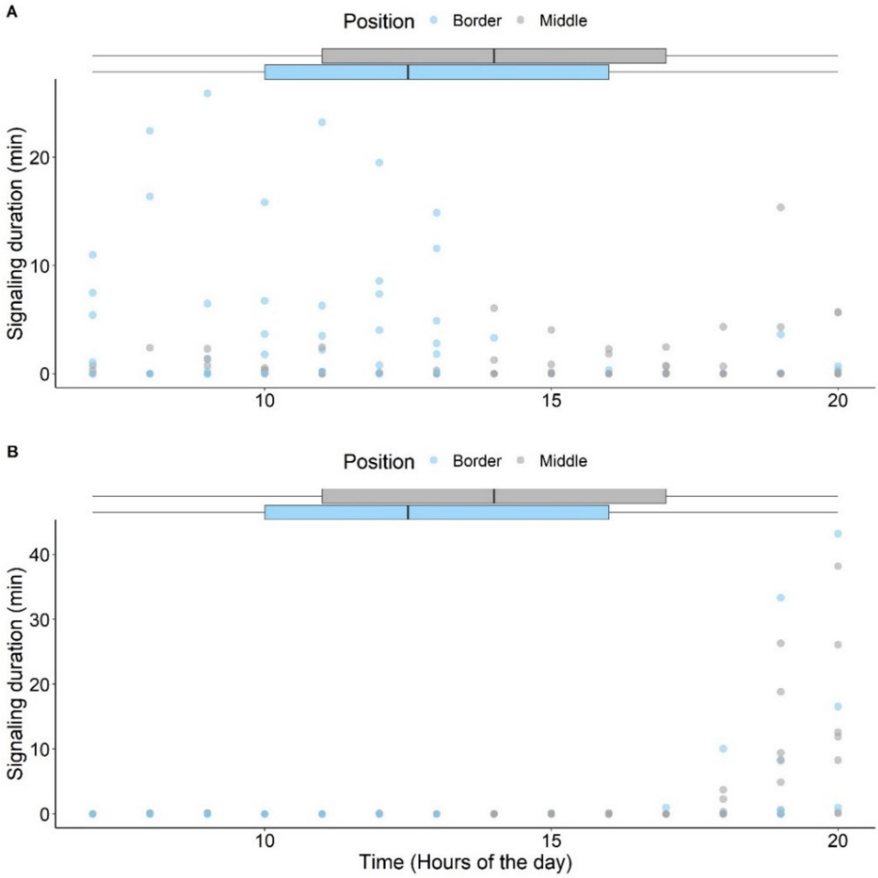

Figure S2: Scatterplots showing the vibrational signaling activity of *H. halys* (**A**) and *S. titanus* (**B**) during that day, according to position of recording in the vineyard as border (blue) or middle (grey)

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Chapter 5

Conclusions and future perspectives

The rapid growth of the field of biotremology has revealed the importance of vibrational signals in diverse insect behaviors and uncovered some constraints that shape the evolution of vibrational signaling systems (Cocroft et al., 2014b, Hill et al., 2019). Hence, considering that biotremology studies have been largely limited to the laboratory-controlled conditions, the aim of the present PhD thesis is to expand the study of vibrational signaling behavior of insects into more ecological systems, simulating natural situations in which they live and therefore provide more reliable information about their behavior. Different methodologies, adapted and suitable to the research objectives, were applied. Overall, the experiments performed throughout this dissertation provided new information about insect vibrational behavior. The different methodologies have allowed us to study a specific behavior, to simulate a high population density scenario, to evaluate seasonal and daily vibrational signaling patterns, and to investigate insect vibrational signaling in natural conditions, all while shedding new light on the possible application of biotremology techniques as a monitoring tool of insect occurrence and diversity. Depending on the investigated behavior and species, an appropriate methodology was chosen. I started from a study conducted in the laboratory where it was more practical to simulate conditions of temperature, relative humidity, and darkness, in order to reproduce the parental care behavior which occurs underground. As for herbivory insects, whose activity depends on photoperiod and other abiotic factors it was necessary to record outdoors.

Substrate vibrations are extremely widespread in animals and especially in insects, yet many behavioral contexts are understudied (Cocroft and Rodríguez, 2005, Cocroft et al., 2014a). Because parental care behavior in burying beetles occurs underground, it is a difficult task to record and study their stridulations in their natural habitat without possible disturbances. The main challenges were to design a common set-up without interferences throughout the period during which the behavior occurs and to find the best design where recording equipment are introduced close to the constantly moving beetles. This experiment was a challenge in that it was not an easy task to avoid interference between insects and recording devices that would have compromised the outcome. The lack of evidence that stridulations are involved in parental care has made research more focused on their chemical signals and cues (Müller et al., 2003, Smiseth et al., 2010, Steiger, 2015, Engel et al., 2016) and the stridulatory signals and corresponding structures have been studied mainly by handling the insects in the laboratory outside the context of parental care (Hall et al., 2013). By reproducing the burying beetles' behavior, we showed that stridulatory signals play an important role in the parental care behavior and more specifically in the post-hatching period. Burying beetles might have evolved such complex behavior to ensure optimal communication in attracting the larvae toward the carrion and/or to coordinate their provisioning. Furthermore, different signal patterns were recorded throughout the life history of the model beetle *N. vespilloides* used in our study, which underlie their social interactions. Beetles, likely, modulate their signals according to the corresponding behavior. The acquired information will serve as a foundation for future studies that address ultimate questions about the significance and evolution of parental care within the use of stridulations, which may display an impressive range of derived parental behaviors. Future studies are necessary to characterize the different types of stridulatory signals and determine their exact function which would improve our knowledge on the complexity and evolution of the parental care behavior in the *Nicrophorus* and other beetle species. The simulation of insect natural behavior as a research model opened new perspectives to explore parental care and possibly other behaviors occurring underground in burying beetles and in other insect species. Irrespective of the specific functions of their stridulations, it is certain that the potential effect of noise on their behavior would be occupying ecologists and conservationists in the future as the burying beetles are beneficial to the ecosystem facilitating decomposition processes and soil nutrient cycling (Scott, 1998, Rozen et al., 2008).

Vibrational signaling underlies many social interactions outside the context of reproduction. Herbivory insects typically live on their host plants along with many conspecific and heterospecific individuals. Such complex vibrational environment includes not only interference from abiotic noise (wind and rain) but also from the signals of competing (e.g.,Yack et al., 2001) or cooperating (e.g., Cocroft, 2005) individuals. In this context, vibrational signaling of multiple individuals of the same sex were recorded to simulate the environment in which they live when occurring in high densities. We characterized the temporal variation in the seasonal and daily vibrational signaling of *P. spumarius* to establish the context in which intrasexual vibrational communication may take place. Females were more active than males and their intrasexual interferences consisted of overlapping signals while male signals were either alternated or overlapping. The meaning of male signaling may refer to competition over space on the same plant whereas in the case of females it seems like a cooperative calling to enhance their chances to attract a suitable mate. Recording vibrational signal in semi-field conditions has provided accurate and valuable information about the behavior of *P. spumarius*. It was further used to evaluate the daily signaling pattern of *S. titanus* (Chapter 4). The methodology applied in this line of research has proven reliable and can therefore be used for other herbivory insects not only to study vibrational signaling, as subjected to seasonal and daily changes, but also to evaluate the effect of population density of the vibrational behavior of insects. The semi-field approach seems suitable in this case, as investigating the effect of population density in laboratory conditions would exclude the effect of environmental conditions.

Several studies have demonstrated correlations between factors of the environment and communication systems. The acoustic modality has been particularly well studied while far less is known about vibrational communication. We proved that vibrational signaling of insects are submitted to the influence of environmental factors. High temperature and wind velocity represented unsuitable conditions for insects in our field trials, which therefore significantly reduced or even stopped their signaling activity. The study of the vibroscape in the vineyard has allowed us to determine the daily signaling pattern of the two vineyard pests, *S. titanus* and *H. halys* and the spatial occurrence of their vibrational signals. Here, we used semi-field recordings as a positive control to those performed in the field. It remains to be ascertained whether the effect of abiotic factors on insect vibrational behavior would be different in the same agroecosystem but in other climatic conditions. It is possible that in hot environments the time window during which insect signaling was reduced would be narrower and signals of well adapted insects will be dominant. It would be interesting to investigate how thresholds of temperature and wind velocity would change in different conditions. By focusing on the communication environment, it becomes clear that there are many ways in which natural selection can affect animal communication. Future research should determine the importance of environmental factors in the evolution of vibrational communication of insects.

Field trials from this dissertation has extended our understanding of the communication environment in the vibrational modality. Given that pest monitoring is imperative for a successful management and traditional surveys of pests are laborious and not highly selective, we believe that such methodology can be used to monitor target insect species in the framework of a sustainable and smart farming system, for instance to detect alien species, pests and disease vectors. Optimizations of this approach were proposed in order to use an optimal setup in monitoring, not only to acquire quantitative information but also to investigate the biodiversity associated to insect vibrational signaling in agroecosystems. In particular, we would be able to compare the environmental quality of cultivated areas at different management systems. This would be feasible by using automated sensors that can be employed in the field as “vibro-traps” to capture and recognize vibrational signals of target species. Fixed or portable sensors can be used in diverse insect habitats for a continuous recording of their behavior.

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Biotremology has contributed to the understanding of multiple intra and interspecific interactions in insects. However, the exchange of vibrational signals in complex communication networks and in ecological contexts represents an understudied dimension of research in biotremology. Therefore, we aimed to expand the study of vibrational signaling behavior of insects into more ecological systems, simulating natural situations in which they live and therefore provide more reliable information about their behavior. Different methodologies were applied according to different research questions. The study consisted of continuous recordings with a laser vibrometer of insect communication throughout a specific behavior, in a multiple individuals' scenario and in natural conditions. Laboratory trials were conducted to reproduce and study the parental care behavior in burying beetles in relation to their stridulations. Comparing the stridulating behavior of Nicrophorus vespilloides between pre- and post-hatching care revealed a higher signaling activity in post-hatching and the occurrence of different patterns of signals. Moving to semi-field conditions, individuals of the same sex of the spittlebug Philaenus spumarius have been recorded throughout their adult stage season. We found a higher and delayed vibrational signaling activity of females compared to males and complex intrasexual interactions consisting of signal overlapping in the case of females and signal alternating, partial or complete overlapping in the case of males. Finally, field recording trials in an organic vineyard showed that low vibrational signaling activity of an insect community was significantly associated with conditions of high temperature and wind velocity. These findings open the possibility to the use of biotremology techniques to detect the presence of insect pests in an orchard as a tool of monitoring. Overall, this thesis provides novel approaches to record and study vibrational signals of insects, which can be used as a basis to perform further experiments in the field of biotremology.

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