

**ORIGINAL ARTICLE**

Special Issue: Biotremology

# The role of substrate-borne vibrational signals in the sexual communication of the painted bug, *Bagrada hilaris*

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**Funding information**

European Union—FSE-REACT-EU, PON Research and Innovation, Grant/Award Number: DM1062/2021

**Abstract**

The invasive alien stink bug *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae), native to eastern-southern Africa and central-southern Asia, poses significant economic threats to agroecosystems due to its polyphagous diet with a preference for brassicaceous crops. Current control methods using broad-spectrum insecticides are expensive and often result in significant negative ecological impacts, as well as posing a health risk to consumers. This study provides a detailed description of the mating behavior of *B. hilaris*, with the aim to ascertain whether substrate-borne vibrations have a role in premating phases. Such knowledge may contribute to the development of alternative biorational control techniques employing vibrational strategies. Virgin adults were tested individually or in pairs on different substrates, that is, a loudspeaker membrane and a bean plant. Vibrations emitted by males and females were recorded using a laser Doppler vibrometer, and the following spectral and temporal parameters were analyzed: fundamental frequency, emission time, repetition time, and signal modulation. Three distinct types of vibration emitted by males and females were identified: a male vibrotype (MV-1), a female vibrotype (FV), and a copula signal exclusively emitted by males (MS-2). MV-1 and FV were emitted prior to genitalia contact, whereas MS-2 was emitted within the mounting and engagement phases. Statistical analysis revealed significant transitions in behavioral phases for couples that achieved mating. By analyzing the walking behavior, there was an inverse relationship between motility and signal emission when comparing the two sexes, which suggests that males might engage in a form of searching behavior. These findings provide insight into the crucial role of vibrational communication to achieve mating in *B. hilaris*.

**KEYWORDS**

agricultural pest, behavioral manipulation, biotremology, Hemiptera, invasive species, IPM, mating behavior, Pentatomidae, stink bug, substrate-borne vibrational communication

**INTRODUCTION**

The global landscape is witnessing a burgeoning trend of biological invasions driven by invasive alien species (Pyšek et al., 2020). These species have significant ecological and economic impacts, posing harm to ecosystems and economies alike (Bellard et al., 2016; Diagne et al., 2021). *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) stands out as one of the most invasive alien species in recent times (Singh & Malik, 1993). Originally found in East Africa, the Middle East, and South

Asia, *B. hilaris* has significantly expanded its geographic range and has established itself as an agricultural pest in various world regions, including California, Mexico, Malta, and Pantelleria (Italy) (Carvajal et al., 2019). This pest poses a significant challenge due to its polyphagous diet, which allows it to infest numerous economically important agricultural crops, primarily those belonging to the Brassicaceae family (Palumbo et al., 2016). *Bagrada hilaris* feeds by a 'lacerate-and-flush' feeding method, inducing circular chlorotic lesions on host plant leaves, leading to apical meristem destruction, and inhibiting

shoot terminal growth, with mechanical damage intensified by gregarious behavior in nymphal instars (Huang et al., 2014). Consequently, affected host plants may be deemed non-marketable (Guarino et al., 2017; Infantino et al., 2007).

Current control techniques involve extensive application of broad-spectrum insecticides such as pyrethroids and neonicotinoids (Palumbo et al., 2016). Besides being expensive and time-consuming solutions, insecticides are often ineffective as the target pest is naturally inclined to develop resistance to periodically used treatments (Dhingra, 1998; Guarino et al., 2007). To achieve sustainable and species-specific control, it is crucial to develop economically viable techniques with no off-target effects. Recent research suggests that classical biological control and the sterile insect technique could be effective strategies for managing this species (Cristofaro et al., 2022; Isman, 2006; Martel et al., 2019). Interestingly, new emerging techniques involving the exploitation of vibrational communication among insect pests are proving to be effective for their control (e.g., Eriksson et al., 2012; Thiery et al., 2023; Zapponi et al., 2023). The study of communication through substrate-borne vibrations belongs to the developing field of biotremology (Hill & Wessel, 2016), which is progressing rapidly with new findings in various insect species (e.g., Mazzone et al., 2017; Nieri et al., 2022). No reports in the literature have evaluated the vibrational behavior of *B. hiliaris*; however, it is very likely that it employs such a communication type, as it is common among pentatomids.

The mating behavior of *B. hiliaris* has been primarily characterized from a chemical communication perspective, where females are attracted to volatile compounds produced by males (Guarino et al., 2008). Once a potential partner is located, the male recognizes the opposite sex mainly by assessing the hydrocarbon-coated cuticular surface through antennation. The actual courtship begins with specific mounting behavior by the male on the female's back and with his antennae in contact with her genitals. Generally, pentatomids rely both on semiochemicals (i.e., pheromones) and semiophysicals (i.e., vibrations) for long- and short-range communication, respectively (Nieri et al., 2022). In this regard, stink bugs have the ability to emit vibrations through body contractions, utilizing the solid surface they inhabit, such as the leaves of a host plant, as a means of dispersing their signals (Cocroft & Rodríguez, 2005). These vibrations are typically used for partner location, recognition, and courtship. Interestingly, among the order of Hemiptera, the Pentatomidae family has been the focus of the majority of research on vibrational signals transmitted through plant substrates (e.g., Čokl et al., 2021; Čokl & Virant-Doberlet, 2003; Virant-Doberlet & Čokl, 2004).

Understanding the communication associated with the mating behavior of an invasive pentatomid species offers the chance of providing a solid foundation for developing monitoring and/or control methods with high

selectivity (e.g., Polajnar et al., 2015). Based on that premise, such an approach might also be applicable for the control of *B. hiliaris*. With this in mind, the present study aimed at providing a thorough description of the courtship behavior and assessing whether vibrational signals are involved during the process of pair formation in *B. hiliaris*. Such knowledge lays the foundation for the possible future development of sustainable control strategies, offering valuable insights into the species' reproductive behavior that could involve targeted and eco-friendly pest management approaches.

## MATERIALS AND METHODS

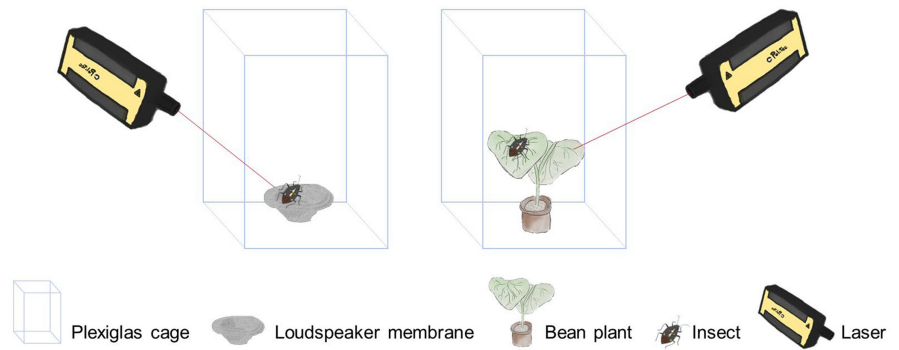
### Insect rearing

In September 2022, *B. hiliaris* nymphs and adults were collected from caper (*Capparis spinosa* L., Capparaceae) fields located near the village of Scauri (36.7722; 11.0606; 22 m above sea level) on the southwestern coast of Pantelleria island in Italy. Subsequently, the insects were relocated to the quarantine facility at the Fondazione Edmund Mach (FEM) in San Michele all'Adige, Italy, and reared in mesh insect cages (30 × 30 × 30 cm; BugDorm, Taichung, Taiwan). The insects were maintained on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*, Brassicaceae) and were provided with one Petri dish (9 cm diameter) filled with a 2 mm layer of fine sand as an oviposition substrate (Taylor et al., 2014). The rearing conditions were: 25 ± 1°C, 60 ± 5% RH, and L16:D8 photoperiod. To obtain and test virgin individuals, daily monitoring was conducted to identify newly emerged adults, which were then isolated in individual cages (15 × 15 × 15 cm, BugDorm) and maintained in the same rearing system described above.

### Recording of vibrational signals and behavior

The vibrational signals of adult males and females (4–8 days old) were recorded with the use of a laser Doppler vibrometer (VibroGo, sensitivity = 5 mm/s/V; Polytec, Waldbronn, Germany). Laser beam reflection was improved by reflective tape located on the recording surface. The laser was connected through a LAN-XI data acquisition system (type 3050-B-040; Brüel & Kjær Sound and Vibration Measurement, Nærum, Denmark) to a computer (HP, EliteBook 8560 p), in which data were digitized and stored with an 8 kHz sample rate and 24-bit depth using the software BK Connect (Brüel & Kjær Sound and Vibration Measurement). Since vibrations' parameters are affected by the substrate's properties (Cocroft et al., 2014), we used two different substrates: a loudspeaker membrane, which served as an inert substrate, and a bean plant (*Phaseolus vulgaris* L., Fabaceae) with two developed leaves, which represented a natural substrate (Figure 1). In order to prevent insects from escaping

**FIGURE 1** Scheme representing the experimental setup with the two substrates.



while recording on the membrane substrate, a modified Petri dish lid was used. The lid base was removed and was substituted by a mesh cover that was glued to the remaining rim. The recordings lasted 15 min per replica and were performed on an anti-vibration table (Astel, Ivrea, Italy). The experiments were conducted in a soundproof chamber, located within the biotremology laboratory of FEM, at  $27 \pm 1^\circ\text{C}$ ,  $70 \pm 1\%$  RH, and L16:D8 photoperiod between 08.30 and 14.00 h. Trials were done using single individuals of both sexes, to detect possible spontaneous sex-specific vibrations, and virgin pairs of a male and a female. Each insect was placed on a distinct leaf, thus allowing them to interact freely and find their counterparts autonomously. Behavioral analyses were performed only for the pairs released on bean plants. To assess the correlation between mating behavior and signal emission and create an ethogram, trials were video recorded with a camera (EOS 80D; Canon, Tokyo, Japan).

## Signals analysis

The acquired data were analyzed and filtered using Raven Pro v.1.6 (The Cornell Lab of Ornithology, Ithaca, NY, USA). To characterize the recorded signals, a fast Fourier transform type Hann was applied, with a window length of 512 samples and 50% overlap. The parameters chosen for the temporal and spectral analyses were: emission time (s), as the total time for each signal/vibration; peak frequency (Hz); fundamental frequency (Hz); signal/vibration modulation (Hz); and signal repetition time (min), which is the time interval between the beginning of two consecutive signals. For the spectral analysis, a filter to reduce the background noise was applied to each file for frequencies below 40 Hz (Polajnar et al., 2016). For the description of the signal or vibration modulation, we measured the fundamental frequency at two or three locations in the signals. For each signal, 10% of the total duration was measured from the beginning ('Mb'), the mid portion ('Mmid'), and at the end ('Me'). The Mmid was measured only for mating signals. Given the gregarious nature of *B. hilaris* and the arena settings, which were far from this species' natural context, we also assessed the relationship between the walking behavior (i.e., motility) and emission of both sexes. For the walking

behavioral analysis, the cumulative time of the vibrational noise associated with walking was used to assess the correlation between motility and signal emission.

## Terminology

The terminology adopted for the behavioral analysis in this paper was the following:

MV-1: male vibration emitted before contact with the female.

FV: female vibration emitted before contact with the male.

MS-2: mating male signal emitted during the mount and the engagement phase, following contact with the female.

Latency time: elapsed time from the beginning of the trial to the onset of the first signal.

Train of signals: a series of signals emitted at regular intervals after contact.

Accessory signal: single signals emitted within a distance between 0.06 and 0.14 min to the nearest train.

Emission time: cumulative time (s) of signal/vibration emission.

Signal/vibration modulation: fundamental frequencies (Hz) measured at 10% of the initial and end portions of the signal/vibration (i.e., 'Mb' and 'Me', respectively). In case of the mating signal, the fundamental frequency of the middle part was measured as well (i.e., 'Mmid').

Signal repetition time: time interval (s) between the beginning of two consecutive signals.

WnM: male wings extension without signal emission.

WnF: female wings extension without signal emission.

Contact phase: first body contact between a male and a female (Guarino et al., 2008).

Distancing: male or female post-contact detachment.

Mount phase: the male mounts the female to mate (Guarino et al., 2008).

Mating attempt: the male tries to hook the female using the copulatory organs.

Engagement phase: the mating takes place (Guarino et al., 2008).

N = number of replicates.

n = number of individuals.

## Statistical analyses

The behavioral analysis was performed using Matlab v.9.13.0 (MathWorks, Natick, MA, USA). Transitions between consecutive phases were analyzed, creating a Markovian matrix (Cro, 2023) using the data collected from the couples on the plant. The MS-2 signals were discriminated between mount and engagement phases in order to highlight differences correlated to the mating behavioral pattern. Transition probabilities were derived by dividing the observed frequency of a transition between two events by the total number of occurrences of the first event. Repeated behavioral transitions were counted as a single event. Using Goodman's (1968) iterative proportional fitting method, the expected values were computed. Subsequently, a G-test (log-likelihood ratio) was conducted to detect significant transitions, and the outcomes were visually depicted in an ethogram. The ethogram was created using the MDPToolBox in Matlab.

To describe the relationship between motility and vibration, a generalized linear model (GLM) was fitted with a linear parameterization negative binomial dispersion using the package 'MASS' on R v.4.1.1 (R Core Team, 2023). The covariates consisted of the interaction between sex and walking (Table 1), and they were optimized using a stepwise algorithm. Moreover, the Akaike information criterion (Sakamoto et al., 1986) was used to select the best-fitting model.

For non-mating vibration, fundamental frequency, emission time, and modulation were compared between sexes and substrates. For mating signals, the fundamental frequency, emission time, signal repetition time, and signal modulation were compared between substrates. In the case of substrates, differentiation between phases (i.e., mount or engagement) was not made during the recordings conducted on the membrane due to the inability to assign the calls to specific phases. All statistical comparisons (i.e., for mating and non-mating signals mentioned above) were obtained by conducting an unpaired two-tailed *t*-test for normally distributed data or a Wilcoxon Rank Sum test

if data were not normally distributed. All plots were done using the 'Tidyverse' and 'cowplot' packages in R (Wickham et al., 2019; Wilke, 2019).

## RESULTS

### Signal description

Three distinct types of vibration, characterized by a modulated harmonic frequency structure, were identified (Figure 2). In the recording setup of single individuals, we observed two types of vibrational production: one from males (MV-1) and one from females (FV). MV-1 and FV were also found in the paired setup before the contact phase, albeit only in two replicates ( $N=30$ ). In the recording setup of pairs, we found a third signal type that we defined as mating signal (MS-2), which was exclusively observed after the initial contact phase, particularly during the mounting and engagement phases.

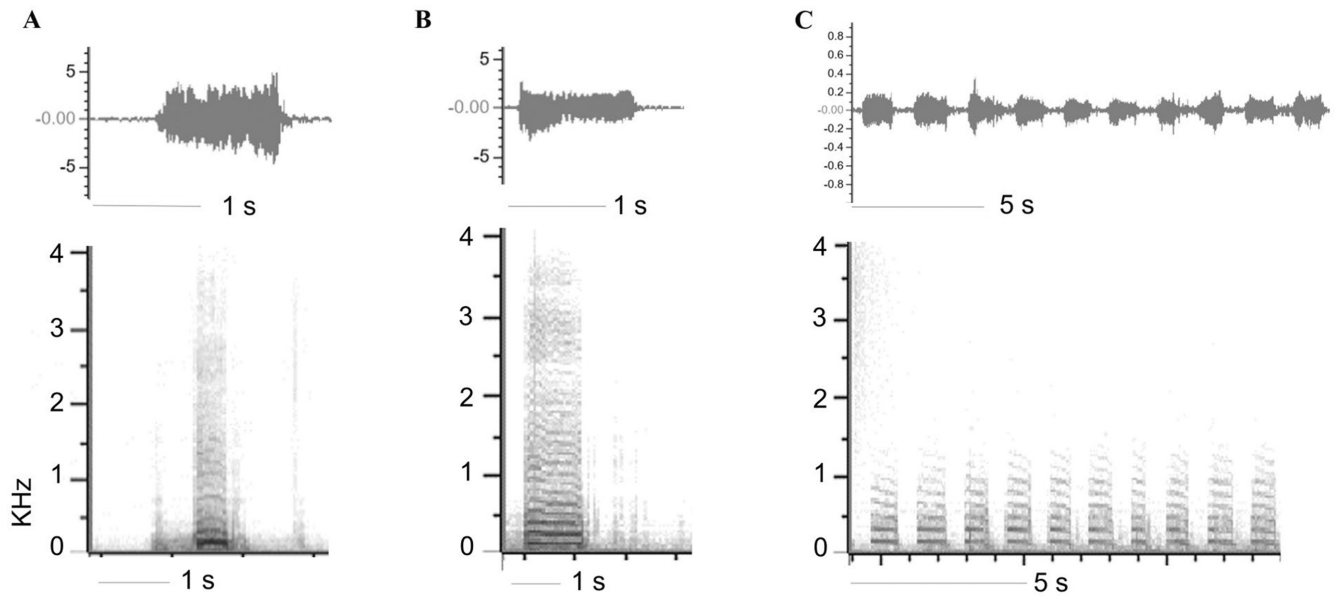
The male vibrotype (MV-1) was emitted while the insect flapped its wings, with a mean ( $\pm$ SD) latency time of  $260.9 \pm 197.2$  s for the plant and  $332.2 \pm 141.2$  s for the membrane (Table S1). The mean duration did not exceed 0.8 s for both substrates, with  $0.68 \pm 0.52$  s and  $0.78 \pm 0.89$  s for the plant and the loudspeaker membrane, respectively (Table S1). The fundamental frequency showed slight differences between the substrates (Figure 3). Specifically, for plants, the mean fundamental frequency was  $130 \pm 18.4$  Hz (Table S1) with a slightly decreasing modulation (Mb:  $138.7 \pm 42.1$  Hz; Me:  $121.3 \pm 24.4$  Hz) (Table S2), whereas for the membrane, it was  $144.9 \pm 6.8$  Hz (Table S1) with an increasing modulation (Mb:  $140.4 \pm 11.3$  Hz; Me:  $205.3 \pm 33.8$  Hz) (Table S2). Moreover, a significant difference was found between substrates in terms of fundamental frequency for MV-1 ( $W=308.5$ ,  $p=0.001$ ,  $n=40$ ) and modulation, but only for the 'Me' portion ( $W=281.5$ ,  $p=0.034$ ,  $n=40$ ) (Figure 3).

In the case of the female vibrotype (FV), the vibration was also emitted while the insect flapped its wings. The FV latency time was higher on the membrane ( $484.5 \pm 331.7$  s)

**TABLE 1** Results of a generalized linear model (GLM) for the effect of walking on the number of calls by male *Bagrada hilaris*: (A) best explanatory model and (B) significant independent variables of the chosen model.

<b>(A) Best explanatory model</b>						
Model synthesis	AIC	Null deviance	Residual deviance	Log-likelihood	df Residuals	Distribution
No. calls ~ walking $\times$ sex	256.09	78.546	72.780	-246.0890	115	Negative binomial
<b>(B) Significant independent variables of the chosen model</b>						
Fixed effects	Estimate	SE	z	<i>p</i> (> z )		
(Intercept)	0.640356	0.528702	1.211	0.23		
Walking	-0.007616	0.003698	-2.059	0.039		
Males	-1.981581	0.816779	-2.426	0.015		
Walking $\times$ males	0.013989	0.005330	2.625	0.0087		

Abbreviation: AIC, Akaike information criterion.



**FIGURE 2** Examples of *Bagrada hilaris* vibration emissions: (A) male vibrotype MV-1, (B) female vibrotype FV, and (C) male train signal MS-2. The top panels show oscillograms, and the bottom panels show spectrograms.

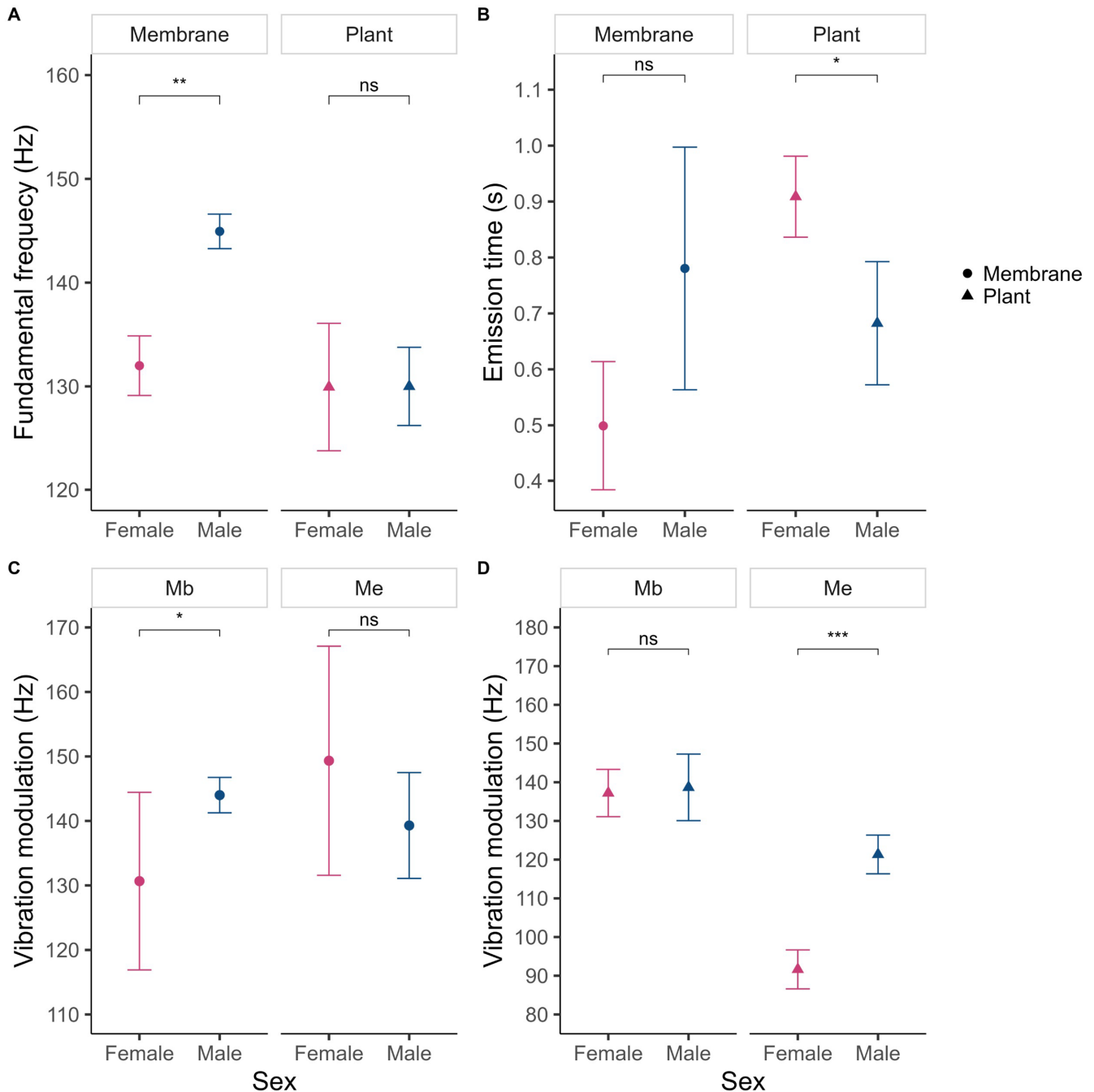
than on the plant ( $269.6 \pm 198.8$  s) (Table S1). The mean duration values were  $0.90 \pm 0.41$  s for the plant and  $0.50 \pm 0.39$  s for the membrane ( $W=89$ ,  $p=0.004$ ,  $n=45$ ) (Figure 3). The fundamental frequency for the plant was  $129.9 \pm 35.2$  Hz, whereas it was  $132 \pm 9.9$  Hz for the membrane substrate ( $W=281.5$ ,  $p=0.019$ ,  $n=45$ ) (Figure 3) (Table S1). Differences in the modulation between the two substrates were noticed for this vibrotype. Indeed, a slightly downward frequency modulation was recorded within the plant substrate (Mb:  $137.2 \pm 35.1$  Hz; Me:  $91.6 \pm 28.9$  Hz), whereas an increasing trend characterized the membrane substrate (Mb:  $130.7 \pm 47.6$  Hz; Me:  $149.3 \pm 61.5$  Hz) (Table S2). A similar pattern was found for FV, as a statistical difference for modulation between substrates was only found in the last portion 'Me' ( $W=312.5$ ,  $p=0.002$ ,  $n=45$ ) (Figure 3).

When comparing MV-1 and FV on the plant, a significant difference was noticed in emission time ( $W=510$ ,  $p=0.030$ ,  $n=56$ ), and for the final portion of the signal (Me) ( $t=4.19$ ,  $df=53.7$ ,  $p<0.0001$ ,  $n=56$ ) (Figure 3). On the other hand, for the recordings performed on the membrane, a significant difference between the two sexes was found only for the fundamental frequency ( $t=3.9$ ,  $df=18.2$ ,  $p<0.001$ ,  $n=29$ ), and in the case of the modulation only for the first portion of the signal (Mb) ( $W=57$ ,  $p=0.037$ ,  $n=29$ ) (Figure 3).

The mating signal (MS-2) is emitted by the male during the mount and engagement phases, right before the mating occurs. In this case, the signals are produced with the contraction of the abdominal part. It is characterized by a sustained emission of short pulses produced in sequence with a signal repetition time between 1.14 and 1.68 s (Table S1). The number of signals included within a train varied from two to a maximum of 15, with the highest number recorded on the membrane. The MS-2 signals

emitted during the mount phase were statistically different from those emitted during the engagement phase. A higher number of signals was recorded for the trains emitted during the engagement phase ( $W=89$ ,  $p=0.009$ ,  $n=132$ ) (Figure 4). The MS-2 repetition time was higher during the engagement phase ( $1.62 \pm 0.24$  s) compared to the mount phase ( $1.14 \pm 0.24$  s) ( $W=0.872$ ,  $p<0.001$ ,  $n=132$ ) (Figure 4; Table S1). The same trend was found for emission time, with higher values within the engagement phase ( $0.75 \pm 0.14$  s) compared to the mount phase ( $0.60 \pm 0.18$  s;  $t=5.02$ ,  $df=111.76$ ,  $p<0.001$ ,  $n=132$ ) (Figure 4; Table S1). Regarding signal modulation, only the middle part (Mmid) was significantly different between the two phases, with higher recorded values within the engagement phase ( $178.8 \pm 88.7$  Hz) and lower within the mount phase ( $165.5 \pm 67.2$  Hz) ( $W=1714$ ,  $p=0.03$ ,  $n=132$ ) (Figure 4; Table S3).

Regarding the differences observed on substrates, the mean fundamental frequency significantly differed between them with  $145.2 \pm 34.5$  Hz and  $162.9 \pm 25.3$  Hz ( $W=20853$ ,  $p<0.001$ ,  $n=373$ ) on the plant and on the membrane, respectively (Figure 5; Table S1). In contrast, the modulation of the signal frequency exhibited a similar pattern in both substrates, with an initial increase followed by a decline (Figure 5; Table S3). Further parameters with observed significant differences were signal repetition time ( $W=0.890$ ,  $p=0.002$ ,  $n=373$ ), emission time ( $W=18529$ ,  $p=0.012$ ,  $n=373$ ), and signal modulation ('Mb':  $W=19739$ ,  $p<0.001$ ,  $n=373$ ; 'Mmid':  $W=20929$ ,  $p<0.001$ ,  $n=373$ ; 'Me':  $W=23263$ ,  $p<0.001$ ,  $n=373$ ), with higher values encountered for the recordings on the membrane (Figure 5). An isolated signal defined as an 'accessory signal' was detected either just before or immediately after the train emission, exhibiting



**FIGURE 3** Differences in vibrotypes of female (i.e., FV) versus male (i.e., MV-1) *Bagrada hilaris* emitted in the pre-contact phase: mean ( $\pm$  SE) (A) fundamental frequency (Hz), (B) emission time (s), and vibration modulation (Hz) (C) on the membrane, and (D) on the plant. Mb represents modulation's beginning, and Me represents modulation's end. Asterisks indicate significant differences between means in pairwise comparisons (Wilcoxon signed-rank test or t-test: \* $0.01 < p < 0.05$ , \*\* $0.001 < p < 0.01$ , \*\*\* $p < 0.001$ ; ns,  $p > 0.05$ ).

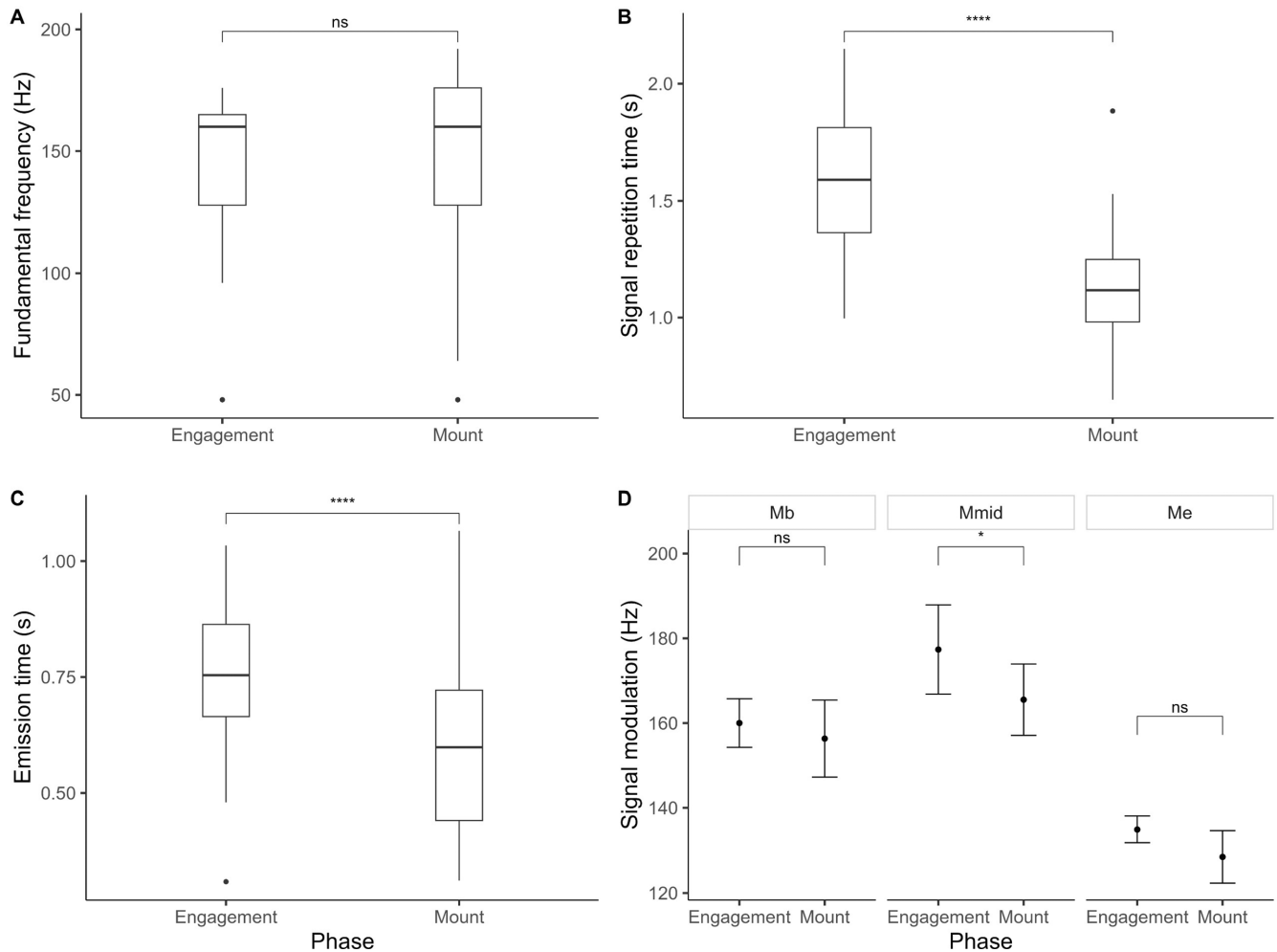
a fundamental frequency of  $160 \pm 33.5$  Hz for the plant and  $147 \pm 36.4$  Hz for the membrane (Table 2).

## Behavioral analysis

To identify recurring behavioral patterns associated with mating success, pairs were distinguished between those that achieved and those that failed mating. For pairs that

failed to mate ( $N=10$ ), no significant transitions between behavioral phases were observed. In total, four main behaviors were identified, including the emission of vibration by both males (i.e., MV-1) and females (i.e., FV), as well as instances where both sexes opened their wings without emitting any signal (i.e., WnM for the male and WnF for the female) (Figure 6).

As for the pairs that achieved the mating ( $N=20$ ), some behavioral transitions were found to be significant



**FIGURE 4** Differences in *Bagra da hiliaris* male train signal (MS-2) emission between the mount and engagement phases on the plant substrate: (A) fundamental frequency (Hz), (B) signal repetition time (s), (C) emission time (s), and (D) mean ( $\pm$  SE) signal modulation (Hz). Mb represents modulation's beginning, Mmid modulation's middle part, and Me modulation's end. The top and bottom boxes in the boxplots represent the first and third quartiles, respectively; the line in between is the median. The whiskers indicate 1.5 $\times$  the interquartile range, and the dots are outliers. Asterisks indicate significant differences between means in pairwise comparisons (Wilcoxon signed-rank test or t-test: \* $0.01 < p < 0.05$ , \*\*\*\* $p < 0.0001$ ; ns,  $p > 0.05$ ).

(Figure 6). Two behaviors, namely the male's vibrational emission, MV-1, and the female's wing opening, WnF, were associated with the onset of a distinct pattern leading to mating. There was a 50% probability of transitioning from these two behaviors to the contact phase (i.e., the male initiating contact with the female). Following the contact phase, a higher percentage (90%) of transitioning to the mount phase (i.e., the male mounting the female) was observed. A less likely transition (10%) consisted of one of the individuals walking away from the other after the initial contact. In general, whenever the pairs were in the mount position, the mating signal by the male was emitted (65% likelihood), subsequently leading to a mating attempt (i.e., the male tries to copulate with the female). In the event of a failed mating attempt, there was a return to the mount phase by the male; however, successful copulation occurred in the most cases (64%) and was accompanied by the production of the mating signal (Figure 6).

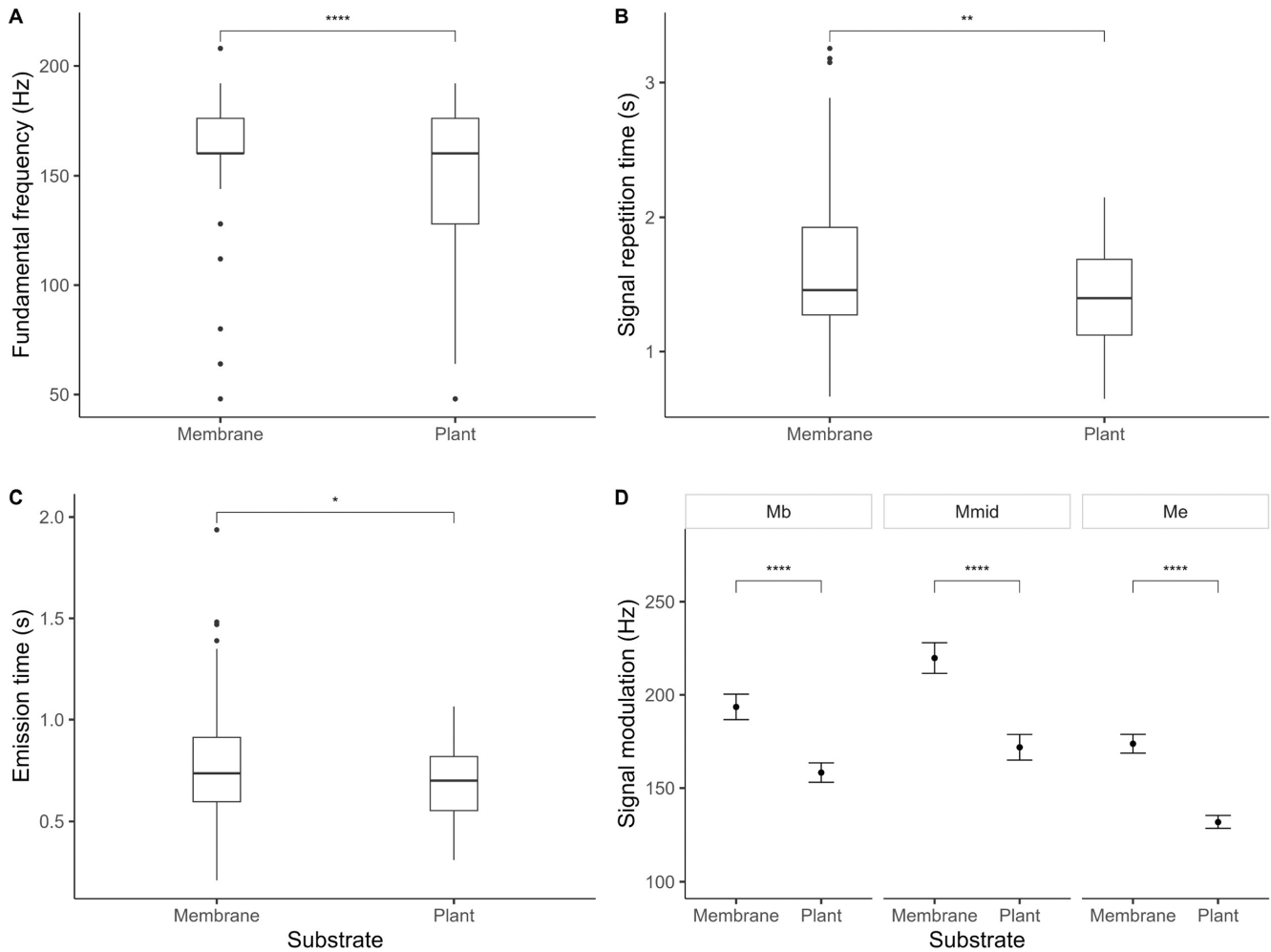
Regarding motility, a general inverse relationship between walking and vibration emission was observed, in

particular for females (Figure 4; Table 1). The significant explanatory factors of the GLM were walking ( $z = -2.059$ ,  $p = 0.039$ ), males ( $z = -2.426$ ,  $p = 0.015$ ), and walking  $\times$  males ( $z = 2.625$ ,  $p = 0.008$ ) (Table 1; Figure 7).

## DISCUSSION

This study gives the first description of the substrate-borne vibrations emitted by *B. hiliaris*, which were recorded from single individuals or during the process of pairing formation. This species presents three main types of vibrational emissions, which we denominated FV, MV-1, and MS-2. The first two are both emitted prior to the contact phase and can be made by both sexes, whereas MS-2 is only emitted by males following the contact phase.

Within the behavioral repertoire observed in this study for *B. hiliaris*, a notable and previously unrecorded pattern involves wing opening without vibration emission (i.e., WnM and WnF). The significance of this behavior, whether



**FIGURE 5** Differences in *Bgrada hilaris* male train signal MS-2 emission between the plant and membrane substrates: (A) fundamental frequency (Hz), (B) signal repetition time (s), (C) emission time (s), and (D) mean ( $\pm$  SE) signal modulation (Hz). Mb represents modulation's beginning, Mmid modulation's middle part, and Me modulation's end. The top and bottom boxes in the boxplots represent the first and third quartiles, respectively; the line in between is the median. The whiskers indicate 1.5 $\times$  the interquartile range, and the dots are outliers. Asterisks indicate significant differences between means in pairwise comparisons (Wilcoxon signed-rank test or t-test: \* $0.01 < p < 0.05$ , \*\* $0.001 < p < 0.01$ , \*\*\*\* $p < 0.0001$ ).

it relates to chemical release or serves as a visual stimulus, remains unclear. Many insects use wing movements for communication, potentially signaling for mates, defending territories, or issuing warnings (Benelli et al., 2012; Gorb, 1998; Kotyk & Varadínová, 2017; Socha, 2004). Wing movements in other species, like silkworm moths and social wasps, are correlated to various roles, from facilitating pheromone detection to diffusing alarm pheromones (Bruschini et al., 2005; Kastberger et al., 1998; Loudon & Koehl, 2000; Togni & Giannotti, 2010). On the other hand, the intricate relationship between the emission of volatile cues and vibrational communication has been well documented for pentatomids (see, e.g., Čokl & Virant-Doberlet, 2003; Miklas et al., 2003; Zgonik & Čokl, 2014). In fact, previous research suggested the involvement of chemical volatiles for the successful mating of *B. hilaris* (Guarino et al., 2008). Considering *B. hilaris*' gregarious nature, wing movement may contribute to intraspecific communication among clustered individuals. However, it's crucial to note that the study's bioassay conditions may not

accurately represent natural conditions; hence, additional studies in natural settings may be needed for a better understanding of this behavior.

Unlike other Pentatomidae, such as *Nezara viridula* (L.) and *Halyomorpha halys* (Stål), our study found no calling or courtship signals in *B. hilaris*. The emission of MV-1 and FV from conspecifics did not elicit a clear behavioral response, suggesting that we could rule out vibrational interactions, such as mating duets (Čokl & Virant-Doberlet, 2003; Polajnar et al., 2016). The absence of such interactions aligns with previous findings on a similar pentatomid species, *Murgantia histrionica* (Hahn) (Čokl et al., 2004), whose adults lack intraspecific identification and/or searching behaviors driven by vibrational signals. Smaller species, such as *M. histrionica*, exhibit a different calling phase from that of larger pentatomids, probably due to shorter leg distances. This could impede their ability to discern signal direction and identify the initial vibration source (Virant-Doberlet et al., 2006). Interestingly, this aspect does not apply for all tiny stinkbugs, as calling signals



**TABLE 2** Mean ( $\pm$ SD) temporal and spectral properties of accessory signals in *Bagrada hilaris*.

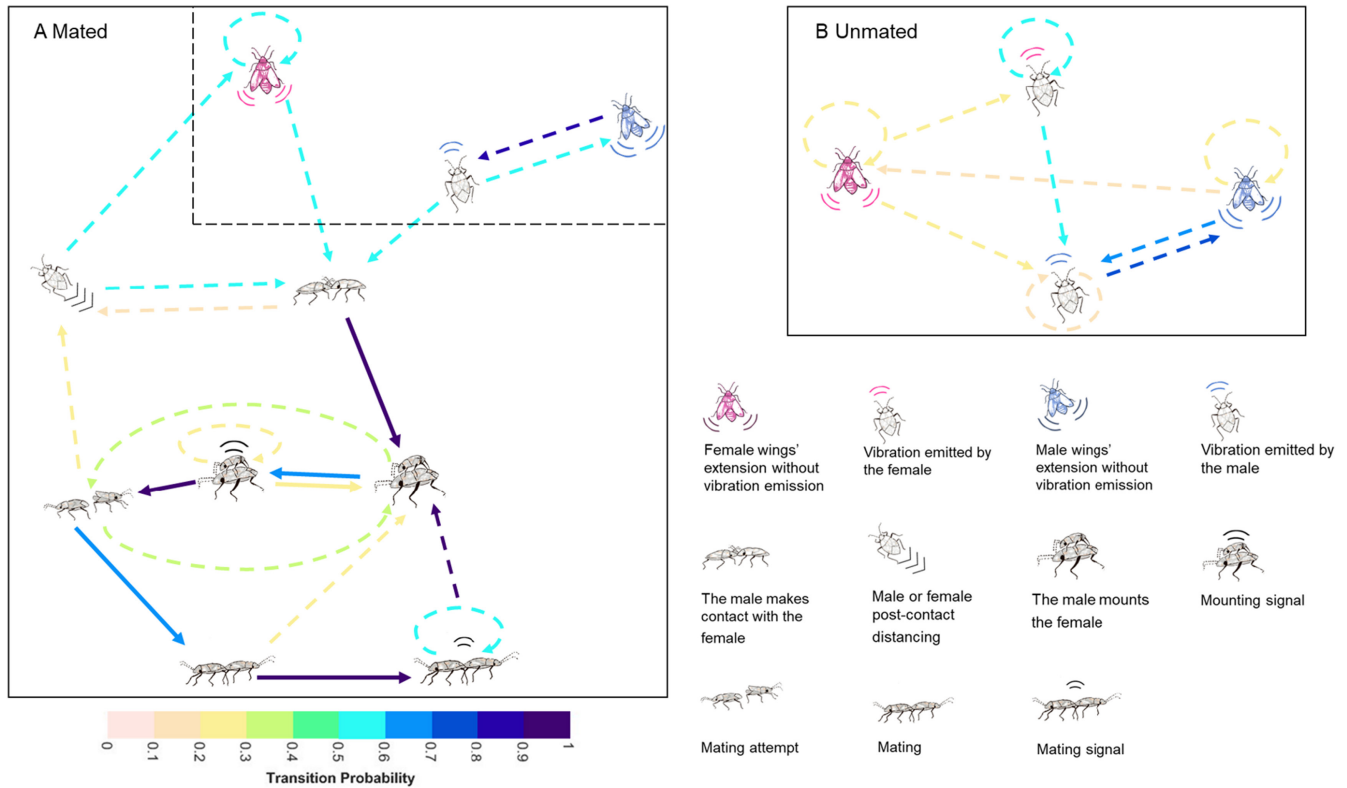
Signal type	Substrate	Emission time (s)	Repetition time (s)	Fundamental frequency (Hz)	Modulation rate (Hz)			n (N)
					Mb	Mmid	Me	
Accessory signal <sub>mount phase</sub>	Plant	0.36 $\pm$ 0.04	4.8 $\pm$ 2.4	160 $\pm$ 37.5	121 $\pm$ 62.5	147 $\pm$ 55.8	102 $\pm$ 33.1	5 (3)
Accessory signal <sub>engagement phase</sub>	Plant	0.75	6	160	160	160	128	3 (1)
Accessory signal	Plant	0.42 $\pm$ 0.16	4.8 $\pm$ 2.4	160 $\pm$ 33.5	128 $\pm$ 58.1	149.3 $\pm$ 50.2	106.6 $\pm$ 31.4	6 (3)
	Membrane	0.67 $\pm$ 0.26	4.2 $\pm$ 1.8	147 $\pm$ 36.4	150.9 $\pm$ 44.6	155.4 $\pm$ 50.6	146.9 $\pm$ 47.4	21 (12)

Abbreviations: Mb, modulation's beginning; Me, modulation's end; Mmid, modulation's middle part; N, number of replicates; n, number of individuals.

have been identified in another small pentatomid species, *Holcostethus strictus* (Fabricius) (Pavlovčič & Čokl, 2001). This suggests that such signal types among smaller species may still exist. In various pentatomid species, calling signals are crucial for finding a mate and are often followed by a courtship phase involving a duet between the two sexes (Čokl & Virant-Doberlet, 2003). Generally speaking, male pentatomids tend to emit a greater quantity of pheromones when exposed to the female's calling song (Miklas et al., 2003). Hence, further studies on *B. hilaris* signals employing playbacks of FV and MV-1 are needed to ascertain their unattractiveness for conspecifics and to explore their possible use as signals. Moreover, studies investigating potential differences in pheromone secretion following vibrational emission are also needed to get a clearer idea on the function of MV-1 and FV.

Regarding the walking behavior, males exhibited a positive correlation between MV-1 production and walking time, whereas the opposite was observed in regard to FV emission with females. This suggests that females might be able to selectively allocate energy for locating males, balancing vibration production and mobility. The energetic cost associated with signal production was already described in the literature, which correlated with indirect physiological costs that had implications for individuals' survival (Fouani et al., 2024; Virant-Doberlet et al., 2023). In addition, the increased mobility of males may be linked to their initiation of courtship behavior.

Once the contact phase is reached, critical transitions with high probabilities dictate the success of mating in *B. hilaris*. This aligns with previous findings highlighting the importance of cuticle hydrocarbons for mating success (Guarino et al., 2008). After contact, the most likely transition is the male mounting the female, though there's a less likely chance of distancing after establishing contact. Subsequently, MS-2 emission is the most likely event to occur while the male is still mounting the female, which could lead to a mating attempt or, to a lesser extent, return back to the mount phase followed by MS-2 emission again. Interestingly, courtship stimuli could be used by females to judge male fitness based on the signals they emit, a phenomenon observed in other species such as *Dendroctonus valens* (LeConte) (Lindeman & Yack, 2015). In *B. hilaris*, the mating attempt is often followed by the engagement phase with high probability, and the return to the mount phase is less likely. Afterwards, the most likely transition to follow is the emission of MS-2. The purpose of the emission of the same signal type during two different phases following contact is unclear. Notwithstanding, copulatory signals have been widely reported for various pentatomid species (Čokl et al., 2001, 2004; Pavlovčič & Čokl, 2001). Intriguingly, for species with internal fertilization, copulation doesn't guarantee insemination, allowing females to exert 'cryptic' choices in sperm precedence or competition, potentially impacting male reproductive success (Thornhill, 1983). Eberhard (1991) suggested that certain insect species employ signals as mechanisms for



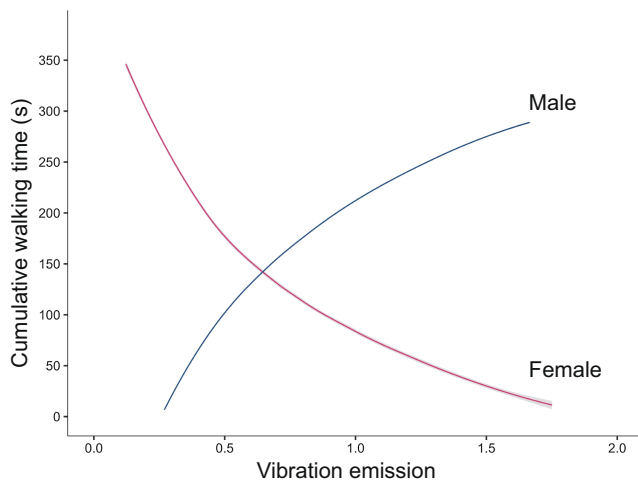
**FIGURE 6** Ethogram representing behavioral transitions for *Bagrada hilaris* (A) mated and (B) unmated couples. For pairs that failed to mate ( $N=10$ ), no significant transitions between behavioral phases were observed (Likelihood ratio test [LRT]:  $p > 0.05$ ); for pairs that achieved mating ( $N=20$ ), some behavioral transitions were found to be significant (LRT:  $p < 0.05$ ). In panel (A), the common part with the unmated insects (B) is delineated with a dashed box. The solid arrows indicate a statistically significant transition, and dashed lines represent those that are not. The different colors indicate the probability of a transition between different phases.

'cryptic' female choices, where females control various processes such as copulation duration, sperm storage, movement, nourishment, and egg maturation. In this regard, Peccerillo et al. (2023) found that irradiating *B. hilaris* males with gamma rays affects the MS-2 signal. This could imply that MS-2 production during the engagement phase may act as a male performance indicator, influencing the female's decision on sperm use. The aspect of female preferences being correlated to the variability of male acoustic characteristics was already demonstrated in the literature (Reinhold, 2011). It is crucial to note that this study focused on signal production and its role in courtship behavior, not assessing the success of copulation. Hence, future studies are needed to investigate the mechanism of sperm utilization by female *B. hilaris* following copulation to assess if they exhibit 'cryptic' choices.

In our study, some discrepancies were observed from recordings performed on the different substrates. Such differences are in line with what was previously reported in the literature (Cocroft et al., 2006, 2014; Polajnar et al., 2016). For instance, Ge et al. (2019) found out that the vibrational communication in the pea leafminer, *Liriomyza huidobrensis* (Blanchard), was severely affected when the insects were recorded on an inert material, such as a glass or plastic surface. Indeed, no vibrational communication

was observed from the recordings performed on these inert surfaces. Moreover, in *N. viridula*, various levels of female responsiveness to male songs were described when the perceived songs emanated from a natural plant environment compared to a loudspeaker system (Miklas et al., 2001).

This study reveals the first evidence of vibrational communication in *B. hilaris*, which differs from other pentatomids. *B. hilaris* emits three types of vibrations, with pre-contact vibrations requiring further investigation. The production of MS-2 during mounting and engagement phases plays a crucial role in mating, possibly influenced by complex interactions between cuticle hydrocarbons and vibrational signals, a theme that deserves additional investigation. This might be further exploited for the integrated pest management of this pest, using chemical pheromones and vibrational playback. Given the gregarious nature of *B. hilaris*, future research should replicate natural conditions more closely and consider potential rival signals. For example, subsequent studies should take into account the diffusion of vibration through the soil, as this is a substrate normally used by *B. hilaris*. Indeed, the absence of calling or searching signals in pre-contact phases and the sole emission of courtship signals following contact might potentially be due to the substrate's (i.e., the soil) unsuitability for long-distance signal transmission. The findings



**FIGURE 7** Model predictions for walking effect on vibration emission prior to the contact phase between *Bagrada hilaris* male and female vibrotypes (i.e., MV-1 and FV).

presented here offer valuable insights for the development of sustainable control methods, such as mating disruption, attract-and-kill, and/or push-and-pull techniques.

### AUTHOR CONTRIBUTIONS

**Marica Scala:** Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); writing – original draft (lead); writing – review and editing (lead). **Chiara Peccerillo:** Data curation (equal); formal analysis (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Jalal M. Fouani:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Rachele Nieri:** Conceptualization (supporting); writing – review and editing (equal). **Nuray Baser:** Supervision (equal); writing – review and editing (equal). **Vincenzo Verrastro:** Supervision (equal); writing – review and editing (equal). **Massimo Cristofaro:** Writing – review and editing (equal). **Gianfranco Anfora:** Supervision (equal); writing – review and editing (equal). **Valerio Mazzoni:** Conceptualization (supporting); supervision (equal); writing – review and editing (equal).

### ACKNOWLEDGMENTS

The study was co-financed by the European Union—FSE-REACT-EU, PON Research and Innovation 2014–2020 DM1062/2021.

### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1.** Mean ( $\pm$  SD) temporal and spectral properties of vibratory types of *Bagrada hilaris*.

**Table S2.** Mean ( $\pm$  SD) vibration modulation of *Bagrada hilaris*.

**Table S3.** Mean ( $\pm$  SD) trains modulation of *Bagrada hilaris*.

**How to cite this article:** Scala M, Peccerillo C, Fouani JM, Nieri R, Baser N et al. (2024) The role of substrate-borne vibrational signals in the sexual communication of the painted bug, *Bagrada hilaris*. *Entomologia Experimentalis et Applicata* 00: 1–13. <https://doi.org/10.1111/eea.13450>