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Use of substrate-borne vibrational signals to attract the Brown Marmorated Stink Bug, Halyomorpha halys

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Response to Reviewers:	Reviewer #1: In this manuscript Polajnar and co-workers investigated the possibility of using substrate vibrations to upgrade the existing pheromone traps in order to trap the Halyomorpha halys males. The study is well-designed and executed and its findings should be of interest not only to practitioners working with H. halys, but also with other	

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Research Article

Use of substrate-borne vibrational signals to attract the Brown Marmorated Stink Bug, *Halyomorpha halys*

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Abstract

Despite the increasing number of studies on the use of acoustic stimuli to control agricultural pests, this approach is still theoretical. Many insect pests, in particular hemipterans, use vibrational signals for mating communication and therefore the application of a control strategy based on acoustic interference is a promising option. The Brown Marmorated Stink Bug, *Halyomorpha halys*, is causing severe economic damage on many crops in the USA and Italy. We tested a female vibrational signal, Female Signal 2 (FS2), to attract males in different settings, such as natural substrate, arenas and a cage representing an acoustic trap. We used video tracking analysis and described the vibrational amplitude field around the individuals to study the male behavior. We found that FS2 can attract more than 50% of males to the source point and has a strong "loitering" effect on searching males that tend to remain in the stimulated area. We concluded that FS2 exhibits good attractiveness to *H. halys* males and that its potential use as a tool integrated in the currently existing pheromone traps should be tested in the field.

Keywords: biotremology, acoustic traps, integrated pest management, behavioral bioassays, Hemiptera

Key message:

- A *Halyomorpha halys* female vibrational signal type, FS2, played back into natural or artificial substrates is significantly attractive to males.
- Once attracted to the source point, males remain near the vibrational source for many minutes.
- FS2 looks promising for the development of an acoustic method to trap the Brown Marmorated Stink Bug in the field.

Author Contribution Statement:

VM, LM, JP, GA and RG conceived and designed research. VM, MB, JP and MVRS conducted experiments. VM and MVRS analyzed data. VM, LM and JP wrote the manuscript. All authors read and approved the manuscript.

Introduction

Application of integrated pest management strategies to control insect pests is achievable if there is adequate knowledge of the ecology and biology of the target species (Pedigo and Rice 2014; Pertot et al. 2016). In particular, the species behavior and the exact role and characteristics of all associated signals must be well understood for setting an efficient method of behavioral manipulation. For example, methods based on communication interference aim at altering a species behavior (i.e. attracting, disrupting, repelling, etc.) by releasing more or less specific stimuli into the environment. The strategies based on pheromones and kairomones operate in this way, as do the strategies that rely on visual stimuli such as light traps and colored sticky panels (Foster and Harris 1997). In theory, thanks to the identification and characterization of key stimuli (e.g. odors, sounds, colors) that trigger specific reactions in individuals it would be feasible to ideate associated control strategies. It follows that the more a signal, or better a sensory mode, is important for guiding a relevant behavioral task, the better candidate it is for developing behavioral manipulation techniques. In biotremology, this was done for the leafhopper, *Scaphoideus titanus* Ball, a species in which vibrational signals are crucial for both identification and location of the potential partner as well as for courtship (Mazzoni et al. 2009). In this case, the interference with the pest's mating behavior was achieved by transmitting a specific disturbance noise into the plant tissues to overpower (= mask) the substrate-borne vibrational signals emitted by duetting couples. In semi-field trials, these signals were sufficient enough to interfere with mating signal reception by individuals and blocked mating (Eriksson et al. 2012; Polajnar et al. 2016a).

Our hypothesis is that the Brown Marmorated Stink Bug (BMSB) *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) is susceptible to vibration-based behavioral manipulation. This insect, originating from Asia, is highly polyphagous and can cause severe economic damage on different crops in the United States (Rice et al. 2014) and Italy (Maistrello et al. 2016) where it was accidentally introduced. Like in other stink bugs, the long range mating communication of BMSB is mediated by male emitted aggregation pheromones (Aldrich 1988; Khrimian et al. 2014) and the short- to mid- range (meant as same plant range) also by the exchange of vibrational signals between potential mates (Čokl and Virant-Doberlet 2003; Virant-Doberlet and Čokl 2004). Although the same approach as in *S. titanus* – mating disruption – is likely not feasible because of BMSB extreme polyphagy and rapid reproduction, attraction for the purpose of mass trapping is an option. The mating process is started by a male call to which females reply with their own vibrational signals, thus triggering male searching (Polajnar et al. 2016b). It is known that searching in pentatomid males is directional (i.e. non-random) and based on perception of regularly repeated female signals (Čokl et al. 1999).Therefore, we hypothesized that the BMSB female signal, previously termed FS2, might be attractive to males as observed in mating trials (Polajnar et al. 2016b). Given that the orientation towards a pheromone is not precise in stink bugs (James et al.

1996; Aldrich et al. 2009), we believe that the continuous emission of FS2 played back into plant or artificial substrates can drive BMSB males to the source. If confirmed, this knowledge could greatly contribute to the development of more efficient pheromone traps complemented with acoustic signals (Nielsen et al. 2011; Leskey et al. 2012; Joseph et al. 2013; Lee et al. 2013). To assess our main hypothesis, we performed four sets of experiments that were designed to prove that FS2 can attract and drive males to the playback source, independently from the substrate (either natural, like plant tissues, or artificial, like plastic). We also tested whether the playback perception caused males to modify the dynamic behavior (i.e. males arrest or loiter nearby the playback source) and if visual cues (i.e. dummy females) could improve or interfere with the playback performance.

Materials and Methods

Insect rearing

Colonies of BMSB were initiated from adults and nymphs collected in the Province of Reggio Emilia, North Italy (44° 41’50" N, 10°37’53”E), during spring and summer 2015. Insects were reared at the Laboratory of Entomology, Dept. of Life Sciences, University of Modena and Reggio Emilia in transparent plastic boxes under controlled conditions (23±0.5°C, 70±10% RH, 16L: 8D). Nymphs and adults were kept in separate containers on a diet of fresh beans, carrots and raw peanuts, with water supplied as soaked cotton, renewed at least twice weekly. Rearing containers were changed and cleaned once per week. Each individual was tested only once.

Signal Playback, Audio/Video Recording and Vibrational Amplitude Field

All tests were conducted in the laboratory of Bioacoustics of Fondazione Edmund Mach (Trentino, Italy) on an anti-vibration table (Astel s.a.s., Ivrea, Italy) within an acoustically insulated chamber kept at 24±1°C, in artificial lighting conditions (50 lux). Individuals (adult virgin males, body length 1.4-1.5 cm, used after at least seven days from the emergence) were stimulated in different contexts (see below) with a playback of a pre-recorded natural BMSB signal that was continuously looped for the total trial time into a substrate using an electromagnetic mini-shaker (mod. 4810, Bruel and Kjaer, Naerum, Denmark). A conical rod was screwed on the top of the mini-shaker and covered with a small amount of blue-was (Surgident Periphery Wax, Australia) to ensure the stable contact with the substrate. The mini-shaker was physically separated from the anti-vibration table with a prong clamp standing on a nearby table. The female playback (pbFS; Fig. 1) tested to assess attractiveness towards males was made of a 11.5 s long BMSB female pulse train (12 pulses, dominant frequency: 80.0±0.6 Hz, recorded from a plant bean leaf), type FS2 (Polajnar et al. 2016b).

The correct transmission of the playback was adjusted to not exceed the insect natural amplitude (max value 1.7 mm/s as substrate vibration velocity; Polajnar et al. 2016b). It was constantly monitored with a laser vibrometer (Ometron© VQ-500-D-V, Brüel and Kjær) and digitized with 48 kHz sample rate and 16-bit resolution, then stored directly onto a hard drive through the LAN XI data acquisition device (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark). The spectral analysis was performed with the software PULSE 14.0 (Brüel and Kjær Sound & Vibration A/S) after applying fast Fourier transform (FFT) with the Blackman–Harris window of length 400 points and 66.7% overlap. This setup was used for describing the vibrational amplitude field (see below) of plants, arenas and cages.

To assess possible differences in the behavior of males stimulated with pbFS in Tests 2 and 3, trials were monitored with the video-tracking tool Ethovision XT (Ver. 7.0, Noldus Information Technologies, Wageningen, Netherlands).

Definitions

Active males: those individuals that left the release point after the acclimation period.

Activation time: from the end of the acclimation period to the moment individuals left the release point.

Audio Sampling Point (ASP): a point on a surface from which the pbFS was recorded with laser vibrometer.

Searching time: from the activation time to the moment a male reached the stimulation point.

Acclimation period: period of 2 minutes from the male release during which the playback was off.

Stimulation Point (SP): point on the substrate in physical contact with the mini-shaker. In Test 1, the SP coincided with the whole vibrated leaf; in Tests 2 and 3 with the associated VSA (SP-VSA).

Vibrational amplitude field: the complex of ASP taken from a substrate (i.e. plant, arena, cage) from which we measured the signal amplitude as substrate velocity (µm/s) at the peak frequency (Hz). The protocol consisted of measuring five randomly chosen pulses of pbFS which was played back for 10 seconds per ASP.

Video Sampling Area (VSA): circular areas (ø = 3 cm and 5 cm in Tests 2 and 3, respectively) on the arena surface used for video track analysis with Ethovision.

Tests

The experimental design was built on four different scenarios: potted bean plants (Test 1), arenas (Tests 2 and 3) and a cage (Test 4). The variability of substrates aimed at assessing the level of efficiency of the playbacks to attract and direct males independently from the system/substrate they were applied to. For each scenario, we measured the vibrational amplitude field to assess whether amplitude gradients towards the SP occurred or not and thus if amplitude could be the cue used by males to find the vibrational source. Furthermore, in test 1, 2 and 3 we also measured the "loitering effect" of the FS2 playback. According to preliminary observations, males did not stop once reaching the SP, but kept circling around it, which we dubbed "loitering effect". This term was borrowed from military jargon and means "circling around the battlefield, waiting for a moment to strike". In test 2, we used a dummy (i.e. a dead female) to assess the possible role of visual cues, in presence or absence of playback stimulation. Finally, in test 2 and 3 we used the software Ethovision to measure possible male behavioural responses related to movements (i.e. tendency to loiter around the playback source, speed and distance moved).

Test 1: Attractiveness on the plant – From leaf to leaf

Test 1 was conceived, primarily, to ascertain whether the pbFS was able alone to attract BMSB males to the SP over the host plant surface. Secondly, we aimed at assessing the loitering effect of pbFS (i.e. to keep BMSB males in the vicinity of the SP, once it was localized). Males (n = 30) were released from a glass vial over a leaf of a potted bean plant (*Phaseolus vulgaris* L.) composed of two leaves (height: 10-15 cm). A second plant, grown from the same pot, was leaned against the first one, the stems being in contact 2-3 cm below the leaf junction (Fig. 2). The playback stimulation was transmitted from a bean leaf (the SP, which was different from the one on which the male was released) after the acclimation period. After each trial, the mini-shaker was randomly moved to another leaf. As a whole, three pots of beans were used to conduct the trials. The trial was discarded if the male left the release leaf during the acclimation period. Males were given 10 minutes to reach the SP. To assess the pbFS loitering effect, these males were further observed for 5 minutes to see whether they stayed on the SP or left it. As a control, we performed trials (n = 23) with identical set-up and protocol but in absence of playback (mini-shaker turned off). The vibrational amplitude field was measured from a total of eight ASPs: the four leaves (on the lamina, at mid leaf length) and the two stems (two points for each stem, above and under the junction point). Video analysis was not performed in Test 1.

Test 2: Attractiveness on the arena (1) – Drive them to the right spot

The aim of Test 2 was to evaluate the influence of pbFS on BMSB male behavior on an artificial substrate. The arena (Fig. 3A) was made with a circular base (ø = 30 cm) of yellow cardboard bordered with a 5 cm tall cardboard strip ("arena wall") to prevent the individuals (n = 20) from leaving the arena. The release point (RP) was inside a hole (ø = 3.5 cm) in which a 50 ml falcon vial cap (depth = 1 cm) was wedged. Before the beginning of a trial, an individual was put in the cap and covered with another identical cap during the acclimation period. The SP was randomly positioned 10 cm from the release point after each trial. Each individual was audio/video recorded for 3 minutes. The video camera was placed exactly above the arena at a distance of 1 m. A prong clamp was used to hold the arena suspended over the table on which the mini-shaker was placed. The prong clamp and mini-shaker were placed on separate tables. We audio-video recorded the males on the arena with (Pb_+) or without playback (Pb_-) and with (Dy_+) or without (Dy_-) a "dummy". The latter was a dead female, washed with dichloromethane to remove epicuticular compounds, and placed next to the SP. We hypothesized that males could have been more attracted by a synergy between vibrations and vision of a conspecific (Pb_+Dy_+) than by vibrations only (Pb_+Dy_-). On the contrary, we did not expect any behavioral difference between vision only (Pb_-Dy_+) and control (Pb_-Dy_-). We monitored four VSA, symmetrically placed on the arena floor, 10 cm away from the center, one of which included the SP (SP-VSA). The vibrational amplitude field was measured from five ASPs: four of them corresponding to the VSAs and one with the releasing point.

Test 3: Attractiveness on the arena (2) - An exit pathway

This test was conceived to assess whether BMSB males (n = 20) could be driven out of the arena, by stimulating the outer end of an exit pathway. Two rods (29.8x0.9 cm) made of red cardboard were added to the arena used in Test 2. Red color was used to increase the contrast with the yellow background. This expedient was necessary to facilitate the video analysis. The proximal end of the two rods was in contact with the arena surface where we placed the two VSAs; the SP was at the rod distal end which was laid on the tip of a mini-shaker. The second rod, not vibrated and used as control, was laid on a second (inactive) mini-shaker. After each trial, vibrated and non-vibrated rods were switched. The rods did not touch the arena wall. The vibrational amplitude field was measured from 19 ASPs, also including the VSAs, the SP (SP-ASP) and the release point (for details see Fig. 3B).

Test 4: The Acoustic Trap – Catch them all

We simulated an acoustic trap in a no-choice scenario and long term stimulation (3 hrs). We used a cubic net cage with 30 cm edge (bugdorm-43030, Megaview Science Co. Ltd, Taichung, Taiwan) and a lateral net sleeve (ø = 18 cm; L = 10 cm). We firmly tied a plastic cylinder (ø = 10 cm; L = 13.5 cm) to the sleeve with some elastic gum. A funnel (ø1 = 10 cm; ø2 = 1 cm; L = 7 cm) was applied between the sleeve and the cylinder, to prevent the individuals from exiting the cylinder once they entered. The cylinder was held up by a metallic prong at the same height as the center of the sleeve hole and was basally connected with the tip of the mini-shaker. Five males were simultaneously released in the cage and four replications were performed. The pbFS was transmitted for 3 hrs. A silent control was also included. The analysis of the vibrational amplitude field was performed based on 45 ASPs, also including the SP (for details on the ASPs positions on the trap see Fig. 4).

Data Analysis

In Tests 1-3, we counted the number of (1) active males and (2) males that reached the SP. Additionally, in Test 2 with the dummy (Dy_+), we counted the males that touched it. In Test 1, we measured the (3) activation time, (4) searching time and the (5) number of males that did not leave the vibrated leaf within 5 minutes from the moment they walked on it, as a measure of the signal loitering effect. In Tests 2-3, we counted the (6) number of males that remained in the arena. In Tests 2-3, the video tracking analysis with Ethovision was used to measure the (7a) total distance moved (cm) and (7b) mean velocity (cm/s). We also measured the (8) number of accesses and the (9) time spent by males on each VSA. In Test 4, we counted the (10) males captured at the end of the trials.

G-test in contingency tables (2x2 or 2x4), Williams corrected, was used to assess the attractiveness of pbFS by comparing treatment (vibrations on) and control (vibration off) for (1), (2), (6) and (10). The Kruskal-Wallis test followed by Mann-Whitney pairwise, Bonferroni adjusted, was used to compare (3) among control and stimulated males that did and did not reach the SP. The same test was used for (7). In particular, we merged all individuals that left the arena and those of Dy_- that remained. The binomial distribution was used to assess differences in (5). Since only one individual reached the target leaf in control trials, we did not perform any statistics on (4). In Test 2, the Friedman test followed by Bonferroni post-hoc test was used to compare (8) among treatments; in Test 3, the Wilcoxon T-test for paired datasets was used to compare (9) among treatments.

As for the vibrational amplitude field, in Test 1, we randomly chose one leaf as SP and then recorded the pbFS from all ASPs. We repeated this protocol for the three pots that were used for the trials. Similarly, in Tests 2 and 3, we recorded all the ASPs and repeated the measurements by transmitting the playback from three different SPs. In Test 4, we repeated the measurements of the vibrational amplitude field three times, on three different days. For the analysis of the signal amplitude, we made an average of the substrate velocity (in μm/s) at the peak frequency of three pulses recorded from each ASP and calculated the mean (±SE) of the three replications. In Tests 1 and 2, differences among ASPs were assessed with the non-parametric (repeated measures) Friedman's test with replication, followed by Bonferroni post hoc test. In Tests 3 and 4, we provided only descriptive statistics, given the high number of ASPs. Figures describing the vibrational amplitude field were created by hand with the freeware graphical software GIMP 2.8 (GNU Image Manipulation Program).

Results

Test 1: Attractiveness on the plant – From leaf to leaf

In Test 1 (Table 1), 77% of males were active (n = 23) in trials with pbFS stimulation, and 61% (n = 14) of which reached the SP. Among these, 70% (n = 10) loitered upon the leaf for a period of 5 minutes. The activation time of males (Fig. 5) that reached the SP was significantly lower than of those males that did not reach it (Kruskal-Wallis test: $X^2 = 11.2$, df = 2; p =0.004). In control trials, we recorded a significantly lower percentage of active males (46%; n = 12) (G-test, p = 0.014) of which only one reached the vibrated leaf (G-test, p = 0.005), without later loitering on it.

The vibrational amplitude field analysis (Fig. 2) indicates a trend of increasing gradient of amplitude towards the SP, on which the pbFS is significantly (Friedman test: $X^2 = 53.5$, df = 7; $p<0.001$) stronger than elsewhere on the plants. In particular, the signal was attenuated by more than 3 dB immediately next to the vibrated leaf, on the upper stem of the vibrated plant, while further losses were recorded from the other ASPs. As a general observation, signals recorded from the leaves were stronger than those from the stems, and those recorded from the upper parts of the plants were stronger than those from the lower ones.

Test 2: Attractiveness on the arena (1) – Drive them to the right spot

We did not observe significant differences among trials in terms of number of active males (G-test: G = 2.2, df = 3; p =0.54). In each of the two trials with pbFS (Pb_+Dy_+ and Pb_+Dy_-), 65% of males (n = 13) remained on the arena for the total duration of the test. This value was significantly higher (G = 21.5, df = 3; $p < 0.001$) than the number of males that remained on the arena without playback, either in presence (Pb_-Dy_+, 10%, n = 2) or absence (Pb_-Dy_-, 25%, n =5) of a dummy (Tab. 2A). Altogether (Tab 2B), males stimulated with playback (Pb_+) did not differ ($G = 1.9$, df = 1; $p < 0.16$) from those not stimulated (Pb_-) in terms of number of active males but the number of individuals that remained on the arena for the total trial duration was significantly higher for Pb_+ ($G = 20.0$, df = 1; $p < 0.001$). On the contrary, when considering all trials in presence (Dy_+) and absence (Dy_-) of a dummy female, they did not differ in either parameter (active males: $G = 0.2$, df = 1; $p = 0.64$; remaining males: $G = 0.2$; df = 1; $p = 0.62$).

Using video analysis (Fig. 6A), we measured a significantly longer distance (Kruskal-Wallis test: $X^2 = 8.2$, df = 2; p =0.016) traversed by males in Pb_+ trials (Pb_+Dy_+ and Pb_+Dy_-) and slower walking velocity of Pb_- males that remained on the arena (Kruskal-Wallis test: $X^2 = 8.4$, df = 2; p =0.02). In the Pb_+Dy_+ trials, the time spent by males inside the SP-VSA was significantly longer (Friedman test: $X^2 = 9.8$, df = 3; p =0.01), and in both Pb_+ trials the number of accesses to the SP-VSA was significantly higher (Friedman test: $X^2 = 12.5$, df = 3; p =0.006) than the number of accesses to other VSAs (Fig. 7). As for the vibrational amplitude field (Tab. 4), the amplitude recorded from the SP-ASP (m±SD: 827.4 ± 16.6 μm/s) was significantly higher (Friedman test: $X^2 = 62.1$, df = 4; $p < 0.001$) (difference over 10 db) than the other ASPs, among which the signal amplitude recorded from the frontal ASP (40.5 ± 8.7 μm/s) was slightly stronger than the ones recorded from the lateral ASPs (23.2 ± 0.4 and 19.9 ± 3.8 μm/s) and the releasing point (29.2 ± 4.7 μm/s).

Test 3: Attractiveness on the arena (2) - An exit pathway

As in Test 2, we did not find significant differences in the number of active males (G-test: $X^2 = 3.8$, df = 1; $p = 0.15$), but differences were found in the number of individuals that remained on the arena during the trials (G-test: $X^2 = 27.1$, df = 1; $p < 0.001$) between pbFS stimulation and the control (Fig. 8). In trials with pbFS, 69% (n = 11) of males that remained in the arena reached the SP located on the external end of the vibrated rod (Video 1), whereas none of them reached the external end of the non-vibrated rod. A significantly (G-test: $X^2 = 12.4$, df = 1; $p < 0.001$) lower number of males (n = 5) remained in the arena in control trials, and only 2 of them (G-test: $X^2 = 8.0$, df = 1; $p = 0.004$) walked to the external end of either rod (Tab. 3). The video analysis revealed a significantly longer walking distance of males stimulated with pbFS, while no differences were found in velocity (Fig. 6B). Males spent a significantly longer time (Wilcoxon T-test: W = 120, p =0.007) in the VSA around the basal end of the vibrated rod, while in the silent control no differences were found between the two VSAs (W = 72, p =0.49). The vibrational amplitude field analysis based on 19 ASPs revealed a rather complex signal amplitude pattern (Fig. 9A, Tab. 4).

The ASP with the highest measured amplitude was that on the arena surface, in front of the internal end of the rods (A1), which reached mean values even higher than the vibrated rod; surprisingly, we measured stimulus amplitude values from the non-vibrated rod (B3, B4 and FR) higher than from the vibrated rod (SP, B1 and B2).

Test 4: The Acoustic Trap – Catch them all

As a whole, 65% (13 out of 20) of the males released in the net cage were collected from the acoustic trap after 3 hrs of trial with pbFS, significantly higher (G-test: $G = 17.2$, $p < 0.001$) than the silent control ($n = 1$).

The vibrational amplitude field analysis (Fig. 9B, Tab. 5) revealed a clear gradient of amplitude from the back to the front of the cage. The highest amplitude values, however, were found on the sleeve and on the funnel, whereas on the plastic cylinder, which was in direct contact with the mini-shaker, they were lower. We found a lack of homogeneity and of symmetry to such extent that the amplitude recorded from one side of the cage was much different from the other.

Discussion

Our research demonstrated that: (1) the BMSB female signal (type 2 or FS2, Polajnar et al. 2016b), which is naturally emitted by females during the process of pair formation, is attractive to males when broadcasted with a mini-shaker; (2) FS2 has a relevant loitering effect as shown by the tendency of males to keep searching in the close vicinity of stimulated areas, either leaves (Test 1) or plastic surfaces (Tests 2 and 3), and by the repeated passages over the stimulation point (Test 2).

In general, males were able to localize the stimulation points both on plants and artificial arenas. As previously observed (Polajnar et al. 2016b), males typically walked and stopped while searching, stretching out the legs before continuing to walk. In Test 3, they used to walk in concentric circles around the rod end and when they finally touched it with the anterior legs, they mounted over it to reach the external end of the rod where the vibrational source was placed. On the contrary, this behavior was not observed when males touched the non-vibrated rod, characterized by vibration velocity values that were even higher than in the vibrated one. This indicates impaired orientation on large flat (2D) surfaces, which raises the general issue of orientation towards vibrational sources. Insects can conceivably use amplitude difference or time delay between sensory inputs (legs) as cues to determine direction of the source. While a definite answer to this question remains to be provided, time difference is a more likely candidate in pentatomids because of unpredictable amplitude patterns associated with the narrow-band signals they use (Virant-Doberlet et al. 2006; Polajnar et al. 2012). This variability is shown by results of the present study where we found a general pattern of increasing amplitude towards the source, but with many exceptions, especially in the arena, which nevertheless did not prevent the active males from locating the source. We therefore assume that time delay was the cue they used, although precise analysis of available cues was out of scope for the present study. Orientation on the basis of either amplitude difference (Polajnar et al. 2016a) or time delay (Hager and Kirchner 2014) was demonstrated in other insect groups, where the strategy of a particular group likely depends on various factors such as body size, signal frequency and bandwidth, and the physical features of the acoustical environment. Apart from that, orientation on a 2D surface would require triangulation regardless of the cue, for which pentatomids are likely not adapted because their usual environment – a tangle of plant branches, leaves and fruits – can be more accurately approximated as a web of 1D and small 2D surfaces (Mazzoni et al. 2014) where triangulation is not necessary. It is therefore not surprising that difficulties were observed with locating a rod on the surface of an arena. Nevertheless, active males did not give up searching despite prolonged search effort, indicating high motivation.

Regardless of the mechanism, males stimulated with pbFS were significantly attracted to the signal source. In Test 4, playback allowed the capture of approximately 50% of released males despite the high heterogeneity of the vibrational amplitude field measured on the acoustic trap. The number of captured males is consistent with the number of males attracted to the SP in all the other tests, which means that males, once stimulated with the female song, can find their way to the source. This result would suggest that FS2 has a good potential to be used for field capturing. Currently, commercial traps are based on two-component aggregation pheromone dispensers which attract BMSB to the vicinity (Khrimian et al. 2014; Weber et al. 2014). The problem arises because not all the individuals enter the traps, likely because the aggregation pheromones are efficient for medium range attraction but much less for precise localization in stink bugs (James et al. 1996; Aldrich et al. 2009). This constraint can cause a tricky contraindication if masses of bugs are attracted to an orchard from outside without capturing many of them (Sargent et al. 2014). Therefore, the use of attractive vibrational signals integrated into the existing trap designs could provide an important synergistic effect, increasing the capture rate. The development of this type of acoustic device would constitute an important innovation of traps based on specific, non-pheromone sexual signals. Indeed, acoustic traps have already been proposed in the past, and some have been recently developed to attract mosquito males (Johnson et al. 2016). Such traps, however, emit pure tone airborne sound to mimic female flight noise. Although such a noise might be considered a species and sexual identifier for males, the mosquito female sound is a constant, unstructured sound and it is involuntary, being simply associated to the flying activity. The function of BMSB female signals, on the other hand, is explicitly to attract males. Another option would be to interfere with the species' sexual communication by blocking the vibrational communication channel with disruptive noise. Signals involved in the mating duet carry information crucial for mate selection, and thus by interfering with perception of vibrational signals in both males and females, would disrupt not only the male search but also the correct identification of the sender. In *S. titanus*, the transmission of a disruptive noise through the vineyard supporting wires let grapevine tissues vibrate and occupy the frequency range used by duetting partners (Eriksson et al. 2012; Polajnar et al. 2016a). However, this technique is not likely to be successful in the case of the BMSB. Unlike *S. titanus* which is monophagous and monovoltine, the BMSB is widely polyphagous and multivoltine. Therefore, to target one or several crops would not be sufficient since mating can occur on a large variety of other hosts where the animals can multiply rapidly. Instead, we consider promising the use of vibrational signals for monitoring and mass trapping by improving the existing pheromone traps.

An important limit of this method is that FS2 can only attract males who are the more active partner, searching for stationary females who do not exhibit any vibration-mediated tropotaxis (Polajnar et al. 2016b). Despite this, a significant increase of the number of captured males would alone represent an important improvement of the trap efficacy. Since both males and females mate multiple times in their life (Lee et al. 2013; Rice et al. 2014), a considerable number of males should be captured to have a measurable effect, but this is an issue shared with the pheromone-based mating disruption methods targeting moths whose efficacy has nevertheless been demonstrated in the field (Witzgall et al. 2010). The use of aggregative vibrational signals could significantly increase the capture rate, also including females and nymphs, but no such signal has been observed so far and pheromones appear to be the only signal covering this role in BMSB. We do recognize, however, that much more research must be done to better characterize and understand the proper function of all BMSB signals (Polajnar et al. 2016b).

We must also consider that a rather conspicuous part of males (from 30 to 50%) did not react at all to vibrational stimulation. In Test 1, for example, 23 males out of 30 moved away from the starting leaf and only 14 of them reached the vibrated leaf. The other nine individuals that did not reach the goal exhibited longer activation time than the successful ones and did not differ in this aspect from the silent control, which means that they probably were walking on the plant without the intention to find the vibrational source. The reason for this low percentage of motivated males is not yet clear, but could be due to either a certain physiological state, perhaps related to age (we did not check the exact age of the tested males, but simply used individuals older than 7 days) or mating history, or to the stimulus quality. In fact, as much as we tried to reproduce a “typical” female signal, we do not know yet the exact spectral and temporal features that would make a female signal more attractive to males. The study of the mating behavior of the planthopper, *Hyalesthes obsoletus* Signoret, revealed that even slight manipulation of the spectral pattern of female pulses could significantly alter the male responsiveness (Mazzoni et al. 2015), and in the case of stink bugs, female signals emitted on different substrates were reported to differ in attractiveness to males of *Nezara viridula* L. (Miklas et al. 2001). Signal quality is, in fact, a cue to males for identification, in first instance, but also for increasing their motivation and thus investing time and energy in mating (Kuhelj et al. 2015). Signaling and searching have a direct metabolic cost, but also incur risks associated with eavesdropping from predators, parasitoids and rivals, so they should be well balanced by any individual (Cocroft and Rodríguez 2005; Virant-Doberlet et al. 2011). Motivated males in our trials were easily identifiable in that they used to remain in the arena, walking most of the time at a relatively high speed, whereas unmotivated males either quickly left the arena or stayed inside but without moving much. Therefore, it seems likely that the male decision to search for the female was mostly, if not exclusively, based on perception of the female vibrational signal. Vision appears to be much less important for this task, although the use of dummy females substantially increased the loitering effect of the signal in Test 3. Males used to continuously enter and exit the SP-VSA in absence of the dummy; on the contrary, the time of permanence in the SP-VSA significantly increased in presence of the dummy. While light-based stimuli have been found to be attractive to BMSB (Leskey et al. 2015), the role of vision (of another individual) during the mating process seems limited to very short distances and thus not useful for improving field traps. The effect of a vibrational stimulus is similar to what is commonly described as arrestant effect, however, the definition of arrestant is “a stimulus that causes the insect to cease locomotion in close contact with the apparent source” (Beck 1965). In the case of BMSB, males did not stop walking, but remained actively moving around the SP, presumably because it lacked other key stimuli provided by a live female. We therefore borrow from military terminology and propose the phrase “loitering” to describe this phenomenon. This fits very well with the typical behavior of insects which use vibrational signals as a cue to locate potential mates (Mazzoni et al. 2014; Polajnar et al. 2014). As an obvious consequence, the loitering effect would eventually cause aggregation and this would reinforce the role of FS2 as an attractant.

In conclusion, we think that the use of FS2 signals as a stimulus integrated into existing pheromone traps could be an important innovation to the current state of BMSB management in the field. By adding the vibrational stimulus, it would be possible to increase the trap efficacy by attracting males inside the traps and thus considerably reducing the male population. However, even without a trap design, the observed loitering effect of the vibratory stimulus might be useful in push-pull strategies. More research is needed to define the signal characteristics which can further improve its efficacy, especially in terms of spectral and temporal parameters that could motivate a higher number of individuals, but also to define thresholds (i.e. of frequency or amplitude) of efficacy. This knowledge is required to set up field experiments and to test acoustic traps.

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Compliance with Ethical Standards

There are no conflict of interest involving the authors.

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

This article does not contain any studies with human participants performed by any of the authors.

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Figure captions

Fig. 1 Oscillogram (above) and spectrogram (below) of the female signal playback (PbFS) used to stimulate the males in all tests. The pbFS, consisting of 12 female pulses, type FS2, was continuously looped for the full duration of each trial

Fig. 2 Scheme and vibrational amplitude field of the bean plants used in Test 1. Two bean plants were grown together in one pot having only one contact point at approximately mid stem length. The mini-shaker (SH) was moved after each trial and thus the stimulated leaf (e.g. Lf1-SP) was randomly changed. The male releasing point was randomized among the non stimulated leaves. The Audio Sampling Points (ASPs) are indicated with black dots. Four of them were placed on the leaves (Lf1-Lf4) and other four on the stems (St1-St4). The mean (±SD) amplitude of the playback signal (as substrate velocity in μm/s) is reported. Different letters indicate significant differences between amplitude values recorded from the ASPs ($p < 0.05$) after Friedman's test with replication followed by Bonferroni post hoc test

Fig. 3 Scheme of the arenas used in Test 2 (A) and Test 3 (B). (A) In Test 2, the mini-shaker was placed in direct contact with the arena surface. Four different Video Sampling Areas (VSA-T2), corresponding with as many Audio Sampling Points (ASP) were defined, one of them at the Stimulation Point (SP) and the others opposite (FR) and laterally (L1 and L2) to it. An additional ASP was placed on the Releasing Point (RP). (B) In Test 3, the SP was set at the external end of a paperboard rod and only two VSAs (VSA-T3) were defined, around the internal ends of the SP and FR rods, respectively. As a whole, the vibrational amplitude field was measured from 19 ASPs, 12 of them on the arena surface (a1-a8 plus L1, L2 and two inside each VSA) and six of them on the rods (SP, b1 and b2 on the vibrated rod, and b3, b4 and FR on the non-vibrated one). In (A), amplitude values (as substrate mean ± SD velocity in μm/s) are reported for each ASP; different letters indicate significant differences between amplitude values recorded from the ASPs ($p < 0.05$) after Friedman's test with replication, followed by Bonferroni post hoc test

Fig. 4 Scheme (3D, above, and flattened diagram, below) of the acoustic trap used in Test 4. As a whole, 45 Audio Sampling Points (ASPs) were placed: 36 ASPs on the upper (Ceiling), lateral (Sides 1 and 2) and back (Back) faces (nine per face) of the net cage. Other four ASPs were placed on the Front face, two on the net Sleeve, one on the plastic Plastic funnel and two on the Cylinder, including the Stimulation Point (SP). Males were released inside the net cage

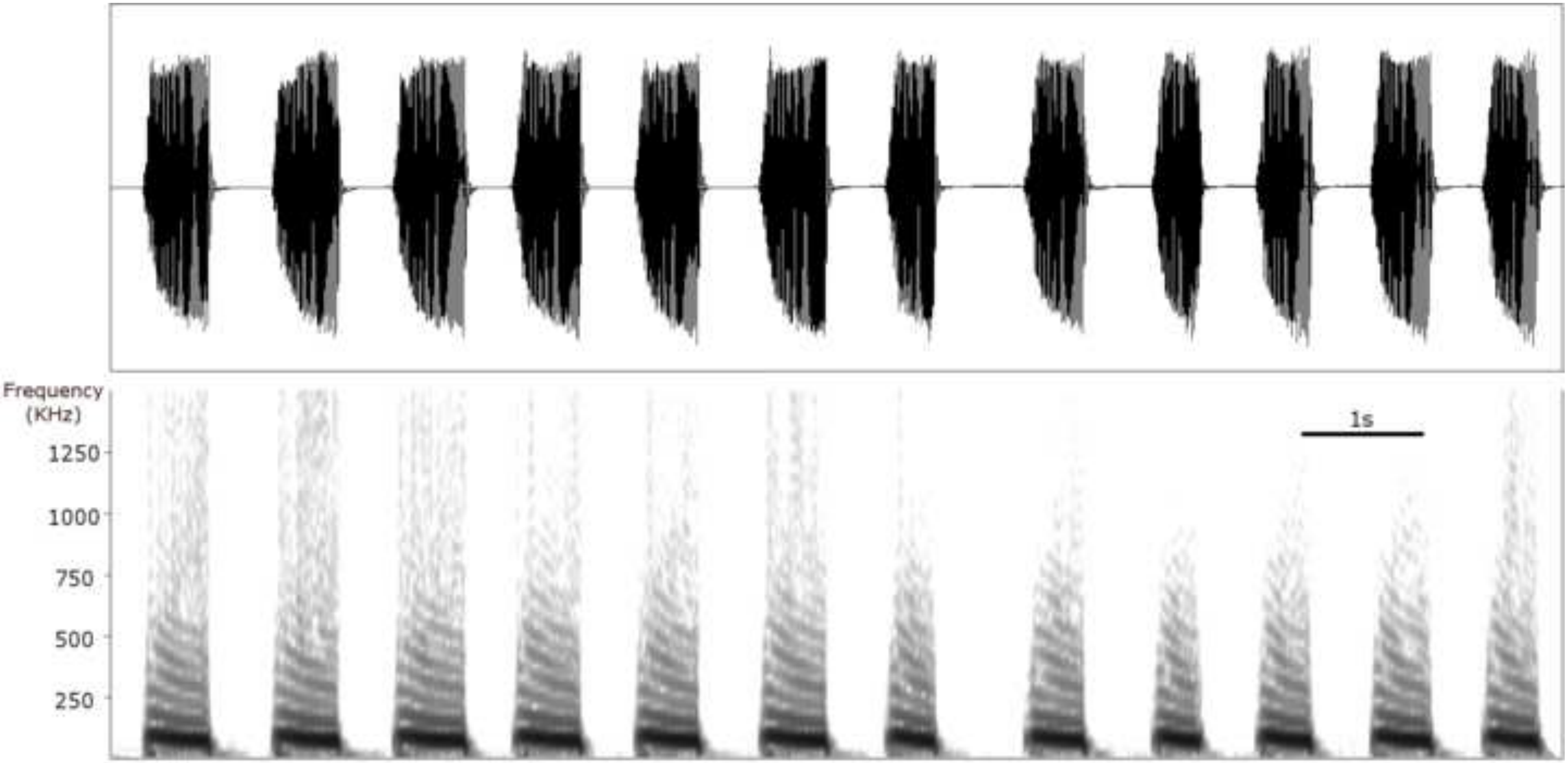
Fig. 5 Box-plot graph of the activation time of males stimulated with pbFS (Pb_+) in Test 1. Pb_- indicates the control trials. Stimulated males were further divided into those that reached (Pb_+SP) and those who did not reach (Pb_+no) the leaf with the Stimulation Point (SP). Different letters indicate significant differences ($p < 0.05$) after Kruskal-Wallis followed by Steel-Dwass multiple comparison test. Plots show median (center line), 75th percentiles (top of box), 25th percentiles (bottom of box), and whiskers connect the largest and smallest values within 1.5 interquartile ranges

Fig. 6 Mean (±SE) distance walked by BMSB males (blue) and their walking velocity (red) in the different trials. (A) In Test 2, the trials were done with both playback and dummy female (Pb_+Dy_+), with only playback (Pb_+Dy_-) and in absence of playback, taking together with and without a dummy (Pb_-). (B) In Test 3, all trials were done in absence of a dummy. “Out” are those individuals that left the arena before the end of the trial time, regardless of the treatment. Numbers in brackets (n) indicate the replications for each treatment. When present, different letters on the same line indicate significant differences after Kruskal-Wallis test followed by Mann-Whitney pairwise post hoc test ($p < 0.05$)

Fig. 7 Mean (±SE) time spent inside (A) and number of accesses to (B) the arena Video Sampling Areas (VSAs) by males in Test 2. The four VSAs are: the vibrated VSA (SP), the one opposite to it (FR) and the lateral ones (L1 and L2). Treatments (Pb = playback; Dy = dummy female; + = on; - = off) and numbers of active males (in brackets) are reported on the X-axis. Different letters indicate significant differences ($p < 0.05$) after Friedman test followed by Bonferroni pairwise post hoc test

Fig. 8 Mean (±SE) time spent inside (A) and number of accesses (B) to the two VSAs by the males in Test 3: the vibrated VSA (SP, white) and the one opposite to it (FR, black). Treatments (Pb_+ = playback on; Pb_- = playback off) and number of active males are reported on the X-axis. ** indicate significant differences after Wilcoxon T test. ns = not significant ($p > 0.05$)

Fig. 9 Vibrational amplitude field of the Test 3 arena (A) and the Test 4 cage (B). The analysis is based on 19 and 41 Audio Sampling Points (ASPs), respectively (see Fig. 3 and 4). SP = Stimulation Point; FR = Frontal (non-stimulated) Rod.


Frequency
(KHz)
1250
1000
750
500
250
1s

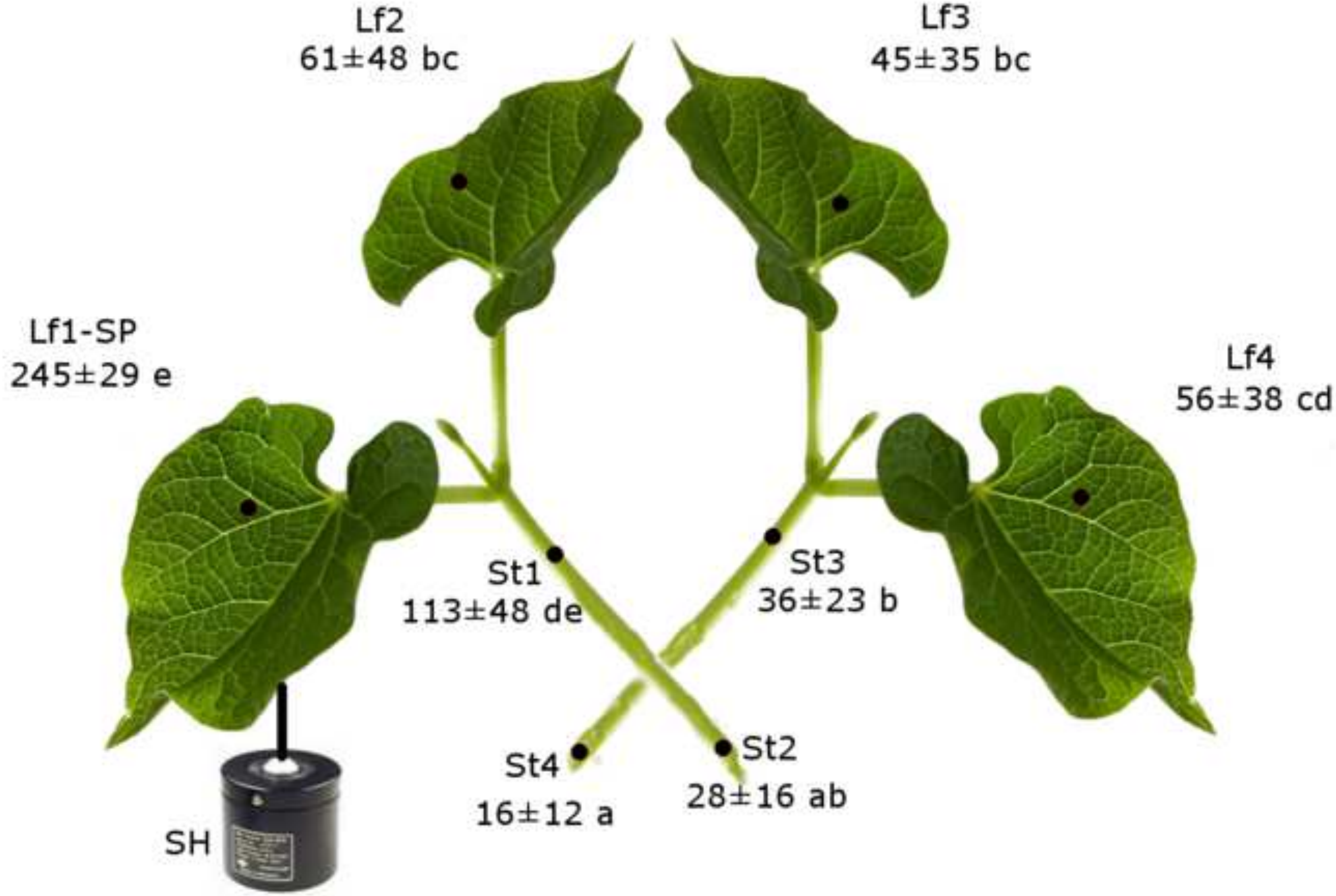

Lf2
61±48 bc
Lf3
45±35 bc
Lf1-SP
245±29 e
Lf4
56±38 cd
St1
113±48 de
St3
36±23 b
St4
16±12 a
St2
28±16 ab
SH

Figure 3

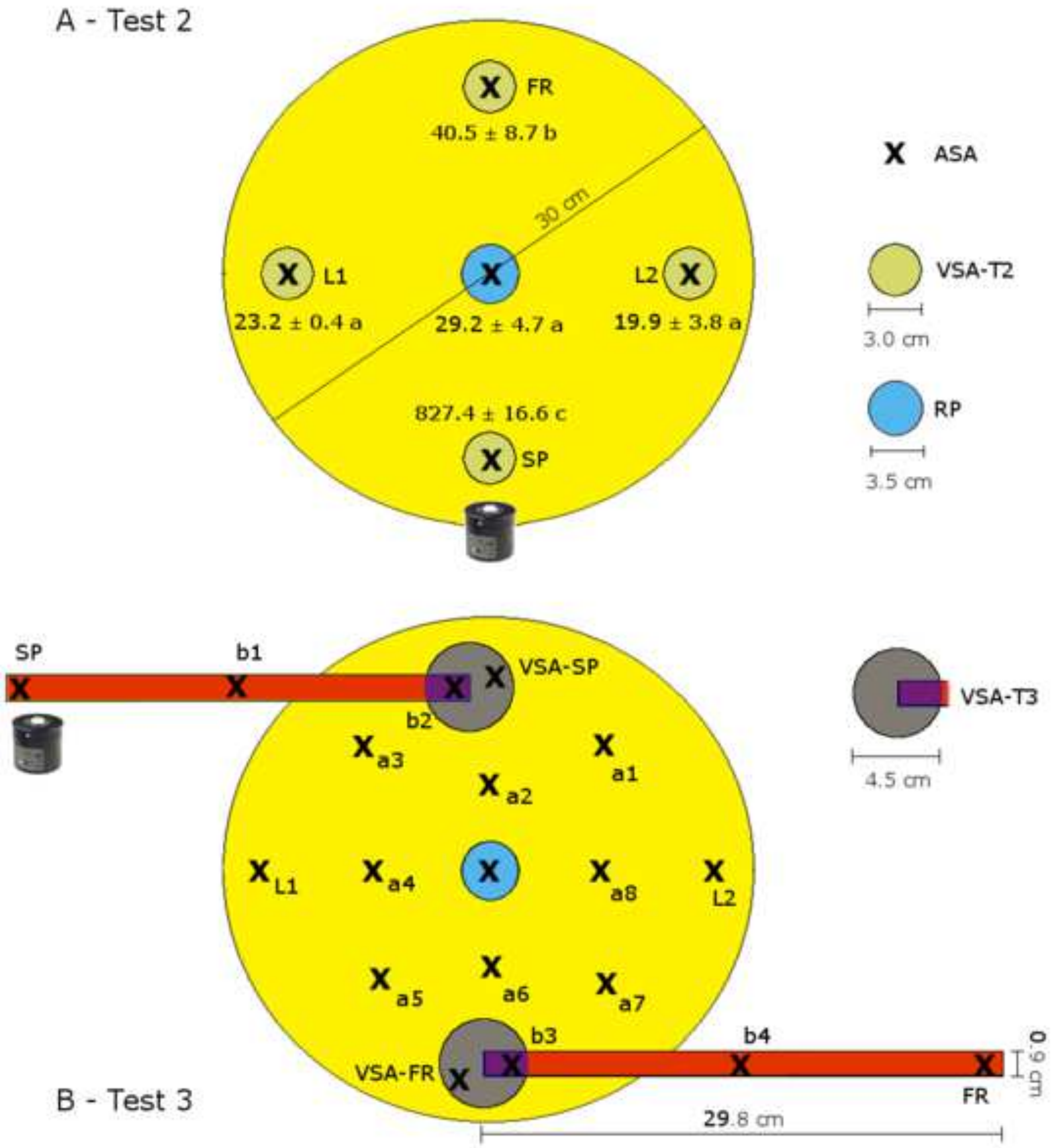
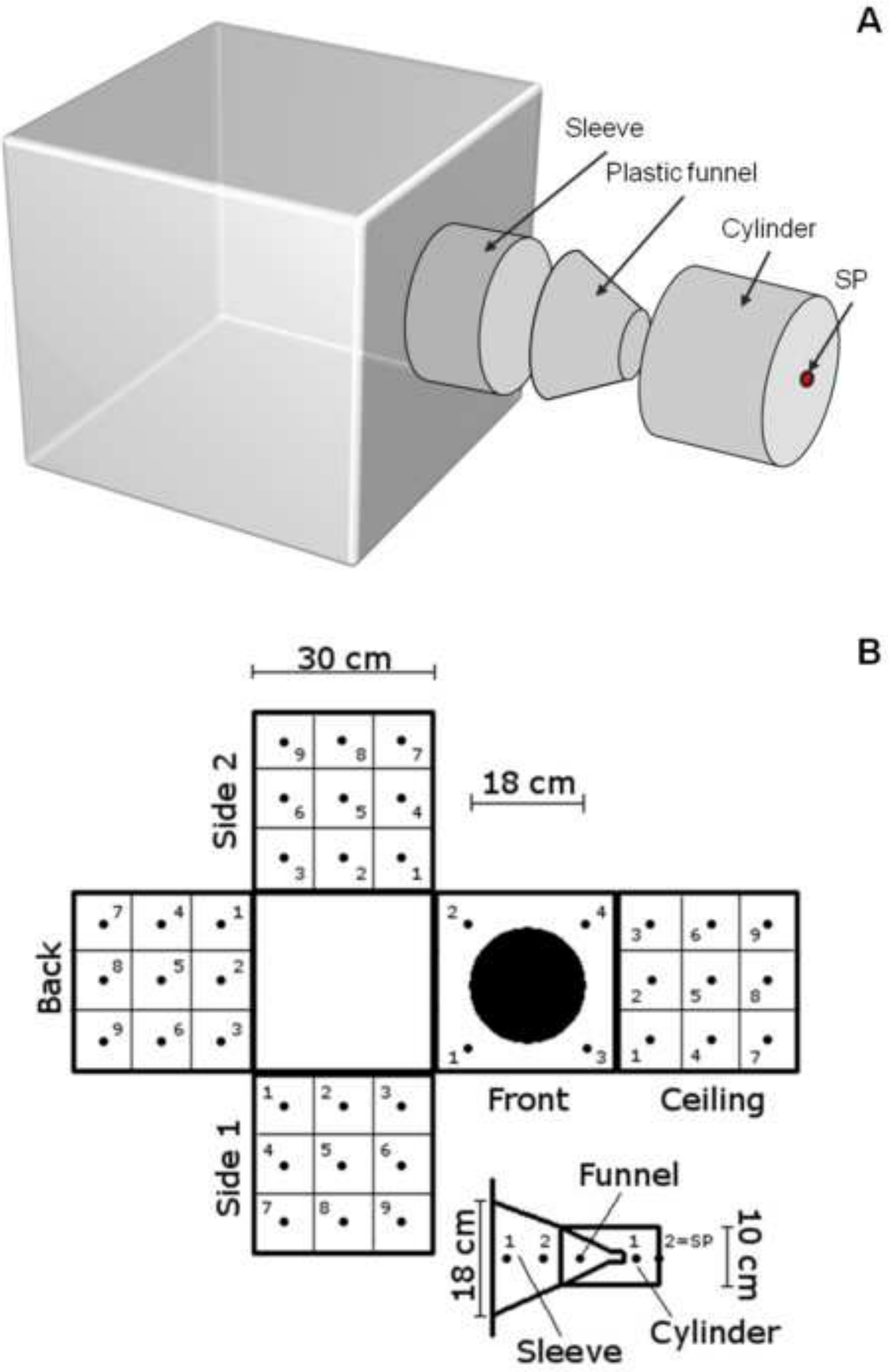

A - Test 2
FR
40.5 ± 8.7 b
30 cm
L1
23.2 ± 0.4 a
29.2 ± 4.7 a
L2
19.9 ± 3.8 a
827.4 ± 16.6 c
SP
ASA
VSA-T2
3.0 cm
RP
3.5 cm
SP
b1
VSA-SP
b2
VSA-T3
4.5 cm
a3
a1
a2
L1
a4
a8
L2
a5
a6
a7
b3
b4
0.9 cm
VSA-FR
FR
29.8 cm
B - Test 3

Figure 4


A
Sleeve
Plastic funnel
Cylinder
SP
B
30 cm
18 cm
Side 2
Back
Front
Ceiling
Side 1
Funnel
2=SP
10 cm
18 cm
Sleeve
Cylinder

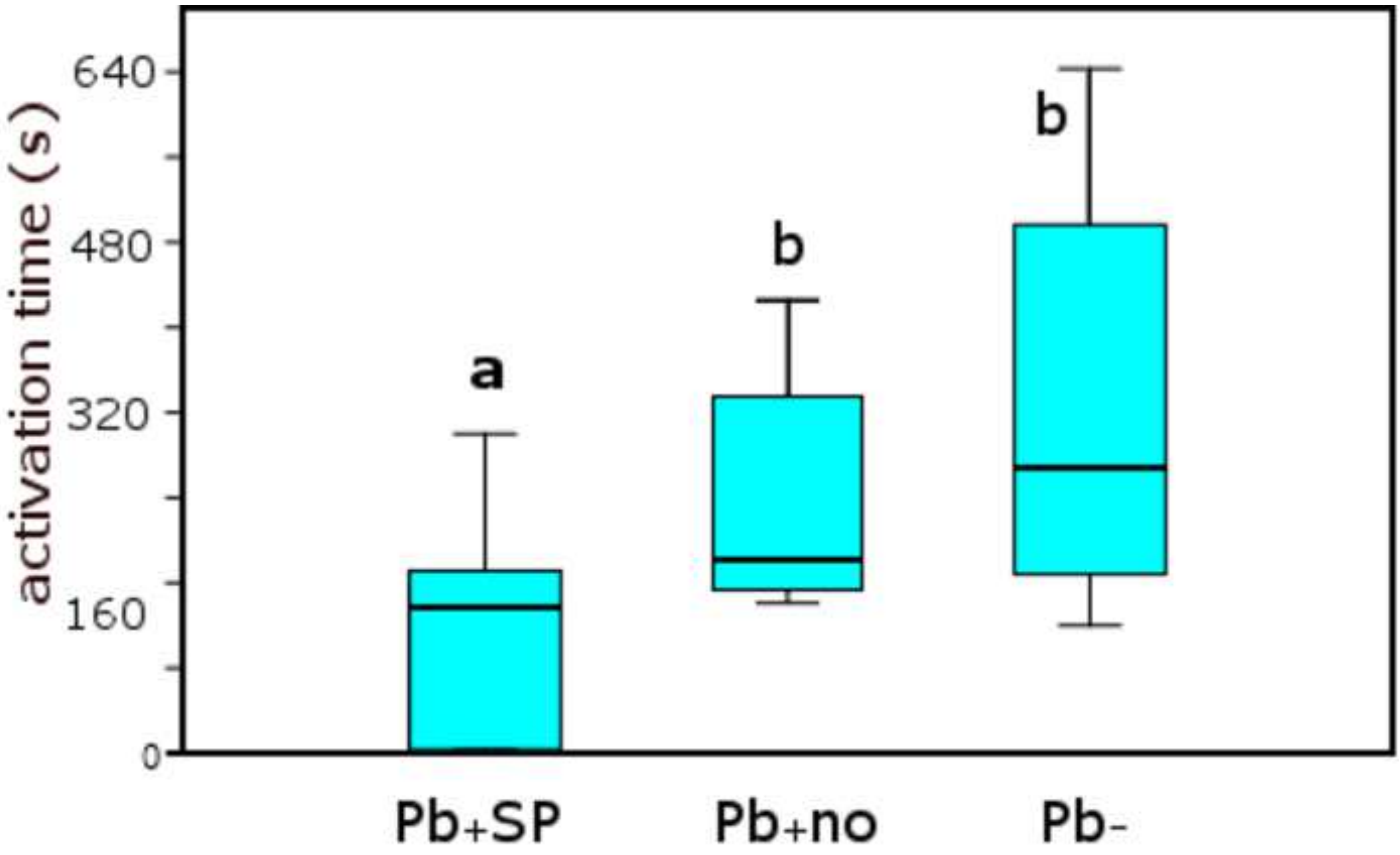
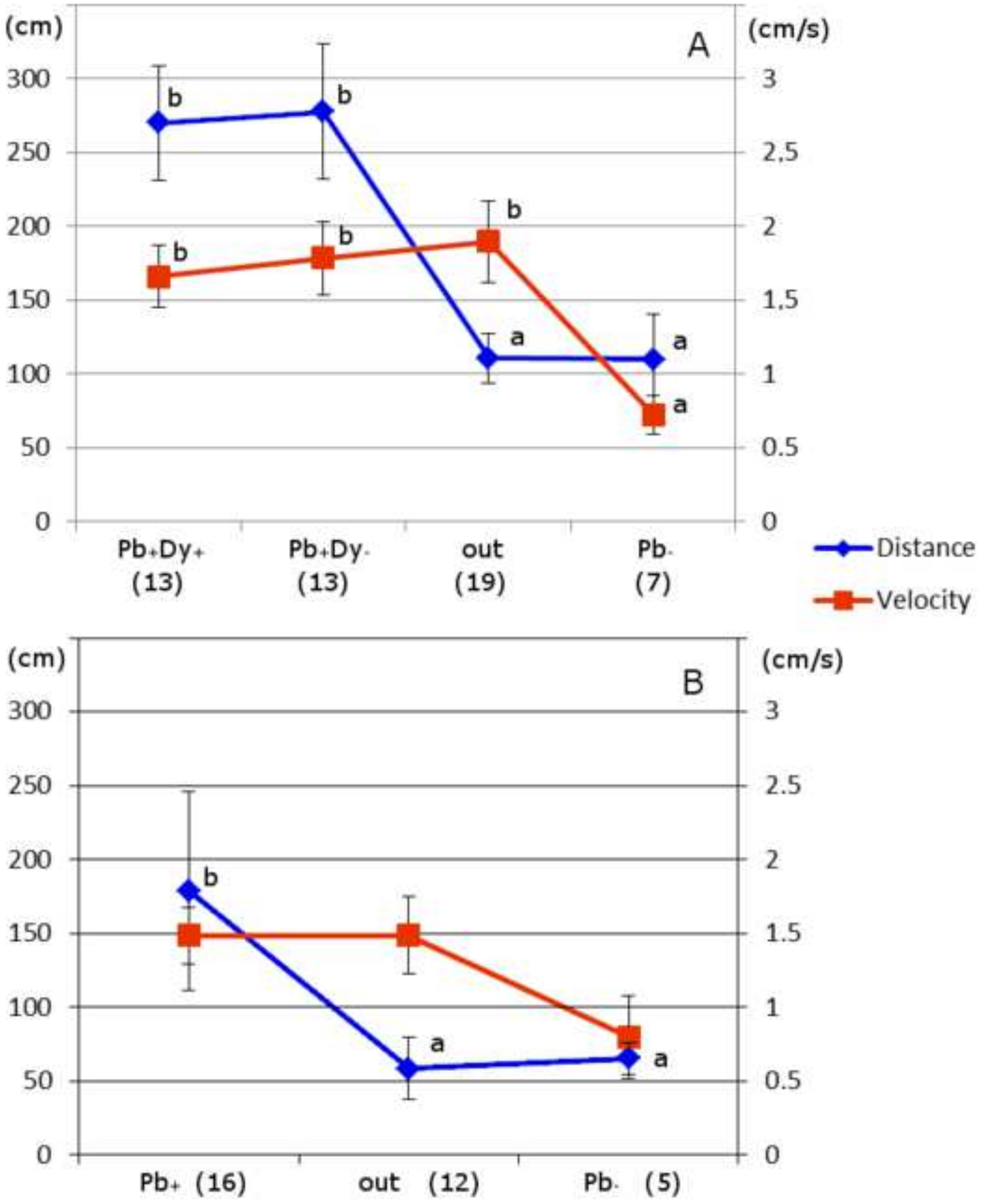
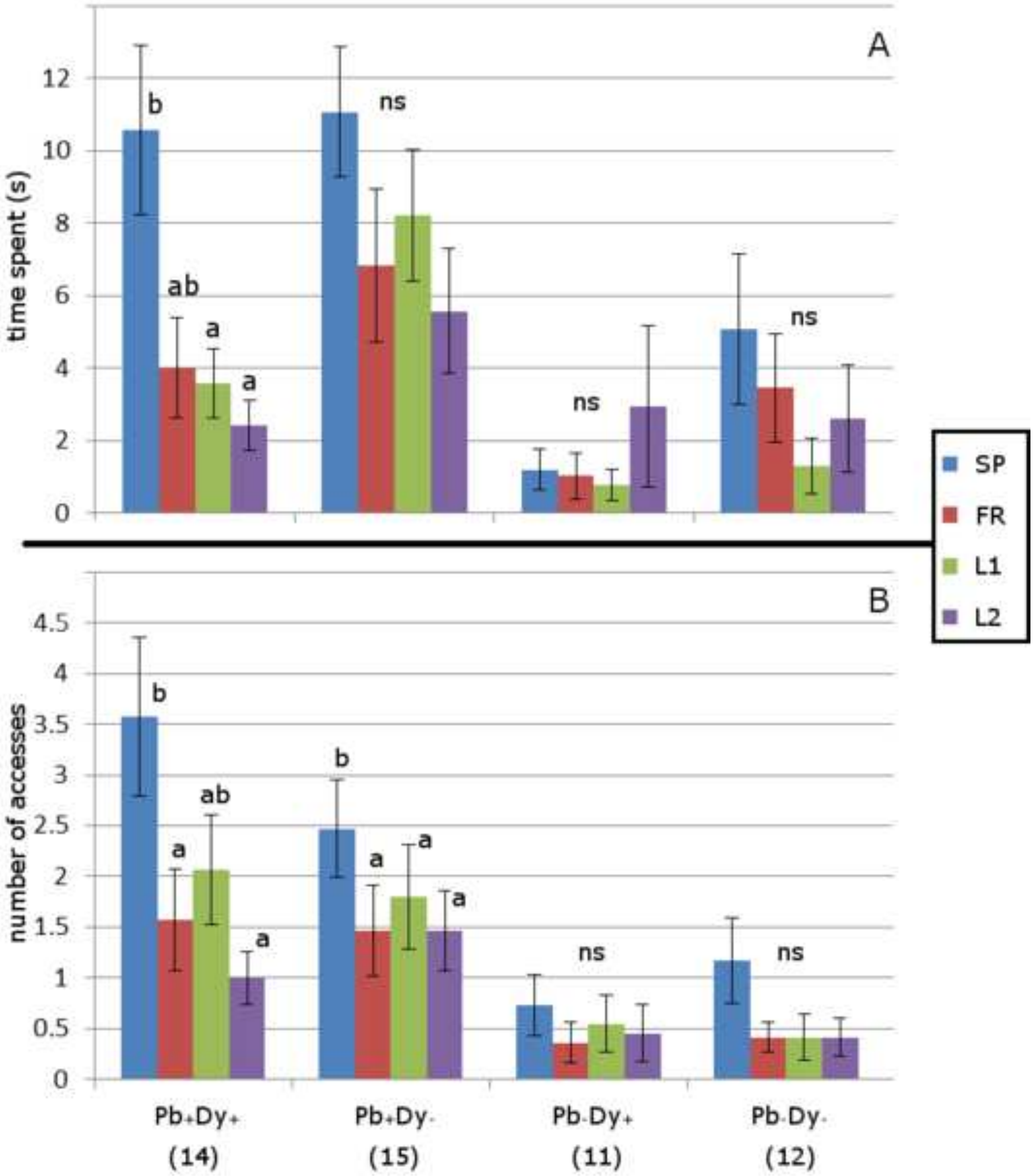

activation time (s)
640
480
320
160
0
a
b
b
Pb+SP
Pb+no
Pb-

Figure 6


A
(cm)
300
250
200
150
100
50
0
(cm/s)
3
2,5
2
1,5
1
0.5
0
b
b
b
b
b
a
a
a
Pb+Dy+ (13)
Pb+Dy- (13)
out (19)
Pb- (7)
Distance
Velocity
B
(cm)
300
250
200
150
100
50
0
(cm/s)
3
2.5
2
1.5
1
0.5
0
b
a
a
Pb+ (16)
out (12)
Pb- (5)


A
time spent (s)
0
2
4
6
8
10
12
b
ab
a
a
ns
ns
ns
B
number of accesses
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
b
a
ab
a
b
a
a
a
ns
ns
Pb+Dy+
(14)
Pb+Dy-
(15)
Pb-Dy+
(11)
Pb-Dy-
(12)
SP
FR
L1
L2

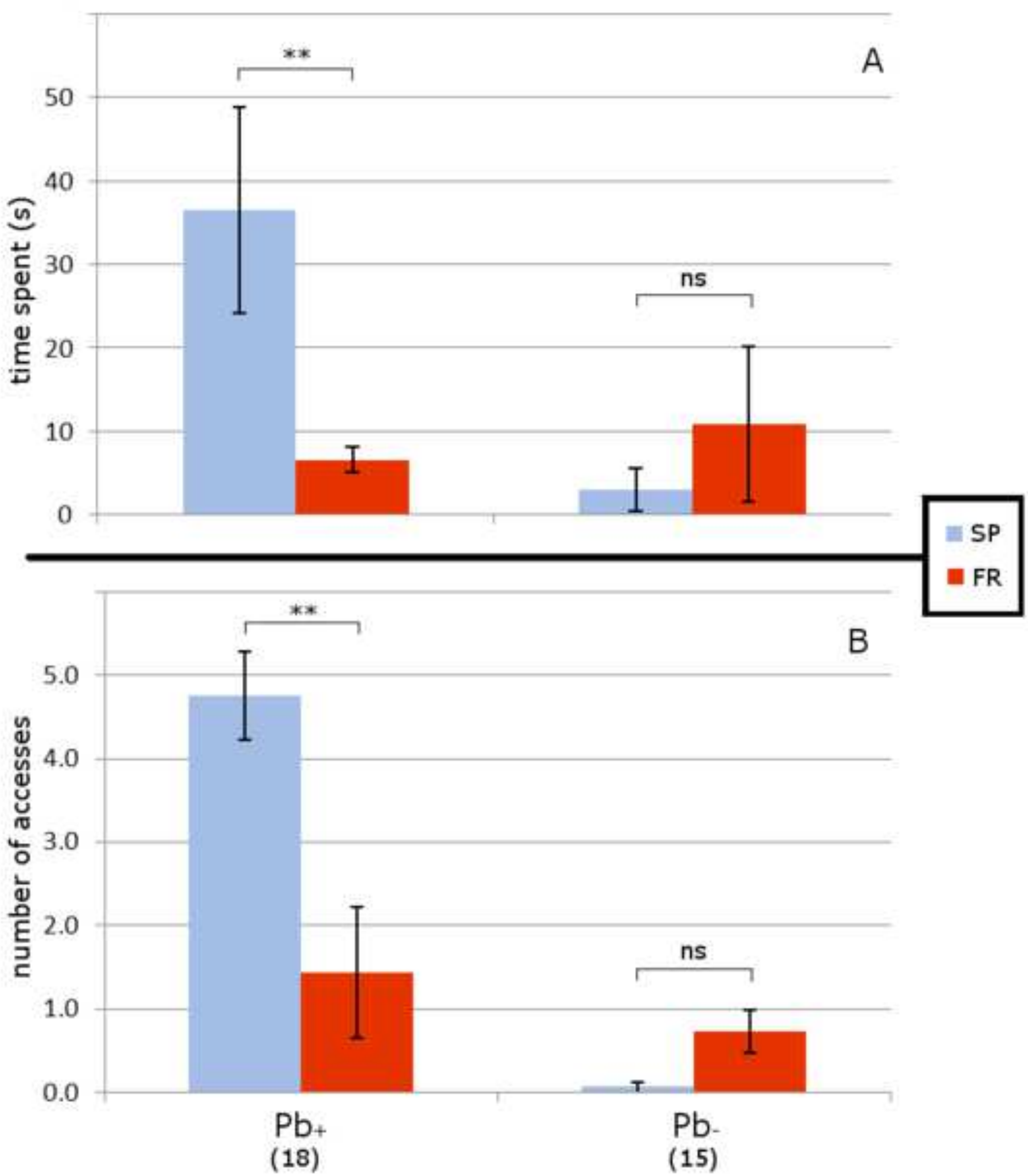
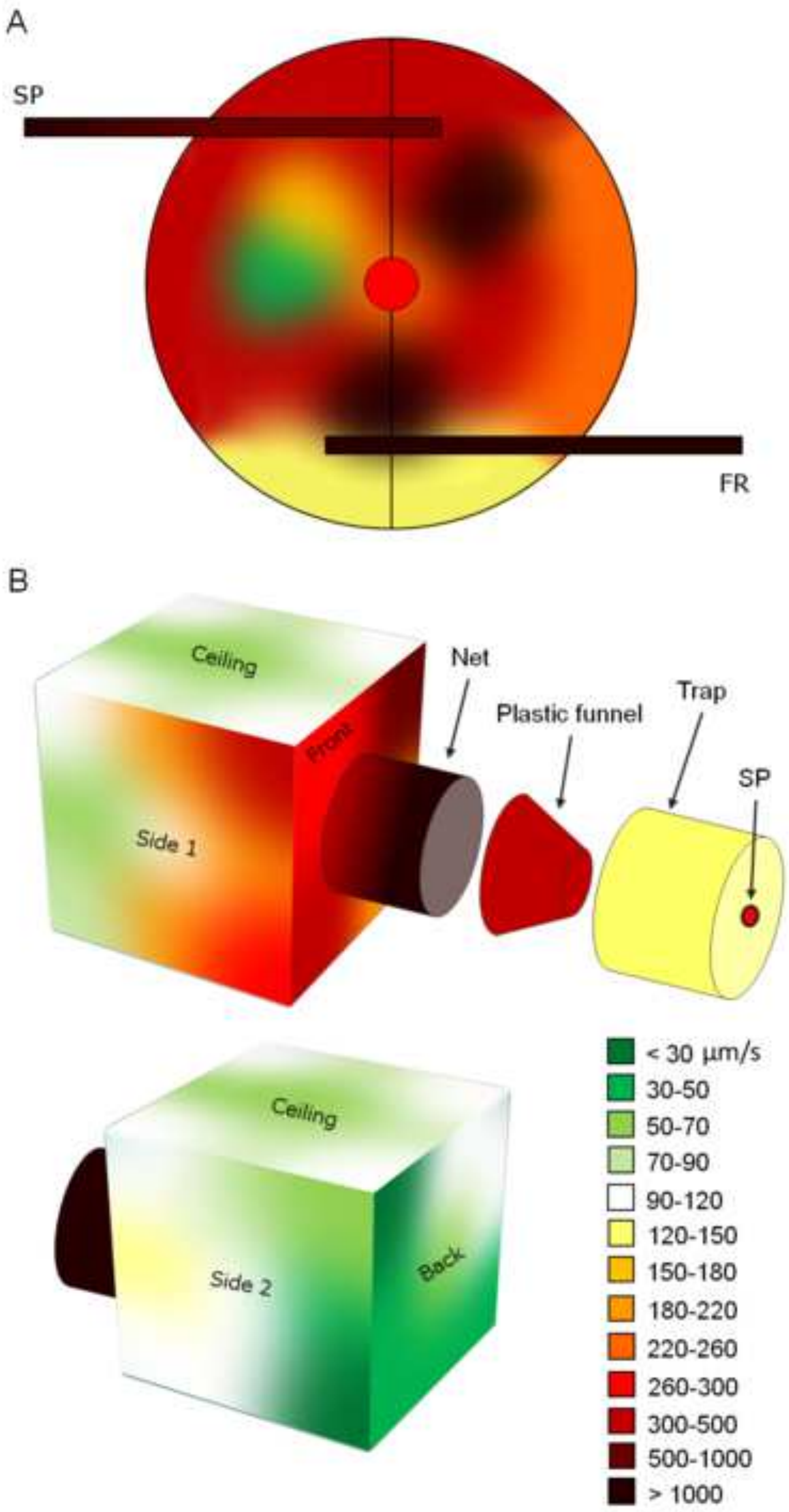

A
time spent (s)
0
10
20
30
40
50
**
ns
SP
FR
B
number of accesses
0.0
1.0
2.0
3.0
4.0
5.0
**
ns
Pb+
(18)
Pb-
(15)

Figure 9


A
SP
FR
B
Ceiling
Side 1
Front
Net
Plastic funnel
Trap
SP
Ceiling
Side 2
Back
< 30 µm/s
30-50
50-70
70-90
90-120
120-150
150-180
180-220
220-260
260-300
300-500
500-1000
> 1000

Tables

Table 1 Results of Test 1 (attractiveness on the plant) for treatment (pbFS stimulation, Pb_{+}) and control (Pb_{-}) trials. The number of active males (Active ♂), of males that reached the Stimulation Point leaf (SP Leaf), that loitered on it for 5 minutes (Loitering) and the male searching time (Search t: m±SD) are reported together with results of G-test (G and p) in a contingency table (2x2)

	Pb_{+}	*Pb_{-}*	*G*	*p*
n	30	23		
Active ♂	23	10	6.0	**0.014**
SP Leaf	14	1	7.7	**0.005**
Search t	245	499		
Loitering	10	0		

Table 2 Results of Test 2 (attractiveness on the arena – spot attraction). In (A), data are divided by treatment (pbFS stimulation, Pb_{+}) and control (Pb_{-}), and by presence (Dy_{+}) and absence (Dy_{-}) of a dummy female. In (B), Pb and Gy data are pooled. The number of active males (Active ♂) and of males that remained (Remained ♂) on the arena for the full trial duration are reported together with results of G-test (G and p) in a contingency table (4x2 and 2x2 in (A) and (B) respectively)

(A)	*Pb_{+}*		*Pb_{-}*			
	Dy_{+}	**Dy_{-}**	**Dy_{+}**	**Dy_{-}**	**G**	**p**
n	20	20	20	20		
Active ♂	14	15	11	12	2.2	0.54
Remained ♂	13	13	2	5	21.5	**<0.001**

(B)	*Pb_{+}*	*Pb_{-}*	*G*	*p*	*Dy_{+}*	*Dy_{-}*	*G*	*p*
n	40	40			40	40		
Active ♂	29	23	1.9	0.16	25	27	0.2	0.64
Remained ♂	26	7	20.0	**<0.001**	15	18	0.2	0.62

Table 3 Results of Test 3 (attractiveness on the arena – exit path attraction). The number of active males (Active ♂), of males that reached the rod end and of those that remained (Remained ♂) on the arena for the full trial duration and those that reached the external end of the vibrated rod (Rod end) are reported together with results of G-test (G and p) in a contingency table (2x2). Data are divided by vibrated (Pb_+) and silent (Pb_-) trials. In the case of Pb-, the rod end value refers to the number of individuals that reached either of the two rod ends

	Pb_+	Pb_-	*G*	*p*
n	20	20		
Active ♂	18	15	3.8	0.15
Remained ♂	16	5	12.4	**<0.001**
Rod end	11	2	8.0	**0.004**

Use of substrate-borne vibrational signals to attract the Brown Marmorated Stink Bug, *Halyomorpha halys*

Online Resource 1, Journal of Pest Science

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The vibrational amplitude field (mean ± SD of amplitude as substrate velocity, in µm/s, measured at the peak of dominant frequency (DF), in Hz) of the Test 3 arena, measured by recording the pbFS from 19 Audio Sampling Points (ASPs). SP = Stimulation Point; B1-B4 = central and internal ASPs on the vibrated rod; FR = external ASP on the non-vibrated rod; A1-A8 = ASPs on the arena surface; VSA-SP and VSA-FR = ASPs included in the Visual Sampling Areas (VSA) on the arena surface, around the basal tip of the two rods, vibrated (VSA-SP) and not vibrated (VSA-FR)

ASP	***Amplitude***	***DF (Hz)***	**ASP**	***Amplitude***	***DF (Hz)***
SP	1275.8±165.2	77.3±5.8	**RP**	303.3±23.2	88.7±1.2
B1	1175.0±61.1	78.7±8.1	**A1**	2800±66.1	88.0±0.0
B2	1086.7±65.2	75.0±1.7	**A2**	604.2±51.0	88.7±1.2
B3	1553.3±137.8	74.0±0.0	**A3**	216.0±1.1	72.0±0.0
B4	1258.3±154.2	74.0±0.0	**A4**	39.8±4.6	72.7±1.2
FR	2579.2±243.8	74.7±1.2	**A5**	645.0±71.4	71.3±1.2
VSA-SP	869.2±99.5	74.0±0.0	**A6**	1389.2±118.4	88.7±1.2
VSA-FR	185.5±5.3	72.0±0.0	**A7**	515.8±61.8	72.7±1.2
L1	413.3±35.1	89.3±1.2	**A8**	936.7±152.0	88.7±1.2
L2	321.7±51.4	72.0±0.0			

Use of substrate-borne vibrational signals to attract the Brown Marmorated Stink Bug, *Halyomorpha halys*

Online Resource 2, Journal of Pest Science

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The (A) vibrational amplitude field (mean ± SD of amplitude as substrate velocity, in µm/s, measured at the peak frequency (Hz), which is reported in (B)) of the Test 4 cage/trap arena. Values were measured by recording the pbFS from the 45 Audio Sampling Points (ASPs) on the Test 4 acoustic trap (see Fig. 4 for more details on the ASPs positions on the trap).

A	*Back*	*Side 1*	*Side 2*	*Ceiling*	*Front*	*Sleeve*	*Funnel*	*Cylinder*
1	102.8±9.0	103.7±10.3	110.7±3.8	115.3±5.1	428.0±18.7	599.0±5.3	753.0±67.0	143.3±6.7
2	45.8±5.1	193.3±17.2	103.7±1.2	88.5±2.7	209.7±22.1	1173.3±95.0		139.7±14.2
3	35.3±3.8	407.3±49.1	27.8±1.0	104.2±9.3	298.7±7.2			
4	39.3±0.9	182.0±12.1	31.5±7.9	77.4±6.9	1150.0±130.8			
5	62.4±1.5	99.3±14.7	93.0±13.9	64.0±5.0				
6	126.7±13.5	65.3±3.2	140.0±1.7	70.9±3.3				
7	25.0±2.9	70.4±7.3	117.7±2.5	100.9±6.3				
8	105.4±13.4	190.0±23.1	88.3±6.3	53.8±2.8				
9	35.1±2.3	285.7±1.5	60.9±1.7	104.2±9.3				

A	*Back*	*Side 1*	*Side 2*	*Ceiling*	*Front*	*Sleeve*	*Funnel*	*Cylinder*
1	82.7±5.8	80.0±0.0	80.7±1.2	80.0±0.0	86.7±1.2	80.7±1.2	88.0±0.0	86.7±2.3
2	78.7±8.1	77.5±0.0	80.0±0.0	77.0±5.8	74.7±1.2	80.7±5.8		86.0±2.0
3	90.0±0.0	145.0±0.0	86.7±1.2	80.0±0.0	88.0±0.0			
4	75.3±1.2	82.0±0.0	52.7±32.3	75.0±1.2	75.3±1.2			
5	74.0±0.0	160.7±3.1	76.7±1.2	78.0±0.0				
6	80.7±1.2	160.8±2.9	80.0±0.0	81.0±2.3				
7	62.7±25.0	78.0±0.0	75.3±1.2	77.0±1.2				
8	73.3±1.2	83.3±1.4	81.3±1.2	89.0±1.2				
9	131.4±37.6	86.0±2.0	82.7±3.1	80.0±0.0				