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Design of ideal vibrational signals for stinkbug male attraction, through vibrotaxis experiments

Design of ideal vibrational signals for stinkbug male attraction

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Authors' contributions:

VM, MT, DM, AB, LM, NMP conceived and designed the research. VC, KW, DM, MT, RM conducted the experiments. VC, DC, VM, AB analyzed the data. VC, VM wrote the manuscript. KEW revised the English language of the manuscript. All authors read and approved the manuscript.

ABSTRACT

BACKGROUND

Many groups of insects utilize substrate-borne vibrations for intraspecific communication. This characteristic makes them a suitable model for exploring the vibrations as a tool for pest control in alternative to chemicals. The detailed knowledge of the species communication is a prerequisite to select the best signals to use. In this sense, this study aimed at exploring the use of substrate-borne vibrations for pest control of the brown marmorated stink bug (BMSB), *Halyomorpha halys* Stål (Heteroptera: Pentatomidae). To this purpose, in a first set of experiments, we identified the spectral and temporal characteristics that best elicit male responsiveness. Bioassays were conducted with artificial signals that mimicked the natural female calling signal. In a second part, we used the acquired knowledge to synthesize new signals endowed with different degrees of attractiveness in single and two choice bioassays using a wooden custom-made T stand.

RESULTS

The results from this study showed that males were attracted to female signals along a high range of amplitudes, specially starting from a threshold of 100 μm/s, a high pulse repetition time (1s) and peak of frequency in correspondence of the first harmonic (76 Hz). This resulted in an “optimal” signal to be used to attract males, while the choice test in the T arena showed this signal elicits searching behavior and attracts males of BMSB towards a stimulation point.

CONCLUSION

We confirm the use of vibrational signal as a strong tool for behaviroal manipulation of males of BMSB and suggest its possible use for the development of field traps and the further management of this pest.

Key-words: biotremology, *Halyomorpha halys*, vibrational communication, playbacks, insects, brown marmorated stink bug.

Declarations

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1 INTRODUCTION

Behavioral manipulation of the target species (with attraction/repellence to/from a source of attractive/repelling signals) and mating disruption are two alternative methods for insect pest management in agriculture to minimize the risk that pesticides pose to human health and the environment[1,2]. Pest control through interference with cues used for inter- and intraspecific communication has been theorized since the 40s with the idea of releasing sexual pheromones in the field to catch moths[3] and is nowadays successfully applied worldwide to control several crop pests, particularly in Lepidoptera[4,5].

More recently, a new technique of mating disruption based on the use of substrate-borne vibrations in place of pheromones has been described and effectively tested in semi-field and field trials[6–8]. In many insect species, vibrational signals (i.e. emission, reception, and correct interpretation of signals) are essential to accomplish mating[9–11]. This is also true for many Hemiptera (i.e., leafhoppers, planthoppers, stinkbugs), in which the mating process is composed of several behavioral steps, after the initial reciprocal (i.e., male-female) identification, passing through mate location, courtship and eventually ending with copulation. As a general rule, the sender (more commonly the male) initially emits a call to elicit a response from the receiver (the potential mate), thus establishing with him/her a vibrational duet[12]. However, a single emission of a call does not automatically elicit a behavioral response in a receiver, and it is fundamental that the emitted signal contains certain spectral/temporal features capable of positively motivating the receiver to respond and search for the sender[13]. Indeed, even small differences in the structure of a vibrational signal (i.e., the secondary components of a song frequency pattern) could drastically affect the motivation of an individual to establish a duet with a potential partner[10,11,14]. The direct correlation between signal and emitter quality is well documented in animals[15]; therefore, it is of interest to define the roles and values of spectral and temporal parameters affecting signal efficacy[16].

In this study, we focused on the brown marmorated stinkbug (BMSB) *Halyomorpha halys* (Hemiptera: Pentatomidae), an insect pest native to east Asia which recently became invasive in North America and Europe, causing severe economic damage to numerous crops[17–19]. In this species, the long-range mating communication is mediated by male-emitted aggregation pheromones[20,21], whereas vibrational signals mediate the behavior interactions at short distances.[7]□ In particular, males emit a low frequency signal (MS1), whose behavioral meaning is not known yet[22], while their interaction with females is associated to a pulsed signal (MS2) to which the females reply with two types of signals (FS1 and FS2)[23]..

Pest control of the species is currently based on commercial traps that use two- component aggregation pheromone dispensers to attract BMSB to the vicinity [21,24]. However, this strategy does not always ensure that the animals will go inside the trap, but instead causes an aggregation of individuals in the surrounding area, once they are efficient for medium range attraction [25,26]. Therefore, the use of attractive vibrational signals towards the inside of the trap is an alternative to cope with this problem. Indeed a recent study[11] demonstrated that males of BMSB can be attracted to an artificial source point (i.e., a mini-shaker) by a playback of FS2 both in plants and artificial arenas. These results suggested that FS2 could be used to capture males for monitoring or mass-trapping purposes. Indeed, integrating pheromone and vibrational traps could increase the capture rate and would constitute an important innovation development for the sector [11].

However, a single emission of signal does not automatically elicit a behavioral response for insects. Vibration signals within a species have a range of spectral and component features variation between individuals [23,27] and failing to send the correct signal could imply a miscommunication. Therefore, the aim of this study was to identify the FS2 exact spectral and temporal components that best trigger the search in BMSB male receivers. In this regard, we stimulated males with different types of FS2 playbacks and designed a new T stand arena for one and two-choice tests of vibrotaxis. Our ultimate goal was to synthesize the optimal attractive FS2 signal for BMSB field trapping.

2 MATERIAL AND METHODS

2.1 Insect rearing

Colonies of BMSB were initiated from adults and nymphs collected in the Province of Trento, North Italy, during spring and summer 2018 and 2019. Insects were reared in transparent plastic boxes in climatic chambers (under a 16:8 h photocycle at 25 ± 1ºC and 60 ± 5 % relative humidity) in a greenhouse at the institute Fondazione Edmund Mach (San Michele all'Adige, Italy), according to the protocol of a previous study[23]. All experiments were performed with sexually mature individuals (7 days after maturation molt).

2.2 Playback experiments

Data collection took place from March to August of 2018 and 2019. Experiment 1 was conducted in 2018 and experiment 2 in 2019. All trials were carried out in the Laboratory of Bioacoustics, Fondazione Edmund Mach, inside an acoustically insulated chamber (24 ± 1ºC in artificial lighting conditions) on an anti-vibration table. The signal, FS2 (taken from our signal library) was used as a template for all the playbacks[11] and consisted of a series of approximately 0.5-seconds long and regularly repeated pulses (pulse repetition time of approx. 1.0 s) with dominant frequency on the first harmonic, 80 Hz and a total duration of 11.5 seconds. We tested male response to FS2 playbacks in three different settings: i) a potted bean plant (*Phaseoulus vulgaris*, 20 cm tall, with two well developed opposite leaves); ii) a custom-made cardstock arena (Fig. 1) and iii) a wooden custom-made T stand (Fig. 2a). The variability of arenas aimed at answering different questions: from more basic parameters following protocol of [11] in experiment 1, to more complex ones, assessing the capacity of individuals to make choices from different directions at the same time in experiment 2 with a proposed new arena (T). Each arena had a "release point" (RP), where the individual was initially released, and a "stimulation point" (SP) in correspondence with the tip of a mini-shaker (model 4810; Bruel & Kjaer, Naerum, Denmark) that was the source of FS2 playbacks.

Each trial began with a BMSB male placed at the RP under a Falcon vial cap (diameter: 3.2 mm). After a time of 30 s (experiment 1) and 1 min (experiment 2), the playback was turned on and the cap was lifted thus freeing the insect (see Table 1 for descriptions of the FS2 used for each test). According to the tests, the trials ended when either the given time ran out (details in experiments session) or the male left the plant/arena or reached the SP. Analysis for each test was primarily based on determining which versions of the female signal mostly triggered the searching behavior. Searching, for this species, is defined as the alternation between walks and stops during pulse emission when the male stretches the legs, presumably in a posture of "listening"[23].

2.3 Design and validation of the T-shaped arena

The T-shaped arena was built of plywood. The three-dimensional (3D) scheme is shown in Fig. 2a: the two arms of the T can oscillate at their free extremity, while two thick pillars, one at the base and the other at the front, support the main stem. The stimulation points SP1 and SP2 are set on the free extremities of the T (red circles in Fig. 2a).

Before performing the experiments, we tested the vibrational signal propagation with laser vibrometer (Polytec PDV 100, Polytec GmbH, Waldbronn, Germany) associated to an acquisition device (LAN XI, Brüel & Kjær, Sound & Vibration A/S, Nærum, Denmark) to verify the symmetry of the setup when the SP was switched from one arm to the other. This preliminary test was also performed to characterize the T-arena by describing the vibrational landscape and the possible occurrence of amplitude gradients. To record, we used a sample rate of 8192 Hz. The spectral analysis was done by applying a fast Fourier transform (FFT) with a Hanning window length of 400 lines, 8 Hz of frequency resolution and 66.7% overlap.

2.4 Bioassays

Two sets of experiments were performed (summarized in Table 1): the first one was composed of 4 different tests, each one targeting a different parameter (either spectral or temporal) of the FS2. They were as follows: Exp1a: amplitude, measured as velocity of substrate vibration (µm/s); Exp1b: continuity of emission (with or without interruption); Exp1c: dominance of the harmonics between the first and the second one; Exp1d: signal emission pattern, measured as pulse repetition time (PRT). After each test, the FS2 playback was adjusted by fixing the parameter that best triggered a positive behavioral response in the male. This means that Exp1b benefited from the experience gained after Exp1a, as well as Exp1c, from information gathered from both Exp1a and Exp1b, and finally Exp1d , thanks to the information gathered from all previous tests. The second set of experiments consisted of one- (Exp2a) and two-choice tests (Exp2b, 2c). FS2 signals were designed to validate the information gathered in experiment 1, and establish which one, between dominant frequency and PRT, was more important to motivate males. In this way, optimal and suboptimal (i.e., deprived of either the optimal frequency or temporal pattern) FS2 signals were designed and played back into the T-arena. Each male was tested only once per test. Different treatments in the same test were randomized, alternating the side of emission, in order to minimize any possible time/position effect as well as the position of them on each side of the T-arena.

2.4.1 Experiment 1

Exp1a: Amplitude of the playback - The aim was to individuate an optimal range of signal amplitude among the tested values. The RP was set on one bean leaf and the SP on the other. Before each trial, the FS2 amplitude was measured, as substrate velocity (um/s), with laser vibrometer in two points: at 1 cm from the RP and 1 cm from the SP. Males were individually tested with randomized velocity (in a scale from 0 to 8000 µm/s, at the RP; see table 1 for detail) of FS2. As a control, we used males placed on the plant without any playback transmission (n=10). Each male was left on the plant for a maximum time of ten minutes. We counted the number of individuals that reached the source (i.e., the mini-shaker).

Exp1b: Continuity of the playback - The aim of this test was to assess whether interruptions of the FS2 affected (i) the motivation in males to express searching behavior and (ii) the accuracy of locating the SP. Therefore, FS2 was either played back in loop as continuous (without any interruption), or discontinuous, with a silent break of 11s between consecutive signals. A third silent treatment was used as a control. The analysis considered both video and audio recordings. Videos were used for measuring the number of “right” (towards the SP) and “wrong” (away from SP) choices along the bean plant in the direction of the SP. The number of MS1 and MS2 signals emitted by the males were also counted. Ten minutes were given as a maximum time.

Exp1c: Dominance of the harmonics - Since the final aim was to use the FS2 playback as attractant in field traps, it was important to assess whether any alteration of the frequency pattern (that can be caused by the different substrates, i.e., matters, size, shape, crossed by the FS2) could affect the male behavioral response. In particular, we created three different FS2 playbacks with variations of the first two harmonics (i.e., 76 and 152 Hz).FS2-76, had the first harmonic (76 Hz) as dominant; FS2-even had the first two harmonics of equal amplitude, by amplifying the second one; FS2-152 has been generated from FS2-even by applying a 20 dB reduction of the first harmonic (Table1; Fig. 3a-c). The arena used in this test was the cardstock arena described above. For each trial, all three signals were played in random order to each insect; each playback was turned on for 1 min and with 30s of silence in between. We counted the number of individuals that showed searching behavior, in correspondence or immediately after each playback.

Exp1d: Pulse repetition time - Exp1a tested the pulse emission rate, or pulse repetition time (PRT), of FS2 and how this variation can affect the male behavioral response. The PRT can be defined as the time between the onsets of two consecutive pulses. The values of PRT, fast and standard, were chosen based on the natural range reported by [28]. We called “FS2-fast” the signal with PRT around 1.0 s which was also used as a model in the previous tests, and we created a “FS-standard” by adding a 0.5 s silent gap between each pulse (PRT ~ 1.5s), which corresponds to the average parameter found in nature (Table1; Fig. 3d-e). Each version of FS2 was randomly played to a male with a 30 s break in between. The total time given to each male was 2 min. We counted the number of individuals that showed searching behavior, in correspondence or immediately after each playback.

2.4.2 Experiment 2

Exp2a: One-choice test - The goal of this test was to measure the accuracy of BMSB to reach the vibrational target males when stimulated with FS2 with optimal parameters (FS2-Best) (Fig. 3f) derived from Experiment 1 (see Table1) in the absence of other stimuli. We used the wooden T-stand arena (Fig. 2a) and we placed 2 mini-shakers (one on and one off) at the end of each of the outstretched arms, one of which was muted and served as a control. Only one of them, randomly chosen, was playing during each trial. The RP was set at the base of the T, at the opposite end from the arms (SP) and was receiving the signal at around 10 um/s in order to elicit the searching behavior of males. The signal increased towards the SP with the shaker arriving up to 2000 um/s (Fig. 2b). Each trial used one male and ran for up to 7 min with the playback playing in a loop. The analysis was based on whether or not the insect showed the searching behavior, moved towards or away from the SP and if it touched the functioning mini-shaker within the stipulated time.

Ex2b: Simple two-choice test - The purpose of this test was to assess if males search towards a preferred signal of FS2 when stimulated by two sources coming from two distinct directions, at the same time, with different spectral characteristics (i.e., the dominant frequency of the harmonics). We used the same setup of Exp2a, but this time both mini-shakers were turned on during the trial. We used the preferred signal FS2-76 that was confronted with FS2-152 (see in Results, Exp1c; Table1; Fig. 3a,c). Before each trial, the playback was switched between the two mini-shakers.

Exp2c: Complex two-choice test - We combined within the same FS2 optimal and non-optimal features (i.e., frequency and PRT) to further test males in two-choice tests. In a first set of trials, we compared two new FS2 in a two-choice test: FS2-Best (FS2-fast + FS2-76) vs FS2-Worst (FS2-standard + FS2-152) (Table1; Fig. 3f-g); in a second set of trials, we compared two suboptimal FS2 versions: FS2-sub1 (FS2-fast + FS2-152) and FS2-sub2 (FS2-standard + FS2-76). The latter set of trials aimed to establish which feature, between frequency and PRT, is more relevant in determining the male choice. Arena and protocols were the same as in Exp2b.

2.5 Data analysis

In Exp. 1a, we explored the effect of signal amplitude measured at the SP invelocity (0-8000μm/s) on the number of BMSB males reaching the mini-shaker with a generalized linear model (glm) with binomial distribution, with velocity (um/s) as explanatory variable. In order to better understand the minimum threshold of the response of BMSB to the signal, we also performed a polynomial regression with binomial distribution to the signal in the range of 0-400 μm/s.

For Exp. 1b (continuity of the signal emission) we used a non-parametric Kruskal-Wallis test comparing differences between treatment, continuous and discontinuous emission, and the silent control for the following parameters: (i) number of movements toward and away from the signal source, (ii) time spent by males in emitting MS1 and MS2, (iii) number of individuals that "search" and that reached the "target". When the test was significant, we applied a post hoc test (Dunn's-Test).

We explored the effect of signal frequency (76Hz, even, 152Hz) on insect responsiveness using a glm with binomial distribution, and frequency as explanatory variable (Exp. 1c). The same analysis was performed for signal speed ("fast" versus "standard" signal) on insect responsiveness with speed as explanatory variable (Exp. 1d).

For the choice tests of experiment 2, we analyzed the effect of the positioning of the signal source (one-choice tests) and of the specific signal source (see Table1 - two-choice tests) on the right or left arm of the T-arena on the number of active individuals that reached the target using G likelihood-ratio test, William's corrected.

We ran the models using packages 'lme4'[29] and "MASS"[30]. We checked the models for residual distribution using the 'car' package[31]. In case of model significance, we additionally performed Tukey test for pairwise comparisons by using the "emmeans" package[32]. Other non-parametric analyses were conducted with Package 'PMCMR'[33]. Figures were built using "seewave", "dyplr", "tidyr" and "ggplot" package. All the analyses were performed in R 3.5.3[34]. All data will be available upon request.

3. RESULTS

3.1 Experiment 1

Exp1a. Amplitude of the playback– As expected no males reached the mini-shaker in the silent control. Signal amplitude emitted by the shaker was a significant factor for the number of BMSB movements toward it (*z=2.373*, p<0.0176). According to the outcome of the glm, there was a significant positive correlation between male responsiveness and signal amplitude, with the best response between the velocity range of 100-200 µm/s, according to polynomial regression (*z=-3.259*, p<0.0011). Individuals were still responsive even at the highest values of amplitude tested (Fig. 4a; Table 2).

Exp1b. Continuity of the playback – Movements toward the signal source were significantly different between treatments (x^2=17.7, p=0.001), lower in the control compared to both the continuous (p=0.002) and discontinuous (p=0.0001) signals, with no differences between the latter (p=0.423) (Fig. 4b; Table 2). Similarly, movements away from the source were significantly different between treatments (x^2=18.56, p=0.001) for both the continuous (p=0.004) and discontinuous (p=0.0001) signals compared with the control (where no movements were observed), with no differences between the continuous and discontinuous (p=0.423). Moreover, no differences were observed among signals in time spent by BMSB in emitting MS1 (x^2=0.38, p=0.823), while MS2 was significantly longer in both continuous (x^2=20.501, p=0.001) and discontinuous (x^2=22.95, p=<0.001) signals compared with the control. The median MS2 time value in response to the continuous (18%) was far greater than in response to discontinuous signal (1.5%). In our experiment, signal continuity played no significant role in terms of number of males that performed "search" (p=0.337) and reached the "target (p=0.408).

Exp1c. Dominance of the harmonics – The best signal frequency in terms of insect

responsiveness was FS2-76 Hz which elicited more often searching behavior in males than FS2-152 (z=-2.478, p=0.0352). On the contrary, we did not find any significant difference between FS2-even and FS2-76 (z=-1.553, p=0.266) or 152 Hz (z=-1.110, p=0.508) (Fig. 4c; Table 2; Supplementary table 1).

Exp1d. Pulse repetition time – BMSB males responsiveness was significantly greater when stimulated with FS2-fast signal compared to FS2-standard (z=2.458, p=0.014) (Fig. 4d; Table 2; Supplementary table 1).

3.2 Validation of the T-shaped arena

Signal propagation tests revealed that most of the signal energy (>99%) dissipated immediately after the signal left the free arm with the active mini-shaker. Along this free arm, there was a clear gradient of amplitude, although the highest value does not necessarily coincide with the SP. The T-stem showed a rather homogenous pattern of amplitude values with amplitude never exceeding 1.0% of the SP, being the lowest values in correspondence with the central part (Fig. 2b) and increasing towards the edges of the T.

3.3 Experiment 2

Exp2a. One-choice test – According to experiment 1, we chose the standard values of the FS2-best parameters for stimulating searching in BMSB males: 100 µm/s of amplitude (as lower threshold of the best response) at 76 Hz peak frequency and 1.0 s PRT, transmitted continuously without any silent break. Twenty-eight out of 59 tested individuals were active, and 22 of these (78%) moved towards the active shaker (signal source) whereas only two moved towards the end with no signal and four did not reach any SP (G test: $G = 24.7$, $df = 2$ $p < 0.001$) (Table 2; Fig. 5a).

Exp2b. Simple two-choice test

FS2-76 vs FS2-156: Of the 24 active males out of 38 tested, 17 (71%) reached the FS2-76 shaker, a number significantly higher than the single male that moved toward the FS2-156 shaker and six that did not reach any SP (G-test: $G = 18.0$; $df = 2$; $p<0.001$) (Table 2; Fig. 5b).

Exp2c. Complex two-choice test

FS2-Best vs FS2-Worst: 20 out of 32 males tested were active. Of those, 70% (14/20) preferred FS2-Best (fast and low dominance frequency), which was significantly higher (G test: $G = 14.9$, $df = 2$; $p=0.001$) than those that reached the shaker emitting FS2-Worst (standard and high dominance frequency) (2/20) and those that did not reach any shaker in the given time (4/20) (Table 2; Fig. 5c).

FS2-Sub1 vs FS2-Sub2: 27 out of 48 males tested were active. We did not find a significant difference (G test: $G = 0.75$, $df = 2$; $p=0.33$) between SP-FS2-Sub1 (10 males), FS2-Sub2 (8 males) and males that did not reach any of the shakers (9 males) (Table 2; Fig. 5d).

4. DISCUSSION

Our study shows that BMSB males responsiveness to playbacks improved proportionally with amplitude, especially with a minimum threshold of 100 µm/s, a peak frequency associated to the first harmonic at 76 Hz and the pulse repetition time fast (1 s). Furthermore, a continuous playback emission did not increase the number of individuals reaching the target, but helped animals to localize it with fewer mistakes. As for the performance of the animals in the T stand arena, we found that males showed a significant orientation towards the female signal, which validates this method for future vibrotaxis studies.

In acoustic signaling systems, the amplitude of a signal can affect male mating success. Higher amplitude signals have greater broadcast distances, and females in many species prefer higher amplitude signals in playback tests[35–37]. For this reason, we first tested signal amplitude in order to establish a standard value of amplitude for the next trials. Signal amplitude strongly depends on the structure and architecture of the substrate through which the signal travels (i.e., a plant)[38]. Consequently, the active space of a species signal also depends on its amplitude[12], thus defining the range of the signal activity (i.e., efficacy to attract the males) to figure out the optimal interspace between attractive systems (e.g., traps). The results of these experiment showed that at low levels of amplitude, few individuals showed searching behavior, but starting from a minimum threshold of 100 µm/s, the behavioral response increased. We did not find a reduction of male responsiveness even at values between 10-20 dB higher. Previous studies with *Nezara viridula* (Hemiptera: Pentatomidae) proved that individuals were responding to playbacks up to 1000 µm/s of signal amplitude[39,40]. Here we found that males were positively reacting to playback up to 8000 µm/s. This is important information for applicative purposes, since it indicates that it will be possible to considerably increase the power of the trap vibration generator to amplify the active space without affecting the male response[40,41]. The next research step should be to further investigate this aspect in order to define the highest values of amplitude with which males could be stimulated without suffering any repelling/saturation effects. However, with this work we have not determined the actual level of the basal threshold of sensitivity. In our trials, although few individuals showed searching behavior at values below 100 µm/s, we believe that the signal should still be perceptible, just not enough to elicit a high searching behavior. We know, indeed, that the threshold curves calculated for neurons in response to vibratory signals in *N. viridula* indicate values around 10 µm/s as the lowest threshold[x42], and this value is suggested to be similar also in BMSB (A. Ibrahim unpublished data). We hypothesize that the male motivation can vary according to the perceived distance from the potential mate. In fact, animals that perceive a mating call from a long enough distance could decide not to search in order to remain inconspicuous to eavesdroppers (i.e., rivals and predators), while they are scanning the surroundings with sensors (chemical and mechanical) to gather more information[43–45]. A choice based on cost/benefit, when the cost of beginning to search outweighs the chance to find a mate[46]. Together with the risks, a distant signal also determines accuracy reduction and thus a waste of energy[43,47]. In this way, we can assume that a threshold of approximately 100 µm/s could be an acceptable and reliable amplitude value to start searching, as showed by the results of the polynomial regression. We must also consider that a natural female signal, measured from the same leaf of the female, was on average 460 μm/s[23]. In this way, values of 100-400 μm/s would presumably indicate to the male the presence of a female in close vicinity, whereas values at lower amplitude, even if perceived, would likely represent a more distant conspecific, thus a more difficult and risky target to reach. This hypothesis needs to be validated with more experiments, also using different substrates.

The second factor we tested was the duration of the playback, in particular the use of continuous and discontinuous signals. Although we have not found any differences between discontinuous and discontinuous with our trials, males may have a better vibrational contact with the sender and thus better directional accuracy with a continuous emission. Thus, we decided to use the continuous signal for the following experiments. Our results would also suggest that males are capable of obtaining directional information while walking and not only during the pauses. In the case of *N. viridula*, the directionality is given by the comparison of the signal simultaneously received from the legs standing at a fork point[48]. According to studies[40,49], the sensitivity of the sensory system of the species, to frequencies higher than 120 Hz, enables the males to detect the higher harmonics. The ability to detect those changes may serve as an orientation cue for the searching male. Furthermore, many species, including leafhoppers and treehoppers, have shown the capacity to correct their direction after choosing the wrong course[12,47,50]. Regarding signal emission by males during this part of the test, individuals exposed to the FS2 playback also emit MS2, which confirms the role of it as an interactive signal with the possible role of maintaining the female motivation to emit FS2 until finding each other[23].

The third analyzed signal feature was the peak frequency. Males were more attracted towards FS2 with fundamental and dominant frequency at 76 Hz, which also corresponds to the one emitted *in situ* by the species. On the contrary, they were significantly less motivated to search when exposed to the FS2-152 (2nd harmonic), while an intermediate result was observed when exposed to FS2-even (1st -2nd harmonics of identical amplitude). Unlike amplitude and continuity of emission, which are temporal (and thus quantitative) parameters, the frequency is a qualitative element. Results from a previous study with the glassy winged sharpshooter, *Homalodisca vitripennis*[10], showed that the shift of energy from the natural dominant harmonic to a different one, or simply the use of two dominant harmonics of identical amplitude, determined a significant reduction of male responsiveness. This observation is important when thinking about the use of mechanical devices to transmit the attractive signal from a constructed trap. In vibrational signals, the transmission of seismic and bending waves is frequency dependent and higher frequencies tend to dissipate before the lower ones[36]□. However, the construction of a trap must consider materials, shapes and position in the site. Underestimating the importance of producing undesired harmonics could make the difference between a successful device and a failure. For this reason, particular attention should be given to both the design of the signal generator (i.e., the shaker) and to the coupling effects between the device and the overall system[51–53].

Finally, the fourth feature that we tested was the pulse repetition time of FS2. We tested the fastest and slowest PRT among those registered inside the natural range of the species[23], and we found a clear prevalence of male responsiveness when they were stimulated with the FS2-Fast. In insects, the signal (i.e., pulses, chirp etc.) emission rate can be associated to different physiological parameters such as the age[54], the size[55] and the nutritional condition[56], therefore it could be an important element to evaluate fitness. Most studies of signal preference and attractiveness focus mainly on the female's choice, while there is less literature about the male's choice and the role of the signal emission rate. Our research indicates that males can also show choosiness since they preferred a fast signal that probably could be associated to a female of higher quality. However, we cannot exclude a reduction of recognition due to the alteration of the signal.

In experiment 2, we found that males exposed to single and double choice tests in the T arena showed a significant orientation towards the female signal. During the single choice test, most of the males exposed to FS2-Best reached the SP. Indeed, several behavioral studies have demonstrated the ability of insects to localize exactly the source of vibrations[44], including Pentatomidae, for either prey location[57] or mate location[58]. The precedent paper[59] demonstrated that the BMSB can also localize the source of an attractive signal but, in addition, in the present study we demonstrated that males can also distinguish between two sources that are transmitting signals with different spectral/temporal features. The "simple" two-choice test gave evidence that males can distinguish between two different types of FS2, when their qualitative difference is enough. This means that males still reached the supposedly preferred FS2, composed of low frequency and high repetition rate (FS2-Best) both in test of "simple" (only one manipulated parameter) and "complex" two-choice (two parameters). However, when "good" and "bad" characters (FS-Sub1 and FS-Sub2) were mixed in additional signals, males did not show a preference and the choices were split between the two SPs. This result can possibly indicate that there is not a hierarchy of parameters and would suggest that frequency and PRT equally affect the FS2 attractiveness to males.

The present study also indicated the importance of using a T stand arena as a powerful tool to study and select which parameters of the vibrational signal are the most efficient in triggering an insect behavior. Indeed, when we switched the stimulation point from one arm to the other of the T-stand arena the male responses remained consistent. In the arena designed, the signal enhances on the two edges towards the shaker (SP) and reduces drastically towards the (RP), so even in two choice test, when emitting signals simultaneously, orientation of males was towards the shaker. In a previous study with *H. halys*[11], the authors mentioned that in general, males were able to localize the stimulation points both on plants and in artificial arenas. Authors assumed that time delay was the cue they used, basing their orientation either amplitude difference[7] or time delay[60]. Furthermore, the plywood, material used for the arena, permitted the emission of the chosen signal with quality, without losing the important parameters that trigger searching in males. Moreover, the application of this setup can be extended to several other species, even adapting its shape and size depending on the needs. The number of potential species testable with a T-arena is large when considering that many insects communicate by means of vibrational signals and of those that use mechanical channels. In particular, among these insects, 80% use vibrational signals alone or in combination with other mechanical signals, and 74% use vibrational signals alone[9].

In conclusion, since animals respond to combinations of signal values that they find attractive[61] we identified some of the features that most elicit the activation of searching behavior in BMSB males. In order to trap insects, it is fundamental to have a signal that not only mimics the natural FS2 emitted by females to sexually attract males, but also indicates a high fitness quality of the artificial calling female. FS2, as well as other vibrational signals, is a multicomponent signal[62] characterized by a range of features in terms of temporal and spectral parameters. The range of variability depends on numerous factors that can be related to the female physiological status (i.e., age, health, etc.) and to the environmental conditions (e.g. temperature and relative humidity). During our experiments, we used all the values reported in the natural range of the BMSB females[11] to ensure the signal compatibility recognition within the thresholds of acceptability by the tested males[61]. In fact, in all the experiments, the exposure of males to our playbacks elicited the response (i.e., searching behavior and or MS2 emission) at least in a part of the individuals that showed a clear preference for certain characteristics. In the present work, we created an "optimal" attractive signal, given by multiple components that interact with each other thus positively affecting the receiver's response[63,64]. Giving the strong directional orientation of males towards the FS2 source, which was clearly demonstrated with the trials conducted on the T-arena, we consider this study to be an important element for the development of new concept traps for BMSB, and furthermore in synergy with the pheromones already available for the commercial devices. The current pheromone traps guide insects towards the surrounding area, many times not succeeding in making the animals come inside the trap. We suggest the combination of long distance pheromones attraction with short distance vibrations (guiding individuals inside the trap) as an interesting design for atrap for *Halyomorpha halys* pest control. Further experiments will be realized in future works to correlate the BMSB female's physiology (i.e., age, health, size, mating status, etc.) and spatial location with signal variability and preferences

5. ACKNOWLEDGEMENTS

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Table 1: Summary of tests and parameters used in each experiment.

	Test	Tested parameter	Type of arena	Sample size	Variations of FS2
Exp 1	**a. Amplitude of the playback**	Amplitude of the signal measured as substrate velocity (µm/s)	Bean plant	42 males	● FS2 (0-8000 µm/s); ● Control – silence
	b. Continuity of the playback	Continuity of the signal (with or without silent breaks)	Bean plant	20 males	● FS2-continuous – FS2 played in loop with no breaks; ● FS2-discontinuous – 11.5s FS2-2 playback with breaks of 11s. of silence; ● Control – silence
	c. Dominance of the harmonics	Dominant frequency (Hz)	Round arena	20 males	● FS2-76 – peak of frequency at the 1^{st} harmonic (76 Hz) ● FS2-even – 1^{st} and 2^{nd} harmonic equally important; ● FS2-152 – peak of frequency at the 2^{nd} harmonic (152Hz)

	d. Pulse repetition time	PRT (s)	Round arena	50 males	• FS2-fast – PRT at 1.0s; • FS2-standard – PRT at 1.5s
Exp 2	**a. One-choice test**	Optimal Exp1 features (a, b, c, d)	T arena	59 males	• FS2-Best – FS2 continuous play, 76Hz and 1s PRT
	b. Simple two-choice test	Dominant frequency (Hz)	T arena	38 males	• FS2-76 – peak of frequency at the 1st harmonic (76 Hz); • FS2-152 – peak of frequency at the 2nd harmonic (152Hz);
	c. Complex two-choice test	Dominant frequency (Hz) and PRT (s)	T arena	80 males	• FS2-Best – FS2 with 100-150 µm/s, continuous play, 76Hz and fast PRT • FS2-Worst – FS2 with 100-150 µm/s, continuous play, 152Hz and standard PRT • FS2-Sub1 – FS2 with 100-150 µm/s, continuous play, 76Hz and standardPRT • FS2-Sub2 – FS2 with 100-150 µm/s, continuous play, 152Hz and fast PRT

Table 2: Summary of the results found in this study.

Test	**Results**
Exp1a. Intensity of the playback	Significant positive correlation between male responsiveness and signal intensity, with the best response between 100-200µm/s.
Exp1b. Continuity of the playback	FS-continuous helps with decision making: males make less mistakes.
Exp1c. Dominance of the harmonics	Male searching is more elicited during FS2-76.
Exp1d. Pulse repetition time	Males preferred FS2-fast – FS2 with PRT 1.0s
Exp2a. One-choice test	Males can easily find a source of a FS2 signal (FS2-Best) on a T-stand arena
Exp2b. Simple Two-choice test	Males preferred FS2-76 more than the altered one (FS2-152).
Exp2c. Complex Two-choice test	Males preferred FS2-Best that FS2-Worst. Males did not show a preference when exposed to FS2 with

	mixed good and bad features (FS2-sub2 vs FS2-sub3).

Supplementary table 1. Estimated regression coefficients, standard errors, and confidence intervals for GLM of variable response to vibratory playback stimuli. The reference category in Dominance of harmonic experiment was 152Hz and for the PRT experiment was Fast playback.

Experiment	**Playback**	**Estimate**	**Std. Error**	**Z value**	**P value**
Dominance of harmonic	*Intercept*	-1.79	0.623	-2.873	0.004
	Signal 76Hz	1.88	0.761	2.478	0.01
	Signal even	0.87	0.788	1.110	0.267
PRT	Intercept	1.20	0.658	1.829	0.067
	Standard	-2.30	0.936	-2.458	0.014

8. FIGURE CAPTIONS

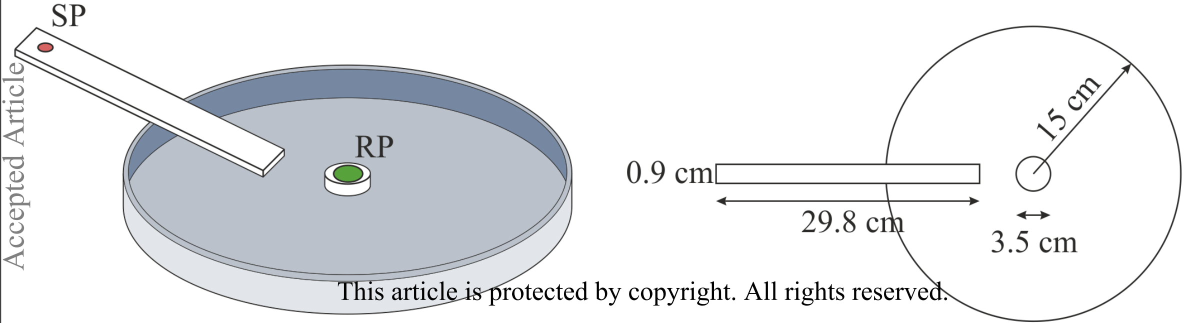
Fig.1 Cardstock arena used for the Exp1c: dominance of the harmonics and Exp1d: pulse repetition time (PRT)

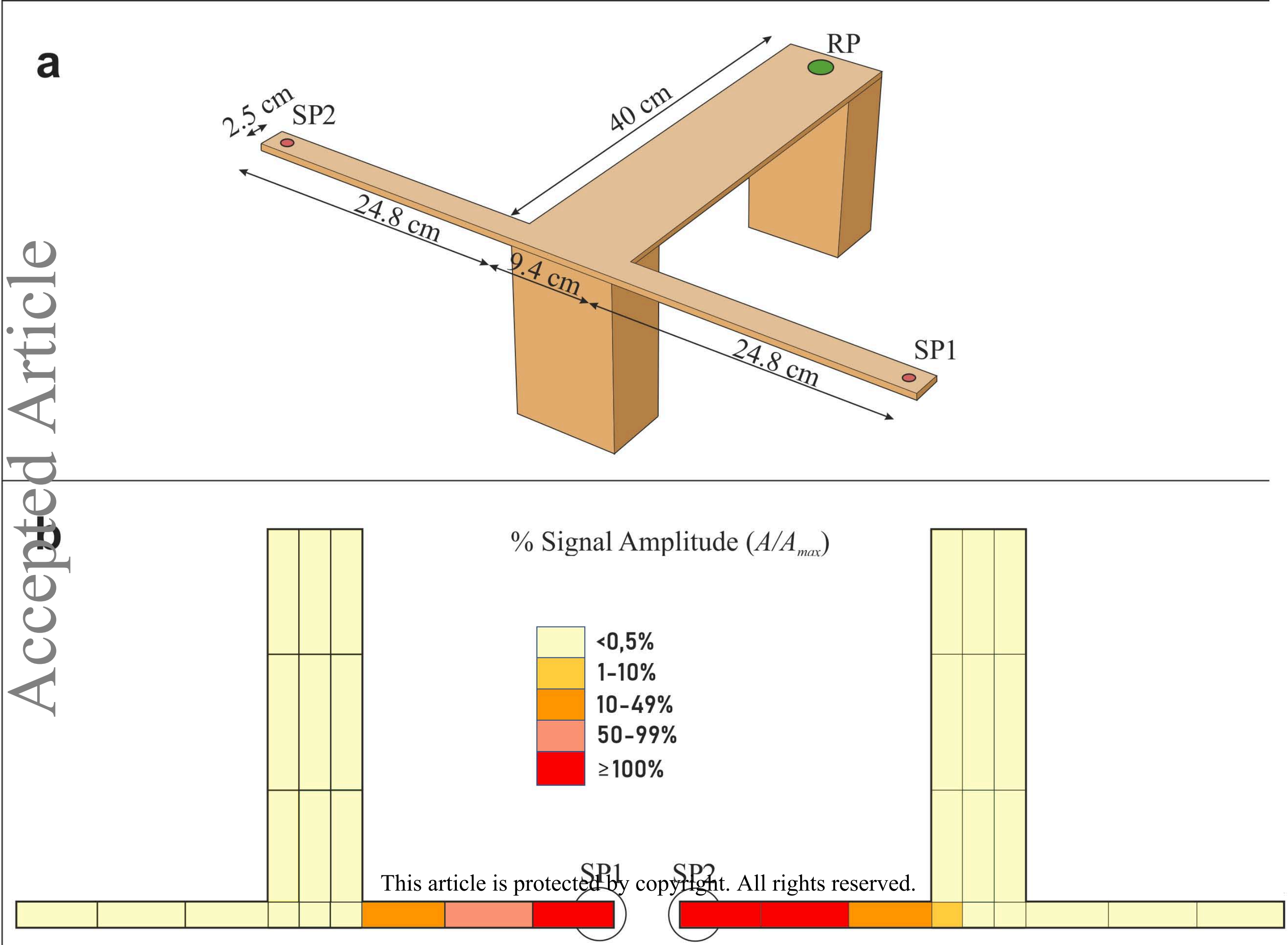
Fig.2 a) Scheme of the plywood T-shaped arena with dimensions; the thickness of the lateral arms is 0.4 cm. The green circle shows the release point (RP), while the red circles identify the stimulation points (SP). b) Results of the signal amplitude propagation by changing the source of the stimulus. The values are normalized by the maximum amplitude recorded on the arena

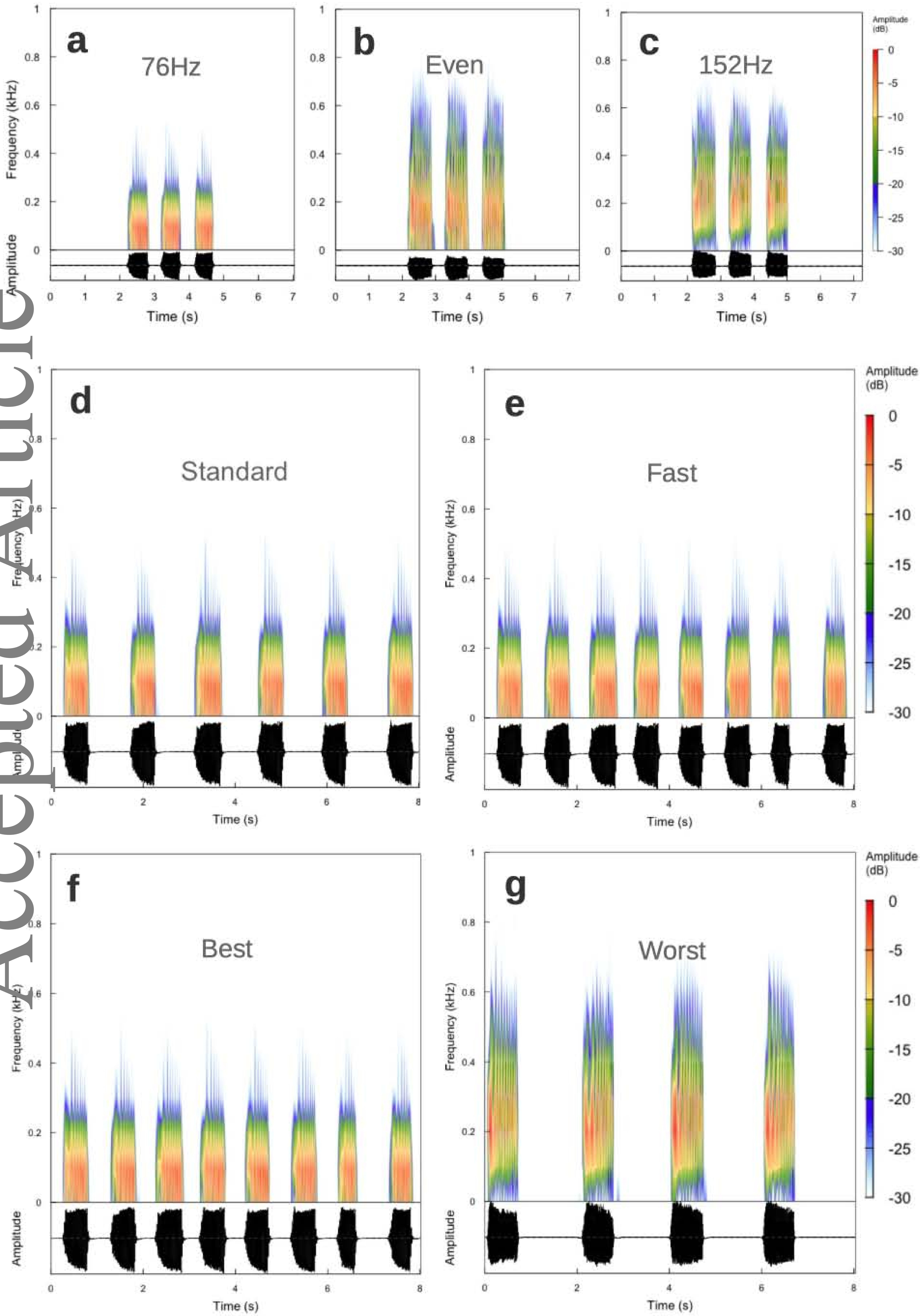
Fig.3 Spectrograms (top) and oscillograms (bottom) of the FS2 signals used in playback experiments. (a) FS2-76 – peak of frequency at the 1st harmonic (76 Hz), (b) FS2-even – 1st and 2nd harmonic are equal; (c) FS2-152 – peak of frequency at the 2nd harmonic (152Hz), (d) FS2-standard – PRT at 1.5;(e) FS2-fast – PRT at 1.0s; standard (f) S2-Best – FS2 with 100-150 µm/s, continuous play, 76Hz and 1s PRT; (g) FS2-Worst – FS2 with 100-150 µm/s, continuous play, 152Hz and 1.5s PRT

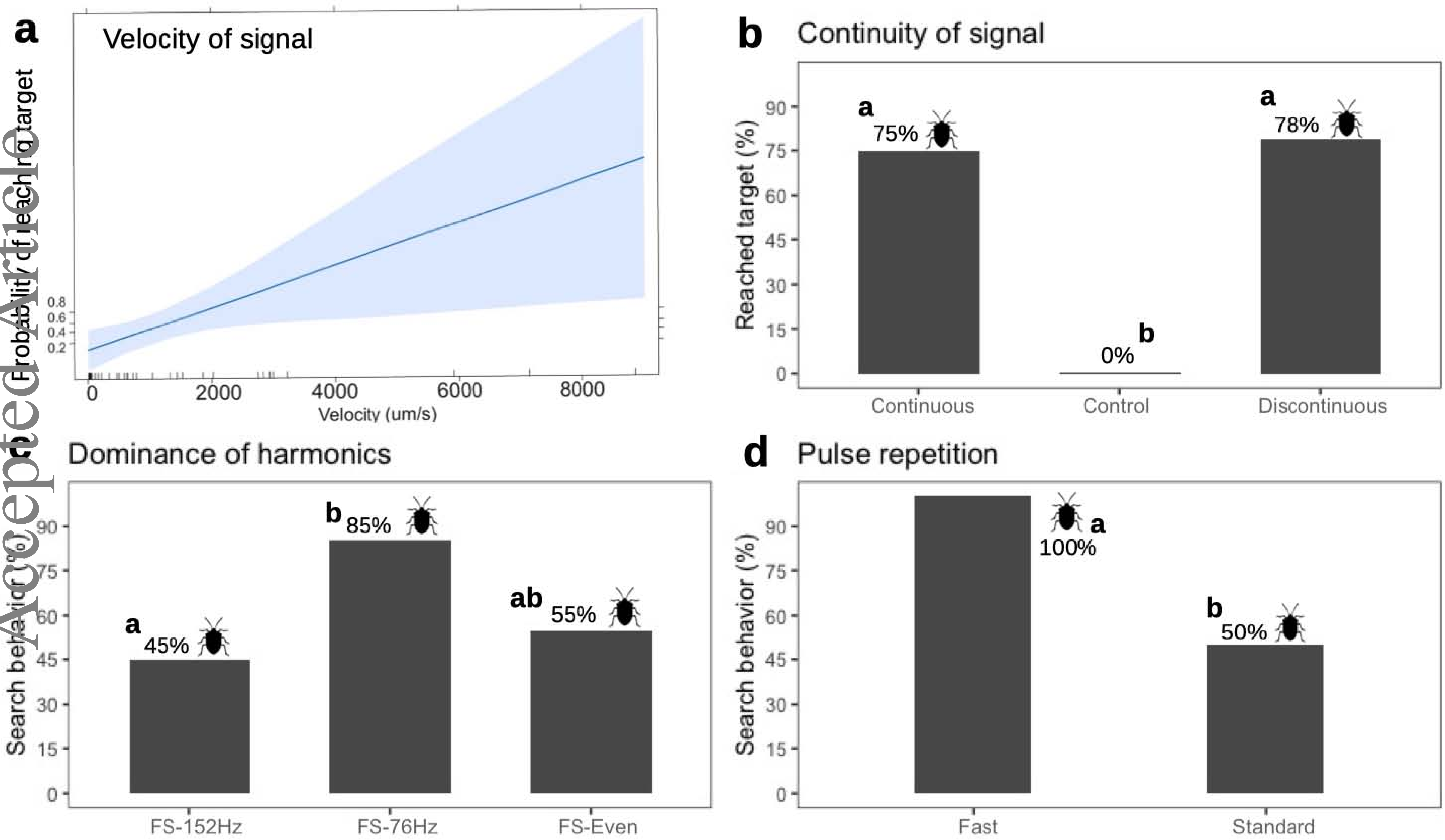
Fig.4 Experiment I: individual behavior according to different signal parameters tested (a) GLM model of the response of individuals to the amplitude of playback (µm/s) with confidence interval (blue shade) confidence interval; (b) Continuity of signal emission; (b) Dominance of harmonics; (d) Pulse repetition time of signal. Silhouette of BMSB next to the percentage represents treatments in which individuals moved towards the emission of a signal. Letters (a-b) represent significant differences between treatments for each parameter tested.

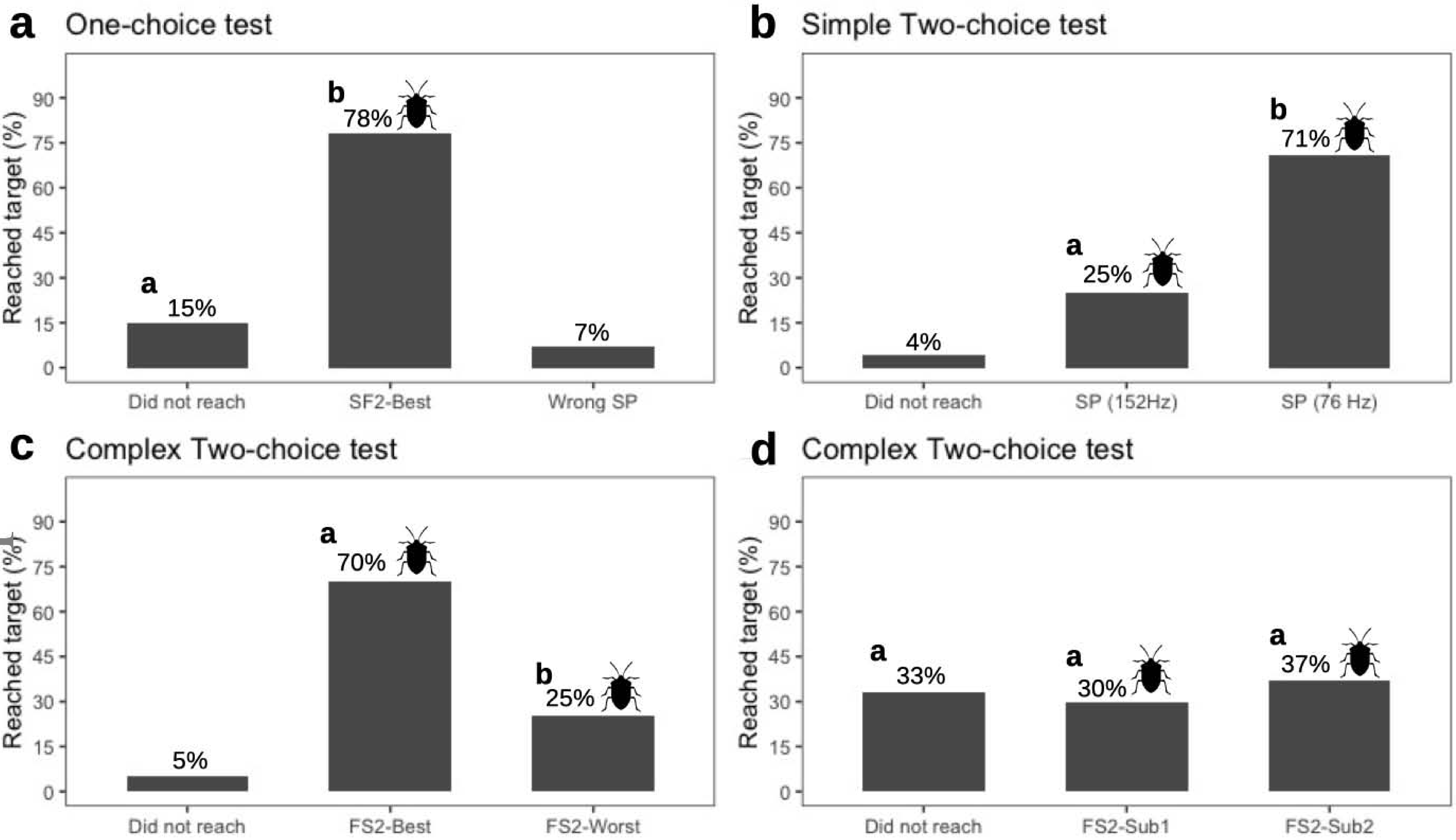
Fig.5 Experiment II: percentage of individuals that arrived to the signal emission target in each of the experiments


SP
RP
0.9 cm
29.8 cm
15 cm
3.5 cm


a
RP
SP2
2.5 cm
40 cm
24.8 cm
9.4 cm
24.8 cm
SP1
b
% Signal Amplitude (A/A_max)
<0,5%
1-10%
10-49%
50-99%
≥100%
SP1
SP2


a
76Hz
b
Even
c
152Hz
d
Standard
e
Fast
f
Best
g
Worst
Frequency (kHz)
Amplitude
Time (s)
Amplitude (dB)


a
Velocity of signal
Probability of reaching target
0.8
0.6
0.4
0.2
0
2000
4000
6000
8000
Velocity (um/s)
b
Continuity of signal
Reached target (%)
90
75
60
45
30
15
0
a
75%
0%
b
a
78%
Continuous
Control
Discontinuous
Dominance of harmonics
Search behavior (%)
a
45%
b
85%
ab
55%
FS-152Hz
FS-76Hz
FS-Even
d
Pulse repetition
Search behavior (%)
100%
a
b
50%
Fast
Standard



a One-choice test
Reached target (%)
90
75
60
45
30
15
0
a
15%
b
78%
7%
Did not reach
SF2-Best
Wrong SP
b Simple Two-choice test
Reached target (%)
4%
a
25%
b
71%
Did not reach
SP (152Hz)
SP (76 Hz)
c Complex Two-choice test
Reached target (%)
5%
a
70%
b
25%
Did not reach
FS2-Best
FS2-Worst
d Complex Two-choice test
Reached target (%)
a
33%
a
30%
a
37%
Did not reach
FS2-Sub1
FS2-Sub2