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The role of signals of different modalities in initiating vibratory communication in *Nezara viridula*

Research Article

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Abstract: Signals of different modalities are involved in the behaviour of the green stink bug, *Nezara viridula* (L.) (Pentatomidae, Heteroptera). Long range attraction is mediated by male pheromones, resulting in aggregation of bugs on the same plant where vibratory signals, vision and various chemical signals become important. Both males and females sing spontaneously. When both are on the plant, males start vibratory communication as often as females. Females induce the exchange of vibratory signals spontaneously or triggered by the male pheromone while males initiate the duet either spontaneously or after seeing the female. Males and females sing spontaneously and respond to signals of different modalities more often in the daylight than in the dark. Long lasting autonomous emission of the female calling song is present when triggered by the male pheromone and males respond to female calling predominantly by the emission of the courtship song.

Keywords: *Vibrational communication • Pheromones • Mating behaviour • Vibrations • Nezara • Spontaneous singing • Green stink bug • Pentatomidae • Heteroptera • Trigger*

1. Introduction

The green stink bug *Nezara viridula* L. is a cosmopolitan, highly polyphagous pest of economic importance for soybean and cotton, and other agriculture crops like tomatoes, potatoes, many legumes and cereals [1]. Its biology has been described in detail by Todd [1] and Panizzi [2].

This species' mating behaviour has been studied in several geographically isolated populations [3-7] and consists of two phases: long range calling and short range courtship behaviour. At long range, male pheromones act as an attractant that brings adults and last stage nymphs together [4,5,8-10]. The composition of the male pheromone has been identified in several populations and the main pheromone components have been synthesized [5,8,9]. Differences between populations are reflected in the different *trans* to *cis* ratios of Z-α-bisabolene epoxide (BAE), the main compound in the pheromone blend [11].

At close range, signals of other modalities become important. While pheromone extracts alone do not elicit short-range courtship behaviour (defined as "butting" and "pivoting") in females [5], pheromones do elicit vibrational communication [12], which enables mate location on the plant [13] and recognition [14]. In *N. viridula* Čokl and co-workers [15] described species specific female and male calling songs, FCS and MCS, and courtship songs, FCrS and MCrS, together with the female repellent (FRS) and male rival (MRS) songs. Temporal characteristics of calling songs differ between geographically isolated populations [7].

Although mating behaviour and communication in *N. viridula* have been described in numerous studies, the question as to which signals trigger vibrational communication remains open. To reveal the role of each modality in triggering communication with vibratory signals we examined the effects of chemical, mechanical (vibrational) and optical (visual) signals. Our hypothesis was that male singing is triggered by females' vibratory and visual stimuli and female singing by males' pheromones. Three different tests were assembled: to reveal the role of visual cues, a dead body of a green stink bug was used as a visual stimulus. Vibrational stimuli were tested

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by playback experiments with song from conspecifics of the opposite sex. Finally different chemical stimuli were tested: conspecific male-emitted odours (from live stimulus male and trapped on filter paper) and synthetic male pheromone (bisabolene epoxide – BAE).

2. Experimental Procedures

2.1 Animals

All experiments were conducted on a laboratory grown adult stinkbug *Nezara viridula* L. (Heteroptera: Pentatomidae) colony that originated from the wild population on the North Adriatic coast of Slovenia. The latter were collected in late summer and autumn (August to October) and kept separated by gender in the laboratory in 30 x 40 x 25 cm terrariums at 8°C in the dark for one to four months. After diapause, animals were kept separated by gender under 16/8 light daily cycle, 22-25°C temperature and 30-60% relative humidity (RH) on a diet of fresh green beans (*Phaseolus vulgaris* L.), small green bean plants, dwarf bean shoots, raw peanuts (*Arachis hypogaea*) and sunflower seeds (*Helianthus annuus*). Laboratory grown populations of the first and second generations were reared under the same conditions in glass boxes (38 x 25 x 25 cm) covered by mesh. Experiments were conducted with naive laboratory reared adults separated by gender one day after the final moult and with wild adult bugs after diapausing under laboratory conditions (see above). To ensure sexual maturity we used laboratory reared males at least 10-14 and females 14-20 days after their final moult [16]. Experimental animals were kept individually in plastic cups (10 cm diameter at the top, 6 cm at the bottom and 14 cm high) provided with the same food in the same conditions as described above.

2.2 Plants

All experiments were conducted on fresh bean plants (*Phaseolus vulgaris* L., var. *etna*) kept at 22°C, 30-60% RH and 16/8 daylight cycle. Bean plants were individually potted in peat soil in plastic cups. For experiments we used 20 – 40 cm high plants with two leaves (5 to 8 cm wide and of about the same length) on each side branching from the stalk on 2 cm long stalks and the central 3-7 cm stalk with two smaller leaves at the top (Figure 1). Plants were kept away from reared bugs to prevent any contact with them before the experiments and a new, individually potted seedling of bean plant was used for each test.

2.3 Signal recording and analysis

All experiments were conducted in the laboratories of the Department of Entomology of the National Institute of Biology in Ljubljana between 9 am and 5 pm (2 to 10h after start of photo phase). Vibrations were recorded for 13 min. from the bean stalks (2 to 4 cm above the soil) by a laser vibrometer (controller 2200-L, sensor head OFV-353, Polytec GmbH, Waldbronn, Germany). For better reflection a 2 mm^2 piece of reflecting tape was fixed at the recording point on the stalk. Recorded vibrations were digitized by Sound Blaster Audigy 4 sound card (Creative Labs Inc., Singapore) and stored in the computer using Cool Edit Pro 2 (Syntrillium Software, Phoenix, AZ, USA) software at sample rate of 44,100 Hz, mono channel and 16 bit resolution. Digitized and stored signals were analyzed by Sound Forge, version 6.0 (Sonic Foundry, Inc. Madison, Ca, USA) software. Behaviour of stink bugs on a plant was analyzed from recordings obtained using a digital camera (GR-DV4000E, JVC, USA).

Communication signals were described as pulses, defined as unitary homogenous parcels of vibrations of finite duration [17], and the term pulse train is used when pulses were arranged into repeatable and temporally distinct groups.

2.4 Experiments with pairs of bugs

Each insect was tested only once per day. The experiment started with a single male or female placed alone on a stalk of a plant. After 5 minutes (control) a

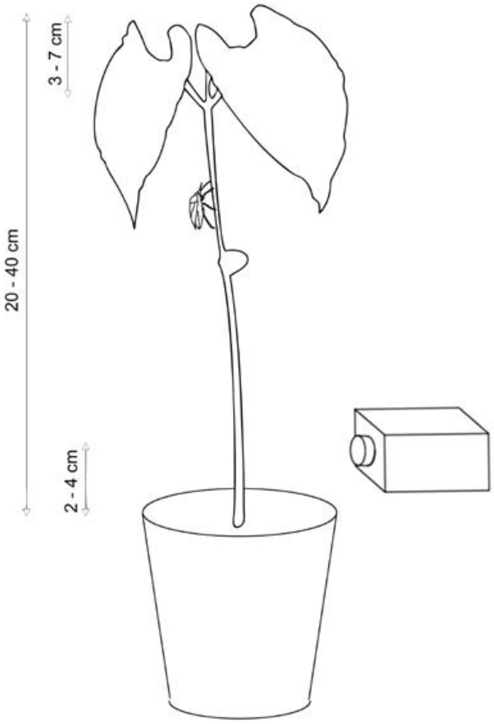

Figure 1. Structure and dimensions of a bean plant (*Phaseoulus vulgaris*) used in experiments and position of laser vibrometer.

bug of the opposite sex was added on the stalk. The experiment was terminated after 25 minutes or just before attempted copulation, to preserve bugs' virginity. In another experiment we omitted the 5 minute control and placed a male and a female at the same time (*i.e.* within a minute) on the opposite leaves. The experiment was terminated after 13 minutes or when the bugs started to sing.

2.5 Experiments with a single animal

A single bug was placed on the plant's stalk and after 1-2 minutes stimuli of different modalities were provided.

2.5.1 Playback experiments

Playback experiments were carried out on a plant (see above) with a vibration exciter (Minishaker 181 type 4810, Brüel & Kjaer, Naerum, Denmark) in touch with the stalk of the plant. A stimulation program was played for 13 minutes.

The stimulation programs consisted of natural female calling (FCS) and male courtship songs (MCrS) pre-recorded by the laser vibrometer (see above) from bugs singing on a low-middle frequency loudspeaker membrane (Conrad Electronic, Germany, impedance 8 Ohm, 4.5 mm diameter, 50–2,000 Hz). Signals were stored on a computer by the sound card (see above) with Cool Edit Pro (see above) software at a sampling rate of 44,100 Hz. Stimulation sequences were assembled from songs of 5 different males or females from a Slovene population with Cool Edit Pro software. The mean duration of stimulus FCS pulse trains was 538.1 ± 25.6 ms, dominant frequency 119.8 ± 1.5 Hz and repetition time (time between onsets of two consecutive signals) 2,559 ± 384 ms, calculated from 62 analyzed pulses of FCS stimulation sequence (Figure 2). The dominant frequency of the MCrS stimulation sequence, analyzed from 64 pulses, was 127.7 ± 7.2 Hz, pulse train duration 2,109 ± 415 ms and repetition time 9,548 ± 2,325 ms. Figure 2 shows a typical pulse train of FCS and MCrS and a section of the stimulation sequences.

Stimulation signals reproduced by vibration exciter were recorded simultaneously with vibrations produced as response.

2.5.2 Experiments with a model

As a model we used live or dead conspecific insects. In the latter case the body of a dead green stink bug was washed with ethanol to remove any odour, glued to a stick and presented to the tested insect at a 2-4 cm air distance. In tests with live bugs as the visual model a female was allowed to climb on a stick that was shown at 2-4 cm distance to the tested bug. In both cases tested bugs were not allowed to touch the model and were not in contact with it over the plant's surface. Vibrations of stimulated bugs were recorded for 13 minutes.

2.5.3 Chemical stimulus experiments

Experiments that excluded visual contact were carried out in the 'dark' under a red light ($\lambda > 610$ nm, $\lambda_{50} = 650$ nm). A bug was placed on a bean plant as in other experiments, left there to acclimate for 1-2 minutes and then stimulated with a filter paper or a live bug in a small cage at a distance 2-4 cm from the bug. The experiment was terminated 13 minutes after applying the stimulus.

Experiments with natural pheromones:

Male in the cage: a small wire mesh cage (4 cm high cylinder, 2.5 cm in diameter) was used to keep a male at the desired distance and to prevent tactile contact and vibrational transmission. Different males were used in the cage for this experiment to minimize the effect of individual variability in pheromone composition, production and release.

Filter paper with male odour: 4-5 male bugs were left for 24 hours on a filter paper to collect their odours. The paper was presented to a male or a female on a plant. The filter paper was placed over air 2-4 cm from the tested animal. The experiment was conducted with several filter papers with odours collected from different males: every day 4-5 males from the laboratory colony were left on a piece of filter paper in a large petri dish for 24 hours and this filter paper was used the next day. The next day other males were used to collect odour.

Experiments with synthetic pheromone:

We used (*Z*)-α-bisabolen epoxide blends with *trans*:*cis* ratios of 3:1, 1.4:1 and 1:1 in n-hexane, at doses from 0.031 to 0.04 μg mL^{-1}, *i.e.* 8-9% of the daily emitted

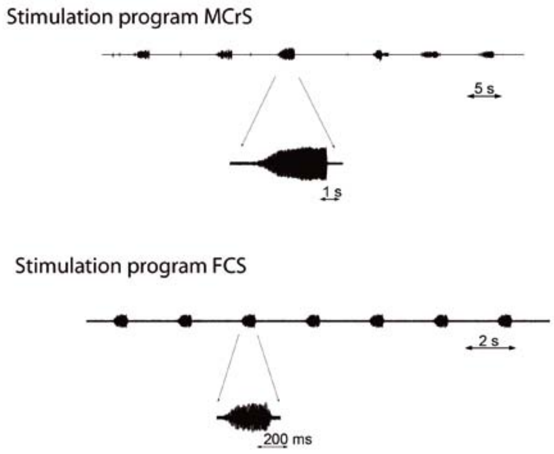

Figure 2. A section of a male courtship song (MCrS) sequence and a typical male courtship song pulse train (above) and part of a female calling song (FCS) sequence with the female calling song pulse enlarged (below) shown in an oscillogram.

dose of an adult male [18]. In order to avoid possible contamination of apparatus, 100 μL of pheromone blend (or pure hexane in control experiments) were dropped onto a 3 x 4 cm piece of filter paper that was presented to the bug on a plant in the same manner as filter paper with male odour (see above). The same filter paper was used for a whole day but the plant and the bug were replaced every time. N-Hexane was used since it is less volatile than other possible solvents.

Controls and spontaneous singing: controls comprised an empty cage, fresh filter paper (in both dark and daylight) and filter paper with pure solvent (n-hexane). Control tests lasted 13 minutes. Controls without any stimuli were also conducted, both in the dark (13 minutes) and in daylight (13 and 5 minutes). These controls provided a level of spontaneous singing.

2.6 Statistical methods

Statistical analysis and graphs were constructed in R (A Language and Environment for Statistical Computing, http://cran.r-project.org/). The χ^2 test was used to compare experiments and controls. Comparisons between different controls were also tested. Bonferroni correction was used where appropriate. The single binomial probability test was used to assess the difference in proportion of males and females initiating the duet in experiments with pairs of bugs. The use of ‘significant’ in such contexts should be taken to signify ‘statistically significant’.

3. Results

Three types of experiments were conducted to reveal the role of signals of different modalities in triggering vibrational communication in *Nezara viridula*. In the first (3.1), singing was monitored in the absence of any applied external stimulus. In the second (3.2), singing of a couple was recorded and analyzed to determine which member started the vibratory communication. In the third (3.3), a single male or female was stimulated by vibratory, visual or chemical signals, in order to identify the stimulus that triggers the emission of the calling or courtship songs. Results of these tests are reported for females (Table 1), males (Table 2) and for males and females combined (Figure 3).

treatment /control	N	control / 2nd treatment	N	χ^2 test	df	P	stat. signif.
stimulation - MCrS	11	control - daylight	41	0.0602	1	0.8062	NS
live f	10	control - daylight	41	0.0171	1	0.896	NS
model - dead m	17	control - daylight	41	0.0600	1	0.8065	NS
m in cage-daylight	17	m in cage-dark	29	0.4234	1	0.5153	NS
m in cage-dark	29	control - empty cage	10	18.1627	1	2,028 e-5	****
m in cage-dark	29	control - dark	14	19.1841	1	1.19e-5	****
fp with male odour	21	control - fresh fp	20	12.3295	1	0.0004459	***
pheromone 4μg BAE(3:1)	14	control - fresh fp	20	5.0891	1	0.02408	*
pheromone 3.1μg BAE(1,4:1)	22	control - fresh fp	20	11.4665	1	0.0007086	***
pheromone 3.3μg BAE(1:1)	17	control - fresh fp	20	8.4158	1	0.00372	**
pheromone 4μg BAE(3:1)	14	control - hexane	14	0.6550	1	0.4183	NS
pheromone 3.1μg BAE(1.4:1)	22	control - hexane	14	3.5080	1	0.06107	NS
pheromone 3.3μg BAE(1:1)	17	control - hexane	14	2.0225	1	0.155	NS
control - daylight	41	control - dark	14	0.0007	1	0.9784	NS
control - empty cage	10	control - dark	14	0.0298	1	0.8629	NS
control - fresh fp	20	control - dark	14	0.2296	1	0.6318	NS
control - hexane	14	control - fresh fp	20	0.8510	1	0.3563	NS

Table 1. Statistical comparison of treatments and controls on females (f): tests are compared with the most relevant controls, the two tests (male (m) in cage in the dark and in daylight) are compared and various controls are tested for differences. N is number of tests/controls; Chi-square values (χ^2 test), degrees of freedom (df) and p-value (P) for χ^2 test are presented and statistically significant difference is shown by *; NS stands for “not significant”. Stimulation - MCrS = stimulation with male courtship song, fp= filter paper, BAE = Z-α-bisabolene epoxide, ratio of *trans:cis* isomers is stated in brackets.

treatment	N	control / 2nd treatment	N	χ^2 test	df	p	stat. signif.
stimulation - FCS	29	control - daylight	25	12.4616	1	0.00042	***
live f -daylight	13	control - daylight	25	7.7757	1	0.0053	**
live f -daylight	13	live f - dark	14	8.3244	1	0.00391	**
model	30	control - daylight	25	0.0374	1	0.8466	NS
model - dead m	17	control - daylight	25	0.2001	1	0.6546	NS
model - dead m	17	model - dead f	13	0.1034	1	0.75	NS
model - dead f	13	control - daylight	25	0.1147	1	0.7349	NS
live m -daylight	11	control - daylight	25	0.0023	1	0.9614	NS
m in cage -daylight	12	control - daylight	25	0.477	1	0.4898	NS
m in cage -daylight	12	m in cage-dark	21	0.1031	1	0.7481	NS
m in cage-dark	21	control - empty cage	10	0.1794	1	0.6719	NS
fp with male odour	12	control - fresh fp	6	1.0045	1	0.3162	NS
pheromone 4μg BAE(3:1)	12	control - fresh fp	6	/	1	/	NS
pheromone 3.1μg BAE(1,4:1)	11	control - fresh fp	6	0.1006	1	0.7511	NS
pheromone 4μg BAE(3:1)	12	control - dark	14	1.1865	1	0.276	NS
pheromone 3.1μg BAE(1,4:1)	11	control - dark	14	0.0817	1	0.775	NS
control - daylight	25	control - dark	14	0.0455	1	0.8311	NS
control - empty cage	10	control - dark	14	0.1805	1	0.671	NS
control - fresh fp	6	control - dark	14	0.2988	1	0.5846	NS

Table 2. Statistical comparison of treatments and controls in males: tests are compared with the most relevant controls, the two tests (male in cage in the dark and in daylight) are compared and various controls are tested for differences. N is number of tests/controls; Chi-square values (χ^2 test), degrees of freedom (df) and p-value (P) for χ^2 square test are presented and statistically significant difference is shown by *; NS stands for "not significant". Stimulation - FCS = stimulation with female calling song, fp= filter paper, BAE = Z-α-bisabolene epoxide, ratio of *trans:cis* isomers is stated in brackets.

3.1 Spontaneous singing

The level of spontaneous emission of vibratory songs was determined by placing a male or female alone on a plant for 13 minutes in daylight or in the dark. In the absence of stimuli, females started to emit the calling song in 12.2% of all cases in daylight (N=41) and in 7.1% in the dark (N=14) (χ^2-test, P=0.98, Table 1). The second female song (FCrS) was emitted by just one female singing spontaneously in the dark.

The level of spontaneous singing was higher in males. In 13 minute experiments, males started to sing spontaneously in 24% of cases (N=25) in daylight and in 21.4% (N=14) in the dark (χ^2-test, P=0.83). In daylight two males emitted MCS, one a transition song from MCS to MCrS, and three produced MCrS. In the dark, one male spontaneously sang MCS and two MCrS.

In 5 minute controls for tests with a couple on a plant (see experimental procedures), 19.8% of males (N=126) sang spontaneously. Under these conditions 5% of females sang spontaneously (N=121). The difference between males and females was significant in a 5 minute test (χ^2 test, P=0.0008) but not in those lasting 13 minutes (χ^2 test, P=0.3640).

3.2 Initiation of vibrational communication in pairs of bugs

Experiments with pairs of bugs were carried out to determine who starts the vibrational communication. When a female was placed on a plant 5 minutes before the male, the male started vibrational communication more frequently than the female. Of 52 pairs, males started vibrational communication in 46.2%, the female in 36.5% and in 17.3% no vibrational communication was recorded; *i.e.* of the 43 couples that exchanged vibratory signals, males started communication in 55.8% and females in 44.2%. However, differences between genders are not statistically significant (test of binomial probability, T=1.2). When a male was placed on a plant 5 minutes before the female, males started vibrational communication in 70% of cases and females in 30% (N=10, all pairs sang) (test of binomial probability, T=1.26). Results of these two tests were not statistically different (χ^2 test: P=0.6428), therefore we combined them for further analysis of initial song emission (Figure 4).

Females first emitted FCS in 86.4% and FCrS in 13.6% (N=22) of cases. FCrS was recorded only after physical (tactile) contact with the male. Males responded with MCS or MCrS, or with a transition from MCS to MCrS, represented by MCS pulses of increasing repetition rate, fusing finally in the same MCrS pulse train. Males' first songs were recorded: MCS in 45.2%, MCrS in 48.4% and in 6.5% a transition MCS-MCrS within the same sequence (N=31).

In the 25 min. experiments (N=145) the differences between genders were even more pronounced. Males started vibrational communication in 58.6% and females in 29.0% of cases; in 12.4%, no vibrational song was emitted. In the 127 couples that exchanged vibratory signals, the males started in 66.9% and females in 33.1% of cases. The difference was statistically significant (test of binomial probability, T=3.8). Females started to sing with FCS in 95.2% of cases when initiating the vibrational communication (N=42) (Figure 4). When starting vibrational communication (N=85) 57.7% of males first emitted MCS and 36.5% MCrS. MCS was transformed to MCrS within the same sequence in 4.8% of cases when the male sang first (Figure 5).

3.3 Responses of single *N. viridula* males or females to stimuli of different modalities

3.3.1 Controls.

The controls within each test were compared and no significant differences were found between them (Tables 1 and 2).

3.3.2 Vibrational stimuli

Vibrational stimuli had significantly different effects on males and on females (Figure 5). Only 9.1% of females (N=11) responded to MCrS and there was no statistically significant difference (χ^2 test, P=0.8062, see Table 1) from the control in daylight (12.2%, N=22). Males however responded to FCS in 75.9% of cases (N=18), with a strong statistical difference (χ^2 test, P=0.0004, Table 2) from controls (24.0%, N=40).

Males (N=29) stimulated with conspecific FCS responded with MCS in 28.6% of cases, with MCrS in 64.3% and, in 7.1% of cases, with MCS transformed into MCrS within the same song sequence. Females (N=11) responded to stimulation with conspecific MCrS only with FCS.

3.3.3 Visual stimuli

To test the effect of visual stimuli we presented a model of a conspecific stinkbug to a single male or female (Figure 3 and Tables 1 and 2). Visual stimuli triggered

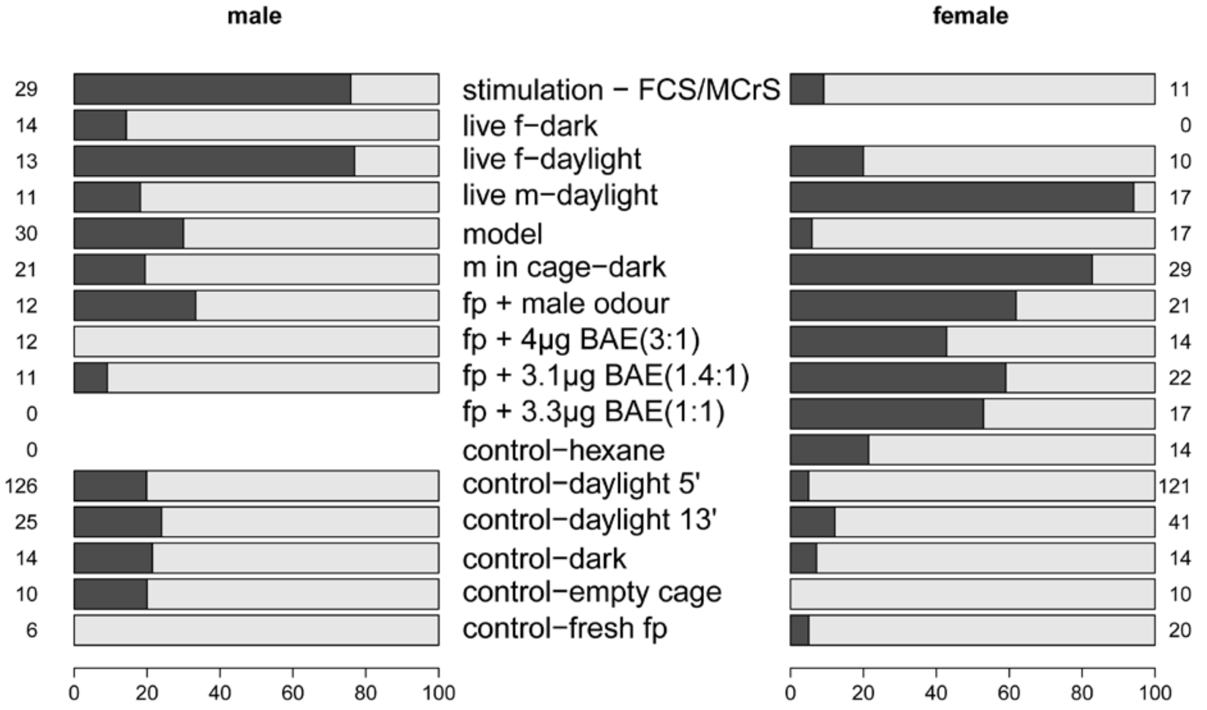

Figure 3. Comparison of males' (m) and females' (f) responses to different stimuli and controls: 13 minute experiments with single animal exposed to stimuli of different modalities. Responses are represented as percentages, male on the left and female on the right; numbers of tests are shown at the sides (left for males, right for females). Stimulation-FCS/MCrS stands for stimulation with either FCS for males or MCrS for females, fp for filter paper, BAE for Z-α-bisabolene epoxide, ratio of *trans:cis* isomers is stated in brackets.

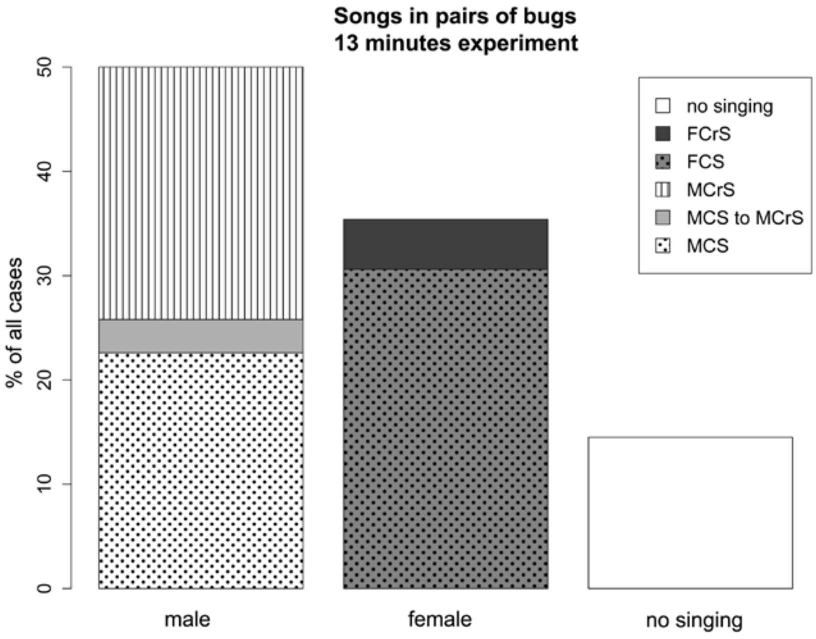

Figure 4. Emission of different songs from pairs of bugs in 13 minute experiments: the bar plot shows the distribution of male and female songs that were emitted first when vibrational communication started. Different songs are represented by different shades (see legend): nosinging = no singing *i.e.* no vibrational communication occurred in these pairs, FCrS = female courtship song, FCS = female calling song, MCrS = male courtship song, MCS to MCrS = transitional song from male calling to male courtship song in the same pulse train, MCS = male calling song.

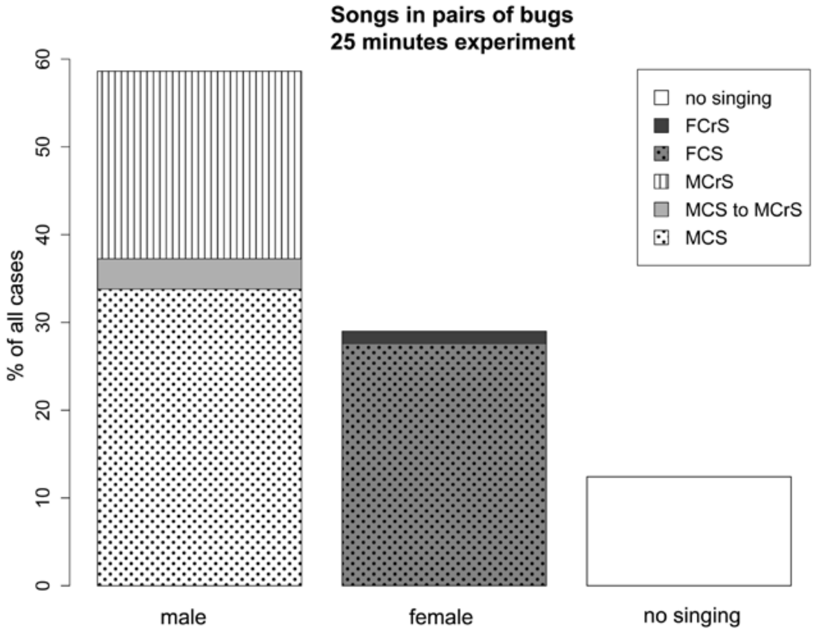

Figure 5. Emission of different songs from pairs of bugs in 25 minute experiments: the bar plot shows the distribution of male and female songs that were emitted first when vibrational communication started. Different songs are presented by different shades (see legend): nosinging = no singing *i.e.* no vibrational communication occurred in these pairs, FCrS = female courtship song, FCS = female calling song, MCrS = male courtship song, MCS to MCrS = transitional song from male calling to male courtship song in the same pulse train, MCS = male calling song.

more males than females to emit vibratory signals. A dead model elicited a very low vibrational response with FCS in females (5.9%, N=15) while males were triggered in 30.0% of cases (N=30). The latter emitted predominantly MCrS (66.7%), MCS transition to MCrS in 22.2% and MCS in 11.1% of cases. No statistically significant difference was observed in responses to male and female bodies used as models (χ^2 test, P=0.75, Table 2). Males' response to the dead models (30.0%, N=30) was not significantly higher (χ^2 test, P=0.8466, see Table 2) than in controls in daylight (24.0%, N=25).

When a live female was presented to a male on a plant in daylight, a very high percentage of males started to sing (76.9%, N=13), statistically different from the control (χ^2 test, P= 0.0053, see Table 2). On the other hand males rarely responded to a female in the dark (14.3%, N=14) (χ^2 test, P=0.00391, see Table 2). Females responded to a live female in 20.0% of cases, N=10 (χ^2 test, P= 0.896, see Table 1).

3.3.4 Chemical stimuli

The influence of chemical stimuli was tested by applying a natural chemical stimulus or a synthetic pheromone (Figure 3 and Tables 1 and 2). More females responded to natural male emitted volatile chemicals than to synthetic pheromones. Nevertheless all female responses to tested chemicals were statistically significantly different from controls in daylight and with fresh filter paper. In contrast males responded to chemical stimuli from males (natural or synthetic) or to live females in the range of controls.

Male in a cage

When a male was presented in a cage, in the absence of all other stimuli, females started to emit FCS in 82.8 % of cases (N=29). The difference from the control (empty cage, when none of 10 females sang) is statistically significant (χ^2 test, P=0.00002; Table 1). The difference is also significant when compared with the control with the female alone in the dark (only 7.1% of females sang, N=14), (χ^2 test, P=0.00001; Table 1). Moreover there is no statistically significant difference between the female response to a male in a cage in the dark and a male in a cage in the daylight (χ^2 test, P=0.5153, see Table 1).

When a male in a cage was brought near a male on a plant under the same conditions (in the dark), the latter elicited vibrational communication in 19.1% (N=12) of cases, which lies in the range of values of the control in the dark (21.4%, N=14) and the control with an empty cage in which 20.0% of 10 tested males sang when an empty cage was placed in their vicinity. When a female in a cage under red light was presented to a male, the latter sang only in 14.3% of cases (N=14) (Figure 3). In both tests males only emitted MCrS. In other experiments performed in the dark, males emitted predominantly MCrS: in controls with an empty cage in the dark only MCrS was recorded and in controls in the dark without any stimuli MCrS was recorded twice and MCS once.

Filter paper carrying male odour

Females responded to odorized filter paper by emitting vibrational signals in 61.9% of cases (N=21), statistically significantly different from the control with fresh filter paper (5% of 20 tested females responded) and from the control in daylight (12.2%, N=41) (Table 1). FCS was emitted in 92.3% of songs and FCrS was recorded in only one case.

Males responded to odorized filter paper with MCS in 33.3% of cases (N=12), higher than controls with fresh filter paper (none of 6 males responded) and higher but not statistically significantly different from controls in daylight (24.0%, N=25) (Table 1).

Synthetic pheromones: Females responded to 0.04 μg ml^{-1} concentration of 3:1 *trans*:*cis* (Z)-α-bisabolene epoxide (BAE) in 42.9% (N=14), always with FCS. Higher response was recorded when 0.031 μg mL^{-1} of 1.4:1 *trans*:*cis* BAE was used (59.1% of all cases; N=22). In this test 76.9% of songs emitted were FCS, others FCrS. When a 0.033 μg mL^{-1} 1:1 *trans*:*cis* BAE was used 52.9% of all females (N=17) produced a vibrational response. 88.9% of songs were FCS. Responding levels in all tests differed significantly from those in controls in daylight and controls with fresh filter paper (Table 1). Controls using solvent hexane stimulated greater response in females (21.4%, N=14) but not significantly different from controls with fresh filter paper (5.0%, N=20). 12 males were tested with 0.04 μg mL^{-1} concentration of 3:1 α-bisabolene epoxide and none responded. On exposure to 0.031 μg mL^{-1} of 1.4:1 *trans*:*cis* BAE, one male (9.1%) out of 12 responded by emitting MCS.

4. Discussion

The results of the present study indicate that females of *Nezara viridula* are triggered to sing predominantly by chemical and males by vibrational and visual stimuli from conspecific mates. Females usually respond by singing FCS and males MCS and/or MCrS.

Females emitted their calling and males their calling and courtship songs in the absence of a mate and during various controls without any obvious external triggering signal in light or in the dark. However the rate of spontaneous singing was significantly lower than in response to a triggering signal. Males have been shown to be more likely to sing spontaneously than females, both in daylight and in the dark. Spontaneous emission of vibratory signals in females included a few signals that usually did not develop into the longer lasting song sequence characteristic of chemically triggered emissions. Since experiments were conducted on fresh plants chemical signals originating from male *Nezara viridula* are excluded as the cause of females singing when alone on a plant but other chemicals might be present on the plant as well. The role of plant-originating chemical signals in inducing vibratory signals described as spontaneous singing can not be excluded as it has been shown to affect insect behaviour [19]. In *Nezara viridula* plant chemicals affect oviposition [20] but their direct triggering of vibrational communication has not yet been studied. Spontaneous singing has been overlooked until now, especially in males, but our experiments show it to occur in a high proportion of males and approximately half as much in females in daylight. The larger number of individuals in 5 minute controls lends stronger support for the conclusion that males are more likely to elicit vibrational signals in the absence of other stimuli than females.

Males and females were shown to sing spontaneously more frequently in the daylight than in the dark. This is not surprising, given that *N. viridula* is a diurnal animal. Although their prolonged copulation [21] can last for several days, searching for mates is more active during the day.

Spontaneous singing could also be one of the key factors in starting vibrational communication; however, under our experimental conditions, females rarely emitted FCS in a steady rhythm of pulse trains as was produced when triggered by male pheromone.

Controls did not differ significantly from one another and were always within the range of spontaneous singing.

Our experiments have shown that a high percentage of males preface vibrational communication by emitting either the calling or the courtship song. We have found no evidence for any external signal, apart from visual stimuli, that underlies the male's singing first in a duet. It cannot be a response to another vibrational song and we have no indication of any female-emitted chemical substances that would induce male calling [10,22-25]. Other vibrations induced, for example, by walking, tapping a plant *etc*. could also participate but we have no information on the role of non-species-specific signals to trigger emission of vibratory communication signals. Nevertheless, there are some differences regarding the circumstances in which a certain song is used. MCS is much more often used in daylight than in the dark (there it was only emitted once). Also, in experiments with pairs of bugs it was observed that MCS is emitted when bugs are already in visual contact and usually in close proximity. MCrS on the other hand is the usual answer to female calling and also the male's song in duets.

It has been shown here that males regularly respond to FCS, mainly by emitting the MCrS, and that female singing relies on male emitted pheromone (FCS) and physical contact (FCrS). Since no direct female response to MCS has so far been observed [26,27] and duets usually consist of the male courtship song (MCrS) and the female calling song (FCS), we used MCrS to stimulate females and likewise, FCS for males.

The question of the role of the first male song in Pentatomidae is still open. No direct female response to a male calling song has been observed so far. It may be regarded as the transitional song to the courtship song that is the usual answer to the female calling. MCrS has generally a more complex and species specific temporal, amplitude and frequency pattern that indicates its important role in mate species recognition. MCS also appears as a transitional form from courtship to rival song. In most cases when a female starts vibrational communication on a plant she emits the calling song. This increases male activity in searching for her on the plant [25], enables vibrational directionality [25], triggers male vibratory responses [28] and increases male pheromone release [12]. FCS is usually the first female song and is the song that enables males to find females and its time parameters are very distinct no matter where she sings [26]. The first emitted female song in most pentatomid species carries less information on species identity due to the latter's similar time and frequency characteristics [28,29]. Recognition runs predominantly at the courtship song level, in close vicinity, where signals of other modalities become important.

Green stink bugs are diurnal insects and the importance of vision during communication at closer distances cannot be excluded. Different visual stimuli have been presented to tested animals to determine the role of sex/size (male or female body), colour and/or distance. No difference in responsiveness was observed between male and female bodies and different colour shades of bodies. The distance and movement of a stimulus, however, played a major role in triggering males. Males responded significantly to a visual stimulus while females' response was in the range of the control.

In experiments conducted in the dark, neither males nor females responded when approached by a live female, which would support the absence of female pheromones [4,9,10,18,22-24].

Males sang more when a live female, rather than a live male, was used as a model. That may be explained by the visual signals produced by females rotating their antennae vigorously when in close vicinity to a male, or to the male's pheromone. Another possibility would be that the pheromone emitted by the model male suppresses the response to the visual stimulus of a tested male. That is, however, less likely, as males also respond to male pheromones to a greater extent than the control, although not differing significantly from the control.

Male *N. viridula* pheromones act as an attractant for both males and females as well as for the fourth stage nymphs and for parasitoids [4], but that was not confirmed in later studies [9]. Many chemical compounds have been found in the secretions of green stink bugs, most of them in the defensive fluid [11,18,30-32]. They are produced by dorsal, abdominal and metathoracic scent glands [8].

Responses to tested chemical stimuli (male *Nezara viridula* pheromone) in females always differed statistically from controls. Females responded to all tested chemical stimuli to a greater extent than males and antennated more often. The rhythm was always steady and the songs lasted longer in contrast to spontaneous singing. When a female (in the cage under red light) was presented to a male, the latter sang in only a very few cases (even lower than the control) whereas, when a male was placed near a female in the dark, the females responded significantly more frequently than the control. To compensate for individual variation in pheromone secretion, a number of males were used in a cage. Nevertheless, significantly more females responded to every one of them, indicating that male pheromone triggers female calling. Females responded highly to a male in a cage in daylight but not statistically differently from a male in a cage in the dark. Also no response to any visual stimulus was observed in females; therefore synergy of visual and chemical stimuli is less likely, although smaller responses were recorded to the male odour trapped on filter paper and to synthetic pheromone blends. All chemical stimulus tests, however, still differed significantly from the control in daylight and from the control with fresh filter paper. The slight difference between the response of females to a male in a cage in daylight (94.1%) and in the dark (82.8%; lower but not statistically different) could be due to the lack of visual stimuli, *i.e.* a general difference occurring between light and dark but, even more likely, it is the result of different amounts of pheromone. Vibratory female signals increase the release of pheromone [12]. The same principle could be applied to visual stimuli that have been shown here to trigger the male's vibratory response. Thus a male in a cage in daylight could release more pheromones, due to visual stimuli, than the one in the dark – similarly to the male's response to FCS. That, however, has to be tested, since there is so far no evidence that visual stimuli trigger an increase in pheromone production.

Females' responses to all chemical stimuli are significantly higher than in controls but the trend from most accurate blend (live male, highest response) to the most simplified trigger (synthetic pheromones, lowest response) is observed.

Females that did not start to sing when exposed to male pheromone often flew to the mesh cage or filter paper. Flying to the vicinity of pheromone is a long-range courtship behaviour elicited by a pheromone that acts as an attractant [5,33]. In this experiment the pheromone was set at a threshold dosage to obtain the short range response of eliciting female calling song. Most females antennated when exposed to chemical stimuli.

In preliminary experiments, different doses of pheromone were tested and 8-9% of daily male production dose [18] was chosen for these experiments. As pheromone emission usually occurs in bursts and not at the same level throughout the day, the concentrations of male pheromone in nature in dense bushes can be higher than expected from aeration data [30,34].

Synthetic pheromones were tested in different ratios of *cis* to *trans* isomers. High inter-individual variation in pheromone composition has been reported [35]. The difference in pheromone composition between populations is reflected in the different ratios of *trans* to *cis* isomers of (Z)-α-bisabolen epoxide (BAE) [11]. Ratios of 3:1, obtained from a USA population, 1.4:1 from a French population and 1:1 found in a Japanese population were used in our experiment [11]. As expected, the highest response was recorded when a 1.4:1 ratio was used since the French population is geographically the closest to the tested (Slovenian) population.

One of the reasons that females were formerly described as the initiators of duets could be the fact that plants in some of the investigations were not changed for every animal in every experiment. Besides airborne pheromones males also emit contact chemicals that adhere to the surface of plants and a female samples them with her antennae on the stalk/stem/leaves while walking and searching for males [36]. What was earlier described as spontaneous singing in females could in part be a response to contact pheromone (or other chemical contact product) or its residues on plants or loudspeakers used in those experiments. Contact chemical signals may have an important effect on behaviour, even at very low doses [36,37], as observed in our experiments where females started to sing earlier when a male was on the same plant than when the male was presented in a cage.

We can conclude that tested chemical and vibratory signals play key roles during calling and courting. Our results open several questions that need further experimental work. For example we have no response to the question of spontaneous singing that appears to be more common in males than in females. It could be a selection-driven outcome similar to the presence of pheromone, so far only found in males. Pheromone emission puts the emitter at risk, as evidenced by the attraction of a parasitoid to sex pheromone. Emitted vibration also constitutes a hazard for the emitter that could be spotted by a predator or parasite. Thus females sing spontaneously less often and only respond with a steady rhythm when triggered by chemical stimuli (male pheromone) and males sing spontaneously more often and are triggered by visual and vibrational stimuli.

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References

[1] Todd J.W., Ecology and Behavior of Nezara viridula, Annu. Rev. Entomol., 1989, 34, 273–292

[2] Schaefer C.W., Panizzi A.R., Heteroptera of economic importance, CRC Press LLC, 2000, ch.13, 421-439

[3] Mitchell W.C., Mau R.F.L., Sexual activity and longevity of the southern green stink bug, Nezara viridula, Ann. Entomol. Soc. Am., 1969, 62, 1246–1247

[4] Harris V.E., Todd J.W., Male-mediated aggregation of male, female and 5th-instar southern green stink bugs and concomitant attraction of a tachinid parasite, Trichopoda pennipes, Entomol. Exp. Appl., 1980, 27, 117–126

[5] Borges M., Jepson P., Howse P., Long-range mate location and close-range courtship behaviour of the green stink bug, Nezara viridula and its mediation by sex pheromones, Entomol. Exp. Appl., 1987, 44, 205–212

[6] Kon M., Oe A., Numata H., Hidaka T., Comparison of the mating behaviour between two sympatric species, Nezara antennata and N. viridula (Heteroptera: Pentatomidae), with special reference to sound emission, J. Ethol., 1988, 6, 91–98

[7] Čokl A., Virant-Doberlet M., Stritih N., The structure and function of songs emitted by southern green stink bugs from Brazil, Florida, Italy and Slovenia, Physiol. Entomol., 2000, 25, 196–205

[8] Aldrich J.R., Chemical ecology of the Heteroptera, Annu. Rev. Entomol., 1988, 33, 211–238.

[9] Brézot P., Malosse C., Renou M., Study of the attractivity of the male pheromone in Nezara viridula L. (Heteroptera: Pentatomidae) [Etude de l'attractivité de la phéromone des mâles chez Nezara viridula L. (Heteroptera: Pentatomidae)], C.R. Aca. Sc Paris, Serie 3, Science de la vie, 1993, 316(7), 671-675 (in French)

[10] Millar J., Pheromones of True Bugs, in: Schulz S. (Eds.), The Chemistry of Pheromones and Other Semiochemicals II, Springer Berlin Heidelberg, 2005, 37–84

[11] Aldrich J., Chemical Communication in the True Bugs and Parasitoid Exploitation, Chemical Ecology of Insects 2, Springer US, 1995, 318–363

[12] Miklas N., Lasnier T., Renou M., Male Bugs Modulate Pheromone Emission in Response to Vibratory Signals from Conspecifics, J. Chem. Ecol., 2003, 29, 561–574

[13] Ota D., Čokl A., Mate location in the southern green stink bug, Nezara viridula (Heteroptera: Pentatomidae), mediated through substrate-borne signals on ivy, J. Insect Behav., 1991, 4, 441–447

[14] Žunič A., Virant-Doberlet M., Čokl A., Species Recognition During Substrate-Borne Communication in Nezara viridula (L.) (Pentatomidae: Heteroptera), J. Insect Behav., 2011, 24, 468–487

[15] Čokl A., Virant-Doberlet M., Stritih N., Temporal and spectral properties of the songs of the southern green stink bug Nezara viridula (L.) from Slovenia, Pflügers Arch., 2000, 439, 168–170

[16] Brennan B.M., Chang F., Mitchell W.C., Physiological Effects on Sex Pheromone Communication in the Southern Green Stink Bug, Nezara viridula, Environ. Entomol., 1977, 6, 169–173

[17] Broughton W.B., Method in bio-acoustic terminology, Acoust. Behav. Anim., 1963, 3–24

[18] Aldrich J., Lusby W., Marron B., Nicolaou K., Hoffmann M., Wilson L., Pheromone blends of green stink bugs and possible parasitoid selection, Naturwissenschaften, 1989, 76, 173–175

[19] Reddy G.V.P., Guerrero A., Interactions of insect pheromones and plant semiochemicals, Trends Plant Sci., 2004, 9, 253-261

[20] Panizzi A.R., Berhow M., Bartelt R.J., Artificial substrate bioassay for testing oviposition of southern green stink bug conditioned by soybean plant chemical extracts, Environ. Entomol., 2004, 33, 1217-1222

[21] McLain D.K., Female choice and the adaptive significance of prolonged copulation in Nezara viridula (Hemiptera: Pentatomidae), Psyche (Stuttg.), 1980, 87, 325–336

[22] Aldrich J.R., Oliver J.E., Lusby W.R., Kochansky J.P., Lockwood J.A., Pheromone strains of the Cosmopolitan pest, Nezara viridula (heteroptera: Pentatomidae), J. Exp. Zool., 1987, 244, 171–175

[23] Aldrich J. R., Numata H., Borges M., Bin F., Waite G. K., Lusby W. R., Artifacts and pheromone blends from Nezara spp. and other stink bugs (Heteroptera: Pentatomidae). Zeitschrift für Naturforschung, Section C. Biosciences, 1993, 48, 73-79

[24] Miklas N., Renou M., Malosse I., Malosse C., Repeatability of pheromone blend composition in individual males of the southern green stink bug, Nezara viridula, J. Chem. Ecol., 2000, 26, 2473–2485

[25] Čokl A., Virant-Doberlet M., McDowell A., Vibrational directionality in the southern green stink bug, Nezara viridula (L.), is mediated by female song, Anim. Behav., 1999, 58, 1277–1283

[26] Čokl A., Bogataj E., Factors affecting vibrational communication in Nezara viridula L., Heteroptera, Pentatomidae), [Dejavniki vpliva na vibracijsko komunikacijo zelene smrdljivke Nezara viridula L.], Biološki vestnik, 1982, 1–20, (in Slovene, abstract and figures in English)

[27] Čokl A., Stink bug communication through plant during mating [Komunikacija rastlinskih stenic prek rastlin med parjenjem], Razpr. IV Razr. SAZU, 2006, 47, 7-35 (in Slovak)

[28] Virant-Doberlet M., Čokl A., Vibrational communication in insects, Neotrop. Entomol., 2004, 33, 121–134

[29] Čokl A., Virant-Doberlet M., Communication with substrate-borne signals in small plant-dwelling insects, Annu. Rev. Entomol., 2003, 48, 29–50

[30] McBrien H.L., Millar J.G., Substrate-borne vibrational signals of the Consperse stink bug (Hemiptera: Pentatomidae), Can. Entomol., 2003, 135, 555–567

[31] Brézot P., Malosse C., Mori K., Renou M., Bisabolene epoxides in sex pheromone in Nezara viridula (L.) (Heteroptera: Pentatomidae): Role of cis isomer and relation to specificity of pheromone, J. Chem. Ecol., 1994, 20, 3133–3147

[32] Borges M., Aldrich J., Instar-specific defensive secretions of stink bugs (Heteroptera: Pentatomidae), Cell. Mol. Life Sci., 1992, 48, 893–896

[33] Borges M., Moraes M.C.B., Laumann R.A., Pareja M., Silva C.C., Michereff M.F.F., et al., Chemical Ecology Studies in Soybean Crop in Brazil and Their Application to Pest Management, Soybean-Biochem. Chem. Physiol. Rij. Croat. Intech Publ., 2011, 31–66

[34] McBrien H.L., Millar J.G., Phytophagous bugs, Pheromones Non-Lepidopteran Insects Assoc. Agric. Plants Cabi Publ. Wallingford UK, 1999, 277–304

[35] Ryan M.A., Moore C.J., Walter G.H., Individual variation in pheromone composition in Nezara viridula (Heteroptera: Pentatomidae): how valid is the basis for designating “pheromone strains”?, Comp. Biochem. Physiol. B Biochem. Mol. Biol., 1995, 111, 189–193

[36] Colazza S., Salerno G., Wajnberg E., Volatile and Contact Chemicals Released by Nezara viridula (Heteroptera:Pentatomidae) Have a Kairomonal Effect on the Egg Parasitoid Trissolcus basalis (Hymenoptera: Scelionidae), Biol. Control, 1999, 16, 310–317

[37] Siljander E., Gries R., Khaskin G., Gries G., Identification of the Airborne Aggregation Pheromone of the Common Bed Bug, Cimex lectularius, J. Chem. Ecol., 2008, 34, 708–718