

Effects of heterospecific and conspecific vibrational signal overlap and signal-to-noise ratio on male responsiveness in *Nezara viridula* (L.)

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SUMMARY

Animals often communicate in environments with high levels of biotic noise that arises from the signals of other individuals. Although effects of background biotic noise on mate recognition and discrimination have been widely studied in air-borne sound communication, little is known about incidental interference between signallers that use substrate-borne vibrational signals. In this study we investigated the ability of males of the southern green stink bug *Nezara viridula* (L.) (Heteroptera: Pentatomidae) to recognize conspecific female song in the presence of biotic noise originating from conspecific and heterospecific vibrational signals. We tested male responsiveness on a bean plant in playback experiments. One leaf was vibrated with conspecific female song, while to the other one we simultaneously applied either heterospecific female signal or various altered conspecific signals with different temporal parameters. We tested males in three levels of biotic noise, ranging from +6 dB to –6 dB and we compared male responsiveness in each treatment with response obtained in unilateral treatment with unaltered conspecific female calling song. Male responsiveness was reduced in the presence of heterospecific signals or when background noise from conspecific signals obscured the species-specific temporal pattern of conspecific female song. By contrast, the presence of two sources of conspecific female songs had a positive effect on male responsiveness, for as long as the signal repetition rate of perceived song did not differ from the species-specific value. In the presence of interfering background signals, searching activity was less affected than male signalling. Increased signal-to-noise ratio restored male responsiveness to the level expressed in unilateral stimulation with conspecific female song. The results are discussed with regard to male behavioural strategies for vibrational communication in a noisy environment.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/213/18/3213/DC1>

Key words: vibrational communication, cocktail party problem, mate recognition, insect.

INTRODUCTION

Insects have evolved a great variety of sophisticated chemical, visual and mechanical (air-borne sound and substrate-borne vibrations) signals for intraspecific communication (Greenfield, 2002). Although the air-borne sound communication is restricted to only few insect groups (Greenfield, 2002; Coccoft and Rodríguez, 2005), all aspects of insect acoustic communication have been extensively studied (reviewed by Gerhardt and Huber, 2002). Although substrate-borne vibratory signalling is prevalent among insects (Greenfield, 2002; Virant-Doberlet and Čokl, 2004; Coccoft and Rodríguez, 2005) it has, nevertheless, received less attention. The role of vibrational signals as part of specific mate recognition systems has been well studied under isolated conditions (reviewed by Claridge and de Vrijer, 1994; Čokl and Virant-Doberlet, 2003), little is known about the influence of background interference signals emitted by con- or heterospecifics on detection, recognition and discrimination of vibrational signals related to sexual behaviour.

Signals used in sexual communication should identify the sender (reveal the species and gender), convey the context (the meaning of the message) as well as the location of the sender (e.g. Bradbury and Vehrencamp, 1998; Pollack, 2000; Gerhardt and Huber, 2002). When choosing a partner belonging to the opposite sex of the same species, under natural conditions, the receiver often faces the problem of simultaneously detecting several conspecific and/or heterospecific signals (e.g. Römer, 1993; Römer and Krusch, 2000; Nityananda et al., 2007) as well as discriminating between signals

that are emitted from several, spatially separated sources (Pollack, 1998; Pollack, 2000; Gerhardt and Huber, 2002). Furthermore, in closely related species signals are often variations on the same basic pattern (Schul, 1998; Čokl, 2008). In most insects using air-borne sounds or substrate vibrations, the main species- and sex-specific information is encoded in temporal parameters of their signals (for as long as the dominant frequency of the signals remains in the appropriate frequency range) (Gerhardt and Huber, 2002; Sueur, 2006).

It has been shown in the field, that many conspecifics, as well as individuals of several insect species, can simultaneously emit vibrational signals on the same plant (Coccoft, 2003; Coccoft and Rodríguez, 2005). The presence of other signallers is the most common source of biotic noise (Forrest, 1994; Brumm and Slabbekoorn, 2005; Bee and Micheyl, 2008). In such situations interference occurs as an incidental by-product either from overlapping or alternating signals. In contrast to the three-dimensional space of air-borne sound communication, vibrational signals are often used in one-dimensional structures such as plant stems. Such situations presents a number of challenges for the receiver, since signal transmission through plants results in degradation of the signal because of frequency filtering (Michelsen et al., 1982; Barth, 1998; Magal et al., 2000) and temporal distortions (Michelsen et al., 1982; Miklas et al., 2001), as well as oscillations of the signal amplitude along the stem (Michelsen et al., 1982; Čokl, 1988; Čokl et al., 2007). Although location decisions on a stem are

relatively simple (a choice between 0 deg and 180 deg), in a one-dimensional environment on a plant, the receiver would perceive signals emitted from distant sources that are all positioned on the same side of the receiver as one compound signal.

Simultaneous bilateral stimulation with conspecific and heterospecific female vibrational signals did not affect discrimination (either recognition or localization) in males of the planthoppers of the genus *Ribautodelphax* (de Winter and Rollenhagen, 1990). By contrast, in such a situation males of the southern green stink bug *Nezara viridula* reduced their signalling activity and did not start searching (Miklas et al., 2003b). Similarly, when males of *N. viridula* were simultaneously stimulated with conspecific signals and synthesized continuous signals of the same frequency, fewer males responded and searched for the source of the female signals (Polajnar and Čokl, 2008).

Here, we investigated the ability of *N. viridula* male to discriminate between vibrational signals emitted simultaneously from two spatially separated sources. *Nezara viridula* is one of the model species for studies of vibrational communication (Virant-Doberlet and Čokl, 2004; Čokl, 2008), transmission properties of their host plants (e.g. Čokl et al., 2005; Čokl et al., 2007), reception and processing of vibratory signals in the central nervous system (Čokl et al., 2006; Zorovic et al., 2008), and male preferences (Čokl et al., 1978; Miklas et al., 2001). In this species the sex pheromone produced by mature males serves as a long-range attractant that enables females to arrive at the same plant (Borges et al., 1987), whereas species- and sex-specific vibrational signals (songs) are used during courtship that takes place on the plant (Čokl et al., 2000). Females respond to male pheromone with their calling song, which in turn stimulates the male to increase pheromone production (Miklas et al., 2003a) and triggers male calling and courtship song (Čokl and Bogataj, 1982) as well as searching behaviour (Ota and Čokl, 1991). The presence of male vibrational signals maintains continuous emission of the female calling song at a stable repetition rate (Čokl and Bogataj, 1982). Female vibrational signals enable the males to locate the female on the plant (Čokl et al., 1999). Pairs stay in copula for up to 3 days (Harris and Todd, 1980). Under natural conditions several females could simultaneously emit calling songs on the same plant (Miklas et al., 2003b). The temporal parameters of female calling song vary significantly among individuals and it has been suggested that variability might play a role in mate choice (Miklas et al., 2003b). *Nezara viridula* is often found syntopically with other stink bug species emitting vibrational signals with similar spectral and temporal characteristics (Čokl et al., 1978; Kon et al., 1988; Čokl et al., 2001; Blassioli Moraes et al., 2005) and interspecific copulations between *N. viridula* and *N. antennata* were observed in the field (Kiritani et al., 1963; Kon et al., 1993). Taking into account the potential predation risks associated with mate searching (e.g. Magnhagen, 1991) and lengthy copulation time, the cost of selecting heterospecific female would reduce reproductive success of the male.

In this study we focus on background interference from conspecific and heterospecific signals as well as discrimination between two conspecific female calling signals that vary in temporal parameters and/or in their amplitude. The aim of the current study was to assess the influence of incidental interfering signals on male responsiveness and to determine the effects of different signal-to-noise ratios. Such information is prerequisite for understanding the sources of selection acting on vibrational communication.

MATERIALS AND METHODS

Insects and plants

All experiments were conducted on adult males of the southern green stink bug *Nezara viridula* (L.) (Heteroptera: Pentatomidae). Adult bugs were collected in late autumn (October to November) at the Slovenian North Adriatic coast. The male and female bugs were kept separately in the laboratory under diapause conditions (8 h:16 h L:D cycle at 5–10°C) for at least one month. The diapausing animals were transferred to a 16 h:8 h L:D cycle at 22–23°C and 70–80% relative humidity at least 10 days before the beginning of behavioural tests to ensure their sexual activity. Each male was kept separately in a plastic pot (14 cm high, 6 cm diameter at the bottom and 10 cm diameter at the top) and fed with raw peanuts (*Arachis hypogaea* L.), sunflower seeds (*Helianthus annuus* L.) and dwarf bean plants (*Phaseolus vulgaris* L.). In addition, some animals were used to establish a laboratory colony. They were reared in glass boxes (38 cm×23 cm×23 cm) on the diet described above. Adult males from the laboratory colony were kept in separate pots as described above and were used for behavioural tests 10 days after the final moult to ensure their sexual maturity.

Bean is one of the preferred host plants of *N. viridula* (Panizzi, 1997) and experiments were performed on dwarf French beans var. etna (*Phaseolus vulgaris* L.) grown in the laboratory. They were planted in peat soil in plastic pots (14 cm high, 6 cm diameter at the bottom and 10 cm diameter at the top) and watered three times a week. Plants used in behavioural tests were at least 10 days old. The test plants were 20–30 cm high and consisted of a middle stem and one pair of leaves (Fig. 1). The place where the petioles were attached to the stem was 4–7 cm below the tip of the plant. At the top of the middle stem was a branch with three leaves. Different plant was taken every 2 days.

Vibrational stimuli

Males were tested with six different stimulation sequences (Table 1). Each stimulatory sequence was composed of elements of natural vibrational signals assembled using the computer programme Cool Edit Pro 2 (Syntrilium Software, Phoenix, USA).

The stimulation sequence for the conspecific female calling song (FCSNv) consisted of an unmodified natural sequence of 39 signals emitted by one female. The spectral characteristics and temporal parameters had mean values that corresponded to the preferred values of males from the Slovenian population (Čokl et al., 1978; Miklas et al., 2001) (Fig. 2A). The female calling song of *N. viridula* was recorded from the membrane of the low-middle frequency loudspeaker membrane (Conrad Electronic, impedance 8W, 4.5 mm diameter, 90–2000 Hz) with a laser vibrometer (controller 2200-L, sensor head OFV-353; Polytech, GmbH, Waldbronn, Germany) and stored on a Sound Blaster Audigy 4 sound card (Creative Labs Inc.) using Cool Edit Pro 2 software at a sampling rate of 44100 Hz. The above mentioned conspecific stimulation sequence was altered using Cool Edit Pro software to obtain sequences with different temporal and amplitude parameters (Table 1). By cutting the middle part of each signal a stimulation sequence was obtained that contained signals of shorter duration (Nv-short). By copying the middle part of each signal we obtained a stimulation sequence composed by signals of longer duration (Nv-long). In each case the duration of the interval was adjusted, so that the signal period (i.e. signal repetition time) stayed the same as in natural sequence. By copying and pasting signals in the middle of each interval we obtained a stimulation sequence with shorter signal repetition time (Nv-fast; Table 1). This sequence represents a noisy environment in which signals of two conspecific females are alternating. Continuous signal

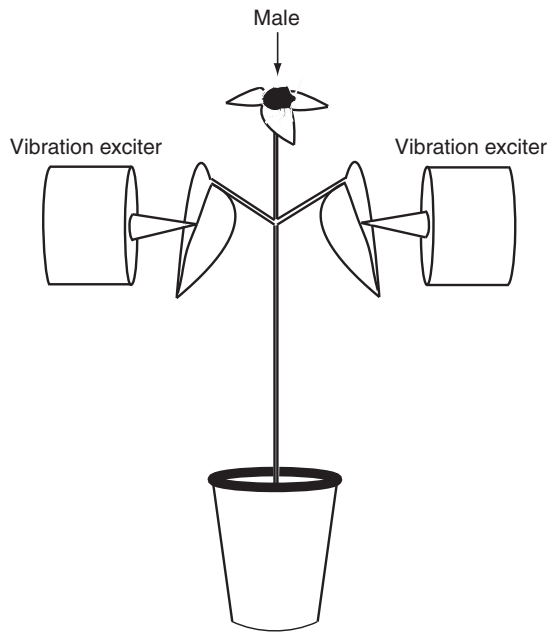


Fig. 1. A schematic drawing of the experimental set-up, showing the initial position of the male on the top of the plant and the positions of vibration exciters (not to scale).

(Nv-con) was constructed by copying and repeatedly pasting the middle part of one natural signal into a one 13 min long signal. This stimulation sequence is an approximation of a noisy environment in which signals of several females are continuously overlapping. As a natural heterospecific vibrational signal we used a female calling song of syntopic pentatomid bug species *Acrosternum hilare* (FCSAh) (Čokl et al., 2001) (Fig. 2B; Table 1). The heterospecific stimulation sequence consisted of four signals emitted by one female. Signals were recorded from a female singing in the presence of a male on the membrane of a low-midrange loudspeaker (10 cm diameter, impedance $8\ \Omega$, 40–6000 Hz, #WS 13 BF, Visaton, Haan, Germany). Signals from the loudspeaker were amplified with a custom-built amplifier, then digitized and recorded directly onto the hard drive of a computer using a Lexicon Core2 PCI recording system for PC (Sweetwater Sound, Fort Wayne, IN, USA), using Cool Edit Pro software (Čokl et al., 2001). Each of the above mentioned stimulation sequences was played back in a continuous loop for the duration of the stimulation period (see below).

For one set of stimulation sequences the amplitude of the signals was adjusted to the level of the recorded signals emitted by responding males (see below). In the second set the amplitude of the signals was reduced by -6 dB by the use of Cool Edit Pro 2 software.

Experimental setup and protocol

All behavioural tests were performed on a bean plant at the room temperature (24°C), 40–50% relative humidity and the same light conditions. We applied vibratory stimuli to the middle vein on the upper third of the left and/or right leaf *via* the tip of the cone screwed firmly into the head of a vibration exciter (Minishaker type 4810; Brüel and Kjaer, Naerum, Denmark; Fig. 1). Care was taken that they were positioned at the same distance from the main branching point. The vibratory stimuli and responses of the bugs were recorded from the main stem 1 cm above the branching point using the laser vibrometer. Signals were digitized with a 48 kHz sample rate and

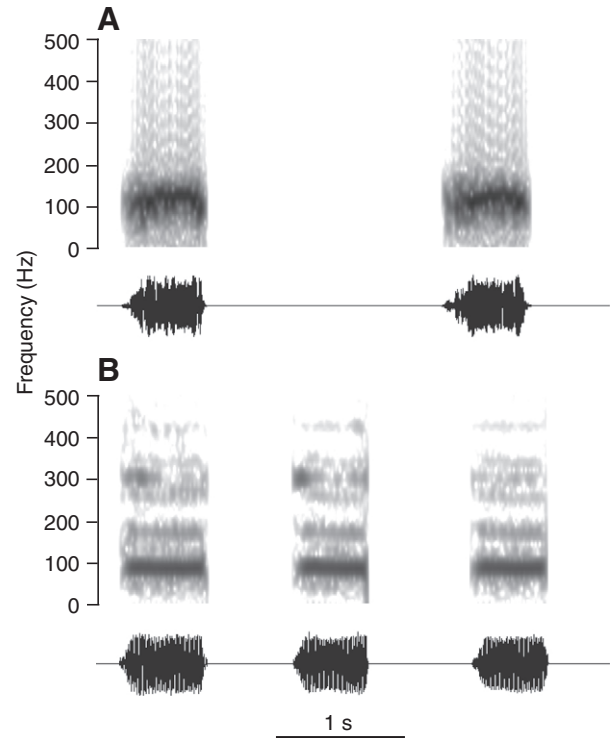


Fig. 2. Sonogram (above) and waveform (below) of the conspecific and heterospecific vibrational signals used for stimulation. (A) Unaltered female calling song of *Nezara viridula*. (B) Female calling song of *Acrosternum hilare*.

16 depth and stored onto a hard drive of a PC computer with an external sound card (Sound Blaster Extigy, Creative Labs Inc.) using Cool Edit Pro 2 software.

Males were tested in three experimental series. Each series was repeated four times with different males. In all experiments we placed a single male on the top of the plant and 2 min later we applied conspecific female calling song for 2 min in order to check daily general responsiveness of each male. After a 2 min silent pause we applied stimulation sequence(s) for 13 min. In all experimental series each male was tested only once per day. In each series each male was tested once with each stimulatory sequence and their order was randomized for each male. We performed the following experimental series that together provided 23 different stimulation treatments.

Unilateral stimulation

We applied the six stimulation sequences described above to one of the side branches. We changed the side from which stimulation was applied four times a day. The amplitude of stimulation was adjusted to the level of the natural signals.

Bilateral stimulation, equal amplitudes

We applied stimulation to both branches simultaneously. The conspecific *N. viridula* female calling song (FCSNv) was either applied to both leaves or one of the leaves was vibrated with one of the other stimulatory sequences. The first signal of each sequence started at the same time. The amplitude of stimulation was adjusted to the level of the natural signals and the amplitudes of both stimulation sequences were equalized at the point of stimulation (signal-to-noise ratio 0 dB).

Table 1. Temporal and spectral characteristics of vibrational signals used in stimulation sequences

	Duration (s)	Interval (s)	Duty cycle	Dominant frequency (Hz)
FCSNv	0.70±0.01	1.91±0.02	0.37	116±0.1
FCSAh	0.81±0.05	0.58±0.02	1.4	77±3
Nv-fast	0.70±0.01	0.61±0.01	1.14	116±0.1
Nv-short	0.38±0.01	2.24±0.03	0.16	108±0.2
Nv-long	1.033±0.02	1.58±0.02	0.65	116±0.1
Nv-con	780	0	–	116±0.1

FCSNv, natural female calling song of *Nezara viridula*; FCSAh, natural female calling song of *Acrosternum hilare*; Nv-fast, modified *N. viridula* song with shorter signal repetition time; NV-short, modified *N. viridula* song composed from shorter signals; Nv-long, modified *N. viridula* song composed from longer signals; NV-con, continuous signal composed from *N. viridula* signal.

Values are means±s.e.m.

Bilateral stimulation, different amplitudes

Stimulation was applied to both branches simultaneously and the amplitude of the stimulation sequence on one side was adjusted to the level of recorded natural signals, while the amplitude of the signals applied to the other side was reduced by 6 dB. In one set of trials the amplitude of conspecific female calling song was kept at the level of the recorded natural signal (signal-to-noise ratio +6 dB), while in the other it was reduced by 6 dB (signal-to-noise ratio –6 dB).

In a control experimental series, a male was placed on the top of the plant and behaviour was observed for 15 min in the absence of stimulation (pre-stimulation control). Then the male was stimulated for 2 min with the conspecific female calling song and afterwards the behaviour was observed for 15 min (post-stimulation control). The 2 min following the stimulation that corresponded to the 2 min silent pause in stimulation treatments were excluded from further analyses. Control experiments were done to determine male activity in the absence of stimulation (pre-stimulation control) as well as to test whether 2 min of pre-stimulation with the conspecific

female song, used to check daily general responsiveness of each male, affected the results.

In all experiments we monitored the following parameters: number of males emitting vibrational signals, male courtship song (MCRS) rate (calculated number of MCRS signals per min during a 13 min stimulation period; signalling rate) and number of males searching [defined as walking while singing (Čokl et al., 1999)].

Analyses

Seventeen animals were used in the control experimental series. In the analyses of stimulation treatments we included only males that showed positive general responsiveness (i.e. they responded to conspecific female calling song on the day of a particular treatment). The numbers of males included in the analyses, after excluding the false negative responses for each treatment, are shown in Table 2.

We compared the results for each treatment with values obtained in unilateral stimulation with an unaltered conspecific female calling signal. To compare the differences in the number of males singing and searching we used χ^2 -tests and contingency tables

Table 2. The effects of different stimulation treatments on signalling and searching activity of *Nezara viridula* males

Stimulation treatment	N	Proportion calling (χ^2 ; d.f.; P)	Proportion searching (χ^2 ; d.f.; P)	MCRS rate (t; d.f.; P)
FCSNv	46	0.91	0.43	3.05±0.37
FCSNv/FCSNv (0 dB)	47	0.98 (2.12; 1; ns)	0.49 (0.3; 1; ns)	3.53±0.45 (0.67; 9; ns)
FCSNv/FCSNv (+/–6 dB)	43	0.98 (1.84; 1; ns)	0.63 (3.36; 1; ns)	5.07±0.56 (2.41; 8; <0.05)
Nv-con	37	0.36 (27.52; 1; <0.001)	0.08 (13.37; 1; <0.001)	1.31±0.75 (–2.19; 24; <0.05)
FCSNv/Nv-con (0 dB)	38	0.53 (14.58; 1; <0.001)	0.33 (1.01; 1; ns)	1.34±0.77 (–2.01; 8; ns)
FCSNv/Nv-con (+6 dB)	44	0.68 (6.90; 1; <0.01)	0.36 (0.43; 1; ns)	2.20±0.42 (–0.86; 24; ns)
FCSNv/Nv-con (–6 dB)	33	0.48 (17.38; 1; <0.001)	0.11 (9.52; 1; <0.01)	0.37±0.14 (–2.69; 24; <0.05)
FCSAh	40	0.26 (37.29; 1; <0.001)	0.02 (20.25; 1; <0.001)	0.17±0.08 (–3.52; 27; <0.01)
FCSNv/FCSAh (0 dB)	57	0.72 (5.63; 1; <0.05)	0.27 (3.1; 1; ns)	2.18±0.48 (–1.76; 9; ns)
FCSNv/FCSAh (+6 dB)	39	0.87 (0.3; 1; ns)	0.56 (1.45; 1; ns)	3.06±0.49 (–0.42; 27; ns)
FCSNv/FCSAh (–6 dB)	36	0.69 (5.99; 1; <0.05)	0.22 (3.89; 1; <0.05)	1.11±0.40 (–1.15; 27; <0.001)
Nv-fast	40	0.71 (5.65; 1; <0.05)	0.24 (3.33; 1; ns)	1.59±0.38 (–3.03; 46; <0.01)
FCSNv/Nv-fast (0 dB)	57	0.74 (4.82; 1; <0.05)	0.12 (13.10; 1; <0.001)	1.09±0.38 (–3.63; 9; <0.01)
FCSNv/Nv-fast (+6 dB)	39	0.89 (0.05; 1; ns)	0.50 (0.38; 1; ns)	2.44±0.56 (–1.13; 46; ns)
FCSNv/Nv-fast (–6 dB)	36	0.81 (1.65; 1; ns)	0.27 (2.28; 1; ns)	1.09±0.50 (–3.98; 46; <0.001)
Nv-short	33	0.59 (10.61; 1; <0.01)	0.19 (5.01; 1; <0.05)	0.99±0.46 (–4.08; 55; <0.001)
FCSNv/Nv-short (0 dB)	59	0.97 (1.49; 1; ns)	0.56 (1.64; 1; ns)	3.43±0.37 (0.98; 9; ns)
FCSNv/Nv-short (+6 dB)	40	1 (3.81; 1; ns)	0.65 (4.01; 1; <0.05)	4.21±0.56 (1.68; 55; ns)
FCSNv/Nv-short (–6 dB)	42	1 (4.1; 1; <0.05)	0.60 (2.6; 1; ns)	4.18±0.50 (1.58; 55; ns)
Nv-long	48	0.96 (0.91; 1; ns)	0.58 (2.11; 1; ns)	3.18±0.34 (0.59; 72; ns)
FCSNv/Nv-long (0 dB)	66	0.98 (3.49; 1; ns)	0.53 (1.02; 1; ns)	4.22±0.44 (2.01; 9; ns)
FCSNv/Nv-long (+6 dB)	40	1 (3.63; 1; ns)	0.71 (6.89; 1; <0.01)	6.10±0.60 (3.16; 72; <0.01)
FCSNv/Nv-long (–6 dB)	39	1 (3.91; 1; ns)	0.80 (12.44; 1; <0.001)	5.58±0.56 (2.94; 72; <0.01)
Pre-stimulation control	17	0.21 (30.74; 1; <0.001)	0.05 (10.53; 1; <0.01)	0.67±0.31 (–2.21; 4; ns)*

For male courtship song (MCRS) rate (number of MCRS signals min^{–1}; signalling rate) values are means±s.e.m.

Values are compared with unilateral stimulation with unaltered conspecific *N. viridula* female calling song (FCSNv).

N, the number of males used for analyses per stimulation treatment after excluding false negatives.

*No significant difference was found due to low number of singing males (N=3) in the pre-stimulation control. For other details, see main text.

(Quinn and Keough, 2002). To compare the differences in MCrS rate, a generalized linear mixed model (GLMM) was used because unilateral treatments were dependent in a nested design. Males were nested in repeats and therefore males and repeats of experimental series were included as random effects. MCrS rate was analysed using penalized quasi-likelihood GLMM with a logistic link function after assuming a Poisson error distribution (McCullagh and Nelder, 1989).

To assess the effectiveness of each treatment we calculated a response index (RI) value:

$$RI = \frac{P_{\text{signal per treatment}} + \text{SMCrS} \times \frac{e^{(-2.223 + 5.6023 \times \sqrt{\text{MCrS rate}})}}{1 + e^{(-2.223 + 2.6023 \times \sqrt{\text{MCrS rate}})}} + P_{\text{search per treatment}}}{3} \quad (1)$$

The response index (RI) describes the relative strength of the male response to each treatment and includes three components: the proportion of signalling males per treatment, the proportion of searching males per treatment and the male courtship song (MCrS) rate (the calculated number of MCrS signals emitted per minute during the stimulation period). In the response index the preference was expressed as the MCrS score (presence or absence of MCrS, SMCrS, 1 or 0) and relationship of the proportion of singing males to the mean of the square root of MCrS rate in each treatment. The former two components indicate the probability that the male will respond (i.e. emit vibrational signals) and search for the source of vibrational signals in the particular treatment. The proportion of responding males is regarded as 'recognition' in Ryan and Rand's terminology (Ryan and Rand, 1993). The proportion of searching males was defined as attractiveness. It indicates that perceived signals were attractive enough that males intended to mate and started to search the source of these signals. The MCrS rate was taken as a measure of preference and it implies a comparison (Rand and Ryan, 1993). Low numbers of emitted MCrSs indicates that although males responded to a particular treatment, the preference was low. The preference for a certain treatment was defined through the following steps: (1) the complete data set for all treatments was square root transformed and averaged per treatment; (2) the means of the square rooted MCrS rate were plotted against the proportion of signalling males (supplementary material Fig. S1). The relationship was tested with a generalized linear model (GLM) with a binomial error distribution with overdispersion (McCullagh and Nelder, 1989) (supplementary material Table S1); (3) the mean of the log transformed parameter signalling latency was plotted against the proportion of signalling males and a significant relationship was found ($t = -3.186$, $d.f. = 21$, $P < 0.01$) using a GLM. However, when signalling latency was included in the GLM together with MCrS rate, it was rejected, compared with a model with only the MCrS rate ($P = 0.07$). The signalling latency was not related to the proportions of signalling and searching males and was therefore not included in the response index.

The response index values range from 0 (no response) to 1 (strongest response). Note that the response index for a particular treatment can exceed the RI value for unilateral stimulation with natural conspecific female song. Differences in RI between unilateral treatment with the *N. viridula* female calling song and other treatments were tested with a Kruskal–Wallis test following a multiple comparison test (Siegel and Castellan, 1988).

RESULTS

In the first part of the control experiments when no stimulation was present, 21% of the *N. viridula* males emitted vibrational signals,

whereas the percentage of signalling males increased to 63% in the second part after 2 min of stimulation with the conspecific female signal. Nevertheless, when compared with unilateral treatment with the conspecific female song, in both controls the proportion of signalling males was significantly lower (Table 2). Although only the signalling rate (MCrS rate) in the post-stimulation control was significantly lower than in unilateral treatment with the *N. viridula* female song, the non-significant difference in the pre-stimulation control is a result of a low number of singing males ($N = 3$). Five percent of the males were signalling and walking when no stimulation was present, whereas 10% were searching after stimulation. In both, the pre- and post-stimulation control, the proportion of searching males was significantly lower than in unilateral treatment with conspecific female signal. Also the response index was significantly lower in both control situations (Fig. 3A). From these results we conclude that the 2 min pre-stimulation with conspecific female song used in experimental stimulation treatments did not significantly affect the results.

The proportion of signalling and searching males and signalling rate were not significantly affected by the simultaneous presence of two conspecific female signals of equal amplitude (Table 2). However, when these criteria were combined in the response index, the bilateral stimulation was significantly more effective than the unilateral treatment (Fig. 3A). When two conspecific songs had different amplitudes, the signalling rate was significantly higher, whereas the proportion of searching males increased non-significantly (Table 2). Consequently, the response index was significantly higher than for unilateral stimulation. These results indicate that there is no significant interference from two sources and that the presence of more than one source of conspecific female signals has a positive effect on the strength of male response.

The presence of a continuous background signal always had a significant negative effect on the strength of the male response (Fig. 3B). When a continuous signal was applied unilaterally, the proportion of signalling and searching males, the signalling rate (Table 2), and consequently the response index were significantly lower than for unilateral treatment with the conspecific female signal. The significantly reduced values for all three response parameters included in the response index were found in the bilateral treatment with a signal-to-noise ratio -6 dB. By contrast, when the amplitude of the conspecific female song was equal or 6 dB higher than the continuous background signal, only the proportions of signalling males and response indices were significantly lower than for unilateral stimulation with the *N. viridula* female calling song.

The presence of heterospecific female song had significant negative effect on male responsiveness, except in the treatment in which conspecific female song had higher amplitude than *A. hilare* female song (Fig. 3C; Table 2). In unilateral stimulation with the heterospecific signal the proportion of signalling and searching males and signalling rate were significantly lower than in the unilateral treatment with the conspecific female signal. The same response pattern was found when in bilateral stimulation the amplitude of *N. viridula* female song was 6 dB lower than the heterospecific song. The proportion of signalling and searching males and signalling rate were not significantly affected by the presence of a heterospecific female signal of lower amplitude. When songs had equal amplitude, only the proportion of signalling males and response index were significantly lower than for unilateral stimulation with conspecific female song.

Similarly, the presence of conspecific female song with faster than normal signal repetition rate had significant negative effect on

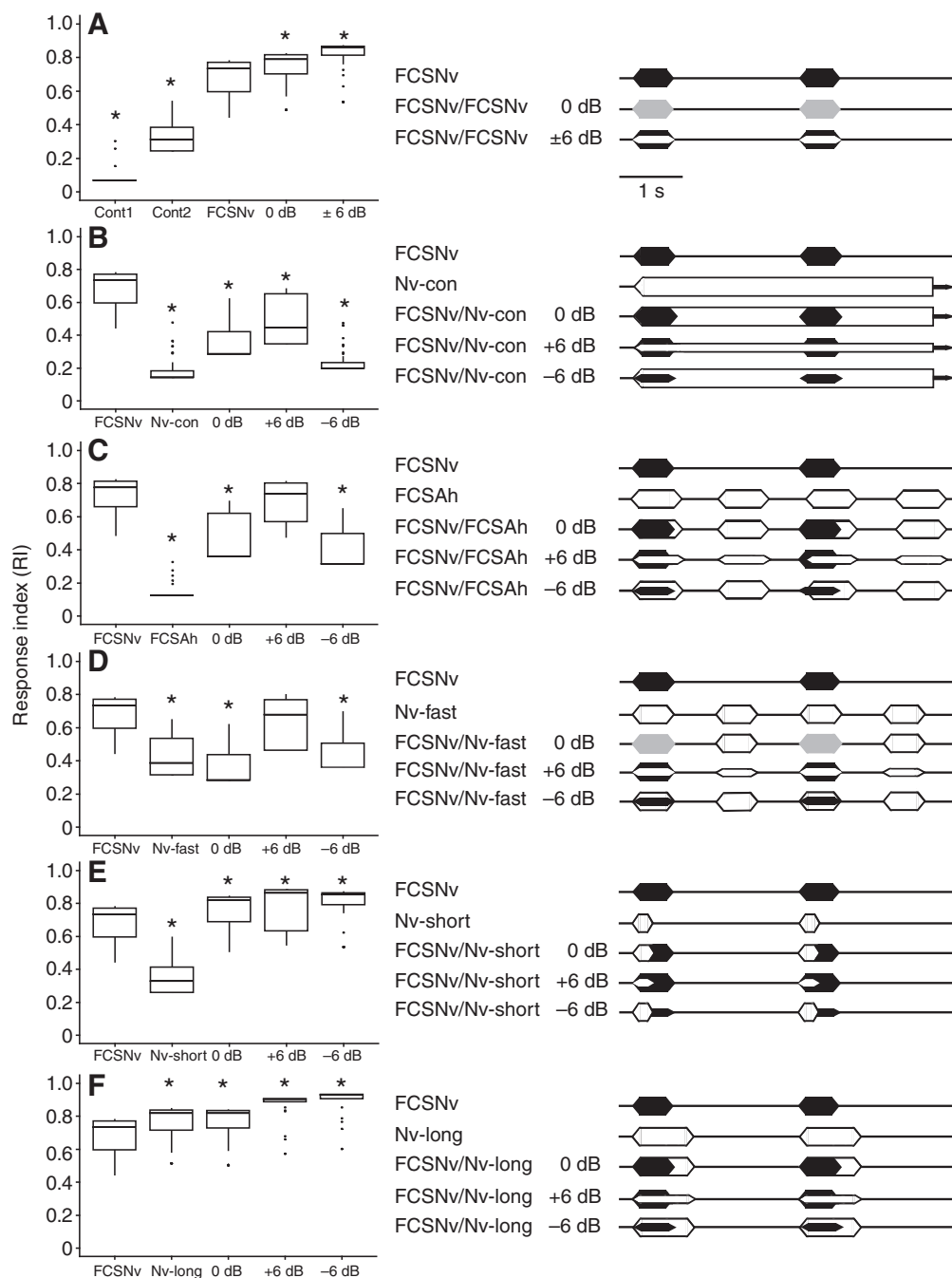


Fig. 3. Response indices for male *Nezara viridula* obtained in each experimental treatment. Box plots show the median, lower and upper quartiles, 95% confidence intervals and outliers. (A) Pre-stimulation (Cont1) and post-stimulation (Cont2) controls and unilateral and bilateral stimulation with conspecific *N. viridula* female song (FCSNv) and different signal-to-noise ratios. (B) Unilateral stimulation with continuous background signal (Nv-con) and bilateral stimulations in combination with FCSNv and different signal-to-noise ratios. (C) Unilateral stimulation with heterospecific female song (FCSAh) and bilateral stimulations in combination with FCSNv and different signal-to-noise ratios. (D) Unilateral stimulation with conspecific female song with faster than normal signal repetition rate (Nv-fast) and bilateral stimulations in combination with FCSNv and different signal-to-noise ratios. (E) Unilateral stimulation with conspecific female song composed of shorter signals (Nv-short) and bilateral stimulations in combination with FCSNv and different signal-to-noise ratios. (F) Unilateral stimulation with conspecific female song composed of longer signals (Nv-long) and bilateral stimulations in combination with FCSNv and different signal-to-noise ratios. In B–F, for comparison, response index values for unilateral stimulation with FCSNv are shown. Schematic presentations of the stimuli are given on the right. Two overlapping identical stimuli of equal amplitude are shown in grey. For numbers of stimulations see Table 2. All values were compared with unilateral stimulation with FCSNv and differences in RI were tested with a Kruskal–Wallis test following a multiple comparison test. *Treatments in which the RI were significantly different ($P < 0.05$). 0 dB: bilateral stimulation with signal of equal amplitude; +6 dB: bilateral stimulation in which FCSNv had a 6 dB higher amplitude; -6 dB: bilateral stimulation in which FCSNv had a 6 dB lower amplitude. For other details see Materials and methods.

the strength of the male response, except in the treatment in which signal-to-noise ratio was +6 dB (Fig. 3D; Table 2). In unilateral stimulation the proportion of signalling males and the signalling rate, but not the proportion of searching males, were significantly lower than in unilateral treatment with natural conspecific female song. By contrast, when in bilateral stimulation with songs of equal amplitudes, the proportion of searching males was also significantly reduced. When unaltered conspecific female song was of lower amplitude (-6 dB), only the signalling rate was significantly lower than for unilateral stimulation with natural *N. viridula* female calling song. These results indicate that pulse repetition time and/or interval duration are important parameters for species recognition.

When conspecific female calling song composed of signals shorter than the preferred value was applied unilaterally, the

proportion of signalling and searching males, the signalling rate and consequently the response index were significantly lower than in unilateral treatment with unaltered *N. viridula* female calling song (Table 2; Fig. 3E). However, in bilateral stimulation the presence of this less attractive female calling song always had a significant positive effect on male responsiveness, regardless of the signal-to-noise ratio (Fig. 3E). When three response parameters included in the response index were analysed separately, significant positive effect was found only in the proportion of signalling males at signal-to-noise ratio -6 dB and in the proportion of searching males when signal-to-noise ratio was 6 dB (Table 2). These results indicate that the presence of additional source of conspecific signals with species-specific pulse repetition time has a positive effect on the strength of male response.

All treatments with conspecific female calling songs composed of signals longer than the mean value, were significantly more effective than unilateral stimulation with the unaltered *N. viridula* female calling song (Fig. 3F). When in bilateral stimulation, signals differed in amplitude, signalling rate and the proportion of searching males increased significantly (Table 2). These results indicate that conspecific signals of longer duration and the presence of more than one female have a positive effect on the strength of the male response.

DISCUSSION

The results of the present study show that recognition and discrimination of conspecific female vibrational signals is reduced in a noisy environment. By contrast, our data also demonstrated that the presence of two sources of conspecific female signals has a positive effect on male responsiveness, as long as the signal repetition time of the perceived signals did not differ from the preferred species-specific value.

In intraspecific communication noise is any signal that interferes with detection of a conspecific signal and transmission of its information (Forrest, 1994). In the field situation two main natural sources of background noise and interference are wind and signals of other individuals (Forrest, 1994; Brumm and Slabbekoorn, 2005; Gröning and Hochkirch, 2008). Animals often emit air-borne sounds or substrate vibrations in aggregations of signalling individuals (e.g. Aubin and Jouventin, 2002; Cocroft, 2003; Greenfield, 2005; Miranda, 2006; Schwartz et al., 2008) and/or in the presence of other signalling species (e.g. Cocroft and Rodríguez, 2005; Marshall et al., 2006; Planqué and Slabbekoorn, 2008). In general, because of matching spectral and temporal characteristics, the most severe masking and interference effects result from acoustic signals emitted by conspecifics (Römer, 1993; Ronacher and Hoffmann, 2003; Bee and Schwartz, 2009). Taking into account that vibrational communication is prevalent in insects (Cocroft and Rodríguez, 2005), it is surprising that there have been few direct studies to determine the influence of background noise on sexual behaviour in species that use substrate-borne vibration for mate recognition and attraction. In general, all four studies known to us showed a negative effect of background noise on mating behaviour. The wind-induced abiotic vibrational noise inhibited male and female signalling behaviour in the *Enchenopa binotata* treehoppers (McNett, 2007). In the presence of interfering vibrational signals males of the leafhopper *Scaphoideus titanus* were less successful in establishing a contact with a female and locating her (Mazzoni et al., 2009). In *N. viridula* interference from continuous background vibrations had a negative effect on male signalling and searching behaviour (Polajnar and Čokl, 2008). In addition, it has been shown that vibrations induced by air-borne synthetic sounds interrupted mating (Saxena and Kumar, 1980). Some indirect information about the incidental interference from heterospecifics has been provided by studies investigating the role of vibrational signals in reproductive isolation. Although the results from two-way choice playback experiments indicate that in males of *Ribautodelphax* planthoppers the presence of heterospecific female signals does not affect recognition and localization of a conspecific female (de Winter and Rollenhagen, 1990), in *N. viridula* signals of heterospecific females reduced male signalling activity and abolished searching for the conspecific female (Miklas et al., 2003b).

The results of our study show that responsiveness of *N. viridula* males is reduced both in the presence of heterospecifics and when the perceived temporal pattern of conspecific vibrational signals differs from the species-specific values characteristic for female

calling signals. Communication in a one-dimensional environment on a plant imposes an important constraint on recognition of conspecific signals in a noisy environment, since the receiver would perceive signals emitted from distant, spatially separated sources, located on the same side of the receiver, as one signal. Although plants have complex three-dimensional geometry, for plant-dwelling insects, stems and branches, nevertheless, still represent a one-dimensional environment. Even insects standing on a two-dimensional surface such as a leaf would perceive vibrational signals that are emitted on other leaves as signals transmitted through a one-dimensional environment of stalks and stems (i.e. a combined signal would reach a receiver from one side). Reduced responsiveness could therefore result from masking due to spectral and temporal overlap of the signals (Römer et al., 1989; Greenfield, 1993; Marshall et al., 2006) and/or degradation of signal parameters resulting from overlap (e.g. Römer, 1993; Römer and Krusch, 2000; Schwartz et al., 2008) and/or obscuring the species-specific signal repetition time due to alternation of signals (Greenfield, 2005). In addition, whether the amplitude of a compound signal decreases or increases at the position of the receiver depends on the frequency filtering, temporal distortions and oscillations of the amplitude of the signals(s) during the transmission.

In structurally complex and heterogeneous substrates such as plants, signal transmission results in spectral (Michelsen et al., 1982; Barth, 1998; Magal et al., 2000; Cocroft et al., 2006) and temporal (Michelsen et al., 1982; Miklas et al., 2001) distortions of the signals. Furthermore, the amplitude of a vibrational signal does not decrease monotonically with distance, but oscillates along the stem (Michelsen et al., 1982; Čokl, 1988; Čokl et al., 2007). Not taking into account differences in signal repetition time, it is likely that interactions between overlapping signals emitted from different positions may cause additional degradation and distortion of spectral and temporal properties of vibrational signals, thus rendering them less attractive. However, spectral characteristics of the signal differ with increasing distance from the source (Cocroft et al., 2006) and consequently, different points on a plant have different ratios between amplitudes of different spectral peaks (Čokl, 2008). When males are changing their positions while searching for a female, the signals perceived at different positions, would differ in their amplitude as well as in their spectral characteristics. It has been shown that spatial separation improved discrimination ability in insects and anurans (reviewed by Gerhardt and Huber, 2002; Bee and Micheyl, 2008) and it is interesting to note that our results show, that in general, in the presence of interfering background noise, searching activity was less affected than signalling.

We found that biotic noise arising from two other, alternating conspecific females significantly reduced male responsiveness to unaltered conspecific female song. Although this result suggests that attractive signals could combine to form an unattractive time pattern, it seems more likely that this situation results from the representation of a compound signal in a one-dimensional environment rather than from neuronal processing in the central nervous system. However, more studies are needed to provide information needed to establish whether the processes of recognition and localization are organized in parallel as in grasshoppers (von Helversen and von Helversen, 1995) or serially as in crickets and bushcrickets (Pollack, 1998; Schul et al., 1998; Gerhardt and Huber, 2002). As in other insects, the majority of the vibration receptors in *N. viridula* are located in all six legs (Čokl et al., 2006). Bugs therefore have a spatial array of mechanoreceptors positioned on the substrate, and on plants a time delay between the arrival of the vibrational signal at each leg is a more likely to be a directional

cue than detected as an amplitude difference (reviewed by Virant-Doberlet et al., 2006). However, in the current study, both signal sources were positioned on the same side of the insect and so as long as the male did not reach the leaf junction, a compound signal did not provide any reliable directional cue. Further studies are needed to establish whether males can discriminate between sources that are positioned on different sides of the body. With only one source present, males of *N. viridula* can accurately locate a vibration source, i.e. a female on a plant (Ota and Čokl, 1991; Čokl et al., 1999; Miklas et al., 2003b). In noisy environments, however, the accuracy of location was reduced (M.deG., unpublished results) and in the presence of heterospecific signals males did not start searching (Miklas et al., 2003b). Detailed studies of interactions between overlapping vibrational signals emitted from spatially separated sources, and transmission of such compound signals in plants are needed to determine the constraints that such interactions impose on signal recognition, discrimination and localization.

Although the results of this study show that in the field incidental interference between several conspecific signallers is likely, they also show that the presence of two synchronous sources of conspecific female songs had a positive effect on male responsiveness (i.e. mate-finding effort). The effect of acoustic signalling in groups (chorusing) on receiver behaviour has been extensively studied in insects and anurans (e.g. reviewed by Gerhardt and Huber, 2002; Greenfield, 2005). It has to be noted however, that little is known about chorusing in communication systems in which potential partners form a duet, and males approach the female (e.g. Tauber, 2001; Tauber et al., 2001; Kotiaho et al., 2004). Since all available information about chorus size (i.e. number of individuals present) on receiver behaviour relates to the air-borne sound communication systems in which silent females approach stationary singing males and therefore includes female preference as well as female choice (e.g. Doolan, 1981; Shelly and Greenfield, 1991; Gerhardt and Huber, 2002), direct comparison with our results is not possible. For these communication systems it has been shown that although larger male groups attracted more females (e.g. Doolan, 1981; Doolan and Mac Nally, 1981; Schwartz, 1994; Gerhardt and Huber, 2002), the attraction *per capita* (e.g. Schwartz, 1994) or mating success (e.g. Doolan, 1981) were not increased. In addition, signalling in aggregations increases the levels of interference (acoustic as well as behavioural).

We did not investigate the mechanisms that enable detection of several sources of conspecific signals and discrimination between them, however, there are several possible mechanisms. One is that due to interactions between two overlapping signals, the increase in male responsiveness results from higher amplitude of a compound signal. However, measurements indicate that even in bilateral stimulation with identical signals of equal amplitude, the amplitude of a combined signal did not differ substantially from the signal amplitude in the unilateral stimulation (amplitude ranged from 1 dB higher to 2 dB lower than in unilateral situation; M.deG., unpublished data). This is supported by observations that in this treatment none of the behavioural response parameters included in the response index differed significantly from the unilateral treatment. Although we cannot exclude the possibility that due to complex vibrational interactions, at some positions along the plant, signals reinforce each other, thus resulting in a combined signal of a higher amplitude, interactions that would increase signal amplitude are also likely under any natural condition when signals are overlapping. Observations that male responsiveness was higher when overlapping signals had different amplitude indicate that interactions other than amplitude reinforcement might be involved. Another possibility is

that at the position of the receiver, frequency filtering and amplitude oscillations that occur during signal propagation result in distortions that enable males to determine that conspecific female signals are emitted from two sources. Another possibility is that processes such as stream segregation (grouping of sounds from several sources into a meaningful stream of information identifying individual sources) (Brumm and Slabbekoorn, 2005; Bee and Micheyl, 2008) might be involved in discrimination. Auditory stream segregation has been mainly described in humans and other vertebrates (reviewed in Bee and Micheyl, 2008), but it has been recently been demonstrated also in insects (Schul and Sheridan, 2006). Detailed experimental data on interactions between overlapping vibrational signals as well as on processing of vibrational signals in the central nervous system are needed before these questions can be answered.

Signalling behaviour should evolve to increase the probability of responses from the receiver (e.g. Endler, 1993). In *N. viridula* volatile male sex pheromone serves to attract females to the plant with a male (Borges et al., 1987) and to trigger emission of female calling song (Miklas et al., 2003a), which in turn, has a positive feed-back effect on emission of male sex pheromone (Miklas et al., 2003a). In the field many individuals often gather on one plant (Clarke and Walter, 1993) and several females could simultaneously emit vibrational signals (Miklas et al., 2003b). With currently available data, we are not yet able to determine whether signalling of *N. viridula* females is coordinated or not. Since substrate-borne vibrational songs are inaudible to humans and because of the technical difficulties, the field studies of vibrational communication are, in general, rare (Cocroft and Rodríguez, 2005). Results from laboratory studies show that females of *N. viridula* continue signalling in the presence of incidental background noise from conspecifics (Polajnar and Čokl, 2008). Although some females did change the signal duration and/or signal repetition time during stimulation, this study suggested that a more important strategy for reducing interference from competitive signallers may be the frequency shift in their vibrational signals. Other observations suggested that females might not synchronize their signals (M.deG., unpublished data). However, the above mentioned studies of female signalling behaviour did not take into account the effects of male pheromone and/or male vibrational signals. It is possible, that in the sustained presence of male sex pheromone and male vibrational signals females would also modulate the temporal parameters of their songs (i.e. synchronize). Further studies of female signalling behaviour under more realistic conditions are needed to elucidate the links between plant transmission properties, constraints of a one-dimensional environment for species recognition and adaptations of the emitter.

Results of the present study show that except in the treatments with continuous background noise, increased signal-to-noise ratio restored male responsiveness to a level that did not differ significantly from that of unilateral stimulation with conspecific female song. Improved recognition and discrimination of signals at higher signal-to-noise ratios is a common phenomenon in air-borne sound communication (e.g. Forrest, 1994; Brumm and Slabbekoorn, 2005; Langemann and Klump, 2005; Bee and Schwartz, 2009). The evidence that emitters increase the signal amplitude in the presence of background noise has been found in mammals, birds and anurans (Brumm and Slabbekoorn, 2005; Penna and Hamilton-West, 2007). Although it has been reported that males of the grasshopper *Ligurotettix coqueillei* sing louder when in aggregations than when alone (Shelly and Greenfield, 1991), it is generally not clear whether insects can control the amplitude of their signals (Brumm and Slabbekoorn, 2005; Fertschai et al., 2007). Adjusting signal amplitude

in response to background noise presents additional challenges for plant-dwelling insects relying on vibrational communication. The intensity of vibrational signals of *N. viridula* measured as a velocity on the plant surface below a singing bug varied between 3 and 18 mm s⁻¹ on a bean leaf (Čokl, 2006), and 0.33 and 0.77 mm s⁻¹ on a sedge leaf (Čokl et al., 2007). The amplitude of the vibrational signal oscillates during the transmission and in a bean stem differences between regularly repeated minima and maxima range between 5 dB below and 10 dB above the value at the source (Čokl et al., 2007). Although higher signal amplitude at the source would certainly increase the broadcast distance, it would not simultaneously assure higher signal-to-noise ratio at the position of the receiver. In the presence of continuous background signal of the same frequency as their vibrational signals females of *N. viridula* did not increase the amplitude of emitted signals, but rather shifted the dominant frequency away from the masking signal (Polajnar and Čokl, 2008). The peak amplitude of male vibrational signals differed between age groups in the treehopper *Umboia crassicornis*, however, observed variation was attributed to physiological factors such as development of motor pattern and/or physical condition associated to age-related changes (De Luca and Cocroft, 2009). More detailed studies are needed to determine the effects of different levels of signal-to-noise ratio on male and female mating behaviour.

Taking into account the number of challenges imposed by a one-dimensional environment and signal transmission through plants, it is likely that in noisy environments males will often encounter ambiguous situations in which recognition of a conspecific female at long range would be difficult. In such situations reduced reproductive success of a male can result either from missing a conspecific female or to mate with heterospecifics. In air-borne sound communication, signalers and receivers use a variety of mechanisms to avoid masking effects of background noise (e.g. Brumm and Slabbekoorn, 2005). Based on the results of the current study and previous work we can make several predictions about the strategies for vibrational communication in a noisy environment. Although, because of the lack of information available for other insect species, predictions are based on knowledge obtained in *N. viridula*, they are relevant also for other plant-dwelling insects. First, males in the presence of background noise from conspecifics males will signal less in response to a conspecific female, but they will keep moving around the plant, even at low signal-to-noise ratio. Moving to different positions along the plant would increase the likelihood of a better signal-to-noise ratio as a result of frequency filtering and amplitude oscillations of the signals occurring during the propagation through the plant. By contrast, we would expect that when heterospecific signals have higher amplitude than conspecific female signals, males will reduce their searching activity. Such strategy would increase the chances of finding a conspecific female and reduce the possibility of interspecific matings. Second, temporal parameters of vibrational signals are crucial for species and sex recognition (Čokl et al., 1978; Hrabar et al., 2004). Thus, the male has to perceive species-specific signal repetition time, and therefore synchronization between females would be adaptive (Greenfield, 2005). Owing to constraints imposed by a one-dimensional environment, males would perceive alternating signals emitted by two or more conspecific females as one signal with repetition time outside the species-specific value. However, under natural conditions it is unlikely that relative positions of the male and several signal sources (i.e. females) would be the same. Furthermore, the amplitude of emitted signals would probably differ substantially between females already at the source (Čokl, 2006; Čokl et al., 2007). Our results suggest that even in the presence of

alternating conspecific female signals, the male would not reduce his searching activity for as long the female signals differed in amplitude. Such strategy would increase the probability of encountering a conspecific female. Third, we would expect that males would increase their signalling and searching activity in the presence of more than one signalling conspecific female. Such strategy would reduce the time needed for localization of a female.

Although our results provide some much needed data regarding the interference of heterospecific and conspecific signals in vibrational communication, further detailed studies are needed to elucidate complex intraspecific interactions indicated by the current study. Although *N. viridula* is one of the best investigated model species for studies of vibrational communication (Virant-Doberlet and Čokl, 2004; Čokl, 2008), little is known about its communication and behavioural strategies in the field. Furthermore, more detailed studies are needed to characterize the sources and relevance of biotic background noise in order to improve our understanding of sources of constraints and selection on evolution of vibrational communication.

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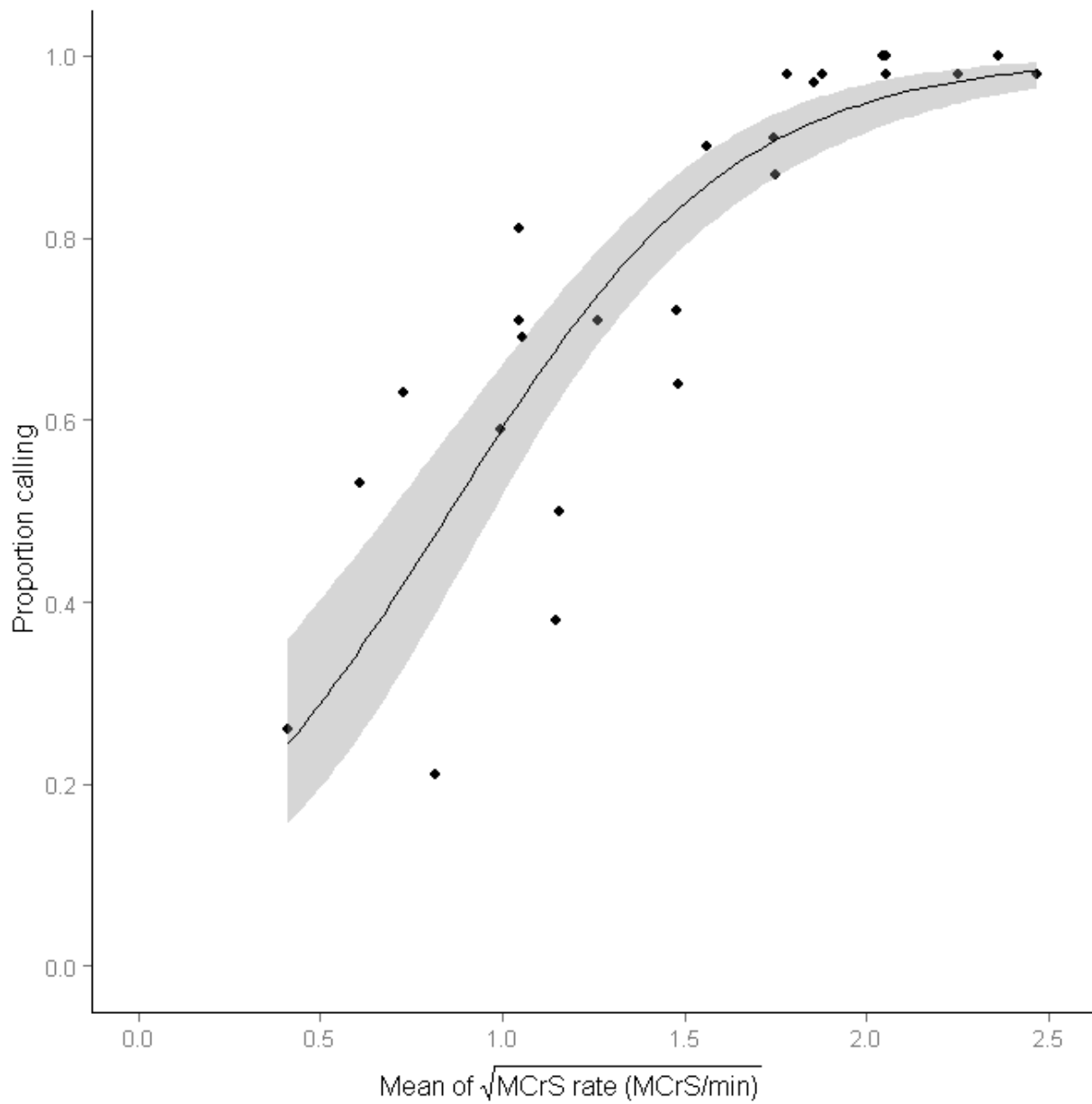


Table S1. Test statistics for the relationship between the proportion of signalling males and the mean of square rooted MCrS rate using a GLM model.

	Estimate	Std. error	<i>t</i> -Value	<i>P</i>
Intercept	-2.223	0.4551	-4.884	<0.0001
MCrS rate	2.6023	0.351	7.413	<0.0001