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Specificity of Male Responses to Female Vibratory Signals in two *Chinavia* Species (Hemiptera: Pentatomidae) is Based on Signal Structure and Narrow Temporal Parameters

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Abstract – In this work, we tested whether variations in temporal pattern and architecture of the vibratory signals favor signals recognition and discrimination in two stinkbug species, *Chinavia ubica* and *C. impicticornis.* To relate the level of species recognition with species-specific vibratory signal we exposed males to natural or artificial signals. Different artificial signals were synthesized by changing the basic structure or temporal parameters of typical female calling signals of each species. Signals were transmitted to bean plants and the response of males was observed and recorded by a piezoelectric accelerometer. Results show that changes in temporal patterns of artificial signals significantly reduced the proportion of males responding by emitting the male song. Our results confirm that specific elements of male vibratory signals are critical for female signal recognition and discrimination by males and could contribute to prezygotic isolation in sympatric *Chinavia* species.

Keywords – Neotropical stinkbugs, Communication, Sexual signals, Reproductive behavior, Signal architecture

Communication during reproductive behavior enables information interchange between signalers and receivers (Greenfield, 2002). The phenotypic architecture of signals enables pair recognition and discrimination of conspecifics. This, in turn could be relevant to maintain behavioral isolation in closely related species (Mendelson & Shaw, 2012; Ryan & Rand, 1993). However, hybridization takes place in closely related species when characteristics of signals or preferences of receivers overlap (Gröning & Hochkirch, 2008). In this way, knowing the specific components of signals that enable conspecific recognition could help us to understand reproductive behavior at neurological, physiological and ecological levels.

Pentatomidae reproductive behavior includes long-range communication with airborne male-produced chemical signals that attract females to herbaceous or shrubby plants occupied by males (Borges & Blassioli-Moraes, 2017). The male sex pheromone triggers emission of the female calling song (Zgonik & Čokl, 2014), which initiates male vibratory responses and searching for the calling female (Čokl, Laumann, & Stritih, 2017).

The relevance of stinkbug signals for recognition (species and sex) and selection (quality) of the right partner is not yet fully understood. Stinkbug male pheromones show extreme diversity in their chemical structure and composition (Moraes, Pareja, Laumann, & Borges, 2008), together with interesting variation in their function. In Neotropical stink bugs, male pheromones selectively attract females; on the other hand, male pheromones in Nearctic species attract both females and nymphs and appear to have an aggregative function (Borges & Blassioli-Moraes, 2017; Weber, Khrimian, Blassioli-Moraes, & Millar, 2017). Despite these differences, the first step in the formation of mates for copulation is triggered by the high species-specificity of the pheromone molecules involved in this process (Borges & Blassioli-Moraes, 2017; Moraes et al., 2008). In some green stink bugs, species of the Nezarini tribe, pheromone constitution is based on the same chemical compounds, and its specificity is achieved by different ratios between them (Aldrich, Oliver, Lusby, Kochansky, & Lockwood, 1987; Blassioli-Moraes et al., 2012; Borges, Jepson, & Howse, 1987; Brezot, Malosse, Mori, & Renou, 1994; McBrien, Millar, Gottlieb, Chen, & Rice, 2001).

Vibratory signals of species-specific temporal and spectral characteristics have been described in more than 30 stinkbug species (Čokl et al., 2017). Temporal characteristics of low-frequency narrow-band stinkbug species-specific vibratory signals determine mate recognition during substrate-borne communication through plants (de Groot, Čokl, & Virant-Doberlet, 2010; Miklas, Stritih, Čokl, Virant-Doberlet, & Renou, 2001; Žunič, Virant-Doberlet, & Čokl, 2011). Disrupting the duration and/or repetition time of species-specific signals by environmental noise and conspecific or heterospecific signals decreases their informational value (de Groot, Čokl, & Virant-Doberlet, 2011; Laumann, et al., 2018; Polajnar & Čokl, 2008).

Chinavia ubica (Rolston, 1983) and *Chinavia impicticornis* (Stål, 1872) are two sympatric Neotropical species of Nezarini with wide geographic distribution in South America; they are found both in natural and agricultural habitats on the same host-plants (Panizzi, McPherson, James, Javahery, & McPherson, 2000; Schwertner & Grazia, 2007). Males and females of both species emit two types of vibratory signals (Laumann, Čokl, Blassioli-Moraes, & Borges, 2016; Moraes, Laumann, Čokl, & Borges, 2005). The species-identified female calling song stimulates the male to emit his calling song and directs him to move on the plant to the calling female. Male vibratory responses stimulate the female to produce another type of calling song signal to establish the duet with the male. At close range, males emit the courtship song that silences the female and starts mating displays that culminate in copulation (Laumann et al., 2016; Moraes et al., 2005).

Species-specific sex pheromones (Blassioli-Moraes et al., 2012) and temporal characteristics (Laumann et al., 2016) of *C. impicticornis* and *C. ubica* vibratory signals play an important role in preventing heterospecific mating. However, there are no data on the specific role of the temporal pattern and architecture of the female calling song in the process of recognizing a mate from the same species during substrate-borne vibratory communication. We hypothesize that the specific architecture (temporal pattern and spectral parameters) of the vibratory signals are fundamental for conspecific signals recognition in *C. ubica* and *C. impicticornis*. The objective of our investigation was to confirm or reject this hypothesis by correlating the level of species recognition with responses to playback signals of various temporal characteristics and different architecture.

Methods

Subjects

Insects. For all experiments we used insects from laboratory colonies of *C. ubica* and *C. impicticornis*, established at Embrapa Recursos Genéticos e Biotecnologia (Brasília, DF, Brazil) from species collected in soybean fields in central Brazil (Federal District and Goiás state). Species were identified following Schwernter and Grazia (2007). Insects were reared for two years in laboratory conditions over more than 20 generations and were maintained on a natural diet based on sunflower seeds, soybeans, and fresh pods of common beans. Nymphs and adults were kept in separate cages in an

acclimatized room (26 ± 10 ˚C, 65 ± 10% RH, photoperiod 14HL:10HD). After the last molt, the adults were separated by sex and kept in separate cages. Adults were used in bioassays when they reached sexual maturity (i.e., after 10 days in the adult stage; Silva, Laumann, Blassioli-Moraes, Aquino, & Borges, 2015).

Plants. The experiments were conducted on common bean plants (*Phaseolus vulgaris* L.). The seeds were planted in plastic pots, 10 cm high and 11 cm in diameter, filled with sterile soil and substrate for plant growth (Tropstrato HA Hortaliças) mixed in equal proportions. These pots were kept in a greenhouse for germination and growth, at 27 ºC and photoperiod of 14 hr of photophase. All plants were used in the vegetative stage, approximately 15 days after germination. At this phenological stage, the bean plants were 15 to 20 cm high and characteristically carried two unifoliolate and two tripholiate leaves (Laumann et al., 2011).

Design and Procedure

All experiments were conducted at Embrapa Recursos Genéticos e Biotecnologia in a sound- and vibration-insulated room. We placed the experimental plants on a shockproof table to reduce environmental vibratory noise. Observations and recordings were conducted between 10:00 h and 18:00 h under artificial light (2 florescent lamps of 40 W) at 25 ± 2 ºC temperature and 70% relative humidity.

Males of each species were placed individually on the plants on one of the trifoliate leaves and their behavior was observed for 20 min. After the male stopped moving, we vibrated the plant by the stimulation program (see below) running continuously throughout the test.

The experimental design was completely randomized (20 males were evaluated for each artificial signal and species) with alternation of the evaluated signals and males of each species in each bioassay. In addition, to evaluate the physiological conditions (the male responding level) on each day of experimentation, the responses of a group of 3 to 5 males of each species were evaluated by stimulation with conspecific female signals (control signals). A sub-sample of responses of 20 males to the latter signals was randomly selected for the statistical analyses.

Insect behavior was monitored by direct observation during all the experimental periods, and behavior and signal characteristics were recorded. We recorded the following parameters: (1) the number of males responding to each reproduced signal, (2) the number of males that oriented to the source of vibration, (3) the male response latency time as the time between start of stimulation and first male emitted vibratory signal (in seconds), (4) the duration of male responses by vibratory signals to playback stimulation and (5) the rate of responses, determined as the number of male response signals per number of playback signals.

Male vibratory response signal frequency and temporal characteristics were analyzed with Sound Forge software version 6.0 (Sonic Foundry, Inc., Madison, California, USA). The variables that were analyzed were: duration of the pulse train (time in milliseconds from the beginning to the end of the pulse train), repetition time (interval in milliseconds from beginning of a pulse train to beginning of the next pulse train) and dominant frequency (frequency with greatest amplitude, in Hz). Pulse was defined as a unitary homogeneous parcel of vibration of finite duration, pulse trains as repeatable and temporally distinct groups of pulses and a song as a sequence of pulses and/or pulse trains with a distinct beginning and end (Broughton, 1963).

Plant vibration and signal recording. Stimulation programs were synthesized by artificial signals or by naturally emitted signals recorded from insects placed on non-resonant substrates (loudspeaker membranes; Laumann et al., 2016). Playback signals were reproduced from a computer with an external sound card (Creative, Sound Blaster X-fi 5.1 Pro) using Sound Forge 4.5 software (Sonic Foundry Inc., Madison, Wisconsin, U.S.A.).The plant was vibrated by an electrodynamic exciter (mini-shaker Model 4810, Bruel & Kjaer, Naerum, Denmark) by attaching a steel pin to the exciter top and a pical tip inserted into the plant stem approximately 10 cm above the soil surface. The horizontally positioned vibration exciter was mechanically isolated from the substrate by an iron support coated with polyurethane foam.

The male vibratory response signals were recorded throughout the whole 20 min period of the test by a piezoelectric accelerometer (1000 mV/g, Brüel & Kjaer, type 4508, Naerum, Denmark), coupled to the stem approximately 5 cm above the point of emission of the stimulatory signals. The recorded signals were digitized by an external sound card (Edirol-Roland, 24 bits, 96 KHZ, model UA-25EX), stored on a computer using Cool Edit Pro software, version 2.1 (Syntrillium Software 2001) at 24 kHz and 100 dB signal-to-noise ratio, and analyzed with Sound Forge software.

Stimulation programs: natural and synthetic signals. Stimulation programs consisted of a sequence of natural or synthetic pulse trains that were looped during all the 20 min test period. For natural stimulation programs (control), a sequence of FS-1b pulse trains of three different individuals were pre-recorded from non-resonant substrate (loudspeaker membrane; Laumann et al., 2016). Thus, the stimulation program consisted of a 3 min sequence of pulse trains of FS-1b of *C. ubica* or *C. impicticornis*, with a total of 42 and 40 pulse trains, respectively.

The artificial stimulatory signals were assembled, with the synthesis function of the software Sound Forge, considering temporal and spectral characteristics of the FS-1b type of both species' female calling song, as described by Laumann et al. (2016). Males were stimulated by five types of artificially synthesized playback signals, built by changing the characteristics of natural FS-1b female calls (Laumann et al., 2016; see Table 1) as:

Artificial signal 1 (AS1): the long pulse with half duration of the mean value of the natural tested signals without the short pulses.

Artificial signal 2 (AS2): the long pulse with half duration of the mean value of the natural tested signal with the short pulses of the same duration as mean values of the natural recorded signals.

Artificial signal 3 (AS3): the long pulse with duration of twice the mean value of the natural tested signal with the short pulses of the same duration as mean values of the natural tested signals.

Artificial signal 4 (AS4): the long pulse with duration of twice the mean value of the natural tested signal, without the short pulses.

Artificial signal 5 (AS5): the long pulse with the mean duration value of the natural tested signal and without the short pulses.

Statistical analyses. The relation between responses and orientation to artificial signals with respect to natural signals was evaluated by calculating the odds ratios between each treatment and control (natural signal). Using the methodology described in Rumel (1986) and Szumillas (2010), the odds ratios and their respective confidence intervals (95%) were calculated to estimate the chance of response to artificial signals in relation to the natural ones. Odds ratios < 1 indicate a lower response probability in relation to the natural signals; odds ratios = 1 indicate the same response in relation to the natural signals; odds ratios > 1 indicate a higher probability of response than natural signals. To evaluate the latency time and duration of the male songs in relation to each artificial signal, analyses of variance (ANOVA) were performed and, when necessary, the mean values between the treatments were compared using Tukey's test, after checking for normality with the Shapiro-Wilk test. The mean values of the time (pulse train duration and pulse train repetition time) and spectral (DF) parameters of the signals emitted by the tested males in each treatment were compared using mixed linear models analyses. In the model, the treatment (type of stimulus signal) and a random factor (individual) were incorporated. To conduct the analyses, the lme4 package was used, and the probability values and confidence intervals were calculated using the Markov chain Monte Carlo method with 10,000 randomizations. The significance between the values of the parameters obtained in each treatment in relation to the control treatment was established with a t-test. A generalized linear model with binomial error distribution and treatment (type of stimulus signal) as a fixed factor was performed to compare the relationship between the pulse trains emitted by the males in relation to the total number of stimulus pulses emitted (treatments). All analyses were performed using R platform 3.4.2 (R Development Core Team, 2012).

Table 1

Time and Spectral Characteristic of Natural and Artificial FS-1b Signals of Chinavia ubica and C.impicticornis used as Stimuli presented to Males in Plant Experiments

Signal	Pulse train duration(ms)	Pulse train repetition time (ms)	Pulse Train interval (ms)	Duty cycle	Pulse train dominant frequency (Hz)	Long pulse duration (ms)	Short pulse duration(ms)	Number of pulses in pulse train
Chinavia ubica								
Natural(1)	1274.94 ± 225.70	2836.33 ± 770.56	--	0.45	110.46 ± 2.62	841.39 ± 132.63	--	3.21 ± 1.11
Natural Cu	847.00 ± 90.66	2497.00 ± 249.53	1653.46 ± 257.77	0.34	112.43 ± 1.27	512.00 ± 69.97	147.38 ± 18.22	3.55 ± 0.55
AS1 Cu	256	1740	1484	0.14	112	256	--	1
AS2 Cu	*1006*	*2364*	1484	*0.42*	112	256	150	*4*
AS3 Cu	1963	*3447*	1484	0.57	112	1024	150	*4*
AS4 Cu	*1034*	*2518*	1484	*0.41*	112	1024	--	1
AS5 Cu	512	1996	1484	0.26	112	*512*	--	1
Chinavia impicticornis								
Natural(1)	1068.20 ± 182.81	3088.15 ± 352.48	--	0.36	81.46 ± 2.86	707.84 ± 243.55	--	2.54 ± 0.99
NaturalCi	1128.53 ± 79.22	2654.58 ± 350.96	1526.05 ± 368.15	0.43	82.43 ± 1.74	892.05 ± 53.34	191.48 ± 27.15	2.08 ± 0.27
AS1 Ci	446	*2229*	1784	0.25	82	446	--	1
AS2 Ci	*1050*	*2834*	1784	0.37	82	446	200	*2*
AS3 Ci	2400	4184	1784	0.57	82	1784	200	*2*
AS4 Ci	1784	3568	1784	0.50	82	1784	--	1
AS5 Ci	*892*	*2676*	1784	0.33	82	*892*	--	1

Note. (1) Data characteristic of the species reported by Laumann et al. (2016). Natural Cu and Natural Ci: data from pulses trains of 3 different females of *C. ubica* (Cu) and *C. impicticornis* (Ci) selected and used to build up the stimulation programs used in the bioassays (Pulse train, N = 40 for *C. ubica* and N = 42 for *C. impicticornis*). See text for description of artificial signals. Values in italic show time or spectral values similar to those of natural signals.

Results

Changes in temporal patterns of artificial signals significantly reduced the proportion of males responding to stimulation by emission of the male song (Figure 1). The probability ratio of the response to artificial signals in relation to the natural signals was significantly lower in males of both species (Table 2), except for stimulation of *C. impicticornis* males with AS4 Ci signals (Table 2). A similar result was found for males of both species when correlating orientated movements (searching) to the source of playback signals with changed temporal pattern characteristics (Figure 1 and Table 2).

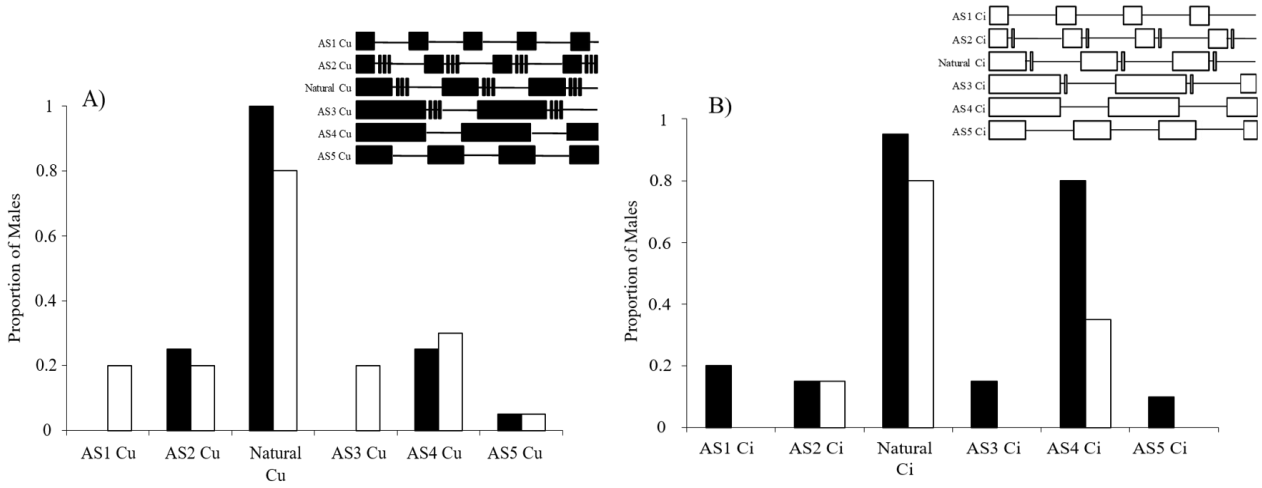

Figure 1. Proportion of males of *Chinaviaubica* (**A**) and *C. impicticornis* (**B**) responding to natural and artificial signals of conspecific female song (FS-1b). Black bars indicate males emitting vibratory signals, and white bars indicate insects walking toward signal sources. See text for description of artificial signals.

Table 2

Odd Ratios and 95% Confidence Intervals for Males of Chinavia ubica and C. impicticornis in Response to Artificial Signals in Relation to Natural Signals (FS-1b) of Females of each Species

Treatment	Response (vibratory signal emission)	Orientated movements (searching)
Chinavia ubica		
AS1 Cu	NO	0.063 (0.013 - 0.29)
AS2 Cu	0.017 (0.002 - 0.17)	0.063 (0.013 - 0.29)
AS3 Cu	NO	0.063 (0.013 - 0.29)
AS4 Cu	0.017 (0.002 - 0.17)	0.107 (0.025 - 0.46)
AS5 Cu	0.003 (0.0002 - 0.05)	0.022 (0.0013 - 0.13)
Chinavia impicticornis		
AS1 Ci	0.0132 (0.0013 - 0.1089)	NO
AS2 Ci	0.0093 (0.0009 - 0.0767)	0.0196 (0.003 - 0.219)
AS3 Ci	0.00929 (0.0008 - 0.0766)	0.0278 (0.0045 - 0.172)
AS4 Ci	**0.211 (0.021 - 2.053)**	0.037 (0.0063 - 0.219)
AS5 Ci	0.0059 (0.00049 - 0.0481)	NO

Note. Odd ratios < 1 indicate reduced responses in relation to responses to natural signals, odd ratio = 1 (or including 1 in the CI 95%) indicate similar responses of males to artificial and natural signals and odd ratios > 1 indicate responses to artificial signals higher than to natural signals. Non-significant responses are marked with bold letters. NO = represents not observed behaviors. Numbers in bold indicate non-significant differences in odds ratios in relation to natural signals.

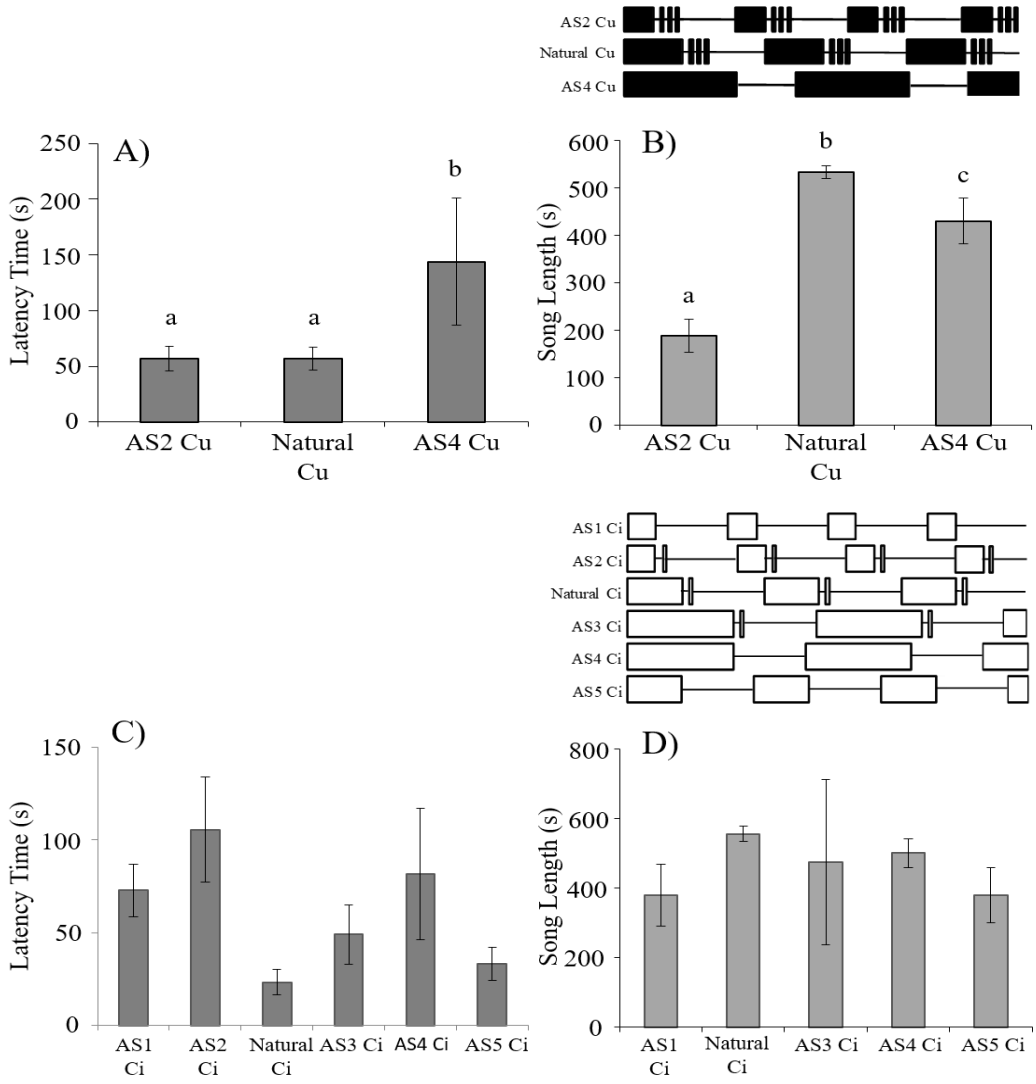

Figure 2. Mean latency time (s) and song length (s) of males of *Chinavia ubica* (panels **A** and **B**) and *C. impicticornis* (panels **C** and **D**) responding to natural and artificial signals of conspecific female song (FS-1b). Bars indicate mean values and lines standard error of parameters. Bars with same lowercase letters indicate non-significant differences in mean values ($p < .05$). For *C. impicticornis* ANOVA showed non-significant effect of treatment for the two parameters. For AS2 Ci signals only two males responded and emitting only one MS1 pulse train so the song length was not calculated.

Males of both species responded to stimulation programs by the emission of MS-1 (Laumann et al., 2016). Only two types of artificial signals stimulated males of *C. ubica* to emit enough pulse trains to be statistically analyzed. In this case, males responded significantly later to artificial signals when the long pulse of the pulse train had a duration (AS4 Cu) twice as long as that of the natural signal, F (2, 26) = 4.18, p = 0.03 and Tukey test $p < .05$; see Figure 2A. Natural signals also stimulated *C. ubica* males to sing longer, F (2, 25) = 48.8, $p < .001$ and Tukey test $p < 0.05$; see Figure 2B). In *C. impicticornis*, latency time and song duration, respectively, were not significantly different between treatments and control, F (5, 42) = 1.11, p = .37; F(4, 40) = 1.62, p = .19; see Figure 2 C and D.

The repetition time of *C. ubica* MS-1 pulse trains increased above the value for the natural signal when stimulated with artificial signals with shorter (AS2 Cu) or longer (AS4 Cu) duration of the long pulse (AS2 Cu, t = 2.75, p = .006 and AS4 Cu, t = 3.07, p = .002, total observations = 509, groups: individual = 155; Figure 3B). The response rate (i.e., the relationship between the number of pulse trains emitted by males and the number of pulse trains of stimulus signals) increased in relation to the natural signal when stimulated by AS2 Cu signals (t (24) = 7.19, $p < .001$; Figure 3D) and decreased by

stimulation with AS4 Cu (t (24) = 3.39, p < .001; Figure 3D). Non-significant differences were observed by comparison of MS-1 pulse train duration and dominant frequency.

Males of *C. impicticornis* stimulated with AS1 Ci, AS2 Ci or AS3 Ci responded with shorter pulse train duration than in responses to natural signals (AS1 Ci t = 3.07, p = .002; AS2 Ci t = 3.05, p = .002; AS3 Ci t = 2.64, p = .008, total observations = 771, groups: individuals = 114; see Figure 4A). We measured higher MS-1 pulse train repetition time by stimulation using long pulses of natural and double duration without the short pulses (AS4 Ci t = 3.36, p = .8, total observations = 771, groups: individuals = 114; AS5 Ci t = 2.65, p = .008, total observations = 771, groups: individuals = 114; see Figure 4B). *C. impicticornis* MS-1 pulse trains showed significantly lower dominant frequency when stimulated with AS3Ci (t = 2.11, p = .03), AS4 Ci (t = 2.97, p = .03) and AS5 Ci (t = 7.16, p < .001; total observations = 771, groups: individuals = 114) when compared with responses to natural signals (Figure 4C). Response rates decreased significantly by stimulation with AS1 Ci (t (40) = 4.22, p < .001) and AS5 Ci (t (40) = 2.97, p < .001) in relation to natural signals (Figure 4D).

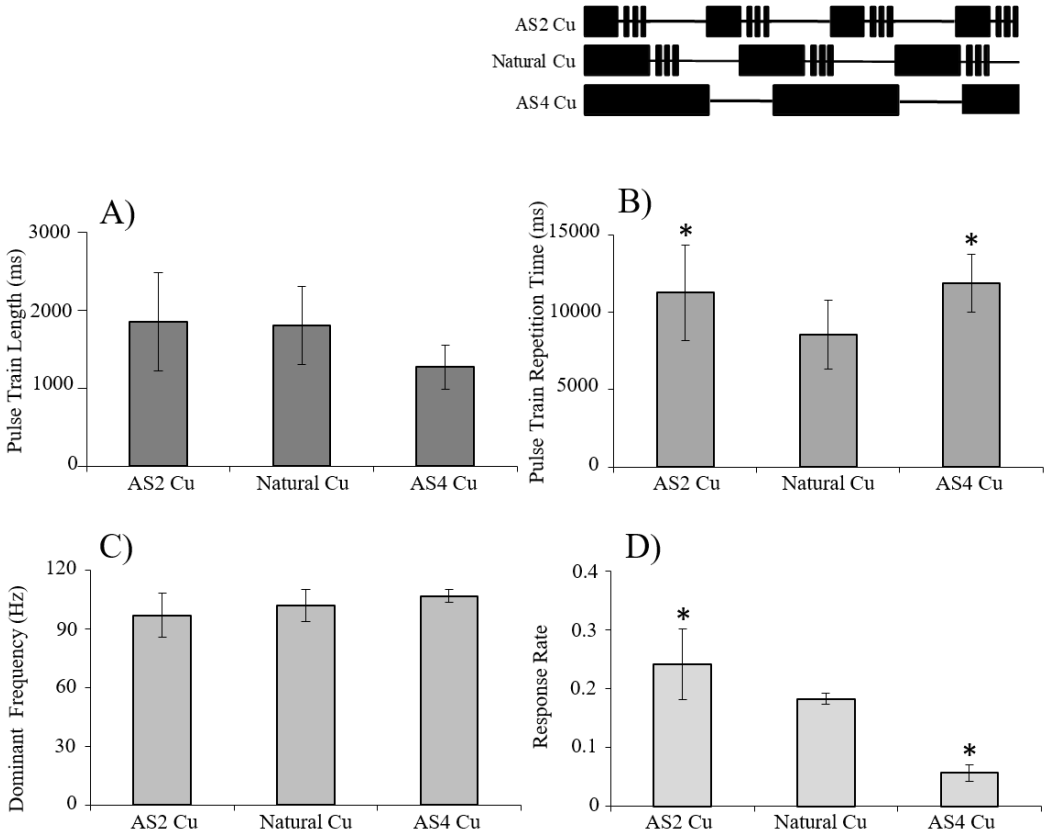

Figure 3. Mean values of pulse train length (**A**), and pulse train repetition time (**B**), dominant frequency (**C**) and response rate (**D**), defined as number of male pulses/number of female pulses, of signals emitted by *Chinavia ubica* males (MS1) in response to natural and artificial signals of conspecific female song (FS-1b). Bars indicate mean values and lines standard error of parameters. * indicates significant differences between parameters in male signals emitted in response to artificial signals, when compared with signal parameters emitted in response to natural signals. Mean values of temporal and spectral parameters were compared using a mixed linear model and t-test, and response rate with a GLM with binomial error distribution (p < .05).

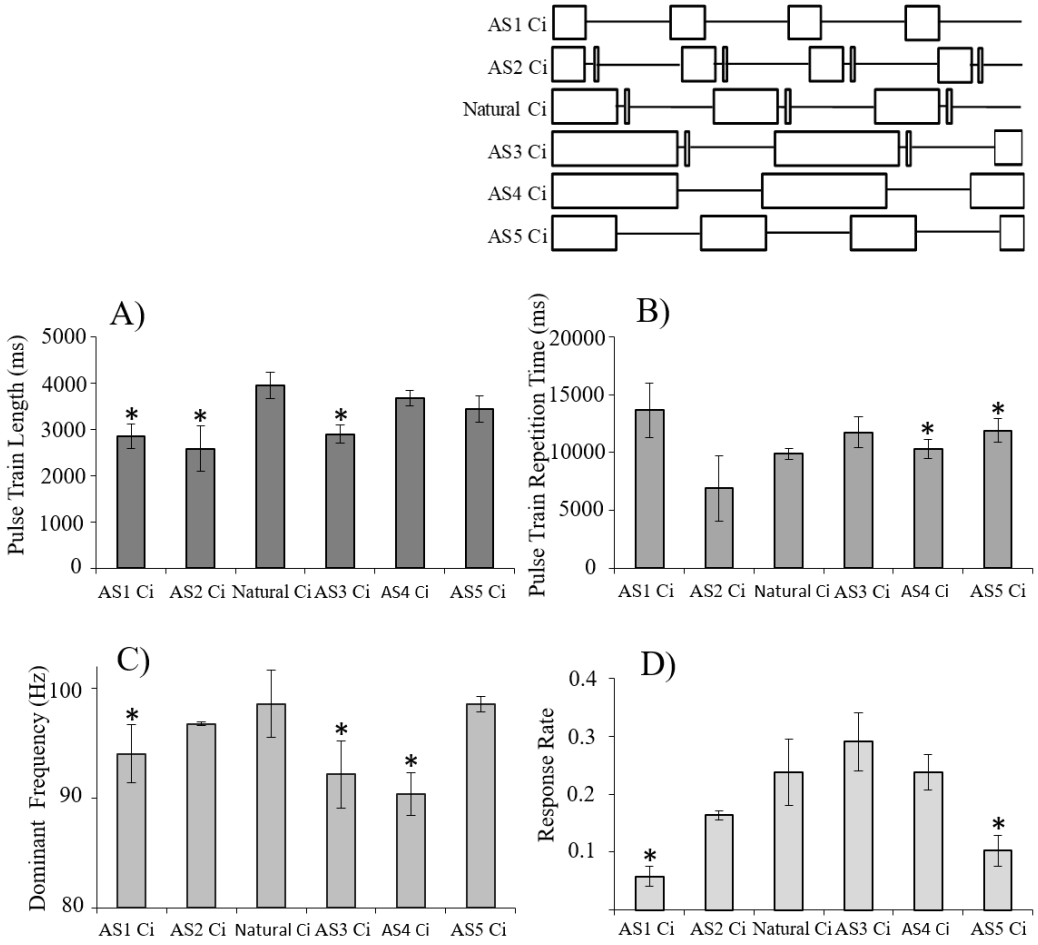

Figure 4. Mean values of pulse train length (**A**), pulse train repetition time (**B**), dominant frequency (**C**) and response rate (**D**), defined as number of male pulses/number of female pulses, of signals emitted by *Chinavia impicticornis* males (MS1) in response to natural and artificial signals of conspecific female song (FS-1b). Bars indicate mean values and lines standard error of parameters. * indicates significant differences between parameters in male signals emitted in response to artificial signals, when compared with signal parameters emitted in response to natural signals. Mean values of temporal and spectral parameters were compared using a mixed linear model and t-test, and response rate with a GLM with binomial error distribution ($p < .05$).

Discussion

Variation of female calling song signal patterns and their temporal parameters significantly modulated male responses in both investigated *Chinavia* species. Pulse trains with reduced or doubled duration of the long pulse, with or without following shorter pulses, decreased the number of males responding by emission of MS-1 and searching behavior. Responses of males also depended on the presence or absence of a shorter FS-1b pulse train, showing the relevance of the signal structure (long and short pulses) for song recognition, as shown in grasshoppers (von Helversen, Balakrishnan, & von Helversen, 2004).

Multivariate analyses that included some temporal and spectral song characteristics of *C. ubica* and *C. impicticornis* as variables showed clear species and sex separation. These results were related to the absence of vibratory communication and duet formation in heterospecific couples (Laumann et al., 2016).

Our results confirm high specificity of song recognition in stinkbugs based on multicomponent time characteristics and signal structure of the conspecific song. In the same way, female calling songs of *N. viridula* from different geographical origins (continents) have different time characteristics (Čokl,

Virant-Doberlet, & Stritih, 2000), and males respond preferentially to those of their own geographical population (Miklas, Čokl, Renou, & Virant-Doberlet, 2003). Žunič et al. (2011) showed that *N. viridula* males recognize conspecific female song by species-specific temporal and spectral characteristics. The authors proposed that the recognition mechanism of males of *N. viridula* relies on multiple female song signal parameters.

FS-1b female song of both investigated *Chinavia* species differs from *Nezara* by complex pulse train structure, characterized by long pulses followed by shorter ones (Laumann et al., 2016; Moraes et al., 2005). Our results show that male recognition and responses in both *Chinavia* species are based on integration of multiple components of female signals, including their different temporal characteristics, as well as their specific pulse train structure. The integrative evaluation of multiple components of signals and the specific responses of receivers to a narrow range of combinations of these components is also reported for chemical (Christensen, Hildebrand, Tomunson, & Doolitle, 1989), acoustic (von Helversen et al., 2004; Reichert & Höbel, 2018; Schul & Bush, 2002) and visual (Tibbetts, Mullen, & Dale, 2017) signals.

Male vibratory response signals differ between investigated *Chinavia* species. *C. ubica* males respond consistently just to AS2 Cu and AS4 Cu type of stimulation signals; compared with natural signals, these have similar pulse train duration and repetition time, but differ in duration and duty cycle of the long pulse. *C. impicticornis* males respond with MS-1 signals to all types of playback stimulation signals.

Males of both investigated *Chinavia* species avoided overlapping of MS-1 with FS-1b signals with changed temporal and spectral characteristics as described in *E. heros* (Čokl et al., 2015; Laumann et al., 2018). However, *C. ubica* and *C. impicticornis* males responded for a shorter time to artificial signals, and in most cases, the response rate was lower compared with natural duets. We can explain it either as reaction to avoid overlapping or as male reaction to disturbed temporal parameters of female signals.

Our results confirm the hypothesis that the specific architecture (temporal pattern and spectral parameters) of the vibratory signals are fundamental for conspecific signals recognition in *C. ubica* and *C. impicticornis* and that this operated in the same way as for chemical signals (sex pheromones) as was previously showed by Blassioli-Moraes et al. (2012). This could help to maintain reproductive isolation of this species at prezigotic level species (Mendelson & Shaw, 2012; West-Eberhard, 1983). Notwithstanding, future works need to be developed to test this hypotheses in a context that includes multimodal signals interaction and neurological and physiological effects of signals parameters variation.

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