The Effect of Timing of Female Vibrational Reply on Male Signalling and Searching Behaviour in the Leafhopper *Aphrodes makarovi*

Anka Kuhelj, Maarten de Groot¤, Andrej Blejec, Meta Virant-Doberlet*

Department of Entomology, National Institute of Biology, Ljubljana, Slovenia

¤ Current address: Slovenian Forestry Institute, Ljubljana, Slovenia
* meta.virant@nib.si

Abstract

Sexual communication in animals often involves duetting characterized by a coordinated reciprocal exchange of acoustic signals. We used playback experiments to study the role of timing of a female reply in the species-specific duet structure in the leafhopper *Aphrodes makarovi* (Hemiptera: Cicadellidae). In leafhoppers, mate recognition and location is mediated exclusively by species- and sex-specific substrate-borne vibrational signals and a female signal emitted in reply to male advertisement calls is essential for recognition and successful location of the female. In *A. makarovi*, males have to initiate each exchange of vibrational signals between partners, and in a duet the beginning of a female reply overlaps the end of the male advertisement call. Results of playback treatments in which female replies were delayed and did not overlap with the male call revealed that in order to trigger an appropriate behavioural response of the male, female reply has to appear in a period less than 400 ms after the end of the initiating male call. Results also suggest that males are not able to detect a female reply while calling, since female reply that did not continue after the end of male call triggered male behaviour similar to behaviour observed in the absence of female reply. Together, our results show that vibrational duets are tightly coordinated and that the species-specific duet structure plays an important role in mate recognition in location processes.

Introduction

Communication enables sexual partners to recognize and find each other and is an essential part of reproductive behaviour [1]. Sexual communication based on acoustic signals often involves reciprocal exchange of signals between partners [2, 3]. The coordinated exchange of air-borne and substrate-borne acoustic signals is usually termed duetting and has been described in many arthropod and vertebrate taxa [2–5]. Duets are characterized by a predictable and stereotyped timing of signals and temporal coordination is expressed in reply latency,

as well as in alternation or overlapping of signals [2, 3, 6]. In comparison with birds [3, 7, 8], duetting in insects has received much less attention [2], most likely because the exchange of signals between partners is not typical in insects communicating with air-borne sounds [9, 10]. However, in the last decade it became obvious that the most common and taxonomically widespread form of acoustic communication in insects is signalling by substrate-borne vibrations [11–14], where partners usually establish a duet [2, 15].

Despite the increasing awareness of the importance of vibrational signalling in animal communication, the structure of vibrational duets has only rarely been systematically studied. In the lacewings of the *Chrysoperla carnea* group (Chrysopidae, Neuroptera) males and females exchange sexually monomorphic species-specific vibrational signals [16]. Partners closely match their response latencies and appropriately timed responses are important in sex recognition [17] and in maintaining reproductive isolation [18]. The exchange of vibrational signals has a stereotyped, species-specific temporal pattern also in other insects relying on vibrational duetting; although, in these groups signals are also sex-specific [19–22]. Besides mate recognition and mate choice [15], the function of a duet has also been associated with localization of a partner [23–26]. Although recent studies suggest that in leafhoppers a precisely coordinated exchange of male and female vibrational signals is essential for mate recognition and successful location of the female [25, 26], the role of species-specific duet structure in mate searching has not been studied so far. Leafhoppers (Hemiptera, Cicadellidae) are one of the most speciose groups of phytophagus insects, with more than 22 000 species [27] and more detailed studies on such diverse group should improve our understanding of mechanisms underlying evolution of duetting behaviour.

Mate searching includes displays to attract and/or to advertise to a potential mate (i.e. emission of advertisement calls), as well as mobility needed to increase the signalling space and/or to locate the partner [28]. In the present study, we investigated the effect of timing of a female reply on male calling and searching behaviour in the leafhopper *Aphrodes makarovi*. As in other leafhoppers, mate recognition and location in this species is mediated exclusively via substrate-borne signals [29–31]. Males use the 'fly/jump/walk-call' strategy to increase their signalling space [32, 33]. Sexual communication is based on a duet initiated by a long and complex male advertisement call to which a sexually receptive virgin female responds (Fig 1), thus triggering the male search for the female on the plant [31]. Female reply is essential for successful mating, since male does not approach the female if she does not respond. Previous research
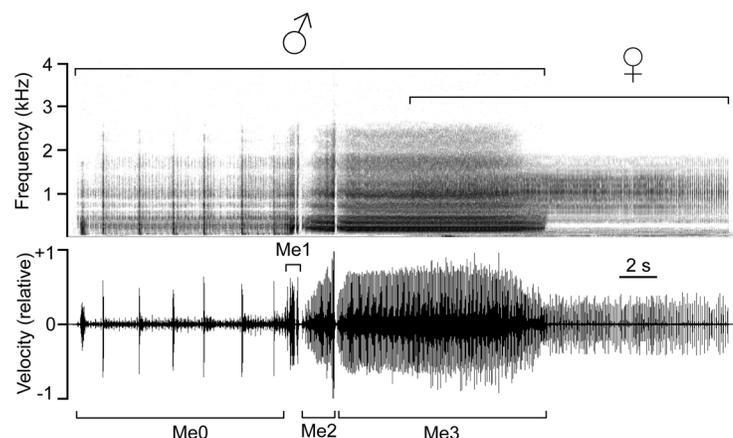

Fig 1. Representative male-female duet in *A. makarovi*. Spectrogram (above) and waveform (below) are shown. Me0-Me3: species-specific elements as described in [25].

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showed that female replies with temporal parameters outside species-specific values had negative effect on recognition, as well as on male's ability to locate the source of female vibrational signal [30]. Furthermore, the duet structure also suggests the existence of a species-specific time window of female reply. In this species, the beginning of a female reply overlaps the end of the male call [31] and although the onset of a female reply in *A. makarovi* is variable, the temporal association between the male call and the female response is nevertheless stereotyped and predictable. The onset of female vibrational reply before the conclusion of the initiating male call has been observed also in other insect taxa relying on vibrational communication [6, 20–22, 34, 35]. The critical response time window associated with the recognition of a female reply and triggering of locomotion related to searching behaviour is characteristic for air-borne sound duets in phaneropterine bushcrickets [36–39]; however, in vibrational communication systems its presence has been so far suggested only in alder flies from the genus *Sialis* (Neuroptera) [40] and in the psyllid *Diaphorina citri* [41].

A duet structure in which a female reply overlaps with his call presents the male with a fundamental problem of detecting the female reply while calling [42]. In *A. makarovi* vibrational signals are generated by a tymbal-like mechanism on the first abdominal segment [43, 44] and are transmitted to the substrate via the legs in which vibration receptors are also located [45, 46]. In this species male call and female reply are both composed of pulses (Fig 1) and there is no difference in the pulse repetition time between the last section of the male call and the female reply [29, 31]. Furthermore, the amplitude of female reply is always below the amplitude of male call [31].

We hypothesized (1) that a delayed female reply that does not overlap with the end of male call and, consequently, does not continue immediately after the end of male call, has a negative effect on male mate searching behaviour and (2) that males are not able to detect a female reply while emitting advertisement calls. To test these hypotheses we used playback experiments and we assessed male signalling and searching behaviour in response to female replies applied with different timing in relation to the male call. We predicted (a) that in the presence of a delayed female reply that does not appear immediately after the end of the call, males' success of finding the female would be reduced (i.e. associated with lower calling rate and lower number of males localizing the source) and (b) that in the presence of a hidden reply that does not continue after the end of the call, male behaviour would be similar to behaviour in the absence of a female reply.

Materials and Methods

Insect collection and maintenance

The study does not involve endangered or protected species and no specific permits were required. Last stage nymphs and adults of *A. makarovi* were collected using a sweep net from alfalfa (*Medicago sativa*) and stinging nettle (*Urtica dioica*) at various localities in Slovenia (46° 03.259'N, 14° 27.719' E; 46° 15.287'N, 13° 28.073' E; 45° 27.300'N, 13° 42.078' E) that are not environmentally protected or privately owned. In the laboratory males and females were kept separately in plastic boxes (38 x 26 x 27 cm) at 23–28°C, 50–70% humidity and 15: 9 h (L: D) photoperiod. They were fed with cut alfalfa placed in vials filled with water and replaced twice a week. Due to morphological similarities between *Aphrodes* species, the species identity of leafhoppers used in behavioural tests was determined prior to the experiments by recording their vibrational signals [29]. Males used in behavioural trials were 3–4 weeks old and were put individually in plastic cups (volume 0.5 L) a day before the start of experiments.

Experimental set-up

For all behavioural tests we used experimental set-up that enabled bilateral playback stimulation and was described previously [30, 31] (Fig 2). All experiments were performed on a
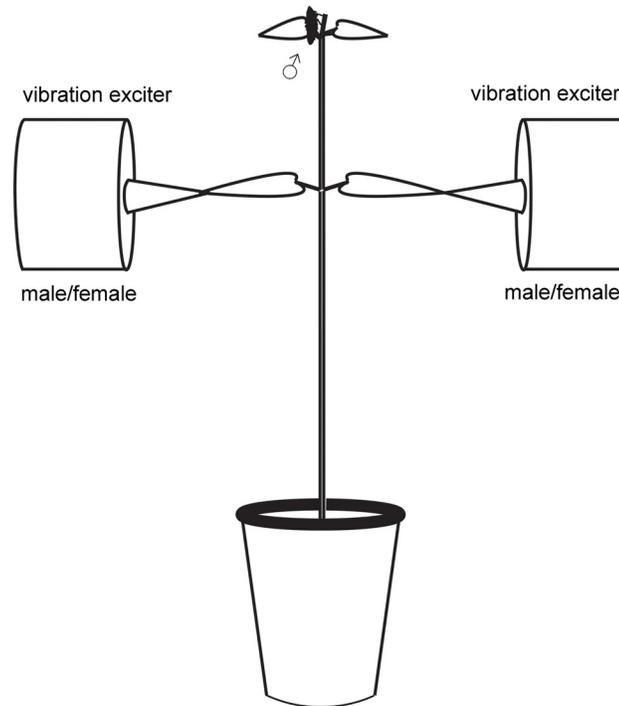
Fig 2. Experimental set-up. A schematic drawing of the experimental setup showing the initial position of the male on the top of the nettle plant and the positions of vibration exciters (not drawn to scale).

stinging nettle (*U. dioica*) at room temperature (20–25°C) and 40–50% relative humidity. The top of the plant (height approximately 25 cm) was cut off and the bottom of the stem was inserted into a vial filled with water (to prevent withering) and placed upright into a jar filled with moist artificial substrate. All inflorescences and leaves, except the pair at the apex and another pair approximately 12 cm down the stem, were removed. Each of the lower leaves was attached to a separate vibration exciter (see below) and a new plant was taken every second day. To prevent males from escaping, a rectangular cage (65 x 65 x 50 cm) without the front panel was put over the plant.

We applied vibrational stimuli (described below) to the lower nettle leaves via the conical tip of the 5-cm metal rod (4 mm in diameter) screwed firmly into the head of a vibration exciter (Minishaker type 4810, Brüel & Kjær, Nærum, Denmark) ([Fig 2](#)) and fixed to the tip of the leaf with Blu-tack. Vibration exciter was driven from the computer via Sound Blaster X-Fi Surround 5.1 pro sound card (Creative, Singapore) by Cool Edit Pro2 (Syntrillium Software, Phoenix, USA). Transmission of vibrations through the plant results in degradation of the signals due to frequency filtering [47–49]. To assure the fidelity of signals perceived by males, we checked each day before starting the playback experiments, as well as several times throughout each experimental day, that the frequency characteristics of playback signals recorded on the plant correspond to the input signal by comparing spectral properties of the applied and recorded female reply. Stimulatory and emitted vibrational signals were registered from the reflective tape placed on the nettle stem 1 cm below the branching point of the lower leaves with a laser vibrometer (PDV-100, Polytech, GmbH, Waldbronn, Germany) and stored in a computer using the above mentioned sound card and Cool Edit Pro2 software at the sampling rate 48-kHz and 16-bit resolution. Male behaviour together with recorded vibrational signals was simultaneously filmed with a 3CCD video camcorder (Canon DM XM2).

Experimental protocol and vibrational stimuli

Males of *A. makarovi* were tested in playback experiments in which we simulated a female response. A single male was placed on the leaves at the apex of the nettle plant (Fig 2). In order to induce the emission of advertisement calls we played him once a pre-recorded duet [30, 50]. We randomly applied to one of the lower leaves a male advertisement call while the other was vibrated with a female reply in order to simulate a male-female duet. Afterwards, whenever the live male emitted an advertisement call, we presented him with a female reply. The application of a female reply was triggered manually [30, 31, 50] (see below). The amplitude of stimulation was adjusted to the level of naturally emitted male advertisement calls and female replies registered at the point of recording (male call: 0.3 mm/s, female reply: 0.2 mm/s). The side from which we applied the female reply changed randomly. In control trials in the absence of female reply (see below) we observed male behaviour for 15 minutes. The trials in which we simulated the female reply continued for 15 minutes after stimulation with a duet or until the male localized the vibration exciter, whichever came first.

We tested males in two experimental series that each included 14 males. In both series males were tested with the same seven playback treatments in which we varied the timing of the female reply (see below). In each series we also included the F_0 control treatment in which males were stimulated once with a duet to induce calling; however, afterwards they received no female replies to their calls emitted during the trial. Males were tested only once per day and each male was tested once with each treatment and their order was randomized for each male. The female signal used in playback experiments was chosen from the signal library at the National Institute of Biology and was composed of natural pulses assembled by the use of Cool Edit Pro 2 computer program [30]. The pulse repetition time (52 ms) corresponded to the mean values determined in previous studies [29, 31] and such composed female reply triggers the same male signalling and searching behaviour as observed in the natural male-female duets [31].

To assess the effect of a delay, i.e. time between the end of male call and the beginning of female reply on mate searching effort, males were tested in five treatments (Table 1) in which they were presented with a 10.4 s long female reply that corresponds to the mean non-overlapped value determined previously [31]. In the F_{10} treatment we simulated a natural duet by timing the onset of the female replies with the typical decrease in the amplitude in the last section (Me3) of the male advertisement call (Fig 1), so that the beginning of the female reply overlapped the end of male call as in a natural *A. makarovi* duet. In the other four treatments,

Table 1. Temporal characteristics (duration and timing) of *A. makarovi* female replies used in playback experiments. Hypothesis 1: A delayed female reply has a negative effect on male mate searching behaviour. Hypothesis 2: Males are not able to detect a female reply while emitting advertisement calls. 0: natural timing of female reply (the beginning of female reply overlapping the end of male call). +: onset of female reply delayed for 400–2000 ms after the end of male call (female reply not overlapping with male call). H: female reply hidden in the last section of male call (female reply completely overlapping with the last section of male call).

	Treatment	Duration (s)	Timing
Hypothesis 1	F_{10}*	10.4	0
	F_{10+400}	10.4	+400
	F_{10+800}	10.4	+800
	$F_{10+1500}$	10.4	+1500
	$F_{10+2000}$	10.4	+2000
Hypotheis 2	F_5	5.2	0
	F_{5H}	5.2	H

*: F_{10} treatment represents a naturally timed female reply of average duration

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the female replies were delayed for 0.4, 0.8, 1.5 or 2 s after the end of male call ([Table 1](#)). In these four treatments, the onset of a playback of a female reply, which in these cases incorporated also the specified reply delay, was timed with the end of male call. Due to unpredictable variations in the duration of the Me3 section and errors in manual triggering of a playback, the actual reply delays varied somewhat; however, the delay ranges did not overlap among treatments (F_{10+400}: 406 ± 42 ms (mean ± SD); F_{10+800}: 816± 57 ms; $F_{10+1500}$: 1503 ± 57 ms; $F_{10+2000}$: 2020 ± 57 ms).

To test whether males perceive a female reply during emission of advertisement calls, males were presented with 5.2 s female replies and the onset of the female responses was timed with the beginning of the Me3 section so that the female signals remained completely hidden in the male calls ([Table 1](#)). For these tests we chose a shorter-than-average female reply within the natural range determined previously [29, 31], since the duration of the Me3 section is often shorter than 10.4 s [31]. In the F_5 treatment, we simulated a natural duet in which only the beginning of such short female replies overlapped with the end of male calls ([Table 1](#)).

In all experiments we monitored the following parameters: number of advertisement calls emitted by males, calling rate (number of calls emitted per min of trial after a duet was established), duration of emitted male calls, number of males searching (searching was defined as leaving the apex of the nettle plant and walking during and/or immediately after female reply), number of males locating the source and searching time (time needed to locate the source after the onset of searching).

Statistical analyses

We included in the analyses only active males that emitted advertisement calls (i.e. calling males) and the numbers of males used in the analyses for each treatment are shown in Figs [3a](#) and [4a](#) for treatments with delayed and hidden replies, respectively. To analyse the success in locating the source of female reply, we took into account the number of calling males, as well as the number of males that were also searching for the female on the plant (i. e. searching males). Only males that located the source of female reply were included in the analyses of searching time.

To assess the effect of a delay of female reply on male signalling behaviour, we compared the values obtained in four treatments in which the female reply was delayed with the values obtained in the F_{10} treatment (naturally timed, i.e. partly overlapped female reply of average duration) using a linear mixed-effect (Lme) model in which male identity was included as a random effect [51, 52] followed by Dunnett's multiple comparisons test with a control [53]. The Ryan-Einot-Gabriel-Welsch (Regwq) multiple comparisons test was used to compare the numbers of searching males and numbers of males locating the source [54].

To test whether males perceive a female reply during emission of advertisement calls, calling parameters obtained in the treatment with a hidden female reply (F_{5H}) were compared with values obtained in the F_5 and F_0 treatments using the above mentioned Lme model followed by Tukey's all pair comparisons test [53]. One-tailed Fisher's exact test was used to compare the numbers of searching males and numbers of males locating the source in the F_5 and F_{5H} treatments. Numbers obtained in the F_0 treatment were not included in these analyses, since in the absence of female reply, leaving the apex of the plant is associated with random movements and cannot be characterized as searching behaviour.

Due to the small numbers of males locating the source in most treatments (see [Results](#)), a meaningful statistical comparison of searching time was possible only for the F_{10} and $F_{10+2000}$ treatments. The values obtained in these two treatments were compared using the Wilcoxon signed rank test.

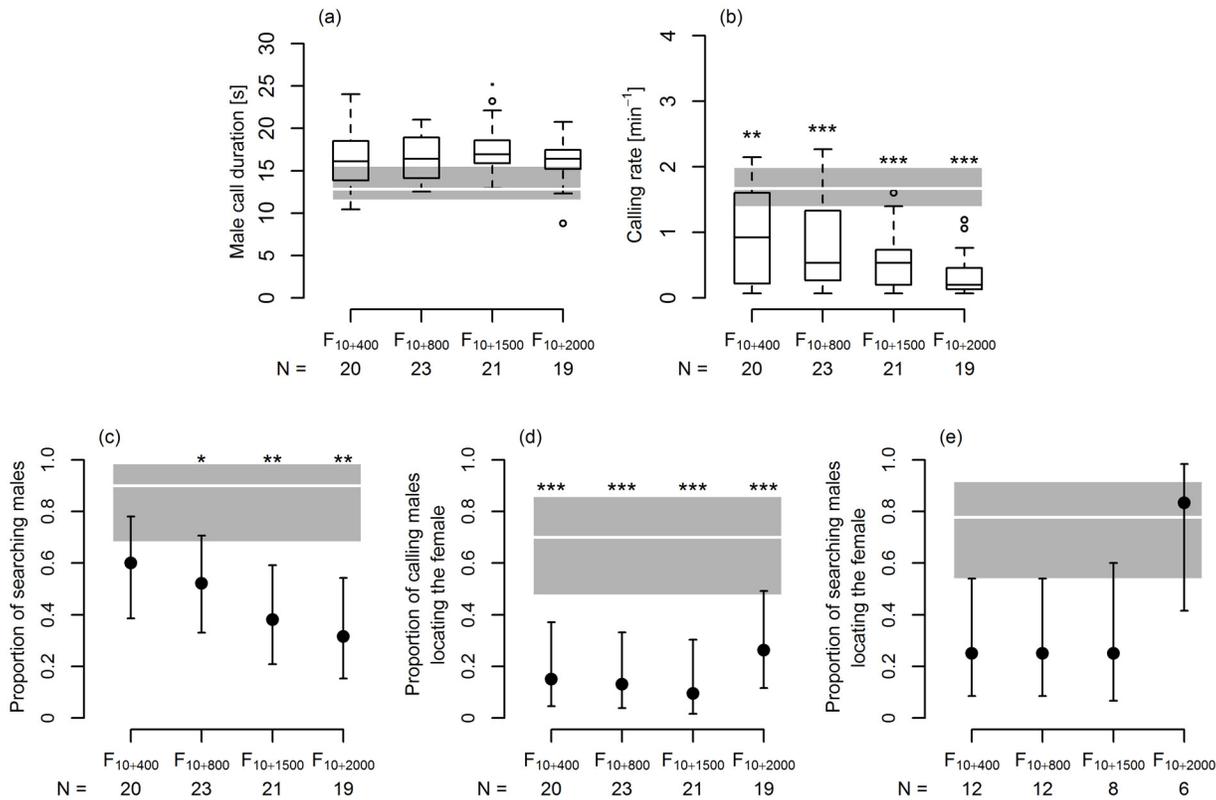All statistical analyses were conducted using R version 3.2.0 [55].

Fig 3. The effect of delay of female reply on signalling and searching behaviour of *A. makarovi* males. (a) male advertisement call duration; (b) calling rate; (c) proportion of males searching for the source of female reply; (d) proportion of calling males locating the source; (e) proportion of searching males locating the source. (a, b) box and whisker plots show the median (black line), the 25–75% interquartile range (boxes), the lowest and the highest data points still within 1.5 of interquartile range (whiskers) and outliers (circles). Values obtained in the F_{10} treatment are shown as median (thick white line) together with 95% confidence interval for median (gray area). *, ** and *** indicate significant difference from the F_{10} treatment (Lme model followed by Dunnett's multiple comparisons test, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). (c-e) determined proportion (black circle) together with 95% confidence interval for proportions is shown. Proportion obtained in the F_{10} treatment (thick white line) is shown together with 95% confidence interval for proportions (gray area). *, ** and *** indicate values that are significantly lower than in the F_{10} treatment (Regwq multiple comparisons test, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). N = number of males included in the analyses. F_{10}: N = 20 (a-d), N = 18 (e).

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Results

The effect of a female reply delay on male signalling and searching behaviour

In comparison with the F_{10} treatment, in which males received the naturally timed (i.e. partly overlapped) female reply, the duration of male advertisement call was significantly longer when female replies were delayed for 800, 1500 and 2000 ms ([Fig 3a](#)). Furthermore, the calling rate was significantly lower in all four treatments with delayed female replies ([Fig 3b](#)). The number of males searching for the female was significantly reduced in correlation with the increasing reply delay ([Fig 3c](#)). Moreover, taking into account the number of calling males, in all treatments with the delayed female response, the number of males locating the source of female reply was significantly lower than when the beginning of female reply overlapped the end of male call ([Fig 3d](#)). Although taking into account only males searching for the female, in the $F_{10+2000}$ treatment higher proportion of searching males located the source than in other treatments with a delayed reply, the numbers of males locating the source in all treatments with delayed replies were not significantly lower than in the F_{10} treatment, probably owing to
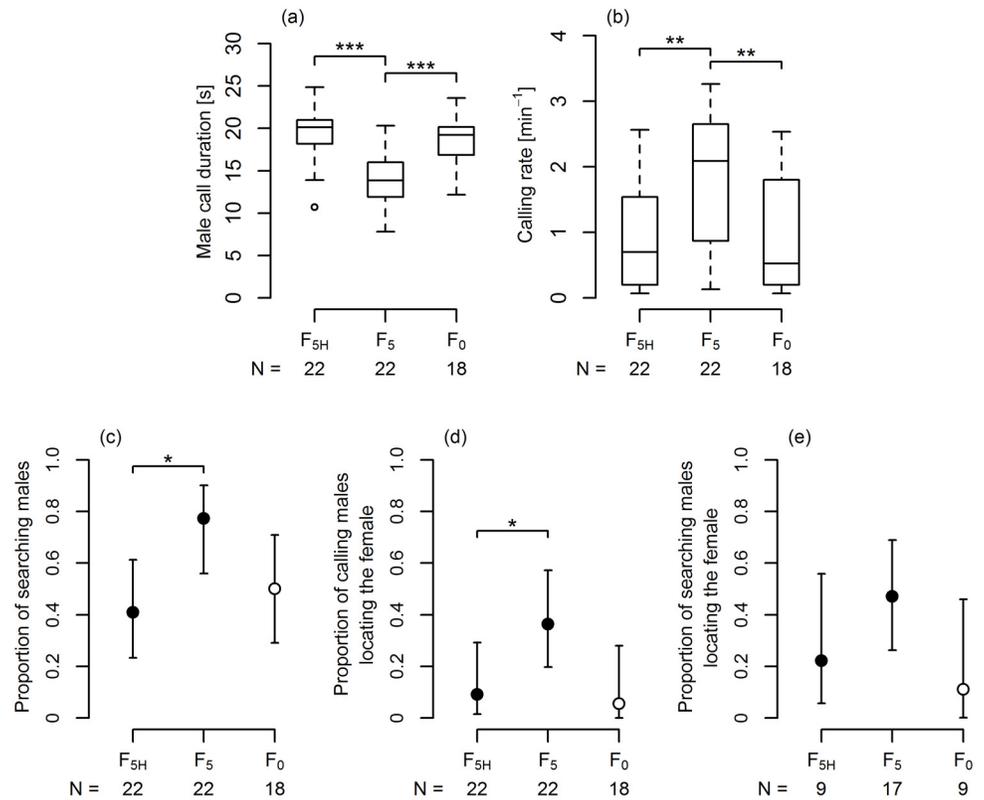
Fig 4. The effect of hidden female reply on signalling and searching behaviour of *A. makarovi* males.
(a) male advertisement call duration; (b) calling rate; (c) proportion of males searching for the source of
female reply; (d) proportion of calling males locating the source; (e) proportion of searching males locating the
source. (a, b) box and whisker plots show the median (black line), the 25–75% interquartile range (boxes), the
lowest and the highest data points still within 1.5 of interquartile range (whiskers) and outliers (circles). *, **
and *** indicate significant difference between treatments (Lme model followed by Tukey's all pair
comparisons test, p < 0.001). (c-e) determined proportion (black circle) together with 95% confidence interval
for proportions is shown. * indicate values that are significantly lower than in the F5 treatment (one-tailed
Fisher's exact test, p < 0.05). Values obtained in the F_0 treatment (white circles) shown for comparison
indicate the number of males changing their position during the trial (c) and the number of males arriving to
the leaves (d, e) and were not included in statistical analyses.

the small number of males searching for the female in some treatments ([Fig 3e](#)). Due to the
small numbers of males locating the source of the delayed female replies a meaningful statisti-
cal comparison of searching time was possible only for the $F_{10+2000}$ treatment, in which search-
ing time did not differ significantly from the F_{10} treatment ([S1 Fig](#)). Taken together, these
results support the existence of male sensory time window immediately after the end of male
advertisement call.

Perception of female reply during calling

When female replies did not continue after the end of male call (F_{5H} treatment), the average
male call duration was significantly longer than in the treatment with partly overlapping female
replies of the same duration (F_5 treatment); however, it did not differ significantly from the F_0
control treatment in which males did not receive any reply ([Fig 4a](#)). Moreover, also the calling
rate in the F_{5H} treatment did not differ from the calling rate in the F_0 treatment, while it was
significantly lower than in the F_5 treatment ([Fig 4b](#)). Significantly fewer males searched for the
source of a hidden reply than for the source of a partly overlapped female reply of the same

duration ([Fig 4c](#)) and only two males arrived at the vibrated leaf in this treatment. In the F_0 treatment, 50% of males moved from the starting position at the apex of the nettle plant during the trial; however, only one of these males walked to the leaf in the allotted time. Taking into account the number of calling males, the number of males locating the source in the F_{5H} treatment was significantly lower than in the treatment with partly overlapping female reply of the same duration ([Fig 4d](#)). However, owing to the small number of males searching for the female, the difference between these two treatments was not significant, when only searching males had been taken into account. Since only two males located the source in the F_{5H} treatment, a meaningful statistical comparison of searching time was not possible ([S1 Fig](#)). Taken together, these results suggest that males did not detect female reply during emission of advertisement calls.

Discussion

Results of the present study show that in *A. makarovi* the timing of a female reply has profound effects on male signalling and searching behaviour and influenced the likelihood of finding the female. Our results indicate that in this species males are not able to detect female reply while calling; however, in order to trigger the appropriate mate searching behaviour female reply has to appear in the time window immediately after the end of male call.

As in other vibrational communication systems in which mate searching has been studied in more detail [24, 26, 32, 33, 56], in *A. makarovi* female reply is essential in triggering searching behaviour, as well as for successful and quick location of the female; however, males have to initiate each exchange of vibrational signals. Males of many small plant-dwelling insects relying on vibrational communication use 'fly/jump/walk-call' strategy to increase their signalling space [25, 32, 33, 57]. Such strategy, however, presents males with a fundamental problem of reliably detecting a replying conspecific female, since her presence in male's local environment is usually unpredictable. It has been hypothesized that a predictable temporal association between the initiating call and the reply signal may be advantageous in communication between partners since it enables mate recognition in the environment with high levels of abiotic and biotic noise [3, 36, 37, 58]. For plant dwelling-arthropods, behaviourally relevant abiotic noise arises predominantly from wind [59–61]. Furthermore, incidental interference from heterospecific signals is common in acoustic communication [62] and individuals of different species living on the same plant can emit vibrational signals simultaneously [12]. *Aphrodes makarovi* leafhoppers communicate in a complex biotic landscape that contains other heterospecific signallers and includes also congeners living syntopically [29] and therefore incidental interference that may result in false alarms is likely. False alarms (i.e. responding to a heterospecific female) are costly due to wasted effort and unnecessary exposure to risk and may ultimately also result in mating with heterospecifics [63]. In *A. makarovi* vibrational signalling incurs direct costs due to eavesdropping predators [64], as well indirect costs due to high energy expenditure, and males with higher calling effort die sooner [50]. Males of this species show high selectivity in their behavioural response to female reply. It should be noted that the number of males arriving at the vibration exciter in most treatments with delayed female replies corresponds to the number of males randomly walking to the leaf in the absence of female reply.

The results of the present study show that *A. makarovi* male has to detect a female reply in a period less than 400 ms after the end of his call in order to respond appropriately. It has been shown in another species in this genus (*A. bicincta*) that males can contribute more than females to sexual isolation between species and that male's contribution to assortative mating resulted from mate recognition as well as from inability to locate the source of an inappropriate

female reply due to a breakdown of a species-specific duet structure [25]. Although a duet structure in *A. makarovi* superficially appears simpler than in *A. bicincta*, in which female replies have to appear in short intervals between continuously repeated elements in male call [25], the results obtained in the present study support the view that vibrational duets are tightly coordinated and that the species-specific duet structure plays an important role in maintaining reproductive isolation.

After they perceive a reply from a conspecific female, males of *A. makarovi* increase their calling rate and commence searching on a plant [30, 31, 50] and therefore calling rate and triggering of locomotion associated with searching for the female could be linked with mate recognition process. However, male has to stimulate each emission of female vibrational signal and as in other duetting systems [15, 24, 65] in *A. makarovi* higher calling rate also increases a probability of locating the female [50]. Although with current data it is not possible to determine to what extent a female reply outside the male's sensory time window selectively affects recognition and/or localization processes, some of our results suggest that different time delays may affect these two processes differently. While 400 and 800 ms time delays appear to have proportionally larger effect on localization, those *A. makarovi* males that were searching in the treatment with the longest female reply delay were highly successful in locating the source regardless of their low calling rate. The success of males locating the source of delayed replies in this treatment may be attributed either to higher motivation or better location ability [66]. These males may also show better behavioural plasticity in switching to alternative tactics, such as satellite behaviour (i.e. silent approach to a female duetting with another male) [57, 67, 68]. Since in a natural situation *A. makarovi* males never overlap their calls further studies should also determine the effect of female reply with a delay that takes into account a duration of rival's call (i.e. delays longer than 10 s).

Calling and listening to other acoustic signals in the environment presents signallers with a fundamental sensory problem of discriminating between their own (i.e. self-generated) and environmental (i.e. external) signals [42]. While insects communicating with air-borne sounds are able to detect external sounds while singing [69, 70], results of the present study indicate that *A. makarovi* male is not able to perceive a female reply during emission of an advertisement call. It should be noted that in this species isolated males emit longer advertisement calls than males duetting with the female [30, 31]. In duetting bushcrickets, when male air-borne call is long and complex, female reply is usually triggered by a particular sound element indicating the end of male call [58, 71–73]. It has also been shown that female reply is triggered by specific elements in vibrational male advertisement calls [21, 35]. In *A. makarovi* the duration of the last section in the call is long and highly variable (between 5–20 s) [31] and currently there is no evidence that the timing of a female reply is correlated with various components of male call. Consequently, by replying before the male call is finished female should maximize the chance that the reply appears in the male's sensory time window [39], especially if the position of male's recognition sensory time window is immediately after the end of his call. While in duetting bushcrickets the sensory time window is often narrow (20–50 ms) [36–38], the suggested time windows in both vibrational communication systems studied so far were wider (400–500 ms) [40, 41]. The lack of detailed information on temporal coordination of duets in other vibrational communication systems precludes more generalized comparisons; however, it is conceivable that due to the relatively low propagation velocities of vibrational signals in plants [47, 74, 75] the resulting transmission time delays are large in comparison with air-borne sound and, consequently, sensory time windows should be wider. Results obtained in the present study suggest that the sensory time window of *A. makarovi* males may be shorter than 400 ms; however, in order to determine the exact position and width of the time window, a development of an automated system, which would reliably distinguish male vibrational calls

from background noise and trigger playback of female replies in real-time with short time delay, is needed. Such system would be invaluable also for testing other species in this genus in which observed female reply delays in natural duets are around 50 ms [76].

Our results suggest that male of *A. makarovi* perceives only that part of a female reply that continues after the end of his call. However, walking associated with searching behaviour is also limited to the duration of non-overlapped female reply and to a period shortly afterwards [30, 31] and leg movements stimulate the same leg mechanoreceptors that also detect vibrational signals. There is no direct information to what extent walking influences perception of female reply; however, it should be noted that males need on average around 1 s after the end of the call to commence searching [30, 31]. Neuronal mechanisms underlying vibrational communication are virtually unexplored [75, 77] and more elaborate behavioural and neurophysiological studies are needed to unravel mechanisms underlying processes of mate recognition and directionality in insects communicating via substrate-borne vibrational signals.

The importance of female signals in the mate recognition process and sender-receiver dynamics is becoming increasingly clear [78] and the results of the present study show that *A. makarovi* provides a good opportunity to explore the importance of female signals in a duet structure. In this species, the duration of a female reply is long and variable [29, 31] and males locate the source of longer female replies faster [30]. Additional studies should elucidate whether observed variation in female reply is related to mate choice [15]. However, males are able to detect and evaluate only the non-overlapped section of female reply, which depends not only on the latency and overall duration of female reply, but also on duration of the initiating male call itself. Further studies should also determine whether males adjust their signalling behaviour according to the duration of female reply in order to optimize their mate searching behaviour.

Supporting Information

S1 Fig. The effect of timing of female reply on searching time of *Aphrodes makarovi* males. (a) treatments with a delayed female response; (b) treatment with a hidden female reply. Raw data are shown. (b) Value obtained in the F_0 treatment (white circle) shown for comparison indicates the time male needed to arrive to the leaf. N = number of trials included in the analyses.
(PDF)

S1 Table. Raw data from playback treatments used to assess the effect of female reply delay. The table summarizes signalling and searching behavioural parameters for each male scored in each treatment.
(PDF)

S2 Table. Raw data from playback treatments used to assess whether males perceive female reply while calling. The table summarizes signalling and searching behavioural parameters for each male scored in each treatment.
(PDF)

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Author Contributions

Conceived and designed the experiments: AK MDG MVD. Performed the experiments: AK. Analyzed the data: AK AB. Wrote the paper: AK MDG AB MVD.

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