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On the function of a female-like signal type in the vibrational repertoire of *Enchenopa* male treehoppers (Hemiptera: Membracidae)

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Abstract

Animals often mimic the behaviours or signals of conspecifics of the opposite sex while courting. We explored the potential functions of a novel female-like signal type in the courtship displays of male *Enchenopa* treehoppers. In these plant-feeding insects, males produce plant-borne vibrational advertisement signals, to which females respond with their own duetting signals. Males also produce a signal type that resembles the female duetting responses. We experimentally tested whether this signal modifies the behaviour of receivers. First, we tested whether the female-like signal would increase the likelihood of a female response. However, females were as likely to respond to playbacks with or without them. Second, we tested whether the female-like signal would inhibit competing males, but males were as likely to produce displays after playbacks with or without them. Hence, we found no evidence that this signal has an adaptive function, despite its presence in the courtship display, where sexual selection affects signal features. Given these findings, we also explored whether the behavioural and morphological factors of the males were associated with the production of the female-like signal. Males that produced this signal had higher signalling effort (longer and more frequent signals) than males that did not produce it, despite being in worse body condition. Lastly, most males were consistent over time in producing the female-like signal or not. These findings suggest that condition-dependent or motivational factors explain the presence of the female-like signal. Alternatively, this signal might not bear an adaptive function, and it could be a way for males to warm up or practice signalling, or even be a by-product of how signals are transmitted through the plant. We suggest further work that might explain our puzzling finding that a signal in the reproductive context might not have an adaptive function.

Keywords: biotremology, *Enchenopa binotata*, sexual mimicry, substrate-borne vibrations, seismic signal, repeatability

Introduction

Many animals have evolved the capacity to perform sexual mimicry, in which individuals of one sex mimic the behaviours, body colouration, pheromones, or signals of individuals of another sex (Field & Keller, 1993; Forsyth & Alcock, 1990; Saetre & Slagsvold, 1996). Males mimic female phenotypes in various contexts to obtain fitness benefits. For instance, sexual mimicry helps cuttlefish males sidestep aggression from dominant individuals to approach females (Hanlon et al., 2005; Norman et al., 1999); it helps scorpionfly males approach other males to steal their resources and offer them to females (Thornhill, 1979); and it helps satellite male bush crickets start duets with females (Bailey et al., 2006; Heller et al., 2011).

Here, we explore the function of a female-like signal type we recently reported in *Enchenopa* treehoppers (Figure 1A) (Escalante et al., 2022). *Enchenopa* are plant-feeding insects that communicate with substrate-borne vibrational signalling (Cocroft & Rodríguez, 2005; Cocroft et al., 2008; Hill, 2009; Rodriguez & Desjonquères, 2019). This modality of communication is relatively unexplored in the study of sexual mimicry.

Mate-searching *Enchenopa* males fly from plant to plant, producing bouts of advertisement signals by vibrating the thorax and abdomen muscles (Miles et al., 2017). These signals consist of a frequency-modulated whine followed by a series of pulses (Cocroft et al., 2008, 2010). Receptive females respond with their own signals, which lack frequency modulation and pulses (Rodríguez & Cocroft, 2006) and establish a duet that continues until mating begins (Cocroft et al., 2008; Rodríguez et al., 2004). Females selectively duet and produce more and longer responses with males they find attractive (Rodríguez et al., 2004, 2006, 2012).

In addition to the above main advertisement signals, *Enchenopa* males often produce an additional signal type in their bouts (Escalante et al., 2022). This signal type is mainly produced immediately after the first advertisement signal and lacks frequency modulation and (often) pulses (Escalante et al., 2022). These features, and the placement after an advertisement signal, where female duetting response signals would occur, make this signal type resemble female duetting responses. Therefore, we have argued that this female-like signal type constitutes a form of sexual mimicry, i.e., that it mimics the duetting signals of conspecific females (Escalante et al., 2022).

To explore the possible sexual mimicry function of the *Enchenopa* female-like signal type, we used vibrational playback experiments to test two non-exclusive hypotheses regarding its effects on receivers. First, one possible reason for males to mimic a female duetting signal might be to induce females to duet with them. Males might perform this signal to "prime" females to respond to them by simulating that they are already in a duet with another female. This female priming hypothesis predicts that a female-like signal experimentally added to a signalling bout in an artificial playback will increase the likelihood of females responding (Stumpner & Meyer, 2001). A second possible reason for males to mimic female signals might be to inhibit other males from signalling.

By simulating that a female is already duetting with them, males might induce other males to move away as that responsive female is already duetting. *Enchenopa* males that receive female responses are known to stay on the plant and continue duetting until they locate the female, whereas males that do not receive female responses cease signalling and leave the plant (Cocroft et al., 2008; Rodríguez & Cocroft, 2006). Thus, the male inhibition hypothesis predicts that a female-like signal experimentally added to a signalling bout will decrease the likelihood that other males will continue signalling (Bailey et al., 2006).

We did not find evidence to support either hypothesis (see *Results* section), leaving the question of the function of
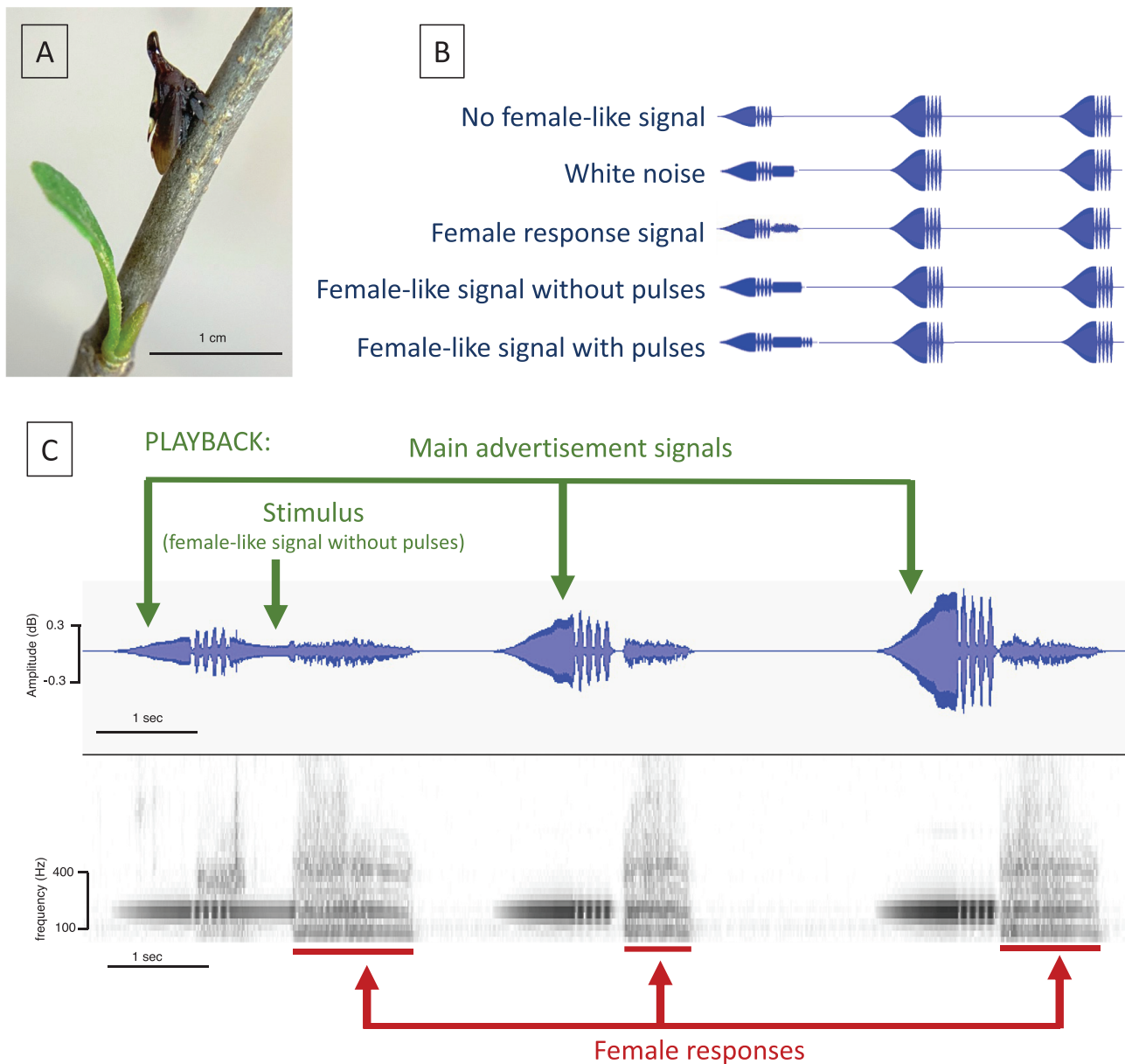
Figure 1. Experimental test of the female-like signal in the vibratory courtship display of *Enchenopa binotata* males. (A) Sample adult male of *E. binotata sp_low*, photo by I. Escalante. (B) The five experimental playback treatments presented to females and males of two species (sp_{low} and sp_{high}) to test the female priming (Figure 2) and the male inhibition (Figure 3) hypotheses. Each playback had three main advertisement signals and one stimulus (or lack thereof as a control) immediately after the first main signal. See *Methods* section for the full description of the playback construction and experimental procedures. (C) Sample recording of a female presented with one playback of a female-like signal without pulses (top arrows). Note the female responses (bottom arrows). This trail corresponds to Supplementary Audio S1. The likelihood of females to respond to each treatment and the mean time of responding are shown in Figure 2B.

the *Enchenopa* female-like signal type open. Therefore, we explored the relationship between the production of this signal type and other aspects of the males' signalling behaviour and morphology. Specifically, we assessed a potential relationship with signalling effort, which prior work has shown males adjust according to the presence of sexual competitors (Rebar & Rodríguez, 2016). We also assessed a potential relationship between body size and condition and the variation between males in the repeatability of producing the female-like signal. By exploring the correlates of this novel signal and morphological and behavioural features, we aimed to identify factors that could explain the adaptive or non-adaptive origin of the female-like signal in these insects.

Methods

Study species

We worked with two members of the *Enchenopa binotata* species complex (Cocroft et al., 2008) that live on *Viburnum lentago* (Adoxaceae) host plants. We collected third-instar nymphs in meadows and forest edges in eight locations in a 50-mile radius in Southwestern Wisconsin, USA, in June of 2018, 2019, 2020, 2021, and 2022 (see Supplementary Table S1 for further information on the field sites). These currently undescribed species can be distinguished by nymph colouration (grey versus black with white stripes) and male signal frequency (~165 and ~315 Hz) (Cocroft et al., 2008, 2010; Rodríguez et al., 2018). Therefore, we refer to these species as sp_{low} and sp_{high}, respectively. After the recordings, we preserved the individuals in 95% ethanol, which is available upon contact with the authors.

We reared the nymphs on potted host plants at the University of Wisconsin—Milwaukee Greenhouse with natural light cycles and temperature variation (air temperature [mean ± *SD*] 24.0 ± 4.4 °C, range: 13.9–36.1 °C) following previous procedures (Escalante et al., 2022). We placed 25–40 nymphs on potted *V. lentago* plants (30–50 cm in height) netted with mesh (30 × 70 cm). When individuals moulted into adults, we separated males and females on different plants (with 12–20 individuals/plant) to ensure that they were virgins and sexually inexperienced during our experiment. Males and females are distinguished in our study species by their sexually dimorphic body sizes, pronotum shapes, and genitalia.

Vibrational playbacks and recording

We tested the female priming and male inhibition hypotheses with vibrational playback experiments on females and males. We tested each individual only once with only one randomly assigned playback. We constructed all playback stimuli to reflect the natural structure of the male signalling displays of each species (see Figure 1C in Escalante et al., 2022): a bout of three main advertisement signals in which we varied the presence and type of signal following the first main signal (Figure 1B and C), where the female-like signal is found in male signal bouts (Escalante et al., 2022). Also, playbacks had mean values for the temporal and spectral features (e.g., whine length and dominant frequency) of signals of each species from the field site where we collected them (Escalante et al., 2022; Rodríguez et al., 2018). We created playbacks with custom-written code in R (R Development Core Team, 2021) using the packages *seewave* V2.1.8 (Sueur et al., 2008, 2022), *tuneR* V1.3.3.1 (Ligges et al., 2022), and *stringr* V1.4.0 (Wickham, 2022).

We used five playback stimuli (Figure 1B): (a) no female-like signal: a bout of main advertisement signals without a stimulus between the first and second signals, as a control for the presence of stimuli; (b) white noise: a synthetic 0.5 s broadband (0–2.2 kHz) segment of white noise placed after the first main advertisement signal in the bout; and (c) female response signal: a bout with a recording of a real female responding to a male (obtained from our library of recordings) placed after the first main advertisement signal in the bout. We included this playback to confirm that actual female signals would prime the listening females to respond, and it also will inhibit the listening males from continuing signalling. This allowed us to compare its effects with the playbacks with artificially generated female-like signals. We also used (d) a playback with a synthetic female-like signal without pulses; and (e) a synthetic female-like signal with four pulses, each placed after the first main advertisement signal in the bout (Figure 1B). We were interested in the potential effect of the presence/absence of pulses in the female-like signal because *Enchenopa* males vary in whether they produce female-like signals with or without pulses (four pulses being the mean in the former case), and because female-like signals without pulses resemble female duetting signals more closely (see the description of the signal in Escalante et al., 2022).

We presented playbacks through a designated playback-recording plant using a custom-written script in MATLAB (v.R2007b. The Mathworks, Natick, MA) that randomly selected one playback of the appropriate species to present to each test individual. We used only one playback and recording plant to avoid variation in the vibration transmission between plant individuals (Cocroft & Rodríguez, 2005; McNett & Cocroft, 2008). We played the stimuli onto the plant with a small (6.5 mm × 6.5 mm × 20.0 mm) piezo-electric actuator (PC4QR; Thorlabs, Newton, NJ) attached to the plant with accelerometer wax (Petro-wax 32227) and controlled by a piezo controller (MDT694A; Thorlabs, Newton, NJ). We calibrated the peak-to-peak amplitude of the playback stimuli at 0.15 mm/s using a Tenma 72-2580 digital oscilloscope (Newark Element 14, Chicago, IL).

We recorded the trials with a portable laser Doppler vibrometer (PDV-100; Polytec, Auburn, MA), with its beam focused on adhesive reflective tape on the plant's stem. We band-pass filtered the output of the laser vibrometer (40–3000 Hz) with an electronic filter (Krohn-Hite model 3202; Brockton, MA) and sent the signal through a USB audio interface (Edirol USB UA-25, Roland, Japan) to a MacBook computer. We recorded at a sampling rate of 44.1 kHz with the program Audacity (v.2.1.2, AudacityTeam). To isolate the playback and recording setup from building vibrations, we placed it on top of a 135-kg iron plank resting on four partially inflated bicycle inner tubes. A shock-absorbing Sorbothane (Edmund Scientific, Tonawanda, NY) sheet isolated the recording plant from the iron plank.

To follow the natural history of these treehoppers, we conducted recordings during the daytime (from 10:00 to 18:00 hours) in July and August. We noted the air temperature near the test individuals using a hygro-thermometer (445702, Extech, Nashua, NH). The temperature ranged between 24.2 and 27.7 C (mean ± *SD*: 26.6 ± 0.7 °C, n = 325 trials).

Two of the playback stimuli included multiple frequency components (the recordings of female signals and white noise), which may be distorted when transmitted along the plant from the piezo stack to where the treehopper perceived

the stimulus, ca. 5–15 cm away (Cocroft, 1996; Cocroft & Rodríguez, 2005; Nieri et al., 2022). All other playbacks had only one frequency. To compensate for potential distortions, we estimated the filtering properties of the playback plant by playing broadband noise (0–2.2 kHz) and recording it with the vibrometer. We then created a compensating digital filter in MATLAB. We applied it to the stimuli such that their frequency components arrived at the target treehoppers without distortion (±3 dB) (Cocroft, 1996; Cocroft & Rodríguez, 2005).

Testing the female priming hypothesis—playbacks to females

To assess the effect of the playbacks, we placed each female on the recording plant and let her acclimate for 1 min. We transferred her from the rearing plant to the recording plant using an Eppendorf tube opened on both ends. Hence, the treehopper walked onto the recording plant, minimizing handling stress.

We first confirmed that the female was responsive by presenting her with a primer of a recording of a male of its own species. The primer had a bout with two signals in the mean preferred frequency for each species (185 Hz for sp_{low} and 290 Hz for sp_{high}).

If the female responded to the primer, we presented her with a randomly assigned playback. If the female did not respond, we gave it 2 additional times. We placed unresponsive females back onto the rearing plant and tried again three days later. For both species, 55% of females responded to the primer the first time, 26% on the second, and the remaining 19% responded between the third and the seventh attempts. We presented only one of the five possible playbacks to each individual. In total, we tested $n = 96$ sp_{low} females and $n = 78$ sp_{high} females (total $n = 174$ females of both species). Of those, 34% responded to only one signal in the bout, 61% to two, and 5% to the three signals.

We visualized the recordings on Audacity and noted whether the female responded with a duetting response to the target stimulus placed in the position of the female-like signal (Escalante et al., 2022) and to the following signals in the playback (Figure 1D, Supplementary Audio S1). We also noted after which signal(s) the female responded (as she could respond 3 times: to the first main advertisement signal + the female-like signal, to the second main signal, or to the third main signal). We considered a response to the stimulus when the female produced the duetting signal within 1.0 s after the stimulus and after the second and third main advertisement signals. Responses to the no female-like signal playback occurred if the female signalled 0.60 s after the end of the first signal in the playback, as this is the average duration of the female-like signal (Escalante et al., 2022) or after the second and third signals. Additionally, to quantify the strength of the female response, we measured the length of each female duetting response signal to the playback. As two thirds of females' responded to more than one signal in the playback bout, we used the mean length for each female. (This measure was correlated with the number of response signals [$r = 0.31$, $p = .005$] and with summed response length [$r = 0.73$, $p < .001$].)

Statistical analysis

To test the prediction of this hypothesis that the female-like signal increases the likelihood of females responding, we ran a logistic regression model using female response to the playbacks (yes, no) as the dependent variable. The explanatory terms were playback treatment (five treatments, Figure 1B), species (sp_{low}, sp_{high}), recording temperature, and the interaction between treatment and species. We included the temperature term to account for the potential effect of variable temperature on the signalling displays of *E. binotata*. To test the prediction of the female priming hypothesis regarding response strength, we used a linear model that included the mean time females spent responding to the signals in the playbacks as the dependent variable. The model had the same explanatory terms as above.

We used four recordings for each species for the playbacks with female responses. These recordings had species-specific temporal and spectral features (Escalante et al., 2022): signal length: $sp_{low} = 0.8 ± 0.2$ s, $sp_{high} = 0.9 ± 0.1$ s; dominant frequency; $sp_{low} = 92.2 ± 3.5$ Hz, $sp_{high} = 134.9 ± 4.5$ Hz. In the above model, we did not include recording identity as a random term, as the other treatments had only one synthetic stimulus. To confirm that the lack of random terms of the stimuli with recordings was not an issue, we ran two linear models using either the likelihood to respond or the response length to the female response signals as the dependent variable. The models had the following explanatory terms: recording identity and its interaction with species as random terms, and species and temperature as explanatory terms. There was no difference in the likelihood to respond (recording identity: Wald $p = .91$; recording identity × species: Wald $p = .65$) nor in the time responding (recording identity: Wald $p = .51$; recording identity × species: Wald $p = .41$). Therefore, we pooled the responses to the four recordings in each species as the female response signal treatment.

Testing the male inhibition hypothesis—playbacks to males

To assess the effect of the playbacks, we placed each male on the plant and waited for him to produce a spontaneous signalling bout; these were the same males analyzed by Escalante et al. (2022). We waited for 5 s to confirm that the bout ended, and immediately afterwards, we manually triggered the MATLAB code to present the playback randomly. If the male did not signal in the first 5 min, we placed it back on the rearing plant and tried again once every 3 days. Of those males, 46% signalled the first time, and the rest in the following 2–7 times.

We recorded the male's behaviour in the following 15 s. We noted (a) if the male responded to the playback by producing a signalling bout. We also noted two additional behaviours as potential indications that the playback inhibits males from continuing to signal: (b) if the male quickly jumped off the plant, which would result in the male flying away to another plant to search for females and (c) if the males produced a wing buzz, a rapid movement of the wings that makes a loud broadband noise. This likely indicates an agonistic display by the males (*pers. obs.*), which can also mask and disrupt the signalling display of another male (cf. Legendre et al., 2012). We presented only one playback to each individual. In total, we tested $n = 38$ sp_{low} males and $n = 59$ sp_{high} males (total $n = 97$ males of both species).

Statistical analysis

To test the prediction of this hypothesis that the female-like signal will decrease the likelihood of males to continue signalling, we ran a logistic regression model as the one described

above. We used whether the male signalled after the playback (yes, no) as the dependent variable. The explanatory terms were playback treatment (five treatments, Figure 1B), species (sp_{low}, sp_{high}), recording temperature, and the interaction between treatment and species. We ran two additional models using whether the males jumped off the plant (yes, no) or produced a wing buzz (yes, no) as the dependent variable (one in each model) and the same explanatory terms.

Behaviour and morphological correlates of the production of the female-like signal

We analyzed the spontaneous signalling bouts males produced before being presented with the playbacks in the above trials. We also analyzed the spontaneous signalling bouts of an additional 110 males that were recorded using the same recording setup but without playbacks (Escalante et al., 2022).

On Audacity, we first noted whether the males produced the female-like signal. We then focused on aspects of signalling behaviour that prior work has shown *Enchenopa* males adjust in response to the social context. Specifically, they increase signal rates and lengths in response to the presence of sexual competitors (Rebar & Rodríguez, 2016). These adjustments constitute increases in overall signalling effort as they increase the duty cycle of the signal bouts (cf. Greenfield, 2002). We analyzed the recordings with Audacity with a 100–2000 Hz band pass filter. We measured signal rate as the inverse of the interval between the ends of two adjacent main advertisement signals. Specifically, we used the third and fourth advertisement signals. Thus, this measure never included female-like signals, as these are often produced between the first and second advertisement signals (Escalante et al., 2022). Previous work has used these landmark signals extensively to explore how signal variation influences mate choice and sexual selection (Desjonquères et al., 2019a; b, 2021; Escalante et al., 2022). The signal rate of the interval between the first and second signals does not differ and is highly correlated with the signal rate of the interval between the third and fourth signals (see Table 2 in Escalante et al., 2022 and $r = 0.63$; $p < .0001$, $n = 203$, respectively). Therefore, the signal rate we report below is a good proxy of the signalling effort of males and allowed us to explore if that effort correlates with the production of the female-like signal. We also measured the length of the fourth signal's whine (in seconds). All these recordings included bouts of at least four advertisement signals.

We explored the relationship between the production of the female-like signal, signalling effort, and body condition with a subset of the above males ($n = 81$), from which we were able to quantify their condition. We wondered whether body condition might limit signalling effort in *Enchenopa*, as signalling is quite costly in other vibrational insects (e.g., Kuhelj & Virant-Doberlet, 2022; Kuhelj et al., 2015). We estimated body condition with the residuals of the regression of body mass on the body size (Hunt et al., 2004; Schulte-Hostedde et al., 2005). Higher values of this measure indicate that males are in better condition (relatively heavy for their size). After each trial, we weighed each male to the nearest 0.001 mg with a Mettler Toledo X6 analytical balance (Greifensee, Switzerland). We then preserved them in 90% ethanol to measure body size. We placed each male in a 3.5 cm Petri dish with sand and ethanol in an Olympus SZ61 microscope (Olympus, Tokyo, Japan). We took a picture of the treehopper on lateral view using Motic Image Plus 2.0.10 (Motic, Richmond, BC, Canada), with a Moticam 2500 camera

(Motic, Richmond, BC, Canada) attached to one eyepiece of the microscope. We used ImageJ (Schneider et al., 2012) to measure the wing length (as the length of the central vein in lateral view), pronotum length, face length, and leg III tibia length (Supplementary Table S2). These are standard morphological traits studied in *Enchenopa* (Cocroft & de Luca, 2006; Rodríguez & Al-Wathiqui, 2011). We measured all variables to the nearest 0.01 mm. We used wing length as an indicator of body size, as it correlated with the other measures ($r > 0.73$ and $p < .001$ for all comparisons).

To explore individual differences in the production of the female-like signal, we estimated the repeatability (Bell et al., 2009) of the production of that signal for a subset of males ($n = 32$), from which we were able to record two spontaneous signalling bouts. For 18 of these males, both bouts occurred in the same recording session, less than 1 min apart (i.e., they produced two spontaneous signalling bouts before we presented them with the above playbacks). For the other 14 males (all sp_{low}), we recorded each signalling bout in different recording sessions, separated by at least 1 day (two males 1 day apart, eight males 2 days apart, and one male each of the following time frames: 7, 10, 19, and 22 days apart). We combined data from both groups since *Enchenopa* male treehoppers maintain consistent features in their signalling display over time (Sattman & Cocroft, 2003).

Statistical analysis

To analyze the relationship between producing the female-like signal and male signalling effort, we ran three separate linear models with either signal rate, whine length, or dominant frequency as the dependent variable. Each model had the following explanatory terms: whether the male's spontaneous signal bout included the female-like signal (yes, no), species, recording temperature, and the interaction between the first two terms. The signal's dominant frequency did not differ between males that produced the female-like signal or not (Supplementary Figure S1). This trait is species-specific. Hence, we did not expect that males could modify it to increase their signalling effort.

To assess whether males differed in body condition according to whether they produced the female-like signal, we ran a linear model with body condition as the dependent variable and the same explanatory terms mentioned above. We further analyzed the role of condition with two generalized linear models having either signal rate or whine duration as the dependent variable. The models had the following explanatory terms: condition, species, and whether the male produced the female-like signal (yes, no), as well as all the two-way interactions between them. We removed the three-way interaction as it was not significant ($F < 0.55$, $p > .46$ for both models).

To assess the repeatability of individual differences in producing the female-like signal, we used logistic regression with the production of that signal in the males' second signal bout (yes, no) as the dependent variable and the following explanatory terms: production of the female-like signal in males' first signal bout (yes, no), species, the interaction between these two terms, and whether both bouts were recorded the same day (yes, no). In this model, the term for producing the signal in the first signal bout tests for a relationship with producing it in the second bout.

Lastly, we compared signal rates and whine lengths between males that showed one of three categories of consistency: (a)
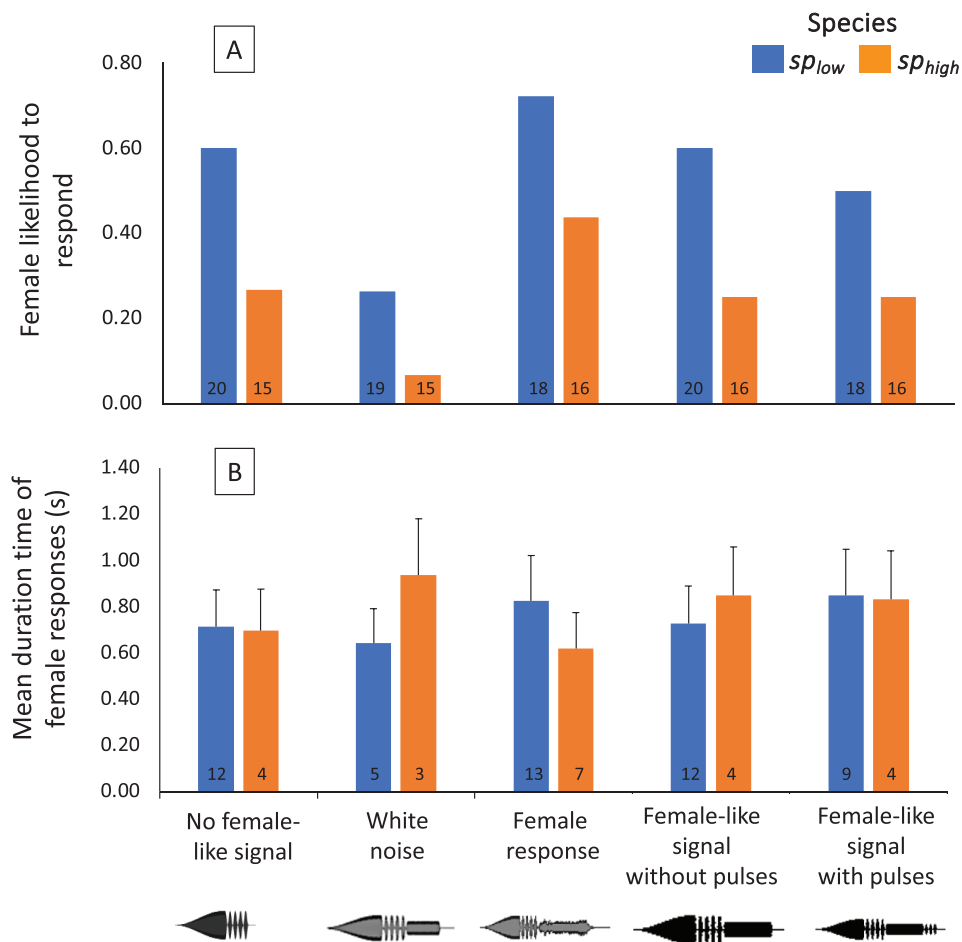
Figure 2. Female priming hypothesis. Responses of female treehoppers of two species of the *Enchenopa binotata* complex to artificial playback bouts with different stimuli after the first main advertisement signal. Sample sizes for each group of individuals presented with a particular stimulus are shown in numbers inside each bar in (A), and the numbers inside bars in (B) reflect only the females that responded to playbacks. Images below categories = stimuli after the first main signal in the playback (the second and third signals in the bout were identical between playbacks and not followed by any stimulus) (see the playbacks and a sample female response in Figure 1 and Supplementary Audio S1). Females were as likely to respond to playbacks with the female-like signals than to playbacks without it, and the same pattern occurred for the duration of female responses (see statistical results in Table 1).

they consistently produced the female-like signal across bouts, (b) they consistently did not produce it, and (c) they were inconsistent and produced it in only one of two bouts. We ran separate models with the signal rate or whine duration as the dependent variable. In these models, the explanatory terms were the above three categories of consistency, species, the signal bout order (first or second), and the interaction between the consistency and signal bout order terms.

We ran the models on JMP v. 16.0.0 (SAS Institute Inc., Cary, NC) and R v. 4.2.1 (R Development Core Team, 2022). The dataset, pictures of males, the playbacks used, and a representative subset of recordings are publicly available on Dryad (https://datadryad.org/stash/share/0svhLIM77Ku-hR-FRCRevXykeB2lbTkrJVq6339kkwm0).

Results

Female priming hypothesis

Females of both species were as likely to respond to playbacks with a female-like signal (regardless of whether it had pulses or not) as to playbacks without it (Figure 2A, Table 1). We found, however, that females had the highest likelihood to respond to playbacks of male advertisement

Table 1. Variation in the responses of females of two treehoppers species in the *Enchenopa binotata* complex (sp_{low}, sp_{high}) to artificial playback bouts of male displays. Results of the models testing the female priming hypothesis.

Term	Likelihood of female response			Mean time responding (s)		
	χ^2	DF	p	F	DF	p
Playback treatments	−2.37	4	.04	0.20	4	.19
Species	1.12	1	.14	0.13	1	.25
Playback × species interaction	0.35	4	.74	−0.25	4	.18
Temperature	−0.11	1	.75	−0.10	1	.11

signals that included a female duetting response signal (Figure 2A, Table 1). Additionally, the time females spent responding to playbacks did not differ between treatments (Figure 2B, Table 1). Given these findings, we found no evidence to support the prediction of the female priming hypothesis. Female-like signals did not increase the likelihood or the strength of females responding to the artificial playbacks.
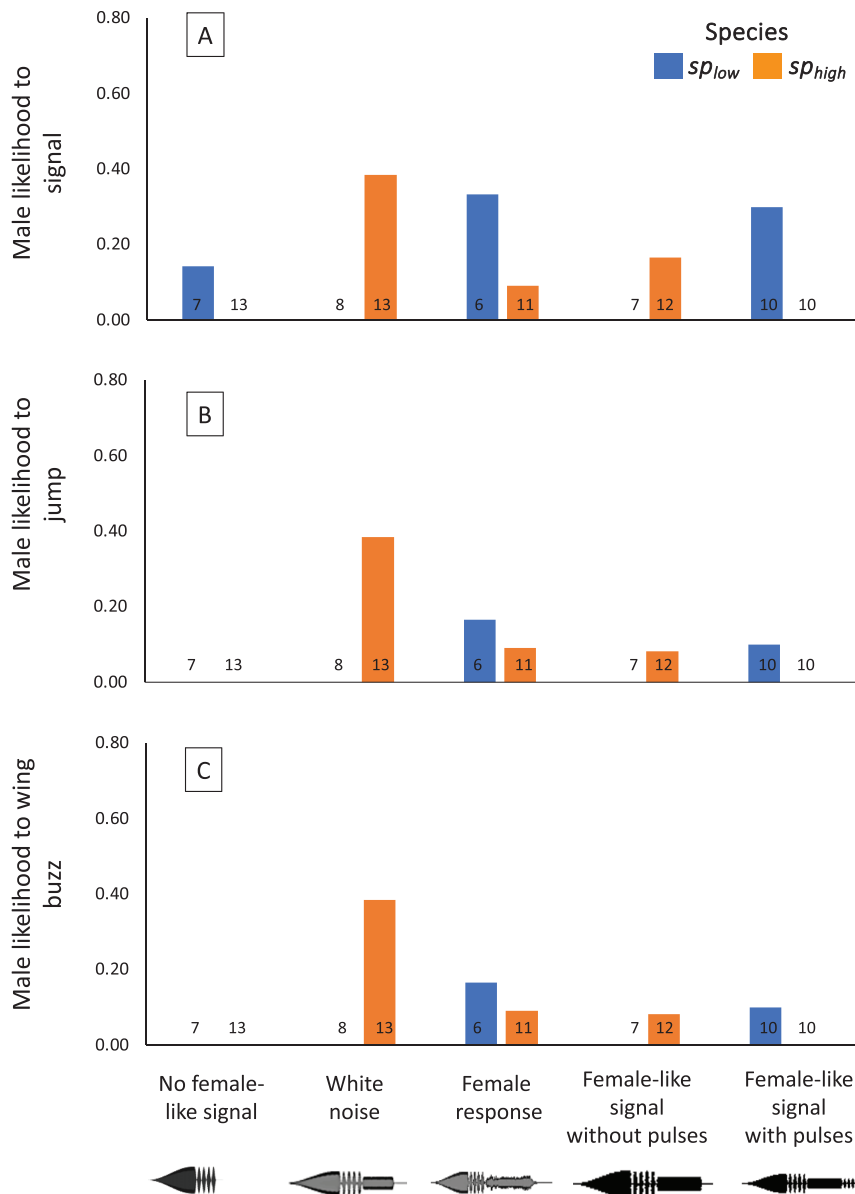
Figure 3. Male inhibition hypothesis. Responses of male treehoppers of two species of the *Enchenopa binotata* complex to artificial playback bouts with different stimuli after the first main advertisement signal. Tests for the predictions that the female-like signal will (A) decrease the likelihood of other males to signal, (B) increase the likelihood that they will jump off the plant, or (C) produce a wing buzz. Sample sizes for each group of individuals presented with a particular stimulus are shown in numbers inside each bar. Images below the categories represent the stimuli after the first main advertisement signal in the playback (the second and third signals in the bout were identical between playbacks, and not followed by female-like signals) (see Figure 1B and C for sample playback treatments). There were no differences in the likelihood of producing any behaviour (see statistical results in Table 2).

Male inhibition hypothesis

Males were unlikely to signal after any type of playback, regardless of whether it had a female-like signal, any other signal, or no additional playback stimulus at all (Figure 3A, Table 2). Only eight males (of 97 of both species) signalled after the playbacks (Figure 3A). Additionally, playbacks with different stimuli did not affect the likelihood of males jumping off the plant or producing a wing buzz (Figure 3B and C, Table 2). Therefore, we found no evidence to support the prediction of the male inhibition hypothesis. Female-like signals did not decrease the likelihood of males to continue signalling and did not seem to affect their overall behavioural response.

Behaviour and morphological correlates of the production of the female-like signal

Approximately two thirds of males of both species produced the female-like signal in spontaneous signalling. Males of both species that produced the female-like signal had higher signal rates (counting only main advertisement signals) (Figures 4A and 5A and B, Table 3). Furthermore, in sp_{low}, those males also produced longer whines than males that did not produce the female-like signal (Figures 4B and 5C and D, Table 3).

In both species, males who produced the female-like signal were in lower body condition than the males who did not (Figure 4C, Table 3). Males of sp_{low} in lower body condition

Table 2. Variation in the responses of males of two treehoppers species in the *Enchenopa binotata* complex (sp_{low}, sp_{high}) to artificial playback bouts of male displays. Results of the models testing the male inhibition hypothesis.

Term	Male likelihood to signal			Likelihood to jump off the plant			Likelihood to wing buzz		
	χ^2	DF	*p*	χ^2	DF	*p*	χ^2	DF	*p*
Playback treatments	19.74	4	.99	19.67	4	.98	−18.60	4	.98
Species	40.19	1	.98	−18.36	1	.99	−18.16	1	.99
Playback × species interaction	5.06	4	.16	4.82	4	.19	3.27	4	.21
Temperature	1.84	1	.47	2.18	1	.40	0.73	1	.71
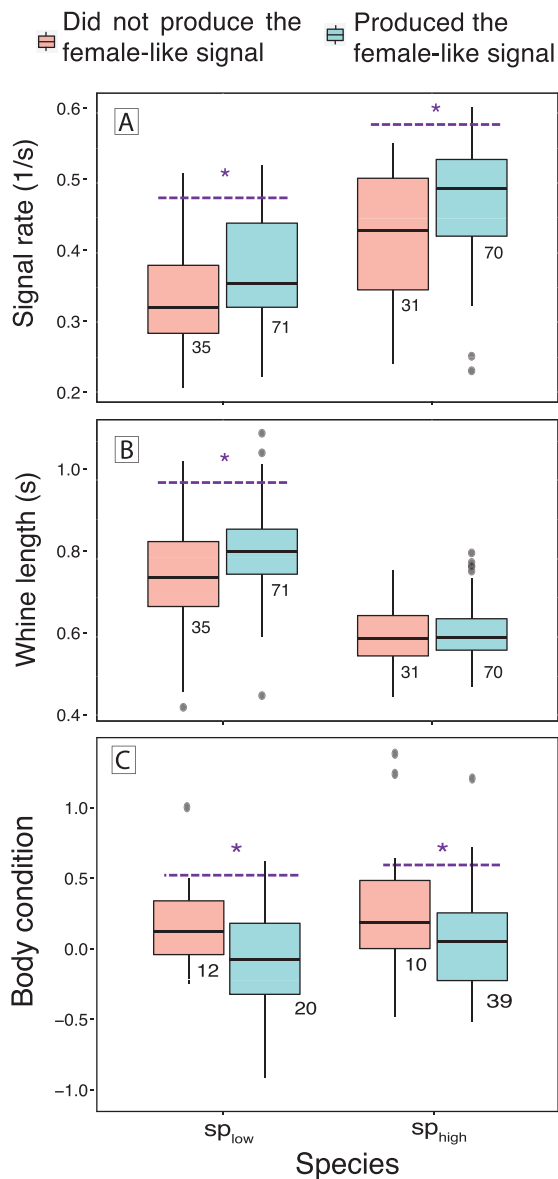
Figure 4. (A and B) Temporal features of the spontaneous vibrational signalling display of *Enchenopa binotata* male treehoppers, based on whether males produced the female-like signal. (A) Signal rate (1/interval between two signals), and (B) the length of the signal's whine. (C) Body condition of male treehoppers, the residuals of a regression of the body weight on body size (wing length). Boxplots = median ± 25% and 75% percentiles, vertical lines = range, and values beyond are outliers. The sample size for all features is shown under boxplots. Dashed lines and * = significant differences between males that produced the female-like signal or not (see statistical results in Table 3).

had higher signal rates (Figure 5A and B; marginally significant interaction between female-like signal produced and species, Table 4). There was no relationship between body condition and whine length (Table 4). As expected from prior work (Escalante et al., 2022), whine length differed between species (Table 4). Lastly, we found no difference in these temporal signal features between the males that consistently produced the female-like signal across bouts, those that did not produce it consistently, or the inconsistent males (Supplementary Figure S2, Supplementary Table S3).

Of 32 males for which we recorded two signal bouts, 28 (88%) were consistent in either producing the female-like signal or not (Figure 6). We found a significant relationship between producing the female-like signal or not in the first and second signal bouts (Table 5). The likelihood of producing the female-like signal in the second bout did not differ between species, the interaction between producing the female-like signal in the first bout and species, or the time interval between the two bouts (Table 5). Together, these findings suggest that the behaviour of producing the female-like signal or not is repeatable across time in these males.

Discussion

We tested two non-exclusive hypotheses regarding the possible sexual mimicry function of a signal in treehoppers: a signal produced by males that appears to mimic female duetting responses (Escalante et al., 2022). We first asked whether the female-like signal induces females to respond. Females responded more often to playbacks of male signals with real female duetting response signals, but the female-like signal produced by males did not increase female response. Therefore, although females could be prime to duet by listening to a real female response, that is not the effect of the artificial playbacks of the female-like signals we used. Consequently, we found no evidence for the female priming hypothesis.

Finding that a signal inside the courtship display of males does not increase female responses is puzzling. Previous work has experimentally shown that the features of the display of *Enchenopa* males are under strong sexual selection due to mate choice (Desjonquères et al., 2020; Rodríguez et al., 2004, 2006, 2013; Speck et al., 2020). The absence of an effect of the female-like signal on the female response could reflect that the initial part of the signalling display (where the female-like signal is typically produced) might not be under selection. The low amplitude of the first main advertisement and the female-like signal could support this possibility. Females might pay less attention to these initial signals than to the ones later in the display (i.e., third and fourth signals, which are also
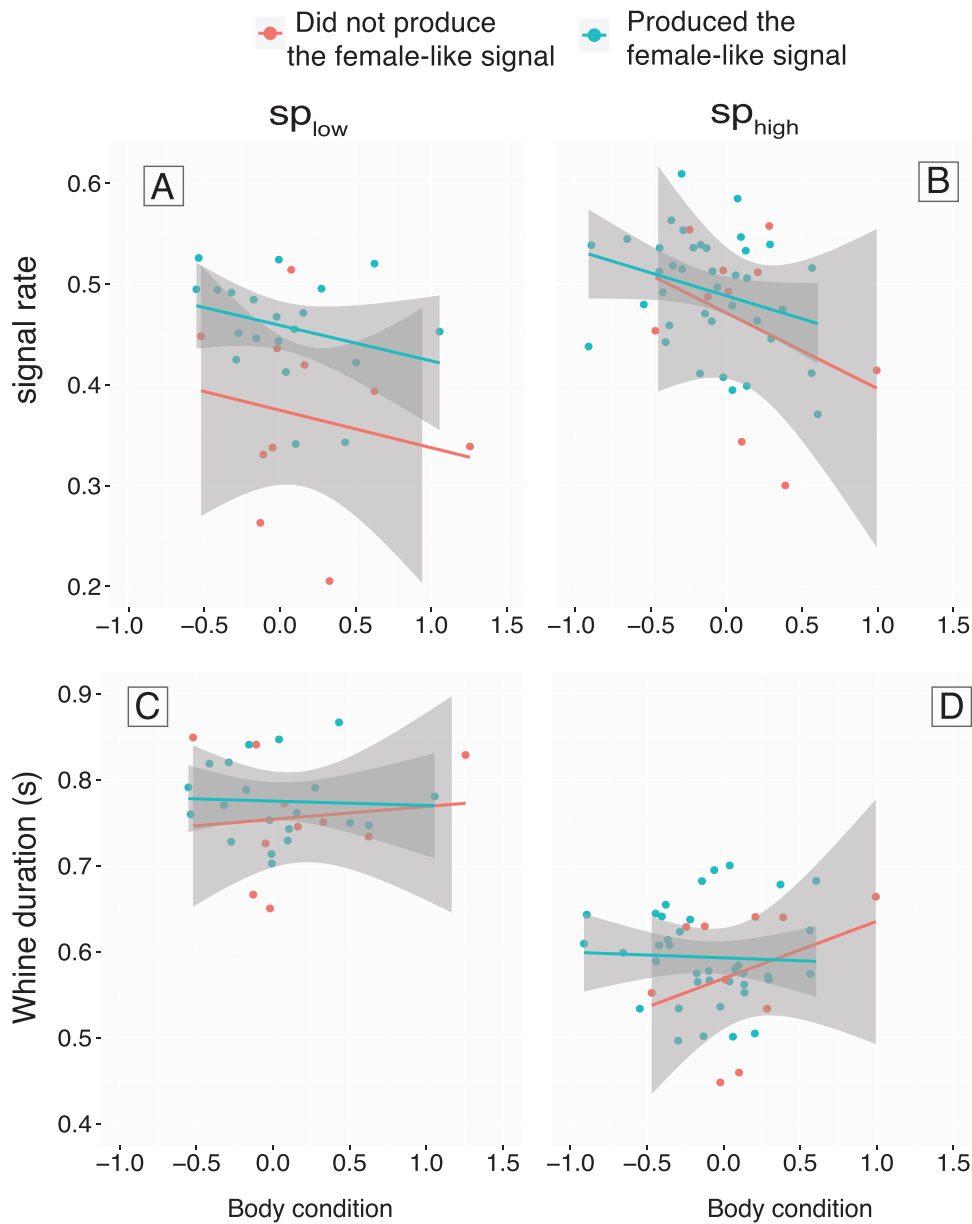
Figure 5. Relationship of the body condition on temporal features of the spontaneous vibrational signalling display of male treehoppers of two species of the *Enchenopa binotata* complex, based on whether males produced the female-like signal. The lines represent the best fit to a linear regression of each signalling phenotype (produced female-like signal or not), and the grey areas around it represent the 95% confidence interval. Males in lower body conditions produced displays with higher signal rates (see statistical results in Table 4).

Table 3. Variation in temporal features of the signal rate and length of spontaneous signal bouts produced by *Enchenopa* male treehoppers according to whether they produced a female-like signal.

Term	Signal rate			Whine length (s)			Body condition		
	F ratio	DF	p	F ratio	DF	p	F ratio	DF	p
Produced female-like signal (yes, no)	19.6	1/197	<.0001	5.1	1/192	.02	4.9	1/77	.03
Species	34.8	1/142	<.0001	110.6	1/110	<.0001	2.1	1/77	.15
Temperature	66.6	1/33	<.0001	1.1	1/44	.29	1.5	1/77	.70
Produced female-like signal × species interaction	0.02	1/197	.80	13.7	1/192	.001	0.1	1/77	.91

louder). Alternatively, our findings might suggest that the female-like signals are less similar to the real female responses than we expected and previously suggested (Escalante et al., 2022). Perhaps there are specific spectral or temporal features of the female responses that are absent in the female-like signals and that prevent females from responding in the same

Table 4. Variation in two temporal features of the spontaneous signalling display of males of two treehoppers species in the *Enchenopa binotata* complex (sp_{low}, sp_{high}), as a function of their body condition and signalling phenotype (producing the female-like signal or not).

Term	Signal rate			Whine duration		
	F ratio	DF	*p*	*F* ratio	DF	*p*
Body condition	5.81	1/72	.019	0.57	1/72	.453
Female-like signal produced (yes, no)	8.18	1/72	.006	2.27	1/72	.136
Species (sp_{low}, sp_{high})	13.41	1/72	.001	135.13	1/72	<.001
Body condition × female-like signal produced	0.11	1/72	.742	1.54	1/72	.219
Body condition × species	0.31	1/72	.577	0.14	1/72	.710
Female-like signal produced × species	3.67	1/72	.059	0.01	1/72	.949
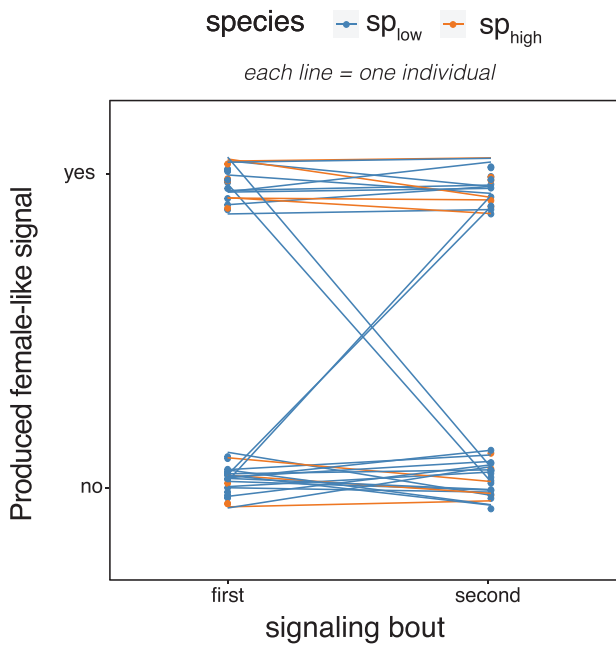
Figure 6. Repeatability in producing the female-like signal or not in the signalling phenotypes of male treehoppers of two species of the *Enchenopa binotata* complex. We recorded 32 males twice, and 88% were consistent in their signalling phenotype. Each line represents one individual.

Table 5. Variation in producing a female-like signal in the second signalling displayed recorded for 32 males of two treehoppers species in the *Enchenopa binotata* complex (sp_{low}, sp_{high}), as a function of whether they produced a female-like signal in the first bout, the species or whether the bouts were recorded the same day or not.

Term	Producing a female-like signal in the second bout		
	χ	DF	*p*
Producing the female-like signal in the first bout (yes, no)	18.9	1	<.001
Species (sp_{low}, sp_{high})	0.07	1	.99
Producing female-like signal in first bout × species	1.09	1	.17
Bouts recorded the same day (yes, no)	0.30	1	.59

based on the quantitative and qualitative features of the preferred displays of each species and from the specific field site where we collected the individuals.

Secondly, we asked whether the female-like signals influence other males' behaviour. Males were as unlikely to signal, jump off the plant, or produce a wing buzz after listening to playbacks with or without that signal. Thus, the female-like signal does not appear to inhibit other males from producing signalling displays, nor does it change their behaviour, as initially expected. This finding is also puzzling, as these treehoppers are very reactive to the signals in the social context they experience. We found no overall pattern of what kind of stimulus would make the listening males change their behaviour. Perhaps males follow exclusively their own motivation and the potential female responses to make decisions of whether to continue signalling or leave the plant, regardless of the signalling behaviour of other males in the plant. These findings have implications for understanding the social context in which males are likely competing for the attention of females.

With these results, the possible function of the female-like signal remains unknown. Below, we consider other potential adaptive and non-adaptive factors that might explain the presence of this signal. First, we consider that our findings of the behavioural and morphological correlates suggest alternative adaptive reasons. We found that males that produced the female-like signals showed higher signalling effort (higher signalling rates and longer signals) despite being in lower body condition. Furthermore, males were highly consistent in whether they produced the female-like signal or not. Consequently, the female-like signal could function to indicate the condition and motivation of the signalling male. Males in poor body condition (perhaps due to a compromised nutritional state) might be motivated to increase their effort in courting. This could be a way to compensate and access mating. We lack knowledge of how fast the energy reserves deplete in these animals. However, producing vibratory signalling displays is energetically costly in other insects (Kuhelj & Virant-Doberlet, 2022; Kuhelj et al., 2015). Whether signalling with higher effort compromises the condition in males is also unknown. Future work can address the relationship between these two processes. The actual benefit males obtain from either of them (a higher signalling effort or a higher body condition) remains to be discovered, and our current data are limited to making further inferences. However, a pilot test showed that males who produced the female-like signal were as likely to mate as males who did not (L. Cirino et al., unpubl.). Whether males are more likely to access mating if they repeatedly produce the female-like signal and display higher signalling effort over time remains unanswered.

way to both. Lastly, our findings could have been affected by methodological constraints. Perhaps the playbacks were not very attractive to females, even though we constructed them

We speculate that the behavioural and morphological correlates we found also hint at another adaptive function: the female-like signal may function to give the males producing it a motivational "self-boost." The effect of mimicking a female response could be similar to the positive feedback between behaviours and internal states that occur when animals win aggressive contests (Goubault & Decuignière, 2012; Lan & Hsu, 2011; Oliveira et al., 2009; Rutte et al., 2006; Sih et al., 2015). For an *Enchenopa* male, duetting with a female might provide a reward akin to winning a contest; e.g., males that receive female responses stay on the plant and continue to duet while locating the female, whereas males that do not receive female responses cease signalling and leave the plant (Cocroft et al., 2008; Rodríguez & Cocroft, 2006). When males perceive that another male is duetting with a female and has located her, they often leave the plant (D. Little, *pers. comm.*). Consequently, producing the female-like signal might increase the male's internal drive to signal, and make him stay in that plant signalling and increasing its chances of getting a duetting response from a female response. Further experimental work can manipulate the levels of motivation of males with agonists and antagonists of dopamine or octopamine, which influences "optimism" in insects (Barron et al., 2010; Peng et al., 2020; Perry & Baciadonna, 2017; Solvi et al., 2016). Then, if experimentally motivated males produce the female-like signal more often, this could support a self-boost hypothesis. This would constitute a novel function of sexual mimicry, as it would involve males exploiting their own positive neurosensory feedback (Reichert & de la Hera, 2022) to modify the behaviour of the signaller, rather than that of receivers, as usually found in sexual mimicry (Bailey et al., 2006; Hanlon et al., 2005; Norman et al., 1999; Thornhill, 1979).

The context in which *Enchenopa* treehopper males produce the female-like signal suggested an adaptive function in pair formation, as has been found in other insects using acoustic and vibrational displays (Bailey et al., 2006; Boumans & Johnsen, 2015; Stumpner & Meyer, 2001). First, other features of the main male advertisement signals, especially but not exclusively dominant frequency, are under strong divergent sexual selection due to mate choice (Rodríguez et al., 2006, 2013) in the *E. binotata* species complex. This has resulted in strong signal-preference coevolution across species in the complex, mainly but not exclusively involving signal frequency and the corresponding mate preferences (Cocroft et al., 2010; Rodríguez et al., 2006, 2013). Second, as advertisement signals, female-like signals are species-specific, differing mainly in dominant frequency, and being similar in quantitative traits to the female duetting responses (Escalante et al., 2022). Third, their production does not appear to be incidental: they are produced by ca. 70% of males in the species we have assessed and show a strong pattern to appear only at the beginning of signal bouts (Escalante et al., 2022). Despite these factors, we found no evidence to support an adaptive function related to reproduction for this signalling trait.

We should also consider the possibility that the signal type we explored here may have no direct adaptive function in signalling. Among the potential non-adaptive functions, it could serve to warm up the muscles associated with the production of the vibrations. It could also be a "practice signal" for males to prepare to perform the signals later on the signalling display. Those signals (for example, the fourth signal) have a higher amplitude and a higher likelihood of being heard by females and other males in the same plant. The female-like signal could also be just a by-product of the signalling display of males. For instance, it could be an artefact of signal transmission along plant stems; e.g., if they arose from echoes due to plant tissue irregularities (Michelsen et al., 1982). We consider this unlikely because then these signals would occur more frequently and be more likely later in the signal bout, where advertisement signals have a higher amplitude (Cocroft et al., 2008, 2010), which is not the case (Escalante et al., 2022). Three sources of evidence we presented here and in Escalante et al. (2022) that might support the possible non-adaptive functions of the female-like signal are (a) that it is mainly only produced after the first signal in a bout, (b) with a lower amplitude than that main male advertisement signals, and (c) this signal might overlap with the actual female duetting response, preventing males from hearing the female and interrupting the formation of the duet. However, future experimental work should test explicit hypotheses regarding potential non-adaptive explanations for these signals. Ultimately, our findings bring a puzzling challenge of unravelling why a female-like signal does not seem to provide direct fitness benefits while being performed in the male signalling bouts, which are under strong sexual selection.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The dataset is publicly available on Dryad (https://doi.org/10.6078/D1QF08).

Author contributions

Ignacio Escalante (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Funding acquisition [supporting], Investigation [equal], Methodology [equal], Project administration [equal], Resources [equal], Software [supporting], Supervision [equal], Validation [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [equal]), Jerald Kellner (Data curation [equal], Funding acquisition [supporting], Investigation [equal], Methodology [equal], Validation [supporting], Visualization [supporting], Writing—review & editing [supporting]), Camille Desjonquères (Conceptualization [equal], Data curation [supporting], Funding acquisition [equal], Investigation [supporting], Software [supporting], Visualization [supporting], Writing—review & editing [supporting]), Gianna Noffsinger (Data curation [supporting], Investigation [supporting], Methodology [supporting], Visualization [supporting]), Lauren Cirino (Data curation [supporting], Investigation [supporting], Methodology [supporting], Writing—review & editing [supporting]), Ariel Noriega Rodriguez (Data curation [equal], Investigation [equal], Methodology [equal]), Sage DeLong (Data curation [supporting], Investigation [supporting], Methodology [supporting]), and Rafael Rodriguez (Conceptualization [equal], Formal analysis [equal], Funding acquisition [equal], Project administration [supporting], Resources [supporting], Supervision

Conflicts of interest

The authors declare no conflict of interest.

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