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Supplementary audio S1_Sample female reponse to playback_HF277.wav

Title page

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

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Conflict of interest

We declare no conflict of interest.

Journal of Evolutionary Biology
August 08, 2023

Dear Author,

Thank you for submitting your manuscript "On the function of a female-like signal type in the vibrational repertoire of<i> Enchenopa</i> male treehoppers (Hemiptera: Membracidae)" (JEB-2023-00160) to the Journal of Evolutionary Biology. Your submission has now been assessed by an Associate Editor and two reviewers, whose comments are included below. As you will see, all found merit in your manuscript and would ultimately like to see it published in our journal. However, they have raised some points that need to be addressed in a major revision of your paper before we can accept it for publication.

Both Reviewers and the Associate Editors do not feel as though all the requested revisions have been adequately addressed, and I share this view. Reviewer 1 and 2 provide a detailed list of what still needs to be addressed so I will not go over these in excessive detail. In short, I believe that the two most important areas that still needs work is (1) to better clarify your methods - this section is still long, repetitive and confusing to follow and (2) greater consideration needs to be given to potential "non-adaptive" explanations for female-like signals. This could be addressed in more detail in the Discussion but I feel this is important to provide a more balanced view of your work. I believe that you should be able to incorporate these changes in a revised manuscript, so am willing to give you a final opportunity to do so.

Please submit your revised paper in an editable format within eight weeks of this letter, otherwise it will be considered a new submission. I hope that you will find our feedback valuable and look forward to receiving your revised manuscript.

Sincerely,

John Hunt
Handling Editor

AUTHOR RESPONSE:
Dear Dr. Hunt
Handling Editor
The Journal of Evolutionary Biology

We greatly appreciate the positive response to our manuscript, and we are very grateful that the associate editor and the two reviewers provided extensive constructive feedback on how to address the main issues in the previous version. Additionally, we appreciate the opportunity to have our findings considered for publication in your journal.

We agreed with all the points raised, addressed all the comments, and made substantial revisions to incorporate the feedback within the manuscript. We consider that this revision successfully addressed the concerns, and the changes substantially improved the clarity, conciseness, and rigor of the presentation of our work. Below, we provide a detailed point-by-point response to the comments. Overall, we hope the revision is suited for acceptance.

Thank you very much in advance for receiving and evaluating our revision. We are very grateful for the opportunity to publish our work in The Journal of Evolutionary Biology. I look forward to hearing from you.

Editor: Nadeau, Nicola
Comments to the Author:

[comment 1]:
I feel the manuscript still gives too much emphasis to the motivational boost hypothesis. I am not particularly convinced by the arguments presented in the introduction that the female-like signals must have an adaptive significance, and I think non-adaptive explanations should be considered in the discussion.
AUTHOR RESPONSE:
Revised as suggested. We removed the mentions of the self-boost hypothesis from the Abstract and the Introduction. In addition, we moved and revised the arguments of the adaptive function of the signal from the introductory paragraph to the Discussion. In the fifth paragraph of the Discussion, we present the revised arguments and contextualize how those were our expectations. Still, given our findings, we found no evidence that the signal has an adaptive function. That section now provides a more balanced view of explaining our results, and considering alternative adaptive and non-adaptive scenarios that could explain the presence of the female-like signal in the display of these males.

[comment 2]:
I also agree with both reviewers that the methods section is unnecessarily long and repetitive and could be structured better to make it easier to follow and reduce repetition.
AUTHOR RESPONSE:
We have addressed all the reviewers' comments and made all the suggested changes. We did additional revisions in ways that we consider our experiment is shown with a better structure, avoiding repetitions, and with an easier flow. Briefly, we condensed sections, reorganized figures, and revised the data analyses. For the latter, we combined the two models that separately tested for assumptions and predictions of each hypothesis into one model for each hypothesis. This reorganization does not affect the theoretical basis of the hypothesis. Additionally, the patterns found with these ‘new’ models are consistent with the previous models. Hence, this revision presents our methods more directly without compromising the reasoning or the data presentation.

Reviewer: 1

Comments to the Author

This is a revision of a previously submitted manuscript, testing hypotheses about the ultimate causes of a female-like signal produced by males in treehopper species.
I previously expressed reservations about one of the hypotheses – that males produce female-like signals to provide themselves with a motivation boost – and references to the signal as a 'female mimicry' without further corroboration. I agree with the authors that their changes have addressed my previous concerns.

[comment 3]:
One small reservation that remains, for me, is the lack of consideration that the female-like signal might not have any adaptive benefit. I do understand the desire to understand the evolution of the unusual signal, but think it is worth considering (maybe in the discussion) that the it might just be, e.g., a consequence of poor condition.
AUTHOR RESPONSE:
We agree. The revision provides a more balanced and extensive mention and discussion of other adaptive and non-adaptive factors that might explain the presence of the female-like signal. We also included mentions to this in the revised abstract. The possibility of this signal not having any adaptive benefit exists, and we appreciate the reviewers' and editor's call to consider it. By incorporating it, we consider that the revised version has stronger and broader implications.
As suggested here (and below in comment 22), we are including the possibility that the body condition of males might explain the presence of this signal. As for other non-adaptive reasons, we include the possibility of this signal to be an indication of muscle warm-up, practice for the subsequent signals, and a byproduct or echo of the signaling bout transmitting across the plant.

I have a few more comments:

[comment 4]:
Stylistically, the Methods section is long and feels quite repetitive. I think it would improve the readability of the paper if the authors could find a way to streamline this. For example, if predictor variables for each of the models are displayed in tables, maybe they do not need to be listed in full for each of the models in the Methods.
AUTHOR RESPONSE:
We agree with this perspective. We have thoroughly revised the Methods section to make it more concise, direct, and less repetitive. We removed the repeated mentions of the models' descriptions (particularly those constructed using the same predictor terms). Additionally, we move the descriptions of the models to each section describing the two hypotheses (see response to Comment 17 below).

[comment 5]:
Next, in the Results section, I think it would be helpful to the reader if the authors could, in each section, briefly summarise the biological interpretation of their results with respect to their assumptions/predictions either at the beginning or end of each section.
AUTHOR RESPONSE:

As suggested, we added explicit summaries of the biological effects of the findings in the first two sections of the Results section. The revised statements now reflect our findings more closely in light of the predictions of each hypothesis.

Specific comments:

[comment 6]:
Line 92: “We found evidence against both hypotheses…”
I would change this to say “We did not find evidence to support either hypothesis”, or similar.
AUTHOR RESPONSE:
Revised as suggested.

[comment 7]:
Line 407: Should “This suggests…” be replaced with “Further inspection suggests…” since the direction of the pattern is not obvious from the presence of a significant interaction?
AUTHOR RESPONSE:
Thank you for this suggestion. We revised the mentions of these findings (previous and following sentences) to clarify the direction of the interaction. We consider the revised paragraph to show the results more succinctly.

[comment 8]:
Line 429: maybe it would be useful for clarity, at the start of the discussion, to specify ‘”female-like signals produced by males”.
AUTHOR RESPONSE:
Revised as suggested. In the first paragraph of the Discussion, we clarify in two instances that we are referring to the female-like signals produced by males.

[comment 9]:
Line 441-458: I feel there is a missing link between describing the effect that duetting with a female may have on male motivation (lines 445-450), and the hypothetical effect that a male producing its own female-like signal might have (raised in lines 441-445, but not rationalised much further).
AUTHOR RESPONSE:
We agree there was a missing link. We have revised the paragraph to clarify our reasoning and added one sentence to link the production of the female-like signal with the motivation obtained by staying on a plant and seeking a response from females to their signaling bout.

[comment 10]:
Line 453: the Lynch & Ryan citation is a bit ambiguous here, and seems to suggest in context that there is precedent for poor condition males producing female-like signals to increase their drive. Is that the case? If not, could the authors expand?
AUTHOR RESPONSE:
We appreciate noting this. We agree on the ambiguous placement of the citation. We removed the citation as there is no precedent that poor-condition males are more likely to produce

female-like signals. We revised the sentence to make our argument more explicit and to allow a better flow in the context of the paragraph.

[comment 11]:
Figures 2 & 3: I am not sure I find the 'assumptions' and 'predictions' headings useful here. The assumptions heading in particular might suggest these are not empirical observations. In Fig. 2, the 'assumptions' heading appears seemingly at random in the middle of panel E. Also, there is inconsistent use of colours for the SPlow and SPhigh in the two figures – why not use the same colours?
AUTHOR RESPONSE:
Thank you for the suggestions. As noted above, we simplified the data presentation (and analysis) so that the assumption/prediction is no longer necessary. The new figures show all five treatments simultaneously, allowing for more direct comparison and avoiding confusion about whether the 'assumption' represents empirical observations.
Additionally, we standardized the color use across all figures. We are not using the same pattern (blue and orange) in all figures that sort the data by species (Figs. 2,3 and 6). The original scheme was aimed to reflect that some data was on females and some on males. However, the suggested revision will be better for readers.

[comment 12]:
Between figures 5 and 6, the same colour scheme is used for two different factors (production of female-like signal, and species, respectively). In Fig. 6, the colour scheme could be replaced by that used in Figs. 2 and 3 (if these are standardized).
AUTHOR RESPONSE:
We revised the color scheme of the figures as suggested. We are now using blue/orange colors in all figures that sort the data by species (Figs. 2, 3, and 6) and pink/light blue for Figs. 4 and 5, which both sort data by whether males did the female-like signal or not.

Reviewer: 2

Comments to the Author
I have carefully read the revised manuscript "On the function of a female-like signal type in the vibrational repertoire of Enchenopa male treehoppers (Hemiptera: Membracidae)". Below, I express my comments and concerns, sometimes referring to particular lines in the manuscript but many of the comments are for the manuscript as a whole.

[comment 13]:
Overall, my main concern is that the methods continue to be confusing and that the discussion jumps from a general stating of the results to over 3 paragraphs of an alternative hypothesis without discussing why, two seemingly robust hypotheses with assumptions and predictions, presumably built on previous literature and knowledge of the system, did not have support. Even when the authors did not find significant results, I believe that the discussion would benefit from exploring why this is the case instead of jumping to another hypothesis.
AUTHOR RESPONSE:

We really appreciate taking the time to provide constructive feedback on our manuscript continuously.

Regarding the methods, we made many substantial changes that alleviate the abovementioned concerns. We have revised the data analyses (models for hypotheses 1 and 2) and presentation (figures) to present the experiment more succinctly.

Regarding the discussion, we also made significant changes and expansions on the potential reasons behind our findings. As expanded below (see responses to Comments 19 and 20), we provide a more thorough discussion of our experiment's findings, detailing the potential causes of not finding an effect of the female-like signal, and considering other alternative adaptive and non-adaptive explanations for this unusual signal.

[comment 14]:

In the introduction, you have included a set of possibilities of why a female-like signal is involved in pair formation. I find that after reading them, I am not convinced. The first reason talks about how the frequency of the main advertisement call is under strong sexual selection but does not speak about the presence or absence of a female-like call. The second reason states that advertisement signals and female-like signals are species-specific, differing in dominant frequency, differing among species? Among advertisement signals and female-like signal? If so, are they critical for pair formation? Are advertisement signals the same frequency as the female-like signals? Perhaps the information is out there, but as it is written now, it is a list of facts regarding signals but does not tell the reader the “why’s” of the alleged function of the female-like signal. For example, do males that do not produce the female-like signal unable to form duets? Are males that produce the female-like signal more successful in acquiring mates? If so, then the third reason holds some weight, but as it is written now, it is not convincing.

AUTHOR RESPONSE:

To address this, we revised and moved that whole paragraph to the Discussion (it is now the 5th paragraph in the section). With this, it is now part of a more balanced Discussion of whether the female-like signal has an adaptive function (which our data cannot resolve at this point). Ultimately, we consider that our manuscript presents a puzzling challenge of unraveling why a female-like signal does not seem to provide direct fitness benefits, despite the fact that it's performed in the male signaling bouts, which are under strong sexual selection.

Also, to address the questions raised regarding the ‘whys’, we expanded on the fact that we had found that the female-like signal is similar in several quantitative traits to the female duetting responses in our previous paper (Escalante et al. 2022 *Behaviour* 159). We also added a mention of a pilot study in which we found that a subset of males that had produced the female-like signal was as likely to mate as males that did not produce it; this is at the end of the 3rd paragraph of the Discussion.

With the revisions from this and many other comments, we consider that this new framework in the Discussion allows for a more balanced and thorough examination of the implications of our findings and proposes future work.

[comment 15]:

In the methods, previous issues raised by the reviewers continue. There is confusion regarding

what the first set of playback is and then what the second set is. For example, the first set has three stimuli and then, the second set has the no-female signal, plus (d) and (e), but later in the manuscript it is stated that there are “5 options”, which ones? The reader has to read the text, and then interpret the figure, which in reality has 6 options, but as stated in the response to the reviewers: “two sets of three (of a total of five playback treatments). Then, you also write “(There is a total of 5 playbacks -instead of 6- because we used one playback [the ‘no female-like signal]”. Why isn’t this explain in the text? This is extremely confusing and requires several reads to actually understand what is going on.
AUTHOR RESPONSE:
We agree that this brought up confusion. We re-framed our data analyses and figures to address this issue. We are now using all five playback treatments in the same analyses. Hence, the description in the Methods sections and the revised Fig. 1B now describe the treatments and how we used them together to test the hypothesis. Using all five treatments in the same statistical models holds the theoretical framework of the hypotheses and tests both assumptions and predictions. Additionally, the revised models show the same biological patterns as the previous sets of models. Therefore, we consider that the changes condense, clarify, and bring a smoother flow to our experiment.

[comment 16]:
You do not introduce the significance of assumptions and predictions until you reach the methods, but they should be introduced earlier and explain why the need to separate the two. The reviewers brought this up in the previous version of the manuscript. The distinction is not clear and this was not resolved in this new version of the manuscript.
AUTHOR RESPONSE:
We appreciate the suggestion. After carefully reading the description of the hypotheses predictions and revising the methods and results section, we revised the manuscript so that we no longer include the assumptions/predictions framework. As noted above in response to Comments 2 and 15, we merged the analyses, and we now compare all five treatments used in only one model to test each hypothesis. This new approach retains the ability to test for the assumption and prediction of each hypothesis by comparing the same treatments. Additionally, the new models yielded the same biological findings as in previous versions of the ms, with the advantage of describing and presenting the results more succinctly and clearly.

[comment 17]:
I am not sure if adding the statistical analysis separate from the main hypotheses actually helped. I would suggest creating subsections for each hypothesis with its respective statistical analysis to avoid redundance.
AUTHOR RESPONSE:
Revised as suggested. We added and summarized the description of the statistical analyses to each of the sections in methods describing the hypotheses and the behavioral and morphological correlates of the female-like signal.

[comment 18]:
In Line 251, the authors explained that to measure signal rate, they used only the third and

fourth signal, and that because of this, there was no female-like signal present. To effectively measure signal rate, wouldn't it be necessary to measure the whole bout? It is not clear what is the rationale behind using just the third and the fourth advertisement signal, other than citing that it has been done in previous work. But is it possible that the timing between the first and second signal is different from the third and the fourth, especially if the males produce a female-like signal? The authors need to explain how these measurements of signal effort relate to the elicitation of female-like signals to be relevant in the current manuscript.

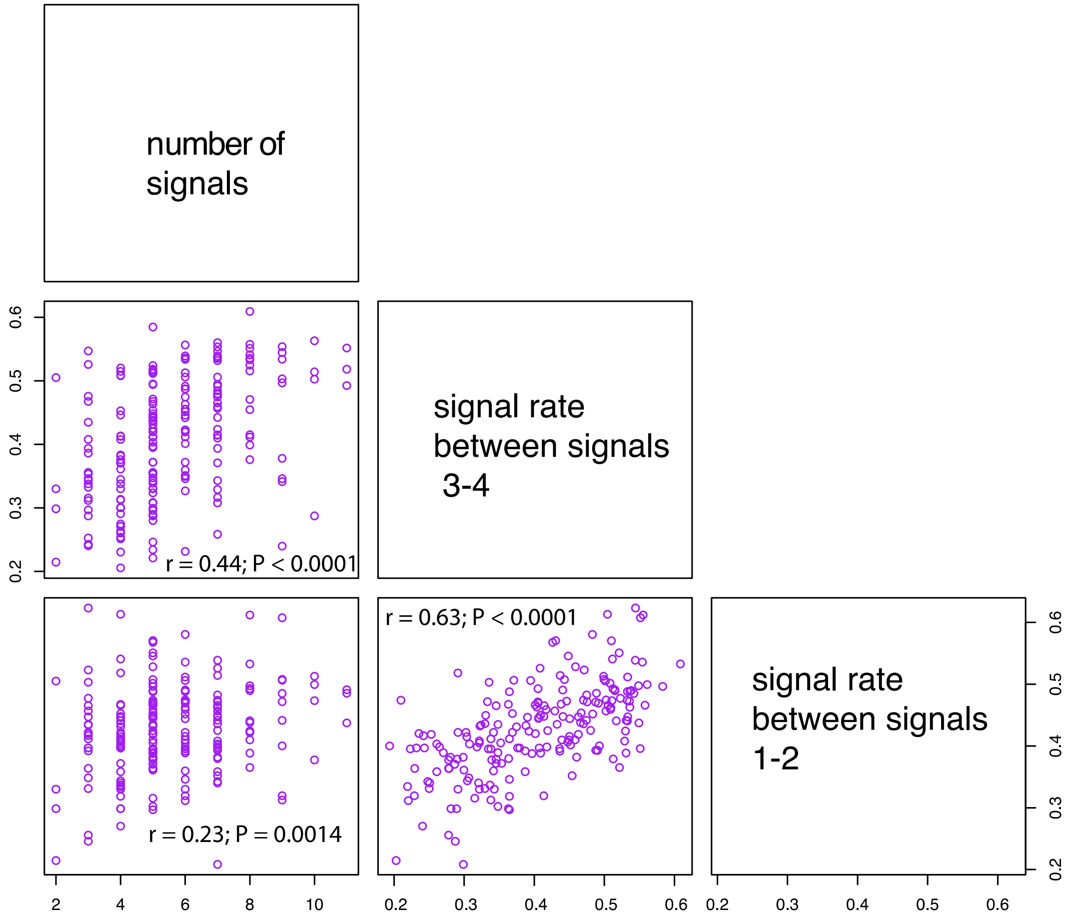
AUTHOR RESPONSE:

We appreciate bringing this up. We agree that the reasoning needed to be clarified in the previous version. We have expanded on this. The third and fourth signals in the bout are more consistent between bouts, and previous research on these insects has shown that sexual selection is a driver of this consistency. Hence, we measured the signal rate similarly to maintain consistency with previous work, which will allow further inter-study comparisons, meta-analyses, and other approaches.

We agree with the point about potential differences in the signal rate between signals 1-2 and 3-4, as the former can contain the female-like signal. However, the signal rate does not differ between those two moments. In a previous paper, we found that the inter-signal duration (the inverse of the signal rate) did not vary between signals 1-2, and signals 3-4 (2.60s and 2.85 s on average, respectively) (See Table 2 on Escalante et al. 2022. *Behaviour* 159).

Additionally, we conducted another analysis and found that both measures of signal rate are highly correlated with each other, as well as with the number of signals in the bout (signal rate for signals 1-2 and signal rate for signals 3-4: $r = 0.63$; $P < 0.0001$, $n = 203$; number of signals and signal rate for signals 3-4: $r = 0.44$; $P < 0.0001$; number of signals and signal rate for signals 1-2: $r = 0.23$; $P = 0.0014$; see Figure below).

Lastly, we expanded on explaining these findings and how they relate to the production of the female-like signal in the first paragraph of the 'Behavioral and morphological correlates…' subsection of the revised Methods section. We consider that the changes clarify and sustain our reasoning.



[comment 19]:
What is repeatability? In the number of female-like signals? In their characteristics (i.e., frequency, duration)? And why is this important? Also, the authors include a citation (Sattman & Cocroft 2003) "male treehoppers maintain consistent qualitative and quantitative features in their signaling display over minutes, days, and even weeks" So why look at repeatability in the first place? Presumably, the authors want to determine if the presence of a female-like signal in the first bout explains the presence of a female-like signal in the second bout. If true, then it does not come across clearly in the text. Throughout the introduction and the methods, the relevance of measuring these traits is not explained. As it reads now, it is just additional measures that the authors explored, but without a foundation of why it would be relevant in the big picture. The lines 362-368 are not particularly easy to understand, and it is unclear what are the expected results.

AUTHOR RESPONSE:

We appreciate this comment. We revised all the instances in which we mentioned the repeatability data to clarify that our motivation was to explore the relationship between the female-like signal and other behaviors related to the signaling displays. As pointed out, we were interested in determining if the presence of the signal in one bout predicts its presence in the second bout. We have clarified this in the Introduction, Methods, and Results, and in the

Introduction, we included a mention to tie this -and other- measures we explore with the big picture of exploring the function of the 'additional' signal we found in these treehoppers. Additionally, we removed the analysis mentioned in lines 362-368 in the previous version. We also improved the quality of the reasoning behind the one model we are presenting to assess repeatability in producing the female-like signal. For this, we edited the third paragraph of Statistical Analyses in the 'Behavioral and morphological correlates…' subsection.
We also removed the mention of that additional model in the last paragraph of Results. All these revisions streamline and highlight the reasoning for exploring the repeatability of producing the female-like signal.

[comment 20]:
The discussion is the main weakness of the manuscript. The current discussion lacks depth and fails to address the implications of your findings effectively. They re-stated their findings in the first paragraph of the discussion, but there is no actual discussion of the findings. For example, they found that females respond to the female signals but not the female-like signals. What does this mean? How different are the female signals from the female-like signals? What are the possible explanations for this behavior, and why they didn't find a similar pattern with the female-like signals?
AUTHOR RESPONSE:
We appreciate flagging this and providing constructive ways to address this issue. We have extensively expanded the Discussion of the experimental part of our work. The Discussion now includes a thorough argumentation of potential explanations for our findings and their implications. The second paragraph addressed the three questions raised here. The revised manuscript has an overall more robust Discussion section.

[comment 21]:
The same applies to the male inhibition hypothesis. Presumably, the assumptions are built in the literature and previous knowledge of the species, and the authors' predictions are constructed based on what they expected, but they did not find supporting evidence. This needs to be discussed. However, the authors skipped this step and jumped to another explanation. Thus, the discussion does not discuss the findings nor considers caveats or alternative hypotheses (other than the self-boost hypothesis). The other reviewer brought this up in the previous version of the manuscript. What other explanations can be considered?
AUTHOR RESPONSE:
We expanded the discussion on the findings of the male inhibition hypothesis and their implications. In parallel, many revisions we made throughout the Discussion section allow us to address the big-picture issue raised here. We have added mentions to other adaptive and non-adaptive reasons that might explain the presence of the female-like signal and potential methodological caveats. We consider that the revisions result in a more thorough and balanced Discussion, and manuscript overall.

[comment 22]:
Body condition was measured after the treehoppers produced their vocalization. How quickly are the energy reserves depleted? Is it possible that because that greater signaling effort they

are in worst body condition? Is it possible that they were in better condition at the beginning, and they can afford to signal more if that ensures a mate? Are female-like signals attractive to females? can both males and females discriminate between female and female-like signals? I believe that all of this should be included and discussed in the Discussion section.

AUTHOR RESPONSE:

These are excellent points, we appreciate the suggestions. We expanded the Discussion of the effect of body condition and its potential correlation with signaling effort in the third paragraph of the Discussion. We added another citation (Kuhelj & Virant-Doberlet 2022) as well as mentions to the questions raised in this comment and the limitation of our data and available information to make inferences to explain the relationship between these mechanisms. Overall, incorporating these mentions allows for addressing this and other comments above, resulting in a stronger Discussion, and delineating how further studies can tackle these aspects.

[comment 23]:

Lastly, in terms of writing style, there are several instances throughout the manuscript in which statements are made in parenthesis or using em-dash and while it is okay in some instances, when repeated too many times it does not help with the flow of the paper. I suggest rewriting sentences to incorporate the information in parenthesis or breaking long sentences with a lot of information in two. Likewise, there is redundancy in cases such as Lines 132–133 (same as 127–128) and in Lines 213–214, repeating information mentioned earlier in the text.

AUTHOR RESPONSE:

We appreciate this stylistic suggestion. The revisions described above resulted in editing and removing most instances of sentence fragments in parenthesis or M-dashes, particularly in the Methods section. As suggested, we also edited some sentences in the Introduction and Discussion, mainly by splitting sentences into 2. Lastly, we removed the redundant mentions noted.

[comment 24]:

In summary, while improvements have been made, the manuscript's biggest weaknesses remain the methods and the discussion, the discussion being critical for consideration for publication. Some parts of the methods remain difficult to follow, and enhancing the overall clarity would significantly improve the reader's understanding of the manuscript. Negative results can be challenging to explain, but providing a clear and comprehensive discussion of these outcomes is crucial for the scientific process. It is critical that the authors address the potential reasons behind these negative results.

AUTHOR RESPONSE:

Regarding the discussion, we have revised the section thoroughly: We expanded our arguments to explain the negative results we found and more detailed reasoning behind linking the behavioral and morphological correlates of the female-like signal with the potential motivational and adaptive functions of this signal. Additionally, we included several non-adaptive factors that might explain the presence of this signal and considerations of why the signal might arise as a by-product of other physiological processes of the treehoppers, or the physical properties of the transmitting substrate.

Regarding the methods, we followed the reviewers' and editor's suggestions, and the revised manuscript presents our experiments more succinctly and directly. Hence, we strongly consider incorporating feedback and re-framing our data analyses and presentation to yield an easier-to-follow manuscript.

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

Abstract

Animals often mimic the behaviors 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 behavior 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 behavioral and morphological factors of the males were associated with the production of the female-like signal. Males that produced this signal had higher signaling 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 signaling, or even be a byproduct 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.

Commented [A1]: Comments 3, 13, 24

Key Words

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 behaviors, body coloration, pheromones, or signals of individuals of another sex (Forsyth & Alcock, 1990; Field & Keller, 1993; 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 (Norman *et al.*, 1999; Hanlon *et al.*, 2005); 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 (Fig. 1A) (Escalante *et al.*, 2022). *Enchenopa* are plant-feeding insects that communicate with substrate-borne vibrational signaling (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 (Rodríguez *et al.*, 2004; Cocroft *et al.*, 2008). 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, this 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 signaling 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 signaling. By simulating that a female is already duetting with them, males might induce other males to move away as that responsive female is already

Commented [A2]: Comments 1,14

Commented [A3]: Comment 23

Commented [A4]: Comment 16

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 signaling and leave the plant (Rodríguez & Cocroft, 2006; Cocroft *et al.*, 2008). Thus, the male inhibition hypothesis predicts that a female-like signal experimentally added to a signaling bout will decrease the likelihood that other males will continue signaling (Bailey *et al.*, 2006).

Commented [A5]: Comment 16

We did not find evidence to support either hypothesis (see Results), leaving the question of the function of 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' signaling behavior and morphology. Specifically, we assessed a potential relationship with signaling 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 behavioral features, we aimed to identify factors that could explain the adaptive or non-adaptive origin of the female-like signal in these insects.

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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 from 2018 to 2022 (see Table S1 for further information on the field sites). These currently undescribed species can be distinguished by nymph coloration (gray versus black with white stripes) and male signal frequency (~165 Hz 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 are 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 to 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 x 70 cm). When individuals molted 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 signaling displays of each species (see Fig. 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 (Fig. 1B, 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, dominant frequency) of signals of each species from the field site where we collected them (Rodríguez *et al.*, 2018; Escalante *et al.*, 2022). We created playbacks with custom-written code in R (R 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).

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We used five playback stimuli (Fig. 1B): (a) no female-like signal: a bout of main advertisement signals without a stimulus between the first and second signal, 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 signaling. 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 (Fig. 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).

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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 x 6.5mm x 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 Scientifics, 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 ± standard deviation: 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 minute. 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 two 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 attempt. 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 (Figs. 1D, Supplementary Audio S1). We also noted after which signal(s) the female responded (as she could respond three 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 signaled 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 2/3 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 [$r = 0.31$, $P = 0.005$] and with summed response length [$r = 0.73$, $P < 0.001$]).

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Statistical analysis

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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, Fig. 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 signaling 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.

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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 lineal 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 = 0.91; recording identity × species: Wald P = 0.65) nor in the time responding (recording identity: Wald

$P = 0.51$; recording identity × species: Wald $P = 0.41$). 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 signaling 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 afterward, 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 three days. Of those males, 46% signaled the first time, and the rest in the following 2 – 7 times.

We recorded the male's behavior in the following 15 s. We noted (a) if the male responded to the playback by producing a signaling bout. We also noted two additional behaviors 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 signaling 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 signaling, we ran a logistic regression model as the one described above. We used whether the male signaled after the playback (yes, no) as the dependent variable. The explanatory terms were playback treatment (five treatments, Fig. 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.

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

We analyzed the spontaneous signaling bouts males produced before being presented with the playbacks in the above trials. We also analyzed the spontaneous signaling 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 signaling behavior 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 signaling effort as they increase the duty cycle of the signal bouts (cf. Greenfield, 2002). We analyzed the recordings with Audacity with a 100-2000Hz bandpass 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 < 0.0001, n =203; respectively). Therefore, the signal rate we report below is a good proxy of the signaling 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.

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We explored the relationship between the production of the female-like signal, signaling 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 signaling effort in *Enchenopa*, as signaling is quite costly in other vibrational insects (e.g., Kuhelj *et al.*, 2015; Kuhelj & Virant-Doberlet, 2022). 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 (Table S2). These are standard morphological traits studied in *Enchenopa* (Cocroft & de Luca, 2005; 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 $P < 0.001$ 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 signaling 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 signaling bouts before we presented them with the above playbacks). For the other 14 males (all sp_{low}), we recorded each signaling bout in different recording sessions, separated by at least one day (two males one day apart, eight males two days apart, and one male each of the following time frames: seven, 10, 19, and 22 days apart). We combined data from both groups since *Enchenopa* male treehoppers maintain consistent features in their signaling display over time (Sattman & Cocroft, 2003).

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Statistical analysis

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To analyze the relationship between producing the female-like signal and male signaling 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 (Fig. S1). This trait is species-specific. Hence, we did not expect that males could modify it to increase their signaling 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* > 0.46 for both models).

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To assess the repeatability of individual differences in producing the female-like signal, we used a 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.

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Lastly, we compared signal rates and whine lengths between males that showed one of three categories of consistency: (i) they consistently produced the female-like signal across bouts, (ii) they consistently did not produce it, (iii) 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 is publicly available on Dryad (https://datadryad.org/stash/share/0svhLIM77Ku-hRFRCRevXykeB2lbTkrJVq6339kkwm0).

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 (Fig. 2A, Table 1). We found, however, that females had the highest likelihood to respond to playbacks of male advertisement signals that included a female duetting response signal (Fig. 2A, Table 1). Additionally, the time females spent responding to playbacks did not differ between treatments (Fig. 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.

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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 (Fig. 3A, Table 2). Only eight males (of 97 of both species) signaled after the playbacks (Figs. 3A). Additionally, playbacks with different stimuli did not affect the likelihood of males jumping off the plant or producing a wing buzz (Figs. 3B, 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 signaling and did not seem to affect their overall behavioral response.

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

Approximately 2/3 of males of both species produced the female-like signal in spontaneous signaling. Males of both species that produced the female-like signal had higher signal rates (counting only main advertisement signals) (Fig. 4A; Fig. 5A, B; Table 3). Further, in sp_{low}, those males also produced longer whines than males that did not produce the female-like signal (Fig. 4B; Fig. 5C, D; Table 3).

In both species, males that produced the female-like signal were in lower body condition than the males that did not (Fig. 4C, Table 3). Males of sp_{low} in lower body condition had higher signal rates (Fig. 5A, 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 (Fig. S2, 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 (Fig. 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 behavior of producing the female-like signal or not is repeatable across time in these males.

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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.

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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 (Rodríguez *et al.*, 2004, 2006, 2013; Desjonquères *et al.*, 2020, 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 signaling 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 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 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 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.

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Secondly, we asked whether the female-like signals influence other males' behavior. 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 signaling displays, nor does it change their behavior, 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 behavior. Perhaps males follow exclusively their own motivation and the potential female responses to make decisions of whether to continue signaling or leave the plant, regardless of the signaling behavior 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.

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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 behavioral and morphological correlates suggest alternative adaptive reasons. We found that males that produced the female-like signals showed higher signaling effort (higher signaling rates and longer signals) despite being in lower body condition. Further, 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 signaling 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 signaling displays is energetically costly in other insects (Kuhelj *et al.*, 2015, Kuhelj & Virant-Doberlet, 2022). Whether signaling 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 (signaling efford and condition) remains to be discovered, and our current data is limited to making further inferences. However, a pilot test showed that males that produced the female-like signal were as likely to mate as males that did not produce it (L. Cirino *et al.*, unpubl.). Whether males are more likely to access mating if they repeatedly

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produce the female-like signal and display with higher signaling effort over time remains unanswered.

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We speculate that the behavioral 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 behaviors and internal states that occur when animals win aggressive contests (Rutte *et al*., 2006; Oliveira *et al*., 2009; Lan & Hsu, 2011; Goubault & Decuignière, 2012; 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 signaling and leave the plant (Rodríguez & Cocroft, 2006; Cocroft *et al*., 2008). 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 signaling 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; Solvi *et al*., 2016; Perry & Baciadonna, 2017; Peng *et al*., 2020). 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

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behavior of the signaler, rather than that of receivers, as usually found in sexual mimicry (Thornhill, 1979; Norman *et al.*, 1999; Hanlon *et al.*, 2005; Bailey *et al.*, 2006).

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 (Stumpner & Meyer, 2001; Bailey *et al.*, 2006; Boumans & Johnsen, 2015). 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 (Rodríguez *et al.*, 2006, 2013; Cocroft *et al.*, 2010). 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 signaling trait.

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We should also consider the possibility that the signal type we explored here may have no direct adaptive function in signaling. 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 signaling 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 byproduct of the signaling display of males. For instance, it could be an artifact 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 (1) that it is mainly only produced after the first signal in a bout, (2) with a lower amplitude than that main male advertisement signals, and (3) 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 unraveling why a female-like signal does not seem to provide direct fitness benefits while being performed in the male signaling bouts, which are under strong sexual selection.

Commented [A31]: Comments 1, 3, 14, 24

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Tables

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.

	Likelihood of female response			Mean time responding (s)		
Term	χ^2	DF	*P*	F	DF	*P*
Playback treatments	-2.37	4	0.04	0.20	4	0.19
Species	1.12	1	0.14	0.13	1	0.25
Playback × species interaction	0.35	4	0.74	-0.25	4	0.18
Temperature	-0.11	1	0.75	-0.10	1	0.11

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

	Male likelihood to signal			Likelihood to jump off the plant			Likelihood to wing buzz		
Term	χ^2	DF	*P*	χ^2	DF	*P*	χ^2	DF	*P*
Playback treatments	19.74	4	0.99	19.67	4	0.98	-18.60	4	0.98
Species	40.19	1	0.98	-18.36	1	0.99	-18.16	1	0.99
Playback × species interaction	5.06	4	0.16	4.82	4	0.19	3.27	4	0.21
Temperature	1.84	1	0.47	2.18	1	0.40	0.73	1	0.71

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.

	Signal rate			Whine length (s)			Body condition		
Term	F ratio	DF	*P*	F ratio	DF	*P*	F ratio	DF	*P*
Produced female-like signal (yes, no)	19.6	1/197	<0.0001	5.1	1/192	0.02	4.9	1/77	0.03
Species	34.8	1/142	<0.0001	110.6	1/110	<0.0001	2.1	1/77	0.15
Temperature	66.6	1/33	<0.0001	1.1	1/44	0.29	1.5	1/77	0.70
Produced female-like signal × species interaction	0.02	1/197	0.80	13.7	1/192	0.001	0.1	1/77	0.91

Table 4. Variation in two temporal features of the spontaneous signaling display of males of two treehoppers species in the *Enchenopa binotata* complex (sp_{low}, sp_{high}), as a function of their body condition and signaling 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	0.019	0.57	1/72	0.453
Female-like signal produced (yes, no)	8.18	1/72	0.006	2.27	1/72	0.136
Species (sp_{low}, sp_{high})	13.41	1/72	0.001	135.13	1/72	<0.001
Body condition x female-like signal	0.11	1/72	0.742	1.54	1/72	0.219

produced						
Body condition x species	0.31	1/72	0.577	0.14	1/72	0.710
Female-like signal produced x species	3.67	1/72	0.059	0.01	1/72	0.949

Table 5. Variation in producing a female-like signal in the second signaling 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.

	Producing a female-like signal in the second bout		
Term	χ	DF	*P*
Producing the female-like signal in the first bout (yes, no)	18.9	1	<0.001
Species (sp_{low}, sp_{high})	0.07	1	0.99
Producing female-like signal in first bout x species	1.09	1	0.17
Bouts recorded the same day (yes, no)	0.30	1	0.59

Figures

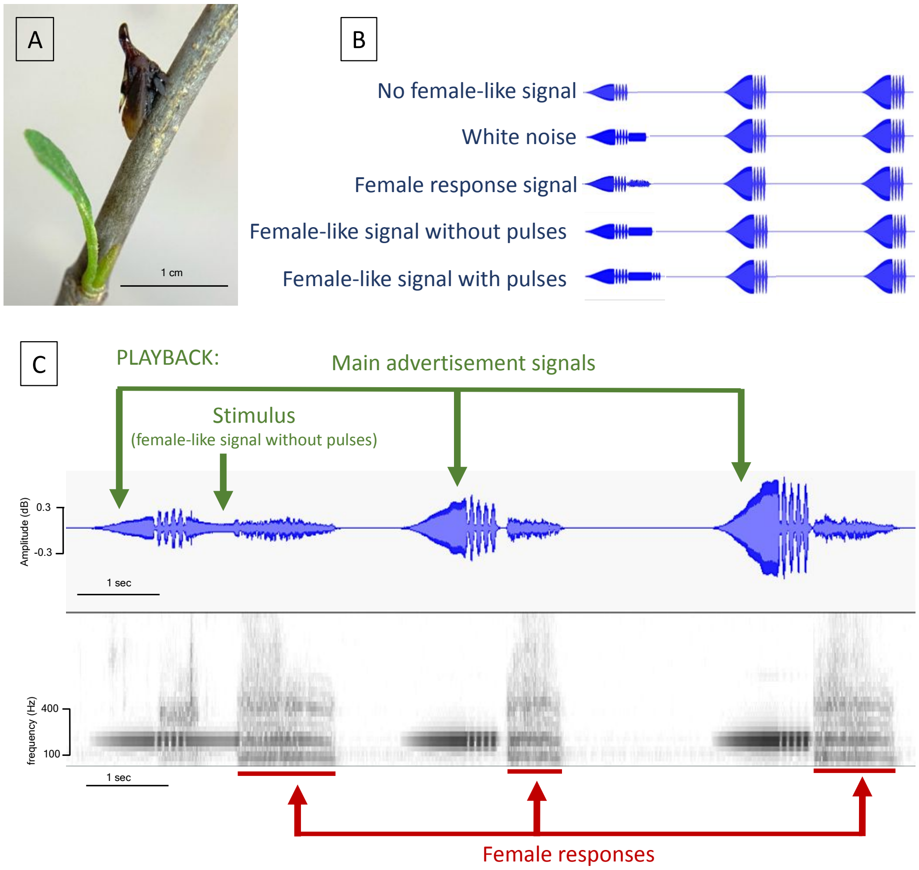


Figure 1. Experimental test of the female-like signal in the vibratory courtship display of *Enchenopa binotata* males. (A): sample adult male of *E. binotata* sp_{low}, photo by I. Escalante. (B): The five experimental playback treatments presented to females and males of two species (sp_{low} and sp_{low}) to test the female priming (Fig. 2) and the male inhibition (Fig. 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 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 Figs. 2B.

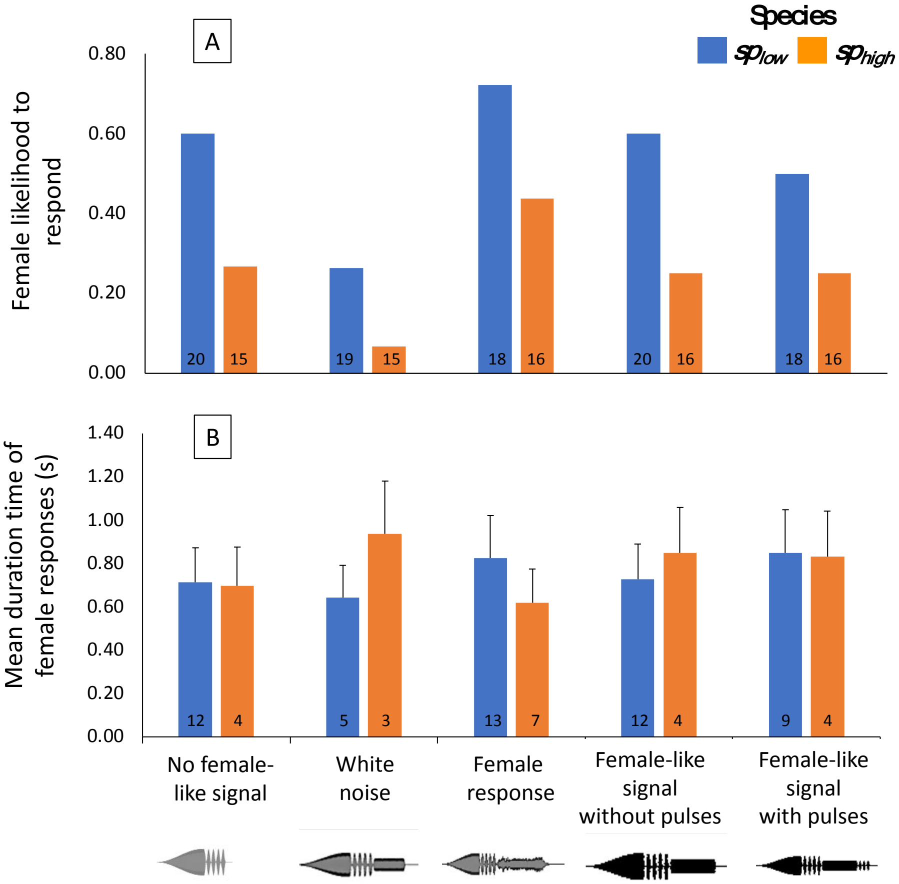
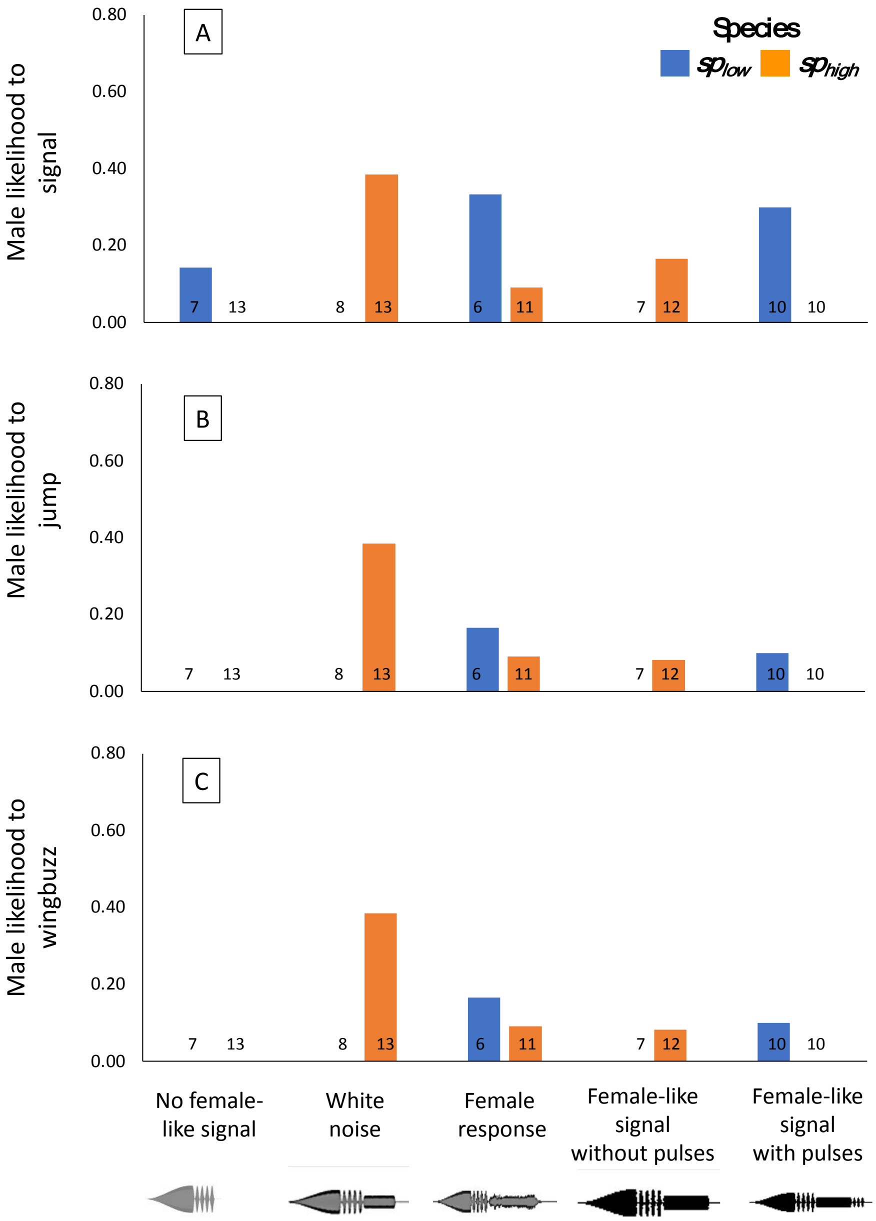


Figure 2. Female priming hypothesis. Responses of female treehoppers of two species of the *Enchenopa binotata* complex to artificial playback bouts with different stimuli after the first main advertisement signal. Sample sizes for each group of individuals presented with a particular stimulus are shown 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 Fig. 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).


A
B
C
Species
*sp*low
*sp*high
Male likelihood to signal
Male likelihood to jump
Male likelihood to wingbuzz
0.00
0.20
0.40
0.60
0.80
7
13
8
13
6
11
7
12
10
10
No female-like signal
White noise
Female response
Female-like signal without pulses
Female-like signal with pulses

683

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 Figs. 1B, C for sample playback treatments). There were no differences in the likelihood of producing any behavior (see statistical results in Table 2).

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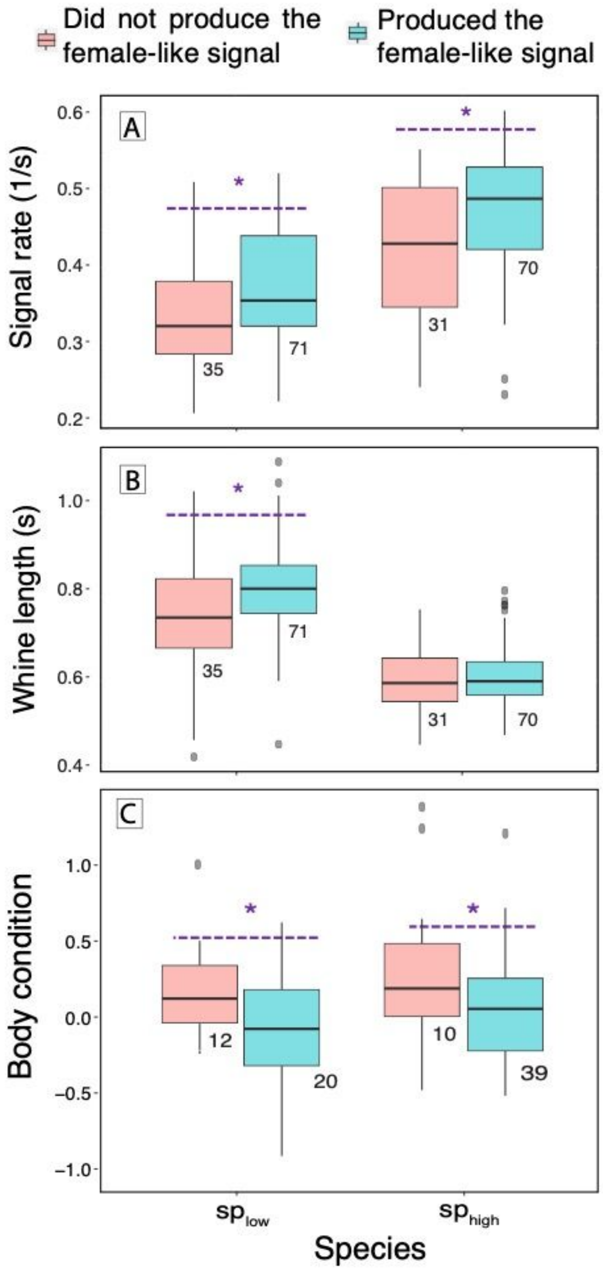

Did not produce the female-like signal
Produced the female-like signal
A
Signal rate (1/s)
0.6
0.5
0.4
0.3
0.2
*
*
35
71
31
70
B
Whine length (s)
1.0
0.8
0.6
0.4
*
35
71
31
70
C
Body condition
1.0
0.5
0.0
−0.5
−1.0
*
*
12
20
10
39
sp_{low}
sp_{high}
Species

694

Figure 4. A, B: Temporal features of the spontaneous vibrational signaling 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).

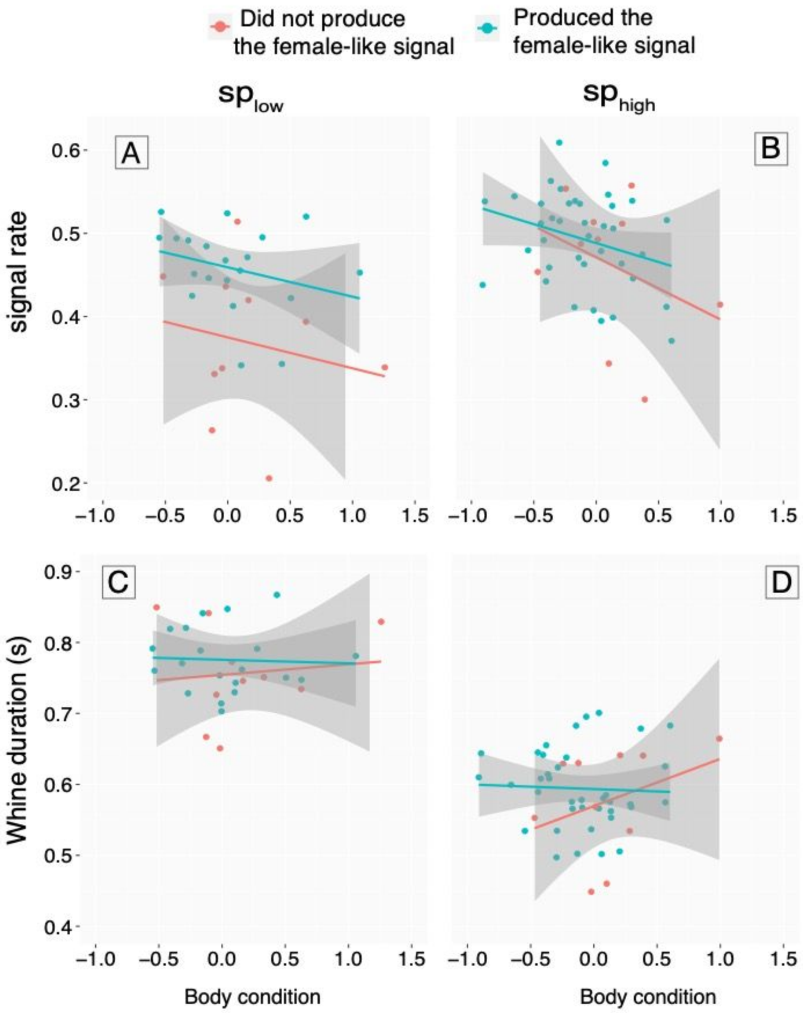


Figure 5. Relationship of the body condition on temporal features of the spontaneous vibrational signaling display of male treehoppers of two species of the *Enchenopa binotata* complex, based on whether males produced the female-like signal. The lines represent the best fit to a linear regression of each signaling 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).

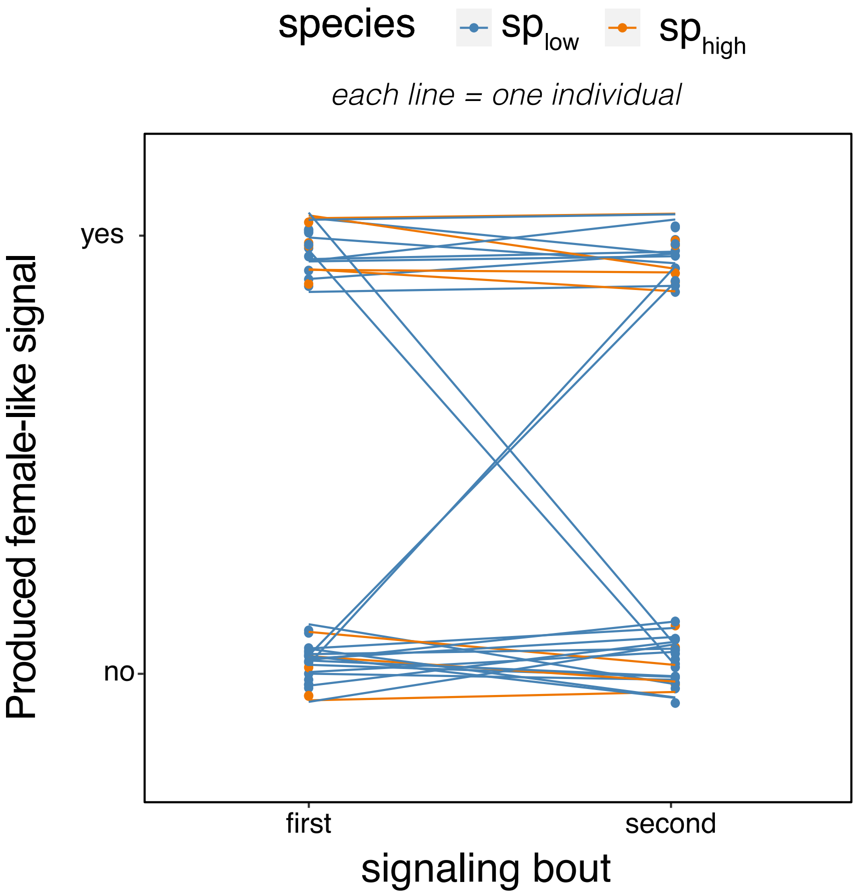


Figure 6. Repeatability in producing the female-like signal or not in the signaling phenotypes of male treehoppers of two species of the *Enchenopa binotata* complex. We recorded 32 males twice, and 88% were consistent in their signaling phenotype. Each line represents one individual.

Commented [A33]: Comment 12

Supplementary Material

Supplementary Table 1. Variation in the morphological features of males of two species of the *Enchenopa binotata* complex, based on whether males produced the female-like signal.

Species	Morphological measure	Did not produce the female-like signal					Produced the female-like signal				
		n	mean	SD	min	max	n	mean	SD	min	max
sp_{low}	Wing length (mm)	12	3.9	0.3	3.5	4.4	20	3.8	0.4	2.8	4.3
	Body length (mm)	12	4.3	0.7	2.2	5.1	18	4.4	0.3	3.8	4.8
	Face length (mm)	12	6.5	3.6	1.0	12.0	18	9.5	5.3	1.0	18.0
	Femur leg III (mm)	12	1.5	0.1	1.3	1.8	15	1.5	0.2	1.2	2.0
	Tibia II width (mm)	10	0.4	0.1	0.3	0.5	15	0.4	0.1	0.3	0.5
	Body weight (mg)	14	4.8	0.6	3.8	5.8	20	4.5	0.4	3.9	5.2
	Body condition (Residuals of wing length and weight)	12	0.3	0.5	-0.5	1.3	20	0.1	0.4	-0.6	1.1
sp_{high}	Wing length (mm)	16	3.9	0.2	3.4	4.3	51	3.8	0.3	3.2	4.6
	Body length (mm)	16	4.9	0.2	4.6	5.4	44	4.8	0.4	3.7	5.7
	Face length (mm)	17	9	5.1	1.0	17.0	44	22.1	12.4	1.0	43.0
	Femur leg III (mm)	12	1.4	0.2	0.9	1.7	39	1.4	0.2	1.0	2.0
	Tibia II width (mm)	11	0.4	0.1	0.3	0.5	34	0.4	0.1	0.3	0.5
	Body weight (mg)	21	4.6	0.5	3.1	5.4	57	4.4	0.5	3.4	5.5
	Body condition (Residuals of wing length and weight)	10	0.1	0.4	-0.5	1.0	39	-0.1	0.4	-1.0	0.6

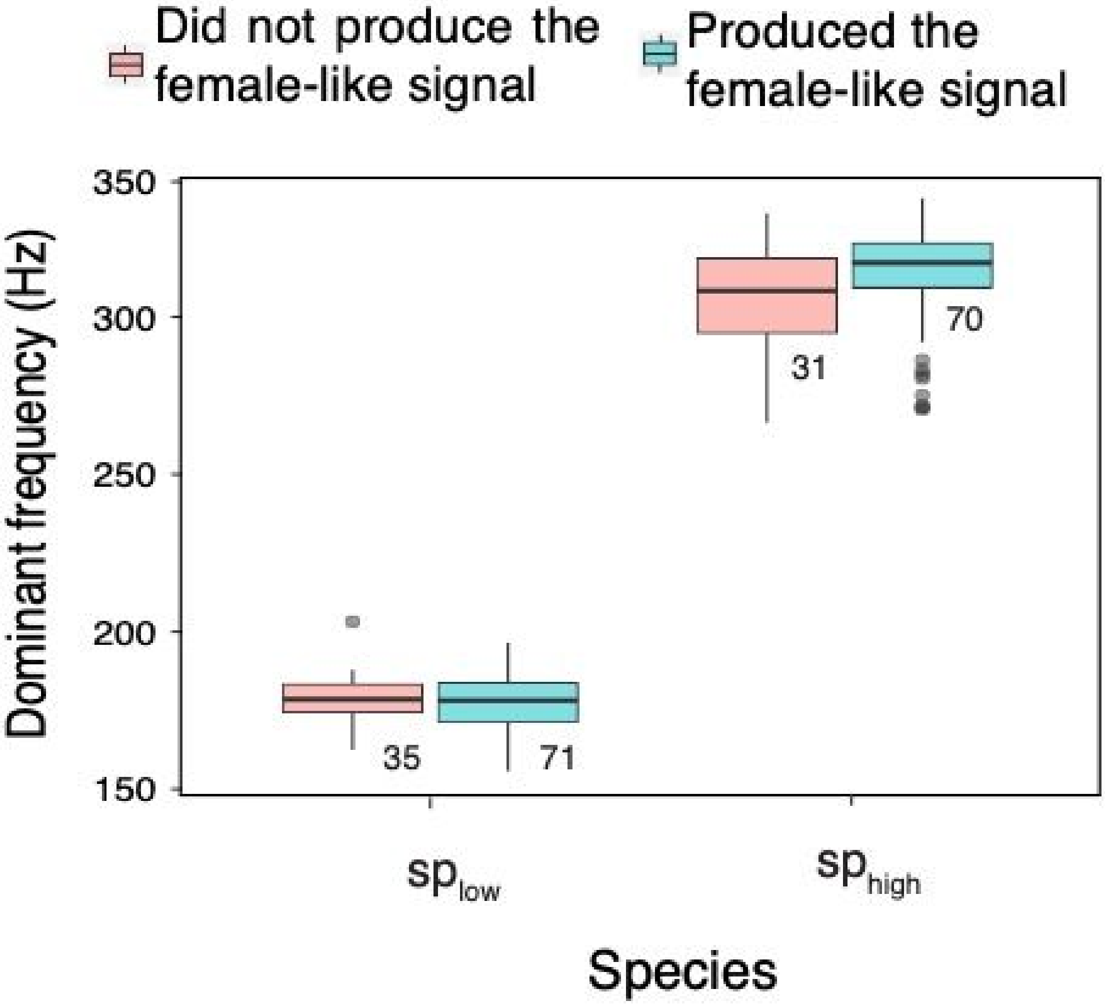
Supplementary table 2. Field collecting of treehoppers 3rd instar nymphs of two species in the *Enchenopa binotata* complex in meadows and forest edges of Ozaukee and Milwaukee Counties, Southeastern Wisconsin, USA. Numbers reflect the sample size of adult males and females used to extract data for different parts.

			Females				Males						
Field site	Coordinates	Year	Female priming hypothesis		Male inhibition hypothesis		Signaling effort		Body condition		Repeatability		More information on the field site
			sp_{low}	sp_{high}	sp_{low}	sp_{high}	sp_{low}	sp_{high}	sp_{low}	sp_{high}	sp_{low}	sp_{high}	
Cedarburg Bog State Natural Area	43.403349 N, 87.998840 W	2021	96	78	38	59	38	81	32	49	5	6	Escalante *et al.*, 2022
		2020					3						
		2019					3						
		2018					7				2	1	
Pleasant Valley Nature Park	43.354702 N, 87.976574 W	2020					36	2					Desjonquères et al. *in revision*
		2019					3				3		
UWM Field Station	43.387128 N, 88.022517 W	2020					1	7					
		2018					9	11					

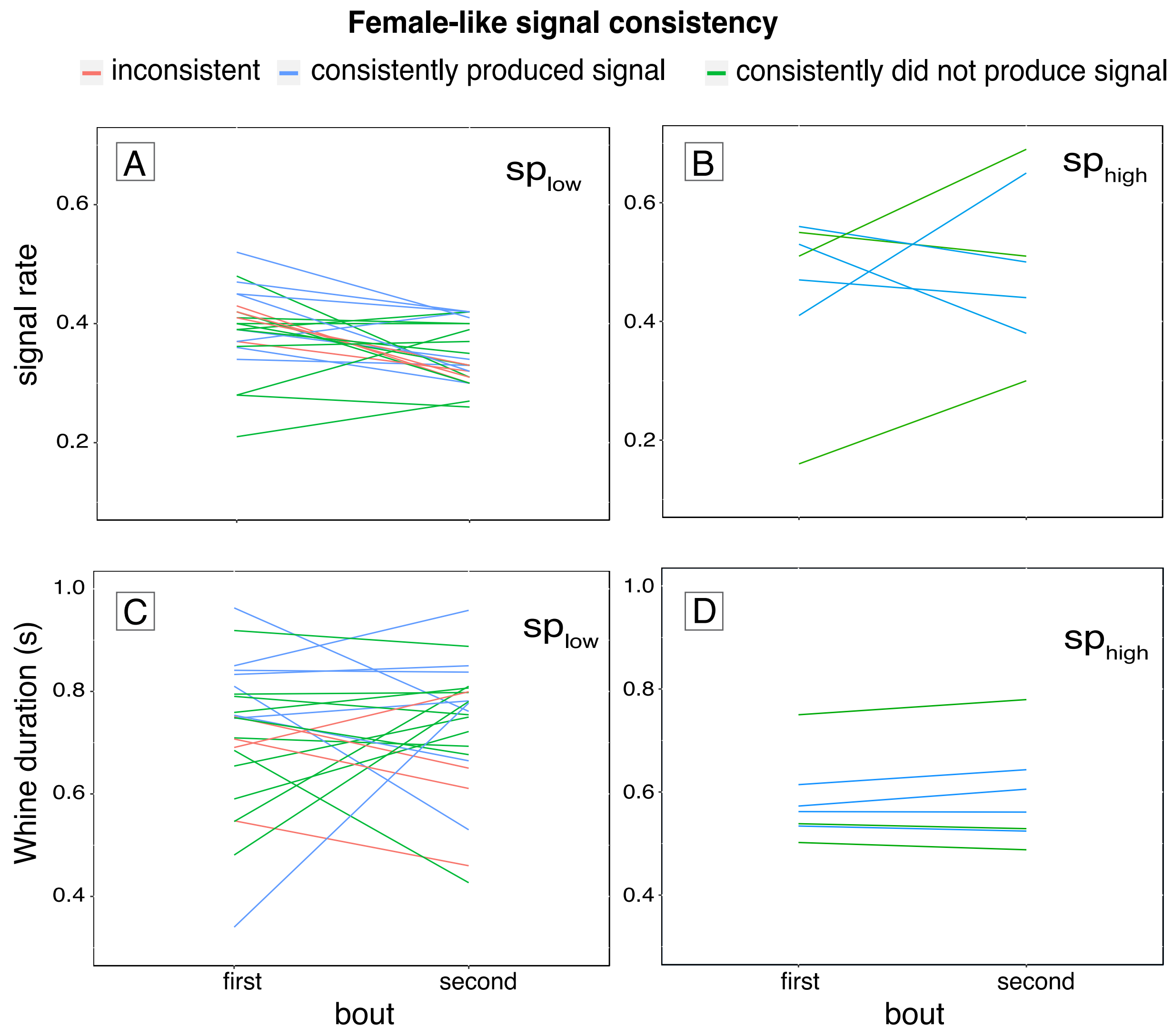
Oak Leaf Trail	43.084865 N, 87.892300 W	2018 2022	7	2	
Kletzsch Park	43.139260 N, 87.923346 W	2022		5	
Lion’s Den Gorge Nature Preserve	43.343406 N, 87.885020 W	2022		3	
Minooka Dog Park	42.980626 N, 88.193144 W	2022		1	Cirino et al. *in prep.*
Warnimont Dog Park	42.932332 N, 87.848587 W	2022		1	
Waubedonia Park	43.468504 N, 87.969024 W	2022		2	

Supplementary table 3. Variation in two temporal features of two spontaneous vibrational signaling displays of males of two species of the *Enchenopa binotata* complex. The model tested for differences in the signal rate or whine duration between males with different signaling phenotypes (inconsistently producing the female-like signal or not across signaling bouts, consistently producing it, or consistently not producing it).

Term	Signal rate			Whine duration		
	F ratio	DF	*P*	F ratio	DF	*P*
Signaling phenotype (legend in Fig. S2)	1.18	2/26	0.323	1.55	2/26	0.232
Species (sp_{low}, sp_{high})	10.11	1/26	0.004	12.60	1/26	0.002
Bout recorded (first, second)	3.90	1/27	0.059	0.02	1/27	0.893
Signaling phenotype * bout	1.92	2/27	0.166	0.33	2/27	0.723



Supplementary Figure 1. The dominant frequency of the main male advertisement signal of the spontaneous vibrational signaling display of male treehoppers of two species of the *Enchenopa binotata* complex, based on whether males produced the female-like signal. Boxplots = median ± 25% and 75% percentiles, vertical lines = range, and values beyond are outliers. The sample size for all features is shown under boxplots. GLM predictor terms: Produced female-like signal (yes, no): $F_{1/194} = 2.89$, $P = 0.09$; Species: $F_{1/52} = 421.93$, $P < 0.0001$; Temperature: $F_{1/1} = 8.49$, $P = 0.43$; Produced female-like signal × species: $F_{1/172} = 1.21$, $P = 0.27$).



Supplementary Figure 2. Consistency in two temporal features of two spontaneous vibrational signaling displays of males of two species of the *Enchenopa binotata* complex. Males displayed one of three forms of phenotypic consistency across the two bouts recorded (legend): (i) they were inconsistent, meaning that they produced the female-like signal in only one of the bouts recorded, (ii) they consistently produced the female-like signal in both bouts, and (iii) they consistently not produced the female-like signal. Each line represents one individual. We found no differences in the signal rate or the whine duration between the signaling phenotypes of males. We only found marginally significant differences in the signal rate between the two recorded bouts for the sp_{low} species (see results in Supplementary table 3).

Supplementary audio 1.

Sample response of a female of *Enchenopa binotata* treehopper the artificial playback presented to test the female priming hypothesis. This audio is the trial depicted in Fig. 1D.