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# Study of communication and acoustic behaviour of Eneopterinae crickets (Orthoptera: Gryllidae)

Jose Luis Benavides-Lopez

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Jose Luis Benavides-Lopez. Study of communication and acoustic behaviour of Eneopterinae crickets (Orthoptera: Gryllidae). Invertebrate Zoology. Muséum National d'Histoire Naturelle, 2022. English. NNT: . tel-04056567

**HAL Id: tel-04056567**

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## **MUSEUM NATIONAL D'HISTOIRE NATURELLE**

Ecole Doctorale 227 Sciences de la Nature et l'Homme : Evolution et Ecologie

Institut de Systématique, Evolution et Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, SU, EPHE, UA

### **THÈSE**

Pour obtenir le grade de

**DOCTEUR DU MUSÉUM NATIONAL D'HISTOIRE NATURELLE**

Présentée et soutenue publiquement par

**Jose Luis Benavides Lopez**

Le 9 Février 2022

**Study of communication and acoustic behaviour of  
Eneopterinae crickets (Orthoptera: Gryllidae)**

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***The day that the encrypted messages of nature and  
the natural logarithm of the language of life  
have been deciphered and coincide,  
we will understand the subtlest threads in the web of life.***

## Acknowledgments

To my dear and beloved Maily and my mother and brother for bringing me the colors to draw and paint my dreams and make them come true on each step, to fly away and never forget the way back home, and for giving me the wisdom to see and hear the natural history book of life.

More than a milestone in my life in this PhD was an inner journey, to discover and find the astonishing invisible threads which plug all the living beings.

To my advisor and friend Tony Robillard who give me all the support even before this time, his big picture on a board showed me a useful path, by fortune, plenty with tons of patience and encouragement to learn and keep walking to achieve the understanding of this discipline.

To Hannah my co-advisor who has taught me each time that I have asked her for some help or any doubt with clear sweetness.

To Carlos, my co-advisor who has shared precious time and experience with me for several hours. Today you are already an associate professor at the UFV. "Parabens voce merece !!! Tudo de bom mestre".

To Anne Marie de Larre de la "Droie" for bringing me a beautiful friendship and a home to live and feel Paris.

To Jerome Sueur, Laure Desutter, Ranjana, Jijia, Marion Guillaume, Natalia Vicente, Michelle Huet, Simon Poulin, Jocelyn, Agniele (ATA), Cassiano, Juan Sebastian Ulloa, Juliette Amara, Paul Aykar, and many other people whose friendship and worth contributions in this path.

To all the members of the thesis committees who have contributed with valuable ways to each previous step to get a better shape.

I really appreciate all the members of my thesis jury, for their acceptance of my defense and their comments and advices: Ms. Anne-Geneviève Bagnères-Urbany (Directrice de Recherche, CNRS Montpellier, Montpellier, France), Ms. Fanny Rybak (Université Paris-Saclay, Paris, France), Mr. Claudio Lazzari, (Université de Tours, Tours, France), Mr. Fernando Montealegre-Z, (University of Lincoln, Royaume-Uni).

Finally, this project was possible by the scholarship 756/2016 COLCIENCIAS-Colfuturo, financial support from Colombia

# Study of communication and acoustic behaviour of Eneopterinae crickets (Orthoptera: Gryllidae)

## Abstract

Behaviour of the subfamily Eneopterinae is explored through different methodological approaches, unremitting hours in the laboratory to achieve records from their acoustic features, responses to different kinds of stimuli, and associated conducts, careful observations that have brought important clues about their diversity in communication systems and the peculiarity of the strategies used by eneopterines. These strategies include almost the whole of the systems described for the Orthoptera order. This thesis aims to understand the acoustic behaviour of the Eneopterinae subfamily. In the first chapter, we explore a Neotropical species, *Ponca hebardi* in the tribe Lebinthini. Male crickets produce high-frequency calls, to which females reply with vibrational signals. Whereas they startle to high-frequency sounds similar to bat echolocation. This novel communication system likely evolved by male sensory exploitation of acoustic startle to high-frequency sounds in females and is therefore likely shared by the whole tribe Lebinthini, dating the origin of this behaviour to coincide with the origin of echolocation in bats. Furthermore, we document male duets involving both acoustic and vibratory signals not previously described in crickets, and we tentatively interpret it as competitive masking between males. In the second chapter, we explore the bioacoustics of the Neotropical *Eneoptera* genus. We have analyzed and described the songs of the four valid species, through a combination of acoustic analysis and measurement of ultrastructures features. This is a remarkable example of acoustic diversity in one genus with four species such as *E. guyanensis* the better-known species which exhibit a movement of their forewings to perform a combination of frequency between high and low with strong amplitude modulation. *E. surinamensis* another species with a loud pure tone that additionally exhibits amplitude modulation. We have described for the first time the calling song

for *E. gracilis* and found a basic loud pure tone in their calling songs. Also, we have described for the first time the calling song for *E. nigripedis* that exhibit a remarkable switching combination of low and high frequencies with amplitude modulated. Besides, their particular bioacoustical behaviours, we were able to observe aggregations of two or more males in the field and the laboratory, with males calling in synchronization known as "chorus". There are multiple cases of sympatry in the *Eneoptera* genus because field as we have observed intrageneric neighbors i.e. *E. gracilis* + *E. surinamensis*; *E. nigripedis* + *E. surinamensis*, and *E. guyanensis* + *E. surinamensis* coexisting in different parts of their distribution. In the third chapter, we have tested the role of high amplitude harmonics in the calling song of Eneopterinae crickets, using *Nisitrus malaya* a tropical species known for its loud pure tone with high amplitude harmonics. We have carried on experiments for females and males, through three synthetic calls treatments with different harmonic content (F1 = zero harmonic, F2 = one harmonic F3 = two harmonics) to elicit phonotactic behaviour and be able to assess the different responses. The results confirm that non-lebinthine Eneopterinae use phonotaxis as in the common system of communication for mate finding in crickets. For females, positive phonotaxis in *N. malaya* is confirmed, in all acoustic treatments. The males were attracted to the male playback song, this result was less expected. Females usually finish the experiments near to the speaker with a considerable proportion of individuals reaching the speaker. Among females performing phonotaxis, those who respond only to the synthetic call (F1) will tend to reach the speaker less often than the females who respond to the synthetic call (F2), and those who respond to the synthetic call (F3). This may suggest that, even though higher harmonics are not necessary to elicit phonotactic behaviour, they may be useful for the female to reach the speaker during the experiments. Few males walked until the speaker, but many walked towards it half the distance to the speaker. This suggests that the phonotactic strategy in males is different from the phonotaxis in females. It is probable that a male hearing another calling male in the field will approach him, but will not approach as to get in close



contact with the calling male. This could be a result of competition between males, similar to the cases of satellite males to intercept females attracted by the calling male. Because males of *N. malaya* never started singing during the experiments. These results suggest that both sexes in *N. malaya* exhibit phonotaxis to a certain degree, and are very probable to be attracted by a calling male. Throughout the experiments of phonotaxis in *N. malaya* we have distinguished another kind of signal linked to conspecific communication, which is a kind of chemical mark behaviour. With some adjustments in the phonotactic setup it was possible to get the chemical samples from *N. malaya*, founding different chemical profiles between sexes. This behaviour seems to be efficient for successful mating because of its inconspicuousness and allows access to continue to explore which could be considered as a multimodal nature of communication system in these crickets. Hereafter, the study of this subfamily has increased our understanding of acoustic behaviours and how some of these crickets have lost or modified their acoustic signaling because of environmental and co-evolutive pressures by returning to chemical, visual, or substrate vibration modes of signaling or even a combination of these channels in a more complex communication network within a plenty animal communication system.

Keywords: Crickets, Acoustic behaviour, Bioacoustics, Ultrasonic, Amplitude modulation, Eneopterinae, Phonotaxis, Vibrotaxis, Eavesdropping, Calling song, Wings ultrastructure, Playback, Lebinthini, Nisitrini, Eneopterini.

## **Etude de la communication et du comportement acoustique des grillons Eneopterinae (Orthoptera : Gryllidae)**

### **Résumé**

Le comportement de la sous-famille des Eneopterinae est explorée à travers différentes approches méthodologiques, des heures incessantes de laboratoire pour réaliser des enregistrements de leurs caractéristiques acoustiques, des réponses à différents types de stimuli et des conduites associées, des observations minutieuses ont apporté des indices importants sur leur diversité dans les systèmes de communication et la particularité des stratégies utilisées par les eneopterines. Ces stratégies incluent la quasi-totalité des systèmes décrits pour l'ordre des Orthoptères. Cette thèse vise à comprendre le comportement acoustique de la sous-famille des Eneopterinae. Dans le premier chapitre, nous explorons une espèce néotropicale, *Ponca hebardi* dans la tribu Lebinthini. Les grillons mâles produisent des appels à haute fréquence, auxquels les femelles répondent par des signaux vibratoires. Alors qu'ils sursautent aux sons à haute fréquence similaires à l'écholocation des chauves-souris. Ce nouveau système de communication a probablement évolué grâce à l'exploitation sensorielle masculine du sursaut acoustique aux sons à haute fréquence chez les femelles. Et il est donc probablement partagé par toute la tribu Lebinthini, datant l'origine de ce comportement pour coïncider avec l'origine de l'écholocation chez les chauves-souris. De plus, nous documentons des duos de mâles impliquant à la fois des signaux acoustiques et vibratoires non décrits auparavant chez les grillons, et nous l'interprétons provisoirement comme un masquage compétitif entre les mâles. Dans le deuxième chapitre, nous explorons la bioacoustique du genre Néotropical Eneoptera. Nous avons analysé et décrit les chants de ses quatre espèces valides, en combinant des analyses acoustiques et de mesures de caractéristiques des ultrastructures. Il s'agit d'un exemple remarquable de diversité acoustique dans un genre avec quatre espèces telles que *E. guyanensis*

l'espèce la plus connue qui présente un mouvement de ses ailes antérieures pour effectuer une combinaison de fréquence entre haute et basse avec une forte modulation d'amplitude. *E. surinamensis* une autre espèce avec un son pur et fort qui présente en plus une modulation d'amplitude. Nous avons décrit pour la première fois le chant d'appel d'*E. gracilis* qui a trouvé un son de base pur et fort dans ses chants d'appel. De plus, nous avons décrit pour la première fois le chant d'appel d'*E. nigripedis* qui présente une remarquable combinaison de commutation de basses et hautes fréquences avec une modulation d'amplitude. En plus de leurs comportements bioacoustiques particuliers, nous avons pu observer des agrégations de deux ou plusieurs mâles sur le terrain et au laboratoire, les mâles appelant en synchronisation appelés « chœur ». Il existe de multiples cas de sympatrie dans le genre Eneoptera car dans les populations sur le terrain nous avons observé des voisins intra-génériques, à savoir *E. gracilis* + *E. surinamensis* ; *E. nigripedis* + *E. surinamensis* ; et *E. guyanensis* + *E. surinamensis*, coexister dans différentes parties de leur distribution. Dans le troisième chapitre, nous avons testé le rôle des harmoniques de haute amplitude dans le chant d'appel des grillons Eneopterinae, en utilisant *Nisitrus malaya* une espèce tropicale connue pour son chant pur et fort avec des harmoniques de haute amplitude. Nous avons conduit des expériences pour les femelles et les mâles, à travers trois traitements de chants synthétiques avec un contenu harmonique différent (F1= zéro harmonique, F2= une harmonique, F3= deux harmoniques) pour susciter un comportement phonotactique et être en mesure d'évaluer les différentes réponses. Les résultats confirment que les Eneopterinae non-lebinthine utilisent la phonotaxis comme dans le système de communication courant pour trouver un partenaire chez les grillons. Pour les femelles, la phonotaxis positive chez *N. malaya* est confirmée pour tous les traitements. Les mâles ont été attirés par la chanson de lecture masculine, ce résultat était moins attendu. Les femelles terminent généralement les expériences près du speaker avec une proportion considérable d'individus atteignant le speaker. Parmi les femelles effectuant la phonotaxis, celles qui répondent uniquement au chant synthétique (F1)

auront tendance à rejoindre le speaker moins souvent que les femelles qui répondent au chant synthétique (F2) et celles qui répondent au chant synthétique (F3). Cela peut suggérer que, même si des harmoniques plus élevées ne sont pas nécessaires pour susciter un comportement phonotactique, elles peuvent être utiles pour que la femelle atteigne le speaker pendant les expériences. Peu des mâles ont marché jusqu'au speaker, mais beaucoup se sont dirigé vers lui et ont atteint la moitié de la distance au speaker. Cela suggère que la stratégie phonotactique chez les mâles est différente de la phonotaxis chez les femelles. Il est probable qu'un mâle entendant un autre mâle appelant dans le champ s'approchera de lui, mais ne s'approchera pas pour entrer en contact étroit avec l'appelant. Cela pourrait être le résultat de compétition entre les mâles, semblable aux cas des mâles satellites pour intercepter les femelles attirées par le mâle appelant. Etant donné que les mâles de *N. malaya* n'ont jamais commencé à chanter pendant les expériences ces résultats suggèrent que les deux sexes chez *N. malaya* présentent une phonotaxis à un certain degré et sont très probablement attirés par un mâle appelant. Tout au long des expériences de phonotaxis chez *N. malaya*, nous avons distingué un autre type de signal lié à la communication conspécifique, qui est une sorte de comportement de marque chimique. Avec quelques ajustements dans la configuration phonotactique, il a été possible d'obtenir les échantillons chimiques de *N. malaya*, trouvant qu'il existe des profils chimiques différents entre sexes. Ce comportement semble être efficace pour un accouplement réussi du a sa discrétion et permet d'accéder à continuer à explorer ce qui pourrait être considéré comme une nature multimodale du système de communication chez ces grillons. Désormais, l'étude de cette sous-famille a augmenté notre compréhension des comportements acoustiques et comment certains de ces grillons ont perdu ou modifié leur signalisation acoustique en raison des pressions environnementales et co-évolutives en revenant à des modes de signalisation chimiques, visuels ou de vibration du substrat ou même une combinaison de ces canaux dans un réseau de communication plus complexe au sein d'un système de communication d'animaux d'abondance.

Mots-clés : Grillons, Comportement acoustique, Bioacoustique, Ultrasons, Modulation d'amplitude, Eneopterinae, Phonotaxis, Vibrotaxis, Ecoute, Chant d'appel, Ultrastructure des ailes, "Playback", Lebinthini, Nisitrini, Eneopterini.

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# General introduction

The study of animal communication has led to significant progress in our general understanding of sensory systems, evolution, and speciation processes. Some of the conspicuous behaviours performed by animals play important roles in their reproduction and survival. Consequently, communication has a central role in studies of behaviour and other mutualistic relationships; leading to answers to the questions about ecology, physiology, development, and evolution, as well as a window into the cognitive world of animals (Otte 1974; Endler 1993; Greenfield 2005; Bradbury and Vehrencamp 2011; Brumm 2013).

Acoustic communication is fundamental for many animals; because it is the main source between the individual and their environment (Truax 1985). The study of acoustic communication has made huge progress through the last century, contributing to, and benefiting from the development of different research areas on the study of behaviour, ecology, evolution, physiology, and neurobiology. Among the most striking behaviours achieved by animals are those linked to acoustic communication, as they are intersected by reproduction and survival strategies expanded by equipment research advances, and their effects focus mainly on the attenuation and scattering degradation of acoustic signals from the signaler to a receiver (Brumm 2013; Stevens 2013; Wiley 2015).

Despite their small size, insects are using diverse sensory channels to communicate. They have evolved these signals several times since they have reach the land 400 million years ago (Warren and Nowotny 2021). They communicate through mechanical and chemical signals in order to generate an inertial force detectable by another individual becoming a sort of useful advertisement signaling. Evolving organs and strategies from surround environment, it is likely that insects were the first animals on earth to evolve sound perception organs. Considering the frequencies reach

by insects in mechanical signals could suffer substantial changes while the signals are produced and spread (Huber et al. 1989; Gerhardt and Huber 2002; Greenfield 2002; Hedwig 2016).

Most studies related to the sound produced by insects have been made based using a few species of the order Orthoptera, beginning with the study of *Gryllus campestris* by (Regen 1913). This is mainly due to practical aspects, including the ability to produce and perceive sound signals also perceived by humans, the fact that they can be studied easily both in the field and laboratory conditions, and to the fact that they live in most regions of the Earth except for the subarctic and arctic. Their easy handling makes them the perfect model to carry out different types of experiments in several research areas, bringing detailed studies on behaviour, acoustics and neurophysiological level which have permitted to reveal crucial aspects of their complex interactions with conspecifics during mating and to understand the mechanisms involved in the production of their calling song at mechanical and neurological levels (Keuper et al. 1988; Montealegre-Z et al. 2009; Schöneich and Hedwig 2011; Schöneich et al. 2015; Warren and Nowotny 2021), and in their sound perception (Bailey 1991; Otte 1992; Hedwig 2016; Horch et al. 2017; Schneider et al. 2017).

Most of the crickets have two pairs of wings. Their forewings (FWs) correspond to thickened tegmina usually carrying the stridulatory structures in males, and the hindwings, when present, are specialized for flight and folded fanlike under the FWs at rest. While many species are both capable of flying and singing, these functions have also been lost several times during the evolution of crickets. There are usually exceptions at subfamily level for species that cannot fly, other that cannot call, and species that cannot fly, call or hear (Dumortier 1963; Bennet-Clark 1989a; Gerhardt and Huber 2002).

The stridulation is a precise mechanism of frequency multiplication that allow to change relatively slow wing muscle movements into higher frequency vibrations, in the acoustic or ultrasonic range

according to the species. Most crickets use relatively low-frequency calls, with dominant frequencies ranging from 2 to 8 kHz, but a few species in the subfamilies Eneopterinae and Trigonidiinae are known to produce higher frequencies, sometimes ultrasonic (10 to 30kHz) (Robillard and Desutter-Grandcolas 2004; Desutter-Grandcolas et al. 2018; Liu et al. 2018; Tan et al. 2021). The movements of stridulation are made through cycles of FW closure and opening, the sound being generated during closure only and the opening being silent. During stridulation, the plectrum, corresponding to the thickened inner edge of the lower FW (generally the left one) hits a row of tiny cuticular teeth located on the ventral face of the right (upper) FW, known as the stridulatory file (Bennet-Clark 1989*b*; Pfau and Koch 1994; Michelsen 1998; Montealegre-Z et al. 2009, 2011). File and plectrum constitute what is called the “stridulum”. When a simple tooth strike occurs between the plectrum and the file, it generates one elementary oscillation with a straightforward clockwork mechanism. Each of these vibrations is then transmitted to the rest of the cricket wings and prolonged by the wing resonance that both amplifies and filters the sound according to the physical properties of the FWs (Koch et al. 1988; Bennet-Clark and Bailey 2002; Montealegre-Z et al. 2011).

The active generation of low frequencies in most of the crickets require large radiators with high resonance to compensate attenuation and scattering degradation which limits effective range communication. A behavioural solution to afford the size and physic boundaries in crickets using plants as baffles and broadcasting from elevated positions on the plants. Such adaptations become important to support the strong link between body size of crickets and how they exploit the surround environment give us clues of each case for acoustic behaviour and strategies used by them (Bailey 1991; Gerhardt and Huber 2002; ter Hofstede et al. 2010).

Therefore, the males crickets sing with their highly compliant wing structures, which produce a near pure tone as each impact strikes the wing at an equivalent rate to the natural vibration frequency of the FWs. The additional tones can be created for the impacts among the sounding

structures at impact rates outside its resonant or natural vibration frequency. The sharpness of spectrum is usually a measure of the generator's ease of motion or an inverse of its degree of damping (Bailey 1991; Greenfield 2002; Robillard et al. 2007, 2013, 2015b).

Besides handling with physics boundaries, low frequencies in calling song proffer distant cues for the females through the different obstacles in the environment, the Eneopterinae subfamily is the only cricket group known for its high-frequency harmonics that can become dominant in the frequency spectrum.

Cricket's songs are typically described as low-frequency, pure-tone signals (Sismondo 1979; Bennet-Clark 1989b; Bailey et al. 2001), although most contain low-amplitude, and high frequency harmonics. Since these harmonics are much less intense than the fundamental frequency of the song, they are often considered unimportant to the behaviour of crickets.

In *Teleogryllus oceanicus* for example, DF occurs around 4.5 kHz and harmonics carry very little energy, but extend above background noise level up to at least 40 kHz (Hutchings and Lewis 1984; Balakrishnan and Pollack 1996). The relevance of harmonics content in the calling song to determine orientation in crickets was discussed based on simple biophysical considerations, by considering that it is evident that the large number of ultrasonic harmonics provide much more directional information than a single pure tone at the fundamental frequency (Hutchings and Lewis 1984; Latimer and Lewis 1986).

### **The Eneopterinae subfamily**

Studies in the cricket subfamily Eneopterinae have documented higher frequencies for their calling songs (Robillard and Desutter-Grandcolas 2004). In this subfamily high-frequency calls are likely to represent a key evolutionary innovation promoting the evolution of a novel distinctive communication system in one tribe where the harmonic frequencies have become dominant (ter Hofstede et al. 2015). Eneopterine males (Gryllidae: Eneopterinae) are the only crickets known

to generate calling songs with intense high-frequency harmonics. In one tribe, the Lebinthini, and in a few species in the genus *Eneoptera* (Robillard 2021), one of the higher-frequency harmonics has repeatedly become the dominant frequency of the call, and this harmonic DF can even be ultrasonic (>20 kHz) in some species (Robillard et al. 2015a; Anso et al. 2016; Tan et al. 2021).

For other tribes of this subfamily, males produce low-frequency loud pure tones in their calling songs, such as in species of the genera *Xenogryllus* or *Nisitrus*, even in some species of the genus *Eneoptera*, but even in these species, the high amplitude harmonic content tend to carry greater energy relatively to the dominant peak than what is currently found in other cricket clades. (Hung and Prestwich 2006). In some of these species, one of the harmonics is even sometimes almost codominant with the fundamental frequency (Robillard and Tan 2013; Jaiswara et al. 2019). These observations led to the hypothesis that having harmonics with enhanced energy may correspond to the ancestral condition of the spectrum of the call in these crickets, and the first step before the harmonics were used as the dominant frequency, as part of the novel system of communication found in the lebinthine species (ter Hofstede et al. 2015; Benavides-Lopez et al. 2020).

### **Thesis main objectives**

The main aim of this thesis is to explore the diversity of communication systems and behaviour in these crickets to unveil the role of the harmonics. To achieve this goal, I have divided this study in three chapters. In the first chapter, I have focus on the study of the genus *Eneoptera* established by Burmeister in 1838. The genus *Eneoptera* constitutes itself the Eneopterini tribe, this tribe has been described with four species, complemented with phylogenetic framework from previous studies as support (Desutter-Grandcolas 1998; Desutter-Grandcolas and Robillard 2003; Robillard et al. 2007). Additionally, the combination of phylogenetic techniques and acoustic

records has given rise to the study of evolution in their communication system in the genus and within the Eneopterinae subfamily (Robillard and Desutter-Grandcolas 2011a; Robillard et al. 2015a). We describe for the first time in this chapter the calling songs of *E. gracilis* and *E. nigripedis*

Within *Eneoptera* genus, the calling songs from two species are known, for *Eneoptera surinamensis* this has been described in 2007 by Miyoshi et al (Miyoshi et al. 2007). The second species which has calling songs described is *E. guyanensis*, this species is an example of the acoustic diversity because its different components in their calling songs, produced in alternance through the stridulatory file composed by simple and double teeth; combined with their wings movement reaching the ultrasonic frequency (Robillard and Desutter-Grandcolas 2005, 2011b; Robillard et al. 2015a). For the remaining two species *E. gracilis* and *E. nigripedis* only morphological features were known at the beginning of this thesis and calling sounds records were not previously available.

Our hypothesis is that the *Eneoptera* genus presents high frequency harmonics in their calling songs. In order to test our hypothesis, we proposed to investigate the *Eneoptera* species and acquiring, analyzing or re-analyzing their calling songs. Through the questions: Do they all produce high frequency calling songs? When they are available records done with the appropriated technical equipment? What is the ultrastructure difference among the species for such contrasting signals in the genus?

The second chapter, I have used the Nisitriini tribe from this subfamily, which fulfill our investigation in the role of the higher harmonics in a low-frequency Eneopterine species. *Nisitrus malaya* (Robillard & Tan, 2021). In this study we carried out playback experiments in the laboratory, together with video monitoring of the cricket in order to investigate his responses to harmonic content. Allow us to understand why these powerful high amplitude harmonics are present and



can become codominant in some species, and dominant in a large part of the subfamily, we investigate whether these harmonics are useful, necessary or accessory for eliciting female phonotaxis. Similarly, we have investigated their possible role in males' interactions.

Even though the positive phonotaxis of *N. malaya* females was experimentally demonstrated by tests made in a previous study (ter Hofstede et al. 2015a), this behaviour needs to be analyzed in more detail to elucidate the role of high frequency harmonics. This known behaviour allow us to contrast this species with the lebinthine species that have lost phonotaxis,

The first possible role of the harmonics is to help female orientation while approaching the male through positive phonotaxis. Males could be attracted by each other phonotactically at some degree but would limit their approach until they start perceiving the harmonics of the neighbor males. Thus, males could also use the harmonic content of their calling songs to space themselves in the environment. Under this hypothesis, the loud harmonics could be necessary in eneopterines due to the vegetation that attenuate these frequencies. With grylline low amplitude harmonics, the male could probably not perceive any trace of harmonics until getting very close to the calling male.

The aim of this chapter was to answer two main questions 1: Is there positive phonotaxis and is phonotaxis different between sexes? 2: For crickets showing positive phonotaxis, are there differences between sex and treatment? Depending on the responses to these two main questions, we will discuss the possible roles of the powerful high-frequency harmonics in the call of *N. malaya*, and more generally in the calls of most non-lebinthine Eneopterinae.

The study of this subfamily increases our understanding of acoustic behaviour and how signals emitted by a sender could be enhanced, transmitted and recognized by a receiver in a more complex communication network within an animal communication system.

In the third chapter, I explore the evolutionary origins of communication signals, focus in the nocturnal neotropical species *Ponca hebardi*, from the Lebinthini tribe, to ameliorate our understanding and knowledge of which requires careful study of multiple species within a known phylogenetic framework as support. As previously exposed most cricket species produce low-frequency calls for mate attraction, although they startle to high-frequency sounds similar to bat echolocation. Male crickets in the tribe Lebinthini are specially known for producing high-frequency calls, to which females reply with vibrational signals. This novel communication system likely evolved by male sensory exploitation of acoustic startle to high-frequency sounds in females. This behaviour was previously described for the Lebinthini from Asia. In this chapter we demonstrate that this novel communication system is found in, *Ponca hebardi*, and is therefore likely shared by the whole tribe Lebinthini, dating the origin of this behaviour to coincide with the origin of echolocation in bats. Furthermore, we document male duets involving both acoustic and vibratory signals not previously described in crickets, and we tentatively interpret it as competitive masking between males.

# Chapter 2

## Acoustic diversity in the Neotropical genus *Eneoptera* Burmeister (Orthoptera, Gryllidae, Eneopterinae)

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Paper in preparation for the journal *Bioacoustics*:

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### Introduction

The cricket stridulation is a precise mechanism of sound generation that involves a step of frequency multiplication that permits converting low-frequency wing muscle movements into acoustic frequencies with values varying across the species (Michelsen 1998). The stridulatory movements are produced by males only, during forewings' closure. Male cricket forewings are more or less symmetrical in morphology, but not in their functioning: each wing has a plectrum on its inner anterior edge, but only the left plectrum is used for stridulation, when the plectrum rubs against a row of tiny cuticular teeth located under a particular vein of the right forewing, known as the stridulatory file (Koch et al. 1988; Bennet-Clark 1989a; Bailey 1991; Montealegre-Z et al. 2009). Together, the file and the plectrum constitute the "stridulum". When a simple tooth strike occurs between the plectrum and one tooth of the file, it usually generates one elementary oscillation, with a straightforward clockwork mechanism, which is immediately prolonged by the wing resonance properties that filter and amplify the sound (Koch et al. 1988; Bennet-Clark and Bailey 2002). As the file teeth are struck at a constant period, they generate a sound syllable with a frequency being equivalent to the tooth strike rate, which also corresponds to the resonant frequency of the harp, a region of the wings acting the main resonator of the system.

Most species in the family Gryllidae have their call dominant frequency ranging between 2-8 kHz, due to physical constraints and their small size (Michelsen 1998). However, this was recently revised for some members of the subfamily Eneopterinae, in particular owing to the improvement in recording equipment and bioacoustical exploration that revealed that many of these crickets, in a least two tribes, use high-frequency harmonic calling songs (Robillard and Desutter-Grandcolas 2011a; Robillard et al. 2013; ter Hofstede et al. 2015).

Within Eneopterinae subfamily, the genus *Eneoptera* Burmeister, 1838, which also corresponds to the only genus in the tribe Eneopterini, currently includes four valid species: *Eneoptera surinamensis* (De Geer, 1773), *E. guyanensis* Chopard, 1931, *E. gracilis* Robillard, 2005 and *E. nigripedis* Robillard, 2005. The combination of phylogenetic information and the study acoustic signals of this genus has given rise to the study of evolution in their communication system, later expanded at the level of the subfamily (Desutter-Grandcolas 1998; Robillard and Desutter-Grandcolas 2006; Robillard et al. 2007).

The best example of the acoustic particularities of this genus is found in the calling song of *E. guyanensis*, which has been described by Desutter-Grandcolas (1998) as having a broad-frequency modulation generated by particular stridulatory teeth. The mechanism at the origin of this species' call has been later explained later explained by the interaction of different components in the call, produced in alternance by two distinct parts of the stridulatory file composed by simple and double teeth (Robillard and Desutter-Grandcolas 2011b; Robillard et al. 2015a).

In contrast, the calling song of *E. surinamensis* has been described as having none of the particular features of *E. guyanensis* (Miyoshi et al. 2007), but Robillard et al. (2015a) suggested that the calling song of this species may reveal hidden high-frequency component if recorded with appropriate equipment.

For the other two species, *E. gracilis* and *E. nigripedis*, they were described based on collection specimens and morphological features only, and no recording was available until now (Robillard and Desutter-Grandcolas 2005).

In this study, we pursued the bioacoustics exploration of this genus and will test the hypothesis that all *Eneoptera* species (= Eneopterini tribe) possess high-frequency harmonics in their calling songs, being either dominant or sometimes co-dominant as in *E. guyanensis*. In order to test our hypothesis, we investigated the bioacoustics of *Eneoptera* species and acquired new data on the two species never recorded before, and analyzed or re-analyzed and compared the calling songs of the four species. We used a combination of different techniques to measure the parameters of the calling songs and stridulatory structures for each species in order to describe and compare the sound production mechanisms across the species, by determining the relationship between the calling song, the stridulatory behaviour and the file ultrastructure among the species in the genus.

## **Materials and methods**

### **Crickets**

The specimens in this study come from previous collections for the species *E. guyanensis* (see Robillard et al. 2015), but new collections were necessary for the three other species:

For *E. nigripedis* and *E. gracilis*, we were able to find a population for each species during a field expedition in Colombia in December 2018. *E. gracilis* specimens were collected at Menegua, municipality of Puerto López, Meta department in Colombia, from 8-10/12/2018 (GPS coordinates: 4°05'32.6"N; 72°48'44.1"W WGS84). *E. nigripedis* were collected at Km 11 vía a

Tarapaca, municipality of Leticia, Amazonas department in Colombia; from 1-4/12/2018 (GPS coordinates: 4°07'01.9"S; 69°57'36.4"W WGS84).

*E. surinamensis* was studied from specimens collected in Brazil at Parque Estadual do Rio Doce (PERD), Marliéria, Minas Gerais, Brazil from 20-24/06/2016 (GPS coordinates: 19°44'58.8"S 42°28'52.3"W & 19°42'07.1"S 42°30'36.1"W). Additional recordings and specimens were collected in Colombia during the 2018 expedition and additional field sessions in La Quebradita, La Buena Esperanza municipality: Belén de los Andaquíes, Caquetá department, Colombia, 16/01/2018 (GPS coordinates: 01°19'28"S; 75°57'49.6"W WGS84); and Finca el Tambor municipality: Honda, Tolima department, Colombia 4/12/2018 (GPS coordinates: 5°10'05.8"N; 74°48'24.4"W WGS84).

### **Recordings of calling songs**

Records from *E. guyanensis* have been previously acquired, from a male collected in Petit Saut (5°03'55.9" N; 53°02'53.3" W), French Guiana by J. Orivel in 2013. This male was reared at the insectarium in the MNHN Paris and was recorded at the acoustic room as described in Robillard et al. (2015).

For *E. surinamensis*, six specimens were recorded at the Orthoptera laboratory in the rearing room under controlled conditions of temperature and humidity (T: 24±3°C, 60±10% RH) in Federal University of Viçosa (UFV), Brazil.

One male of *E. nigripedis* and *E. gracilis* were recorded in captivity in the field, at 31.2°C, 85% Relative humidity and 26°C, 72% relative humidity, respectively. Posteriorly of been recorded, the specimens were euthanatized and preserved in ethanol for morphological studies.

All the recordings were obtained with a modified condenser microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) with a frequency range of 3-150 kHz  $\pm$  6 dB (R. Specht, pers. comm.). Each male was put individually in a suspended textile net cage overnight with the lights on and the microphone suspended 30 cm above the cage. Sound-triggered recordings were made using Avisoft Recorder software version 2.97 (Specht 2008) and an 8-Pro MOTU sound card at a sampling rate of 96 k-samples per second (16 bit). To generate audio files with accurate power spectra, we applied a user-defined finite impulse response (FIR) filter in Avisoft-SASLab Pro version 4.40 that corrected for the microphone frequency response provided by the manufacturer.

## **Morphology**

For each species two crickets were selected from dry or alcohol-preserved specimens. The right wings were dissected and mounted a piece of cardboard. Stridulatory files were photographed with a HIROX RH-2000 microscope at MNHN at 250x magnifications.

We used the ImageJ software to measure the parameters of the files, including length of the stridulatory file, total number and distribution of simple teeth (all species), and double teeth (*E. guyanensis* and *E. nigripedis*).

The functional part of the stridulatory file was defined as the teeth that are effectively hit by the plectrum during the stridulation process. We measured distances between each tooth in consecutive order, from one crest to the next one, in order to obtain the distance between the teeth along the file for each species. The distance between the tooth increase progressively from anal to basal zone of the stridulum. The functional part corresponds to the more or less straight area of the file. This increase tends to compensates the relative increase of speed of the wings during the stridulatory movement: as the forewings close, their speed slightly increase as the movement progresses; the increasing distance between the successive teeth allows to

compensate the speed of the wings during the closure movement (Gu et al. 2012). As a consequence, the increasing inter-teeth distances allow to maintain a relatively constant time between two teeth, therefore the vibration produced have a similar period, and in turn a frequency relatively constant during the sound syllable.

### **Calling song analyses**

These calling songs were analyzed with the software Avisoft-SAS Lab Pro version 5.2 (Specht, 2008). Temporal and spectral song features were measured using the automatic parameter measurements feature in Avisoft-SAS Lab Pro (FFT length 256, rectangle window, 50% overlap).

We followed the basic cricket song terminology proposed by Ragge & Reynolds (1998): a syllable is generated during a complete movement opening and closure of the forewings; an echeme is a group of syllables emitted with a determined pattern; this is the unit of the song which is repeated at length during calling, and an echeme sequence, or song bout, corresponds to one uninterrupted series of echemes. The term “trills” was used to describe a continuous train of rapidly repeated, ungrouped syllables lasting indefinitely during a song bout. Trills, thus, differ from continuous, dense echeme-sequences, where short echemes are repeated rapidly and continuously. The syllable rate is the number of syllables emitted per second within an echeme.

We measured the following variables: syllable duration, syllable period (time from start of one syllable to start of next) and dominant frequency (frequency with maximum energy, kHz) using four graphic representations of the sound: oscillograms, envelope amplitude, logarithmic or linear power spectrum (Hamming) and sonogram (Hann). With the oscillograms, envelopes and sonograms we could characterize the temporal patterns of the calling songs.



The power spectrum and sonograms feature are used to study the spectral properties of the calling songs. In the calling songs we define the dominant frequency ( $F_d$ ) as being the frequency peak in the spectrum having the maximum energy among the fundamental frequency ( $F_1$ ) and its second to fifth upper harmonics, which correspond to multiple integers of  $F_1$ , designated as  $F_2$ ,  $F_3$ ,  $F_4$ , and  $F_5$  respectively.

Amplitude of frequency peaks in energy spectra were measured for each species using the spectrogram analysis after a careful manual selection of 750 for *E. guyanensis*, 685 for *E. surinamensis*, 600 for *E. gracilis* and 620 for *E. nigripedis* of marked syllables respectively. We then measured temporal and spectral features of each syllable to setup the parameters of measurements and generate the spectrograms for each call using Avisoft-SAS Lab Pro.

### **Stridulation analysis**

To determine whether the dominant frequency of the song corresponds to the natural fundamental frequency or to a higher harmonic, we compared wing velocity measurements, calculated as the length of functional region of the file divided by the whole FW closing time (= syllable duration), with instantaneous song frequency, calculated by dividing the average intertooth distance within the functional region by the time separating two subsequent waveforms in the song, under a candidate value of fundamental frequency ( $1/F_n$ ). The instantaneous song frequency that most closely matches the wing velocity measurement is then supposed to correspond to the tooth impact rate (hereafter named tooth-strike rate, TSR).

Irrespective of the relative energy of each peak of the spectrum, if the first peak matches the tooth impact, it means that each functional file tooth produces one sound elementary waveform and that  $F_d$  is directly produced by the stridulatory mechanism, as described for Gryllinae (e.g. Montealegre-Z et al. 2009). However, if the estimated wing velocity matches a higher

instantaneous frequency, it suggests that more than one dominant waveform is produced per tooth strike, as a result of the harmonic vibration of the wings, as previously suggested for some other eneopterine crickets (Robillard et al. 2007, 2013).

## **Results**

The males of the different species of the genus all require a short warm-up period before calling at maximum intensity (Figure 1). Once warmed, the males of all the species produce calling songs corresponding to continuous trills with stable syllable rates in absence of regular rest intervals, sometimes for song bouts of several hours in *E. surinamensis* and *E. nigripedis*.

There is no echeme structure in the temporal pattern of the calling songs of any species of *Eneoptera*. Thus, for the present analysis we refer to "sections" of trill groups of syllables characterized by their amplitude and frequency features.

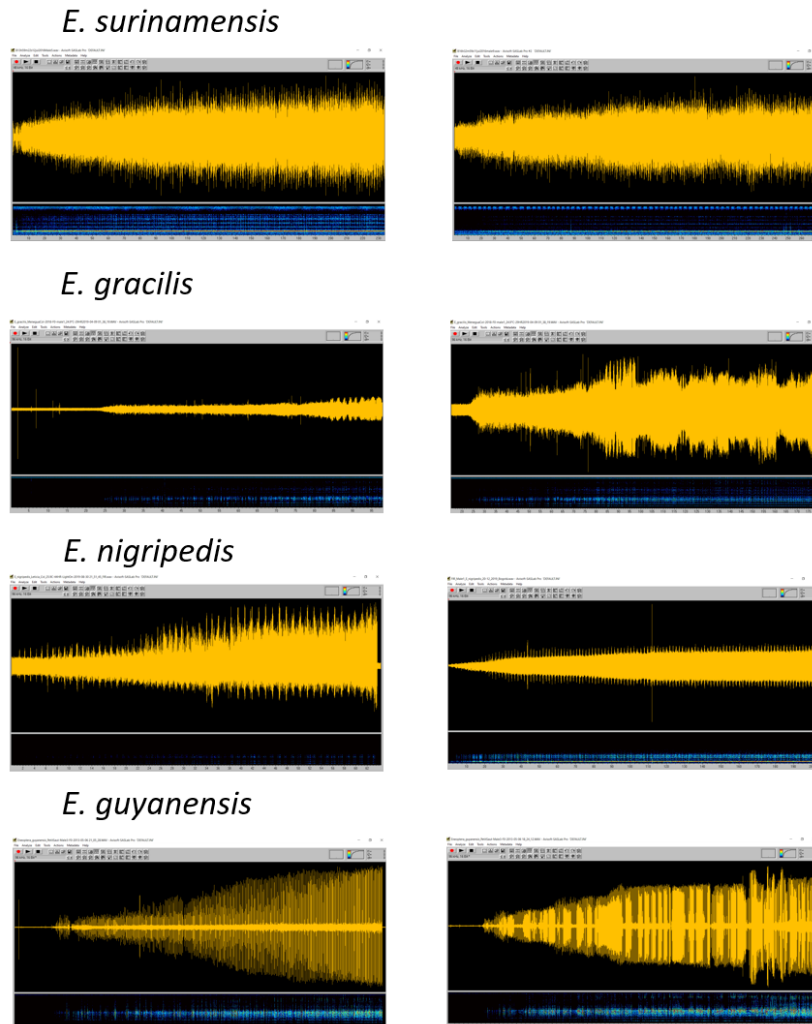


Figure 1. Oscillogram of the initial warm up in the *Eneoptera* genus

### ***Eneoptera guyanensis***

The species *E. guyanensis*, is the best studied species in the genus. Its known distribution ranges from Suriname to French Guiana (Robillard and Desutter-Grandcolas 2005). The first acoustic recordings and analyses of signals of this species have led to a functional hypothesis involving broad-frequency modulation produced by a particular file morphology including two distinct regions (Desutter-Grandcolas 1998), which were reanalyzed with additional behavioural and acoustic data in Robillard & Desutter-Grandcolas (2011) and Robillard et al. (2015).

Robillard et al (2015) have interpreted the mechanism of sound production in *E. guyanensis* using integration of data based on high quality recording, behavioural study of FW movement and details on the ultrastructure of the stridulum. Their study confirmed the complex acoustic behaviour of this species, with a unique trilling song that alternates two parts with different frequency ranges, with a continuous trill settled by HF ( $19.96 \pm 0.09$  kHz) section; and LF ( $3.83 \pm 0.04$  kHz) section with F5 codominance at (20 kHz).

These all including high frequencies, produced by two different movements of the FWs: the large teeth that look like normal cricket teeth are located in the basal section of the file, and the distal section composed by double teeth, each one separated by a median furrow into two parallel crests, giving approximately the double of the crest density in this region compared with the section with normal teeth (Figure 2).

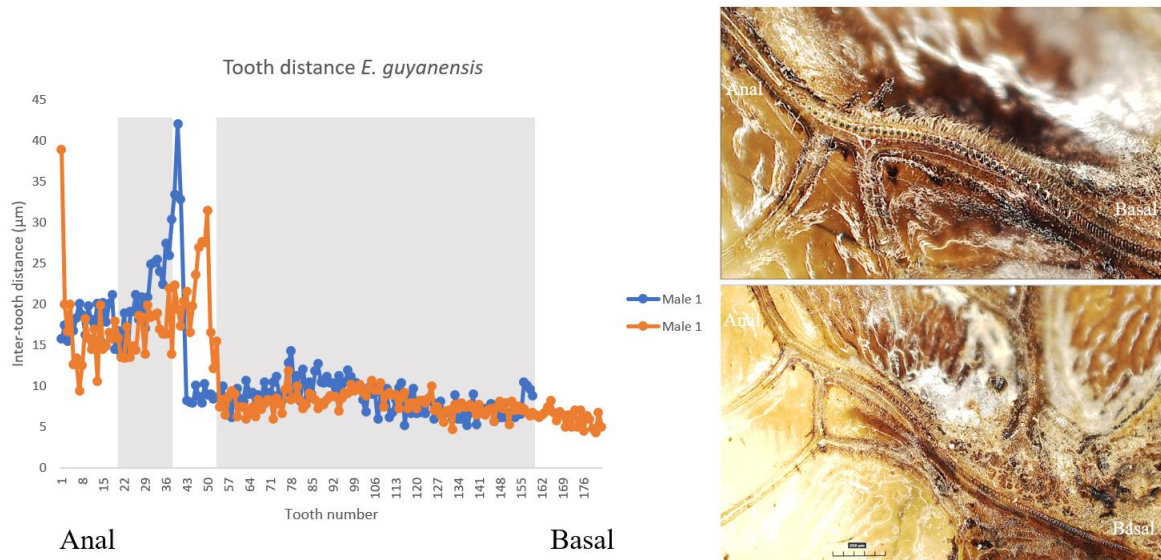


Figure 2. Tooth distance for two males of *E. guyanensis*; pictures stridulatory file (250µm scale).

*Acoustic characterization:*

The calling song of *E. guyanensis*, is re-analyzed here with two additional sound records in order to quantify its harmonic content. The present results confirm that the species has a continuous trill constituted by two different “high” frequency sections (3.9 kHz and  $20.2 \pm 0.750$  kHz) (Table 1).

The first section of syllables has lower amplitude (formerly “low-frequency” section in Desutter-Grandcolas, 1998 and Robillard & Desutter-Grandcolas, 2011). Our new analyses follow as proposed by Robillard et al. in 2015 a dominant frequency at 3.9 kHz, corresponding for the first peak F1 (F1 amplitude =  $-64.78 \pm 1.46$  dB), although, the fifth peak F5 is co-dominant, the F5 (20 kHz, F5 amplitude =  $-53.631 \pm 2.816$  dB).

The second, higher-amplitude section, formerly mentioned as the “higher-frequency” section, has a broadband frequency peak corresponding to the first peak of the spectrum and is dominating at  $20 \pm 0.75$  kHz. There is also less powerful a high-frequency harmonic at 41 kHz, suggesting that this peak constitute a multiple integer (F2) of the fundamental frequency generated by the crests of the double teeth (Figure 2 and 3).

In order to complement the analysis done in this species, we have measured the short transition section between the low-amplitude and high amplitude sections. It suggests that F4 is dominant at  $17.2 \pm 0.65$  with codominance in F5 at  $20.4 \pm 0.8$  kHz (table 1, figure 4).

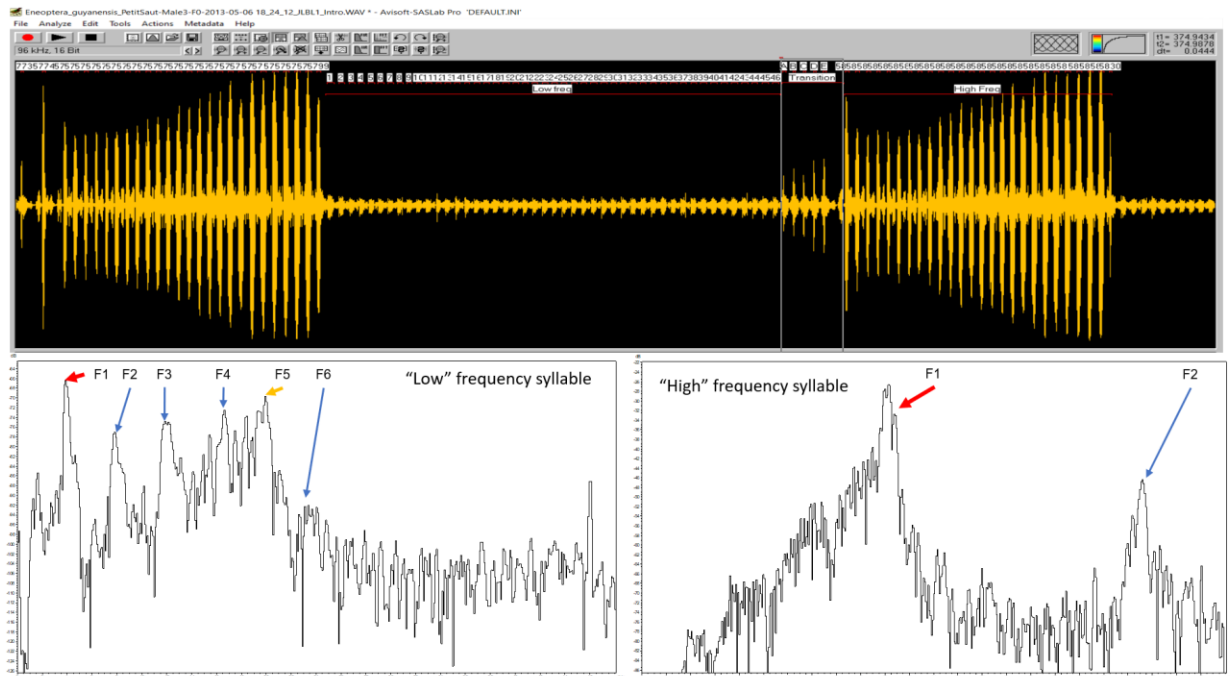


Figure 3. Spectrogram with “low” and “high” frequency, composed by simple and double tooth respectively of *E. guyanensis*. Also, power spectrum with low and high frequency syllables left and right respectively. Left: Dominant frequency (red) co-dominant (orange) frequency and blue harmonic content. Right: Dominant frequency (red) harmonic (blue).

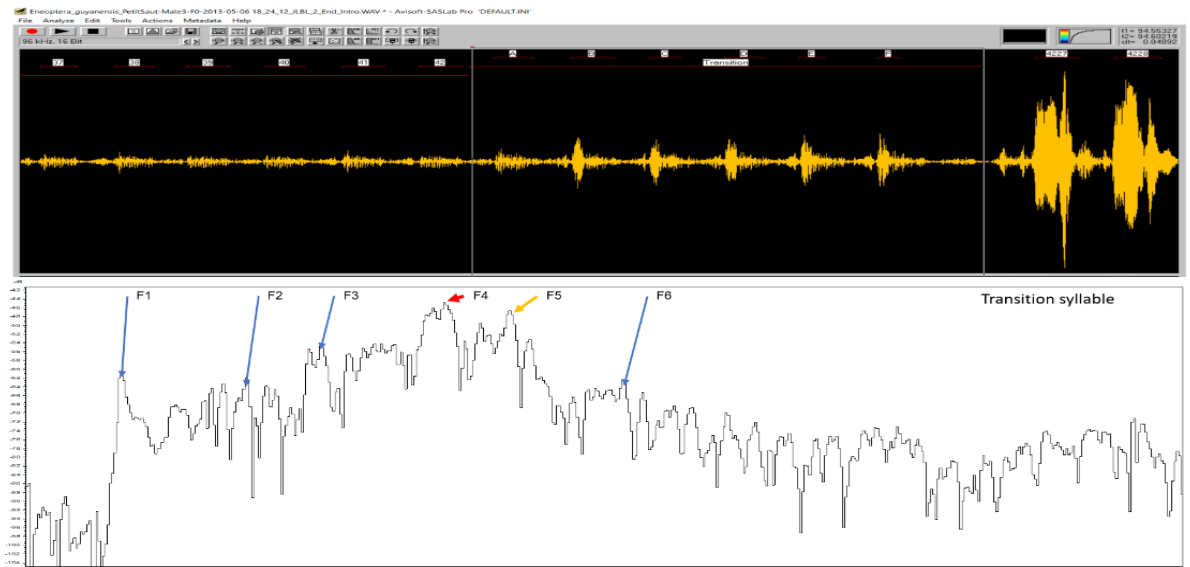


Figure 4. Spectrogram with the transition teeth of *E. guyanensis* dominant frequency (red) co-dominant (orange) frequency and blue arrows harmonic content.

Table 1. Calling song analysis for two records with high and “low” frequency sections, harmonic content and amplitude detailed. The Gray squares represent the Fd dominant or co-dominant frequency peak(s); several peaks codominate for the “low-frequency” part of the call.

<i>E. guyanensis</i>	Freq. 1 (kHz)	Ampl. 1 (dB)	Freq. 2 (kHz)	Ampl. 2 (dB)	Freq. 3 (kHz)	Ampl. 3 (dB)	Freq. 4 (kHz)	Ampl. 4 (dB)	Freq. 5 (kHz)	Ampl. 5 (dB)	Freq. 6 (kHz)	Ampl. 6 (dB)
“Low frequency”	3.891 ±0	-64.788 ±1.460	7.597 ±0.15	-66.621 ±1.6206	11.800 ±0	-52.994 ±0.940	15.700 ±0.123	-51.677 ±3.775	19.918 ±0.455	-53.631 ±2.816	25.447 ±1.692	-70.978 ±2.984
High frequency	20.207 ±0.749	-19.576 ±5.651	41.565 ±0.985	-46.153 ±8.035								
Transition frequency	3.9 ±0	57.119 ±0.846	9.139 ±0.647	-59.439 ±1.91	12.585 ±0.918	-49.596 ±1.39	17.297 ±0.649	-41.224 ±1.44	20.4 ±0.8	-46.245 ±5.095	24.319 ±0.823	-58.791 ±2.986

The calling song of *E. guyanensis* exhibits remarkable swap on its frequencies. And their broad amplitude modulation is remarkable; the number of syllables differ among its sections, for the simple tooth make low amplitude syllables in “low” frequency section, we found ca. 45 syllables with a syllable duration of  $3.5 \pm 1.8$  ms, for a period of  $10.6 \pm 0.4$  ms. Meanwhile, for the double tooth with very high amplitude syllables in “high” frequency section we found ca. 25 syllables, with syllable duration of  $3.5 \pm 0.5$  ms and a period of  $11.4 \pm 1.6$  ms; finally, in the transition section we found ca. 5 syllables, syllable duration of  $2.79 \pm 0.2$  ms, and period of  $10 \pm 0.3$  ms. Thus, the duty cycle for the "low" frequency section is 33%, and 30% for the "high" frequency section, and 26% for the transition section (Table 2).

Table 2. Syllable features in the “low”, “high” and transition sections of the calling song of *E. guyanensis*.

	Number of syllables	Syllables duration (ms)	Interval syllables (ms)	Syllables period (ms)	Frequency (kHz)
“Low” frequency	43.8 ±1.81	3.579 ±0.245	7.029 ±0.291	10.608 ±0.406	3.9 ±2.43
“High” frequency	23.8 ±0.63	3.526 ±0.553	7.916 ±1.576	11.442 ±1.671	20.06 ±0.605
Transition syllables	5.3 ±0.8	2.796 ±0.244	7.281 ±0.55	10.065 ±0.382	17.29 ±0.649

We have marked and analyzed the sections and syllables of 10 different recording song bouts with complete low and high frequency sections, we count a total of  $75.3 \pm 4.2$  syllables with a total time of  $563 \pm 25$  ms by entire section, in the “high” sections a period  $203.06 \pm 8.66$  ms, in the “low frequency” with  $321.5 \pm 21.5$  ms, and transition with  $39.07 \pm 7.67$  ms (Table 3).

Table 3. Recording song bouts analyses of *E. guyanensis* discriminated by “low, high, and transition”.

Syllables “low frequency”	Period (ms)	Syllables transition	Period (ms)	Syllables “high frequency”	Period (ms)	Total syllables (L+T+H)	Total time (ms) (L+T+H)
45.75 $\pm 3.01$	321.5 $\pm 21.5$	4.85 $\pm 0.93$	39.07 $\pm 7.67$	24.8 $\pm 1.54$	203.06 $\pm 8.66$	75.3 $\pm 4.28$	563.1 $\pm 25.21$

### ***Eneoptera surinamensis***

The calling song of *E. surinamensis* was re-analyzed with new sound recordings for six males (five from Brazil and one from Colombia), all recorded using a microphone allowing high-frequency recordings. As previously found by (Miyoshi et al. 2007), the calling song consist of a continuous trill emitted in bouts that can last a few hours when a male is not disturbed. We have found a slight amplitude modulation in the calling song generating a characteristic temporal pattern. When several males call together in their natural habitat, and even in laboratory conditions, they usually synchronize their amplitude modulations to form a chorus (Pers. Obs.).

#### *Acoustic characterization:*

The new sound recordings confirm that the dominant frequency corresponds to the low fundamental peak of the spectrum,  $F1 = 2.95 \pm 0.099$  kHz. Our study allows to correctly records the upper part of the frequency spectrum, while it could not be estimated previously.



The results reveal that the calling song exhibits a power spectrum with five clear harmonics above F1, at integer multiple values of approximately 6 kHz (F2), 9 kHz (F3), 12 kHz (F4), 15 kHz (F5) and 18 kHz (F6); for which we also estimated the relative amplitudes (Table 4 and Figure 5).

These harmonics in the calling song are low in energy compared to F1 (Table 5); none of them never dominate or co-dominate the spectrum contrary to our predictions.

We observed that the call does not have any notable particularity in terms of frequency spectrum. However, it includes a noticeable amplitude modulation generating a regular pattern within the continuous trill including two distinct sections with slightly different syllables. For the low amplitude sections (ca. 67 syllables), syllables have a duration of  $5.4 \pm 1.2$  ms, and a period of  $20.7 \pm 4.9$  ms. Meanwhile, for the high amplitude sections (ca. 45 syllables), we found that syllable duration is  $4.9 \pm 1.4$  ms, with a shorter period of  $17.4 \pm 3.1$  ms. Thus, the main difference between the low and high amplitude sections is the syllable duration and period longer in the low amplitude sections, with shorter and closer syllables in the high amplitude sections. The syllable duty cycles are not clearly different between the two types of sections, being 26 % for low amplitude, and 28 % for high amplitude sections (Table 4). The difference between the two sections is appreciable by human hearing mostly because of the amplitude modulation (Figure 5).

For *E. surinamensis* we have marked and analyzed 5 calls with complete sections for the six males, we found a total of  $114.5 \pm 5.6$  syllables with a total time of  $1686 \pm 73.5$ ms by entire section, in the high amplitude sections a period  $48.1 \pm 6.8$ ms, in the low amplitude with  $1133.9 \pm 58.6$ ms, (table 7).

#### *Ultrastructure features:*

The two males of *E. surinamensis* studied for ultrastructure possess 71 stridulatory teeth. The teeth are simple and arranged along the stridulatory file as typically observed in crickets, which allows the production of loud pure tones. In the most anal zone, the distance between the teeth

is quite random, suggesting that this region is not used during the stridulation, but the median region of the file shows a constant increase in the inter-tooth distance toward the basal zone, following the direction of travel of the plectrum. To the functional part of the stridulatory file ( $0.7845 \pm 0.015$  mm) (Figure 6).

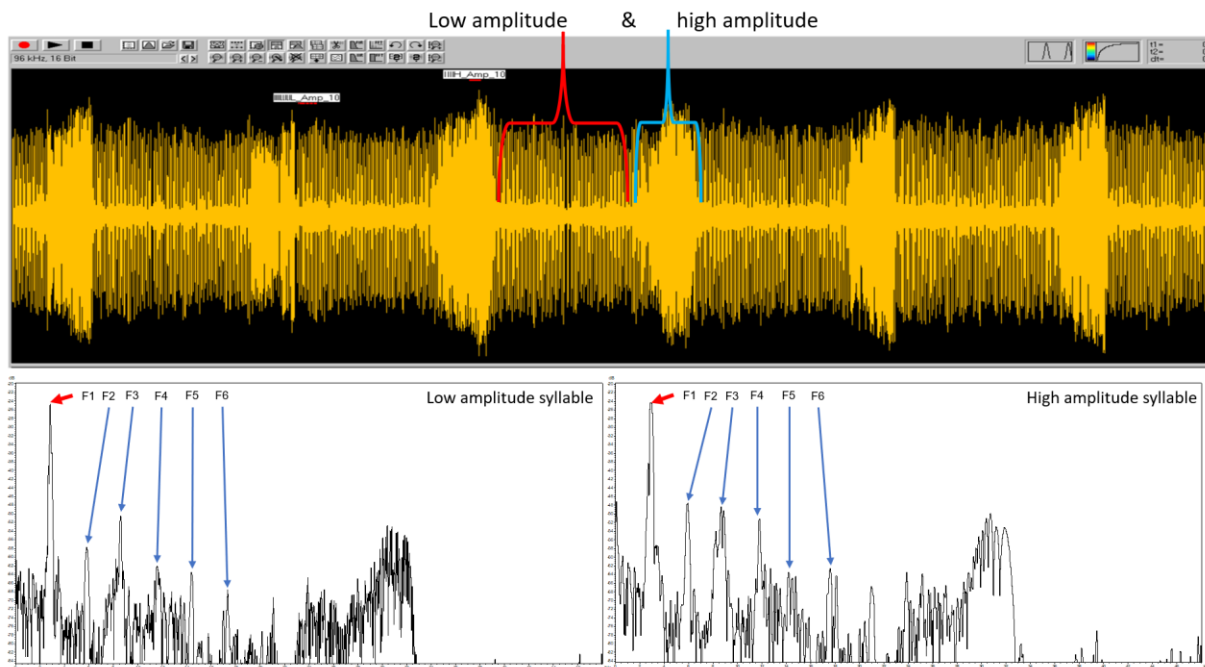


Figure 5. Calling song of *E. surinamensis*. Spectrogram showing low and high amplitude sections and power spectrum for low and high amplitude syllables; the fundamental frequency is indicated by red arrows, and blue arrows indicate harmonic peaks.

The mean inter-tooth distance for the functional part is  $35.66 \mu\text{m}$ , with a tooth density of approximately  $38/\text{mm}$  (Figure 6). Based on the null hypothesis that TSR produces the lowest peak in the spectrum (i.e.  $F1 = fd$ ), the instantaneous speed of the system is  $109 \text{ mm/s}$  ( $35.66 \mu\text{m} / (1/F1)$ ). The global speed of the stridulatory movement deduced from syllable duration and the total length of the functional part of the file, is  $121 \text{ mm/s}$ , which also more or less corresponds to the null hypothesis that TSR is the low frequency peak of the spectrum (Table 6). We could

deduce that one tooth impact generates the vibration in the primary wave at low frequency as the dominant peak (fd).

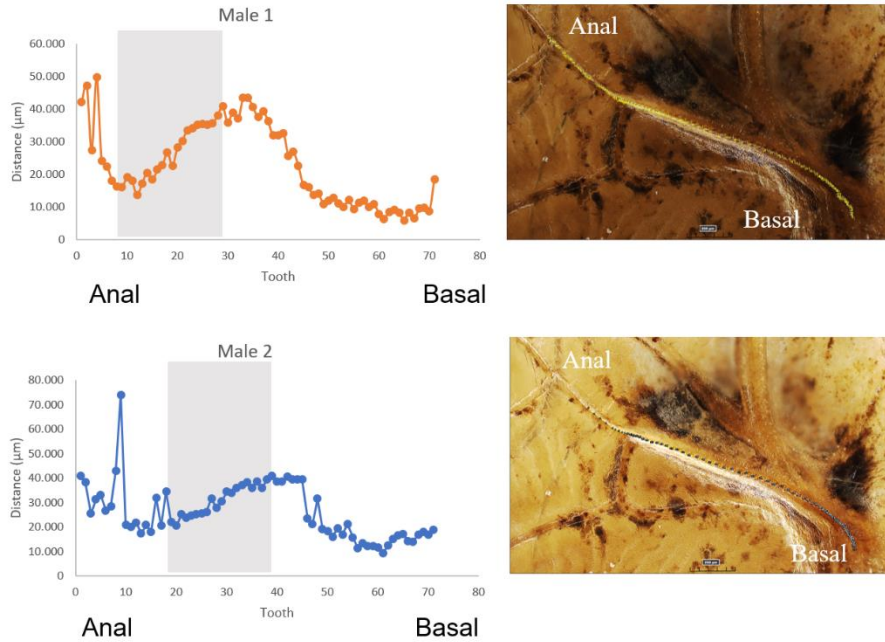


Figure 6. Tooth distance for two males of *E. surinamensis*, pictures stridulatory file (250 µm scale).

Table 4. Song parameters and acoustic analysis of calling song of six males for *E. surinamensis*.

<b>LOW AMP</b>	Syllable	Syllable duration (ms)	Interval (ms)	Period (ms)	<b>HIGH AMP</b>	Syllable	Syllable duration (ms)	Interval (ms)	Period (ms)
Male 1	63 ±4.18	7.51 ±0.87	18.43 ±2.35	25.485 ±5.042	Male1	52 ±6.28	6.29 ±0.89	11.04 ±1.54	17.677 ±2.332
Male 2	67.2 ±1.48	4.63 ±1.09	15.52 ±2.32	19.939 ±3.294	Male 2	41.6 ±9.21	4.45 ±1.13	10.85 ±2.2	15.086 ±2.922
Male 3	70 ±3.39	5.45 ±0.78	15.8 ±1.87	20.253 ±5.055	Male 3	38.8 ±12.27	4.87 ±1.12	10.42 ±1.35	15.050 ±2.338
Male 4	71 ±0.7	5.49 ±1.9	14.9 ±6.03	19.994 ±6.815	Male 4	50 ±3.67	4.53 ±1.88	19.64 ±1.94	23.882 ±3.673
Male 5	71.2 ±1.64	5.07 ±1.86	13.67 ±5.58	18.426 ±6.301	Male 5	45.2 ±5.07	5.17 ±2.22	13.67 ±4.09	18.594 ±4.775
Male 6	67 ±1.22	3.98 ±1.31	16.75 ±1.88	20.481 ±3.009	Male 6	42.6 ±6.10	3.75 ±1.23	11.03 ±1.63	14.542 ±2.595
<b>LOW</b>	<b>68.23</b> ±2.10	<b>5.42</b> ±1.24	<b>15.86</b> ±3.34	<b>20.763</b> ±4.919	<b>HIGH</b>	<b>45.03</b> ±7.10	<b>4.93</b> ±1.4	<b>12.78</b> ±2.12	<b>17.472</b> ±3.106

Table 5 Calling song analysis with frequencies, harmonic content, and amplitude measurements in *E. surinamensis*.

<b>Low amp.</b>	<b>FD (kHz)</b>	<b>Ampl.1 (dB)</b>	<b>Freq.2 (kHz)</b>	<b>Ampl.2 (dB)</b>	<b>Freq.3 (kHz)</b>	<b>Ampl.3 (dB)</b>	<b>Freq.4 (kHz)</b>	<b>Ampl.4 (dB)</b>	<b>Freq.5 (kHz)</b>	<b>Ampl.5 (dB)</b>	<b>Freq.6 (kHz)</b>	<b>Ampl.6 (dB)</b>
Male1	2.97 ±0.07	-25.8 ±2.74	5.91 ±0.23	-48.37 ±3.46	8.58 ±0.54	-54.61 ±3.13	11.29 ±0.454	-52.69 ±2.65	14.33 ±0.67	-57.063 ±4.33	18.6 ±1.33	-66.80 ±3.056
Male2	2.93 ±0.06	-17.28 ±1.6	5.81 ±0.05	-43.58 ±1.75	8.79 ±0.22	-54.22 ±2.137	11.58 ±0.182	-46.36 ±2.0	13.768 ±0.55	-56.951 ±2.25	20.58 ±0.42	-68.94 ±2.443
Male3	3.0 ±0.024	-20.47 ±1.89	6.06 ±0.91	-54.76 ±2.18	9.0 ±0.05	-41.03 ±1.79	12.04 ±0.020	-52.78 ±1.39	14.88 ±0.557	-58.125 ±3.046	18.259 ±0.73	-63.36 ±2.768
Male4	2.8 ±0.016	-24.37 ±1.65	5.95 ±0.26	-46.33 ±1.84	8.53 ±0.15	-44.876 ±1.115	11.83 ±0.43	-49.505 ±2.198	14.42 ±0.785	-55.37 ±3.165	18.22 ±1.13	-59.76 ±2.471
Male5	2.97 ±0.52	-27.36 ±1.19	6.019 ±0.38	-54.85 ±1.98	8.885 ±0.96	-41.545 ±1.177	11.56 ±0.39	-49.650 ±2.166	14.389 ±0.5	-54.66 ±2.396	18.66 ±1.31	-61.69 ±3.655
Male6	2.99 ±0.012	-30.17 ±1.925	6.04 ±0.12	-61.17 ±2.876	8.98 ±0.028	-49.147 ±1.48	11.77 ±0.32	-59.662 ±1.979	14.52 ±0.685	-63.475 ±2.82	18.53 ±1.21	-62.48 ±2.416
<b>Mean</b>	<b>2.94 ±0.35</b>	<b>-24.24 ±1.83</b>	<b>5.96 ±0.19</b>	<b>-51.51 ±2.351</b>	<b>8.797 ±0.181</b>	<b>-47.57 ±1.805</b>	<b>11.68 ±0.33</b>	<b>-51.77 ±2.06</b>	<b>14.386 ±0.625</b>	<b>-57.609 ±3.002</b>	<b>18.81 ±1.024</b>	<b>-63.84 ±2.802</b>
<b>High amp.</b>	<b>FD (kHz)</b>	<b>Ampl.1 (dB)</b>	<b>Freq.2 (kHz)</b>	<b>Ampl.2 (dB)</b>	<b>Freq.3 (kHz)</b>	<b>Ampl.3 (dB)</b>	<b>Freq.4 (kHz)</b>	<b>Ampl.4 (dB)</b>	<b>Freq.5 (kHz)</b>	<b>Ampl.5 (dB)</b>	<b>Freq.6 (kHz)</b>	<b>Ampl.6 (dB)</b>
Male1	2.97 ±0.62	-24.1 ±4.067	5.9 ±0.52	-46.32 ±3.646	8.43 ±0.88	-48.33 ±4.273	11.26 ±0.74	-47.479 ±3.396	14.269 ±0.68	-51.467 ±5.937	18.98 ±1.56	-63.96 ±3.587
Male2	2.95 ±0.039	-16.89 ±0.877	5.98 ±0.34	-43.66 ±2.168	8.96 ±0.25	-52.64 ±2.244	11.5 ±0.4	-46.47 ±2.526	13.517 ±0.369	-53.076 ±5.336	19.48 ±0.68	-64.55 ±1.737
Male3	2.95 ±0.305	-20.7 ±3.88	6.03 ±0.787	-52.84 ±8.483	8.86 ±0.94	-40.958 ±5.538	11.86 ±1.49	-48.067 ±8.52	14.085 ±1.848	-50.945 ±10.326	18.409 ±2.6	-61.24 ±10.625
Male4	2.84 ±0.075	-24.5 ±2.21	6.13 ±0.49	-44.66 ±2.115	8.69 ±0.346	-44.58 ±2.079	11.8 ±0.289	-46.37 ±3.27	14.13 ±0.71	-52.367 ±4.237	18.68 ±1.57	-60.128 ±4.279
Male5	2.99 ±0.077	-26.99 ±2.51	6.65 ±0.629	-51.63 ±2.791	8.94 ±0.136	-42.561 ±1.53	11.55 ±0.65	-43.32 ±2.74	14.3 ±0.95	-48.218 ±5.526	19.09 ±1.36	-61.67 ±3.561
Male6	2.98 ±0.40	-29.73 ±2.81	5.75 ±1.2	-56.885 ±3.655	8.99 ±0.029	-49.976 ±2.011	11.41 ±0.729	-55.40 ±2.73	13.94 ±0.629	-54.832 ±3.7	18.88 ±1.4	-63.218 ±2.95
<b>Mean</b>	<b>2.95 ±0.099</b>	<b>-23.83 ±2.72</b>	<b>6.076 ±0.66</b>	<b>-49.33 ±3.81</b>	<b>8.816 ±0.429</b>	<b>-46.509 ±2.946</b>	<b>11.56 ±0.719</b>	<b>-47.85 ±3.865</b>	<b>14.046 ±0.866</b>	<b>-51.817 ±5.844</b>	<b>18.92 ±1.53</b>	<b>-62.46 ±4.456</b>

Table 6. Morphological and acoustical measurements associated with stridulation for *E. surinamensis*.

	Total file length (mm)	No. of teeth (total)	No. of teeth (used)	Tooth density (No./mm)	Total distance used (mm)	Average tooth distance ( $\mu\text{m}$ )	TSR (strikes/s)	Syllable duration (ms)	Candidate fundamental frequency (kHz)	Global speed syllable duration (mm/s)	Instantaneous speed from frequency (mm/s)
<b>Male 1</b>	1.832	71	21	38	0.7949	36.136	2641.5	L: 7.517 H: 6.294	L: 2.793.6 H: 3.336.5	L: 105.75 H: 126.30	109.6
<b>Male 2</b>	2.116	71	21	33.55	0.7740	35.185	2713.17	L: 5.493 H: 5.075	L: 3.823.04 H: 4.197.33	L: 140.90 H: 152.51	108.285
<b>MEAN SD</b>	<b>1.974</b> <b><math>\pm 200.8</math></b>	<b>71</b> <b><math>\pm 0</math></b>	<b>21</b> <b><math>\pm 0</math></b>	<b>35.77</b> <b><math>\pm 3.15</math></b>	<b>0.7845</b> <b><math>\pm 0.015</math></b>	<b>35.661</b> <b><math>\pm 0.672</math></b>	<b>3845.49</b> <b><math>\pm 1103.66</math></b>	<b>L: 6.505</b> <b><math>\pm 1.43</math></b> <b>H: 5.684</b> <b><math>\pm 0.86</math></b>	<b>L: 3.308.32</b> <b><math>\pm 727.92</math></b> <b>H: 3.766.9</b> <b><math>\pm 608.7</math></b>	<b>L: 123.32</b> <b><math>\pm 24.85</math></b> <b>H: 139.4</b> <b><math>\pm 18.5</math></b>	<b>108.94</b> <b><math>\pm 0.93</math></b>

Table 7. Sections of *E. surinamensis*; differentiated in high and low amplitude

	Count total syllables	Number of call	# syllables High Amp	Period (ms)	#syllables Low Amp	Period (ms)	Total time (ms)
Male 1	121 $\pm 3.674$	5 $\pm 0$	49.75 $\pm 3.674$	541.45 $\pm 45.05$	71.25 $\pm 0.707$	1107.37 $\pm 37.332$	1649.5 $\pm 29.885$
Male 2	115 $\pm 3.937$	5 $\pm 0$	52 $\pm 6.285$	591.3 $\pm 75.544$	63 $\pm 4.183$	1163.1 $\pm 87.357$	1754.4 $\pm 53.849$
Male 3	109.6 $\pm 6.025$	5 $\pm 0$	42.6 $\pm 6.107$	449.58 $\pm 54.371$	67 $\pm 1.225$	1057.78 $\pm 25.949$	1526.98 $\pm 50.077$
Male 4	107.4 $\pm 9.236$	5 $\pm 0$	43.8 $\pm 11.777$	488.02 $\pm 130.343$	63.6 $\pm 3.847$	1037.5 $\pm 53.762$	1525 $\pm 99.592$
Male 5	120.8 $\pm 7.014$	5 $\pm 0$	52.8 $\pm 8.348$	691.8 $\pm 140.952$	68 $\pm 3.535$	1338.72 $\pm 61.104$	2029 $\pm 152.559$
Male 6	113.2 $\pm 4.147$	5 $\pm 0$	48 $\pm 5.049$	534.96 $\pm 61.495$	65.2 $\pm 5.63$	1099.14 $\pm 86.405$	1633.4 $\pm 55.12$
<b>Mean SD</b>	<b>114.5</b> <b><math>\pm 5.672</math></b>	<b>5</b> <b><math>\pm 0</math></b>	<b>48.158</b> <b><math>\pm 6.873</math></b>	<b>549.518</b> <b><math>\pm 84.626</math></b>	<b>66.341</b> <b><math>\pm 3.188</math></b>	<b>1133.935</b> <b><math>\pm 58.652</math></b>	<b>1686.38</b> <b><math>\pm 73.514</math></b>

## ***Eneoptera gracilis***

The calling song of *E. gracilis* was studied based on one male collected in Menegua, Puerto Lopez-Meta. The corresponding population was located based on the labels of one specimen from the collections of the Natural Sciences Institute (ICN) in Bogotá.

### *Acoustic characterization:*

We were able to record for the first time the calling song of this species and characterize its acoustic features. The calling song is a continuous trill, with a dominant frequency of  $3.58 \pm 0.04$  kHz corresponding to the F1 of the spectrum (Table 7). We measured the harmonic peaks as follows F2:  $7.3 \pm 0.013$ kHz; F3:  $10.8 \pm 0.011$ kHz; F4:  $14.35 \pm 0.3$  kHz; F5:  $16.1 \pm 0.4$  kHz; F6:  $21.7 \pm 3.9$ kHz. The three first harmonics exhibit similar amplitude reaching the half of dominant frequency peak, these harmonics are notorious in comparison with the spectrum of *E. surinamensis*, which present three clear harmonics with low amplitude (Table 10 and Figure 7).

Table 8. Song parameters and acoustic analysis of three calling songs of one male of *E. gracilis*.

	Syllables	Duration (ms)	Interval (ms)	Period (ms)	Dominant freq.(kHz)
<b>Call1</b> Mean SD	60.8 $\pm 1.09$	8.223 $\pm 0.618$	16.456 $\pm 0.3675$	24.683 $\pm 0.7692$	3.54 $\pm 0.082$
<b>Call2</b> Mean SD	58.8 $\pm 3.3$	6.725 $\pm 0.847$	17.110 $\pm 1.1998$	23.832 $\pm 1.2325$	3.69 $\pm 0.04$
<b>Call3</b> Mean SD	61.6 $\pm 2.8$	9.515 $\pm 1.394$	16.405 $\pm 0.5462$	25.694 $\pm 2.4093$	3.5 $\pm 0.012$
<b>Mean</b> <b>SD</b>	<b>60.4</b> <b><math>\pm 1.44</math></b>	<b>8.155</b> <b><math>\pm 0.953</math></b>	<b>16.657</b> <b><math>\pm 0.7045</math></b>	<b>24.737</b> <b><math>\pm 1.4704</math></b>	<b>3.58</b> <b><math>\pm 0.045</math></b>

The calling song in *E. gracilis* does not have any particularity in terms of frequency spectrum. Its amplitude exhibits a slight modulation quite imperceptible, not allowing to distinguish sections as in previous species. Syllable duration is  $8.1 \pm 0.9$  ms, for a period of  $24.7 \pm 1.4$  ms. Thus, the syllable duty cycle 33.3 % (Table 8) (Figure 7).

For *E. gracilis* we have marked and analyzed 3 different recording song bouts each one composed by 200 syllables, we found in a distinct part within 10 syllables periods with  $177.9 \pm 11.4$  ms, in a total time of  $3355.8 \pm 135.5$  ms by the entire section, this species in particular do not exhibit any change in the amplitude or frequency (table 11).

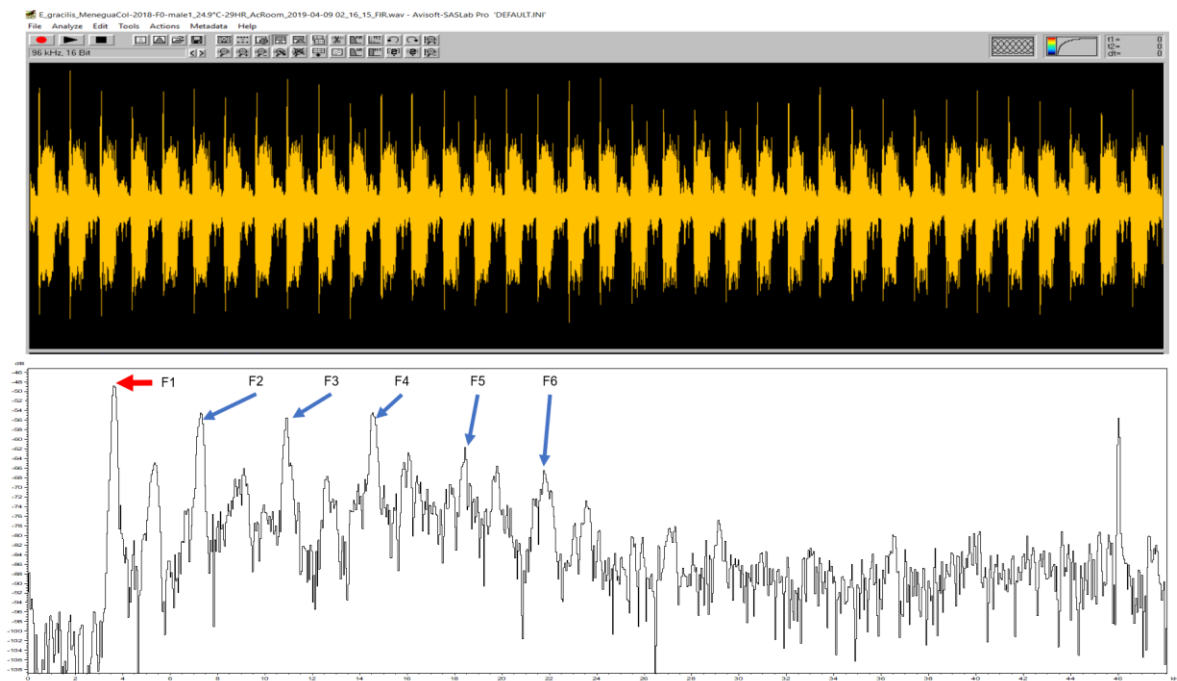


Figure 7. Spectrogram section, and power spectrum for one syllable, the F1 (fd) fundamental frequency red arrow, and blue arrows for harmonic content in *E. gracilis*.

*Ultrastructure features:*

The stridulatory file consists of 64 teeth in simple structure, 26 are functional teeth, with a length of  $0.814 \pm 0.06$  mm; the mean inter-tooth distance of the functional part is  $35.66 \mu\text{m}$  (tooth density =  $35.4 \pm 1.4$  teeth/mm) (Table 9).

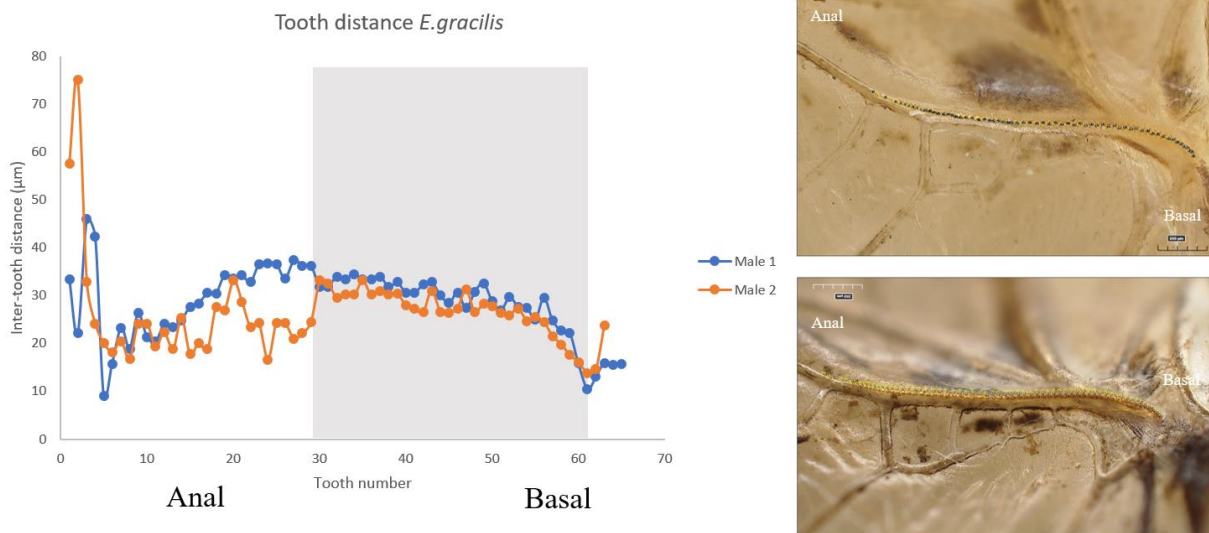


Figure 8. Tooth distance for two males of *E. gracilis*, pictures stridulatory file (250 µm scale).

The mean inter-tooth distance for the functional part is  $31 \mu\text{m}$ , with a tooth density of 35 teeth/mm approximately (Figure 8). Based on the null hypothesis that TSR produces the lowest peak in the spectrum ( $F1 = fd$ ), the instantaneous speed of the system is  $103.59 \text{ mm/s}$  ( $29.599 \mu\text{m} / (1/F1)$ ), The global speed derived from the syllable duration is  $109 \text{ mm/s}$ . Which is a similar value in terms to corresponds to the null hypothesis that TSR is the low frequency peak of the spectrum (Table 9). This result suggests that one tooth impact generates one primary wave at low frequency as the dominant peak.



Table 9. Morphological and acoustical measurements associated with stridulation for *E. gracilis*.

<i>E. gracilis</i>	Total file length (mm)	No. of teeth (total)	No. of teeth (used)	Tooth density (No./mm)	Total distance used (mm)	Average tooth distance ( $\mu$ m)	TSR (strikes/s)	Syllable duration	Candidate fundamental frequency (kHz)	Global speed syllable duration (mm/s)	Instantaneous speed from frequency (mm/s)
<b>Male 1</b>	1.810	65	26	35.911	0.8571	30.613	3161.86	8.223	3.161	104.22	107.145
<b>Male 2</b>	1.804	63	27	34.92	0.7717	28.584	3565.29	7.573	3.565	114.73	100.044
<b>MEAN SD</b>	<b>1.807</b> <b><math>\pm 0.004</math></b>	<b>64</b> <b><math>\pm 1.41</math></b>	<b>26.5</b> <b><math>\pm 0.7</math></b>	<b>35.41</b> <b><math>\pm 0.70</math></b>	<b>0.8144</b> <b><math>\pm 0.06</math></b>	<b>29.599</b> <b><math>\pm 1.43</math></b>	<b>3363.57</b> <b><math>\pm 285.27</math></b>	<b>7.898</b> <b><math>\pm 0.459</math></b>	<b>3.363</b> <b><math>\pm 0.285</math></b>	<b>109.47</b> <b><math>\pm 7.43</math></b>	<b>103.595</b> <b><math>\pm 5.021</math></b>

Table 10. Calling song analysis with frequencies, harmonic content, and amplitude measurements for *E. gracilis*.

Calls	FD (F1) (kHz)	Amp 1 (dB)	F2 (kHz)	Amp 2 (dB)	F3 (kHz)	Amp 3 (dB)	F4 (kHz)	Amp 4 (dB)	F5 (kHz)	Amp 5 (dB)	F6 (kHz)	Amp 6 (dB)
1-Mean SD	3.500 $\pm 0$	-44.401 $\pm 0.587$	7.297 $\pm 0.042$	-48.234 $\pm 0.763$	10.801 $\pm 0.016$	-50.001 $\pm 0.478$	14.387 $\pm 0.232$	-47.228 $\pm 1.012$	16.046 $\pm 0.295$	-53.491 $\pm 1.463$	21.877 $\pm 3.995$	-61.348 $\pm 6.077$
2-Mean SD	3.500 $\pm 0$	-44.173 $\pm 0.125$	7.300 $\pm 0$	-48.102 $\pm 0.174$	10.800 $\pm 0$	-49.524 $\pm 0.203$	14.300 $\pm 0.489$	-47.522 $\pm 1.985$	16.106 $\pm 0.416$	-53.180 $\pm 1.022$	22.041 $\pm 3.807$	-61.821 $\pm 6.459$
3-Mean SD	3.500 $\pm 0.011$	-44.043 $\pm 0.194$	7.300 $\pm 0$	-48.111 $\pm 0.201$	10.802 $\pm 0.019$	-49.283 $\pm 0.186$	14.385 $\pm 0.283$	-47.257 $\pm 1.255$	16.244 $\pm 0.506$	-52.151 $\pm 1.559$	21.262 $\pm 4.047$	-59.579 $\pm 6.012$
<b>Mean SD</b>	<b>3.500</b> <b><math>\pm 0.031</math></b>	<b>-44.206</b> <b><math>\pm 0.302</math></b>	<b>7.299</b> <b><math>\pm 0.014</math></b>	<b>-48.149</b> <b><math>\pm 0.379</math></b>	<b>10.801</b> <b><math>\pm 0.012</math></b>	<b>-49.602</b> <b><math>\pm 0.289</math></b>	<b>14.357</b> <b><math>\pm 0.334</math></b>	<b>-47.336</b> <b><math>\pm 1.4180</math></b>	<b>16.132</b> <b><math>\pm 0.406</math></b>	<b>-52.941</b> <b><math>\pm 1.348</math></b>	<b>21.726</b> <b><math>\pm 3.949</math></b>	<b>-60.916</b> <b><math>\pm 6.183</math></b>

Table 11. Analysis based on three different song bouts from *E. gracilis*.

	Count total syllables	Number of call	# syllables	Period (ms)	Total time (ms)
Call 1	200	1	10	176.452 $\pm 6.592$	3259.952 $\pm 43.785$
Call 2	200	2	10	186.986 $\pm 20.376$	3485.886 $\pm 267.028$
Call 3	200	3	10	170.532 $\pm 7.252$	3321.778 $\pm 95.658$
<b>Mean SD</b>	<b>200</b> <b><math>\pm 0</math></b>		<b>10</b> <b><math>\pm 0</math></b>	<b>177.99</b> <b><math>\pm 11.407</math></b>	<b>3355.872</b> <b><math>\pm 135.490</math></b>

## ***Eneoptera nigripedis***

We were able to record for the first time the calling song of *E. nigripedis*, this was analyzed with specimens collected from Colombia.

### *Acoustic characterization:*

The calling song of *E. nigripedis* is a continuous trill, that exhibits a notable difference in the dominant frequency combined with high and low amplitude modulation sections. This species possesses a bisinuated stridulatory file with a dominant frequency ( $F_d$ ) for the double teeth section corresponds to the fourth peak of the spectrum,  $F_4 = 12.9 \pm 0.7\text{kHz}$ ; and for the simple teeth section the dominant frequency at  $3.06 \pm 0.047\text{ kHz}$  which corresponds to the first peak of the spectrum ( $f_d$ ). Thus, by a double call strategy, this species performs a frequency switch within the syllables due to the teeth distribution, complemented by an amplitude modulation, which represents a striking acoustic behaviour (Figure 9).

The number of syllables contrast between the two types of sections of the call. For the low amplitude sections, we found ca. 73 syllables, with a syllable duration of  $3.574 \pm 0.7\text{ ms}$ , and a syllable period of  $15.26 \pm 0.67\text{ ms}$ ; meanwhile, for the high amplitude section we found ca. 30 syllables, with a syllable duration is  $4.14 \pm 0.7\text{ ms}$ , we found a shorter period with  $11.5 \pm 0.97\text{ ms}$ . (table 12).

Additionally, the duty cycle for low amplitude is 23.3 % in low amplitude section, and 35% in high amplitude sections. The difference is appreciable by hearing the cricket calls, and by comparison between sections in the spectrogram (Figure 10).

The calling song exhibits for the simple teeth a power spectrum with five clear harmonics above  $F_1$  (simple  $F_1: f_d$ ), these multiple integrals frequencies of  $F_1$  approximately near of codominance in  $F_2$  at 6 kHz, followed on importance by the next three peaks  $F_3: 9.5\text{kHz}$ ,  $F_4: 12.9\text{kHz}$ ,  $F_5:$

15.7kHz; and F6: 19 kHz. The double teeth exhibit a power spectrum with five harmonics, the three first in F1: 3 kHz; F2: 6.8 kHz; and F3: 9.6 kHz; below the dominant peak f4 (double 12.9 kHz, F4: fd) and nearby the fifth peak F5 at 15.6 kHz, and F6: 19.5 kHz (Table 13). Although the bandwidth values have similar values, the main reliable aspect to approach is the amplitude modulation of the two sections of the trill (Figure 9).

Table 12. Song parameters and acoustic analysis of calling song of high and low amplitude in *E. nigripedis*.

Amplitude	Syllables	Duration double (ms)	Duration simple (ms)	Total duration (ms) (couple)	Interval (ms)	Period (ms)
High SD	30 ±2.154	1.783 ±0.1157	2.36 ±0.25	4.14 ±0.7013	7.421 ±0.8494	11.564 ±0.97
Low SD	73 ±3.928	1.504 ±0.1116	2.07 ±0.1545	3.574 ±0.7204	11.688 ±0.7208	15.262 ±0.675

*Ultrastructure features:*

We measured the stridulatory file of *E. nigripedis* in two specimens. In both cases, the file is divided in two well differentiated sections. First, the anal section includes double-crest teeth each one likely behaving as a simple tooth, with its two crests hit by the plectrum. It results in remarkably different inter-tooth distances between the double-teeth file section compared to the next file section, that includes only simple teeth.

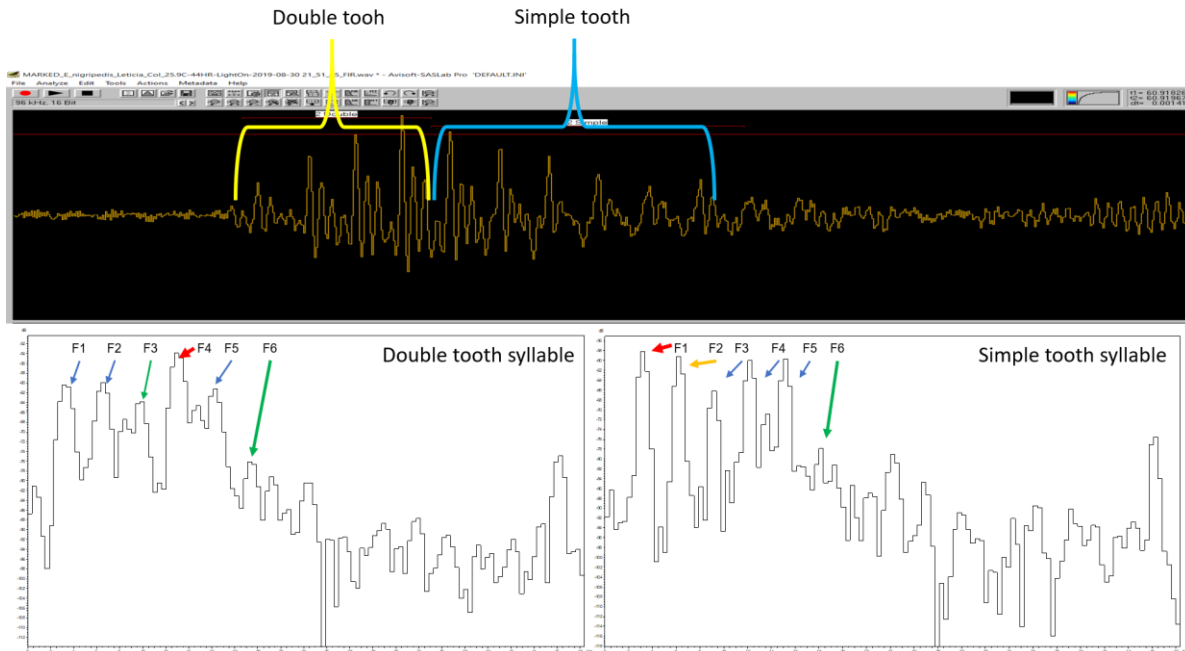


Figure 9. Spectrogram of *E. nigripedis* with double and single tooth syllables. Also, power spectrum with Double and simple tooth syllables left and right respectively. Left: Dominant frequency (red) co-dominant (blue) frequency and green harmonic content. Right: Dominant frequency (red) co-dominant (orange) and (blue), (green) harmonic content.

The basal section presents 40 simple teeth, that are arranged well separately the one from the others. The anal section presents 54 double-crest teeth, these are arranged closer. This is explained because each tooth of the anal section is subdivided by a longitudinal furrow over its whole length, which separates it into two parallel crests (Table 14). This results in a set of equidistant crests likely acting as secondary teeth, which doubles the number of stridulatory crests implied in the stridulation process. Approximately, in the middle of the stridulatory file is located the division between the two teeth sections (Figure 11).

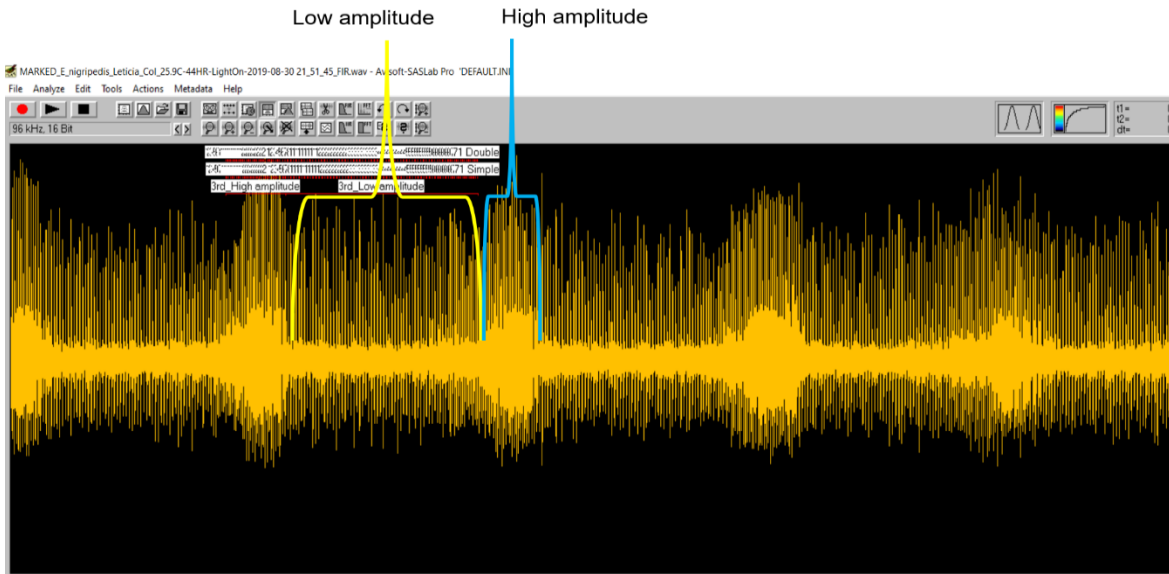


Figure 10. Spectrogram with high and low amplitude sections in *E. nigripedis*.

We have measured and plotted as function of teeth/crests numbers (Figure 11). The mean inter-tooth distance for simple teeth is  $21.07 \pm 3.5 \mu\text{m}$ , and for the double teeth the mean inter-crest distances of  $8.30 \pm 1.4 \mu\text{m}$  (or inter-crest distances for double teeth). The functional parts for each part of the stridulum have been measured, in the simple section is  $0.4209 \mu\text{m}$ , the double teeth section is  $0.6405 \mu\text{m}$ . Besides with a tooth density in 45 and 102 teeth respectively (Table 14).

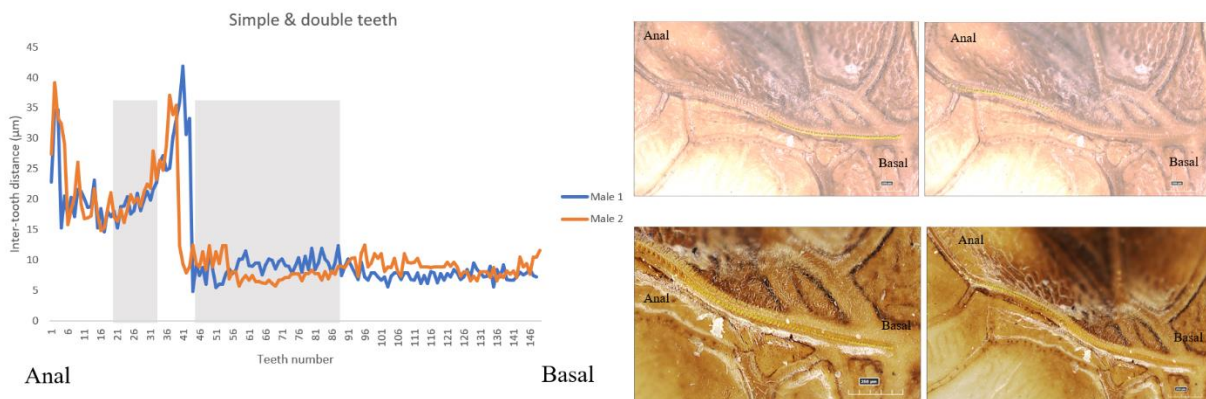


Figure 11. Tooth distance for two males of *E. nigripedis*; pictures stridulatory file (250µm scale).

Since the TSR produces the lowest peak in the spectrum, the instantaneous speed of this system will be differentiated in the two file sections, combined with low and high amplitudes. Because the speeds change for each part of the stridulatory file correspond to the two amplitude sections. For simple teeth is 31mm/s in the low amplitude section and 45 mm/s in the high amplitude section, since it would be 161.38 mm/s in high 181.66 mm/s. The value in terms of TSR is near the second peak, this fits with the value of harmonic F2, which is near to codominance with F1 for the simple teeth. Besides, for the double teeth is 48.70 mm/s in the low amplitude section, and 67.85mm/s for the high amplitude section, being in the global speed 94 mm/s and 109.68 mm/s respectively, those are values near of the F3 which exhibit codominance with F4 (fd) follow by F5 peak.

Table 13 Calling song analysis frequency measurements, harmonics and amplitude measurements.

<b>High amp.</b>	Freq-1 (kHz)	Amp-1 (dB)	Freq-2 (kHz)	Amp-2 (dB)	Freq-3 (kHz)	Amp-3 (dB)	Freq-4 (kHz)	Amp-4 (dB)	Freq-5 (kHz)	Amp-5 (dB)	Freq-6 (kHz)	Amp-6 (dB)
Call 1 mean SD	3.04 ±0.05	-66.32 ±5.76	6.57 ±0.841	-77.45 ±6.84	9.98 ±0.836	-76.83 ±4.62	12.92 ±0.68	-65.20 ±4.394	15.84 ±0.836	-73.36 ±6.63	18.95 ±1.21	-77.64 ±6.54
Call 2 mean SD	3.081 ±0.04	-64.95 ±6.75	6.22 ±0.527	-74.85 ±5.68	9.33 ±0.399	-75.46 ±5.99	12.78 ±0.785	-64.05 ±3.49	15.48 ±0.408	-64.26 ±3.205	19.8 ±1.35	-80.36 ±6.4
Call 3 mean SD	3.052 ±0.05	-60.28 ±4.39	6.23 ±0.411	-74.12 ±7.49	9.67 ±0.657	-76.14 ±4.932	13.04 ±0.728	-63.19 ±5.79	15.7 ±0.421	-64.39 ±4.569	19.81 ±1.5	-78.08 ±9.135
<b>Mean SD</b>	<b>3.058 ±0.047</b>	<b>-63.85 ±5.63</b>	<b>6.34 ±0.593</b>	<b>-75.47 ±6.67</b>	<b>9.66 ±0.631</b>	<b>-76.14 ±5.184</b>	<b>12.91 ±0.731</b>	<b>-64.14 ±4.557</b>	<b>15.68 ±0.555</b>	<b>-67.33 ±4.802</b>	<b>19.52 ±1.37</b>	<b>-78.69 ±7.35</b>
<b>Low amp</b>	Freq-1 (kHz)	Amp-1 (dB)	Freq-2 (kHz)	Amp-2 (dB)	Freq-3 (kHz)	Amp-3 (dB)	Freq-4 (kHz)	Amp-4 (dB)	Freq-5 (kHz)	Amp-5 (dB)	Freq-6 (kHz)	Amp-6 (dB)
Call 1 mean SD	30.03 ±0.045	-68.36 ±5.98	6.29 ±0.585	-79.69 ±6.78	9.87 ±1.029	-80.72 ±4.09	12.96 ±0.783	-70.30 ±7.021	15.67 ±0.816	-75.33 ±8.009	18.98 ±1.07	-81.54 ±5.26
Call 2 mean SD	3.05 ±0.098	-66.41 ±7.25	6.14 ±0.306	-75.50 ±6.31	9.17 ±0.384	-75.59 ±2.94	12.79 ±0.668	-65.17 ±4.107	15.67 ±0.577	-69.35 ±7.246	19.06 ±0.66	-79.46 ±8.56
Call 3 mean SD	3.07 ±0.05	-63.77 ±7.20	6.2 ±0.339	-76.19 ±10.21	9.42 ±0.231	-74.85 ±1.90	13.12 ±0.743	-63.88 ±5.755	15.94 ±0.677	-69.77 ±9.787	19.35 ±1.2	-78.56 ±10.41
<b>Mean SD</b>	<b>3.051 ±0.064</b>	<b>-66.18 ±6.81</b>	<b>6.21 ±0.41</b>	<b>-77.12 ±7.76</b>	<b>9.49 ±0.548</b>	<b>-77.05 ±2.98</b>	<b>12.96 ±0.731</b>	<b>-66.45 ±5.628</b>	<b>15.76 ±0.69</b>	<b>-71.48 ±8.347</b>	<b>19.13 ±0.98</b>	<b>-79.85 ±8.08</b>

Furthermore, the amplitude modulation is similar to that of *E. surinamensis* in terms of timing: the high amplitude section is shorter than the low amplitude sections. The high amplitude sections have a mean of 60 syllables for a total duration of  $581 \pm 33.5$  ms for the section. The low amplitude sections have a mean of 146 longer syllables and a total duration of  $703 \pm 51$  ms for the section, giving a total  $1305 \pm 51$  ms for both sections together (Table 15).

Table 14. Morphological and acoustical measurements associated with stridulation for *E. nigripedis*.

<i>Eneoptera nigripedis</i>	Total file length (mm)	No. of teeth (total)	No. of teeth (used)	Tooth density (No./mm)	Total distance used (mm)	Average tooth distance ( $\mu$ m)	TSR (strikes/s)	Syllable duration (ms)	Candidate fundamental frequency (kHz)	Global speed syllable duration (mm/s)	Instantaneous speed from frequency (mm/s)
<b>Male 1</b> Simple	0.861	42	24	48.78	0.4246	20.820	5652.38	L: 2.780 H: 2.575	L: 8.633 H: 9.320	L: 152.73 H: 164.89	L: 32.21 H: 45.46
Double	1.086	53 (106)	32 (64)	48.80 (97.60)	0.6488	8.319	9864.36	L: 6.992 H: 5.744	L: 9.153 H: 11.142	L: 92.79 H: 112.95	L: 49.33 H: 68.72
<b>Male 2</b> Simple	0.925	39	24	42.16	0.4173	21.325	5751.25	L: 2.454 H: 2.103	L: 9.799 H: 11.412	L: 170.04 H: 198.43	L: 31.66 H: 44.63
Double	1.021	55 (110)	32 (64)	53.86 (107.73)	0.6323	8.294	10121.77	L: 6.639 H: 5.942	L: 9.640 H: 10.770	L: 95.24 H: 106.41	L: 48.08 H: 66.98
<b>Simple SD</b>	<b>0.893</b>	<b>40.5</b>	<b>24</b>	<b>45.47</b>	<b>0.4209</b> $\pm 0.0051$	<b>21.072</b> $\pm 3.57$	<b>5701.81</b> $\pm 69.91$	L: 2.617 $\pm 0.23$ H: 2.339 $\pm 0.33$	L: 9.216 $\pm 0.824$ H: 10.366 $\pm 1.479$	L: 161.38 $\pm 12.24$ H: 181.66 $\pm 23.71$	L: 31.93 $\pm 4.62$ H: 45.04 $\pm 0.58$
<b>Double MEAN SD</b>	<b>1.053</b>	<b>54</b> <b>(108)</b>	<b>32</b> <b>(64)</b>	<b>51.33</b> <b>(102.56)</b>	<b>0.6405</b> $\pm 0.0116$	<b>8.306</b> $\pm 0.017$	<b>9993.06</b> $\pm 182.01$	L: 6.815 $\pm 0.249$ H: 5.843 $\pm 0.14$	L: 9.396 $\pm 0.344$ H: 10.956 $\pm 0.263$	L: 94.01 $\pm 1.73$ H: 109.68 $\pm 4.62$	L: 48.70 $\pm 0.88$ H: 67.85 $\pm 1.23$

*Sound production mechanism:*

*E. nigripedis* exhibits a similar bisinuated stridulatory file with two distinct file sections as in *E. guyanensis*. Our results suggest that there are two kinds of transitions in the call of this species: the first within the syllable, and the second between the high and low amplitude sections of the call. The constant frequency modulation combined with the amplitude modulation make this species exhibit unique acoustic features.

This species exhibits frequency modulation within the analyzed syllables due to two functional parts of the file. Thus, within each syllable there are high and low frequency components together, produced sequentially by the double teeth and the simple teeth during each FW closure. The high frequency syllable parts reach 12.9 kHz corresponding to the harmonic F4. Likewise, the low-frequency syllable parts reach the dominant frequency of 3.05 kHz corresponding to F1. At the level of the syllable, the species displays almost codominant F1 and F4 frequencies (Table 13).

Table 15. Sections *E. nigripedis* high & low amplitude sections.

Number of call	Count total syllables	High amp. # syllables	High amp. sections (ms)	"Couple" High amp. Period (ms)	Low amp. # syllables	Low amp. sections (ms)	"Couple" low period (ms)	Total time (ms)
1st	110	33	603.54	10.393 ±0.704	76	753.31	13.936 ±0.591	1356.85
2nd	100	29	598.83	10.505 ±0.891	71	706	13.580 ±0.759	1304.83
3rd	100	29	543.23	10.406 ±0.828	71	650.58	13.556 ±0.429	1254.12
<b>Mean</b>	<b>103.3</b>	<b>30.66</b>	<b>581.86</b>	<b>10.434</b>	<b>73</b>	<b>703.296</b>	<b>13.690</b>	<b>1305.26</b>
<b>SD</b>	<b>±5.547</b>	<b>±2.618</b>	<b>±33.543</b>	<b>±0.061</b>	<b>±3.928</b>	<b>±51.418</b>	<b>±0.212</b>	<b>±51.366</b>

## Discussion

We studied the calling song and sound production mechanism in all the species of the genus *Eneoptera*. We have analyzed and described the songs of the four valid species, based on new data, new acoustic recording and combining techniques of acoustic analyses and measurement of ultrastructures, in order to interpret or reinterpret the calling signals of this genus and discuss about its particular bioacoustical behaviours.

For *Eneoptera* male crickets the peripheral mechanism of sound production is similar as for other crickets for production of long-distance calling signals. It involves relatively slow muscular



contractions driven by the central nervous system and leading to peripheral structures to reach higher frequencies of vibration (Michelsen et al. 1994; Gerhardt and Huber 2002; Montealegre-Z et al. 2011). Thereupon, within *Eneoptera* the length of the stridulum and the number of file teeth are generally inversely correlated with syllable rate, thus calling songs differ among the four species in their dominant frequency, syllable rate, and amplitude modulation.

The males of all the species in the *Eneoptera* genus remain stationary while they perform their calling songs (Personal observation). For *E. surinamensis*, we were able to observe aggregations of two or more males in the field and the laboratory, with males calling in synchronization. These aggregations are also known as chorus, and were described as synchronized stridulation in different species of Gryllidae and Tettigoniidae. It was suggested that this behaviour is related to the ability to hear of these insects (Fulton 1928; Walker 1957a, 1957b). Recently studies have been made based on species of insects and anurans, suggesting that establishment of choruses occurs usually near where conditions are propitious to feed, refuge and breed. Competition tend to become intense among males that form choruses during seasonal time, these periods being favorable for courtship and mate in areas where females are concentrated, also as a strategy reducing predation of risks (Gerhardt and Huber 2002; Greenfield 2002, 2015; Greenfield et al. 2017).

There are numerous harmonics detectable over a close range (Latimer and Lewis 1986). By general rule the harmonic content is not acquired in most of acoustic records, probably due to limitations of equipment. The harmonic content found in *Eneoptera* demonstrate that these may play a role in terms of communication over several meters. In these crickets the song amplitude spectrum is almost a pure sinusoidal wave, amplitude modulated with two parts, composed by one low and one high amplitude section. For *E. guyanensis*, and *E. nigripedis* the frequency experiences changes in low frequency and the other in high frequency, and amplitude modulation,

due to the different movements of FWs closing–opening cycles, to reverberation, scattering, and environmental degradation of signal in both parts (Speaks 1996; Greenfield 2002).

As mentioned above, males tend to aggregate while calling, which generates a synchronized chorus which may, in turn, improve the attractiveness of females to this assemblage, and aid to reduce predators and parasites (Bailey et al. 2010). This kind of crowd exerts a considerable effect on how neighbors correspond to rhythm and time adjustments (Gerhardt and Huber 2002; Greenfield 2005, 2015). The cricket responses may help to thermoregulation, find resources, and enhance reproductive chances (Breed and Moore 2012). Aggregations of calling crickets became “acoustic leks”, these displays of active males interest females mainly for mating because the males exhibit on vegetation taking fixed positions on territory distribution, feeding patches, and better signaling spots effectively (Hoglund and Alatalo 1995). In these leks exploit information of other males take place; likewise females can make comparisons among the active males and decide based on acoustic feedback from higher-quality males (Kokko 1997).

There are multiple cases of sympatry in the *Eneoptera* genus, because in the populations we have observed intergeneric neighbours i.e. *E. gracilis* + *E. surinamensis*; *E. nigripedis* + *E. surinamensis*, and *E. guyanensis* + *E. surinamensis* in different parts of their distribution. The genus exhibits in general a continuous trill and the overlap of related harmonic frequencies when they share the same place, and how they deal with the scattering and diffraction effects. This could be explained based on the acoustic records and the features of the analyzed species. Because a more continuous and synchronous furthermore the powerful high-frequency harmonics in their calling songs could make that the sound behaves better across the surrounding vegetation, avoiding the signals dissipation (table 16).

Narrowly related, vegetation assemblies that are denser in the middle than at the edge, tend to accelerate the sound waves traveling faster throughout the midline, and a receiver bestride on

the sideways would perceive lengthened modulation envelopes. Intricate branching structures in vegetation, imposes several variations in perception either responses to bending sound waves, these alterations must to be explored in further studies (Greenfield 2002).

Table 16. Comparative amplitude levels (low and high) among the four species in *Eneoptera*, related to frequencies for each one.

<b>Species</b>	<b>Freq1 (kHz)</b>	<b>Ampl. 1 (dB)</b>	<b>Freq. 2 (kHz)</b>	<b>Ampl. 2 (dB)</b>	<b>Freq. 3 (kHz)</b>	<b>Ampl. 3 (dB)</b>	<b>Freq. 4 (kHz)</b>	<b>Ampl. 4 (dB)</b>	<b>Freq. 5 (kHz)</b>	<b>Ampl. 5 (dB)</b>	<b>Freq. 6 (kHz)</b>	<b>Ampl. 6 (dB)</b>
<b>suriname Low amp.</b>	DF2.945 ±0.035	-24.243 ±1.835	5.966 ±0.189	-51.511 ±2.351	8.797 ±0.181	-47.572 ±1.805	11.679 ±0.330	-51.777 ±2.064	14.386 ±0.625	-57.609 ±3.002	18.814 ±1.024	-63.842 ±2.802
<b>suriname High amp.</b>	DF2.951 ±0.099	-23.829 ±2.727	6.076 ±0.663	-49.334 ±3.810	8.816 ±0.430	-46.509 ±2.946	11.567 ±0.719	-47.851 ±3.865	14.046 ±0.866	-51.817 ±5.844	18.922 ±1.528	-62.461 ±4.456
<b>gracilis</b>	DF3.500 ±0.038	-44.206 ±0.302	7.299 ±0.014	-48.149 ±0.379	10.801 ±0.012	-49.602 ±0.289	14.357 ±0.334	-47.336 ±1.4180	16.132 ±0.406	-52.941 ±1.348	21.727 ±3.950	-60.916 ±6.183
<b>nigripedis High amp.</b>	3.058 ±0.047	-63.85 ±5.634	6.343 ±0.593	-75.47 ±6.669	9.662 ±0.631	-76.146 ±5.184	DF12.918 ±0.731	-64.145 ±4.557	15.679 ±0.555	-67.336 ±4.802	19.525 ±1.375	-78.693 ±7.357
<b>nigripedis Low amp.</b>	3.051 ±0.064.	-66.184 ±6.812	6.214 ±0.410	-77.128 ±7.769	9.491 ±0.548	-77.057 ±2.98	12.959 ±0.731	-66.452 ±5.628	15.764 ±0.690	-71.487 ±8.347	19.135 ±0.979	-79.859 ±8.083
<b>guyane Low frequency</b>	3.891 ±0.042	-64.788 ±1.460	7.598 ±0.015	-66.621 ±1.6206	DF11.800 ±0	-52.994 ±0.940	15.700 ±0.123	-51.677 ±3.775	19.918 ±0.460	-53.631 ±2.816	25.447 ±1.692	-70.978 ±2.984
<b>guyane High frequency</b>	DF20.207 ±0.750	-19.5763 ±5.651	24.129 ±0.414	-50.842 ±2.763	28.18.9 ±0.859	-61.547 ±2.568	32.371 ±0.652	-63.647 ±1.929	37.252 ±1.488	-61.533 ±4.470	41.565 ±0.985	-46.153 ±8.035

Amplitude modulation is explained by Greenfield (2002) shows for many cases how individuals through alterations in some of their fundamental features by increasing uninterruptedly a varying feature of a signal and incites a different receiver answer. This kind of behaviour are linked in sexual contexts. For example, a wooed female may exhibit different levels of acceptance

proportional with the amplitude or frequency of the male calling songs. Three of the species of *Eneoptera* produce a remarkable amplitude modulation (*E. gracilis* males have a slight modulation which is nearly imperceivable) (Table 16).

Among eneopterines the advertisement signals with powerful high-frequency harmonics, frequency modulation and amplitude modulation are remarkable features recently described and confirmed, through the acquisition of acoustic records in the field and laboratory as several hours of experimental analyses, observations, equipment advances, evolutive related to bioacoustic features and encouragement of all whose not just sight but also hear the “zoophonic” details in the web of life (Florence 1876; Shaw 1995; ter Hofstede et al. 2015; Desutter-Grandcolas et al. 2018; Dong et al. 2018; Benavides-Lopez et al. 2020; Robillard 2021; Tan et al. 2021).

Future studies must continue in order to bring new clues and forecast the calling songs acquiring a clear understanding of the acoustic diversity in crickets. Advances in technology has opened several possibilities to explore in detail aspects like movements of wings, ultrastructure associated, analyses of callings songs with more powerful equipment and software. Further research on acoustic fingerprints interrelated to the behaviour of each species is becoming indispensable to elucidate features and acquire a better understanding of these species in their acoustic niche. The present study becomes an entrance for further research to connect environmental conditions and their calling songs which allow us to have a better understanding of the acoustic behaviour of this genus and unravel the complex communication systems in the Eneopterinae subfamily and Orthoptera order.

## **Acknowledgements**

We thank the Alexander von Humboldt Institute (IAVH) from Colombia, for logistical assistance during field records and collections in Colombia. Specimens were collected under scientific agreement of collaboration between the MNHN from Paris and IAVH from Colombia No.16-253.

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# Chapter 2

## Testing the role of high amplitude harmonics in the calling song of Eneopterinae crickets (Orthoptera, Gryllidae)

Paper in preparation for the journal *Ethology*:

Benavides-Lopez J. L., Tan, M. K., Sperber, C., ter Hofstede H., Robillard T. Testing the role of high amplitude harmonics in the calling song of Eneopterinae crickets (Orthoptera, Gryllidae).

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### Introduction

Reproductive behaviours are essential to the evolutionary fitness of animals. In the animal kingdom, each species has its own sets of courtship rituals to attract a mate. Courtship behaviours evolve in response to selection from potential mates, eavesdroppers, and environmental factors. Males and females can find each other through diverse ways such as dancing, uttering calling songs, and gesturing to make themselves more detectable and more attractive to potential mates. These features are passed on from generation to generation through sexual selection (Endler 1993; Penn and Smith 2007; West 2009; Gadagkar 2011).

These features in animal courtship fit into two basic types: physical traits and behaviours. Physical traits are related to an animal's body, such as colors or accessory appendages; while behaviour is an answer of an organism to signals on its environment, such as a calling song, chemical response, or movements (Greenfield 2002; West 2009). Likewise, pheromones in Lepidoptera or sounds in Orthoptera, anurans, and birds are known to get the attention of couples

(Martin 1996; Gerhardt and Huber 2002; Ball and Ketterson 2008; Casas et al. 2009). Males and females need to find each other, by using different behaviours mainly use of communication signals to make themselves more attractive to potential mates. Even the use of a combination of physical features with mating calls for some birds (Marler and Slabbekoorn 2004; Moreno-Rueda 2017; Garcia et al. 2020).

For example, in anurans the evolutive and behavioural dynamics of signals are perceived reasonably well; this, implicate for both major shifts in signal features in the clade reflecting adaptations for improved signal efficacy as a potential result (Ryan 1983; Endler 1993; Gerhardt and Huber 2002; Page and Ryan 2008).

For couple formation in crickets, acoustic signals are usually broadcast by males and received by females which reply to these species-specific calling songs (Doolan and Pollack 1985; Greenfield 2002). Once the female is attracted to this call, this is considered positive phonotaxis, it has been widely shown since very early studies (Regen 1913; Walker 1957*b*; Huber et al. 1989; Otte 1992; Pollack 2000). Crickets songs are typically described as low-frequency with pure-tone signals (Sismondo 1979; Bennet-Clark 1989*b*; Bailey et al. 2001); although most contain low amplitude and high-frequency harmonics.

Since these harmonics are much less intense than the dominant frequency of the song, they are often considered unimportant to the behaviour of crickets. In *Teleogryllus oceanicus* for example, dominant frequency occurs around 4.5 kHz and harmonics carry very little energy but extend above background noise level up to at least 40 kHz (Hutchings and Lewis 1984; Balakrishnan and Pollack 1996).

The frequency spectrum of most cricket calls typically consists of a low fundamental or carrier frequency  $f_1$ , followed by a series of higher-frequency harmonics ( $f_2, f_3, f_4 \dots f_n$ ) being multiple integrals of  $f_1$  (Speaks 1996). In most crickets, the dominant frequency ( $f_d$ : carrying most energy) of the sound produced by this mechanism can transmit important information about the

identity and biological state of the emitter, although much information relies on the temporal features of the calls (Bertram and Rook 2011).

Recently, studies in the cricket subfamily Eneopterinae have documented higher frequencies for their calling songs (Robillard and Desutter-grandcolas 2004). In this subfamily, high-frequency calls are likely to represent a key evolutionary innovation promoting the evolution of a novel distinctive communication system in one tribe where the high amplitude harmonic frequencies have become dominant (ter Hofstede et al. 2015). Eneopterinae males (Gryllidae: Eneopterinae) are the only crickets known to generate calling songs with intense high amplitude in high-frequency harmonics. In one tribe, the Lebinthini, and a few species of the genus *Eneoptera* (Robillard 2021) one of the higher-frequency harmonics has repeatedly become the dominant frequency of the call, and this high-amplitude harmonic dominant frequency can even be ultrasonic (>20 kHz) in some species (ter Hofstede et al. 2015; Anso et al. 2016; Tan et al. 2021).

In the other tribes and species of this subfamily, males produce low-frequency songs, such as in species of the genera *Xenogryllus* or *Nisitrus*, in some species of the genus *Eneoptera*, but even in these species, the harmonics tend to carry greater energy relatively to the dominant peak than what is currently found in other cricket clades (Hung and Prestwich 2006). In some of these species, one of the high-amplitude harmonics is even sometimes almost codominant with the fundamental frequency (Robillard and Tan 2013; Jaiswara et al. 2019). These observations led to the hypothesis that having high-amplitude harmonics with enhanced energy may correspond to the ancestral condition of the spectrum of the call in these crickets, and the first step before the harmonics were used as the dominant frequency, as part of the novel system of communication found in the Lebinthinae species (ter Hofstede et al. 2015; Benavides-Lopez et al. 2020).

The relevance of high-amplitude harmonics content in the calling song to determine orientation in crickets was discussed based on simple biophysical considerations, by considering

that it is evident that a large number of ultrasonic harmonics provide much more directional information than a single pure tone at the fundamental frequency (Hutchings and Lewis 1984; Latimer and Lewis 1986).

In this paper, we investigate the role of the high-amplitude harmonics in a low-frequency eneopterine species, *Nisitrus malaya* (Robillard & Tan, 2021) by exposing individuals to three different treatments of synthetic calls with different harmonic composition. We also address the question of why did high-amplitude harmonics evolve in the Eneopterinae?

To understand why these powerful high-amplitude harmonics are present and can become codominant in some species, and dominant in a large part of the subfamily, we will investigate whether these harmonics are useful, necessary, or accessory for eliciting female phonotaxis. Similarly, we will investigate their possible role in males' interactions.

The first possible role of the high-amplitude harmonics is to help female orientation while approaching the male through positive phonotaxis. Given that eneopterines tend to live in the more complex environment of plants rather than the ground, where field crickets live, and that dense vegetation attenuates more high frequencies than low frequencies (Gerhardt and Huber 2002; Rajaraman et al. 2015; ter Hofstede et al. 2015; Benavides-Lopez et al. 2020), the harmonic contents may firstly be used by the female as a cue to find the male more efficiently, which could be achieved either by accelerating the phonotaxis and by making it more accurate in terms of male localization or both.

Males could also use the high-amplitude harmonic content of their calling songs to space themselves in the environment. Males could be attracted by each other phonotactically to some degree but would limit their approach until they start perceiving the harmonics of the neighbor males. Under this hypothesis, these high-amplitude harmonics could be necessary for eneopterines owing to the vegetation that attenuates these frequencies. Within grylline low

amplitude harmonics, the female could probably not perceive any trace of harmonics until getting very close to the calling male.

Even though the positive phonotaxis of females *N. malaya* was experimentally demonstrated by tests made in a previous study (ter Hofstede et al. 2015), which allowed contrasting this species with the lebinthine species that have lost phonotaxis, this behaviour needs to be analyzed more in detail to elucidate the role of high-amplitude harmonics. In this study, we have used playback experiments carried out in the laboratory, and video monitoring of the cricket behaviour to investigate this behaviour. We aim at answering two main questions: 1. Are there positive phonotaxis and are phonotaxis different between sexes? 2. For crickets showing positive phonotaxis, are there differences between sex and treatment?

Depending on the responses to these two main questions, we will discuss the possible roles of the high-amplitude harmonics in the call of *N. malaya*, and more generally in the calls of most non-lebinthine Eneopterinae.

## **Material and Methods**

### *Insects*

Specimens of *N. malaya* were collected as eggs in Singapore in 2014 and reared under controlled conditions at the insectarium of the Muséum national d'Histoire Naturelle (MNHN) in Paris. Crickets were fed ad libitum with dog food, a common ivy plant, and water, and they were maintained under controlled conditions of temperature and humidity ( $22.5 \pm 2^\circ\text{C}$ ;  $30 \pm 15\%$  humidity). The crickets used in experiments were nymphs of generations F4 and F5 that were separated as pre-adults into individual boxes, noting the date of separation to calculate their age as adults. A total of 59 individuals were used for the experiment.



### Acoustic stimuli

We used the features of the natural calling song of the species *N. malaya* from a previous study (Robillard and Tan 2013) to generate synthetic calls for playback experiments. The calling song of *N. malaya* contains echemes that are 33 ms in duration and have a period of 51 ms (figure 1).

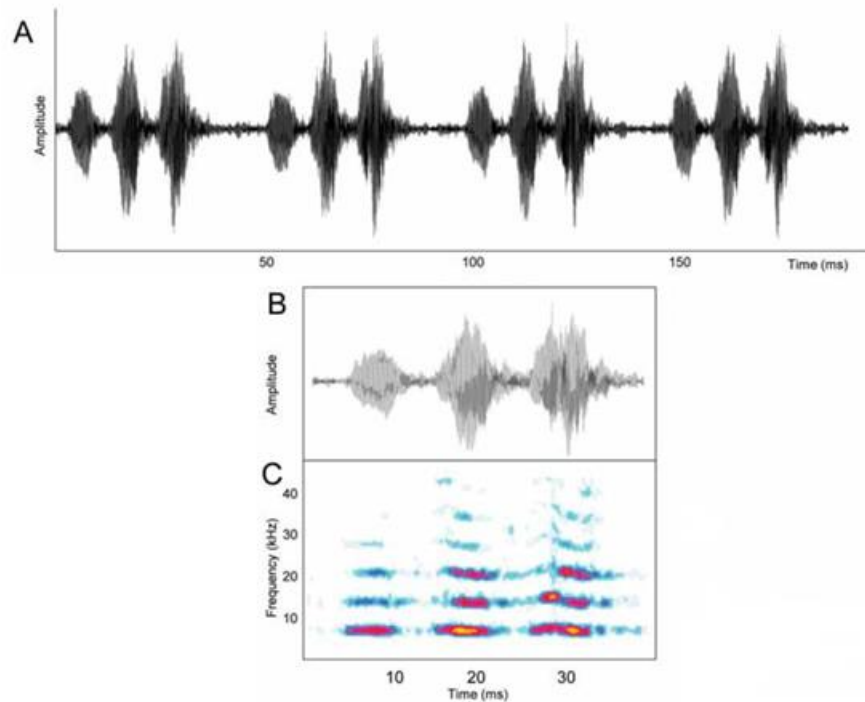


Figure 1. Calling song of *Nisitrus malaya*. Oscillogram of 4 echemes (A); oscillogram (B) and sonogram (C) of 1 echeme; linear power spectrum of 1 syllable. (From Robillard & Tan, 2013, used with permission)

The basic cricket song terminology follows (Ragge and Reynolds 1998). Echemes are the repeated subunit of a song. Each echeme has three syllables, and each syllable has a duration of  $6.9 \pm 1.4$ ms. The echemes are repeated to produce a continuous trill, with a fundamental frequency of 7.3 kHz. The frequency spectrum of the natural call shows significant energy in several harmonic peaks, while the fundamental frequency is slightly dominant (figure 1).

Three synthetic calls were generated using Avisoft-SAS Lab Pro software version 5.2 (Specht 2008). Using the mean values of the features of the natural call (given above), we created three synthetic calls repeated at the natural period for the playback experiments. The three

synthetic calls only differ in their frequency spectrum: the first synthetic call (F1) had no harmonics beyond the fundamental frequency, the second synthetic call (F2) had one harmonic, and the third synthetic call (F3) had two harmonics in addition to the fundamental frequency (Figure 2). The echemes in each playback treatment were ca. 51 ms (F1: 54.5 ms, F2: 51.2 ms, F3: 53.6 ms).

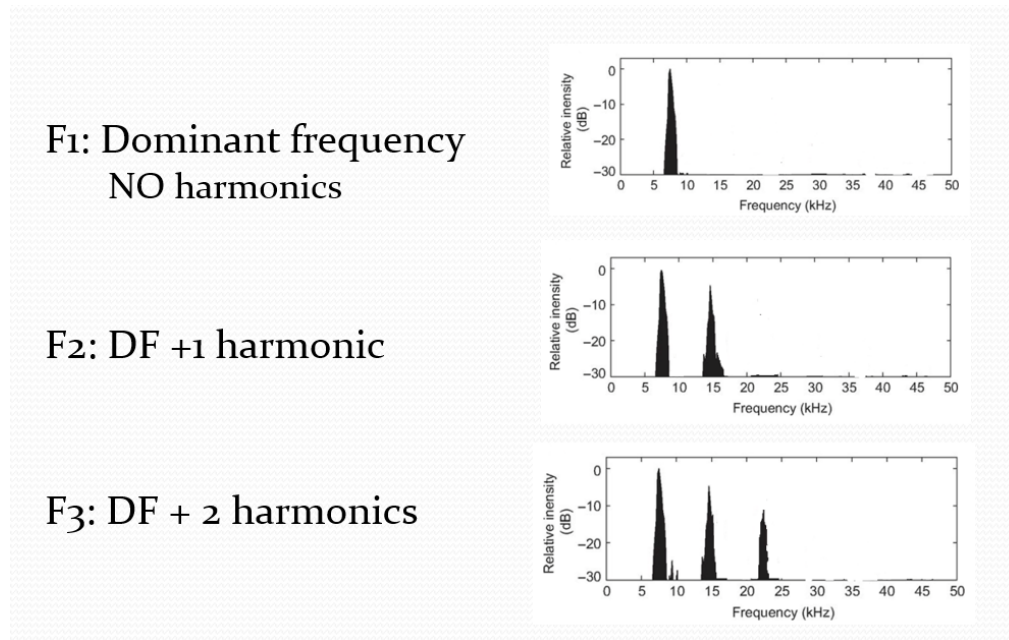


Figure 2. Graphical representation of synthetic calls with harmonic content.

### *Experimental protocol*

We conducted playback experiments in a room lined with sound attenuating foam. The temperature in the room was maintained at  $23.5 \pm 1^\circ\text{C}$  and the relative humidity at  $40 \pm 15\%$  during experiments. The arena used for playback experiments measured 1.54 m x 0.65 m and was covered with white Kraft paper (figure 3). The paper was changed for each new treatment to remove potential chemical cues. Acoustic stimuli were broadcast in the arena using an ultrasound gate Avisoft USG216H and two Avisoft ScanSpeak Ultrasound Speakers through the Avisoft

software (Specht 2008). The speakers were placed at either end of the arena equidistant to the center (distance from center to speaker= 67cm) and were pointed toward the center of the arena.

For each playback treatment (F1, F2, or F3), the cricket was placed under a plastic (in 2018) or glass (in 2019) container in the center of the arena.

The cricket was left undisturbed for five minutes in darkness to allow the cricket to acclimatize. The container was previously cleaned with alcohol to avoid biases from potential chemical cues from previous tests. After five minutes, we turned on the lights, started the acoustic playback, and lifted the container, allowing the insect to walk freely on top of the arena. Each playback trial lasted until the cricket reached the speaker, the cricket left the arena, or ten minutes had elapsed. Each trial was video recorded with a webcam (Logitech HD C525) from above the arena. The sound pressure level (SPL) of the playback experiments was calibrated at 75 cm of distance, using a 971 Svantek sonometer, to maintain approximately 70 dB at the center of the arena (except for silence).

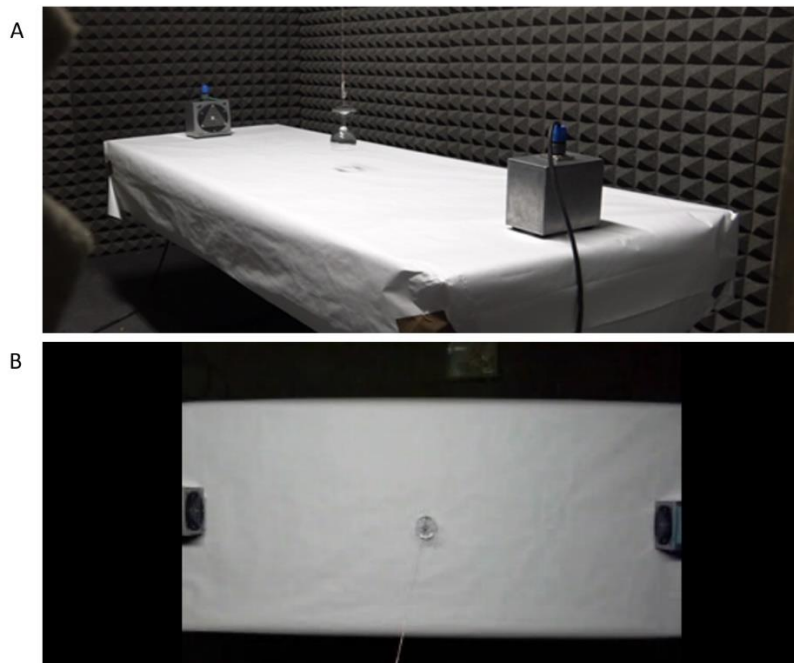


Figure 3. Arena and equipment placement in acoustic room A: lateral view; B: view from above.

One treatment (F1, F2, or F3) was played to a given individual per day, with a pause of one day between trials to avoid the effects of habituation. We also randomly changed the side of the active speaker for each experiment to avoid potential side biases; the active speaker broadcasts acoustic stimuli whereas the other speaker was silent. Because this species is diurnal and has good vision, we left both speakers in place at all times so that the visual stimuli were the same on both sides for all trials.

### *Video analysis*

Video analysis was conducted using EthoVision XT version 10 video analysis software (Noldus Information Technology Inc.). Ethovision software automatically tracks the movement of objects in videos and provides the 2-dimensional coordinates for the object in each frame of a video. The cricket was automatically detected and tracked by the software using the differencing function, which compares each frame of video to a still image from the video in which the cricket is absent. The position of the center of the speaker relative to the cricket in each video was identified using a frame of the video and comparing it to a frame image in which the center of the speaker is covered with a white circle. Distance measurements were calibrated in each video by providing the software with the known length of the speaker.

Analysis began when the cricket was released from the container in the center of the arena. For each frame of the video, Ethovision provided the position of the cricket (X-Y coordinates, mm), the time since the start of analysis (s), the distance between the cricket and the center of the active speaker (mm), and the distance moved by the cricket between frames (mm). Videos in which the cricket did not leave the container or immediately jumped away from the arena were not analyzed.

### *Statistical analyses*

To test whether males and females showed positive phonotaxis (moved closer to the speaker), data for all three acoustic treatments were combined and two separate linear mixed-effects models (LMMs) were fitted with (1) minimum euclidean distance to the speaker and (2) final euclidean distance to the speaker as response variables. The sex of the cricket was fitted as a fixed effect and cricket identity (ID) nested within the year of the experiment as a random effect. Cricket ID was used as a random intercept because each cricket was subjected to more than a single trial. The year of the experiment was also used as a random intercept because different generations of crickets were used for the experiments and there were minor differences in the experimental design; the plastic container was changed for a glass container to avoid any kind of mark and to be able to clean the container more effectively after each trial. Minimum distances less than 100 mm were set to 100 mm because the resolution of Ethovision did not allow for more accurate measurements.

To investigate the effects of the different acoustic treatments (F1, F2, and F3) on phonotaxis behaviour, four separate LMMs were fitted with (1) minimum euclidean distance to speaker, (2) time to reach minimum distance and, (3) speed to the speaker as response variables. For these tests, we only included individuals that demonstrated positive phonotaxis to assess whether additional harmonics improved signal efficacy. Positive phonotaxis was defined as crickets that achieved a minimum or final distance within 375 mm of the speaker (halfway between the center of the arena and front of the speaker). For all models, the interaction between the acoustic treatment and the sex of the cricket was fitted as a fixed effect and cricket ID was nested within the year of the experiment as a random effect.

All analyses were run in R (R Core Team 2018) and LMMs were fitted using the “lmer” function from the R package “lme4” (Bates et al. 2014) using Restricted Maximum Likelihood estimation (REML). The significance of model parameters was assessed either by comparing a

model to a model with only the intercept using likelihood ratio tests (lrtest function from lmerTest package; figure 4A) or using estimated marginal means (emmeans function from the emmeans package; figure 4B). For each model, values for the marginal R squared ( $R^2_m$ ; for fixed effects) and the conditional R squared ( $R^2_c$ ; for both fixed and random effects) are reported.

## Results and Discussion

We tested 29 female and 30 male crickets in two series of experiments carried out in 2018 and 2019. Sample sizes by year and acoustic treatment are provided in Table 1.

Table 1. Experiment sample sizes by treatment and year in *N. malaya*

Acoustic Treatment	Year	Females	Males	Trials
F1	2018	15	6	21
	2019	5	9	14
	Total F1:	20	15	35
F2	2018	14	5	19
	2019	8	15	23
	Total F2:	22	20	42
F3	2018	16	4	20
	2019	3	10	13
	Total F3:	19	14	33
	Trials:	61	49	110

**Question 1: Does *Nisitrus malaya* show positive phonotaxis, and if so, does phonotaxis differ between males and females?**

*Minimum euclidean distance to the speaker*

This variable measures the minimum distance between the cricket and the active speaker during each trial, independent of the final position of the cricket during the trial (for example, a cricket

may come very close to the speaker then walk away). When data for acoustic treatments (F1-F2-F3) were pooled, the median values for the minimum euclidean distance to the speaker (figure 4A) suggest that female (100.0 mm) individuals usually showed positive phonotactic responses, whereas male individuals usually did not move closer than halfway to the active speaker (360.4 mm). Sixteen of the 29 female crickets walked directly onto the active speaker during at least one trial, whereas only 6 of the male crickets did so during a trial. There was a significant difference between models when sex was included or excluded as a fixed effect (Likelihood ratio test,  $\chi^2 = 14.2$ ,  $P < 0.001$ )

For both sexes, some individuals did not clearly respond to the playback sound and some just remained in the initial position, but the overall results support positive phonotaxis independent of the number of harmonics in the sound stimulus for female crickets. Females usually moved closer to the active speaker than males (coefficient for the treatment variable “sex” in the full model is not zero: = 123.8, 95% CI of 14.2 - 249.5), and their responses were also more consistent than that of males, as shown by the smaller difference in quartiles in figure 4A. There was a larger variance in males’ than females’ responses, with most males having a minimum distance just greater than halfway to the active speaker. Interestingly, however, this median value suggests that males are usually moving closer to the sound source, rather than away from it. In fact, 6 of the 30 male crickets tested walked directly onto the active speaker.

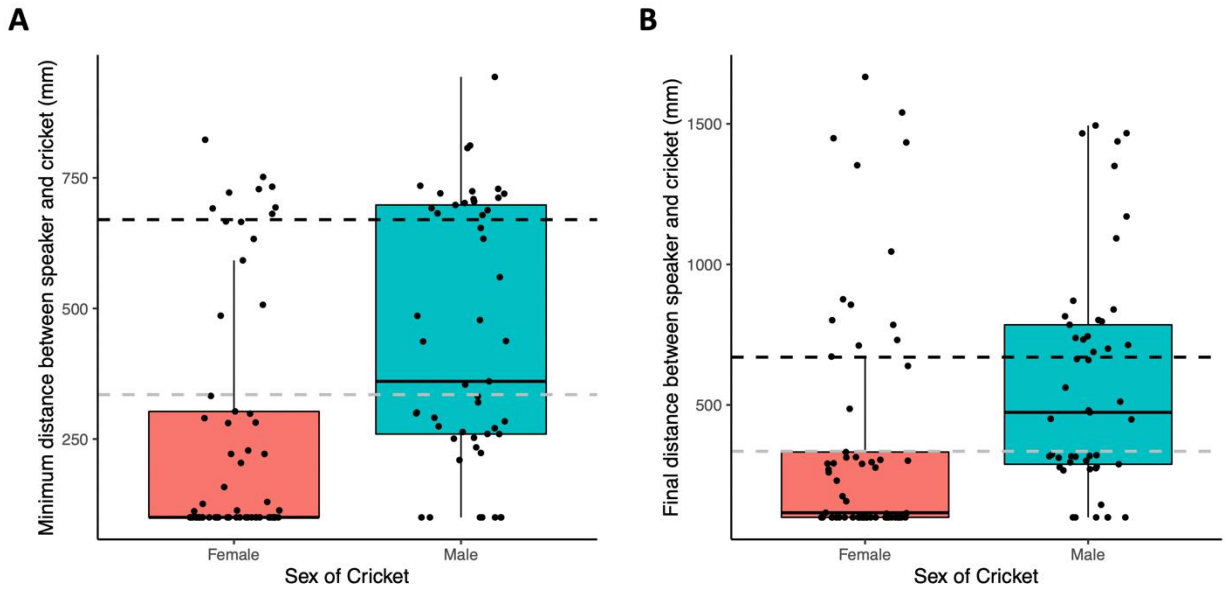
These results suggest that individuals of both sexes interact acoustically to a certain degree, and are likely to be attracted by a calling male. Positive female phonotaxis in *N. malaya* is confirmed, independent of the treatment, which is consistent with previous results (ter Hofstede et al. 2015) and expectations. It confirms that non-lebinthine Eneopterinae use phonotaxis as in the common system of communication for mate finding in crickets.

The fact that males might be attracted to the male song was less expected, but this is not unknown from the literature on other crickets (Bailey and Simmons 1991). In this case, that the

median minimum distance is close to half the distance to the speaker suggests that the male's phonotaxis is not equivalent to the female phonotaxis. Few males walked all the way to the speaker, but many walked towards it. It is thus likely that a male hearing another calling male in the field will approach him, but will not approach as to enter in close contact with the calling male. This could be a result of competition between males, similar to the cases of satellite males approaching a calling male in the species *Gryllus integer*, *Bufo calamita*, and *Pseudacris crucifer* (Blum and Blum 1979; Arak 1988; Bailey 1991; Lance and Wells 1993) to intercept females attracted by the calling male. This mating strategy could be used by *N. malaya*. Note that males of *N. malaya* never started singing during the assays, contrary to what occurred in playback experiments in Lebinthini species, for example (Benavides-Lopez et al. 2020).

It is possible that males might indeed start calling in response to the playback after a longer period of time than the experiment. Further studies, experiments, and field observations are needed to understand if males remain silent when another one is calling in passive-listener attitude, or if males use acoustic information to space themselves in their habitat (Blum and Blum 1979; Bailey 1991; Greenfield 2002).





**Figure 4. Phonotactic behaviour of *Nisitrus malaya*** (A) Minimum distance to speaker attained by a female (orange) and male (blue) crickets during playback experiments;  $R^2m= 0.05$ , and  $R^2c= 0.58$ . (B). Final euclidean distance to speaker attained by crickets during playback;  $R^2m= [1,] 0.03$ ;  $R^2c= 0.45$ . Each trial is represented by a black dot. In ordinates, 0 mm represents the center of the active speaker, and 100 mm the position directly in front of the speaker, corresponding to the position where “reached speaker” is checked; 670 mm is the initial position of the crickets during trials (black horizontal dashed lines). Color box margins indicate the 25th and 75th percentiles; the horizontal line inside the box represents the median; the ends of the vertical lines indicate the minimum and maximum. Points beyond the lines can be considered as outliers. The grey horizontal dotted line at 335 mm represents half the distance between the initial position and the active speaker. Individuals showing the minimum distance to the speaker beyond 670 mm performed negative phonotaxis, while individuals moving within 335 mm of the speaker performed positive phonotaxis.

#### *Final euclidean distance to the speaker*

The results are similar when we compare the final distance to the active speaker (Figure 4B), which represents the position of the cricket at the end of the experiment, independently of how close the individual approached the speaker or explored the arena before this. This distance covers three different situations ending the experiments: (1) the cricket reached the active speaker, (2) the cricket left the arena (in this case the last position on the arena is the final distance), or (3) the cricket was somewhere in the arena at the end of the trial (10 minutes).

With this variable, the individual results are scattered along the length of the arena, from one end to the other. Although the final distance to the active speaker was not drastically different

between the males and females (estimate = 147, 95% CI of -29, 367), females usually ended the experiment close to the speaker (median final distance to the speaker = 117 mm), with a large proportion of female individuals reaching the speaker. In contrast, the male's median final distance to the speaker (i.e., 474 mm) is less than halfway to it, although the 25<sup>th</sup> percentile is more than halfway to the speaker. There was a significant difference between models when sex was included or excluded as a fixed effect (Likelihood ratio test,  $\chi^2 = 13.2$ ,  $P < 0.001$ ). That final euclidean distance does not give the same results as minimum euclidean distance suggests that crickets may arrive close to the speaker but leave the area when they do not encounter another cricket.

***Question 2: For crickets showing positive phonotaxis, are there differences between sexes and treatment?***

*Minimum distance to the speaker*

In the second set of analyses, we focused on the crickets that exhibit positive phonotaxis to look for differences between sexes and treatments. We selected a subset of data including only those crickets with a minimum distance of 335 mm or less to the active speaker (figure 5). It is aimed at comparing only individuals that are “motivated” to perform phonotaxis.

When looking at the minimum distance reached from the speaker, the  $R^2$  values for the final model indicates that neither sex nor treatment had a strong influence on the minimum distance reached from the speaker ( $R^2_m = 0.20$ ,  $R^2_c = 0.30$ ). Pairwise comparisons of contrasts for estimated marginal means only show two significant differences: 1) between males and females for the F2 treatment ( $P = 0.026$ ), and 2) between the male F2 and the female F3 treatments ( $P = 0.005$ ). The median values and 25-75th percentiles, however, show some interesting trends that are worth discussing. For females, the median value was 100 mm (reached the speaker) for all

three acoustic treatments. The variability of the responses (shown by the 25-75th quartile boxes), however, greatly differ among the treatments for females, with F1 having the greatest variability, F2 intermediate variability, and F3 the least. This result suggests that among females performing phonotaxis, those hearing only the low-frequency peak (F1) will tend to reach the speaker less often than the females hearing the two-harmonic call (F2), and those hearing three harmonics (F3).

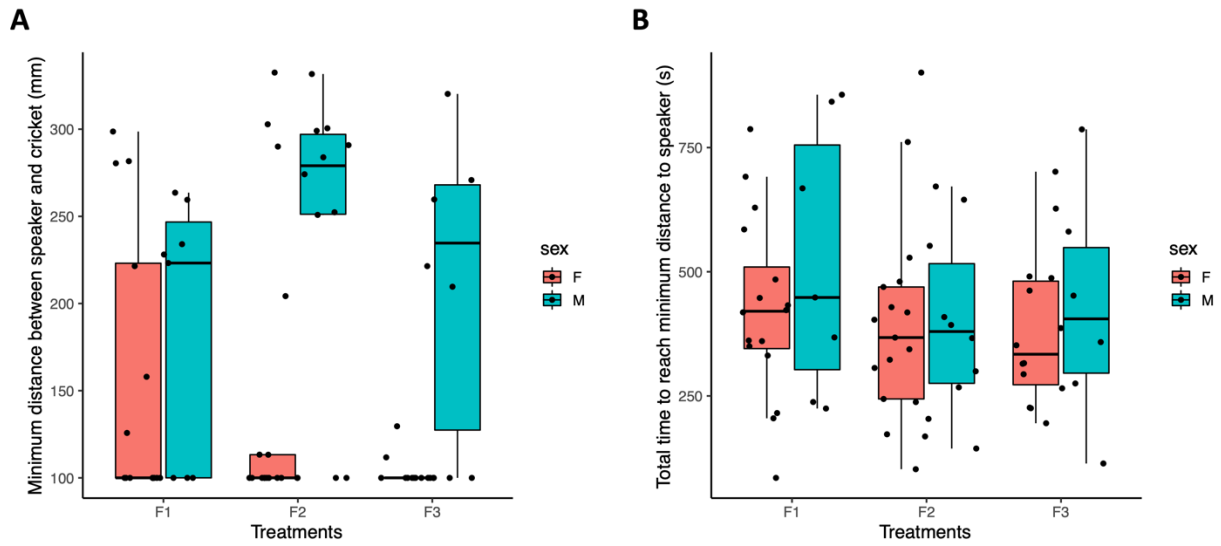
It may suggest that, even though higher harmonics are not necessary for the female to reach the speaker during the experiments, harmonics may help them anyway. In more natural conditions than the very simple experimental ones, where no obstacle was placed between the speaker and the subjects, harmonics may play a more crucial role in the phonotaxis, not by motivating the female to walk toward the source of the call, but by helping her find it. More elaborate experiments will need to be performed to test this hypothesis, by placing different kinds of obstacles on the arena, to test if significant results arise between treatments in situations more similar to natural ones.

This trend is not found for males, for which the treatments F1 and F3 are very similar, both in terms of the median (F1: 223 mm, F3: 234 mm) and the large variability. Treatment F2, however, has a higher median (279 mm) and lower variability than the other two treatments. The three treatments suggest that, unlike females, males usually do not walk all the way to the sound source (although 2 or 3 males did reach the speaker in each treatment). The difference between F1 / F3 vs F2 is difficult to interpret. It may be linked with what the different harmonics represent for the male-male acoustic interactions related to the estimation of the distance of the neighboring males: hearing only one harmonic may represent a situation where the calling male is relatively far (its high harmonics being “filtered” by the distance and the vegetation). Alternatively, the seemingly different response to the F2 treatment could be a simple size issue because the number of males that performed positive phonotaxis was much less than for females.

The comparison of males and females for each treatment suggests that they have different responses to the three treatments, in particular when comparing the 25-75% percentile interval. For treatment F1, the range of male and female results are similar, despite the much higher median value for males. For treatment F2, the results are significantly different, with most males remaining at ca. 280 mm from the speaker, while females reach it (100 mm). For treatment F3, while the median values are not significantly different among sexes, the 25-75% are strikingly different, suggesting differences in the individuals' behaviours, the females consistently reaching in front of the speaker (25-75% interval is negligible), while males show highly variable responses (25-75% interval spanning ca. 200 mm).

#### *Time to reach a minimum distance*

This metric assesses if the previous results are linked with differences in how quickly crickets respond to the acoustic treatments. The same minimum distance may be reached among sexes and treatments, but with very different timing. For example, females that are motivated to mate might reach a similar minimum distance much faster than males. The final model, however, did not find any significant differences between any of the treatments and sexes for the time taken to reach the minimum distance (all contrasts with  $P > 0.05$ ). Therefore, there does not appear to be a relationship between how likely a cricket is to reach the sound source and how much time the cricket takes to get there.



**Figure 5. The behaviour of female (orange) and male (blue) crickets in response to different acoustic treatments. (A)** Minimum distance to speaker attained by individual performing phonotaxis; coefficient =  $R^2m= 0.20$ ;  $R^2c= 0.30$ . **(B)** Time to reach minimum distance; coefficients:  $R2m=0.05$ ,  $R2c= 0.51$ . Color box margins indicate the 25th and 75th percentiles; the horizontal line inside the box represents the median; the ends of the vertical lines indicate the minimum and maximum. Points beyond the lines can be considered as outliers.

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# Chapter 3

## Novel system of communication in crickets originated at the same time as bat echolocation and includes male-male multimodal communication

Paper published in the journal *The Science of Nature*:




Benavides-Lopez J. L., ter Hofstede H., Robillard T. (2020). Novel system of communication in crickets originated at the same time as bat echolocation and includes male-male multimodal communication. *The Science of Nature*, DOI: 10.1007/s00114-020-1666-1.

The Science of Nature (2020) 107:9  
<https://doi.org/10.1007/s00114-020-1666-1>

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ORIGINAL PAPER

## Novel system of communication in crickets originated at the same time as bat echolocation and includes male-male multimodal communication

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### Abstract

Understanding the evolutionary origins of communication signals requires careful study of multiple species within a known phylogenetic framework. Most cricket species produce low-frequency calls for mate attraction, whereas they startle to high-frequency sounds similar to bat echolocation. Male crickets in the tribe Lebinthini produce high-frequency calls, to which females reply with vibrational signals. This novel communication system likely evolved by male sensory exploitation of acoustic startle to high-frequency sounds in females. This behaviour was previously described for the Lebinthini from Asia. Here we demonstrate that this novel communication system is found in a Neotropical species, *Ponca hebardi*, and is therefore likely shared by the whole tribe

Lebinthini, dating the origin of this behaviour to coincide with the origin of echolocation in bats. Furthermore, we document male duets involving both acoustic and vibratory signals not previously described in crickets, and we tentatively interpret it as competitive masking between males.

**Keywords:** Orthoptera, Predation; High-frequency calls; Vibrational signals; Multimodal duets

## **Introduction**

How and why new communication signals evolve are enduring questions in evolutionary biology (Searcy and Nowicki 2005; Bradbury and Vehrencamp 2011). To understand the evolutionary origins of a communication system, studies must test hypotheses with experiments that integrate detailed observations of behaviour across multiple species in a phylogenetic framework (Shaw 1995). Evidence from diverse taxa have shown that new mate advertisement signals can arise when novel signals tap into a pre-existing perceptual bias of females (Ryan and Cummings 2013), a process referred to as sensory exploitation. In most cases, males produce signals that are similar to environmental cues that attract females, such as food or shelter (Fleishman 1992; Proctor 1992; Rodd et al. 2002; Christy et al. 2003). Mate attraction signals that resemble predator cues are rare, likely because typical responses to predator cues involve freezing or fleeing and not movement towards the cue. Interestingly, the two taxa in which sensory exploitation of predator cues for mating has been documented are both insects in which males produce high-frequency sounds in the range of bat echolocation calls (moths (Nakano et al. 2013); crickets (ter Hofstede et al. 2015)). A key assumption of these studies is that the signal evolved after the evolution of echolocation in bats. Here we compare new behavioural data with dating from previously published phylogenies to support the hypothesis that the timing of high-frequency calls in the cricket tribe Lebinthini coincided with the timing of the origin of echolocation in bats.

Convergence in timing would suggest a rapid change in communication at the same time as the emergence of a new and dangerous predator.

Males of most cricket species in the family Gryllidae produce low-frequency (3-8 kHz) calling songs as intraspecific communication signals for mate attraction (Bennet-Clark 1989). Female crickets find males by following the sound signal (positive phonotaxis). Alternatively, when crickets hear high-frequency sounds in the range typically produced by bats for echolocation, they show stereotyped anti-predator behaviours, including negative phonotaxis in flight (Wytenbach et al. 1996) and acoustic startle responses (running or jerking the body) when perched on a surface (ter Hofstede et al. 2015). A recent study (ter Hofstede et al. 2015) revealed that an alternative communication system evolved in crickets of the Lebinthini tribe (Eneopterinae), involving major changes in every component of the usual cricket system of communication: males produce high-frequency signals (10-28 kHz) (Robillard and Desutter-Grandcolas 2004a); females lack phonotaxis and instead produce vibrational signals in response to male calls; and males locate females via vibrotaxis. The vibrational signal produced by lebinthine females in response to the male's high-frequency call is remarkably similar in latency and structure to the vibrations produced by acoustic startle responses observed in closely related crickets when they hear high-frequency sounds typical of bat calls (ter Hofstede et al. 2015). Therefore, it is hypothesized that the lebinthine communication system evolved through male sensory exploitation of the acoustic startle response to high frequency sounds in females (ter Hofstede et al. 2015).

The lebinthine communication system was previously described for three species of different genera from Asia and islands from the Pacific region (Fig. 1). The second major branch of the Lebinthini, however, is found in the Neotropics (Fig. 1) (Vicente et al. 2017), but communication has not been studied in these species. If species in the neotropical clade demonstrate the same acoustic-vibrational duet as seen in the Asian-Pacific species, it would support the hypothesis that this novel communication system evolved in the ancestor of the entire

tribe. The tribe Lebinthini is estimated to have diverged ~55 million years ago (Ma) (95% highest posterior density: 44.43–69.53 Ma (Vicente et al. 2017), which coincides with the time estimated for the origin of an echolocating ancestor in bats ~58 Ma (Shi and Rabosky 2015; Thiagavel et al. 2018). Here, we test whether the species *Ponca hebardi* Robillard, 2005 from the Neotropical Lebinthini lineage (Fig. 1) demonstrates the alternative acoustic-vibrational communication system found in species from the Asian-Pacific clade of the Lebinthini. We recorded the calling song of this species for the first time and used the recorded calls for playback experiments to test the behavioural responses of both sexes.

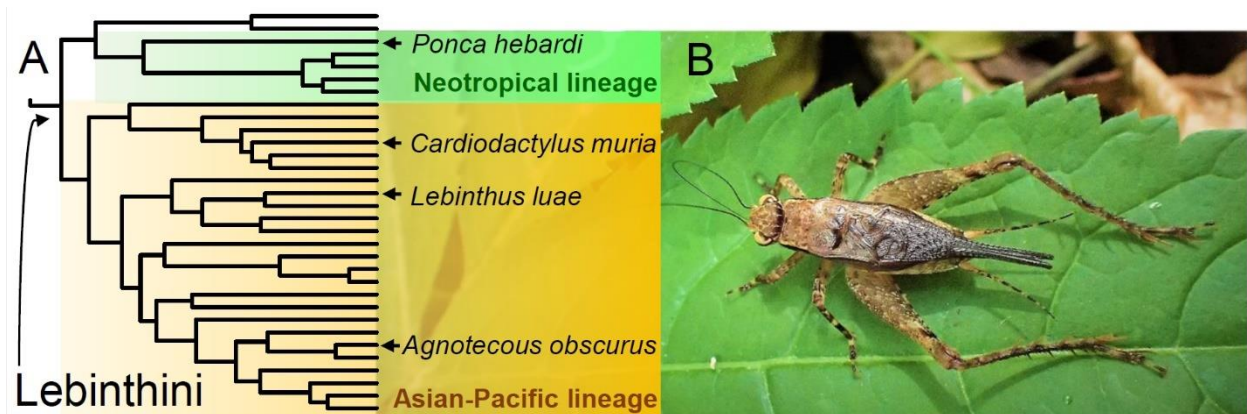


Figure 1. Evolutionary relationships within the cricket tribe Lebinthini. (A) Phylogenetic tree of the Lebinthini inferred through Bayesian and maximum likelihood approaches based on four mitochondrial and three nuclear gene sequences (modified from Vicente et al. (2017), with arrows pointing to the phylogenetic positions of *Ponca hebardi* and the Asian-Pacific lebinthine species previously analyzed by ter Hofstede et al. (2015). (B) Photo of male *P. hebardi* on vegetation.

## Methods

### Study animals

*Ponca hebardi* is a nocturnal eneopterine cricket species (figure 1) previously known by only two type specimens collected in 1954 on Barro Colorado Island, Panama (Robillard and Desutter-Grandcolas 2005). We collected juveniles in the same locality in March 2017 and maintained them in the laboratory. Crickets were separated by sex before final moult and were tested in playback experiments two to four weeks after final moult. All male call recordings and playback tests were conducted in a room lined with sound attenuating foam at controlled temperature and humidity



conditions (temperature:  $23.5 \pm 1^\circ\text{C}$ , humidity:  $60 \pm 15\%$ ). Specimens were deposited in the Muséum national d'Histoire naturelle (MNHN-EO-ENSIF4124-4127) and recorded .wav files were deposited in the sound library of MNHN under accession numbers MNHN-SO-2019-87 to MNHN-SO-2019-90 (<https://sonotheque.mnhn.fr/>).

### **Male recordings and acoustic analysis**

Call recordings were obtained from four males with a modified condenser microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) with a frequency range of 3-150 kHz  $\pm$  6 dB (R. Specht, pers. comm.). Each male was put individually in a suspended textile net cage overnight with the lights off and the microphone suspended 30 cm above the cage. Sound-triggered recordings were made using Avisoft Recorder software version 2.97 (Specht 2008) and an 8-Pro MOTU sound card at a sampling rate of 96 k-samples per second (16 bit). To generate audio files with accurate power spectra, we applied a user-defined finite impulse response (FIR) filter in Avisoft-SASLab Pro version 4.40 that corrected for the microphone frequency response. Temporal and spectral song features were measured using the automatic parameter measurements feature in Avisoft-SASLab Pro (FFT length 256, rectangle window, 50% overlap). We measured syllable duration, syllable period (time from start of one syllable to start of next) and dominant frequency (frequency with maximum energy, kHz).

### **Playback experiments**

The responses of female and male *P. hebardii* to male calls were tested within a dark arena (1.54 m x 0.65 m). Playback experiments were conducted using an UltraSoundGate Player 216H with Avisoft recorder USGH software and Avisoft Ultrasonic Dynamic Speaker Vifa. We selected five calls from one male recording with acoustic parameters similar to the mean values of the four recorded males. These calls were broadcast at an amplitude of 65 dB at the cricket, matching the natural amplitude of the call at 80 cm, measured with a SVAN971/Svantek sonometer. Playback



experiments were monitored using a SONY Handycam HDR-HC3 video camera using the night-shot vision function.

Six unmated females were tested for behavioural responses to male calls. In a first set of experiments, phonotactic response was assessed with free moving individuals in an arena. The floor of the arena was covered by white filter paper that was changed after each experiment to remove any odor cues left by previously tested individuals. We placed each cricket in the middle of the arena, broadcast male calls for 10 minutes and observed whether the female walked towards the speaker (positive phonotaxis). In a second set of experiments, females were placed on a foam base covered by a layer of filter paper and covered by a nylon mesh cage. For two of the six females, a custom accelerometer was placed underneath the filter paper to record vibrational signals. Using a microphone pointed at the speaker and the accelerometer below the female, we simultaneously recorded the male call playback and the female vibrational responses on two channels using Avisoft Triggering Hard-disk Recorder. From these recordings, we measured the dominant frequency (kHz), duration (ms) and time delay after the male call (ms) of the female vibrational signals using Avisoft-SASLab Pro.

In a third set of experiments, two unmated males were tested for behavioural responses to male calls. Males were placed in the middle of the arena and covered by a nylon mesh cage. A microphone was pointed at the cricket to record both the calls of the focal cricket and the playbacks from the speaker behind the male. Due to the microphone orientation, the focal male's calls and the broadcast calls differed in amplitude on the oscillogram. From these recordings, we measured the dominant frequency (kHz), duration (ms) and time delay after the playback (ms) of the male calls using Avisoft-SASLab Pro. Videos monitoring playback experiments were analyzed frame by frame to document the timing of the vibration behaviour of the focal male.

## Results and Discussion

The call of *P. hebardii* consists of a single syllable with a mean duration of  $51.2 \pm 8.6$  ms (mean  $\pm$  SD), syllable period of  $4.6 \pm 2.2$  s and a dominant frequency of  $17.6 \pm 0.3$  kHz (N = 4 crickets, n = 160 calls). The dominant frequency corresponds to the third harmonic peak (Fig. 2A-B). The frequency structure is similar to other Lebinthini species, and the call particularly resembles that of the species *Cardiodactylus muria* (ter Hofstede et al. 2015). Calls are emitted in bouts of  $8.9 \pm 4.2$  syllables, lasting  $35.7 \pm 16.7$  s, with a bout period of  $52.6 \pm 0.1$  s.

Playback experiments revealed that female *P. hebardii* (N = 6 crickets) exhibit the same type of communication behaviour as the species of the Asian-Pacific clade: females produce vibrational signals at a specific time interval after male calls and show no phonotactic activity (SI-Video\_part1). The female vibrational signal (N = 2 crickets, n = 200 signals) occurs  $327.8 \pm 14.6$  ms after the male's call and has a duration of  $169.8 \pm 5.8$  ms, with a dominant frequency of  $97.0 \pm 3.9$  Hz. These values are similar to previously studied Lebinthini species (Table 1). Therefore, this novel communication system likely evolved in the ancestor of the Lebinthini tribe, representing a key innovation leading to the evolutionary diversification of these crickets (Robillard and Desutter-Grandcolas 2004b; ter Hofstede et al. 2015).

**Table 1:** Male and female communication signal features for previously studied Asian-Pacific lebinthine species and the Neotropical species *Ponca hebardii*. Values are means  $\pm$  standard deviations. Sample sizes are reported in brackets (number of crickets; number of vibrational signals).

Species	Male dominant frequency (kHz)	Female vibration frequency (Hz)	Female vibration delay (ms)	Male vibration delay (ms)
<i>A. obscurus</i>	15	$48 \pm 5$ (8; 42)	$141 \pm 8$ (7; 7)	?
<i>C. muria</i>	14	$38 \pm 3$ (9; 88)	$631 \pm 43$ (10; 10)	?
<i>L. luae</i>	17	$84 \pm 4$ (9; 76)	$138 \pm 8$ (9; 9)	?
<i>P. hebardii</i>	17	$97 \pm 3$ (2; 200)	$327 \pm 14$ (2; 200)	$351 \pm 509$ (1; 59)

A fascinating and unexpected result of the playback experiments demonstrates that male *P. hebardii* (N = 2 crickets, n = 177 ± 4 playbacks per cricket) also respond to calls of other males, both by alternating their own call between playbacks and by producing vibrational signals similar to those documented in females (SI-Video\_part2). These male vibrational signals have not been looked for in previously studied species. The mean delay between a male call and the male vibrational signal (351 ± 509 ms; N = 1 cricket, n = 59 vibrational signals) was similar to the delay measured for female signals (328 ± 15 ms). However, the delay between male call and male vibrational signal was much more variable (range: 30-2,490 ms) compared to the delay between male call and female vibrational signals (range: 271-380 ms). Interestingly, most of the male vibrational signals (49 of 59, 83%) had shorter delays than the mean female delay, with five very long delays generating the higher mean and much larger standard deviation for male than female delays. Male vibrational signals are the first replies to call playbacks and are usually followed by a call after 2.1 ± 0.6 s (Fig. 2).

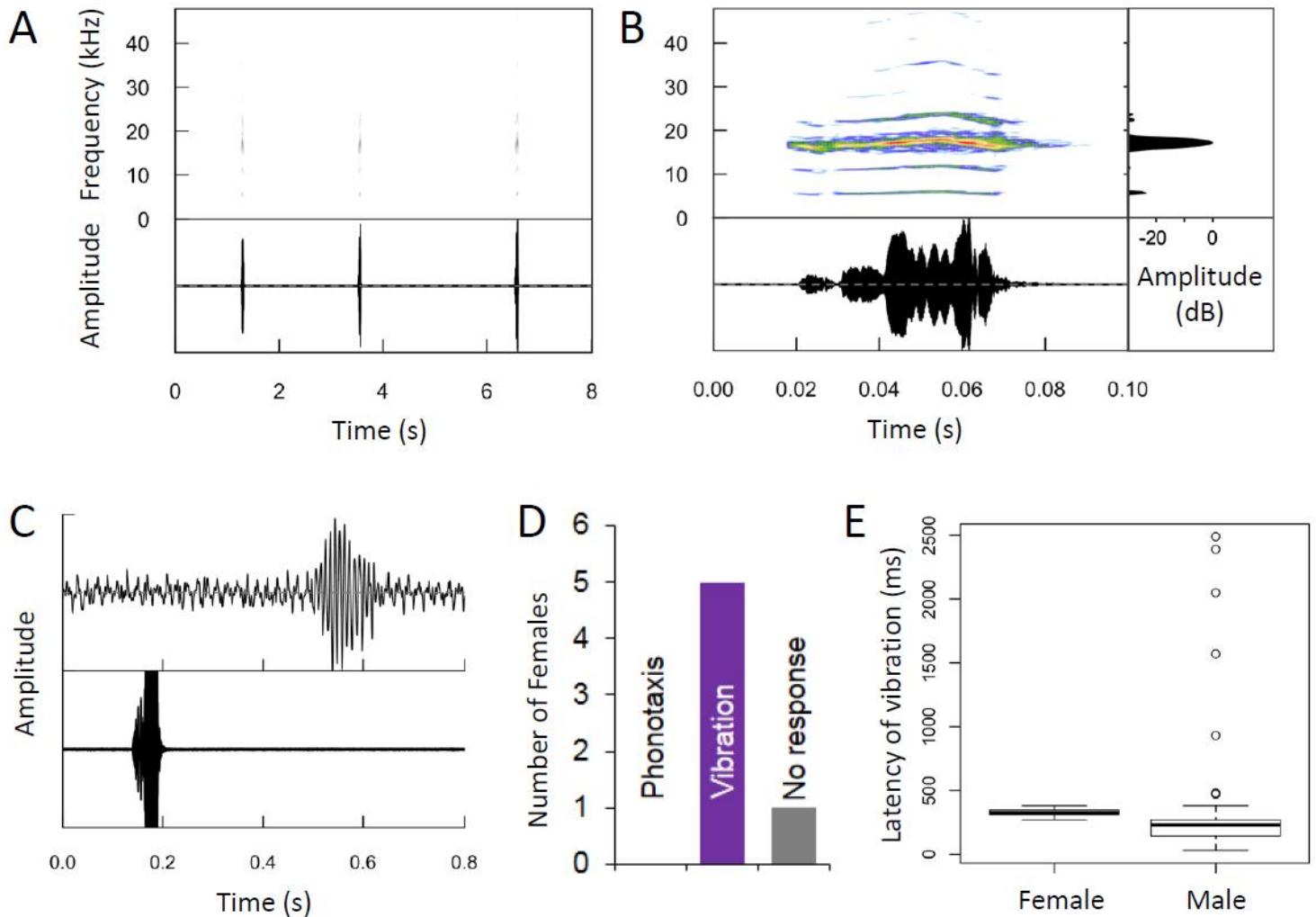


Figure 2. Communication signals of the species *Ponca hebardii*. (A, B) Spectrograms (top trace) and oscillograms (bottom trace) of the calling song of *P. hebardii*: (A) three syllables over 8 s; (B) detailed view of one syllable. (C) Example of accelerometer recording of a female vibrational reply (upper trace) to the male call (lower trace). The male call is clipped in this recording due to the high gain needed to get a suitable signal-to-noise ratio for the vibrational signal recording, but the call itself was not distorted during playback. Graphs made with the R package “seewave” (Sueur et al. 2008). Results of playback experiments with the species *Ponca hebardii*. (D) Behavioural responses of *P. hebardii* females to a speaker broadcasting conspecific male calling song. (E) Delay between the male call and the female (n = 200) and male (n = 56) vibrational reply.

Considering that *P. hebardii* male vibrational signals are similar to female responses and occur in response to male calls, we suggest that the male vibrational signal evolved to mask

female replies to a rival male's calls, thereby preventing rival males from detecting and locating females. This disruptive male strategy of mimicking the female signal in response to another male's signal has been documented in other vibrationally duetting insects (Tauber 2001; Bailey et al. 2006; Mazzoni et al. 2009a, b; Legendre et al. 2012; Polajnar et al. 2014). The delay between male call and male vibration signal was usually shorter than the delay measured in females, perhaps ensuring that the male signal reaches the rival male before the female reply. However, males occasionally produced vibrational signals at very long intervals after another male call, suggesting that spontaneous vibrational signaling might also be part of this species' signaling repertoire. In addition, *P. hebaridi* males produce their own acoustic signal between acoustic signals of the rival male, allowing them to maintain a duet with the female. This behaviour has the potential to increase a male's mating success if the male can produce a masking signal that increases the time required by the rival male to reach the female (Bailey et al. 2006; Legendre et al. 2012; Cocroft and Hill 2014) without decreasing his own call production (Bailey et al. 2006; Legendre et al. 2012).

In crickets, male-male interactions such as aggressive songs, fighting, phonotaxis and victory displays are all known in behavioural contexts linked to male rivalry (Shaw et al. 1990; Brown et al. 2006; Bertram et al. 2010; McCarthy et al. 2013). Nevertheless, those interactions almost exclusively rely on acoustic signals occurring at long range, or consist of multimodal short-range aggressive behaviours. The novel long-range male-male bimodal interactions described here significantly increase the behavioural repertoire of cricket male-male communication and adds to the complexity of the communication system of the Lebinthini. Under the hypothesis that the Lebinthini's communication system evolved by sensory exploitation of a startle response in females, it is likely that male vibrational signals originated through a similar mechanism, by tapping into preexisting sensory biases both in male and female receivers (ter Hofstede et al. 2015). These interactions suggest selection pressure in the form of competition among males for

detecting female responses and contending with eavesdropping rival males (Bailey 1991; Mc Gregor 2005; Cocroft and Hill 2014).

This study supports an origin of the acoustic-vibratory duet in the ancestor of both the Paleo- and Neotropical Lebinthini clades, and demonstrates a potential novel male strategy for thwarting rival males while communicating with a female. Both vibrational and acoustic communication are common and widespread in insects and arthropods (Cocroft and Hill 2014), but only a handful of species are known to use bimodal acoustic-vibrational signals in reciprocal interactions between and among sexes (Rajaraman et al. 2015; ter Hofstede et al. 2015). Understanding how these communication systems function can help us understand conditions that favor multimodal communication and competitive interactions within these systems. The presence of the novel acoustic-vibrational duet in the Neotropical lineage of the Lebinthini supports the hypothesis that it evolved in the ancestor of this tribe and provides a time estimate of ~55 Ma for the origin of this novel communication system. This coincides remarkably well with the estimated time for the origin of echolocation in bats (~58 Ma), suggesting a potentially rapid adaptation to a new predator and subsequent effects on communication within crickets. As methods for molecular dating improve, more accurate estimates will reveal how closely these events occurred in time and provide answers about the rate of evolutionary change in a novel communication system.

#### **List of supplementary information**

Excel file with datasets: DATA\_Ponca.xlsx

SI-Video: Video file of female (Part 1) and male (Part 2) responses to acoustic playback.

#### **Authors' contributions**

TR collected the specimens. J.L.B.-L. conducted behavioural recording experiments. All authors contributed to the conception, design, analysis and writing.

**Competing interests**

We have no competing interests.

**Funding**

The study was realized as part of the PhD project of JLBL, funded by Colciencias scholarship (756-2016). Field work in Panama was possible thanks to a grant from Action Transversal du Museum (MNHN).

**Acknowledgements**

We thank the Smithsonian Tropical Research Institute staff for logistical assistance during field collections on Barro Colorado Island (Panamá). Specimens were collected under scientific permit No.SEX/A-27-17 from the Ministerio de Ambiente de Panama. We thank Stefan Schöneich for advice and Marion Guillaume for cricket maintenance assistance in MNHN.

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### Additional observations

Amid our phonotactic acoustic experiment, we observed an abdomen mark behaviour in *N. malaya* males (figure 6) which constitutes a chemical channel of communication. Therefore, we proceed to take lateral view video records with a Sony Handycam (HDR-CX700VE) camera for both males and females and used Whatman paper to sample the potential chemical mark that was confirmed through gas chromatography (GC) analysis. GC analyses were carried on direct samples from cuticle and genitalia plates for 8 specimens and for 22 samples collected during the phonotactic acoustic experiment. Gas chromatographic analyses were conducted in the laboratory of chemical analyses in Ecology (PACE) Labex CeMEB in Montpellier.



Figure 6. Male (left), and female (right) of *N. malaya* performing mark behaviour.

Based on the notes taken in 2018 and 2019, mark behaviour is predominant in males, although it is present also in females (Table 2). In both sexes of *N. malaya* we have found that there are different chemical profiles (figure 7).

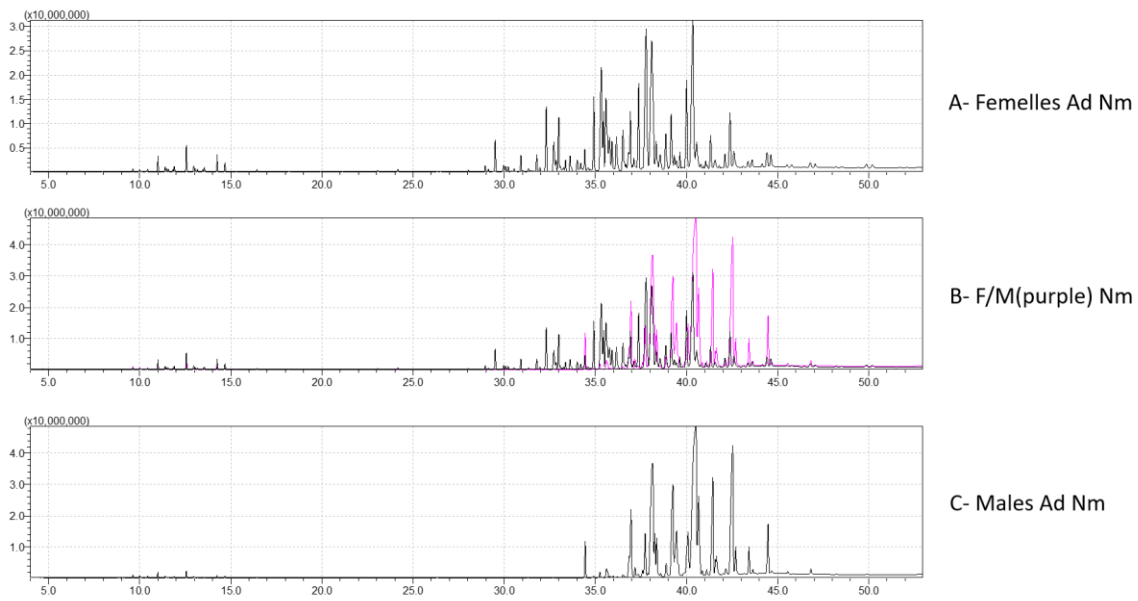


Figure 7. Chemical profiles from cuticular samples in adult stages. (A) Female adult profile. (C) Male adult profile. (B) Comparison of chemical profiles of *Nisitrus malaya* among females and males (in purple).

Chemical profiles are also different between cuticular and genitalia plates samples (figure 8). Remarkably, the chemical profile recorded from the phonotactic acoustic experiment corresponds to genitalia plates chemical profile (figure 9)

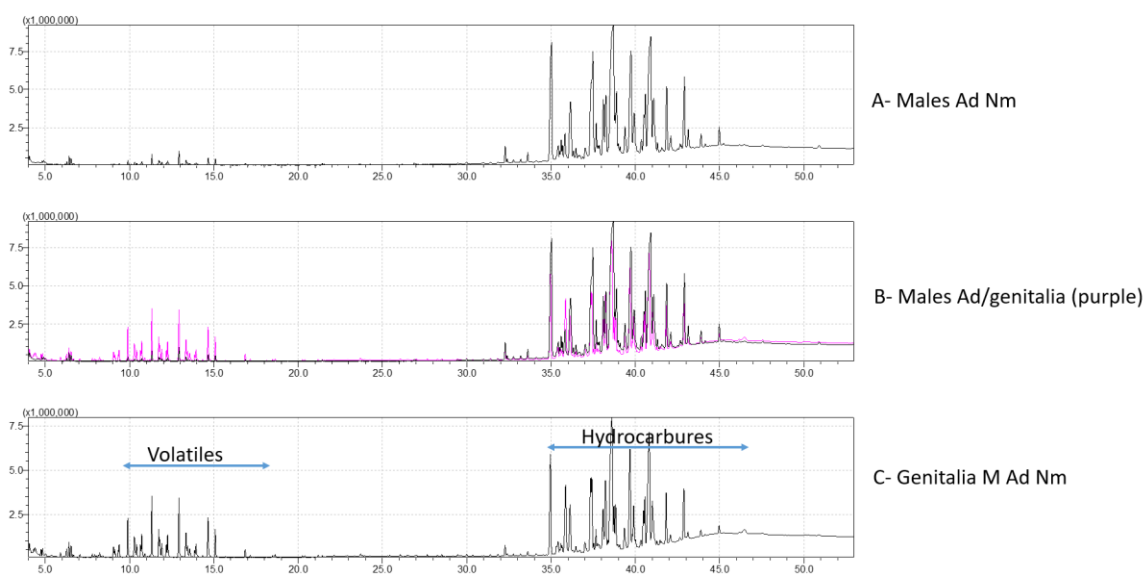


Figure 8. Chemical profiles from cuticular and genital plate in adult males. (A) Male cuticular adult profile. (B) Comparison of chemical profiles of *Nisitrus malaya* among cuticle and genital plate from males (in purple). (C) Genital plate from adult male profile.

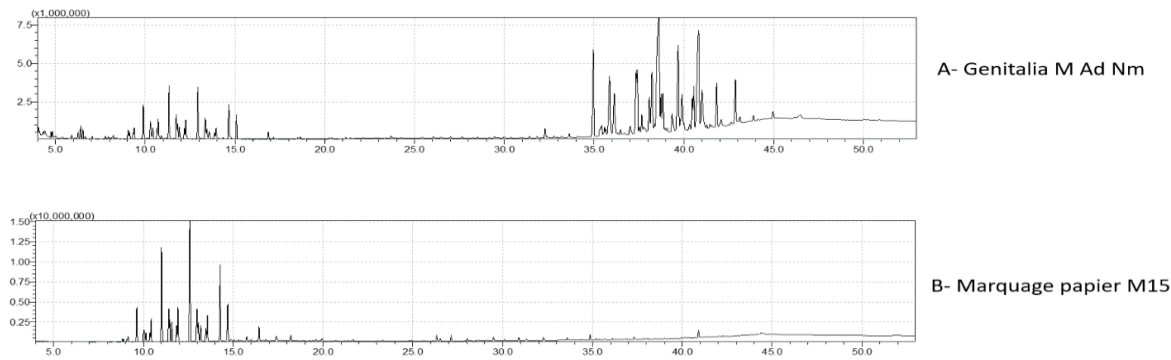


Figure 9. Chemical profiles from genitalia and Whatman paper samples in adult stages. (A) Male genitalia adult profile. (B) Whatman paper profile marked by a male adult of *Nisitrus malaya*.

Table 2. Count observations of mark behaviour discriminated by week, year, treatment and sex.

2018	F1		F2		F3	
	Males	Females	Males	Females	Males	Females
	total sample 58	total sample 75	total sample 45	total sample 59	total sample 45	total sample 69

Week 1	1	1	4	1	4	
Week 2	7		4		2	1
Week 3	4		4		3	
Week 4	4		2		4	
Week 5	3		3		1	
Week 6	1					
<b>Total marks 2018</b>	20	1	17	1	14	1
<b>2019</b>	<b>total sample 22</b>	<b>total sample 24</b>	<b>total sample 21</b>	<b>total sample 24</b>	<b>total sample 21</b>	<b>total sample24</b>
Week 1	2		4		2	
Week 2	2		3		1	
Week 3				1		
Week 4					1	
Week 5	1		1	3	1	
<b>Total marks 2019</b>	5		8	4	5	
<b>General marks</b>	<b>25</b>	<b>1</b>	<b>25</b>	<b>5</b>	<b>19</b>	<b>1</b>

It is necessary to increase the number of samples to conclude on the role of the chemical mark in *N. malaya* but is an interesting perspective for future work on the species that could be considered to have a multimodal communication system (Calvert et al. 2004; Rajaraman et al. 2015; Benavides-Lopez et al. 2020).

These marks seems to be efficient for successful mating because this behaviour is inconspicuous (Casas et al. 2009). Part of the animal communication is when a signal causes mutual benefit that could influence the receiver behaviour and also is perceived by conspecifics, furthermore,

chemical stimuli are detected by cuticular contact with compounds that benefit pair formation becoming pheromonal signals (Greenfield 2002). Nevertheless, complementary to our observations this behaviour will need further research in order to accumulate a bigger sampling number to analyze the association to mating behaviour or even satellite strategies by marks positions spacing.



# General conclusion

In this thesis, we have outlined the main aspects regarding the bioacoustics features and behaviours associated with the Eneopterinae subfamily. The calling songs of *P. hebardii* males have been recorded and described for the first time, these calls exhibit high frequency in their acoustic features. Besides, the vibrational reply of females; which constitutes the startle behaviour; likewise, the "vibrotaxis" in males. This novel communication system is likely shared by the whole tribe Lebinthini. Dating the origin of this behaviour to coincide with the origin of echolocation in bats. Additionally, we have documented in the cricket male duets involving both acoustic and vibratory signals not previously described in crickets, and we have tentatively interpreted it as competitive masking between males, to avoid eavesdroppers neighbors. We have explored the calling songs of the four valid species of the Neotropical *Eneoptera* genus. Accomplished in a combination of field trips, acoustic records, analysis, re-analysis, and measurements of ultrastructures features. This genus exhibits remarkable bioacoustic diversity in each of them. We were able to observe synchronous "chorus" of males. Additionally, we observed multiple cases of sympatry in different parts of their distribution. These results and these kinds of observations and notes help us to complement and understand the diversity in bioacoustical behaviours of the genus, the subfamily and the crickets in general. How the coexistence of these close related species affects the diversity of acoustic behaviours remain to be addressed. We show the role of high amplitude harmonics in the calling song of Eneopterinae crickets, using *Nisitrus malaya*. Through the experiments carried on for females and males, we were able to assess different responses that confirm the use of phonotaxis in non-lebinthine eneopterines at least for this species. Positive phonotaxis was obtained independent of the treatment applied. Females usually finish the experiments closer to the speaker with a considerable proportion. Even

though high amplitude harmonics are not necessary to elicit phonotactic behaviour, could be useful to guide them. Results show that males were attracted to the playback songs, this result was less expected. Interestingly, few males walked to reach the speaker, but many keep halfway the distance to the speaker. This could be explained as satellite males which suggest a different phonotactic strategy in males. Thus, males avoid getting in possible confrontations by close contact with the calling male and intercept females attracted by the calling male. These results suggest that both sexes in *N. malaya* exhibit phonotaxis to a certain degree, and are very probable to be attracted by a calling male. For *N. malaya* we noticed another remarkable signal linked to conspecific communication, a chemical mark. We were able to get the chemical samples from *N. malaya* found different chemical profiles between sexes that there are. Overall, phonotaxis and the chemical mark suggest a multimodal nature of the communication system in *N. malaya*. This inconspicuous behaviour seems could be potentially efficient successful for mating. Although further research with bigger sample size and another set of experiments is necessary to confirm the chemical mark behaviour, our observations open a new path for the Eneopterinae crickets research. Hereafter, the study of this subfamily has increased our perspectives and comprehension of acoustic behaviours of these crickets bringing a solid basis for further studies in this subfamily, Orthoptera and even other arthropods. Within a plethora of animal communication systems, the high amplitude harmonic studies have been underrated, we are called to recognize the importance of this feature in bioacoustics, evolutive, behavioural, or even a combination of these to go deeper in a more complex communication network. Through this thesis, I have aimed to understand the acoustic behaviour of a portion of the Eneopterinae subfamily, and certainly, the whole of the systems described for the Orthoptera order.

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