ACOUSTIC COMMUNICATION IN *dendroctonus adjunctus* BLANDFORD (CURCULIONIDAE SCOLYTINAE): DESCRIPTION OF CALLS AND SOUND PRODUCTION MECHANISM

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ABSTRACT: Acoustic communication system (ACS) in bark beetles has been studied mainly in species of the genera *Dendroctonus*, *Ips*, and *Polygraphus*. Specifically, ACS of the roundheaded pine beetle, *Dendroctonus adjunctus*, has been little studied. In this study, we described the stridulatory apparatus of this beetle using optical and scanning electron microscopy and recorded the call types produced by males in three behavioral contexts: stress, male-female, male-male interactions. From the spectrograms and waveforms, call types, as well as temporal (tooth strike, tooth strike rate and intertooth strike interval) and spectral features (minimum, maximum and dominant frequency) were determined. Males have a functional elytro-tergal stridulatory apparatus, females not, consisting of a file for the pars stridens and two lobes for the plectrum. Most of spectro-temporal features were statistically different between single- and multi-noted calls and across the three behavioral contexts. In the male-male interaction, a new type of call named "withdrawal" was produced by the male withdrawing or fleeing. Our results suggest that the spectro-temporal features of single- and multiple-noted calls in the three behavioral conditions are specific and different from each other. Yet, the combination of single and multiple calls determinate an overall calling pattern characteristic of the tested behaviors and, therefore species-specific.

KEYWORDS: bioacoustics, bark beetle, elytro-tergal, single and multiple-noted calls, withdrawal call.

INTRODUCTION

The acoustic system (AS) (signals, auditory organs, stridulatory devices) is a key innovation that evolved independently in many insect groups [1,2]. Around 92% over 195,000 described insect species produce mainly vibrational signals and other forms mechanical signaling involved in different intra- and interspecific functions [3], such as disturbance and alarm, aggregation, aggression, courtship, copulatory, postcopulatory, and aggressive mimicry [4-8]. Yet, the AS does not consist of a set of evolutionarily independent components, as they are likely to be functionally related to components of other communication systems, such as chemical and visual [9-11]. The importance of each system depends on the insect group and on the environment in which they live [1,12].

The pattern of sound signals in insects is varied, complex and generally species-specific [13]. Sound signals are continuous or discontinuous vibrations with physical properties dependent on its dispersion across a medium and its reception by auditory organs [14,15]. The most common signaling device in insects is an intersegmental stridulatory apparatus, followed by mesonotal- pronotal and other less common apparatuses, such as elytro-tergal, vertex- pronotal and gula-prosternal [16,17].

Bark beetles (Curculionidae: Scolytinae) are a diverse group of herbivorous insects that play an important role in the ecosystem, because they contribute to the structure, composition, dynamics, and vitality of the forests by colonizing and killing old, damaged or physiologically weakened trees [18,19]. Moreover, bark beetles create food webs because generate habitats by killing trees which promotes biodiversity (fungi, mites, bacteria and many other invertebrates and vertebrates use this new resource) [20]. Furthermore, these beetles have established complex ecological interactions with their symbionts, thereby constituting a true holobiont [21,22]. Yet, they are also considered important disturbance agents, because the outbreaks of some bark beetle species can kill thousands of health trees and caused a negative impact on ecosystem services for humans and significant economic losses to timber producers [23,24].

Bark beetles are essentially olfactory insects, since they use specific mixtures of semiochemicals to differentiate, select and colonize host trees and attract mates and conspecifics [25,26]. Inside plants, bark beetles live in a subcortical environment where they build their galleries, mate and grow their offspring [24]. This is a dark and chemically saturated environment where a chemical communication system could not be reliable [5,15,27]. In the subcortical environment, the AS may be of paramount importance, since wood is a good mediator of sonic signals transmission for communication [27].

Studies of the AS in bark beetles have been carried out mainly in species of the genera *Dendroctonus*, *Ips*, *Hylurgus* and *Polygraphus* [28-32]. From these studies, the manner in which sound is produced in these beetles has been delineated (*e.g.*, elytro-tergal, vertex-pronotal, and gula-prosternal); specifically in the *Dendroctonus*-bark beetles, the mechanism found is the elytro-tergal, which is composed of two structures: the pars stridents located on the inner face of the declive elytral and the plectrum, situated at the seventh abdominal tergite, which generate sound sequences or stridulations [16,28,33]. Moreover, a general catalogue of sounds under stress conditions has been outlined [16], as well as the spectral and temporal characteristics of sound in different biological contexts (e.g. stress, courtship, and female-male interactions) and the effect that some morphological features (e.g., beetle size) have on the sound type produced during courtship [29,30,34-36].

The roundheaded pine beetle, *D. adjunctus* Blandfort, is an aggressive species that colonize around 18 pine species, preferentially *Pinus hartwegii*, across its distribution range from the United States to Central America [37,38]. Studies of acoustic communication in this bark beetle are scarce and were conducted under a stress condition; in addition, dimorphism was observed in the sound production because only males produced it [16]. Based on this information, we investigated the variability and specific degree of the spectral and temporal features of male calls. We first described the morphology of stridulatory apparatus using optical and scanning electron microscopy and, later, we recorded the call types produced by males as well as temporal and spectral features of sounds under different behavioral conditions, such as stress and male-female and male-male interactions.

MATERIALS AND METHODS

Pinus hartwegii trees infested and non-infested by the roundheaded pine beetle were felled in May 2023 at the Parque Nacional Volcán Nevado de Colima, Jalisco State, Mexico (19°35’15.2” N, 103°36’7.33” W; 3414 m elevation). Trees of each condition were sectioned into logs (20 cm length x 30 cm diameter) and sealed at the ends with paraffin to avoid dehydration. Once in the laboratory, uninfected logs were stored in the freezer at 4 °C, while infested logs were enclosed in cloth bags and stored at room temperature. Emerged adults were collected daily, placed in Magenta™ vessels GA 7 (Magenta Corp, Sigma-Aldrich-Merck, Darmstadt, Germany) containing wet filter paper, and stored at 4 °C to keep them alive until needed. The sex of the organisms was ascertained by the shape of the seventh abdominal tergite [39].

Optical and scanning electron microscopy of stridulatory structures

The seventh abdominal tergite and the left elytra from 30 males were removed using a pair of fine forceps and fine needles. Both structures were cleared by incubating them for 3 h at 70 °C in 10% KOH solution. Thereafter, structures were immersed in 10% acetic acid solution to neutralize the KOH solution, rinsed with 96% alcohol, and later mounted on slides in Hoyer's medium [40]. The pars stridens (Figure 1a) on the left elytra and the plectrum (Figure 1c) in the seventh tergite were observed by optical microscopy (Prime Star 1, Carl Zeiss, Jena, Germany), and the length and width of both structures were measured with a graduated eyepiece and a 0.01 mm calibration slide (Walfront, Micrometer Calibration Slide). The variables measured of these structures were as follows: The length of the pars stridens was the distance between the ridge closest to the anterior edge of the elytra to the ridge farthest from the posterior edge (Figure 1a-1); the width was the distance from the midpoint of the pars stridens closest to the sutural margin to the farthest midpoint of this structure (Figure 1a-2), and the ridge width was the distance between grooves, calculated from the beginning of one groove to the beginning of the next (Figure 1b-3). The external width of the plectrum was the distance between the outer edge of the right lobe to the outer edge of the left lobe in the anteroposterior position of the insect body (Figure 1d-5). The internal width was the distance between the inner edge of the right lobe to the inner edge of the left lobe (Figure 1d-6), and the lobe width was the distance between the edges of the right lobe (Figure 1d-7). In addition, the stridulatory apparatus of males was observed and photographed in a variable pressure scanning electron microscope under low vacuum (0.6 mbar) and acceleration voltage of 5 kV (FEI Quanta 250 ESEM, FEI Company, Hillsboro, United State) at the Centro de Nanociencias y Micro y Nanotecnologías, Instituto Politécnico Nacional.

Sound recording

In this study, we referred to the sounds produced by insects as calls [16], which present different temporal and spectral features depending on the type of interaction. To avoid noise disturbance, all calls produced by the roundheaded pine beetle under stress, male-female and male-male interactions were recorded at night, inside a purpose-built soundproofed wooden box (width x length x depth, 20 x 40 x 30 cm) to minimize stray noise. Calls were recorded with an omnidirectional condenser microphone model ECM8000 (Behringer, Willich, German) and an audio interface model U-PHORIA UM2B (Behringer, Willich, German) in an ASUS laptop, using the virtual audio editor Audacity at 96 kHz, 48-dB gain, and 24 bits sampling rate.

Stress calls of thirty males were recorded while holding them with the thumb and forefinger for 2 min and placing the microphone at 5 mm distance, leaving the elytra free, and lightly pressing their head and pronotum. Male- female interaction calls were repeated 24 times. For this assay, a 0.9 mm diameter hole was drilled in the non-infested *P. hartwegii* logs bark with a drill and a 5/16*4'' drill bit to go through the bark and avoid reaching the phloem. Subsequently, each female was confined in logs for 1 h placing an empty gel capsule over the hole. Once the female began to build a gallery, which was confirmed by the accumulation of frass in the entrance hole, the gel capsule was removed and a randomly selected unmated male was placed at the entrance of the gallery [29]. Male-male interaction was repeated 15 times. For this assay, a circular arena of two cm diameter was made in the bark of non- infested logs with the help of a knife and a drill with a 5/16 drill bit.

Subsequently, the first male was placed in the arena with the help of fine-tipped tweezers. After 1 min the second male was quickly placed in the arena, covering it with a 0.2 mm mesh to avoid escape. Calls from male-female and male-male interactions were recorded for two minutes or until no signals were detected, the microphone was placed at 2 cm from the hole and arena, respectively [29].

Analyses

Spectro-temporal features

From the sounds file, call types (single or multiple notes), ratio, and spectro- temporal features were measured using spectrograms and waveforms in Raven Pro™ 1.4 (www.birds.cornell.edu/raven) following the nomenclature from [16,29,40].

A sound call is a train or set of stridulatory impulses provoked by the tooth strike, that is, the rubbing of the plectrum against a tooth of the pars stridens. In bark beetles, single-note calls are characterized by a series of regularly spaced strikes and multiple-noted calls by two or more pulses spaced by brief periods of silence [41]. In this study, multiple-noted calls were considered as sounds spacing with a duration longer than two and a half milliseconds, and at least three strikess in each part of the train. The call ratio was calculated as the relationship between single-noted calls or multiple-noted calls with respect to the total number of calls observed, this feature was expressed in percentage.

The temporal features recorded included: the tooth strike rate (estimated as the strike number delivered per second), the intertooth strike interval (calculated as the silence time at milliseconds between each tooth strike), and the call duration (estimated as the time which elapsed between the first and last tooth strike from a call). The spectral features evaluated include the maximum frequency (measured as the highest number of repeated waves with respect to time), the minimum frequency (measured as the lowest number of repeated waves), and the dominant frequency (calculated as overlapping of the frequency with the highest amplitude) [30].

The spectro-temporal features of the single-noted and multiple-noted calls of the three conditions assayed were compared using a paired t-test for independent data and different sample sizes. To estimate significant differences of the spectro-temporal features of single-noted and multiple-noted calls among the three conditions assayed (stress, male-male interaction, and female-male), an one-way ANOVA and post hoc test of Tukey-Kramer were conducted. All analyses were performed with Past 4.03 [42].

RESULTS

Stridulatory apparatus of D. adjunctus

The shape of the pars stridens is a row of ridgs arranged longitudinally with respect to the inner margin of the elytra. The row has ridges aligned perpendicular to the margin of the elytra, which are highly developed on the left elytra and marginally on the right; in fact, when the right elytra is closed it overlaps the left (Figure 1a-b, e-f). The pars stridens is characterized in that most of its ridges are continuous, and only a minority of them are observed fused at some points (Figure 1b-4). The size of this structure was variable among individuals, with a length of 550-690 ± 7.1 μm, width 170-260 ± 4.2 μm, and a crest width 6.25-8.3 ± 0.1 μm (Table 1).

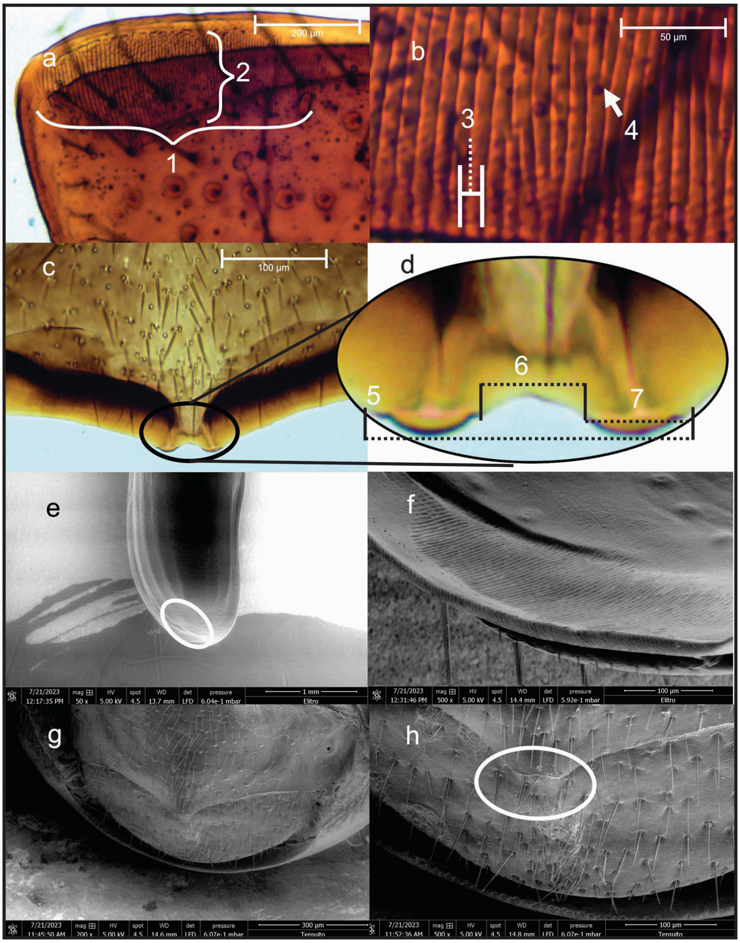

Figure 1. Micrografies of stridulatory apparatus of male *D. adjunctus*. (**a**) pars stridens observed by optical microscopy (**b**) zoom of pars stridens, (c) plectrum observed by optical microscopy, (**d**) zoom of plectrum (**e**) right elytra and (**f**) pars stridens observed by scanning electron microscopy, (**g** and **h**) plectrum by scanning electron microscopy; the white circles indicate the pars stridens and the plectrum. (**1**) length of pars stridens, (**2**) width of pars stridens, (**3**) ridge width, (**4**) division of a ridge, (**5**) external width and (**6**) internal width and (**7**) lobe width of the plectrum.

***Pars stridens**	**Width (µm)**	**Length (µm)**	**Ridge width (µm)**
Range	170-260	550-690	6-8
Media	219.71	610.85	6.71
Standar error	25.26	42.31	0.78
***Plectrum**	**Internal width (µm)**	**External width (µm)**	**Lobe width (µm)**
Range	26-31	57-71	15-21
Media	26.54	65.12	19.56
Standar error	3.74	4.73	2.64

Table 1. Measurements of the stridulatory apparatus, pars stridens and plectrum males of *D. adjunctus*.

* see Figure 1 for information about plectrum and pars stridens features.

The plectrum is in the middle part of the posterior margin of the seventh tergite, it consists of two conspicuous lobes projecting towards the eight tergite which, when rubbed voluntarily by friction against the pars stridens, produces the sound (Figure 1c-d, g-h). The plectrum was also variable, with an external width of 57-71 ± 0.7 µm, an internal width of 26-31 ± 0.6 *µ*m, and a lobular width of 15-21 ± 0.4 µm (Table 1).

Sounds recording

Stress calls

Stressed males emitted a call train at an average rate of 2.25 ± 0.07 calls/s and an output of 10-60 dB, until the insect was released. The call type was predominantly single-noted in most of the insects (30 specimens) analyzed; however, some of them (13 specimens) also emitted multiple-noted calls (Figure 2a-d; Table 2). The total calls ratio was 90% single-noted and 10% multiple-noted (Table 2). The tooth strikes number and tooth strikes rate per second were higher in single-noted calls than multiple-noted calls; the intertooth strike interval was greater for multiple-noted calls than for single-noted calls.

The duration of single-noted calls was slightly shorter than multiple-noted calls (Table 2). Meanwhile, the minimum frequency of single-noted calls was approximately half that of multiple-noted calls, the maximum and dominant frequencies were similar between both noted calls (Table 2). The plot of the power spectrum showed a single-noted call with a maximum amplitude (dB) between one and nine kHz (Figure 2e).

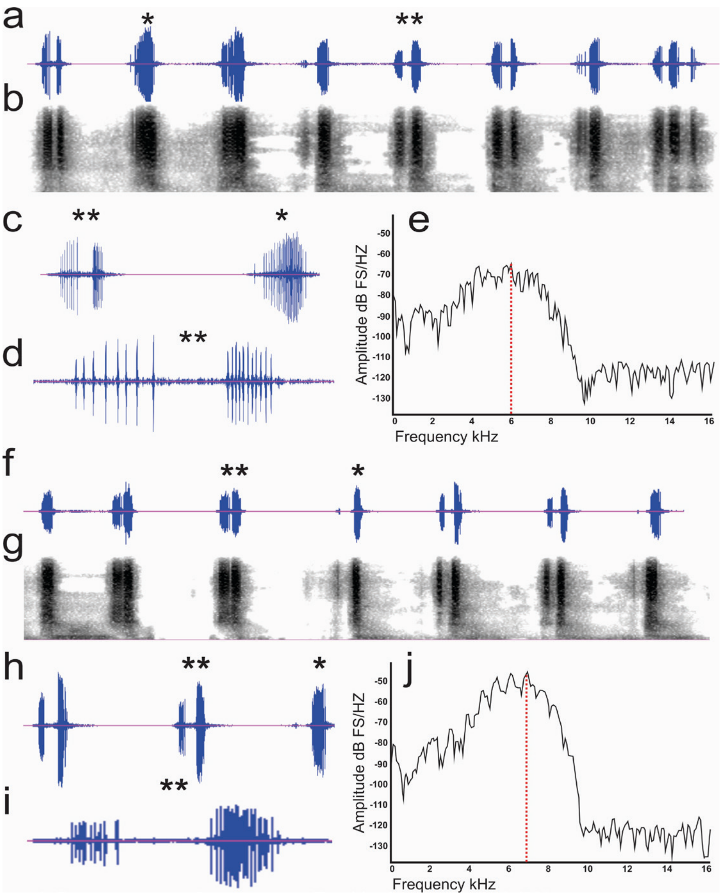

Figure 2. Wave diagram and spectrogram of the stress (**a**-**e**) and male-female (**f**-**j**) condition. (**a**) wave diagram and (**b**) spectrogram of the stress condition, (**c**) amplification of a single chirp (*) and an interrupted chirp (**), (**d**) amplification of an interrupted chirp, (**e**) relative power diagram, (**f**) wave diagram and (**g**) spectrogram of the male-female interaction, (**h**) amplification of a single chirp (*) and an interrupted chirp (**), (**i**)amplification of an interrupted chirp, (**j**) relative power diagram.

Female-male interaction

In the female-male interaction, twenty four beetles produced single-noted call, but twelve of them produced also multiple-noted calls in this context (Table 2) (Figure 2f-i). The calls rate was 74% single-noted and 26% multiple-noted (Table 2). During courtship, the male initially emitted single-noted calls, but as it approached the female, it produced multi-noted calls. The call train emitted by males was at an average rate of 7.8 ± 0.27 calls/s and an output of 10-65 dB.The number of tooth strikes, the tooth strike per second, and the intertooth strike interval were higher in multiple-noted calls than single-noted calls. The duration of both types of calls was double in multiple-noted calls than single- noted calls (Table 2); the minimum, the maximum and dominant frequencies of spectral parameters were similar between single-noted and multiple-noted calls (Table 2). The plot of power spectrum showed a single-noted call with an amplitude (dB) between two and ten kHz (Fig. 2j).

				Temporal				Spectral		
Call context	**Call type**	**Proportion** n	(%)	**Tooth strike (no./call)**	**Tooth strike rate (no./s)**	**Intertooth strike interval (ms)**	**Call duration (ms)**	Minimum frequency (kHz)	Maximum frequency (kHz)	Dominant frequency (kHz)
Stress	Single	30	0.9	41.4±0.7	*1034.3±26.5	0.9±0.03	*40±1	2.1±0.14	8.6±0.03	a6.5±0.06
	Multiple	13	0.1	27.6±6.5	585.7±75.2	1.6±0.23	47.2±5.5	4.3±0.04	a8.7±0.08	6.7±0.3
Female-male	Single	24	0.74	23.8±1.8	675±20.7	1.4±0.04	35.3±3.7	5.6±0.07	7.8±0.08	6.7±0.07
	Multiple	12	0.26	36.9±1.9	532±27.4	1.8±0.09	69.5±1.3	4.3±0.1	b7.1±0.07	b 6±0.06
Male-male remains	Single	15	0.857	16.4±1	611.7±15.5	1.6±0.04	26.8±2	* 2.7±0.19	a8.4±0.07	c6.6±0.06
	Multiple	7	0.143	21.9±0.3	549.6±24	1.8±0.07	40±1.5	2.5±0.41	a8.5±0.26	c6.2±0.11
Male-male withdrawal	Single	10	1	24±1.9	616.3±24	1.6±0.05	39±2.8	5±0.21	b7.3±0.11	d 6.3±0.11
Significance level Paired t test										
Comparison between Call type	Stress		$t=$	3.32 (p<0.05)	4.68 ($p<0.05$)	5.42 ($p<0.05$)	1.84 ($p=0.06$)	3.75 ($p<0.05$)	1.05 ($p=0.2$)	1.04 ($p=0.3$)
	Female-male		$t=$	3.68 (p<0.05)	11.44 ($p<0.05$)	17.63 ($p<0.05$)	15.54 ($p<0.05$)	8.41 ($p<0.05$)	4.83 ($p<0.05$)	5.88 ($p=0.3$)
	Male-male remains		$t=$	1.68 (p<0.05)	1.66 ($p=0.1$)	1.58 ($p<0.1$)	2.11 ($p<0.05$)	0.44 ($p=0.6$)	0.66 ($p=0.5$)	2.65 ($p<0.05$)
Significance level one ways ANOVA										
Comparison between context	Single		$F=$	153 ($p<0.05$)	76.4 ($p<0.05$)	78.58 ($p<0.05$)	12.29 ($p<0.05$)	68.26 ($p<0.05$)	37.59 ($p<0.05$)	2.7 ($p=0.06$)
	Multiple		$F=$	3.4 ($p<0.05$)	17.76 ($p<0.05$)	42.69 ($p<0.05$)	238.3 ($p<0.05$)	19.58 ($p<0.05$)	42.71 ($p<0.05$)	4.1 ($p=0.08$)

Table 2. Temporal and spectral measurements of single-noted and multiple-noted calls of *Dendroctonus adjunctus*, in three interactions stress, male-female, and male-male interactions.

*Condition with significant difference for each temporal characteristic, and treatments associated with the same lowercase letter did not differ significantly for spectral characteristics, through the Tukey- Kramer test.

Male-male interaction

Males displayed two behaviors during the interaction: both males stayed in the arena and later one of them left, the latter behavior was observed in most insects (ten out of fifteen) and the time in which the males retreated from the arena was between 30 to 60 s. The males that remained in the arena emitted a calls train at an average rate of 3.7 ± 0.27 calls/s, while males withdrawing emitted a calls train at an average rate of 2.56 ± 0.22 calls/s. Calls in both behaviors showed a range of amplitude of 10-60 dB. Single-noted calls were the primary call type in male-male interactions (Table 2) (Fig. 3a-d). Males which remained in the arena also produced multiple-noted calls (14.3% of the time; Table 2, Figure 3e). For males that remained in the arena, their temporal and spectral characteristics were maintained during the interaction (Table 2).

These temporal and spectral characteristics were like those of the males that remained in the arena until one withdrew from the arena. However, during the insect retraction, the amplitude of the single-noted call decreased significantly.

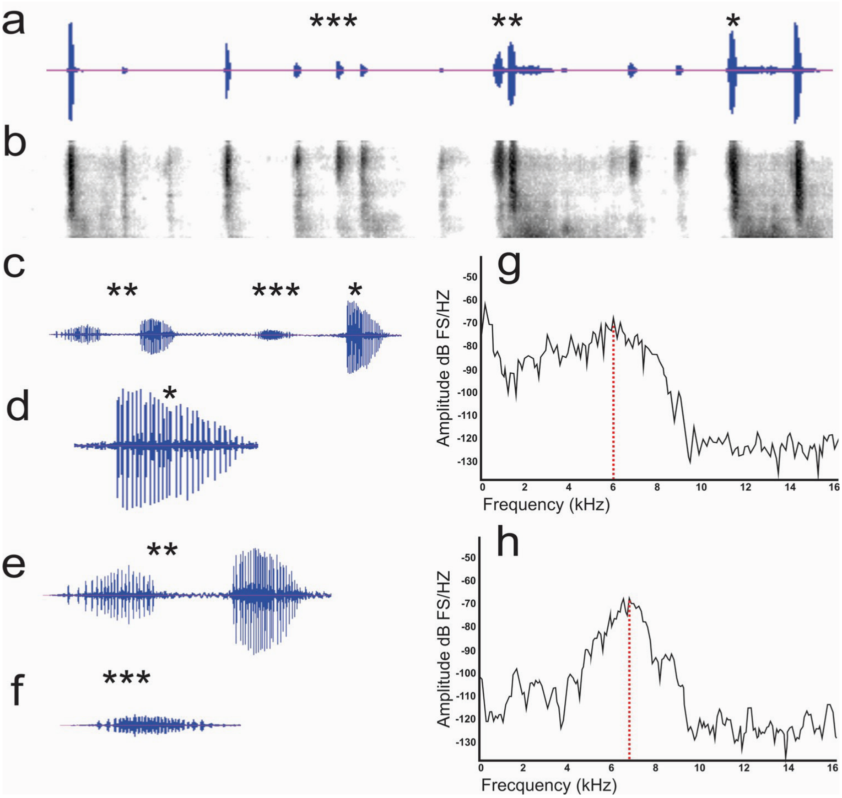

Figure 3. Wave diagram and spectrogram of the male-male condition. (**a**) wave diagram and (**b**) spectrogram, (**c**) amplification of a single-noted call (*), multiple-noted call (**), and "withdrawal call" (***) (**d**) amplification of an single- noted call, (**e**) amplification of an multiple-noted call, (**f**) amplification of "withdrawal call"(***), (**g**) single-noted call relative power diagram, and (**h**) single-noted call relative power diagram of a "withdrawal calls".

Significant differences were found between several spectral and temporal features of single-noted and multiple-noted calls generated in each assayed condition. The features tooth strike ($t = 3.32$, $p = 0.001$), tooth strike rate ($t = 4.68$, $p < 0.05$), inter-tooth strike interval ($t = 5.42$, $p < 0.05$), and minimum frequency ($t = 3.75$, $p < 0.05$) were different in the stress condition; tooth strikes ($t = 3.68$, $p = 0.001$), tooth hit rate ($t = 11.44$, $p < 0.05$), intertooth hit interval ($t = 17.\ 63$, $p < 0.05$), call duration ($t = 15.54$, $p = 0.04$), minimum frequency ($t = 8.41$, $p < 0.05$) and maximum frequency ($t = 4.83$, $p < 0.05$) in the male-female interaction, and only call duration ($t = 2.11$, $p = 0.04$) and dominant frequency ($t = 2.65$, $p < 0.05$) in the male-male interaction (Table 2). ANOVA and Tukey- Kramer test showed significant differences in the temporal features of the tooth strikes ($F = 153.0$, $p = 0.001$), the frequency of the tooth strikes ($F = 76.4$, $p = 0.001$), the interval between tooth strikes ($F = 78.58$, $p = 0.001$), and the call duration ($F = 12.29$, $p = 0.001$), as well as in the spectral features of minimum ($F = 68.26$, $p = 0.001$) and maximum ($F = 37.59$, $p = 0.001$) frequency both of single and multiple notes among stress, male-female and male-male conditions (Table 2).

DISCUSSION

This is the first report of the acoustic diversity of male *D. adjunctus* under stress conditions, and female-male and male-male interactions. Roundheaded pine beetle has a stridulatory elytro-tergal apparatus whose general morphological organization aligns with the description of males from *D. frontalis*, *D. pseudotsugae*, *D. brevicomis* [43], *D. ponderosae* [29, 43], *D. rufipennis*, *D. valens* [36,43], *D. terebrans* [44], *D. approximatus* [30], as well as *D. rhizophagus*, *D. mexicanus*, *D. mesoamericanus*, and *D. vitei* [45]. No sound was recorded in females of the roundheaded pine beetle under stress condition, which agrees with the report by [16] for this same species, but not in the female- male interaction. Previous studies have demonstrated that other female *Dendroctonus* have a second stridulatory apparatus called the "terminal abdominal sternite" located in the wall of posterior margin of the last sternite [33,44], from which, it was assumed that they can generate sounds.

To confirm the presence or not of the terminal abdominal sternite, we analyzed 30 females of the roundheaded pine beetle. Interestingly, females have this structure, in agreement with that reported for other species of *Dendroctonus* [44-47], but they also have a structure analogous to the male stridulatory apparatus on the left elytra consistent of a file whose ridges are apparently not well developed and arranged as in males (Figure S1). This structure is also present in females of other *Dendroctonus* species, but it is not known whether they produce sounds with this structure as a true stridulation mechanism [46], as it has been reported for *D. terebrans* [44]. It remains to be resolved whether the sounds produced by females are true calls or are acoustic reminiscences of an atrophied morphological structure in the course of evolution, which could be associated with the ecological role that females play as the sex that initiates host tree selection and colonization, as previously suggested [16].

Acoustic sound generation by male *D. adjunctus* includes single and multi- noted calls in stress tests and female-male and male-male interactions, which are consistent with studies of other *Dendroctonus* bark beetles under the same conditions [16, 29,30,36,43,47]. In both call types of this bark beetle, the significant differences observed in spectral and temporal features indicate that these sounds are linked to specific behaviors, as it has been suggested in other *Dendroctonus* species [29,30,36].

Calls produced in stress conditions by the roundheaded pine beetle were mainly 90% single-noted and 10% multiple-noted, these serve as evidence of two different morphotypes: two-noted and three-noted calls, which is consistent with that reported for other *Dendroctonus*-bark beetles [16,29,47], including *D. approximatus* that apparently only generated single-noted calls in stress [30], but our spectrograms indicate that they can also produce multiple-noted distress calls (Figure S2). In addition, our findings also showed that the intraspecific variation of spectro-temporal features in the roundheaded pine beetle was low, and independent of note type and multi-note morphotypes.

However, significant differences were found between spectro-temporal features in both call types (Table 2), except maximum and dominant frequencies, and call duration. Furthermore, the interspecific comparison of some spectral- temporal features (e.g., maximum, minimum and dominant frequencies, and) of different species generated in stress (Table 3) suggest that the patterns of single-noted distress calls are similar among them, except for the call duration records reported for *D. approximatus* and *D. terebrans* [16]. Unfortunately, the absence of multi-noted call data does not allow us to evaluate whether the spectro-temporal features of these call types are similar or different between species.

Significant differences between the spectro-temporal features of single- noted and multiple-noted calls produced by *D. adjunctus* males in the female- male interaction and in the stress condition indicate that the general pattern of these calls corresponds with different biological behaviors. Our results show a higher prevalence of single notes than multiple notes in this female-male interaction, which does not coincide with that observed in *D. ponderosae* and *D. approximatus* where multiple-note calls are predominant [29,30]. Unfortunately, it is also not possible to compare spectral and temporal features between species, as these data have not always been reported.

It has been suggested that the production of interrupted calls is indicative of the vigor and fitness of males, which determines their preference for females [36]. Nevertheless, the ratio of both types of calls, the emission pattern and their temporal combination or alternation may also be a mechanism of intraspecific recognition and of interspecific reproductive isolation, especially when several species coexist in sympatry or syntopy. This is because it is widely recognized that *Dendroctonus* species can coexist in space and time in the same locality and tree. Some studies have shown that males approaching the female's gallery produce calls that cause the female to stop producing aggregation pheromones [46]. This may be associated with the colonization and mass attack strategy of these species.

Species			Temporal				Spectral			
			Tooth strike (no. /call)	**Tooth strike rate (no./s)**	Intertooth strike interval (ms)	Call duration (ms)	Minimum frequency (kHz)	Maximum frequency (kHz)	Dominant frequency (kHz)	Reference
Dendroctonus adjunctus	Stress	Single-noted	41.4±0.7	1034.3±26.5	0.9±0.03	40±1	2.1±0.14	8.6±0.03	6.5±0.06	Our data in this study
		Multiple-noted	27.6±6.5	585.7±75.2	1.6±0.23	47.2±5.5	4.3±0.04	8.7±0.08	6.7±0.3	
	Male-female	Single-noted	23.8±1.8	675±20.7	1.4±0.04	35.3±3.7	5.6±0.07	7.8±0.08	6.7±0.07	
		Multiple-noted	36.9±1.9	532±27.4	1.8±0.09	69.5±1.3	4.3±0.1	7.1±0.07	6±0.06	
	Male-male	Single-noted	16.4±1	611.7±15.5	1.6±0.04	26.8±2	2.7±0.19	8.4±0.07	6.6±0.06	
		Multiple-noted	21.9±0.3	549.6±24	1.8±0.07	40±1.5	2.5±0.41	8.5±0.26	6.2±0.11	
Dendroctonus ponderosae	Stress	Single-noted	17.4±1.8	828.5±59.8	1.4±0.1	21.8±1.7	ND	ND	15.6±2.8	[29]
		Multiple-noted	27.9±4.1	593.2±89.5	2.2±2.8	56.3±8.0	ND	ND	18.3±5.8	
	Male-female	Single-noted	21.3±3.5	786.0±63.7	1.4±0.1	30.0±6.8	ND	ND	26.0±4.6	
		Multiple-noted	35.0±3.5	433.1±18.8	2.6±0.1	90.0±6.5	ND	ND	21.9±5.9	
	Male-male	Single-noted	16.9±2.2	709.5±40.2	2.6±1.0	28.8±6.3	ND	ND	17.4±1.6	
		Multiple-noted	22.2±2.2	464.0±44.7	2.6±0.2	56.1±8.5	ND	ND	26.4±3.4	
Dendroctonus approximatus	Stress	Single-noted	55.1±0.6	517.6±23.0	ND	108.6±1.2	ND	ND	5.2±0.1	[30]
	Male-female	Single-noted	61.7±1.2	505.7±12.0	ND	124.5±2.7	ND	ND	5.6±0.12	
Dendroctonus adjunctus	Stress	Single-noted	ND	ND	ND	39.0±12.2	2.99±0.13	10.3±1.68	5.94±1.94	
Dendroctonus brevicomis	Stress	Single-noted	ND	ND	ND	68.1±27.4	3.86±0.74	14.3±4.21	6.03±1.50	
Dendroctonus frontalis	Stress	Single-noted	ND	ND	ND	59.9±19.2	3.85±0.73	18.2±3.84	7.99±4.36	
Dendroctonus pseudotsugae	Stress	Single-noted	ND	ND	ND	36.4±8.8	2.86±0.42	8.16±2.03	4.92±1.25	
Dendroctonus terebrans	Stress	Single-noted	ND	ND	ND	99.3±21.9	6.08±2.23	39.2±3.57	22.6±6.05	[16]
Dendroctonus terebrans	Stress	Single-noted	11.3±0.6	375±79	ND	31±6	ND	ND	ND	[47]

Table 3. Temporal and spectral measurements of the single-noted and multiple-noted calls in different species of the genus *Dendroctonus*.

Male-male interaction is a special case of stress to avoid physical aggression, which can impede the access of another male to the female or to block his entry into the gallery (territoriality). Although the proportion of single and multiple calls between the stress condition (90%-10%) and the male-male interaction (85%-15%) was similar in this study, significant differences between both conditions were mainly concentrated in temporal characteristics (e.g. rate of tooth strikes, interval between tooth strikes, call duration), suggesting the configuration of different calling patterns in these two stress conditions. The pattern of calls in the male-male interaction could be due to combinations of intimidation or deterrence to avoid physical contact. In fact, the proportion of single- and multi-note calls was similar in males that remained in the arena, but not in males that withdrew, which were only single-note. Similar results were obtained in *D. ponderosae* and were interpreted as a characteristic of rivalry between individuals of the same sex, regardless of the presence of a female or of maintaining or blocking the entrance to the gallery when occupied by a pair [29]. In contrast, in *D. valens*, the sound pattern produced by males was related to their body size, as males competing for females displayed two types of calls, the first was to prevent direct fighting with potential competitors of equivalent size, while the second was to scare away and deter small-sized competitors [48].

In the male-male interaction, both males *D. adjunctus* produced both types of calls while staying together, but when one of the males withdrew, not only did its aggressive behavior change, but also the call sound changed as they were 100% one-noted. The minimum frequency of retreat calls was twice the calls of males while interacting in territoriality, the maximum frequency was lower in retreat calls compared to calls of males that stayed together, and the dominant frequency was very similar in all interactions (Table 2). Differences between the calls of males that remained and those that withdrew showed a wider repertoire of signals compared to those known so far, introducing a new type of sound, the “withdrawal calls”.

These calls when exhibited in conjunction with flight behavior, may be an indicator of surrender by the male. Future studies may provide clarity on the behavioral implications of this type of calls.

Lastly, while AS (signals, auditory organs, stridulatory devices) in other insect orders (e.g., Orthoptera, Hymenoptera, Hemiptera) have been widely studied and associated with a wide range of biological behaviors and environmental factors, in bark beetles (Coleoptera: Curculionidae: Scolytinae) its integration with the chemical communication system, behavior and reproductive ecology has received little attention [1,33,49,50]. In particular, it would be desirable that future studies focus on aspects related to acoustic signals, which, being apparently species- specific, could be involved in isolation and reproductive behavior, as well as in pheromone synthesis, especially in species that produce these compounds *de novo*.

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SUPPLEMENTARY MATERIALS

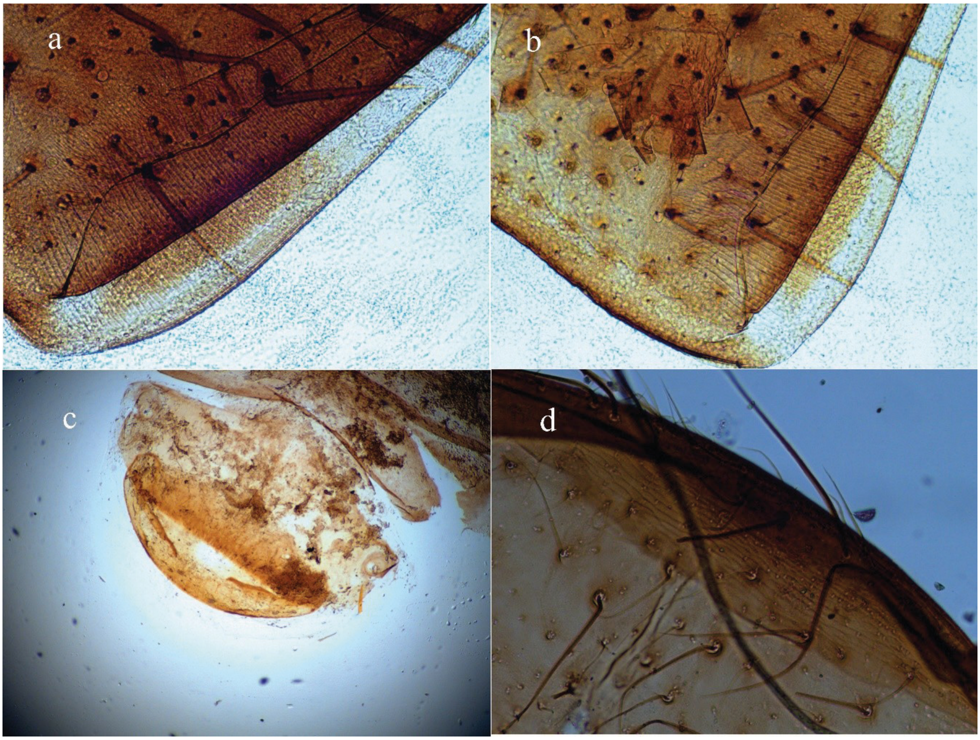

Figure S1. Structure of the stridulatory apparatus of *D. adjunctus* female. (**a** and **b**) left elytra, (**c**) absence of plectrum, and (**d**) pars stridens in the last sternite.

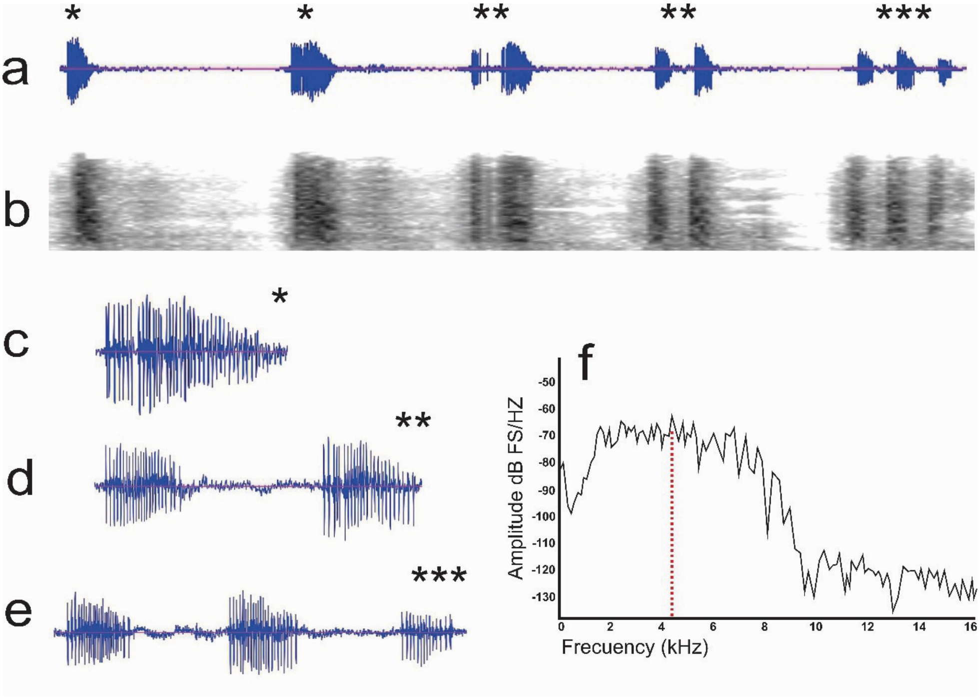

Figure S2. Wave diagram and spectrogram of the male of *Dendroctonus approximatus* in stress condition. (**a**) Wave diagram, single-noted call (*), multiple- noted call (two note ** and three note ***), and (**b**) spectrogram of the stress condition, amplification of a (**c**) single-noted call and multiple-noted call of (**d**) two note and (**e**) three note, and (**f**) single-noted call relative power diagram.