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ORIGINAL ARTICLE

Acoustic segregation of five sympatric and syntopic species of genus *Pristimantis* (Anura: Strabomantidae) from Western Colombia

Fray Geovanny Arriaga-Jaramillo [a,b*], Oscar Mauricio Cuellar-Valencia [a,b*], Isabella García-Gómez [a,b], Irene Ceballos-Castro [a,b], Wilmar Bolívar-García [a,b], David Andrés Velásquez-Trujillo [a,b], Ayda Susana Ortiz-Baez [a,c] and Jhon Jairo Ospina-Sarria [a]

[a]Calima, Fundación para la Investigación de la Biodiversidad y Conservación en el Trópico, Cali, Colombia; [b]Departamento de Biología, Grupo de investigación en Ecología Animal, Universidad del Valle, Cali, Colombia; [c]Marie Bashir Institute for Infectious Diseases and Biosecurity, Charles Perkins Centre, School of Life & Environmental Sciences and Sydney Medical School, University of Sydney, Sydney, Australia

ABSTRACT
The Andean Cordilleras of Colombia, especially the Cordillera Occidental, are among the areas with the highest diversity of *Pristimantis* frogs in the world. Within the Cordillera Occidental, the Serranía de los Paraguas is famous for its high diversity of sympatric species of *Pristimantis* frogs. In this study, we investigated acoustic frequency segregation in five sympatric and syntopic species of the genus *Pristimantis* inhabiting this Serranía. It is important to point out that three of the five species' calls are described for the first time: *Pristimantis brevifrons, P. ptochus*, and *P. silverstonei*. Our results showed that acoustic frequency segregation occurs among these five *Pristimantis* species. Another notable result was the inverse relationship between dominant frequency and male body size, which was corroborated in all species except in *P. alius*. Finally, we suggested that for improving our knowledge on species coexistence in *Pristimantis*, future research should also focus on species history and character evolution, given that the evolutionary history is also fundamental for explaining species coexistence.

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Introduction

Most works on amphibian acoustics have been concerned with signals and the structure of the communication system, either in a single species or in groups of species inhabiting the same area. In the case of acoustic signals among species with overlapping ranges ('sympatric' in its original evolutionary sense), one of the topics that has received more attention is acoustic segregation (Hödl 1977; Duellman & Pyles 1983; Herrick et al. 2018). Acoustic segregation is defined as all physical structural differences of the advertisement call among species in the same environment (Martins et al. 2006). Empirical research has documented acoustic segregation among sympatric congeneric species (Bignotte-Giró et al. 2019) as well as among sympatric non-congeneric species (Lüddecke et al. 2000). A very common assertion in the literature of acoustic segregation is that differences in mating calls may serve as an important mechanism in preventing mismatings and competition, thus promoting species coexistence within anuran communities (Hödl 1977; Lüddecke et al. 2000; Gröning & Hochkirch 2008).

Tropical anuran communities are of particular interest to study the mechanisms underlying species coexistence mainly because they are species-rich. In particular, one of the most diverse groups among land-living vertebrates in the tropics is the genus *Pristimantis*, in which approximately 570 species are recognized (Frost 2021). Notably, one of the areas with the highest diversity of *Pristimantis* frogs is the Cordillera Occidental of Colombia (Lynch 1998; Lynch & Grant 1998; Cuellar-Valencia et al. 2021). The Serranía de los Paraguas is a mountainous region situated in the central part of the Cordillera Occidental of Colombia and is known to harbor 38 *Pristimantis* species, of which 25 inhabit the Cerro El Inglés Nature Reserve, a peak in the Serranía de los Paraguas (Lynch 1998; Cuellar-Valencia et al. 2021).

Recent fieldwork in the Cerro El Inglés Nature Reserve yielded eight species of *Pristimantis* vocalizing sympatrically and syntopically – *Pristimantis alius* Cuellar-Valencia et al. 2021, *P. brevifrons* (Lynch 1981), *P. ingles* Cuellar-Valencia et al. 2021, *P. myops*

CONTACT Jhon Jairo Ospina-Sarria sarriajhon@gmail.com

*These authors contributed equally to this work

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(Lynch 1998), *P. phalarus* (Lynch 1998), *P. quantus* (Lynch 1998), *P. ptochus* (Lynch 1998), and *P. silverstonei* (Lynch & Ruiz-Carranza 1996). Despite spending a great effort to secure acoustic data for all eight species, we were able to secure advertisement calls for five species (except *P. myops, P. phalarus* and *P. quantus*). More importantly, although these eight species belong to the *Pristimantis* genus, their phylogenetic relationships are still poorly understood given that only *Pristimantis brevifrons* has been incorporated in phylogenetic studies involved in overhauling the taxonomy of this genus (e.g. Waddell et al. 2018). *Pristimantis* species deposit terrestrial eggs that undergo direct development, and the only ecological mechanism recognized for explaining coexistence among both related and non-related sympatric species is in habitat use (Lynch & Duellman 1997; Guayasamin & Funk 2009). Considering that acoustic segregation is recognized as an additional mechanism of coexistence (Gröning & Hochkirch 2008), here we test the occurrence of acoustic segregation among five sympatric and syntopic species of the genus *Pristimantis* inhabiting Cerro El Inglés Nature Reserve, Colombia.

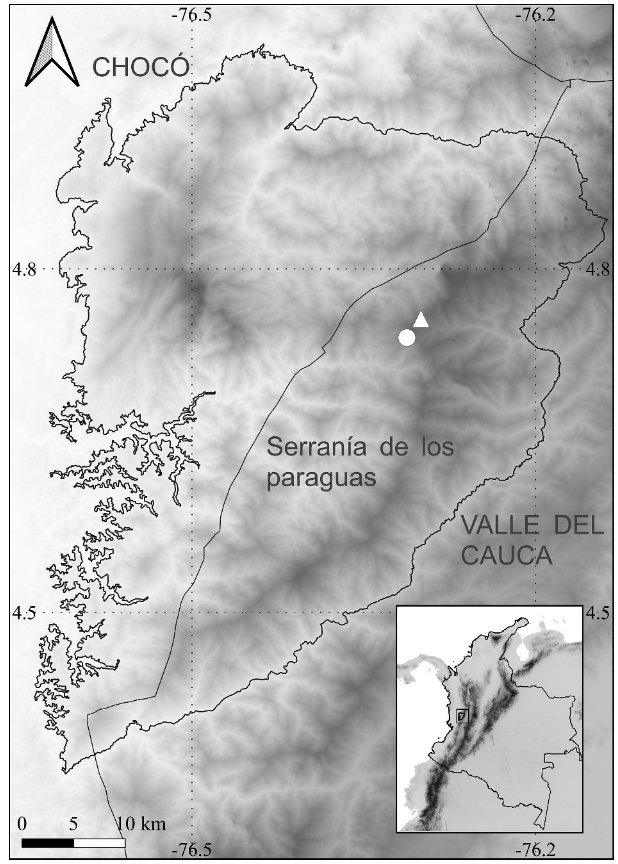

Figure 1. Map of southwestern Colombia (inset) showing sites surveyed in the Serranía de Los Paraguas: Boquerón (white circle) and Santicos (white triangle).

Materials and methods

Study area

The study was conducted at the Cerro El Inglés Nature Reserve, a protected area located in the Serranía de los Paraguas on the frontier between Chocó and Valle del Cauca departments, Western Colombia (04°44.2'N, 76° 18.3'W; datum = WGS84) (Figure 1). The reserve occupies an area of approximately 756 hectares between 1800 and 2500 m above sea level. This area is characterized by rainy weather with frequent fog and high humidity, and by a high diversity of wildlife (Cuellar-Valencia et al. 2021). In addition, the Cerro El Inglés provides a variety of ecosystem services. For example, Las Amarillas microbasin originates in this protected area, which provides water supply to local communities and it is an affluent of the Garrapatas River in the San Juan River macrobasin.

Field techniques

Acoustic recordings were carried out during a 9-day fieldwork exploration (19–27 July 2019) at the Cerro El Inglés Nature Reserve, a peak in the Serranía de los Paraguas. During the expedition, we visited two sites (Figure 1): Boquerón (4° 44'23.5"N, 76°18'44.2"W; datum = WGS84) and Santicos (4° 45'23.4"N, 76°17'59.0"W; datum = WGS84), which were surveyed along transects in daylight from 0900 to 1300 h and darkness from 1900 to 0100 h. During these surveys, we recorded individuals of five *Pristimantis* species vocalizing: *P. alius, P. brevifrons, P. ingles, P. ptochus*, and *P. silverstonei.*

The calls were recorded under field conditions using a TASCAM DR-100mkll LINEAR PCM recorder without external microphone. The recorder was placed at approximately 30 cm from each of the recorded specimens. All the recorded calls were digitized with a sampling rate of 44,100 Hz and 16 bits per sample in the mono pattern. At the end of each recording, the body temperature of the frog and the temperature were measured using a Testo 810 Infrared thermometer. Each frog was captured and tagged for positive association with the recording. Voucher specimens were euthanized by immersion in a 2% xylocaine solution and fixed in 10% formalin, and preserved in 70% ethanol. Snout-vent length (SVL) was taken with a digital caliper (± 0.01 mm) under Leica dissecting microscope. In the field, geographic coordinates were determined using two handheld GPS satellite receivers. All tapes and preserved voucher specimens are deposited in the Colección de Prácticas Zoológicas Universidad del Valle (CPZ-UV).

Identification of species was achieved using original descriptions, available redescriptions of species, and direct comparison of the collected material with type specimens. All specimens examined are listed in the Appendix A. Institutional abbreviations are as follows: CPZ-UV (Colección de Prácticas Zoológicas, Universidad del Valle, Cali, Colombia), ICN (Instituto de Ciencias Naturales, Museo de Historia Natural, Universidad Nacional de Colombia, Bogotá, Colombia), KU (Biodiversity Institute, University of Kansas, Lawrence, Kansas, USA).

Data analysis

The acoustic analyses were performed on Raven Pro v1.5 for Windows (Cornell Laboratory of Ornithology, Ithaca, NY) with a Blackman algorithm and a window size of 1024 samples. We used the note-centered approach (defining uninterrupted units of sound as notes and their entirety as call) and the terms and definitions for the acoustic parameters as defined by Köhler et al. (2017). Eleven variables of the temporal structure of the advertisement calls of the species were quantified: 1) notes per call, 2) note duration (in seconds = s), 3) internote duration (s), 4) note period (s), 5) call duration (s), 6) note repetition rate (s), 7) amplitude modulation, 8) pulse per note, 9) pulse repetition rate (s), 10) call repetition rate (in minutes = m), and 11) intercall intervals (s). Likewise, we quantified two variables in the spectral structure of the advertisement calls: 1) dominant frequency of the call (i.e. the frequency containing the highest sound energy; Hz), 2) bandwidth where 90% of the call energy is concentrated (difference between frequencies 95% and 5%).

To estimate all variables, the advertisement calls were visualized and measured in oscillograms and spectrograms. All results were expressed in the format average ± standard deviation (x ± SD). For exploring differences in acoustic variables among species, we implement a Principal Component Analysis (PCA) using all quantitative variables of temporal structure and the dominant frequency of the call (i.e. notes per call, note duration, internote duration, note period, call duration, note repetition rate, pulse per note, pulse repetition rate, call repetition rate, intercall intervals, and dominant frequency of the call). The other spectral structure variables were not included because they may be affected by background noise or technical artifacts (Köhler et al. 2017). Considering that the dominant frequency is often inversely correlated with the male body size (e.g. Hödl 1977; Duellman & Pyles 1983; Lüddecke et al. 2000; Wells 2007), a correlation analysis was performed by directly computing the Spearman correlation coefficient among these variables. The oscillograms and spectrograms were generated using the R programming version 4.0.5 (R Core Team 2021) through R package Seewave 2.1.4 (Sueur et al. 2008) in the RStudio software 1.1.442/Windows (2009–2018 R Studio, Inc.).

Results

The advertisement calls of *Pristimantis brevifrons, P. ptochus*, and *P. silverstonei* are described here for the first time, while the advertisement calls of *P. alius* and *P. ingles* were described by Cuellar-Valencia et al. (2021). Although all five species of *Pristimantis* were observed actively vocalizing in both sites, we only obtained call recordings for *P. ptochus* at both sites. In total, seven males were recorded as follows: Site 1–Boquerón: *P. ingles* (n = 1), *P. ptochus* (n = 1), and *P. silverstonei* (n = 1). Site 2–Santicos: *P. alius* (n = 2), *P. brevifrons* (n = 1), and *P. ptochus* (n = 1). A ninth *Pristimantis* species was found syntopically at Site 1 – *P. restrepoi* (Lynch 1996); however, as far as it is known, the species is mute.

Call descriptions

The advertisement call of *P. brevifrons* (Figure 2b) resembles a 'bell' sound. It is composed of a short tonal single note. The notes are separated by intervals of silence (i.e. 100% amplitude modulation). The call is 0.031–0.057 sec in duration (0.041 ± 0.007; n = 20). The call repetition rate is of 19.353 calls per minute (n = 1). The inter-call interval is of 2.532–3.9871 sec in duration (3.06 ± 0.39; n = 19). The bandwidth 90% of the call corresponds to 344.5–818.3 Hz (557.72 ± 115.63; n = 20), which is concentrated between a maximum frequency at 95% energy of 6503 Hz and a minimum frequency at 5% of energy of 5684.8 Hz. These calls were obtained from one male (CPZ-UV 6851) that was found calling at night above vegetation approximately 190 cm high, at an environmental temperature of 16.2°C and a body temperature 15.8°C.

The advertisement call of *P. ptochus* (Figure 2d) consists of 4–7 equal tonal notes (4.51 ± 0.74; n = 43) widely separated. The notes are separated by intervals of silence (i.e. 100% amplitude modulation). The call is 0.436–1.031 sec in duration (0.577 ± 0.136; n = 26), with notes of 0.024–0.057 sec in duration (0.037 ± 0.008; n = 194), and inter-notes of 0.082–0.177 sec in duration (0.116 ± 0.018; n = 151). The call repetition rate is 4.870–6.409 calls per minute (5.385 ± 0.694), the note repetition rate is 5.814–7.557 notes per second (6.552 ± 0.534). Inter-call interval is 4.412–24.115 sec in duration (9.766 ± 3.637; n = 39). The dominant frequency is 3316.1–4091.3 (3712.933 ± 254.088; n = 43). The bandwidth 90% of the call corresponds to 172.3–387.6 Hz (219.419 ± 44.416;

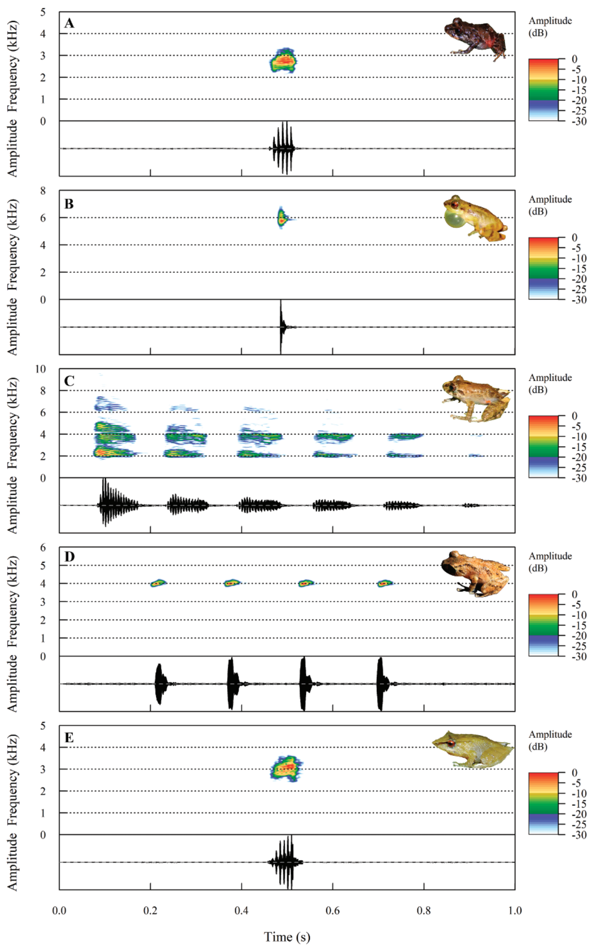

Figure 2. Oscillograms and spectrograms in a 1-seconds time-window showing temporal features of representative advertisement calls of the five species in this study. A. *Pristimantis alius*, B. *Pristimantis brevifrons*, C. *Pristimantis ingles*, D. *Pristimantis ptochus*, E. *Pristimantis silverstonei.*

n = 42), which is concentrated between a maximum frequency at 95% energy of 4220.5 Hz and a minimum frequency at 5% of energy of 3186.9 Hz.

These calls were obtained from two males (CPZ-UV 6852, 6853) that were found calling at night above low vegetation up to 100 cm in height. The body temperature was 14.7°C and 13.5°C, whereas the environment temperature was 17.7°C and 20.2°C, respectively.

The advertisement call of *P. silverstonei* (Figure 2e) is similar to 'cricket' sounds. It is composed of a short single note distinctly pulsed, with 6–10 pulses per note (7.33 ± 1.5; *n* = 9). The notes are separated by intervals of silence (i.e. 100% amplitude modulation). The call is 0.047–0.073 sec in duration (0.059 ± 0.007; *n* = 9), with a call repetition rate of 0.791–0.869 calls per minute (0.829 ± 0.053), and an inter-call interval of 46.4–85.07 sec in duration (70.071 ± 14.465; *n* = 7). Pulse repetition rate is 111.111–191.489 pulses per second (147.478 ± 31.291; *n* = 9). The dominant frequency is 2971.6–3402.2 Hz (3215.6 ± 153.757; *n* = 9). These calls were obtained from a single male (CPZ-UV 6857) that was found calling above low vegetation up to 100 cm height. A peculiarity of the advertisement calls of *P. silverstonei* is its low volume compared to natural background noise, for which it was difficult to accurately measure the bandwidth where 90% of the energy of the call concentrates, so we decided not to report this value. Body and environmental temperatures were not registered.

Acoustic segregation

All species show clearly separated calls (Figure 3). These results also show that 89.6% of variance is explained by two components, where the first component accounted for 51.3% and the second explained 38.3% of total variance (Table 1). Correlation analysis between frequency and male body size showed that the dominant frequency decreases in larger species (Spearman correlation r^2 = 0.734694; P-value = 0.02381; Figure 4a). The only exception to this trend was observed in *P. alius*, which presented a lower dominant frequency than expected for its body size.

Evident frequency segregation was found among the five species of *Pristimantis* (Figure 4b). Frequency ranges are as follows: *P. alius* 2670.1–2885.4 Hz, *P. brevifrons* 5814–5857 Hz, *P. ingles* 2368.7–2454.8 Hz, *P. ptochus* 3316.1–4091.3 Hz, and *P. silverstonei* 2971.6–3402.2 Hz. The results also showed distinctive variation in dominant frequency range for *P. ptochus* between sites, being 3962.1–4091.3 Hz in Boquerón and 3316.1–3574.5 Hz in Santicos (Figure 4c).

Discussion

Our results revealed that acoustic frequency segregation occurs in five sympatric and syntopic species of the genus *Pristimantis* inhabiting the Cerro El Inglés Nature Reserve, Western Colombia. Similar findings have been reported among sympatric congeneric species of the genus *Eleutherodactylus* from Puerto Rico and Cuba (Villanueva-Rivera 2014; Bignotte-Giró et al. 2019). Of particular interest was the lack of overlap in frequency ranges among sites for *Pristimantis ptochus* (Figure 4c). Although our sample size is limited, our results agree with those reported in the literature (i.e. dominant frequency varies with temperature and elevation Narins & Meenderink 2014). Our study sites are separated by 2.3 km in distance and have a difference of 136 m in elevation (2315 m in Santicos and 2179 m in Boquerón). Contrary to

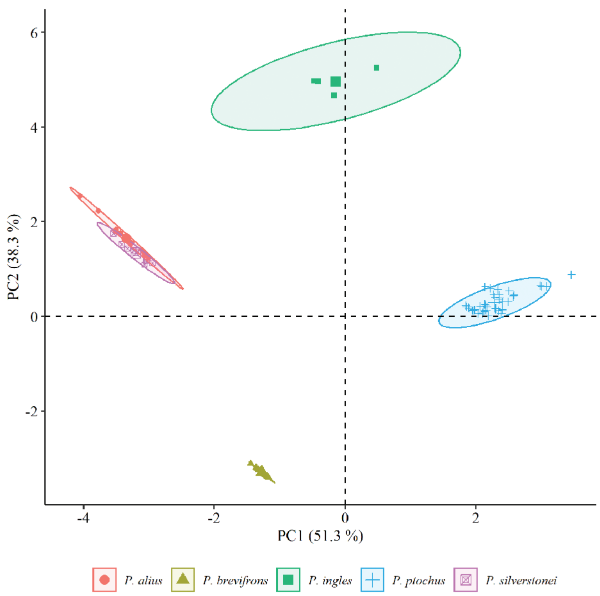

Figure 3. A principal component analysis plot showing (dis) similarity among the respective five species of *Pristimantis* based on eleven variables of the advertisement call (call duration, notes per call, note duration, internote duration, note period, note repetition rate, pulses per note, pulse repetition rate, call repetition rate, intercall intervals and dominant frequency).

Table 1. Contribution of the call variables in percent (%) to first two principal components of the PCA.

Call variables	PC1 (51.3%)	PC2 (38.3%)
Call duration	11.54	5.96
Notes per call	12.87	4.73
Notes duration	8.39	7.08
Internote duration	15.86	1.65
Note period	15.13	3.11
Note repetition rate	14.93	2.77
Pulses per note	6.08	12.03
Pulse repetition rate	8.19	10.3
Call repetition rate	0	20.76
Intercall intervals	6.99	10.34
Dominant frequency	0.01	21.28

what was expected, that is to say, temperatures decrease with elevation, the higher environmental temperature was recorded at the higher site – Santicos, probably due to differences in vegetation structure. Since Boquerón has less vegetation cover, it is likely to exhibit a higher fluctuation in temperature than Santicos. The body temperature of *P. ptochus* was higher in the specimen recorded in Boquerón (14.7°C) than in that recorded in Santicos (13.5°C). That is, our results suggest that differences of 1.2°C in body temperatures change the call frequency range in *P. ptochus*. However, as noted above, the sample size is too small to draw reliable conclusions. Further research is in need to corroborate or refute these findings.

Another notable result was the inverse relationship between dominant frequency and male body size, which was corroborated in all species except in *P. alius* (Figure 4a). In terms of the general structure ("cricket" sounds composed by single short notes distinctly pulsed) and male body size, *P. alius* resembles *P. silverstonei* (Figure 2a, e). Thus, the noteworthy finding registered in *P. alius* could be interpreted as an explanation for non-overlapping with *P. silverstonei*, since given their body size, they are predicted to overlap in frequency (Figure 4). A similar result was reported by Bignotte-Giró et al. (2019) for *Eleutherodactylus gundlachi* in Cuba. At the moment, no data are available about temporal and spatial segregation of calls among the species studied here. Nevertheless, during the field surveys, we only heard calling activity *P. silverstonei* throughout the day. Therefore, it is possible that in addition to the acoustic segregation, some degree of temporal and spatial segregation may also be occurring among these species at the Cerro El Inglés Nature Reserve.

Conclusions

There is accumulating evidence that acoustic segregation occurs in a wide range of anuran taxa. More importantly, such evidence has been mainly suggested as a way to prevent reproductive interference, thereby promoting species coexistence. Herein, we provided evidence of acoustic segregation in five sympatric and syntopic species of one of the most diverse genera among land-living vertebrates in the tropics – *Pristimantis*, for which the most obvious segregation among both related and non-related sympatric species is in habitat use (Lynch & Duellman 1997; Guayasamin & Funk 2009). Considering that all the species studied here are arboreal, we hypothesized that in addition to acoustic segregation, some degree of temporal and spatial segregation could be expected among these species given that segregation in time and space is a widespread mechanism of coexistence (Gröning & Hochkirch 2008). Thus, future studies should also focus on other ecological mechanisms, such as temporal and spatial segregation, as well as the occurrence of other systems of communication such as movements and color signals for courtship behavior and mate-choice in this megadiverse clade of frogs. Finally, it should be pointed out that it is certainly challenging to distinguish currently acting mechanisms from evolutionary responses to the former selective pressures associated with species coexistence (Gröning & Hochkirch 2008). Therefore, if we are going to improve our knowledge on species coexistence, future research should also focus on species history and character evolution, given that the evolutionary history is also fundamental for explaining species coexistence.

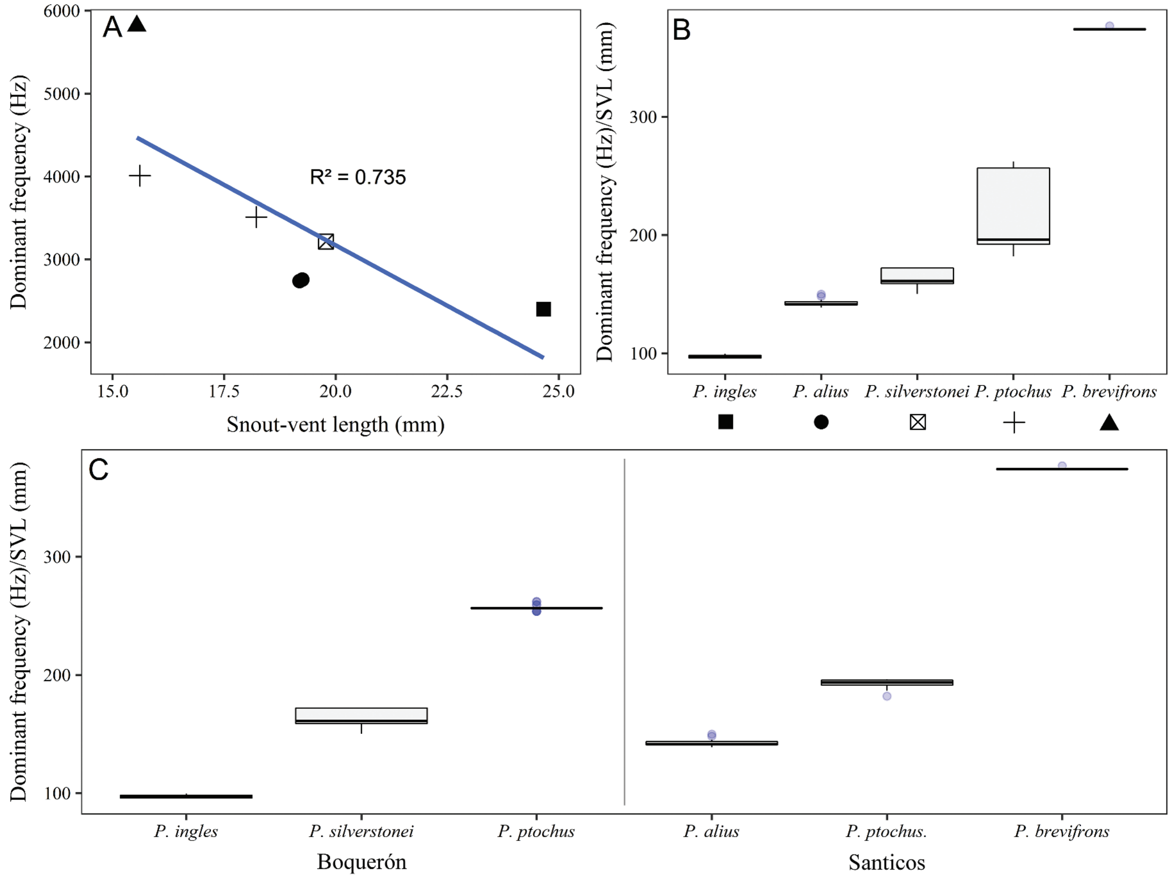

Figure 4. Analyses showing body sizes against dominant call frequencies and frequency range occupied by males of the five frog species in the Serranía de Los Paraguas. (A) Dominant frequencies plotted versus male body sizes (mean SVL of each species); (B) Dominant frequency range controlled by body sizes for the five *Pristimantis* species; (C) Dominant frequency range controlled by body sizes for the five *Pristimantis* species among sites.

Acknowledgments

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Data availability statement

The data that support the findings of this study are openly available in Figshare at https://figshare.com/s/7e4bf70be70fad8b02ab, reference number [22275873, 22276050, 22276236].

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Fray Geovanny Arriaga-Jaramillo http://orcid.org/0000-0002-3015-564X
Oscar Mauricio Cuellar-Valencia http://orcid.org/0000-0003-1285-716X
Isabella García-Gómez http://orcid.org/0000-0003-3326-0290
Irene Ceballos-Castro http://orcid.org/0000-0001-7478-7836
Wilmar Bolívar-García http://orcid.org/0000-0002-9395-721X
David Andrés Velásquez-Trujillo http://orcid.org/0000-0001-9971-8431
Ayda Susana Ortiz-Baez http://orcid.org/0000-0003-0080-0633

Jhon Jairo Ospina-Sarria http://orcid.org/0000-0002-9099-7793

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Appendix

Appendix A. Specimens examined from Colombia

Pristimantis alius. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Reserva Natural Comunitaria Cerro El Inglés (CPZ-UV 6123-30).
Pristimantis brevifrons. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Reserva Natural Comunitaria Cerro El Inglés (CPZ-UV 6851). CAUCA: Parque Natural Nacional Munchique, W slope Cerro Charquagayo, 38 Km NW Uribe, 2240 m (KU 169006).
Pristimantis ingles. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Reserva Natural Comunitaria Cerro El Inglés (CPZ-UV 6121-22).
Prisitmantis myops. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Vereda las Amarillas, El Boqueron (ICN 36925, 39684).
Pristimantis phalarus. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Vereda las Amarillas, El Boqueron (ICN 36934, 39678).
Pristimantis ptochus. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Reserva Natural Comunitaria Cerro El Inglés (CPZ-UV 6852-53). COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Vereda las Amarillas, El Boqueron (ICN 39780).
Pristimantis quantus. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Vereda las Amarillas, El Boqueron (ICN 29340).
Pristimantis silverstonei. – COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Reserva Natural Comunitaria Cerro El Inglés (CPZ-UV 6857). COLOMBIA: VALLE DEL CAUCA: Municipio El Cairo, Vereda las Amarillas, El Boqueron (ICN 29045).