Variation in Bioacoustic Characteristics in *Eleutherodactylus coqui* Thomas, 1966 and *Eleutherodactylus antillensis* (Reinhardt and Lutken, 1863) (Anura: Eleutherodactylidae) in the Puerto Rico Bank

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**Abstract:** Bioacoustics is an interdisciplinary science that combines biology, acoustics, and mathematics. This discipline can be used to study population ecology and behavior. Furthermore, we can use this tool to assess a population and suggest if a species of interest may be in a transitional state of becoming a new species by allopatric speciation. Amphibians communicate via sound and the environment has a key role in metabolism and sound dispersion. By analyzing temporal and spectral properties of acoustical communication in anurans, we can understand better how these animals are evolving to cope with their ever-changing environment. We studied the variation in acoustic parameters among five populations each of the red-eye coqui, *Eleutherodactylus antillensis* (Reinhardt and Lutken, 1863) and the common coqui, *E. coqui* Thomas, 1966 across the Puerto Rico Bank. These species are changing their vocalizations. Some populations have higher sound frequencies than other conspecific populations; other nocturnal species have populations with different temporal patterns of sound production. We found strong variation among the five populations examined for each species. In, *E. antillensis*, the size of the organism relates to temporal variation in sound production (i.e., inter-note interval and total call duration) and did not relate to spectral differentiation. In *E. coqui*, the population living at highest elevation above sea level assessed had a spectral footprint no other population shares, probably due to geographic isolation from other conspecific populations that live in lower elevations.

**Keywords:** Bioacoustics, *Eleutherodactylus*, *Eleutherodactylus coqui*, *Eleutherodactylus antillensis*, Puerto Rico, microevolution, communication, sound spectrogram, speciation,

**Introduction**

**Natural History**

Amphibians are important components of many ecosystems because their total number and biomass affect ecosystem function through complex trophic interactions (Dodd 2010). However, these vertebrates are not immune from the ravages that are happening on Earth. Herpetofaunas are susceptible to sudden environmental changes (Sala et al. 2000, Cushman 2006), and these changes can either diminish their populations or, in extreme cases, extinguish them. Some of
the factors that affect animal populations are land use, such as agriculture, recreational uses, construction of urban areas and military training sites; all leading to habitat fragmentation (Gibbons et al. 2000, Thrush et al. 2008, Ríos-Franceschi et al. 2016). In such cases, many amphibians are unable to escape from the area that has been fragmented or modified, mainly because of their low mobility (Vredeburg and Wake 2007).

Puerto Rico lies within the Caribbean biodiversity hotspot (Cox and Moore 2000). Puerto Rican diversity consists of approximately 26,410 species of plants, fungi and animals (Joglar 2005). These species, within the Puerto Rican Bank have been isolated, by physical barriers, such as by the rising sea levels after the last glacial maximum (Heatwole et al. 1981 and Figure 1, below) for approximately 8,000–10,000 years, creating different niches and augmenting the diversity in the archipelago. This can provide a privileged location to study evolution in a microscale for many different taxa, including amphibians.

![Figure 1](image-url)  
**Figure 1.** Map of the Puerto Rican Bank (a and b) and St. Croix (c) illustrating the topography of the islands and the approximate geographic location of the sampling localities of *Eleutherodactylus antillensis*. The outermost line in a, b and c indicates the approximate land configuration at maximum sea level (-120 m, Siddall et al. 2003), during the Last Glacial Maximum (circa 26.5-19 kya). The thicker line depicts the current extent of land area. Map was modified from Barker et al. 2012 by José R. Almodóvar.

Overall morphological similarity and intrapopulation variation of morphological features make some species identification challenging (Angulo and Reichle 2008, Bickford et al. 2007). Species descriptions for a variety of taxa have traditionally relied primarily on morphological traits (Mayden 1997, Angulo and Reichle 2008). Some cryptic species cannot be identified by morphological analysis alone and scientists need to use other characteristics. Acoustic signals are the main vehicle of communication in most anurans (Ryan 1988, Gerhardt and Huber 2002, Röhr and Juncá 2013). Herpetologists often use acoustic, or sonic, signals in species identification and diagnosis for taxa that use such signals to communicate (Angulo and Reichle 2008). For example, advertisement calls have
been used to resolve the systematics of several groups within the family Eleutherodactylidae (Heyer 1984, Angulo and Reichle 2008). Although most species may have more than one kind of vocalization, the advertisement call is the most widespread and it is the focus of most bioacoustics’ studies of frogs (Gerhardt 1994, Gerhardt and Huber 2002, Röhr and Juncá 2013). Advertisement calls are conspicuous acoustic signals emitted by males of most species of anurans (Erdtmann et al. 2011). These calls generally have multiple purposes, such as female attraction, territory defense against conspecific males (Duellman and Trueb 1994, Wells 2007; Erdtmann et al. 2011, Papes and Ladich 2011, Röhr 2013, Velázquez et al. 2013) and informing about a male’s physical condition. On the other hand, animal communication signals have an important role in species divergence, promoting reproductive isolation and speciation (Coyne and Orr 1998, Velázquez et al. 2013). Variation of behavioral characters across their geographical distribution has been the subject of studies aiming to establish the degree of divergence among populations (Coyne and Orr 1998; Stafförd et al. 2001, Coyne and Orr 2004, Quispe et al. 2009, Velázquez et al. 2013).

Clinal variation of advertisement calls related to geographical distance between populations (Nevo and Capranica 1985; Wilczynski and Ryan 1999; Castellano et al. 2002; Giacoma and Castellano 2001; Bernal et al. 2005; Pröhl et al. 2006, 2007; Velázquez et al. 2013) or the relevance of adaptations to different environmental conditions (Hunter and Krebs 1979, Boughman 2002, van Dongen et al. 2010) has been reported for different anurans. These conditions have been affected by climate change and these effects force species to meet dynamic thermal environments and may jeopardize the performance of their basic physiological functions, especially in ectothermic organisms, like frogs (Pörtner and Knust 2007, Huey et al. 2009, Kearney et al. 2009, Duarte et al. 2011, Llusia et al. 2013). Predictions of the compensatory response of ectotherms to climate change, which is an urgent and challenging task in current biology, depend upon comprehending the ecological performance of key biological functions under different thermal environments (Pörtner and Knust 2007, Buckley 2008, Huey et al. 2009, Kearney et al. 2009), and the mechanisms that underlie the thermal adjustments of the species (Helmuth et al. 2005, Angilletta 2009, Llusia et al. 2013).

The effects of temperature on anuran vocalizations

Ectothermic animals are dependent on environmental heat sources and control their body temperature through external means (Papes and Ladich 2011). Compared to endothermic animals, they conserve relatively low metabolic rates. In general, the speed of all metabolic processes is influenced by the body temperature, which, for ectothermic organism, depends on the ambient temperature (Cossins et al. 1977, Salem and Omura 1998, Navarro 2002, Andersson 2003, Itoi et al. 2003). Ambient temperature affects various physiological processes, such as neuronal and muscular activities, including all

Studying contemporary populations may help reveal selective forces that have shaped and maintained, vocal traits (Gerhardt and Huber 2002), thereby providing a better understanding of inter- as well as intraspecific variation in anuran vocalization (Röhr 2013). Some of this variation may be attributed mainly to sexual selection in a strict sense (Wilbur et al. 1978, Gerhardt 1991, Márquez 1995, Rosso et al. 2006, Röhr and Juncá 2013) and some to environmental limitations (e.g., Ryan et al. 1990; Ryan and Wilczynski 1991, Kime et al. 2000, Castellano et al. 2003, Röhr and Juncá 2013). For example, the transmission of a sound signal can be affected by its acoustic characteristics (Morton 1975, Marten et al. 1977, Ryan and Sullivan 1989, Kime et al. 2000) as well as by a variety of other factors such as habitat and vegetation structure (Wiley and Richards 1978, Sorjonen 1986, Ryan et al. 1990, Endler 1992), ambient noise (Feng et al. 2006, Preininger et al. 2007), climatic conditions (Wiley and Richards 1978), and height of vocalization perch (Wiley and Richards 1978, Wilczynski et al. 1989, Kime et al. 2000, Papes 2011). Furthermore, ambient temperature affects hearing in invertebrates and ectothermic vertebrates (Narins 2001, Papes 2011). Such effects have been tested in amphibians (Benedix et al. 1994, Egert and Lewis 1995, Long et al. 1996, Van Dijk et al. 1997) and reptiles (Eatock and Manley 1981, Smolders and Klinke 1984, Papes 2011). For these reasons, we analyzed the variation in temporal and spectral properties of these populations and how these variation affects anuran communication by the means of sexual selection.

**Methods**

The sound patterns of two common species of eleutherodactylid frogs *Eleutherodactylus coqui* Thomas, 1966 and *Eleutherodactylus antillensis*
(Reinhardt and Lutken, 1863) (Joglar 1998, Rivero 1998) (Figure 2), were recorded across Puerto Rico during 2016-2018. A total of 731 minutes from ten different sites (approximately 73.0 minutes for each site) were recorded and analyzed (Figure 3).

**Study localities**

From these results, six major localities were selected and documented: (1) Mayagüez (University of Puerto Rico, Mayagüez Campus’, Urban Forest. Elevation: 24.0 m), (2) Utuado (Río Abajo State Forest. Elevation: 426.0 m), (3) Arecibo (Islote, near Caño Tiburones Reserve. Elevation: 3.0 m), (4) Río Piedras (University of Puerto Rico, Río Piedras Campus. Elevation: 26.0 m), (5) Jayuya (Coabey, near Tres Picachos Reserve. Elevation: 996.0 m), and (6) Culebra Island (Mount Resaca. Elevation: 130.0 m). These sites were selected for their variation in elevation and climatic conditions. The other four sites were eliminated due to duplicity of data collection near two sites: Utuado (three sites) and Mayagüez (one site) area.

**Sonic data collection**

We used in-site audio recordings (.WAV format). This technique allows the researcher to record a specific individual while calling, which adds specificity and precision to the research. For this task a hand-held recorder, H6 Sound Recorder from Zoom Laboratories® was used. Data was analyzed using Raven Pro Sound Analysis Software® from Cornell University’s Ornithology Laboratory. This software processes the field data and extracts information such as spectrogram, frequency, call length, power (dB), energy (dB) and amplitude.

**Morphometric measurements**

Five morphometric measurements were taken from each frog recorded: snout-vent-length (SVL), head diameter (from eye socket to eye socket), tibio-
fibula length (TL), tympanic diameter (TYM) and weight. Measurements were taken using a digital caliper (Calipro®, 0.02 mm), for weight a spring scale (Pesola®, 100.00 g) and for body temperature a laser thermometer (Etekcity®, <100°C ±2.0°C).

**Statistical analyses of morphometric measurements**

A Student’s t-test was used to observe differences in morphometry (SVL, TL, TYM, head diameter, and weight), of the collected individuals. Furthermore, to estimate if there were differences in acoustic variables across populations, we used ANOVA. For the latter, we used the Shapiro-Wilks normality test (n = 10, p > 0.05) and Q-Q plots to test whether our data was normally distributed, reducing “type 1” error (Figure 4). Moreover, to examine which populations had similarities between these variables, the Fisher’s Least Significant Difference (LSD) test (Meier 2006) was used to estimate these differences by comparing mean values of acoustic variables in the ANOVA such as duration of each note, inter-note interval, and frequencies. As a result, these populations can be clustered according to their vocal characteristics. These analyses provide information about the possible plasticity or microevolution occurring in the species.

Figure 4. Q-Q plot for normal distribution data using morphometric variables. A, *Eleutherodactylus antillensis* and, B, *E. coqui*. 
**Sonic data collection**

In each site, two species of the genus *Eleutherodactylus* were recorded between 20:00–02:00. After encountering a calling male, we recorded individuals calls with a shotgun microphone (Zoom® SGH-6) data from 1.0 m away (Figure 5) as proposed by Meenderink et al. (2010), Narins and Meenderink (2014).

![Figure 5. Recording distance between the shotgun microphone and the target amphibian (1.0 meter).](image)

Every sample was analyzed using *Raven Pro®* software to extract the best recordings (e.g., highest signal-to-noise ratio). Each recording was an individual frog calling at the site. At that point, 30 recordings of one minute each within each site were selected and analyzed to determine the lowest frequency, highest frequency, power (dB), energy (dB) and call length of each individual note of the call of each species for a total of 150 recordings for *E. coqui* and 151 recordings for *E. antillensis*. Sound spectrographs were used in identifying the species courtship notes, which vary depending on the behavioral context of the caller (e.g., another male or female nearby) (Figure 6). Also, a rare form of a call was found during the analysis, this call was found only in one specimen of *E. coqui* in the Utuado area (Figure 7).

Statistical analyses of sonic data

The following statistical analyses were performed using (Info Stat®, 2012). An analysis of variance (ANOVA) was performed for *Eleutherodactylus antillensis* and for *E. coqui* to examine if the localities or different environmental factors investigated were associated to the differences in the variables of interest, such as lowest frequency, highest frequency and call length (n =150 for *E. coqui* and n = 151 for *E. antillensis*, p-value < 0.0001; each value represents a recording from a male).

Furthermore, we wanted to test if the variables described above (“CO” “QUI” and “CHU” “RI”) were being affected in the same way in each locality. To do this, we used a Fisher’s LSD test with an alpha = 0.05. This test compares each variable independently with each locality and groups them with similar variances. This test helps us estimate which populations are most related. The “coqui” and “churi” calls were divided into two distinct notes. These notes were selected as independent and were analyzed as follows: highest and lowest frequency, energy (d), power (dB), call length (s) and amplitude. Also, the entire call length and time between each note was measured for each call (Figure 8). Additionally, linear regressions were performed to establish relations between body morphology (SVL) and frequency.
Results

Morphology, body temperature, and acoustics of Eleutherodactylus coqui

Common organisms, such as Eleutherodactylus coqui, can be more amenable for research owing to its widespread distribution and environmental diversity that can be suited for comparative analysis. For this species, five localities were selected, from coastal areas to high elevation mountains. Morphological, body temperature, (Table 1), as well as acoustic data (Tables 2 and 3) were garnered. For acoustic data, ANOVA yielded significant differences between all populations across Puerto Rico (n = 150, p-value < 0.0001) for all variables, including a small difference for the highest frequency for the “CO” note (n = 150, p-value < 0.0004). Temperature data did not relate to any other variable.

By analyzing these notes individually, we can assess small fluctuations in sound that can affect intraspecific communication. Calling behavior plays a key role in frog reproduction and defense of territory. The “CO” note refers to a territorial call by which males defend their territory against other males. The “QUI” note functions as a call to attract females (Narins and Capranica 1978, Narins and Smith 1986). Additionally, as part of the analysis, total call duration was measured. Call duration (seconds), can be correlated to anuran metabolism. Individuals located in colder environments tend to have a slower call than its cohorts in higher temperatures due to changes in metabolic rate. Here, we measured the notes independently, “CO” and “QUI” and in their entirety, “CO QUI”.
Table 1. Mean and standard deviation for morphological traits and body temperature of *Eleutherodactylus coqui* for localities with n = 30.

<table>
<thead>
<tr>
<th>Locality</th>
<th>SVL (mm)</th>
<th>TL (mm)</th>
<th>Head width (mm)</th>
<th>Tympanum diameter (mm)</th>
<th>Body temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utuado</td>
<td>28.35 sd = 2.40</td>
<td>15.25 sd = 1.85</td>
<td>11.62 sd = 3.02</td>
<td>2.25 sd = 0.33</td>
<td>21.21 sd = 1.01</td>
</tr>
<tr>
<td>Mayagüez</td>
<td>28.21 sd = 3.51</td>
<td>12.83 sd = 2.63</td>
<td>11.33 sd = 2.04</td>
<td>1.63 sd = 0.07</td>
<td>21.65 sd = 0.79</td>
</tr>
<tr>
<td>Jayuya</td>
<td>29.6 sd = 2.67</td>
<td>15.84 sd = 2.50</td>
<td>12.31 sd = 1.79</td>
<td>2.29 sd = 0.42</td>
<td>20.27 sd = 0.92</td>
</tr>
<tr>
<td>Arecibo</td>
<td>21.00 sd =3.67</td>
<td>13.50 sd = 0.92</td>
<td>11.5 sd = 0.04</td>
<td>2.10 sd = 0.01</td>
<td>21.38 sd = 0.85</td>
</tr>
<tr>
<td>Río Piedras</td>
<td>26.10 sd = 2.20</td>
<td>12.10 sd = 2.25</td>
<td>11.20 sd = 0.64</td>
<td>2.30 sd = 0.46</td>
<td>21.56 sd = 0.95</td>
</tr>
</tbody>
</table>

We analyzed the “CO” and “QUI” notes in five localities which include: Mayagüez, Arecibo, Jayuya, Utuado and Río Piedras. As shown in Figure 7, the note was analyzed by measuring highest and lowest frequency of the note in a spectrogram (96kHz, 24bit, 1 channel).

**Advertisement “CO-QUI” note**

Beginning with the lower part of the “CO” note, the Fisher’s LSD shows three distinct populations denoted by letters in Table 2. Jayuya had the lowest mean frequency of all populations, followed by Río Piedras and Utuado (no significant differences) and finally Mayagüez and Arecibo with the highest frequency of the note (24.0 m and 3.0 m elevation respectively). Furthermore, the results of the upper part of the “CO” note also shows three distinct populations, although with less differentiation between them. Interestingly, Jayuya still has the lowest frequency but this time Río Piedras instead of Arecibo has the highest frequency.

**Courtship “CO-QUI” note**

For the lower part of the “QUI” note, the Fisher’s LSD shows four distinct populations, Jayuya being the lowest frequency and Río Piedras being the highest of the group. Also, the Fisher’s LSD shows that when we observe the upper part of the “QUI” note, Jayuya still has the lowest frequency and Río Piedras the highest (Table 2).
Table 2. Fisher's Least Significant Difference (LSD) for frequencies in *Eleutherodactylus coqui* in different localities in Puerto Rico. Dissimilar populations are denoted by different letters (n = 150).

<table>
<thead>
<tr>
<th>Note</th>
<th>Parameter</th>
<th>Locality</th>
<th>Mean (sec)</th>
<th>Populations</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>“CO”</td>
<td>Lowest Frequency</td>
<td>Jayuya</td>
<td>1.05</td>
<td>A</td>
<td>Alpha = 0.05, LSD = 0.09397, Error = 0.0339, df = 145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rio Piedras</td>
<td>1.27</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Utuado</td>
<td>1.31</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mayagüez</td>
<td>1.48</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arecibo</td>
<td>1.54</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>“QUI”</td>
<td>Lowest Frequency</td>
<td>Jayuya</td>
<td>1.83</td>
<td>A</td>
<td>Alpha = 0.05, LSD = 0.12460, Error = 0.0596, df = 145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Utuado</td>
<td>2.24</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mayagüez</td>
<td>2.33</td>
<td>B C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arecibo</td>
<td>2.45</td>
<td>C D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Río Piedras</td>
<td>2.49</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>“CO”</td>
<td>Highest Frequency</td>
<td>Jayuya</td>
<td>1.89</td>
<td>A</td>
<td>Alpha = 0.05, LSD = 0.11618, Error = 0.0518, df = 145</td>
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<tr>
<td></td>
<td></td>
<td>Arecibo</td>
<td>2.01</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Utuado</td>
<td>2.03</td>
<td>B C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mayagüez</td>
<td>2.11</td>
<td>B C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Río Piedras</td>
<td>2.14</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>“QUI”</td>
<td>Highest Frequency</td>
<td>Jayuya</td>
<td>3.15</td>
<td>A</td>
<td>Alpha = 0.05, LSD = 0.12460, Error = 0.0596, df = 145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Utuado</td>
<td>3.44</td>
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<td>Arecibo</td>
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<td>B C</td>
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</tr>
<tr>
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<td></td>
<td>Mayagüez</td>
<td>3.62</td>
<td>C D</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Río Piedras</td>
<td>3.69</td>
<td>D</td>
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</table>
Table 3. Fisher's Least Significant Difference (LSD) for the length of independent notes an entire call duration in *Eleutherodactylus coqui*. Dissimilar populations are denoted by different letters (n = 150). Means with a common letter are not significantly different (p > 0.05).

<table>
<thead>
<tr>
<th>Note</th>
<th>Parameter</th>
<th>Locality</th>
<th>Mean (sec)</th>
<th>Populations</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>“CO”</td>
<td>Duration</td>
<td>Río Piedras</td>
<td>0.08</td>
<td>A</td>
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<tr>
<td></td>
<td></td>
<td>Mayagüez</td>
<td>0.10</td>
<td>A</td>
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<tr>
<td></td>
<td></td>
<td>Arecibo</td>
<td>0.12</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jayuya</td>
<td>0.12</td>
<td>B</td>
<td></td>
</tr>
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<td>Utuado</td>
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<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>Arecibo</td>
<td>0.18</td>
<td>B C</td>
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<td>Jayuya</td>
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<td>B C</td>
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<td>Utuado</td>
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<td>“CO”</td>
<td>“QUI” Inter-</td>
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</tr>
<tr>
<td>“QUI”</td>
<td>note interval</td>
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<tr>
<td></td>
<td></td>
<td>Jayuya</td>
<td>0.14</td>
<td>C</td>
<td></td>
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<tr>
<td>Total</td>
<td>duration of call</td>
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<td>A</td>
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<td>Arecibo</td>
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<td>“CO”</td>
<td>“QUI” Duration</td>
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<td>“QUI”</td>
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<td>C D</td>
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<td></td>
<td></td>
<td>Jayuya</td>
<td>0.48</td>
<td>D</td>
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</tr>
</tbody>
</table>
Call duration

The area with the shortest territorial note, “CO”, was the population located in Río Piedras followed by the populations in Mayagüez, Arecibo, Jayuya, and Utuado with the longest note as expected. The Fisher’s LSD separates this populations in three distinct groups (Table 3). The courtship note, “QUI”, had the exact Fisher’s LSD order as the territorial note, being Río Piedras the shortest note and Utuado the longest. Also, the inter-note interval between the “CO” and the “QUI” notes was measured (Table 3). Additionally, Utuado and Jayuya had the shortest and longest inter-note interval respectively, leaving the coastal populations with intermediate pauses between the first two. Finally, the entire call was measured to observe which population had the shortest and the lowest call of all the populations studied. As expected, lower elevation populations such as Río Piedras, Mayagüez and Arecibo, had shorter calls than the populations located in higher mountainous areas like Jayuya and Utuado (Narins 1995) (Table 3).

Morphology, body temperature, and acoustics of Eleutherodactylus antillensis

As Eleutherodactylus coqui, E. antillensis is also widespread in Puerto Rico but it was thought that it could be found more commonly in disturbed low elevations areas. These characteristics make it possible for researchers to study the species without negatively affecting their populations. As with E. coqui, we analyzed the E. antillensis two-note call (“CHU-RI”), for frequencies and body morphometry (Table 4) in different populations across the island of Puerto Rico. For this species, Río Piedras was substituted for Culebra Island due to the absence of the species in recordings.

<table>
<thead>
<tr>
<th>Locality</th>
<th>SVL (mm)</th>
<th>TL (mm)</th>
<th>Head width (mm)</th>
<th>Tympanum diameter (mm)</th>
<th>Body temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utuado</td>
<td>26.52 sd = 1.01</td>
<td>12.47 sd = 0.66</td>
<td>9.88 sd = 1.07</td>
<td>2.01 sd = 0.06</td>
<td>24.70 1.12</td>
</tr>
<tr>
<td>Arecibo</td>
<td>27.66 sd = 0.89</td>
<td>10.03 sd = 0.33</td>
<td>10.75 sd = 0.54</td>
<td>3.0 sd = 0.78</td>
<td>24.66 sd = 0.45</td>
</tr>
<tr>
<td>Mayagüez</td>
<td>27.28 sd = 1.13</td>
<td>10.03 sd = 0.09</td>
<td>12.00 sd = 1.50</td>
<td>2.28 sd = 0.16</td>
<td>21.50 sd = 0.65</td>
</tr>
<tr>
<td>Culebra</td>
<td>23.45 sd = 1.42</td>
<td>11.17 sd = 1.89</td>
<td>9.47 sd = 1.26</td>
<td>1.73 sd = 0.56</td>
<td>22.65 sd = 0.66</td>
</tr>
<tr>
<td>Jayuya</td>
<td>22.8 sd = 1.34</td>
<td>10.30 sd = 1.37</td>
<td>8.70 sd = 1.99</td>
<td>2.40 sd = 0.22</td>
<td>22.18 sd = 0.89</td>
</tr>
</tbody>
</table>
“CHU-RI” note
Frequency measurements from the lower part of the “CHU” note spectrogram shows a lower frequency on the populations found in the mountainous region, being the Jayuya population the lowest. On the other hand, the Culebra population had the highest frequency of all (Table 5). Also, with the latest population, the Fisher’s LSD acknowledged this population as a separate group. Moreover, results from the upper part of the “CHU” note spectrogram shows an interesting pattern displaying the highest and lowest frequencies belong to the populations in high elevations (e.g., Jayuya and Utuado, respectively) and the intermediates to the lower elevation localities (e.g., Mayagüez, Culebra, and Arecibo).

“CHU-RI” note
Within the call, the “RI” note is something that this species can modulate to emit the unmistakable “QUI, QUI, QUI…” thrill call that Ovaska and Caldbeck (1997) described. The sound spectrogram presented similarities with the first note such as, frequency patterns with populations. For example, the high elevation populations still have the lowest frequency of all populations and as we go down in elevation, their frequencies get progressively higher (Table 5).

Table 5. Fisher's Least Significant Difference (LSD) for frequencies in *Eleutherodactylus antillensis*. Dissimilar populations are denoted by different letters (n =151). Means with a common letter are not significantly different (p > 0.05).

<table>
<thead>
<tr>
<th>Note</th>
<th>Parameter</th>
<th>Locality</th>
<th>Mean (sec)</th>
<th>Populations</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Jayuya</td>
<td>1.26</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Utuado</td>
<td>1.31</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>“CHU”</td>
<td>Lowest</td>
<td>Arecibo</td>
<td>1.54</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frequency</td>
<td>Mayagüez</td>
<td>1.56</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Culebra Is.</td>
<td>1.87</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Utuado</td>
<td>1.95</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jayuya</td>
<td>1.95</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>“RI”</td>
<td>Lowest</td>
<td>Mayagüez</td>
<td>2.26</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frequency</td>
<td>Arecibo</td>
<td>2.32</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Culebra Is.</td>
<td>2.57</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>
Call Duration

Examination of the duration of individual notes (CHU-RI), inter-note intervals and total call duration demonstrates that populations at lower elevations have shorter calls than populations at higher elevations. More importantly, populations such as Utuado and Jayuya appear to be a separate group from the others (Table 6).

Table 6. Fisher's Least Significant Difference (LSD) for the length of independent notes an entire call duration in *Eleutherodactylus antillensis*. Dissimilar populations are denoted by different letters (n = 151). Means with a common letter are not significantly different (p > 0.05).
### Table 1

<table>
<thead>
<tr>
<th>Location</th>
<th>Duration of Call</th>
<th>Alpha</th>
<th>LSD</th>
<th>Error</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utuado</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jayuya</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mayagüez</td>
<td>0.12</td>
<td></td>
<td>0.08782</td>
<td>0.0228</td>
<td></td>
</tr>
<tr>
<td>Arecibo</td>
<td>0.15</td>
<td></td>
<td>0.05133</td>
<td>0.0102</td>
<td></td>
</tr>
<tr>
<td>Culebra Is.</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Discussion

Vocalization evolves as the result of a variety of selection pressures in the environment (Drewry and Rand 1983). *Eleutherodactylus* is one of the few genera of anurans that can use the same notes to engage in courtship and advertisement calls rearranging the notes (Narins and Capranica 1978, Stewart and Rand 1991, Stewart and Bishop 1994). A study conducted on the genus *Eleutherodactylus* in the main island of Puerto Rico showed that *E. antillensis* has a high activity peak call during midnight and low activity at dusk and dawn (Drewry and Rand 1983). In Culebra Island, the pattern of activity was different as this species was more active during dusk and dawn (Ríos-Franceschi et al. 2016). These differences may function in defense of a calling territory to other males (Ovaska and Caldbeck 1997). In the case of Culebra Island, the frequency of calls by *E. antillensis* was broader in range compared to its conspecifics at El Yunque Rain Forest (Drewry and Rand 1983), being 1.05 kHz higher (Ríos-Franceschi et al. 2016). It is known that frogs can modify the amplitude of the call depending of the environment, depending on how open or closed is their habitat space and the intensity of interspecific competitors (Drewry and Rand 1983, Lopez et al. 1988). For visual reference, see Figures 9-10 and 12.
Figure 9. Representative waveforms (amplitude vs. time) of advertisement calls of *Eleutherodactylus antillensis* in four localities in Puerto Rico. A, Arecibo; B, Jayuya; and C, Río Piedras. Each plot was generated using Raven Pro1.5®, Cornell University.
Figure 9 (continuation). Representative waveforms (amplitude vs. time) of advertisement calls of *Eleutherodactylus antillensis* in Puerto Rico. D, Utuado. Each plot was generated using Raven Pro1.5®, Cornell University.

*Eleutherodactylus antillensis*. Spectrographs showed that *Eleutherodactylus antillensis* frogs usually trill (aggressive calls), which are used in defense of a territory (Ovaska and Caldbeck 1997) when *E. coqui* is present. It is well known that these two species share the same habitat, thus they may compete for the same resources. Narins (1995) stated that frog calling assemblages can exhibit time-sharing on a millisecond time scale or on a diel time scale, restricting their calling to particular times of the day (Villanueva-Rivera 2014, Ospina et al. 2013). *Eleutherodactylus coqui* does not seem to be occurring in Culebra Island because both species were recorded calling at the same time in almost every single recording from Culebra Island. On the other hand, at the other data collection localities there seems to be evidence of time-sharing (Narins 1995) between *Eleutherodactylus antillensis* and *E. coqui*. 


Figure 10. Representative waveform of *Eleutherodactylus antillensis* in Puerto Rico. A. Amplitude vs. time. B. Spectrograph of “thrill” call.
Environmental factors such as, humidity, temperature, elevation and body size are known to affect the call frequencies and rates with which a male can communicate. By examining populations across several localities (e.g., in this study, all secondary forests, Figure 15) using an elevation gradient, we can analyze these factors. For example, a recent study testing the hypotheses surrounding vicariance and dispersal using mtDNA sequences from *E. antillensis*, found support for a hypothesis of colonization of the islands east of Puerto Rico from sources located in eastern Puerto Rico during the penultimate and last glacial period. This occurred when a land bridge united the Puerto Rican Bank, thus dividing populations in different haplotypes (Barker et al. 2012), probably contributing to speciation. Using linear regressions, we found that the size (SVL) of *E. antillensis* frogs tends to be negatively correlated to call frequency (kHz) although this trend is not statistically significant. High “CHU” and “RI” frequencies were significantly correlated with SVL, the larger the frog, the lower the frequency, but these correlations were not considered strong ($R^2 = 0.48$ and $R^2 = 0.52$, respectively, Figure 11). The reason could be to low frog collection in each locality ($n = 30$, per locality).

As is known, the larger the organism, the lower its call frequency, but in this case, Arecibo (Puerto Rico), is located nine meters above sea level and, as expected, their population had one of the highest sonic frequencies of all, but more importantly, it had the largest individuals of all populations (mean SVL = 27.66 mm, $n = 30$). Morphological features such as the size of the larynx may play a key role in frequency production in each population (Ríos-Franceschi, unpublished data). Also, for *E. antillensis* spectrogram analysis revealed highest energy (dB) in Jayuya, Puerto Rico with a mean dB = 132 and max dB = 170, $n = 10$.

If we combine the ethological and morphological data collected, we can suggest that each population of *E. antillensis* has a different accent, in such cases, some populations vary so much that we could speculate that sexual selection could be in play. This suggests that selection has likely favored the evolution of a specialized neural auditory mechanism for extracting time and frequency information from such calls (Hall 1994, Richards 2006).
Figure 11. Linear regressions of *E. antillensis*. A, SVL* High frequency “CHU” note. B, SVL* High frequency “RI” note.
Figure 12. Representative waveforms (amplitude vs. time) of advertisement calls of *Eleutherodactylus coqui* in five localities in Puerto Rico. A, Arecibo and B, Jayuya. Each plot was generated using Raven Pro1.5®, Cornell University.
If we compare the mountainous populations, Utuado and Jayuya, they can call at the same lower frequencies of both notes (e.g., CHU-RI), but the population located in Utuado has a higher frequency range. Furthermore, the Utuado population has shorter calls than the Jayuya population. We can assume that the Utuado and Jayuya populations can communicate using different accents. However, if we compare the populations at a lower elevation locality, such as Figure 11. Linear regressions of *E. antillensis*. A, SVL* High frequency “CHU” note. B, SVL* High frequency “RI” note.
Frogs from Culebra Island and Jayuya are so sonically dissimilar (Figure 13) that they may communicate in different spectral and temporal properties that they may not recognize each other if they are placed together, promoting sexual divergence and isolation, which could lead to speciation. Research on anuran papillae demonstrates spectral sensitivity to distinct frequencies (Meenderink et al. 2010); in other words, each isolated population varies in spectral and temporal properties in which case, the auditory papillae can be fixed to specific frequencies. This can be tested by doing playbacks in every population to observe intra-sexual and inter-sexual response.

![Figure 13. Total frequency range of *E. antillensis* at each locality (elevation, in meters, above sea level).](image)

*Eleutherodactylus coqui*. For the call frequencies of *E. coqui*, the population in Jayuya, seems to emit territorial and courtship call at different frequencies that frogs at the other localities analyzed. This is important because this species has specific frequency sensitivity, so the probability that the population in Jayuya can communicate effectively with any other population analyzed in this research is low (Figure 14). This hypothesis has to be tested by playbacking recordings in each location.

![Figure 14. Total frequency range of *E. coqui* at each locality (elevation, in meters, above sea level).](image)
For the acoustic communication to be successful, the individuals must be sensitive to spectral and temporal signals in the call (Gerhardt and Schwartz 2001, Meenderink et al. 2010). Spectral and temporal properties of a call can be affected by temperature and morphology, such as body size (Littlejohn 1977, Narins and Smith 1986). The call frequency in *E. coqui* varies with an altitudinal gradient, rather than temperature (a 6°C decrease every 1,000 meters, El Yunque National Forest), SVL was suggested to be the reason of these variations in calling frequencies within the altitudinal gradient in El Yunque National Forest (Meenderink et al. 2010). Linear regressions testing SVL and frequencies revealed a weak ($R^2 < 0.55$) negative correlation between the two variables, but none was statistically significant, probably caused by low data collection. Background noise such as the El Yunque National Forest can influence amphibian diel calling patterns and temporal properties of their calls in presence of sound interference (Narins 1982, Narins and Smith 1986). For them, to detect calls in noisy environments requires the appropriate sensitive mechanism (Ryan 1986, Meenderink et al. 2010). Also, for *E. coqui* spectrogram analysis revealed highest energy (dB) in Utuado, Puerto Rico with a mean dB = 127 and max dB =155, n = 10.

Intraspecific divergence in mating signals, whether generated by sexual selection, pleiotropic effects, drift, or other evolutionary processes when correlated with divergence in female preference can lead to reproductive isolation and speciation (Richards 2006). Females often maintain this isolation through selection of male calls. Furthermore, anurans adapt to these environmental changes by modifying their behavior and possibly their inner ear anatomy to adjust to these changes in acoustical signals. Although ear anatomy is an important part of signal reception, this paper has focused on signal production.

Acknowledgments

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Literature Cited


Figure 15. An example of a secondary forest where this study was conducted. These photos depict one of the sites located in the Rio Abajo State Forest, Utuado, Puerto Rico. A. Before Hurricane Maria hit Puerto Rico (September 20, 2017). B. Same site after the vegetation was destroyed by the hurricane. More information on the devastation caused by Maria to Puerto Rican forests can be found here: 3-D Views of Puerto Rico’s Forests After Hurricane Maria, available in this link: https://www.youtube.com/watch?v=QeGFaqwDY3s