Research Article

Serenading for ten thousand years: The mating call of insular populations of the green treefrog *Aplastodiscus eugenioi* (Anura: Hylidae)

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Abstract

Islands are natural laboratories for evolutionary studies due to their small areas, structural complexity (compared to continents), and their different degrees of isolation. The present study involved four distinct areas, one continental and three insular, on the southern coast of the State of Rio de Janeiro, Brazil. These islands were formed by coastal flooding at the end of the last glacial maximum, approximately 10 thousand years ago. We investigated acoustic parameters in the advertisement call of the green tree frog, *Aplastodiscus eugenioi* (Hylidae), looking for variations that could be associated with geographic isolation in the insular populations. We measured six acoustic parameters of the advertisement calls of 59 males, 15 specimens from the continent and 44 from the islands. We found significant variation in acoustic parameters within the population from Ilha de Itacuruçá, suggesting that isolation, despite the relatively short period, favored differentiation of acoustical parameters. In fact, call evolution may involve a series of changes in both females and males in a synchronous way, so that the signal producer and receiver can behave accordingly for successful reproduction.

Keywords: advertisement call; amphibians; evolution; island biogeography; variation

Resumo

Devido a seu tamanho diminuto, complexidade estrutural (quando comparadas a continente) e seus diferentes graus de isolamento, ilhas são consideradas laboratórios naturais para estudos sobre evolução. Vários aspectos relacionados à fauna e flora insulares podem estimular percepções as quais nos permitem compreender diversos fenômenos biológicos relevantes em diferentes escalas, desde populações até comunidades, em sistemas continentais ou oceânicos. O presente estudo envolveu quatro áreas distintas, sendo uma continental e três insulares, na costa Sul do Estado do Rio de Janeiro, Brasil. Estas ilhas foram formadas como resultado da inundação da costa ao final do último máximo glacial, há aproximadamente 10 mil anos trás. Nós investigamos os parâmetros acústicos do canto de anúncio de *Aplastodiscus eugenioi* (Hylidae) buscando por variações as quais pudessem ser associadas ao isolamento geográfico das populações insulares. Nós medimos seis parâmetros do canto de anúncio de 59 machos, sendo 15 indivíduos do continente e 44 oriundos das ilhas. Nós encontramos variação significativa nos parâmetros acústicos da população da Ilha de Itacuruçá. Estes resultados podem indicar que o isolamento, apesar do curto período de tempo, favoreceu a diferenciação destes parâmetros. De fato, a evolução do canto deve envolver uma série de mudanças em ambos, machos e fêmeas, numa maneira de alguma forma sincronizada, assim, o gerador e o receptor do sinal podem se comportar de acordo no contexto reprodutivo.

Palavras-chave: canto de anúncio; anfíbios; evolução; biogeografia de ilhas; variação

Introduction

Observations of insular populations clearly influenced Darwin's [1] and Wallace's [2] perceptions of population changes and biological evolution. Because of their small size compared to continents and geographic isolation, islands are considered natural laboratories of evolutionary phenomena, even over relatively short periods [3, 4]. On islands, it is possible to evaluate the processes that affect species' physiology [5], demography [6], and even community structure [7]. Studies of insular fauna and flora reveal biologic phenomena relevant at different scales, from populations to communities. The relationship between islands and biological diversity [8] has provided some of the basic concepts and principles of conservation biology [9 - 11].

Two types of islands are distinguished by their geologic history: oceanic and continental. Oceanic islands usually are older, formed by tectonic processes, and distant from continents [12]. These islands are colonized by plant and animal species that have dispersed from the continents or nearby islands [8]. Continental islands were formed near the coast by marine transgressions caused by global warming; similar processes may also occur in large continental lakes [13]. Animal and plant communities were already present in the areas where transgressive sea-level changes produced continental islands. Because amphibians' intolerance of salinity prevents overseas dispersal, they are seldom found on oceanic islands (*e.g.*, [14]). Therefore, investigations of insular amphibian populations typically involve continental islands.

Insular founder effects (genetic drift and bottlenecks) are better studied than behavioral divergences. Although behavior has been studied in birds and flies, where the female plays a predominant role in selection of phenotype and male displays [15, 16], similar studies of frogs are less common [17 - 19]. Female anurans exert strong selection on males, based on the cost of their calls [20], which might be correlated to body size [21,

22], vitality [21], or certain genes present in males [23]. In many anurans, the advertisement call strongly influences the female choice of male partners. The advertisement call may vary geographically among populations of the same species [24 - 26], indicating both potential future evolutionary changes and the history of selection and constraints [24].

Studies of insular amphibian populations have revealed the effects of isolation, even in recently isolated continental islands ([27 - 29] and references therein). Some studies addressed the breeding behavior of frogs from isolated populations on the mainland, such as those on mountaintops [30 - 34]. Few studies have addressed the correlation between insulation and variations in the advertisement call of frogs. Littlejohn [35, 36] is still the best reference, showing that advertisement calls may diverge among populations isolated for 12 thousand years (at the end of the last glacial maximum).

We used recordings of calling frogs to test the hypothesis that variations in the advertisement call of the green tree frog, *Aplastodiscus eugenioi* [37] are due to geographical isolation of three insular populations from a continental population, in the State of Rio de Janeiro. Although these islands are near the coast and to each other, features associated with breeding biology of this species (breeding in forest streams and adjacent areas; see [37]) make the possibility of dispersion and connection among populations quite remote. Thus, each population is considered isolated from the others and all of them from the continent.

Methods

Study Area

This work was conducted between 2005 and 2015 at four study sites: three on islands and one on the mainland. The continental site is near Represa do Itingussú (22° 54' 3.44"S; 43° 53' 34.59"W, about 160m above sea level), in the Municipality of Itaguaí, at about 10 km from type locality of the species (Serra do Piloto, Mangaratiba, Rio de Janeiro State) and also the nearest locality to the continental islands sampled (Fig. 1). Islands were chosen based on previous knowledge of the presence of *Aplastodiscus eugenioi* [29]. The islands sampled were: Ilha Grande, Vila do Abraão (23° 08' 02,7''S; 44° 10' 23,3'' W, sea level), Municipality of Angra dos Reis; Ilha da Marambaia (23° 04' 23''S; 43° 58' 12''W, sea level), Municipality of Mangaratiba; and Ilha de Itacuruçá, Vila da Gamboa (22° 56' 01''S; 43° 53' 00''W, sea level), also in the Municipality of Mangaratiba. Marambaia and Itacuruçá are located in the Sepetiba Bay, and Ilha Grande is located at the entrance of two contiguous bays, Ilha Grande and Sepetiba. All three islands have similar landscapes, with permanent streams and forests in good states of conservation [29], except for Ilha de Itacuruçá, which is the smallest of the islands, the closest to the continent, and the one that appears to have suffered the most anthropogenic alteration. Within forests, *A. eugenioi* calls, builds its nests in the riparian clay, and reproduces along small to mid-size streams (see [38, 39]; personal observation).

The process of insular formation on the coast of Rio de Janeiro (and for most similar islands along the coast of Brazil) is commonly linked to events during the last ice age. Ice accumulation in glaciers throughout the planet (mainly in the poles) caused sea level to

drop about 100 m, exposing nearly the entire continental shelf of Brazil [41]. During this period, the Atlantic rain forest may have covered the whole area, as evidenced by the vegetation and forest-dependent frog fauna, even on more distant islands [40]. About 17,500 YBP, glaciation had receded and sea level rose again, reaching the levels we observe today (see [42]). With sea level rise some mountain summits near the coast became isolated by water, creating the coastal continental islands included in this study [28]. Because sea-level fluctuation happened gradually, the processes that isolated the islands from the mainland occurred at different times, resulting in islands with different ages, depending on the distance and the depth of the channels separating them from each other and from the mainland. Therefore, with a channel of approximately 55 m depth that separates Ilha Grande from the mainland, this was the first island to be isolated, about 10,000 YBP, followed by Ilha da Marambaia and Ilha de Itacuruçá, at about 7,000 YBP. Although Ilha da Marambaia has been connected to the continent by a narrow sand strip since the last marine regression (~ 3500 YBP), the restinga environment of the sand bar does not seem to provide a bridge for dispersion of forest species, especially species such as the green tree frog that need fresh water streams [43]. Therefore, for our purposes, the paleo-island of Marambaia is considered to be an island as it has been by other researchers [28, 29, 40].
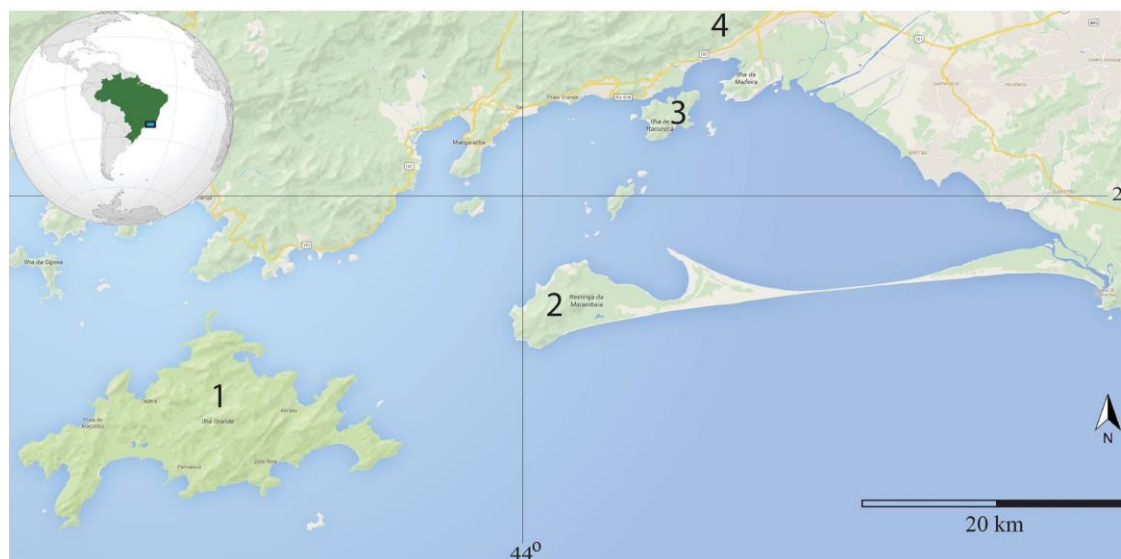
Fig. 1: Map of the Rio de Janeiro State presenting the localities where the recordings of *Aplastodiscus eugenioi* calls were made: (1) Ilha Grande, (2) Ilha da Marambaia, (3) Ilha de Itacuruçá, (4) Itaguaí.

Recording and Call Analysis

Calls of *A. eugenioi* were recorded during three-minute sequences using a Marantz PMD 660 digital recorder with a Sennheiser (e835S) microphone. Voucher specimens from Itaguaí, Ilha Grande, Ilha da Marambaia and Ilha de Itacuruçá are deposited at Coleção Herpetológica da Universidade Federal Rural do Rio de Janeiro. Calls were recorded at

44 kHz frequency sampling and 16 bits of resolution, and then analysed using the software Raven Pro Version 1.4 and SoundRuler Version 0.9.4.1.

Six acoustic parameters of 579 advertisement calls, of each individual recorded, were measured: call duration (s), peak time (s) (following [44]: time from the beginning of the call to the point of maximum amplitude), call shape (ratio between peak time and call duration, which provides envelope form: linear, exponential or inverse exponential; see [45]), fundamental frequency (kHz), dominant frequency (kHz), and frequency modulation (kHz: difference between the final dominant frequency and the initial dominant frequency). The first three variables are temporal parameters and the last three are spectral. Call rate (a temporal variable) was not used because it is highly influenced by temperature [17, 22] or social context (such as number of calling males in the surroundings, females approaching, etc.), as reported by Zina and Haddad [46] for two species belonging to the *Aplastodiscus* genus. Waveforms, sonograms and power spectra were produced using the Sound Ruler Version 0.9.4.1 (FFT = 256; O = 90%; Hanning).

The variation among individuals from the same population for each acoustic parameter was calculated using the inter-individual coefficient of variation [$CV_{inter} = (\bar{x}_{DP}/\bar{x}_{means})$ * 100]. The coefficient of variation was used in order to compare the variability of behavioral attributes that differ in mean values [21]. Acoustic parameters with low variation among individuals were ranked as statics (below 5 % variation). Parameters with values between 5 % and 12 % were considered intermediate, whereas parameters with high variability were ranked as dynamics (superior to 12 %; see [21]). Because frequency modulation is a variable recorded as positive and negative values, sometimes with a wide variation of values, we decided to exclude it from the calculations of the inter-individual variation.

Statistical Analysis

Traditional descriptive analyses were performed with acoustic variables based on the mean, median, standard deviation and amplitude. To test the null hypothesis, in which advertisement calls are not significantly divergent between mainland and islands, we applied multivariate techniques. First, all data were normalized. For each variable, the mean was subtracted and then divided by its respective standard deviation. This is usually necessary for environmental data where variables are often on completely different scales with arbitrary origins. It is then possible to derive meaningful distances between samples, using Euclidean distances. The Plymouth Routines in Multivariate Ecological Research (PRIMER) software package [46] Version 6 was used to create a Normalized Euclidian Distance matrix. This matrix was subjected to a Principal Components Analysis (PCA) seeking distribution patterns in samples to explain data variability.

Results

The advertisement calls of *A. eugenioi* consist of a single tonal note, with harmonic structure, repeated at irregular intervals. On the continent, the calls had median duration (data did not fulfil all the normality requirements) of 0.08 s (N = 146 calls) with

a mean of 13 calls per minute and dominant frequency ranging between 2.41 and 2.93 kHz. On the islands, the advertisement call had a median duration of 0.09 s (N = 433), mean of 17 calls per minute and dominant frequency ranging between 2.06 and 3.10 kHz. Results are summarized in Table 1.

The advertisement calls of *A. eugenioi* had an inverse exponential envelope form (Fig. 2). The energy peak was temporally at the beginning of the call, and decayed slowly after this peak (Fig. 2). Analyses of the sonogram and power spectrum indicate that the second harmonic is the dominant (90 % in the mainland and 92 % in the islands).
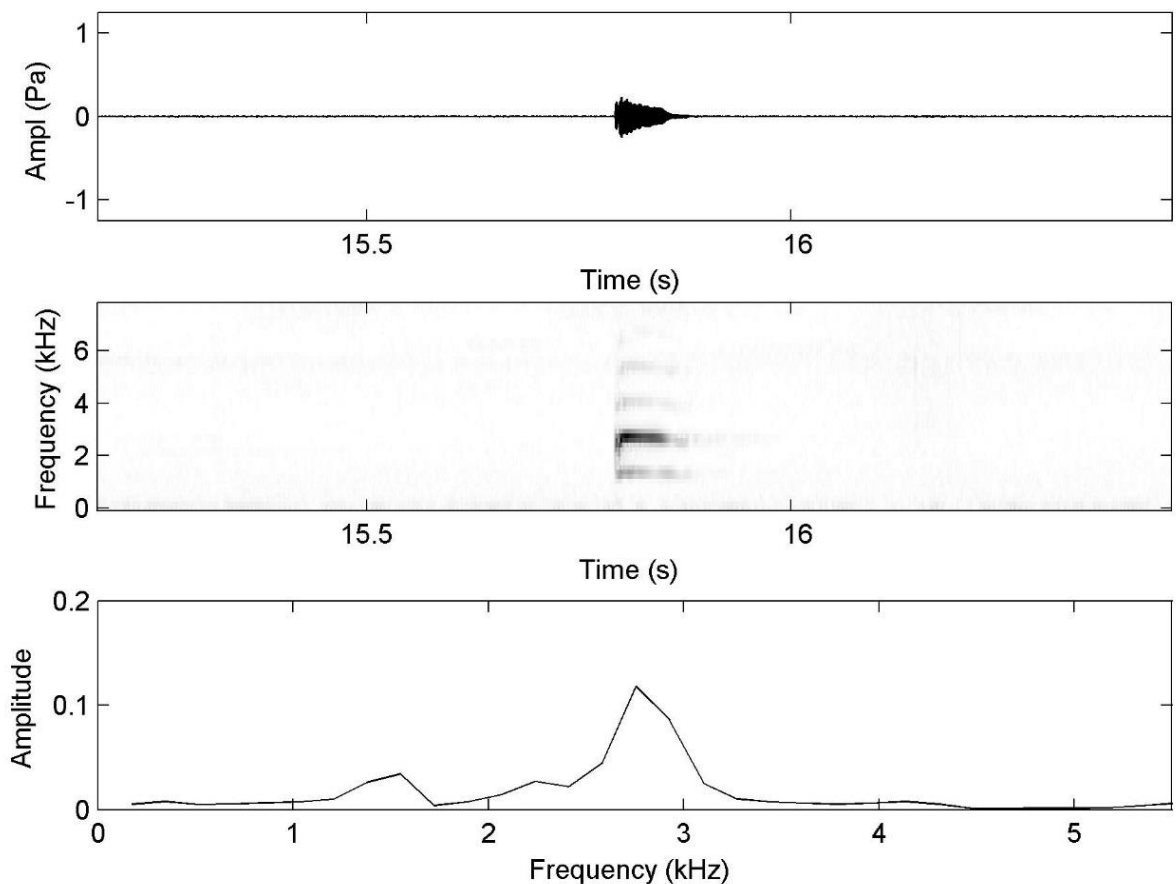

Fig. 2. Advertisement call of *A. eugenioi* recorded in Itaguaí, Rio de Janeiro State: (TOP) oscilogram, (MIDDLE) sonogram and (BOTTOM) power spectrum.

Table 1. Acoustic parameters of the advertisement call of *A. eugenioi* for four localities (Itaguaí, Ilha da Marambaia, Ilha Grande e Ilha de Itacuruçá). Results presented as mean ± standard deviation (amplitude); except frequency modulation, dominant and fundamental frequency, which values correspond to the median values.

	Continent	Island		
	Itaguaí	Marambaia	Ilha Grande	Itacuruçá
Call duration (s)	0.10 ± 0.02 (0.06–0.14)	0.09 ± 0.03 (0.06-0.21)	0.09 ± 0.04 (0.05-0.22)	0.09 ± 0.01 (0.06-0.14)
Call Peak time (s)	0.01 ± 0.01 (0.001–0.03)	0.01 ± 0.01 (0.001-0.03)	0.01 ± 0.01 (0.001-0.04)	0.02 ± 0.01 (0.002-0.03)
Call shape	0.13 ± 0.08 (0.02–0.30)	0.12 ± 0.06 (0.01-0.33)	0.13 ± 0.09 (0.01-0.44)	0.18 ± 0.13 (0.02-0.70)
Fundamental frequency (kHz)	1.38 (1.29–1.70)	1.33 (1.20-1.46)	1.37 (1.19-1.90)	1.47 (1.20-1.56)
Dominant frequency (kHz)	2.67 (2.41–2.93)	2.58 (2.84-2.35)	2.58 (2.25-2.93)	2.76 (2.06-3.10)
Frequency modulation (kHz)	0 (-0.34–0.34)	0 (-0.17-0.17)	0 (-0.17-0.34)	0 (-0.34-0.34)
N (calls; individuals)	146; 15	154; 16	140; 14	139; 14

The three PCA axes were obtained by the ordination of the six variables measured (call duration, call peak time, call shape, fundamental frequency, dominant frequency and frequency modulation), which explained 99.5 % of total variance (Table 2). Component I included frequency modulation as the highest loading (positive), followed by fundamental frequency (negative), and explained 49.9 % of the total variation. Component II included call peak time and call shape as the highest loading (both positive), and explained 30.6 % of total variation. Component III had call duration with the highest loading, and explained 16.0 %. Results are summarized in Table 2.

Fig. 3 shows a graphic representation of the PCA results, in which there are clear differences among some of the populations. Ilha de Itacuruçá (IC) is partially separated from the other three populations: Itaguaí (IT; mainland), Ilha Grande (IG) and Ilha da Marambaia (IM).

The coefficient of variation (CV_{inter}) indicated that higher variation was found for call shape, whereas the lower variation was detected in dominant frequency, followed by fundamental frequency. These two last parameters we considered to be static parameters, as well as call duration. Call shape and call peak time showed high levels of

variation. Thus, we considered these as dynamic parameters. Table 3 presents, with more details, the coefficient of variation (CV_{inter}) results for *A. eugenioi*, in Itaguaí.

Table 2. Acoustic parameters weights for the three first principal components.

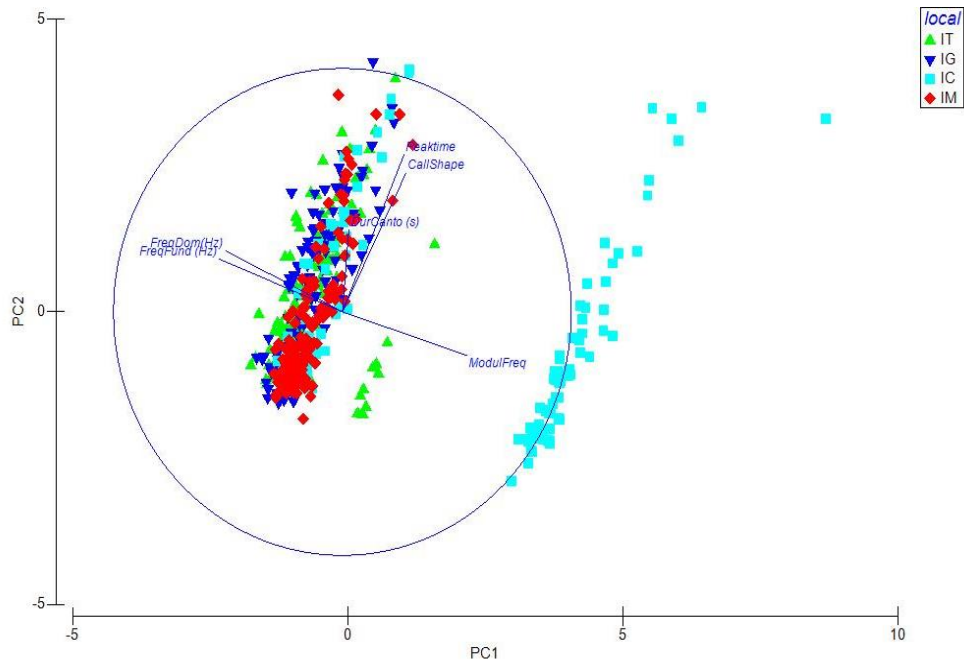| Variables	Component I	Component I	Component III
Call duration (s)	0.028	0.335	0.907
Call peak time (s)	0.270	0.647	-0.049
Call shape	0.279	0.572	-0.404
Fundamental frequency (kHz)	0.544	-0.179	0.053
Dominant frequency (kHz)	-0.510	0.250	-0.067
Frequency modulation (kHz)	-0.541	0.218	-0.070
Eigenvalues	3.00	1.84	0.96
Variance	**49.9 %**	**30.6 %**	**16 %**

Fig. 3: PCA plot of acoustic parameters of *A. eugenioi* advertisement call from IT: Itaguaí; IC: Ilha de Itacuruçá; IG: Ilha Grande; IM: Ilha da Marambaia.

Table 3. Interindividual variation values within the Itaguaí *A. eugenioi* population. Amplitude of values in brackets.

Parameter	Type	CV$_{inter}$(%)
Call duration (s)	Intermediate	6.4 (0.06–0.15)
Call peak time (s)	Dynamic	48.0 (0.001–0.03)
Call shape	Dynamic	50.5 (0.02–0.30)
Fundamental frequency (kHz)	Static	2.3 (1.29–1.68)
Dominant frequency (kHz)	Static	1.7 (2.41–2.89)

Discussion

Although sea level fluctuation is a relatively recent process in overall geological time scales, the reshaping of coastal areas, formation of islands, and resultant changes in biological diversity are apparent at various levels [27, 28, 3]. A number of studies address the effects of geographic isolation of populations due to sea level rise. Wu [27] reported an increase in body size of the rice frog *Rana limnocharis* (= *Fejervarya limnocharis*; Anura, Dicroglossidae) on islands in the Zhoushan archipelago, China. Measey [14] used mitochondrial DNA sequences to refute the hypothesis that endemic amphibians from the Guinea Gulf islands were introduced anthropogenically; developing evidence that natural dispersion took place instead. Bell [47] found genetic evidence of bottlenecks in insular populations of *Scinax perpusillus* (Anura, Hylidae) tree frog group from São Paulo State coast. Montesinos [28], using morphometric data, detected dwarfism in *Rhinella ornata* (Anura, Bufonidae) populations in two (Ilha da Marambaia and Ilha Grande) of the three islands in our study.

Studies concerning acoustic variation among insular populations are less common. Our results are in accordance with those of Littlejohn [35, 36] who studied an insular system with a history of formation quite similar to our own system and discovered variation in advertisement calls. Our data offer significant support for the hypothesis that the advertisement call of *A. eugenioi* does vary due to the geographic isolation of islands. Geographic isolation appears to have produced divergence among acoustic parameters. The population from Ilha de Itacuruçá presented a higher variation in call parameters than the other three populations investigated, suggesting that this population has experienced some sort of acoustic divergence. Environmental conditions and local differences in communities may affect divergences among geographically isolated populations [34]. Andersen [48] demonstrated that habitat loss and fragmentation in a Danish island caused inbreeding and bottlenecks in *Hyla arborea*. Hoskin [32] documented significant divergences in body size and call in allopatric populations of *Litoria genimaculata* isolated for less than 8,000 years. Furthermore, previous studies

suggested that call is the first to diverge during speciation [49, 23], even over significantly small geographic scales [34].

Although studies with reptiles found divergence in ecological and/or morphological characters in insular populations [50 – 52, 53], indicating that the time of insulation of similar islands is sufficient to fix some variation, the frog populations we studied seem more similar regarding advertisement call among islands. In our survey of call variation, Ilha da Marambaia and Ilha Grande had no significant divergence in call parameters compared to the mainland. These results may indicate that *A. eugenioi* sexual selection upon call variation, exercised by females, may prevent variability. Females call choice could be acting as a stabilizing force on the species call in insular populations.

Ilha da Marambaia and Ilha Grande present ecologic gradient and habitat diversity, and a relatively large area, important features for maintenance of large populations [54, 55]. On Ilha de Itacuruçá, the smallest in area and the most densely occupied by people, *A. eugenioi* is losing habitat due to anthropogenic activities, and populations are confined mostly to intermittent streams and rivulets. Since the early 16th century, the human occupation on these islands associated with sugar cane and coffee cultivation has caused impacts on local fauna [40]. Our study provides more evidence of the rapid response of advertisement calls to a fragmentation effect, in this case fragmentation of the island itself.

Another factor to be considered is the sea level fluctuation. As discussed by Bittencourt-Silva and Silva [40] sea level fluctuation are responsible for the insular formation along the Brazilian platform. By the end of the last glacial period, about 20,000 YBP, sea level rose inundating the exposed platform. About 10,000 YBP Ilha grande was already insulated from the continent and the other islands, near the present coastline, formed latter. By approximately 5,000 YBP, the sea level rose another 3.5 m [56, 57]. This event, which reduced the area of the islands, likely affected areas where we now find calling males and tadpoles of *A. eugenioi*, and reduced the area available for reproduction of the species. This may have caused population reduction, pushing the species through a bottleneck process.

Bottlenecks cause loss of genetic diversity and the reduced potential of small populations to react to selective pressures [58], as well as increased inbreeding that can reduce population viability [59]. Mayr [60] associated bottleneck events with the formation of new species through behaviors related to premating reproductive isolation, and previous studies suggest that divergence in sexual communication systems might be a major cause of speciation in many animal groups [49, 61, 62]. There are cases related to acoustic and other behavioral parameters influencing prezygotic isolation. Similar results are reported for the *Drosophila willistoni* group [49], the salamander *Desmognathus ochrophaeus* [63], and the Túngara frog *Physalaemus pustulosus* [23]. Ohmer [34] detected regional phenotypic differences in behavioural (calls) and morphological characters (color and body size).

The call coefficient of variation within the *A. eugenioi* population from Itaguaí, reveals that the two factors with lower variation are spectral: dominant frequency and fundamental frequency, both static. The dynamic parameters with higher variation are call shape and call peak time, both temporal variables. The spectral acoustic parameters

are influenced by morphology (body size, for example), whereas temporal acoustic parameters are influenced by environmental (as temperature) and social elements [44]. Although spectral parameters tend to vary little and temporal variables are more variable, in various combinations both these static and dynamic parameters are potential indicators of male reproductive fitness [21].

Our results indicate that in *A. eugenioi* sexual selection exercised by females is acting upon premating factors to reduce their variability. If so, the lower variability of calls observed on two islands and on the continent, may have been kept stable by that process. This would also explain lower variability in the call of widespread species ([64] and references within). In the case of the population of *A. eugenioi* on Ilha de Itacuruçá we suggest that it may have passed through a process of acoustic differentiation from the other three populations due to some sort of bottleneck process. Population in this particular island may have shrunk in the past and, as a result, variability may have favored differentiation. Because of its lower altitude this island may have suffered more area shortage during a transgression that happened about 5,000 YBP, when the ocean was higher than the present three meters [65]. Further analysis is required to determine the level of divergence among these populations and whether other features are diverging as well. We stress that a combination of tools is the ideal way to search for evidence of evolutionary modification of behavior, in a manner similar to the approach presented by Ohmer [34] with the study of *D. ebraccatus*. In order to confirm the variation in acoustic parameters of *A. eugenioi* due to geographic isolation of land-bridge islands, we suggest complementary studies with the same populations using morphometry and/or morphology, with molecular markers as tools.
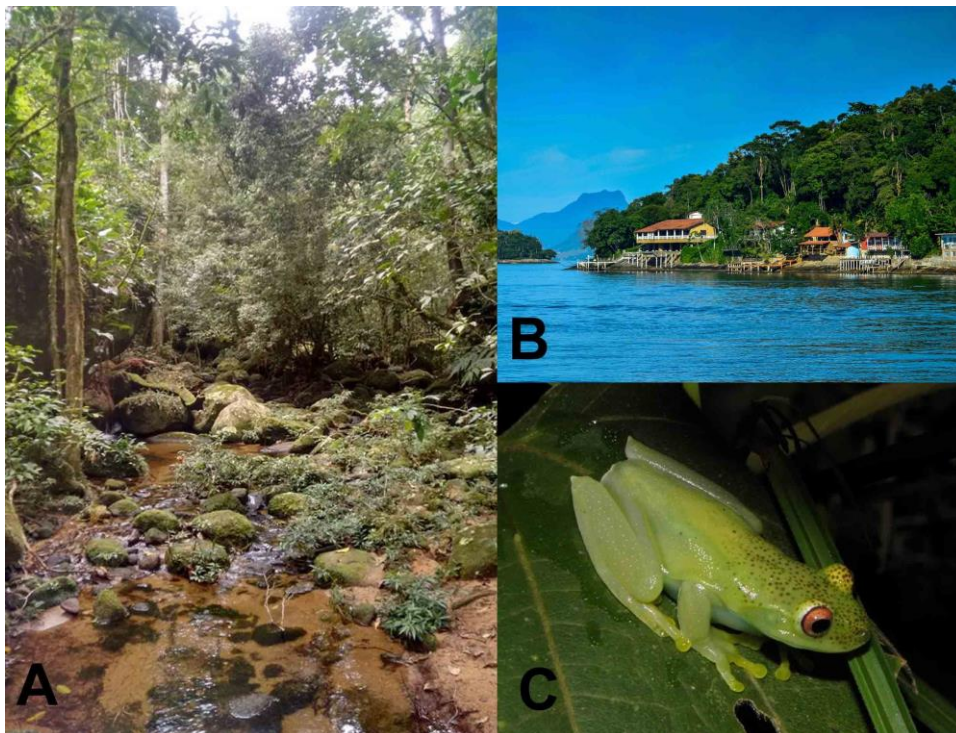

Fig.4: (A) *Aplastodiscus eugenioi* breeding habitat; (B) Ilha de Itacuruçá panoramic south view; (C) *A. eugenioi* (male; 35mm).

Implications for conservation

MacArthur and Wilson [8] described the relationship between islands physical features and biological diversity. Recent studies [9 - 11] showed the significance and correlation of the concepts they described for the conservation of insular flora and fauna and those from fragmented continental areas. Ilha de Itacuruçá is under great anthropic pressure because of its small area and the high human land usage. As we mentioned before, in this particular island, because of human activities, *A. eugenioi* is losing habitat in a dramatic way. Colonization of the area dates back to the arrival of the Portuguese to Brazil, about 500 years ago, which long impacts on local fauna [40] and flora. In the particular case of *A. eugenioi*, the most important impact relates to sand removal from the rivers, for house construction and usage of the scarce water from these rivers. Thus, we suggest that further investigations on this and other frog populations from these islands so we can better invest in conservation strategies for this system and gain insights on evolution at this temporal scale.

Acknowledgments

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