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The advertisement calls of *Epipedobates anthonyi* (Noble, 1921) and *Epipedobates tricolor* (Boulenger, 1899) (Anura: Dendrobatidae: Colostethinae): intra- and interspecific comparisons

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Abstract. Vocal communication is the main mechanism to exchange information in many groups of animals, and anurans represent an impressive example of complex acoustic communication signals. To date several calls with different and partly multiple meanings have been described for numerous species. Anuran calls were found to be species-specific, providing important taxonomic information, and advertisement calls are frequently used for systematic assignments. The closely related poison dart frogs *Epipedobates anthonyi* and *Epipedobates tricolor* inhabit geographically disjunct areas in Ecuador and Peru. Both species are very similar in appearance and have frequently been confused. Consequently, multiple scientific publications on *E. tricolor* refer to *E. anthonyi* instead and vice versa. This is also true for the descriptions of the species' advertisement call. So far, no comparative study on the species' calls exists. Here we analyze and compare the calls of *E. anthonyi* and *E. tricolor*. We found the advertisement calls of both species to be very similar in general but to differ in pulse frequency and interval. Thus, they can be distinguished with certainty. However, a comparison of characteristic call patterns between populations revealed intraspecific variation to be higher than interspecific variation, which questions the usefulness of calls for species delimitation of frogs for the genus *Epipedobates*.

Keywords. Acoustic communication, anurans, bioacoustics, species delimitation, systematics.

INTRODUCTION

Communicational signals are primarily visual or acoustic, but can also be chemical, seismic, electrical or tactile (Pough 2001, Janik & McGregor 2014) and often combinations of different signals are used (Starnberger et al. 2014). Anurans primarily use acoustic communication which plays a significant role in reproduction and social interactions (Salthe & Mecham 1974). Capranica & Rose (1983) showed that the anuran auditory system is highly adapted to the species-specific dominant frequencies and furthermore functions as a filter, improving the detection of important signals and suppressing distracting background noises. According to Wells (2007) the whole female sensory system is tuned to detect certain spectral and temporal characteristics of calls, including frequency and pulse rate, whereas the ranges of the characters depend on the species. This ability also allows discrimination between conspecific and heterospecific calls (Fritsch et al. 1988) through recognition of specific spectral and temporal components (Salthe & Mecham 1974; Wells 1977; Ryan 1985; Cocroft & Ryan 1995).

Many different types of calls and their respective functions have been described (e.g., Borgert 1960; Littlejohn 1977; Hödl 1996; Wells & Schwartz 2006; Wells 2007). In most anuran species only males produce advertisement calls during the breeding season to attract females (Bogert 1960; Wells & Schwartz 2006; Wells 2007). However, acoustic signals may also have territorial (Bogert 1960) or competitive (Wells & Schwartz 2006; Wells 2007) meaning, are used for localization (Whitney & Krebs 1975; Wells 1977, 1978), or as defense, distress, or alarm signal (Bogert 1960; Wells 2007). Previous research reveals that quantitative and qualitative parameters of anuran advertisement calls are species-specific and thus facilitate systematic assignments, particularly in poison-arrow frogs (Wells 1977; Zimmermann & Zimmermann 1988; De la Riva et al. 1996; Lötters et al. 1999; Napoli & Cruz 2005; Padial et al. 2008, Mayer et al. 2014).

In this study, we characterize and analyse the advertisement calls of two species of poison dart frogs, *E. anthonyi* and *E. tricolor*. The distributional range of the poison dart frog *Epipedobates anthonyi* stretches from southwestern Ecuador to neighboring areas of northwestern Peru (Zimmermann 1983a; Lötters et al. 2007). Within this range twelve different populations with distinct morphotypes exist; however, precise information on the distribution of most is lacking (Ostrowski & Mahn 2016). The species' natural habitat covers hot and humid forests, gallery forests and rivers which are surrounded by dry and sparsely vegetated landscapes at an elevation of up to 1,800 m (Lötters et al. 2007). Different call types have

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Fig. 1. Photographs of selected specimens of all study populations of *Epipedobates anthonyi* and *Epipedobates tricolor*. A: *E. anthonyi* I: "Buena Esperanza". B: *E. anthonyi* II: "Rio Jubones". C: *E. anthonyi* III: "Rio Saladillo". D: *E. anthonyi* IV: "Tierra alta". E: *E. tricolor* V: "Moraspungo". F: *E. tricolor* VI: "Rio Soloma".

been reported for this species and besides the known functions (courtship behavior, territorial behavior, competition, localization, defense signal, distress signal and alarm signal) (e.g., Bogert 1960; Whitney & Krebs 1975; Wells & Schwartz 2006; Wells 2007), their advertisement call also triggers specific short-term behavior (females show positive phonotactic responses on the day of oviposition, which are head lifting, persevering, orienting by head and body movements, zigzag hopping towards sound source, tactile contact to sound source) and reproductive state (Zimmermann & Rahmann 1987). According to Hermans et al. (2002), specific calls might also serve to identify already occupied territories. The closely related species *Epipedobates tricolor* inhabits premontane forests and sparsely vegetated margins of streams in western and central Ecuador (Graham et al. 2004; Lötters 2007).

Silverstone (1976) recorded a population of *E. anthonyi* from the province Azuay in Ecuador which he erroneously identified as *E. tricolor*. Graham et al. (2004) corrected the mistake several decades later by detecting significant genetic differences that correspond to the geographically disjunct distribution ranges, separated by a corridor of 200 km. Based on the assignment by Silverstone (1976), multiple scientific papers confused the two species including the description of the advertisement call of *E. tricolor* which actually corresponds to *E. anthonyi* in Zimmermann & Rahmann (1987). For their studies Zimmermann & Rahmann (1987) used specimens from their own breeding stock (Zimmermann 1983b), which were identified following Zimmermann (1983a).

According to Zimmermann & Rahmann (1987), the advertisement call, emitted by males during the breeding season, is a long trilling high-pitched sound with a duration of 2.15–5.2 s, on average 40–115 pulses per trill, and a dominant frequency ranging between 4,160–4,530 Hz. The pulses last about 15–25 ms, whereas the pulse intervals show a length of 30–50 ms. However, the advertisement call of *E. tricolor* remained yet undescribed. Here, we provide a description of the advertisement call of *E. tricolor* and compare spectral and temporal call characteristics between the closely related species to assess whether acoustic-based species discrimination is feasible. In addition, we quantify intraspecific variation in call patterns by comparing calls of geographically isolated populations.

MATERIAL AND METHODS

To compare acoustic signals of *Epipedobates anthonyi* and *Epipedobates tricolor*, advertisement calls of four populations of *E. anthonyi*, and two populations of *E. tricolor* were recorded. Study populations for *E. anthonyi* comprise I: “Buena Esperanza” (n=5), II: “Rio Jubones” (n= 6) and III: “Rio Saladillo” from geographically ranges in the vicinity of El, Oro, Ecuador (Ostrowksi & Mahn 2016) and IV: “Tierra alta” (n=4) which occurs along the border of Loja and Peru (Ostrowski & Mahn 2016). Study populations for *E. tricolor* comprise V: Moraspungo (n=6) from Cotopaxi and VI: “Rio Soloma” (n=4) from Bolívar (Ostrowski & Mahn 2016) (Fig. 1). All frogs were obtained from the pet trade and represent F_2 from wild caught specimens (Understory Enterprises Inc. [www.understoryenterprises.com] via Peruvian frog import [www.peruvianfrogimport.com now available at https://nasuta.nl/] and Raymond Kuijf, both the Netherlands). Populations were held in separate enclosures measuring 70 x 60 x 50 cm (H x W x D) or 40 x 40 x 50 cm. Enclosures were densely vegetated with tropical plants such as *Pilea* sp., bromeliads like *Neoregelia schultesiana*, some mosses and tendrils and equipped with a small pond of water. The bottom was covered with wet sponges and walls were lined with cork for all populations except “Tierra alta”, for which Hygrolon was used instead. The air temperature was kept between 22 and 24 °C with constant conditions during all recordings; rain was simulated by irrigation systems running three times per day for 30 minutes, and water was sprayed three times per day for 30 seconds, to maintain humidity.

The calls were recorded using a PMD620MKII Professional Handheld Recorder (Marantz Professional, Kawasaki, Japan) by placing the recorder on the sound-permeable ceiling of the enclosures and maintaining a distance of several meters from the enclosures to keep disturbance to a minimum. To ensure suitable recordings, the irrigation system was deactivated during recordings. All calls were recorded, cut and processed using Audacity V. 2.0.5. (Audacity Development Team, available at http://audacity.sourceforge.net). For visualization we used the seewave package for Cran R 3.3.2 (Sueur et al. 2008). Spectrograms were utilized to measure call length, pulse length, pulse intervals and number of pulses; frequencies of each call were plotted using a Hanning Window with a size of 512 and a linear representation to determine dominant frequencies, fundamental frequencies and harmonies. Images from oscillograms and spectrograms were produced for each population. In addition, minima, maxima and mean values of important call features (call duration, number of pulses, pulse duration, pulse frequency, pulse interval length and important frequencies) were computed. Furthermore, one-way ANOVA tests were performed to test for significant differences between important call parameters of *E. anthonyi* and *E. tricolor*; post hoc Tukey HSD tests were used to assess intra- and interspecific differences.

RESULTS

A total of 401 calls were recorded and analyzed. For *E. anthonyi* 282 calls were recorded (89 for I: “Buena Esperanza”, 39 for II: “Rio Jubones”, 64 for III: “Rio Saladillo”, 90 for IV: “Tierra alta”), 119 calls were recorded for *E. tricolor* (68 for V: “Moraspungo”, 51 for VI: “Rio Soloma”).

The advertisement calls of both species were found to be high-pitched and trilling with numerous pronounced pulses per call, six harmonies located above and a fundamental frequency located below the dominant frequency

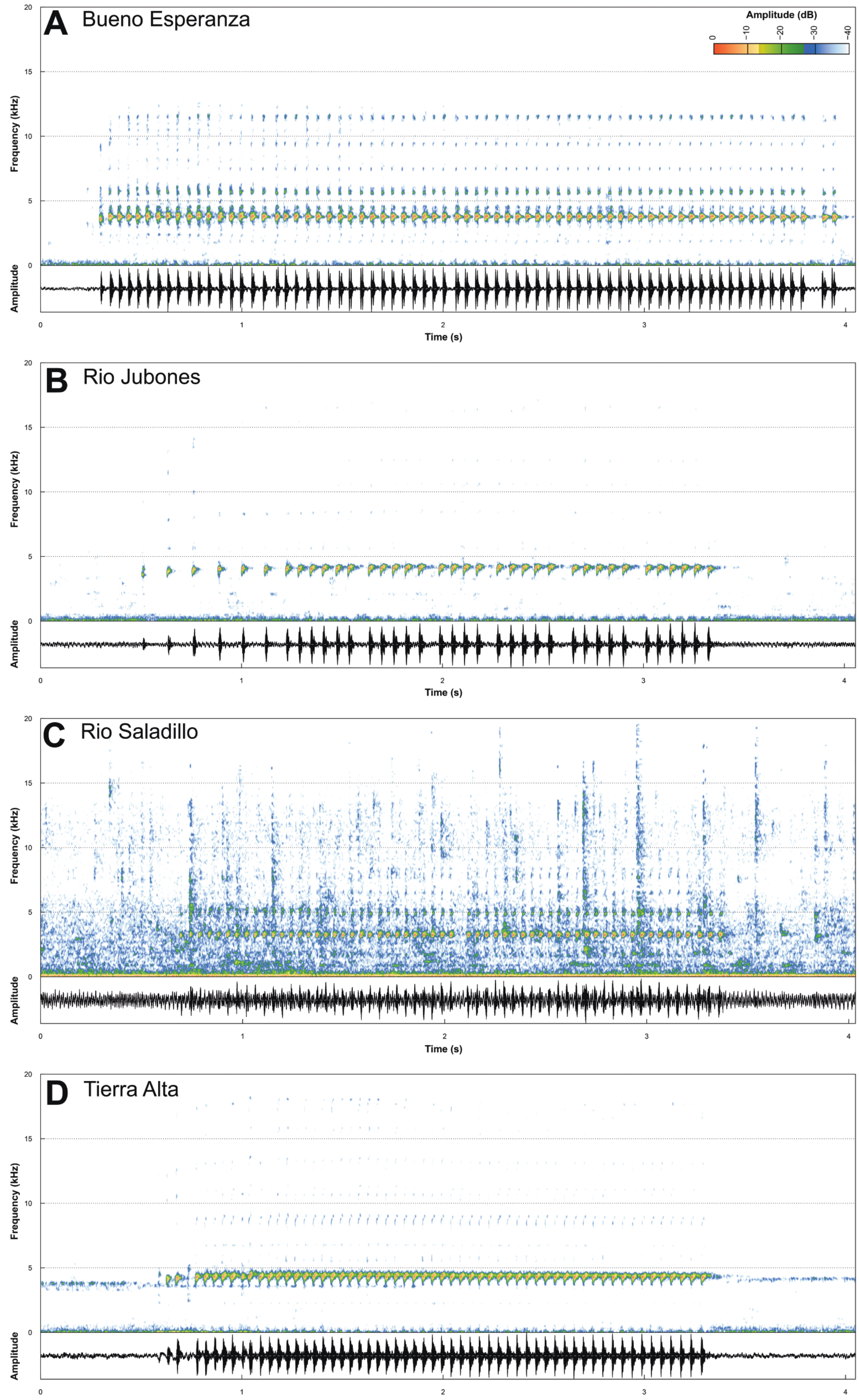

Fig. 2. Spectrograms and oscillograms of the recorded advertisement calls of *Epipedobates anthonyi* A: "Bueno Esperanza", B: "Rio Jubones", C: "Rio Saladillo" and D: "Tierra Alta".

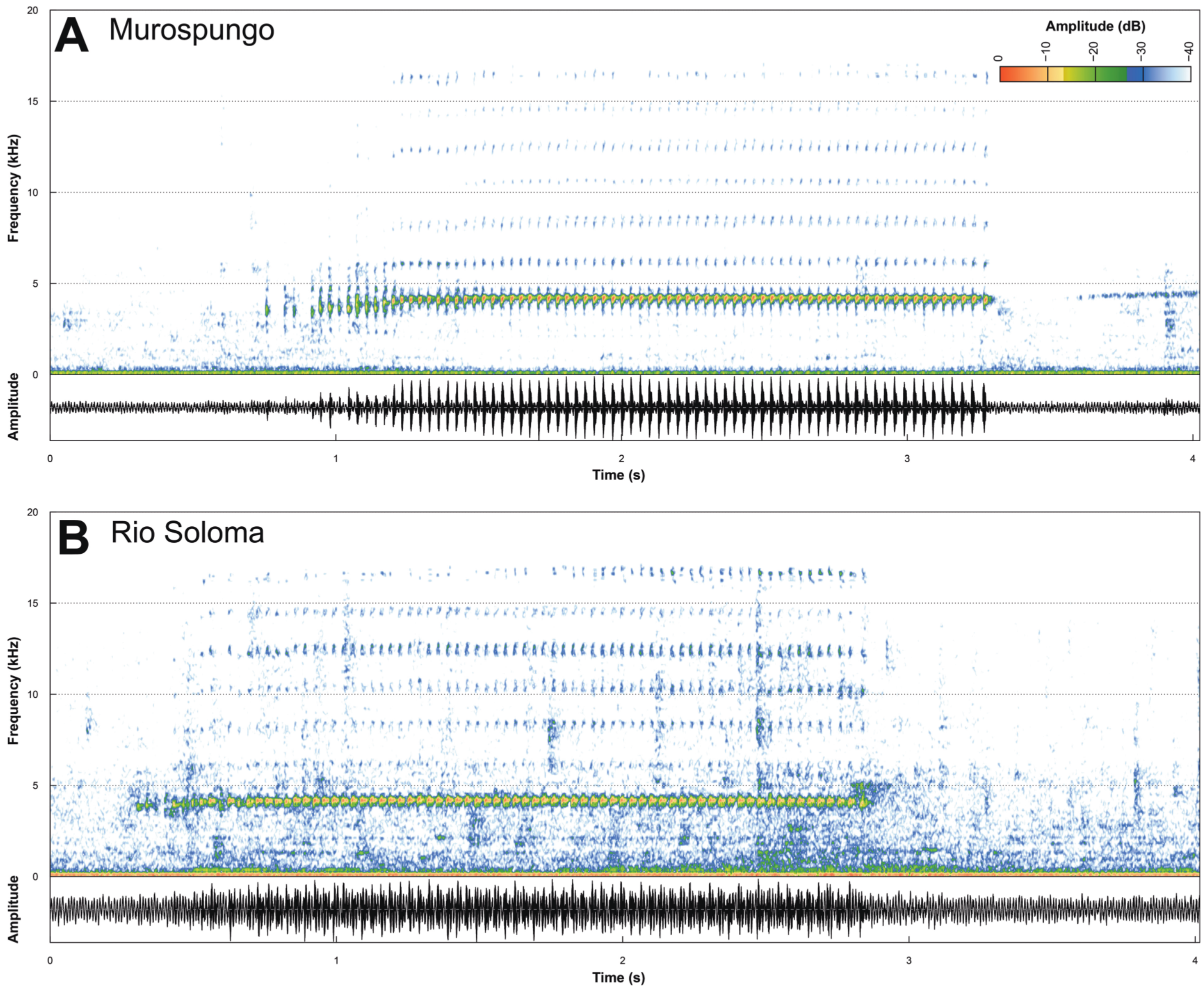

Fig. 3. Spectrograms and oscillograms of the recorded advertisement calls of *Epipedobates tricolor*. A: "Moraspungo" and B: "Rio Soloma".

(Figs. 2, 3). In general, dominant frequencies, fundamental frequencies and harmonies remained constant from the beginning to the end, but for some calls slightly lower initial frequencies could be recognized followed by stabilization at higher values. Comparable to this, pulse frequency and arrangement of pulses remained relatively regular maintaining similar distances to the neighboring pulses during the whole call, but sometimes this was modulated. We observed that pulse frequency for some calls was lower at the beginning and increased after an initial phase. Also, in the final stage an increasing pulse frequency can be recognized for some signals. However, the pulse pattern is occasionally irregular with two or more pulses forming groups which are clearly separated from other groups of pulses, so that calls consist of several notes with a variable number of pulses per note. Although this was observed in both species, it remains an exception. Due to automatic recording, calls could not be related to specific individuals within the monitored tank.

The trilling signals of *E. anthonyi* had an average duration of 2.55 s ± 1.267 (range: 0.5–5.6 s; *n*=282), were composed of 48.521 pulses ± 20.971 (range: 9–99 pulses; *n*=282) per call and had a pulse frequency of 20.293 pulses/s ± 5.02 (range: 8.776–40 pulses/s; *n*=282; computed for each call by dividing the number of pulses through call length). Every pulse possessed a length of 17.091 ms ± 4.041 (range: 9–33 ms; *n*=282), and the average length of intervals between pulses was 32.645 ms ± 7.618 (range: 14–71 ms; *n*=282). The dominant frequency with an average value of 4.24 kHz ± 0.672 (range: 3.126–5.67 kHz; *n*=282) was located above the fundamental frequency of 2.196 kHz ± 4.737 (range: 1.456–4.584 kHz; *n*=229) and below up to six harmonies occupying a frequency band of 4.497–18.135 kHz. The first harmony averaged on 5.918 kHz ± 0.483 (range: 4.441–7.151 kHz; *n*=262), whereas the last harmony was observable at a frequency of 16.997 kHz ± 1.138 (range: 12.330–18.135 kHz; *n*=150). Detailed harmony frequency values are depicted in Table 1.

Table 1. Properties of the advertisement calls of *Epipedobates anthonyi* and *Epipedobates tricolor* in comparison. Values are given as mean ± standard deviation (SD).

Feature	*Epipedobates anthonyi*	*Epipedobates tricolor*
Call duration (s)	2.550 ± 1.267 (0.5-5.6) *n*=282	2.016 ± 0.337 (0.6-2.6) *n*=119
Total number of pulses per note	48.521 ± 20.971 (9-99) *n*=282	60.765 ± 11.682 (14-79) *n*=119
Pulse frequency (pulses/s)	20.293 ± 5.02 (8.776-40) *n*=282	30.051 ± 3.039 (17.5-33.75) *n*=119
Pulse duration (ms)	17.091 ± 4.041 (9-33) *n*=282	13.958 ± 2.584 (9-22) *n*=119
Pulse interval length (ms)	32.645 ± 7.618 (14-71) *n*=282	19.748 ± 2.324 (13-26) *n*=119
Dominant frequency (kHz)	4.240 ± 0.672 (3.126-5.67) *n*=282	4.081 ± 0.218 (3.551-5.431) *n*=119
Fundamental frequency (kHz)	2.196 ± 4.737 (1.456-4.584) *n*=299	2.229 ± 0.153 (2.036-2.814) *n*=96)
1. Harmony (kHz)	5.918± 0.483 (4.441-7.151) *n*=262	5.941 ± 0.336 (4.719-6.567) *n*=119
2. Harmony (kHz)	8.154 ± 0.619 (6.364-9.659 kHz, *n*=250)	8.267 ± 0.345 (7.103-9.985) *n*=114
3. Harmony (kHz)	10.210 ± 0.773 (8.131-12.469) *n*=211	10.365 ± 0.348 (9.36-10.879) *n*=116
4. Harmony (kHz)	12.171 ± 0.958 (9.174-13.493) *n*=213	12.240 ± 0.480 (10.664-13.06) *n*=114
5. Harmony (kHz)	14.400 ± 1.066 (11.157-15.819) *n*=191	14.428 ± 0.350 (13.579-15.131) *n*=105
6. Harmony (kHz)	16.997 ± 1.138 (12.330-18.135) *n*=150	16.289 ± 0.302 (15.45-16.869) *n*=97

Table 2. Properties of the advertisement calls of populations I–VI of *E. anthonyi* and *E. tricolor* in comparison. Values are given as mean ± standard deviation (SD).

Feature	I: "Buena Esperanza"	II: "Rio Jubones"	III: "Rio Saladillo"	IV: "Tierra alta"	V: "Moraspungo"	VI: "Rio Soloma"
Call duration (s)	3.750 ± 1.343	2.190 ± 0.815	1.940 ± 1.004	2.312 ± 0.527	2.115 ± 0.316	1.884 ± 0.317
	(0.5-6.2) *n*=100	(0.7-3.9) *n*=40	(0.5-4.4) *n*=129	(0.7-3.2) *n*=112	(0.6-2.6) *n*=68	(0.8-2.5) *n*=51
Total number of pulses	63.970 ± 24.349	35.800 ± 15.482	39.500 ± 15.068	47.580 ± 11.623	64.382 ± 10.661	55.941 ± 11.233
per note	(9-99) *n*=100	(11-68) *n*=40	(15-78) *n*=129	(12-64) *n*=112	(17-79) *n*=68	(14-75) *n*=51
Pulse frequency (pulses/s)	17.198 ± 3.153	16.153 ± 2.980	22.875 ± 6.284	20.500 ± 1.944	30.461 ± 2.482	29.505 ± 3.581
	(8.776-32.759) *n*=100	(9.444-21.765) *n*=40	(10-40) *n*=129	(9.333-22.800) *n*=112	(18.636-33.750) *n*=68	(17.5-33) *n*=51
Pulse duration (ms)	19.160 ± 3.343	19.625 ± 6.155	15.680 ± 2.880	15.794 ± 3.314	13.912 ± 2.369	14.020 ± 2.846
	(12-28) *n*=100	(11-35) *n*=40	(10-25) *n*=128	(9-26) *n*=112	(9-21) *n*=68	(10-22) *n*=51
Pulse interval length (ms)	35.220 ± 6.424	38.875 ± 9.152	31.590 ± 11.486	33.186 ± 5.753	19.947 ± 2.099	19.882 ± 2.587
	(23-57) *n*=100	(27-71) *n*=40	(14-92) *n*=128	(18-66) *n*=112	(13-26) *n*=68	(14-26) *n*=51
Dominant frequency	3.649 ± 0.264	4.336 ± 0.263	4.226 ± 1.162	4.324 ± 0.124	4.008 ± 0.189	4.179 ±0.217
(kHz)	(3.019-5.413) *n*=100	(3.985-5.787) *n*=40	(3.041-5.670) *n*=129	(3.682-4.750) *n*=112	(3.551-4.233) *n*=68	(3.776-5.431) *n*=51
Fundamental frequency	2.116 ± 0.306	2.151 ± 0.287	1.903 ± 0.937	2.184 ± 0.054	2.267 ± 0.150	2.147 ± 0.125
(kHz)	(1.732-3.043) *n*=79	(1.735-3.747) *n*=37	(0.869-4.454) *n*=71	(1.995-2.411) *n*=111	(2.036-2.658) *n*=66	(2.081-2.814) *n*=30
1. Harmony (kHz)	5.560 ± 0.194	6.061 ± 0.470	5.164 ± 0.849	6.247 ± 0.330	5.805 ± 0.329	6.123 ± 0.246
	(4.727-5.948) *n*=100	(5.186-7.151) *n*=38	(3.673-6.879) *n*=91	(4.969-8.985) *n*=112	(4.719-6.369) *n*=68	(5.456-6.567) *n*=51
2. Harmony (kHz)	7.430 ± 0.432	8.576 ± 0.269	7.444 ± 1.244	8.454 ± 0.300	8.200 ± 0.375	8.8358 ± 0.273
	(6.894-8.938) *n*=69	(8.075-9.344) *n*=38	(4.696-9.659) *n*=102	(6.461-8.985) *n*=111	(7.103-9.985) *n*=66	(7.752-8.867) *n*=48
3. Harmony (kHz)	9.360 ± 0.375	10.750 ± 0.392	8.378 ± 1.060	10.736 ± 0.396	10.279 ± 0.352	10.474 ± 0.311
	(8.131-10.434) *n*=81	(9.974-12.469) *n*=38	(5.747-10.401) *n*=46	(8.673-11.188) *n*=112	(9.360-10.743) *n*=65	(9.790-10.879) *n*=51
4. Harmony (kHz)	11.084 ± 0.531	12.840 ± 0.328	11.033 ± 0.764	12.850 ± 0.500	12.165 ± 0.481	12.563 ± 0.373
	(9.174-11.678) *n*=70	(12.078-13.329) *n*=37	(9.654-12.184) *n*=25	(10.804-13.493) *n*=111	(10.664-12.739) *n*=64	(11.856-13.060) *n*=50
5. Harmony (kHz)	12.304 ± 0.486	14.946 ± 0.420	13.605 ± 0.235	15.075 ± 0.392	14.328 ± 0.284	14.541 ± 0.383
	(11.157-13.087) *n*=25	(13.980-15.752) *n*=37	(13.164-14.006) *n*=39	(13.577-15.890) *n*=107	(13.662-15.131) *n*=56	(13.579-15.065) *n*=49
6. Harmony (kHz)	14.664 ± 0.904	17.124 ± 0.292	14.258 ± 0.823	17.582 ± 0.307	16.182 ± 0.270	16.430 ± 0.278
	(12.330-16.442) *n*=24	(16.243-17.761) *n*=35	(14.258-15.903) *n*=2	(16.208-18.135) *n*=91	(15.450-16.745) *n*=56	(15.553-16.869) *n*=41

Table 3. Properties of the advertisement calls of *Epipedobates anthonyi* and *Epipedobates tricolor* in comparison. Values are given as mean ± standard deviation (SD).

Comparison		Call	Pulses				Frequencies	
		Length	Number	Length	Frequency	Interval lenght	Fundamental	Dominant
Intraspecific		p adj	p adj	p adj	p adj	p adj	p adj	p adj
E. anthonyi	Rio Jubones-Bueno Esperanza	**<0.01**	**<0.01**	**1.000**	0.599	**<0.05**	0.989	**<0.01**
	Rio Saladillo-Bueno Esperanza	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	Rio Saladillo-Rio Jubones	**<0.01**	0.977	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	Tierra alta-Bueno Esperanza	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.104	0.802	**<0.01**
	Tierra alta-Rio Jubones	0.957	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.999	0.999
	Tierra alta-Rio Saladillo	**<0.01**	**<0.01**	0.574	**<0.01**	**<0.01**	**<0.01**	**<0.01**
E. tricolor	Rio Soloma-Muraspungo	0.619	**<0.05**	1.000	0.654	1.000	0.738	0.281
Interspecific								
	Et Muraspungo-Ea Bueno Esperanza	**<0.01**	1.000	**<0.01**	**<0.01**	**<0.01**	0.135	**<0.01**
	Et Muraspungo-Ea Rio Jubones	0.994	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.705	**<0.05**
	Et Muraspungo-Ea Rio Saladillo	**<0.01**	**<0.01**	0.592	**<0.01**	**<0.01**	**<0.05**	**<0.01**
	Et Muraspungo-Ea Tierra alta	0.527	**<0.01**	**<0.05**	**<0.01**	**<0.01**	0.751	**<0.01**
	Et Rio Soloma-Ea Bueno Esperanza	**<0.01**	**<0.05**	**<0.01**	**<0.01**	**<0.01**	0.995	**<0.01**
	Et Rio Soloma-Ea Rio Jubones	0.411	**<0.01**	**<0.01**	**<0.01**	**<0.01**	1.000	0.787
	Et Rio Soloma-Ea Rio Saladillo	**<0.05**	**<0.01**	0.773	**<0.01**	**<0.01**	**<0.01**	**<0.01**
	Et Rio Soloma-Ea Tierra alta	**<0.05**	0.065	**<0.05**	**<0.01**	**<0.01**	0.999	0.357

For the advertisement calls of *E. tricolor* an average length of 2.016 s ± 0.337 (range: 0.6–2.6 s; *n*=119) was measured. The total number of pulses per note was 60.765 ± 11.682 (range: 14–79; *n*=119) with 30.051 ± 3.039 pulses per second (range: 17.5–33.75 pulses/s; *n*=119). The single pulses on average persisted for 13.958 ms ± 2.584 (range: 9–22 ms; *n*=119) and maintained intervals for 19.748 ms ± 2.324 (range: 13–26 ms; *n*=119). A dominant frequency of 4.081 kHz ± 0.218 (range: 3.551–5.431 kHz; *n*=119) and a fundamental frequency of 2.229 kHz ± 0.153 (range: 2.036–2.814 kHz; *n*=96) was measured. As for *E. anthonyi*, six harmonies above the dominant frequency could be recognized, occupying a frequency band of 4.719–16.869 kHz. For detailed results see Table 1. In general, observed variations were lower in *E. tricolor* than in *E. anthonyi*.

One-way ANOVA tests were used to assess statistically significant differences between all local variants of *E. anthonyi* and *E. tricolor* for the most relevant call properties: call length ($F_{(5,395)}$=77.16; p<0.001), fundamental frequency ($F_{(5,319)}$=6.031; p<0.001), dominant frequency ($F_{(5,395)}$=62.97; p<0.001), pulse interval ($F_{(5,395)}$=117.8; p<0.001), number of pulses per call ($F_{(5,395)}$=46.8: p<0.001), pulse frequency ($F_{(5,395)}$=200.4; p<0.001), pulse length ($F_{(5,395)}$=33.84; p<0.001).

Intraspecific differences between the populations of *E. anthonyi* and *E. tricolor* were detected in terms of standard deviation. While there are very slight differences between *E. tricolor* V: "Moraspungo" and VI: "Rio Soloma" in matters of some harmony frequencies (first, second, fourth and fifth harmonies), huge discrepancies were found between the populations of *E. anthonyi*. Comparing the means and standard variations of call properties our results suggested that the variation between the *E. anthonyi* populations was much greater than the differences between *E. anthonyi* and *E. tricolor*. With a call duration of 3.750 s ± 1.343, the call of I: "Buena Esperanza" was significantly longer than for the remaining populations, which all lasted on average 2 s. Furthermore the total number of pulses per call is highest for I as well (63.970 s ± 24.349). For III: "Rio Saladillo" and IV: "Tierra alta" we found a slightly elevated pulse frequency (22.875 s ± 6.284 and 20.500 s ± 1.94) in comparison with I (17.198 s ± 3.153) and II (16.153 s ± 2.980). The dominant frequency of I (3.649 kHz ± 0.264) is lower than the dominant frequencies of the other populations, all averaging above 4.000 kHz. Additionally, smaller values for the fifth and the sixth harmonies were recognized for I, and III also showed a decreased average for the sixth harmony. Besides, we noticed slight variations between all populations of *E. anthonyi* in terms of the first and the fourth harmony. In general, population I was most variable showing the highest variability across all populations in terms of standard deviation. Intraspecific differences are depicted in Table 2.

Post hoc Tukey HSD tests revealed *E. tricolor* to show statistically significant differences across both populations in the number of pulses per call (p<0.05), however, dominant and fundamental frequency, pulse frequency, pulse length, pulse interval and call duration do not vary significantly. In *E. anthonyi* there were statistically significant differences between population I and III for all parameters that were included (p<0.01 each). In contrast, population II and IV only statistically significantly differ in number of pulses per call, pulse frequency, pulse length and pulse interval length (p<0.01 each), whereas call duration (p=0.957), dominant frequency (p=0.999), and fundamental frequency (p=0.999) do not show statistically significant differences. Pairwise comparison of all remaining combinations of the populations proved at least five statistically significant different parameters of included call properties. For detailed results see Table 3.

DISCUSSION

Properties of the advertisement call of *Epipedobates anthonyi* in the present study were generally in accordance with the physical characteristics determined by Zimmermann & Rahmann (1987), but call patterns were revealed to be more variable. Although the note length (2.15–5.2 s) described by Zimmermann & Rahmann (1987) matches the computed average call duration observed in this study, we found that the lower limit reported by Zimmermann & Rahmann (1987) was higher than observed here. This was also true for the total number of pulses per note, which Zimmermann & Rahmann (1987) reported to range from 40 to 115 but was found to range from 9 to 99 in the present study. Due to very few short calls (0.5 s) the average call length was slightly shorter than in previous studies (Zimmermann & Rahmann 1987). However, this might be attributed to elevated levels of disturbance in our animal keeping facility which may have sometimes led to premature interruption of calls. Zimmermann & Rahmann (1987) reported pulse durations between 14–25 ms with intervals of 30–50 ms. Although pulse duration and intervals determined in this study were both longer, results are comparable in general. While Zimmermann & Rahmann (1987) studied a single population we studied four, which could account for the variation in recorded pulse duration and intervals. Zimmermann & Rahmann (1987) found most calls recorded to exhibit only a single harmony with a frequency around 8 kHz while up to six distinct harmonies were present in the majority of the calls in the present study. The few cases in which no or only few harmonies could be identified were caused by background noises or low calls. Pulse frequency and fundamental frequency were not reported previously.

A comparison of the call characteristics of both species yielded small variations. While patterns in *E. anthonyi* were very similar in terms of standard deviation in note duration, number of pulses per note, pulse duration and all important frequency values, standard deviations of *E. tricolor* revealed similarity of calls to be restricted to calls to note duration, number of pulses per call, and all important frequencies except for the last harmony. As we studied four populations of *E. anthonyi* but only two populations of *E. tricolor*, these discrepancies between the standard deviations might be attributed to sample size.

Despite the similarities, calls of both species clearly differ in pulse interval length (*E. anthonyi*: 32.645 ± 7.618 vs. *E. tricolor*: 19.748 ± 2.324) which results in a significantly higher pulse frequency for *E. tricolor* than for *E. anthonyi* (*E. anthonyi*: 20.293 ± 5.02 vs. *E. tricolor*: 30.051 ± 3.039), which might be attributed to local variation across their different distribution areas. Pulse frequency is known to be important for anuran communication (Cocroft & Ryan 1995) and according to Cocroft & Ryan (1995) this feature seems to be the most divergent character in advertisement calls for the genera *Bufo* and *Pseudacris* and is different in almost every species included in their study. Given the clear differences in pulse frequency and interval length, which were found between *E. anthonyi* and *E. tricolor*, these characteristics might prove to be relevant characters for species delimitation in *Epipedobates* (significantly different in intraspecific comparisons: 100%, intraspecific comparisons: 71.5 %).

Only few descriptions of the advertisement calls of other species of *Epipedobates* exist, except for a comprehensive description for *E. boulengeri* (Barbour, 1909) (Lötters et al. 2003) who found distinct advertisement calls for two populations from Colombia and Ecuador, respectively. While the population from Colombia was found to exhibit calls consisting of 1–3 notes, calls of the population from Ecuador comprised 6–12 significantly shorter notes with lower numbers of pulses per note. Calls consisting of several notes were occasionally recognizable for *E. anthonyi* and *E. tricolor* as well, but these calls remained exceptions. The dominant frequencies of 5.192 kHz (Colombia) and 5.198 kHz (Ecuador) for *E. boulengeri* are higher than in the present study, thus, their calls differ in at least two characteristics from *E. anthonyi* and *E. tricolor*. Further studies are required to facilitate a more thorough comparison. Because of their different advertisement calls, populations of *E. boulengeri* were thought to represent a species complex and Grant et al. (2006) proved *E. boulengeri* not to be monophyletic based on genetic data.

We found huge intraspecific disparities across populations of *E. anthonyi* with population I being the most divergent. However, as the geographic distribution of the species is poorly known, a character comparison relating to geographical distribution and environmental influences was not possible. Given that previous research on various taxa revealed varying signals between and within geographically separated units and conspecific populations and that variations increase with geographical distances (Nei 1972; Wöhrmann & Jain 1990; Hutchinson & Templeton 1999), it appears likely that population I exhibits the largest geographic distance to all other populations of *E. anthonyi.* Intraspecific differences of *E. tricolor* were very low and their distribution is poorly known as well. Considering huge call similarities, both populations might show small geographical distance.

Previous research demonstrated that depending on the species, some call characteristics are more important for species discrimination than others, and sometimes only a single feature varies between two closely related species, for example, in some species of *Bufo* (Cocroft & Ryan 1995). Thus, calls can be very helpful for taxonomic matters, facilitating acoustic signal- based species discrimination (Wells 1977; Zimmermann & Zimmermann 1988; De la Riva et al. 1995, 1996; Lötters et al. 1999; Padial et al. 2008). However, calls are not always suitable to discriminate between closely related taxa in amphibians (Lötters et al. 2001; Napoli & Cruz 2005; Tsuji-Nishikidoet et al. 2012). Tsuji-Nishikidoet et al. (2012) showed that differentiation is not possible based on acoustic signals for the closely related species *Allobates nidicola* and *A. masniger* (Aromobatidae), which occupy similar areas along the Madeira River. Additionally, previous research on frogs of the genus *Allobates* (Aromobatidae) supports that discrepancies in call characters are often associated with genetic differences in amphibians but do not necessarily indicate different species (Simões et al. 2008). Advertisement call characteristics were shown to be very different even within a species depending on temperature, distribution area and other aspects (Bernal et al. 2005; Márquez & Eekhout 2006; Lötters et al. 2009); this is now also shown across different populations of *E. anthonyi*. Huge differences in call features within *E. anthonyi* question the suitability of advertisement call characteristics for species identification. Between *E. anthonyi* and *E. tricolor* significant intraspecific variation could be detected for pulse frequency and interval length which can be used for species identification. Thus acoustic based classification should be regarded with suspicion for at least those two species of *Epipedobates*.

The present research raises new questions, which require further investigation. First, to show if pulse frequency and interval length, which differ between *E. anthonyi* and *E. tricolor*, are sufficient for females to discriminate between species supporting the genetic assignment by Graham et al. (2004), further behavioral studies should be conducted: According to the biological species concept, a species is a group of potentially interbreeding individuals in time, thus, mating experiments between *E. anthonyi* and *E. tricolor* could be investigated to detect if pulse frequency and pulse interval length are relevant for female attraction and species recognition. Besides, acoustic comparison of more populations would help to determine whether observed variation in pulse frequency and interval length was attributed to the restricted sample size or whether these characteristics still differ from each other in a more extensive study and thus proving them to be relevant for species identification. Further advertisement call comparison of more species of the genus *Epipedobates* would help to detect if pulse frequency and pulse interval length are important for species discrimination for *Epipedobates* in general. In addition, investigations in more populations of both species are required to determine whether intraspecific differences are also observable across other populations. Ecological mating experiments could help to indicate if females are attracted by calling males from other populations despite intraspecific differences and thus support the populations belonging to the same species.

Sometimes species are difficult to distinguish solely based on their genetic or morphological characters (Rubinoff et al. 2006). Thus the anuran advertisement call characteristics can be useful to help classify taxa. To determine which and how many acoustic characters need to be used to identify diverging species, more extensive call comparison studies have to be conducted. Due to intraspecific variations for *E. anthonyi* and *E. tricolor*, acoustic based discrimination should be interpreted with care. Although we did not find out whether the observed different call characteristics facilitate species discrimination between *E. anthonyi* and *E. tricolor* or if identification based on advertisement calls is possible, this study is a starting point for further investigations in acoustic based-identification of *Epipedobates*.

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