Comparative Call Characteristics in the Anuran Subgenus *Nenirana*

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It is important that species descriptions be accurate. This is especially true with threatened and endangered species, where the scientific literature provides context for species identification and management decisions. In 1953, Bragg documented the advertisement calls of both Southern Crawfish Frogs (*Lithobates a. areolatus*) and Northern Crawfish Frogs (*L. a. circulosus*) and concluded *L. a. areolatus* have lower frequency calls. One of us (M.J.L.) has long felt this observation was incorrect, because adult *L. a. areolatus* are smaller than *L. a. circulosus*, and anurans with smaller body sizes tend to produce higher, not lower, frequency calls. Bragg's observations are not only the sole comparative assessment of advertisement call characteristics of *L. areolatus* in the primary literature, but also represent the only assessment of the advertisement calls of any of the four species comprising the subgenus *Nenirana*, which consists of threatened and endangered Gopher Frogs (*L. capito* and *L. sevosus*) and Pickerel Frogs (*L. palustris*). Given this dearth of information, the reliability of this information, and the conservation concerns surrounding this group, we felt it was necessary to reassess the advertisement calls of Crawfish Frogs and give them context by describing the calls of the *Nenirana* species *L. capito* and *L. palustris*. Our data revise Bragg's conclusions, but just as importantly, we show that the call characteristics of *L. capito* and *L. palustris* are similar by having long calls with long interpulse intervals, while both subspecies of *L. areolatus* have shorter calls with less interpulse separation. These results do not align with morphological and phylogenetic clustering within the *Nenirana*, but are consistent with call character displacement within this group. Additional data will be necessary to test this inference.

CRAWFISH Frogs (*Lithobates areolatus*) have long been considered the most secretive ranid in North America (Wright and Myers, 1927), due in large part to their obligate use of crayfish burrows and consequently their miniscule (~0.05 m^2) home ranges (Heemeyer and Lannoo, 2012; Heemeyer et al., 2012). This secrecy has led to sampling challenges, which in turn have produced a number of contradictory statements in the scientific literature about the basic biology of this species (Hoffman et al., 2010). As the conservation status of *L. areolatus* becomes more tenuous—of the 243 United States counties known to have been inhabited across their historic range, today Crawfish Frog populations remain in 159, a 35% decline in occupancy (Lannoo et al., 2017)—there is an urgency to resolve these inconsistent reports in order to understand these highly specialized animals.

One such question concerns the advertisement (breeding) call characteristics of Crawfish Frogs. Bragg (1953) documented the advertisement calls of both Southern Crawfish Frogs (*L. a. areolatus*) and Northern Crawfish Frogs (*L. a. circulosus*) in eastern Oklahoma and observed that compared with *L. a. circulosus*, *L. a. areolatus* have deeper pitched (lower frequency) calls, with a longer duration, that rise in frequency and often terminate in a low whistle. One of us (M.J.L.) has long thought Bragg's (1953) observation about comparative call frequencies was incorrect, because adult *L. a. areolatus* are substantially smaller than *L. a. circulosus* (Wright and Wright, 1949; Lannoo, 2005), and anurans with smaller body sizes tend to produce higher, not lower, frequency calls (Blair, 1958). Bragg's (1953) observations continue to be not only the sole comparative assessment of advertisement call characteristics of *L. areolatus* in the primary literature, but also are the only assessment of the advertisement calls of any species within the four-species subgenus *Nenirana* (Hillis and Wilcox, 2005; see Elliott et al. [2009] to hear the calls of *L. a. circulosus* [listed as *L. a.*

areolatus], *L. palustris* [Pickerel Frogs], and *L. capito* [Gopher Frogs]).

Hillis and Wilcox (2005) erected the subgenus *Nenirana*, a single clade consisting of four species arranged as follows: (Pickerel Frogs + (Gopher Frogs [2 spp.] + Crawfish Frogs)). Engbrecht et al. (2011) showed that in contrast to the small, slender Pickerel Frogs, Gopher Frogs and Crawfish Frogs are large, heavy bodied ranids, which—despite their preference for small, underground retreat sites—have no morphological specializations for burrow excavation. Gopher Frogs are named for their occupancy of Gopher Tortoise (*Gopherus polyphemus*) burrows, although they will inhabit a variety of retreat sites including pine stump hollows and crayfish burrows. In contrast, Crawfish Frogs are obligate crayfish burrow dwellers. Individuals will inhabit the same burrows for up to five years (M.J.L., unpubl. data), and the tight fit of frog to burrow facilitates the specialized predator avoidance behavior of these frogs (Altig, 1972; Engbrecht et al., 2012).

Given the lack of data on advertisement call metrics in this group, the reliability of this information (critical for species/ subspecies identification in the field), and the uncertain future of Crawfish Frogs and Gopher Frogs (Lannoo, 2005), we felt it was necessary to 1) reassess the call metrics of Crawfish Frogs, 2) formally describe the calls of Gopher Frogs and Pickerel Frogs, and 3) compare them to each other (a within-*Nenirana* assessment) as well as to Southern Leopard Frogs (*L. sphenocephalus*), to provide an outgroup comparison.

MATERIALS AND METHODS

Call sampling.—We acquired advertisement calls from several ongoing research or monitoring programs, as follows. We recorded *L. a. circulosus* at Hillenbrand Fish and Wildlife Area West, in southwest Indiana; *L. a. areolatus* and *L. sphenocephalus* at Attwater Prairie Chicken National Wildlife Refuge in southeast Texas; *L. capito* at the Joseph W. Jones Ecological

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Table 1. Means and 95% confidence intervals of call metrics (dominant frequency, call duration, interpulse period, and pulses/call) for *Lithobates sphenocephalus* (outgroup comparison), and three species (*L. palustris, L. capito,* and *L. areolatus*) representing the subgenus *Nenirana*. Within *L. areolatus*, we consider both the northern (*L. a. circulosus*) and southern (*L. a. areolatus*) subspecies. Phylogenetic relationships among these taxa are indicated by the left-side cladogram (*sensu* Hillis and Wilcox, 2005).
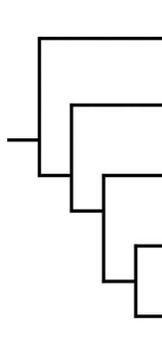
Species		Dominant Frequency (Hz)	Call Duration (sec)	Interpulse Period (sec)	Pulses/ Call
L. sphenocephalus	Mean	1338.2	0.52	0.098	6.7
	± 95% CI	111.7	0.05	0.006	0.4
L. palustris	Mean	871.9	1.95	0.028	72.5
	± 95% CI	127.4	0.11	0.002	6.9
L. capito	Mean	708.8	1.82	0.045	42.4
	± 95% CI	56.3	0.20	0.007	12.2
L. a. areolatus	Mean	1133.7	0.51	0.017	32.9
	± 95% CI	54.2	0.04	0.001	2.4
L. a. circulosus	Mean	806.4	0.84	0.019	40.0
	± 95% CI	72.7	0.08	0.002	2.5

Research Center in southwest Georgia; and *L. palustris* in Houston County in east Texas. We could not obtain adequate numbers of recordings of the federally endangered *L. sevosus* to include in our analyses. At all sites, we recorded vocalizations with Song Meter® models SM1 and SM2 (built-in microphone sensitivity at –35 dBV/pa and –40 dBV/pa, respectively; Wildlife Acoustics Inc., Concord, Massachusetts).

We sampled advertisement calls according to the protocols established by the local research or monitoring programs, as follows: *L. a. circulosus* from 1900 to 0300 hr at two wetlands in Indiana during March and April 2010 (Williams et al., 2013); *L. a. areolatus* and *L. sphenocephalus* during the first minute of each hour throughout the day, year-round during 2013 and 2014 at 21 wetlands in Texas; *L. capito* from 2000 to 0400 hr during February 2014 at four wetlands in Georgia; and *L. palustris* for the first minute of each hour from 2100 to 0200 hr year-round from 2010–2014 at three wetlands in Texas. We recorded ambient temperature at the time of each call and selected the subset of call bouts made at 15±0.5°C. From each call bout, we analyzed the first non-overlapping call we encountered. We analyzed a total of 30 calls for each taxon (species or subspecies) considered.

Call analysis.—To analyze calls we used Song Scope® call recognition software version 3.4 (Wildlife Acoustics Inc.) and Raven Pro® Interactive Sound Analysis Software version 1.4 (Bioacoustics Research program, 2011, The Cornell Lab of Ornithology, Ithaca, New York). For each breeding call, we measured dominant frequency, duration, and interpulse period (window type: Hann, size: 256). We used Audacity version 2.0.5 (open source software available at http://audacity.sourceforge.net) to remove interference when noise inhibited analysis but excluded calls from the analysis when noise interference precluded measurement ($n = 98$). We calculated the number of pulses per call by dividing call duration by interpulse periods, then adding one. We have deposited our call files in The Cornell Laboratory of Ornithology's Macaulay Library, where they may be accessed at https://search.macaulaylibrary.org/catalog?view=List&catId=230113-230137.

In order to assess the effects of body size (snout–vent length; SVL) on call metrics (see below), we used male SVLs from the dataset published in Engbrecht et al. (2011) for *L. a. circulosus* ($n = 33$, 96.7±2.7 mm, mean±95% confidence) and *L. capito* ($n = 28$, 79.3±3.9 mm). For male *L. a. areolatus* ($n = 58$, 74.6±1.7 mm), *L. palustris* ($n = 24$, 50.8±1.5 mm), and *L. sphenocephalus* ($n = 74$, 59.4±1.5 mm), we measured SVLs of all males in the museum collections at Texas A&M University, the University of Texas, and Stephen F. Austin State University.

Statistical analysis.—We assessed call metrics for the four species and two subspecies as follows. We first applied the Bartlett's test of sphericity ($\chi^2 = 251.3$, df $= 6$, $P < 0.0001$) and Kaiser-Meyer-Olkin test of sampling adequacy (score $= 0.460$), which determined that variances were equal and variable reduction was warranted (Friel, 2009). Using a Principal Components Analysis (PCA; Program R Package Psych; R Core Team, 2016), we reduced the number of potential variables to two principal components (PC1 and PC2), which explained 86% of the variance in our data (Table 1). We then used a Varimax rotation to define the variables associated with each component (Table 2). Next, we compared the rotated loadings with the average male SVL (natural-log transformed) calculated for each species using linear regressions in Program R. Our preliminary results revealed that body size influenced both PC1 and PC2 (see below). Because of this finding, we compared size-adjusted call metrics by analyzing the residuals of the preliminary regressions comparing size and the rotated loadings with Analysis of Variance (ANOVA) and Tukey's honest significant difference (HSD) tests. Residuals represent the deviations from the expected call metrics for a frog of a given size.

RESULTS

Call metrics varied as follows (Fig. 1; Table 1). Call frequencies were highest in *L. sphenocephalus* (~1340 Hz) and *L. a. areolatus* (~1100 Hz) but otherwise similar (between 700 and 900 Hz) in *L. palustris*, *L. capito*, and *L. a. circulosus*. Call durations were longest in *L. palustris* and *L. capito* (1.8–2.0 sec) and shortest in *L. sphenocephalus* and *L. a. areolatus* (0.51–0.52

Table 2. Results of the Varimax rotation on the retained principal components (PC1 and PC2, Table 1). High rotated values are bolded and best explain those components.

Call characteristics	Loadings		Varimax rotation	
	PC1	PC2	PC1	PC2
Dominant frequency	−0.762	0.049	**−0.522**	**0.558**
Duration	0.796	0.582	**0.979**	−0.121
Interpulse period	−0.761	0.613	−0.134	**0.968**
Pulses/call	0.961	0.042	**0.729**	**−0.628**

sec). Interpulse periods varied from 0.017 (*L. a. areolatus*) to 0.098 sec (*L. sphenocephalus*). The number of pulses per call ranged from 7 (*L. sphenocephalus*) through 33–42 (*L. a. areolatus*, *L. a. circulosus*, and *L. capito*) to 73 (*L. palustris*). In short, *L. sphenocephalus* had the shortest, highest frequency calls with the greatest interpulse periods, while *L. capito* and *L. palustris* had the longest, lowest frequency calls. Both *L. a. circulosus* and *L. a. areolatus* had intermediate call metrics, with the calls of *L. a. circulosus* being longer and lower than *L. a. areolatus* (Tukey's HSD, $P < 0.0001$; Fig. 1; Table 1).

Principal components analysis confirmed the trends indicated by our descriptive statistics. PC1 defined longer calls (both duration and pulses/call) with lower frequencies, while PC2 defined shorter calls with greater interpulse periods and higher frequencies (Fig. 2). Size-adjusted call metrics represented by both PC1 and PC2 revealed significant differences among species independent of their differences in body size (ANOVA results, $F_{4,71} = 136.9$, $P < 0.0001$; $F_{4,71} = 90.8$, $P < 0.0001$, respectively). (Body size explained 14% of the variation in PC1 and 39% of the variation in PC2.) The Varimax rotation of our principal components axes revealed three distinct species clusters. The first consisted of only *L. sphenocephalus*, which exhibited shorter calls with higher frequencies and greater interpulse periods. The *Nenirana* contained two clusters: *L. palustris* and *L. capito* grouped together based on their longer, lower frequency calls, and the two subspecies of *L. areolatus* grouped together based on their intermediate frequencies, shorter call durations, and shorter interpulse periods (Fig. 2).

DISCUSSION

Our data correct several conclusions drawn by Bragg (1953), who documented (with the aid of recording devices but without analytical software) the advertisement calls of both Crawfish Frog subspecies. While Bragg (1953) determined that *L. a. areolatus* had lower dominant frequency calls with a longer duration that rise in frequency and often terminate in a low whistle, our sonograms show essentially the opposite, that *L. a. areolatus* have higher frequency calls of shorter duration. Further, while Bragg (1953) observed, correctly, that calls of *L. a. areolatus* rise in frequency, so do the calls of *L. a. circulosus* (Fig. 1). We did not observe a terminal, higher-frequency whistle in either subspecies.

Phylogenetics.—Our data suggest a clear separation in call metrics between *L. sphenocephalus* and the species that comprise the *Nenirana*. Compared with *L. sphenocephalus*, species of *Nenirana* exhibited longer calls with lower frequencies and shorter interpulse periods (Figs. 1, 2; Table 1).

Within the *Nenirana* (Pickerel Frogs + (Gopher Frogs [2 spp.] + Crawfish Frogs)), there is little difference between the calls of *L. palustris* and *L. capito* (Figs. 1, 2; Table 1). In contrast, there are considerable differences in call metrics between the *L. palustris* + *L. capito* grouping and *L. areolatus*. Specifically, while demonstrating a similar interpulse period, *L. areolatus* exhibited a lower dominant frequency and decreased call duration (Figs. 1, 2; Table 1). The difference in dominant frequency can be explained by larger body size of *L. areolatus* (linear regression, $P < 0.001$).

Within the *Nenirana*, morphology (size and shape) follows phylogeny (Pickerel Frogs + (Gopher Frogs [2 spp.] + Crawfish Frogs)). Engbrecht et al. (2011) showed that Pickerel Frogs are small and slender, while Gopher Frogs and Crawfish Frogs are large, heavy bodied ranids. Gopher Frogs and Crawfish Frogs are similar; as recently as 35 years ago, *L. capito* and *L. areolatus* were considered to be the same species (Altig and Lohoefener, 1983). In fact, differences between Pickerel Frogs and Gopher Frogs + Crawfish Frogs are sufficient to place them in separate genera by the criterion of Inger (1958): "each genus should represent the same kind of entity: a distinct mode of life and a distinct evolutionary shift."

Predictions about call metrics based on either morphology or phylogenetic position would cluster *L. capito* and *L. areolatus* to the exclusion of *L. palustris* (Table 1). But, as noted above, what we observe instead is the clustering of *L. capito* with *L. palustris* to the exclusion of *L. areolatus* (Fig. 2). We were curious about this incongruity and offer the following hypothesis as a potential explanation. One of the primary drivers of the study of anuran advertisement calls
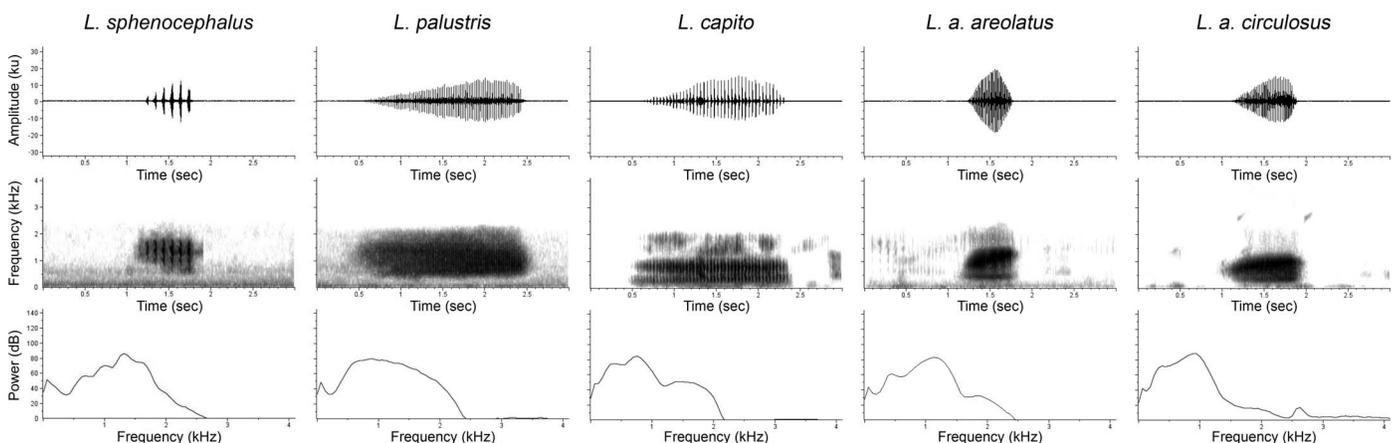
Fig. 1. Song oscillograms (amplitude), sonograms (frequency), and power outputs of *L. sphenocephalus, L. palustris, L. capito, L. a. areolatus,* and *L. a. circulosus* recorded at 15±0.5°C.
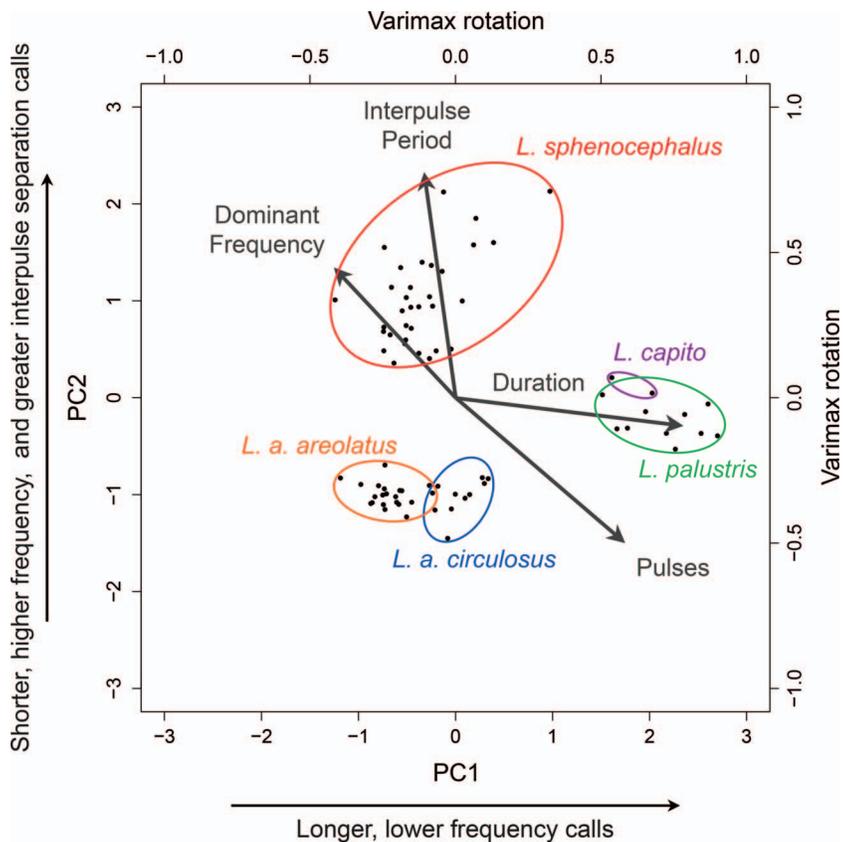
Fig. 2. The Varimax rotation of our principal components axes revealed three distinct species clusters based on call variables, as follows: *L. sphenocephalus*, *L. palustris* and *L. capito*, and the two subspecies of *L. areolatus*.

has been the idea of reproductive character displacement (Blair, 1955; Ball and Jameson, 1966; Gerhardt, 1994; Höbel and Gerhardt, 2003; Lukanov et al., 2015). As Blair (1958) elucidated, because the main function of advertisement calls is to attract females, these vocalizations are useful for understanding speciation and general evolutionary dynamics. Further, because advertisement calls serve as an important species isolating mechanism (Blair, 1955; Wells, 2007), sympatric species are expected to differ in their mating calls (Blair, 1964). With the exceptions of sympatry in South Carolina and North Carolina, *L. capito* and *L. palustris* are allopatric across most of their range (Green et al., 2014), so character displacement is unnecessary. In contrast, the distribution of *L. areolatus* is largely sympatric with *L. palustris* (Green et al., 2014), where we observe substantial call divergence. Reproductive character displacement is characteristic of closely related species in areas of sympatry and has been described in the families Microhylidae (Blair, 1955), Hylidae (Ball and Jameson, 1966; Gerhardt, 1994; Höbel and Gerhardt, 2003), and Ranidae (Lukanov et al., 2015). While call divergence between sympatric *L. areolatus* and *L. palustris*, but not allopatric *L. capito* and *L. palustris* suggests reproductive character displacement, as Gerhardt and Huber (2002) emphasize, merely observing this pattern is inconclusive without evidence that selection against mating has contributed to it. A true test of this idea would come from female choice experiments.

ACKNOWLEDGMENTS

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