

Population Variation of Complex Advertisement Calls in *Physalaemus petersi* and Comparative Laryngeal Morphology

KATHRYN E. BOUL AND MICHAEL J. RYAN

Populations of *Physalaemus petersi* show extraordinary variation in the expression of facultative complex calls. Previous studies have shown that a structure in the larynx, the fibrous mass (FM1), is correlated with variation in production of the complex call among taxa in the *Physalaemus pustulosus* species group. We tested the hypothesis that the variation in the presence of call complexity that occurs within *P. petersi* mirrors the same laryngeal variation associated with complex calls among other species of the genus. We examined calls and larynges of *P. petersi* from populations on a north-south transect on the western side of its Amazonian distribution. All four populations of *P. petersi* (Peru and Brazil) in the south produce complex calls, whereas only one population of five in the north (Ecuador) produce complex calls. We compared larynges of four populations of *P. petersi*, two that produce complex calls and two that do not. We then compared them to larynges of *P. pustulosus*, which produces complex calls, and to *Physalaemus coloradorum*, which does not produce complex calls. The FM1 is significantly larger in populations of *P. petersi* that produce complex calls than those without complex calls. FM1 sizes of populations of *P. petersi* were more similar to other species with the same call type than they were to populations of their own species with a different call type. These data suggest several interpretations of the evolution of complex calls and the associated underlying morphology.

COMMUNICATION is critical to initiate mating in many animals. Variation among communication signals can result from the interaction of natural and sexual selection (Tuttle and Ryan, 1981; Endler, 1993). Just as features of signals should evolve in response to selection, so should the mechanisms that produce them. Limitations, such as those imposed by morphological (Maynard Smith et al., 1985) and phylogenetic (Podos, 1997) constraints, however, might further influence the direction of signal evolution. Understanding the mechanistic constraints on signal production has provided insights into signal evolution in songbirds (Greenewalt, 1968; Podos, 1997), insects (Otte, 1992), and anurans (Martin, 1971; Ryan, 1986).

Most male frogs advertise to females using vocalizations (Gerhardt and Huber, 2002). Sound is produced by pulmonary air passing through the larynx (Fig. 1A–C), where vocal folds and arytenoid cartilages vibrate (Martin, 1971; Gans, 1973). Frogs of the family Leptodactylidae often possess thickenings of the vocal folds, or fibrous masses (Martin, 1971; Drewry et al., 1982; Fig. 1A). In some *Physalaemus* species, these fibrous masses (FM1s) are freely suspended and occlude the bronchial passages (Martin, 1971; Drewry et al., 1982; Ryan and Drewes, 1990) (Fig. 1B–D).

Acoustic communication in *P. pustulosus* consists of a simple call, the whine, and a secondary

component, the chuck, added facultatively during vocal interaction (Rand and Ryan, 1981). The simple call coupled with a secondary component is known as the complex call. The facultative complex call is unusual in the genus, because it has been recorded in only two species, *P. pustulosus* and *P. petersi* (A. Cardoso, D. Cannatella, S. Rand, M. Ryan unpubl.). Comparative laryngeal studies of *P. pustulosus* with two distantly related congeners (Drewry et al., 1982) and among more closely related *Physalaemus* species (Ryan and Drewes, 1990) showed qualitatively that a larger FM1 size was correlated with the presence of complex calls. Behavioral studies have shown that complex calls are favored over simple calls by sexual selection (Ryan, 1985). Therefore, it can be hypothesized that the corresponding structures in the larynx resulted from the same sexual selection pressures.

Exploratory studies revealed substantial variation among *P. petersi* in their ability to produce complex calls. *Physalaemus petersi* is found throughout the Amazon (Lynch, 1970; Cannatella and Duellman, 1984; Kok, 2000), although it might represent two species: a northern species in Ecuador (*P. petersi*), and a southern species in Peru, Bolivia, and Brazil (*Physalaemus freibergeri*; Cannatella et al., 1998). Because the taxonomy is not resolved, we will use the epithet “*petersi*” for both taxa. Although complex calls

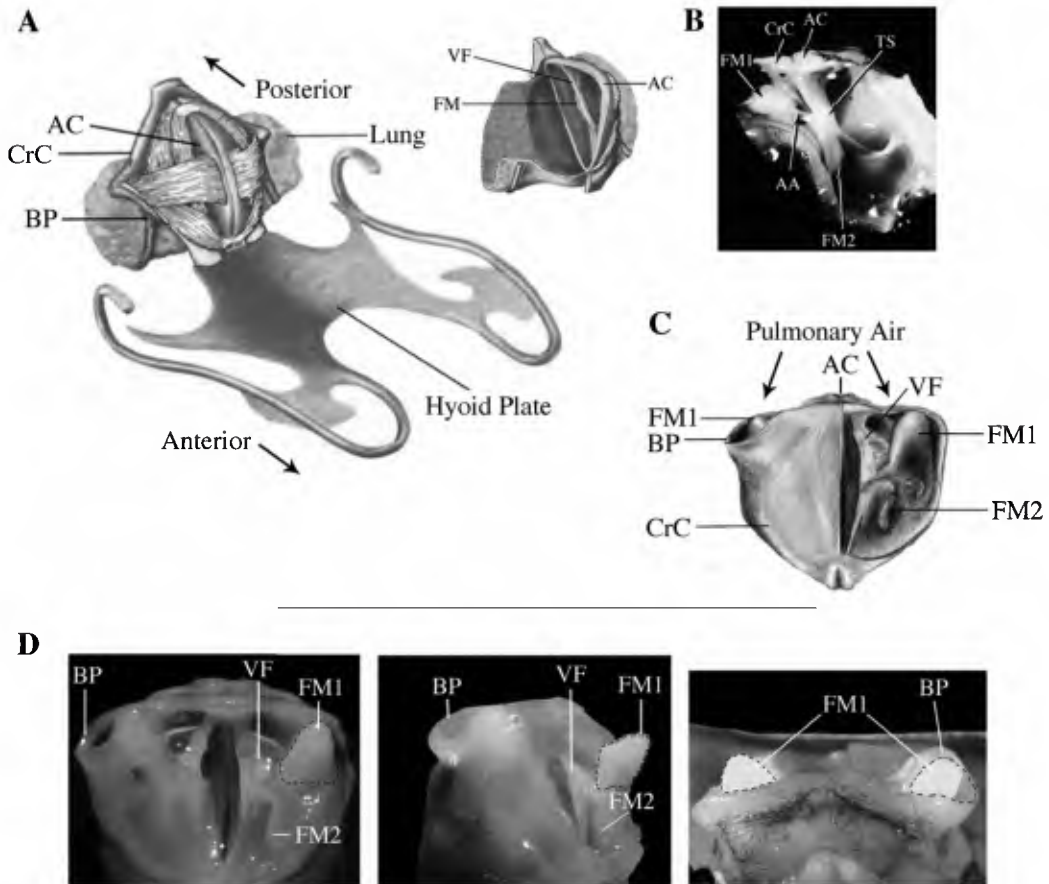


Fig. 1. Images of anuran larynges. (A) Drawing of an anuran hyolaryngeal structure, taken from Duellman and Trueb (1986). AC = arytenoid cartilage, CrC = cricoid cartilage, BP = bronchial process. To the right is a cross-sectional view of the anuran larynx, adapted from Martin (1972) and Duellman and Trueb (1986). The vocal folds, VF, and a fibrous mass, FM, can be seen in this view. (B) Cross-sectional view of a larynx of *Physalaemus pustulosus*, from Ryan and Drewes (1990). The primary fibrous mass, FM1, occludes the airway. A transverse thickening, TS, of the vocal folds is also shown here. (C) Drawing of the ventral or cardiac view of a *Physalaemus petersi* larynx without the musculature and hyoid plate, showing the opposite side of the larynx to (A), with the AC hidden from view. The BP is at the entrance to the lungs and the FM1 occludes the airway. The CrC is removed on the right side of the larynx, exposing the FM1 embedded in the vocal folds, VF. The smaller FM2 is positioned anterior to the FM1. (D) Photographs of a Yasuni male's larynx. From left to right: ventral, side, and posterior view. The dotted lines represent the measured FM1 and BP areas.

of *P. petersi* had been reported in Peru and western Brazil (R. Cocroft and R. McDiarmid; Crombie, personal communication cited in Ryan and Rand, 1993), an analysis of over 1500 calls of *P. petersi* in the vicinity of Jatun Sacha, in Ecuador (Fig. 2) by Ryan and Rand (1993) revealed no complex calls. Fieldwork by K. Boul in 1998 (unpubl.), however, revealed complex calls in the northern extent of the range of *P. petersi*.

The current study has two motives: (1) to examine the presence or absence of complex calls among populations of *P. petersi* along a north-south transect of its northwestern range; and

(2) to test the hypothesis that the correlation of variation in call complexity and laryngeal morphology among *Physalaemus* species occurs among populations of *P. petersi*. Thus, we compare within species variation of calls and laryngeal morphology to calls and laryngeal variation between species.

MATERIALS AND METHODS

Advertisement calls.—We recorded advertisement calls of individuals from three populations of *P. petersi* near La Estación Científica Yasuni, La Selva Hostería, and Cando (Fig. 2; Appendix 1),

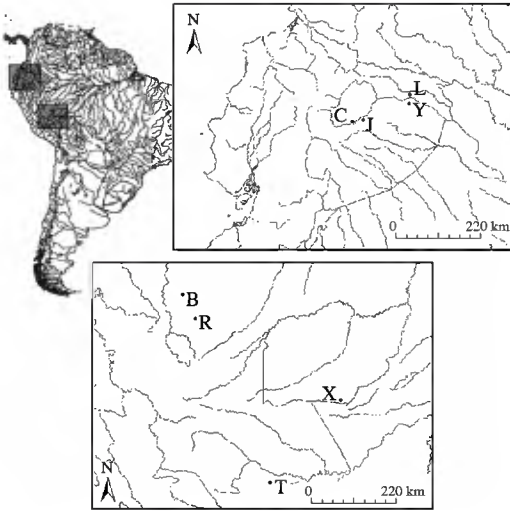


Fig. 2. Map of northwestern South America with locations of *Physalaemus petersi* populations. The two shaded areas represent the northern and southern regions, enlarged on the right. From north to south: L = La Selva, Y = Yasuni, J = Jatun Sacha, C = Cando, B = Boca do Tejo, R = Restauração, X = Xapuri, T = Tambopata.

using a Sennheiser SE 66 microphone and a Sony Walkman WM-D6C professional stereo cassette recorder. For each male recorded, we noted the snout-vent length and temperature of the water in which he was calling. We also used calls and temperature data previously recorded by A. Cardoso for populations in Boca do Tejo, Restauração, and Xapuri, Brazil and by R. Crocroft, for the population in Tambopata, Peru. We digitized calls using Signal at 20 kHz.

In Yasuni, La Selva, and Cando, we broadcast simple and complex calls of *P. pustulosus* and *P. petersi* to several calling males to facilitate the production of complex calls (Ryan and Rand, 1993). These experiments were conducted from March to June 1998 and 2001 in choruses of over five males. If no complex calls were produced, complex calls were noted as absent. If at least one individual produced a complex call, complex calls were marked as present. Calls were broadcast 1 m from each calling male and adjusted by ear to the level of the male calling. In some frogs, the sound level of a call may alter the response of the male. In *P. pustulosus*, however, males produced more complex calls as the amplitude of the broadcasted call increased (Rand and Ryan, 1981). We realize that the lack of complex calls during our surveys does not definitively support the null hypothesis that complex calls are never produced.

Laryngeal dissection and analysis.—Two populations of *P. petersi* in which males produce complex calls (Yasuni and Tambopata) and two populations which do not produce complex calls (Cando and La Selva) were used to compare male laryngeal variation between populations of the two call types (simple and complex). For further comparison, we measured larynges of male *P. pustulosus*, a species in which all populations studied produce complex calls, and *P. coloradorum*, a species in which all populations studied are known only to produce simple calls.

Specimens were collected from Ecuador and borrowed from Museo de Zoología, Centro de Biodiversidad y Ambiente, Pontificia Universidad Católica del Ecuador (QCAZ), Texas Memorial Museum (TNHM) and National Museum of Natural History (USNM). All specimens were fixed in formaldehyde and maintained in 70% ethanol.

We have used terminology from Trewavas (1933) and Martín (1971) to describe the laryngeal structures. To examine the larynges ex situ, we excised the hyo-laryngeal apparatus. We removed the cricoid cartilage on the cardiac side from one side of the larynx, allowing for full view of the FMI. Each larynx was soaked for 15 min in Weigert's Lugol solution to increase the contrast of the structures. We took digital pictures using a SPOT microscope camera with a millimeter scale in three different views: cardiac (ventral), side, and posterior (Fig. 1D). Using Image J (vers. 1.27; National Institutes of Health), we measured the surface areas of the larynx. We summed the three measures of the FMI and measured the bronchial process (BP) area (Fig. 1D), larynx area in the ventral view, and snout-vent length. We also qualitatively compared the anchoring of the FMI to the arytenoid cartilage.

Statistical analysis.—Statistical analyses were conducted in Systat. We analyzed laryngeal measurements using an analysis of covariance with body size and larynx area as covariates, to ensure that the variation of the structures was not due to body or laryngeal size. In all analyses, the four treatment groups were *P. petersi* (simple calls), *P. petersi* (complex calls), *P. pustulosus* (complex calls), and *Physalaemus coloradorum* (simple calls).

RESULTS

Advertisement call variation.—Three populations did not exhibit complex call production: Cando, La Selva, and Jatun Sacha. The Yasuni population was the only population of *P. petersi* re-

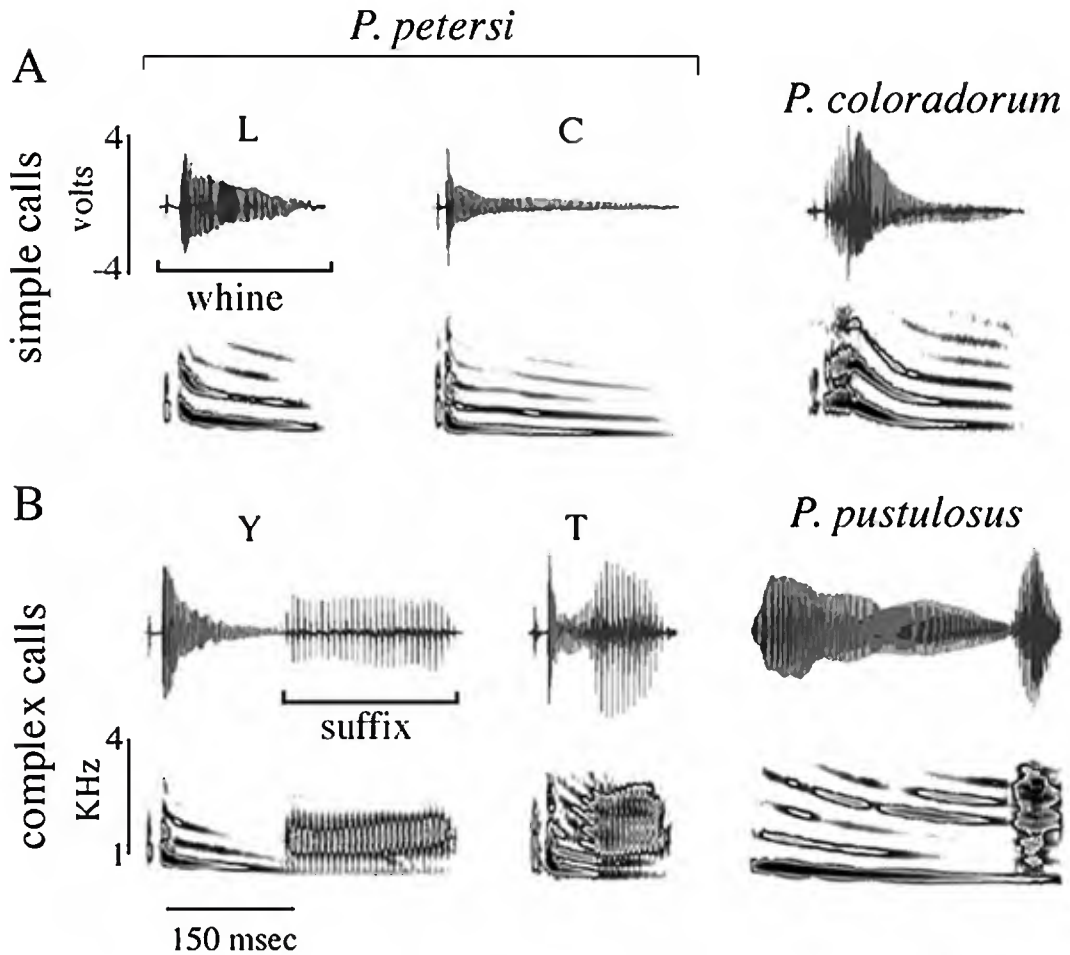


Fig. 3. Oscillograms and sonograms of *Physalaemus petersi*, *Physalaemus pustulosus*, and *Physalaemus coloradorum*. All calls are uniformly scaled. (A) Representatives of simple calls from populations of *P. petersi* and *P. coloradorum*. L = La Selva, C = Cando. (B) Representatives of complex calls from populations of *P. petersi* and *P. pustulosus*. Y = Yasuní, T = Tambopata.

corded in the north with a complex call (Fig. 3). The complex call was present in all the southern populations surveyed (Tambopata, Peru; Restauração, Boca do Tejo, and Xapurí, Brazil).

General description of larynges.—Larynges of four populations of *P. petersi* and two closely related species (*P. pustulosus* and *P. coloradorum*) all exhibited vocal folds with a large fibrous mass (FM1), a small, sausage-shaped fibrous mass (FM2) and transverse thickenings of vocal folds (TS; Fig. 1C–D).

Size of laryngeal structures, relative to body size.—Male *P. petersi* from populations that produce complex calls were significantly larger in body size than populations of *P. petersi* with simple

calls (ANOVA $P < 0.001$). This difference was due to the large males from Yasuní. Although *P. pustulosus* males were larger than *P. coloradorum* males, all males in populations of *P. petersi* were larger than *P. coloradorum* and *P. pustulosus* (Appendix 1).

Populations of *P. petersi* with complex calls had larger FMIs for all measures than conspecific populations with simple calls when adjusted for body size (ANCOVA ventral: $P < 0.001$; side: $P < 0.001$; posterior: $P < 0.01$; summed: $P < 0.001$; Fig. 4A; Appendix 1). The same differences were apparent when *P. petersi* was compared to the other two species. The sizes of the FM1 in populations of *P. petersi* with complex calls and in *P. pustulosus* were significantly larger than the FM1 of *P. petersi* populations with simple calls and *P. coloradorum* (ANCOVA, body size

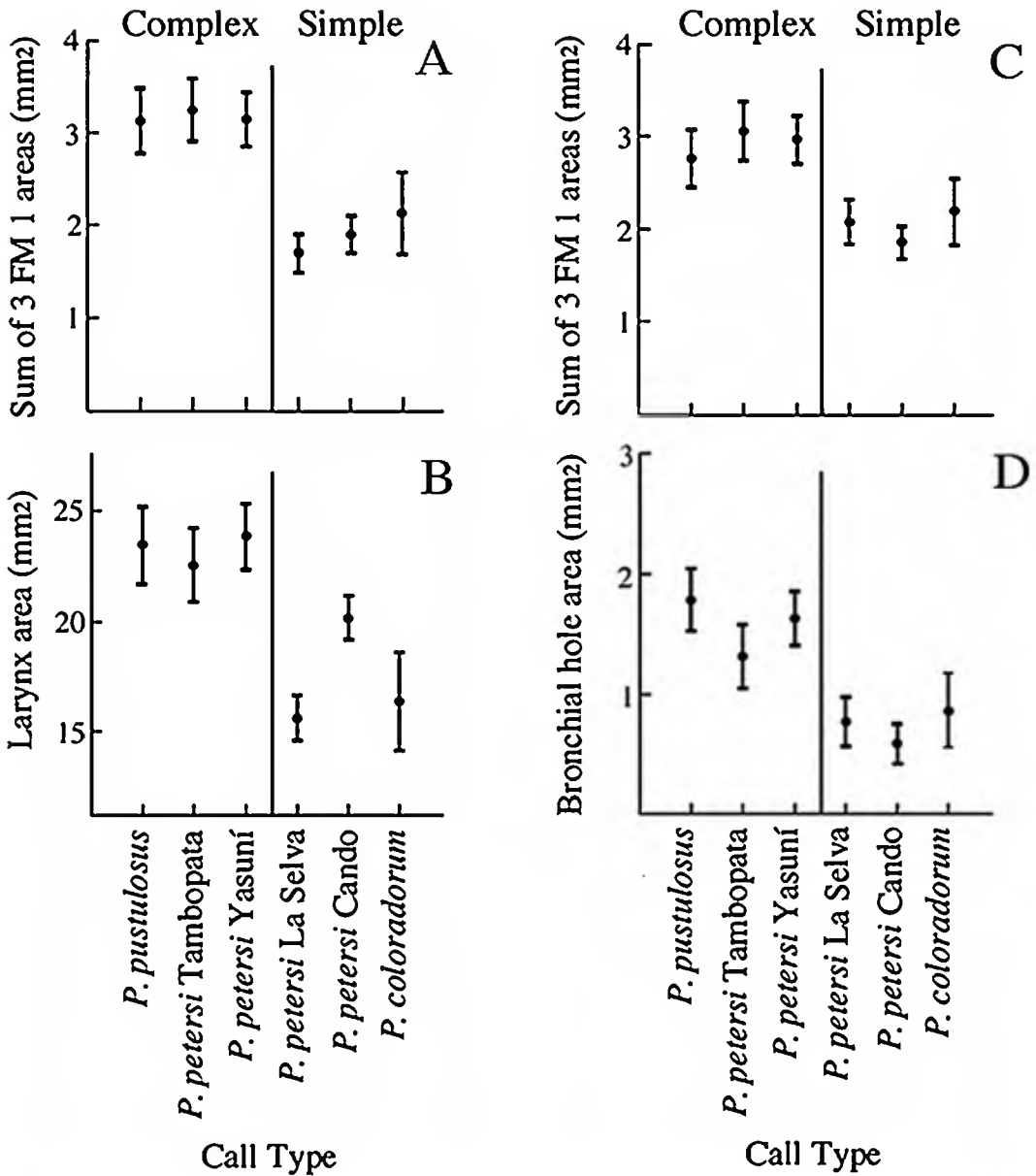


Fig. 4. Mean and standard error plots of measurements for each population. Populations of *Physalaemus petersi* with complex calls are on the left side of the line and the populations with simple calls are on the right side on the X-axis. On the far right is a species with simple calls, *Physalaemus coloradorum* and on the left is *Physalaemus pustulosus*, a species with complex calls. (A) Measurements of the fibrous mass size summed in three views, adjusted for SVL body size. (B) Measurements of larynx size, adjusted for body size. (C) Measurements of the fibrous mass area summed in three views, adjusted for larynx area. (D) Measurement results of the bronchial process (BP) area from the posterior view, adjusted for larynx area.

as covariate: ventral: $P < 0.001$; side: $P < 0.001$; posterior: $P < 0.01$; summed: $P < 0.001$; Fig. 4A). The FM1 of populations of *P. petersi* that produce only simple calls were more similar for all measures to *P. coloradorum* than they were to other *P. petersi* populations with complex calls

(Fig. 4A). The FM2 did not vary with simple or complex calls in any comparisons (ANCOVA, body size as covariate: ventral: $P = 0.097$).

The area of the BP in the posterior view was comparatively larger in populations of *P. petersi* with complex calls compared to *P. petersi* with

simple calls (ANCOVA, body size as covariate: $P < 0.001$; Appendix 1). The BP area of *P. pustulosus* was more similar to populations of *P. petersi* with complex calls than *P. coloradorum* and populations with simple calls. (ANCOVA, body size as covariate: $P < 0.001$).

Size of laryngeal structures, relative to larynx size.—*Physalaemus pustulosus* and populations of *P. petersi* with complex calls had larger larynges than *P. coloradorum* and populations of *P. petersi* with simple calls (ventral view: ANOVA, $P < 0.001$; body size ANCOVA, $P < 0.001$; Fig. 4B). We compared sizes of laryngeal structures by controlling for differences in larynx size. The FMI and BP area in the side view and summed measurements were larger between populations and species with complex calls (ANCOVA: side area, $P = 0.025$; summed, $P = 0.012$, Fig. 4C; BP $P = 0.005$, Fig. 4D).

Significant differences in FMI size were absent in the ventral view of the FMI for intra- and interspecific comparisons when adjusted for larynx area (ANCOVA: ventral view $P = 0.078$). *Physalaemus coloradorum* was not significantly different from complex calling populations of *P. petersi* in this view although there was a nonsignificant trend in the expected direction (ANCOVA, larynx area as covariate: posterior: $P = 0.077$). However, we did find differences between the two groups of *P. petersi* in the posterior view. The FM2 of Yasuní was significantly larger than the conspecific populations and heterospecific species (ANCOVA, larynx area as covariate: ventral view $P = 0.007$), but there was no difference between other comparisons.

FMI anchoring.—The FMI was anchored by two attachments, one of which is in the dorsal area of the BP and extends dorso-posteriorly. All specimens examined showed variation in the thickness of this FMI attachment, masking any potential intra- or interspecific differences in this feature. We did not observe any difference in the anchoring of the FMI inside the BP either intra- or interspecifically.

DISCUSSION

Advertisement call variation.—Males of *P. petersi* produced complex calls in all populations in the southern extent of its range (Peru and Brazil) but in only one population in the north (Ecuador). This contrasts with *P. pustulosus*, where all populations surveyed over a 5000 km transect throughout its range produced complex calls (Ryan et al., 1996). The variation of call complexity within *P. petersi* allowed for a com-

parative study between populations and closely related species.

Laryngeal variation and correlations of size to vocal production.—*Physalaemus pustulosus* and populations of *P. petersi* with complex calls had larger FMIs, larynges, and BPs than *P. coloradorum* and populations of *P. petersi* with simple calls. Between populations with the two distinct call types, the FMI shows more variation in the side view than the ventral or posterior views, suggesting that the side measurement is a more important component in the correlation of complex call production. Ryan and Drewes (1990) compared the larynges of four species in the *P. pustulosus* species group: *P. pustulosus*, *P. petersi*, *P. coloradorum*, and *P. pustulatus*. *Physalaemus petersi* specimens were examined from Ecuador, Peru, and Alto Paraiso, Brazil. They concluded that the FMI of *P. petersi* was smaller than *P. pustulosus* but that both were larger than the FMI of *P. coloradorum* and *P. pustulatus*. Our results concur with Ryan and Drewes but show additional variation among populations of *P. petersi* corresponding to complex call production. This variation is extended to the closely related species, *P. pustulosus* and *P. coloradorum*, which produce complex and simple calls, respectively.

We found no difference in the dorsal attachment of the FMI to the BP, which does not concur with Ryan and Drewes (1990) who found that populations with complex calls had an attachment similar to *P. pustulosus* and larger than other populations of *P. petersi* they examined. We did detect a general difference in the protrusion of the FMI into the BP. Species and populations with simple calls had FMIs that protruded less into the BP, away from the airflow, than those with complex calls.

The average FMI size was also significantly larger in Yasuní than the La Selva population just 20 km north. These populations are found on either side of the Rio Napo, which has a width of 1300 m, suggesting that the Rio Napo forms a biogeographic barrier. Gascon et al. (1998) report, however, that the Amazonian Juruá River, which can be 500 m in width at its mouth, was not a genetic barrier to several species of frogs, including *P. petersi*. Although the mouth of the Juruá River is smaller in width than the Rio Napo, it suggests caution in assuming that the Rio Napo would be a substantial barrier to gene flow between the Yasuní and La Selva populations.

The FMI as a mechanism for producing complex calls.—The question remains as to how, or if, the FMI mediates complex call production. It has

been suggested that the FMI vibrates to produce the secondary component (Drewry et al., 1982; Ryan and Drewes, 1990). This proposition would require the FMI to be removed from the direct flow of pulmonary air while the primary component is produced and then brought back to the airflow to vibrate, producing the secondary component. This could occur through a change in larynx shape (Drewry et al., 1982) mediated by laryngeal muscles rotating the arytenoid cartilages anteroventrally, thereby tightening the space around the FMI (Ryan and Drewes, 1990). We propose, alternatively, that the FMI might change the path of air to the vocal folds. Instead of the FMI vibrating, the large FMI size and the rotation of the arytenoid cartilages could serve to redirect the airflow to another point on the vocal folds than that, which produces the simple call.

The complex call and the comparatively large fibrous masses that correspond to complex call production are derived states for the genus, because these two characters are not found in other congeners or probably even in other frogs (Ryan and Drewes, 1990). In *P. petersi*, the sizes of the larynges and FMIs of populations lacking complex calls more closely resemble *P. coloradum* than its own species with complex calls. Ryan and Drewes (1990) propose that the dorsal attachment inside the bronchial wall was necessary for complex call evolution. Because we did not see a correlation between this attachment and complex calls, we suggest that the attachment is not required and that a larger fibrous mass would be the necessary change. A detailed resolution as to how complex calls in *Physalaemus* are produced, however, needs to come from biomechanical experimentation rather than structure-function correlations.

SPECIMENS EXAMINED

Physalaemus petersi: Estación Científica Yasuní, Orellana, Ecuador 00°40'46S 76°23'83W [QCAZ 11957, 11644, 14737, 15121–22, 15124, 15133, 15135–38]; Cando, Napo Province, Ecuador 01°04'20"S 77°55'99"W [QCAZ 15102–03, 15105–07, 15082, 15161, 15162, 15176]; Jatun Sacha, Napo Province, Ecuador 01°02'24"S 77°21'36"W; La Selva Hostería, Sucumbios, Ecuador 00°29'90"S 76°22'39"W [QCAZ 11486, 15166–67, 15169–70, 15184–86]; Boca do Tejo, Acre, Brazil 08°58'53S 72°43'04"W; Xapurí, Acre, Brazil 10°39'00"S 68°31'00"W; Restauração, Acre, Brazil 09°13'00"S 72°14'00"W; Tambopata, Madre de Dios, Perú: 12°44'00"S 69°11'00"-W [USNM 268995, 268997, 343006].

Physalaemus pustulosus: Gamboa, Panamá [TNHC 62667–69].

Physalaemus coloradum: Santo Domingo de los Colorados, Ecuador [QCAZ 19416, 19418, 7173–74, 2964].

ACKNOWLEDGMENTS

We thank the Museo de Zoología, Centro de Biodiversidad y Ambiente, Pontificia Universidad Católica del Ecuador (QCAZ), the Texas Memorial Museum, and the National Museum of Natural History for loaning specimens. We are grateful to the Ministerio del Ambiente of Ecuador for issuing permits (004-IC-FAU-DFP). We thank A. S. Rand and R. Heyer for comments on the manuscript, D. Cannatella for assistance with the analysis, S. Ron for assisting with the map, and F. Ayala, R. Boul, J. Guayasamin, D. Lombeida, K. Newcomb, K. Koe, and K. Mott for assisting with fieldwork. Animal care procedures were approved by University of Texas (0302170). Research was funded by the National Science Foundation (IBN 99–81631).

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- SECTION OF INTEGRATIVE BIOLOGY C0930, UNIVERSITY OF TEXAS, AUSTIN, TEXAS 78712. E-mail: (KEB) kathyboul@mac.com; and (MJR) mryan@mail.utexas.edu. Send reprint requests to MJR. Submitted: 4 June 2003. Accepted: 26 Feb. 2004. Section editor: M. J. Lannoo.

APPENDIX 1. LARYNGEAL MEASUREMENTS FOR POPULATIONS OF *Physalaemus petersi* AND RELATED SPECIES. Ventral = ventral view; Side = side view; Post = posterior view; All = three views combined; SVL = snout-vent length (mm); FM1 = primary fibrous mass area (mm²); BP = bronchial process area (mm²); Lar = larynx area (mm²).

Populations	Stats	Ventral FM1	Side FM1	Post. FM1	All FM1	Post. BP	Ventral Lar	SVL Body
Simple: Cando	# of ind	9	9	9	9	7	9	9
	Mean	0.829	0.683	0.287	1.800	0.594	19.638	25.73
	SD	0.159	0.317	0.276	0.694	0.281	2.298	1.01
	CV	0.191	0.464	0.961	0.386	0.473	0.117	0.04
Simple: La Selva	# of ind	8	8	8	8	8	8	8
	Mean	0.797	0.601	0.255	1.654	0.627	15.375	26.24
	SD	0.188	0.154	0.109	0.358	0.325	2.316	2.18
	CV	0.236	0.256	0.428	0.216	0.519	0.151	0.08
Complex: Yasuní	# of ind	11	11	10	11	10	11	11
	Mean	1.414	1.368	0.852	3.556	1.837	27.332	30.19
	SD	0.403	0.234	0.378	0.766	0.558	4.784	1.69
	CV	0.285	0.171	0.444	0.216	0.304	0.175	0.06
Complex: Tambopata	# of ind	3	3	3	3	3	3	3
	Mean	1.444	1.118	0.751	3.313	1.414	23.362	27.17
	SD	0.261	0.101	0.244	0.411	0.059	1.344	1.04
	CV	0.180	0.091	0.324	0.124	0.041	0.058	0.04
Simple: <i>P. coloradorum</i>	# of ind	5	5	5	5	5	5	5
	Mean	0.677	0.484	0.276	1.438	0.595	11.456	20.87
	SD	0.102	0.159	0.063	0.152	0.127	1.418	0.96
	CV	0.151	0.329	0.228	0.106	0.213	0.124	0.05
Complex: <i>P. pustulosus</i>	# of ind	3	3	3	3	3	3	3
	Mean	1.159	0.988	0.759	2.907	1.844	22.111	24.63
	SD	0.156	0.077	0.199	0.407	0.961	2.583	0.40
	CV	0.135	0.078	0.262	0.140	0.521	0.117	0.02