

Geographic variation in the laryngeal morphology of a widely distributed South-American anuran: behavioural and evolutionary implications

CARLOS LÓPEZ¹, MARICEL QUISPE², ALDO VILLALÓN³, MIGUEL L. CONCHA^{4,5,6}, MARIO PENNA² and NELSON A. VELÁSQUEZ^{1*} 

¹Laboratorio de Comunicación Animal, Facultad de Ciencias Básicas, Universidad Católica del Maule, San Miguel 3605, 3480112, Talca, Chile

²Laboratorio de Neuroetología, ICBM, Universidad de Chile, Independencia 1027, 8380453, Santiago, Chile

³Health Sciences Department, Universidad de Aysén. Obispo Vielmo 62, Coyhaique, Chile

⁴Laboratory of Experimental Ontogeny, Institute of Biomedical Sciences, Facultad de Medicina, Universidad de Chile, PO Box 70031, Santiago, Chile

⁵Biomedical Neuroscience Institute, Independencia 1027, Santiago, Chile

⁶Center for Geroscience, Brain Health and Metabolism, Santiago, Chile

Received 13 March 2019; revised 1 December 2019; accepted for publication 10 December 2019

Sound-producing organs generate acoustic signals that have a fundamental role in communication systems. In species exhibiting different biogeographic patterns, variations of these structures can explain a large part of interpopulation differences of their signals. *Pleurodema thaul* is an anuran with an extensive geographic distribution in Chile and presents an evident divergence in its acoustic signals among three genetic/bioacoustic groups (i.e. northern, central and southern). By means of classic histology and 3D-reconstructions, we study the geographic variation in the larynx of *P. thaul* males from these three groups. In addition, volumes of six laryngeal structures are used as predictors of acoustic characteristics of advertisement calls recorded in previous studies for the same subjects used in the current study. After removing the effect of body size, the arytenoid cartilage, dilator muscle and vocal cords show significant differences between the three bioacoustic groups. Furthermore, arytenoid cartilage and dilator muscle volumes predict some temporal parameters and also the dominant frequency of advertisement calls. Our results show important geographic variation in laryngeal morphology, which is in correspondence with acoustic, behavioural and genetic variation in this species.

ADDITIONAL KEYWORDS: 3D-reconstruction – advertisement call – animal communication – intraspecific variation – larynx frog – *Pleurodema thaul* – sound – voice.

INTRODUCTION

Animal communication operates when a sender generates a signal that causes a behavioural change in a receiver (Green & Marler, 1979; Bradbury & Vehrencamp, 2011). This process allows conspecific and/or heterospecific interactions such as kin recognition, danger alert, mate attraction or territory defence (Bossert & Wilson, 1963; Alexander, 1967; Marler, 1967; Alcock & Bailey, 1995). As signals are

involved in important processes and stages along lifetimes of animals, they usually exhibit species-specific (reviewed in: Gerhardt & Huber, 2002; Wells, 2007) and optimum characteristics for transmission in the environments where they are produced (Endler, 1980; Wiley & Richards, 1982). Because of the close relationships between these traits and the environment (Marler, 1967), many signals show important intraspecific geographic variation (Verrell & Arnold, 1989; Ryan *et al.*, 1990; Ryan & Wilczynski, 1991; Mitani *et al.*, 1999; Brooks & Endler, 2001), which has been accounted for by the action of diverse

*Corresponding author. E-mail: nelson.velasquez@gmail.com

evolutionary forces: genetic drift (Barton, 1996; Roy, 1997), natural (Morton, 1975) and sexual selection (West-Eberhard, 1983; Price, 1998).

Variation or alteration in any of the components of the communicative process (i.e. sender, receiver, environment and signal) can generate evolutionary changes and speciation as long-term outcomes. Diverse instances have been reported in which variation in signals (Levine & MacNichol, 1979; Verrell & Arnold, 1989; Fleishman, 1992; Buesching *et al.*, 2002; Johansson & Jones, 2007), environment (Naguib & Wiley, 2001; Ord & Stamps, 2008; Velásquez *et al.*, 2018) or sensory systems (Levine & MacNichol, 1979; Fleishman, 1992; Ryan *et al.*, 1992; Guillamón & Segovia, 1997; Briscoe & Chittka, 2001; Narins, 2001; Woolley *et al.*, 2006; Hunt *et al.*, 2009) have important consequences for communicative processes. In addition to these sources of variation, drastic effects on communication occur as a consequence of structural modifications of signal-producing organs (Quay, 1959; Griffiths, 1978; Ballintijn *et al.*, 1994; Endler & Houde, 1995; Galeotti *et al.*, 2003; Dale, 2006).

Anurans provide excellent models for studies on acoustic communication in general (Ryan, 2001; Gerhardt & Huber, 2002; Wells, 2007) and, in particular, variation in the acoustic signals of anurans has been supported by variation in sound-producing organs (McClelland *et al.*, 1996, 1998). Intraspecific differences in the acoustic signals of anurans result from dissimilar configurations of laryngeal components, namely muscles and cartilages (Martin, 1971). These structural configurations determine differences in acoustic characteristics of anuran calls, as the temporal characteristics of these sounds are generated by movements of the laryngeal muscles and arytenoid valves combined with abdominal muscle contractions, while the spectral characteristics of the vocalizations are associated with the arytenoid cartilages and vocal cords (McClelland *et al.*, 1996; Boul & Ryan, 2004; Suthers *et al.*, 2016; Lagorio *et al.*, 2019).

The four-eyed frog, *Pleurodema thaul* (Lesson, 1826), has a broad geographic distribution in Chile, from the Atacama Desert (Correa *et al.*, 2007) to the Patagonian region (Veloso, 2006). This species occurs across a wide range of habitats having different climatic characteristics (Hajek & Di Castri, 1975). Northern populations are subjected to high temperatures, but these are attenuated by the influence of the Pacific Ocean at coastal locations, and annual rainfall is below 140 mm. Central populations experience a Mediterranean climate (i.e. hot, dry summers and cool, wet winters), with an annual precipitation of ~450 mm, while populations inhabiting southernmost localities are subjected to a temperate forest regime, where

annual rainfall is ~2600 mm. During the reproductive season (July to December), males of *P. thaul* call on the surface of slow-water streams, inflating their vocal sacs and emitting advertisement calls to attract conspecific females. Penna & Veloso (1990) described these calls as long sequences of individual pulses lasting about 50 ms and having a dominant frequency centred at about 2 kHz. Subsequent studies identified three bioacoustic groups in correspondence with three different latitudinal geographic areas: northern, central and southern (Velásquez *et al.*, 2013, Velásquez, 2014). Advertisement calls differ broadly among these three bioacoustic groups, as the individuals from the northern and central populations emit calls composed of longer pulses, also having a higher number of pulses, slower pulse rates and higher intrapulse modulation depths than those of individuals from southern localities (Velásquez *et al.*, 2013). In addition, a recent study demonstrated that males of this species follow Bergmann's rule, as body size increases with latitude in this species (Rivas *et al.*, 2018). Based on the geographic variation of the acoustic characteristics of the calls of *P. thaul* studied previously, we here test the hypothesis that laryngeal structures vary in correspondence with the extant acoustic variation between populations of this species. We thereby expect to unveil morphological correlates for a comprehensive appraisal of intraspecific variation of the sound communication system of a species having an extensive geographical distribution.

MATERIAL AND METHODS

Male frogs were collected from eight populations of *P. thaul* along the geographical distribution of this species in Chile: Totoral (27° 54' S, 70° 56' W; *N* = 3), Socos (30° 43' S, 71° 29' W; *N* = 4), Torca Lagoon (34° 46' S, 72° 2' W; *N* = 5), Hualqui (36° 55' S, 72° 51' W; *N* = 3), Elicura (37° 55' S, 73° 6' W; *N* = 1), Coñaripe (39° 32' S, 71° 57' W; *N* = 6), Máfil (39° 41' S, 72° 57' W; *N* = 7) and Osorno (40° 35' S, 73° 3' W; *N* = 6). Subjects were transported to the laboratory and their snout–vent length (SVL) and weight measured. All individuals were euthanized by immersion in a 1% tricaine methanesulfonate solution (MS-222; Sigma-Aldrich Inc., USA). After performing an abdominal incision, the entire bodies of the animals were fixed in 4% paraformaldehyde (PFA) for ten days. Subsequently, larynges were extracted and post-fixed in fresh 4% PFA until they were dehydrated, paraffin-embedded and processed for routine staining. Slices of 10–20 µm thickness were obtained with a rotatory microtome (MICROM GmbH, HM 310, Walldorf, Germany) and stained with haematoxylin–eosin. The complete series

of slices was digitalized with a digital camera (Leica MC 120 HD) mounted on a dissection microscope (Leica S6D), using the Leica Suite LAS EZ 3.0.0 software (Leica Microsystems, Germany). These series were stacked, registered and segmented. Contours of six laryngeal structures were drawn following the nomenclature of previous studies (McClelland *et al.*, 1996; Suthers *et al.*, 2016): arytenoid cartilage, vocal cords, anterior constrictor muscle, posterior constrictor muscle, external constrictor muscle and dilator muscle. Three-dimensional-reconstructions of laryngeal structures were assessed for volumetric analysis with Fiji software (<https://fiji.sc>; Fig. 1). This volume was computed from 2D slices (contour) as the sum of 2D areas multiplied by the Z spacing (considering voxel size). Bilateral symmetry was assumed and only the left halves of the larynges were considered.

To determine the geographic variation of the laryngeal structures measured in *P. thaul* males, we grouped the populations in three biogeographic zones according to their genetic closeness and behavioural proximity (Velásquez *et al.*, 2013, 2014; Velásquez, 2014): northern (Totoral, Socos), central (Torca Lagoon, Hualqui) and southern (Elicura, Coñaripe, Máfil, Osorno). To evaluate the effect of biogeographic zone on the joint variation of laryngeal structures, we utilized multivariate general linear models (GLM) from raw matrix data of the laryngeal volumes as dependent variables and the biogeographic zone as categorical predictor. Due to the high correlation

between volumes of the laryngeal structures and body size measured in this study ($P < 0.05$), we used the SVL as continuous predictor in the multivariate GLM. Subsequently, raw matrix data of the laryngeal were standardized by body size, dividing each value by SVL^3 to remove the effect of this variable. Subsequently, we repeated the multivariate GLM but without the continuous predictor. Tukey's tests were employed as post hoc analyses.

Furthermore, acoustic data extracted from advertisement calls of the same individuals used in the current study and published previously (Velásquez *et al.*, 2018) were used to perform multiple linear regressions. Measurements of vocalizations of all individuals for which larynx measurements were obtained were carried out ($N = 35$). Raw volumes of the laryngeal structures obtained in the current study were used as predictor variables of each of the acoustical parameters of the advertisement calls from our previous study. All analyses were carried out with IBM SPSS Statistics 24 (IBM Corp., NY, USA).

RESULTS

Raw volumes of the laryngeal structures of males of *P. thaul* present a remarkable variation among the three biogeographic zones studied (i.e. northern, central and southern). Biogeographic zone has effects on the overall volume of the laryngeal structures as

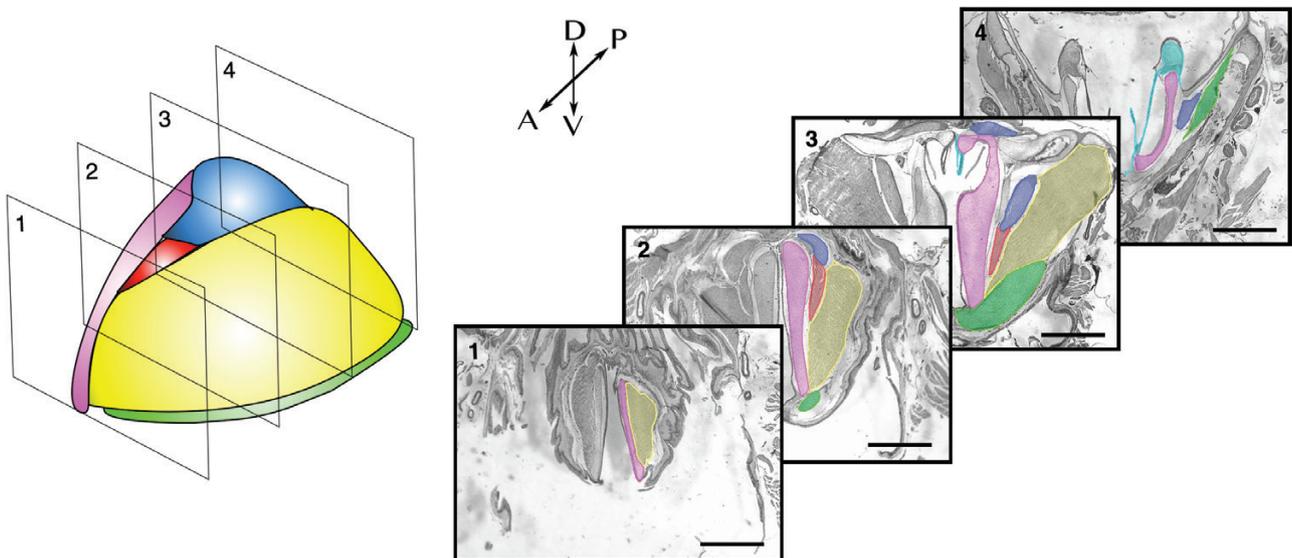


Figure 1. A, schematic representation of a left half of larynx of a *Pleurodema thaul* male showing the location and orientation of muscles and cartilages (colours) and the frontal planes corresponding to four histological sections shown in B are indicated. B, photomicrographs of frontal sections along the larynx. External constrictor muscle (green), dilator muscle (yellow), arytenoid cartilage (purple), anterior constrictor muscle (red), posterior constrictor muscle (blue) and vocal cords (turquoise) are shown. Scale bars: 500 μ m.

analysed by multivariate GLM (Wilks' $\lambda = 0.444$, $F_{12,52} = 2.168$, $P = 0.028$). In addition, the covariate (i.e. SVL) also shows a significant effect on this variation (multivariate GLM: Wilks' $\lambda = 0.443$, $F_{6,26} = 5.447$, $P < 0.001$). Biogeographic zone has specific effects on arytenoid cartilage volume (univariate GLM: $F_{2,31} = 5.283$, $P = 0.011$). Individuals from the southern zone show arytenoid cartilage volumes larger than those from northern and central biogeographic zones (Tukey's test: $P_s < 0.05$). However, the rest of laryngeal structures do not show significant differences among the three zones: dilator muscle (univariate GLM: $F_{2,31} = 2.825$, $P = 0.075$), vocal cords (univariate GLM: $F_{2,31} = 1.275$, $P = 0.294$), external constrictor muscle (univariate GLM: $F_{2,31} = 0.812$, $P = 0.453$), anterior constrictor muscle (univariate GLM: $F_{2,31} = 1.374$, $P = 0.268$) and posterior constrictor muscle (univariate GLM: $F_{2,31} = 0.858$, $P = 0.434$; Fig. 2A).

After standardizing by body size, the raw matrix data of the laryngeal volumes and the effect of biogeographic zone on the overall volume of the laryngeal structures persist (multivariate GLM: Wilks' $\lambda = 0.249$, $F_{12,54} = 4.517$, $P < 0.001$). The effect on arytenoid cartilage volume also persists (univariate GLM: $F_{2,32} = 6.010$, $P = 0.006$) but, in addition, significant differences occur for dilator muscle volume (univariate GLM: $F_{2,32} = 10.043$, $P < 0.001$) and vocal cord volume (univariate GLM: $F_{2,32} = 6.875$, $P = 0.003$). Post hoc comparisons show that arytenoid cartilage volume is similar between northern and central zones, but these values are low relative to the southern zone. The dilator muscle volume is similar between northern and southern zones, but these values are higher relative to the central zone (Tukey's test: $P_s > 0.05$). The vocal cord volume is similar between the northern and central zones, but these values are high relative to the southern zone (Tukey's test: $P_s < 0.05$; Fig. 2B). Furthermore, when volumes of the laryngeal structures are analysed as predictor variables for the variation of the acoustical parameters, some of these are predicted by joint variation of the morphological predictors, as shown in Table 1. As such, pulse duration (multiple linear regression: $F_{6,28} = 4.673$, $P = 0.002$), pulse rate (multiple linear regression: $F_{6,28} = 3.094$, $P = 0.019$) and dominant frequency (multiple linear regression: $F_{6,28} = 3.051$, $P = 0.020$) are predicted by variation of volumes of arytenoid cartilage and dilator muscle volumes as follows: pulse duration (arytenoid cartilage: $\beta = -0.530$, $P = 0.043$; dilator muscle: $\beta = 1.076$, $P < 0.001$) and pulse rate (arytenoid cartilage: $\beta = 0.676$, $P = 0.020$; dilator muscle: $\beta = -1.009$, $P = 0.002$). In addition, dominant frequency is significantly predicted by dilator muscle volume only ($\beta = -0.894$, $P = 0.006$).

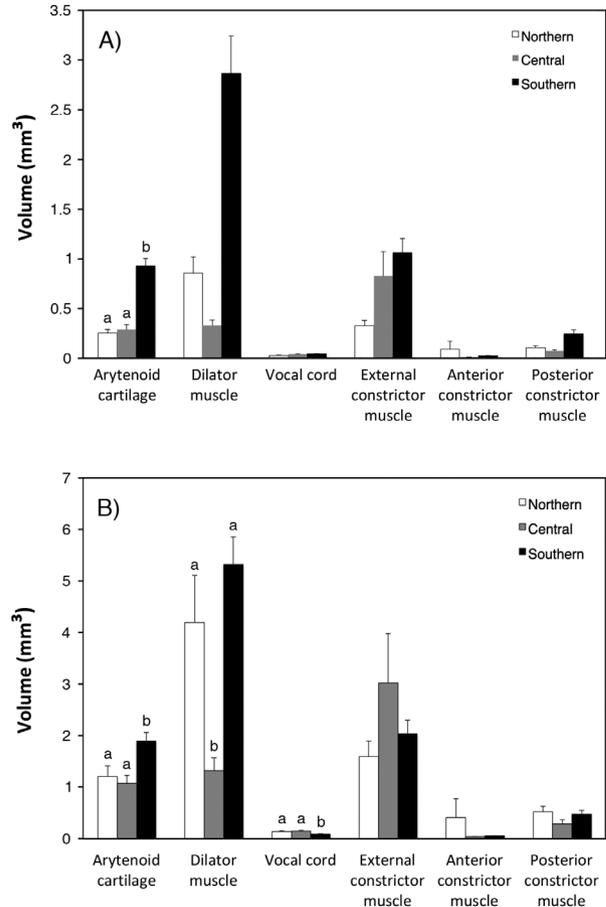


Figure 2. Volumes of six laryngeal structures of *Pleurodema thaul* males from different biogeographic zones. A, shows volumes non-standardized by SVL; B, shows volumes standardized by SVL. Bars correspond to averages and whiskers to standard errors. Different letters and asterisks indicate significant differences.

DISCUSSION

Our results show a remarkable geographic variation in the laryngeal morphology among populations along the extensive geographic distribution of *P. thaul* (Fig. 3). In particular the arytenoid cartilage, dilator muscle and vocal cords allow us to discriminate between the three bioacoustic groups (northern, central and southern) proposed for this species (Velásquez *et al.*, 2013). However, only the arytenoid cartilage shows significant differences with SVL included as covariate. As such, the morphological variation observed is in general agreement with the acoustic, behavioural and genetic variation reported in previous studies for this species (Velásquez *et al.*, 2013, 2014). In addition, two laryngeal structures (i.e. arytenoid cartilage and dilator muscle) predict the variation of some advertisement call parameters (i.e. pulse duration, pulse rate and

Table 1. Multiple regressions between laryngeal structure volumes and acoustic parameters of advertisement calls of *Pleurodema thaul* males. Multiple Rs and standardized coefficients of regression for the analyses carried out with the raw data of anatomical structures. Values listed for individual structures correspond to β values. Significant β values are in bold characters

	Call duration	Number of pulses	Pulse duration	Pulse rate	Dominant frequency	Modulation depth
Multiple R	0,485	0,506	0,707**	0,631*	0,629*	0,214
Arytenoid cartilage	-0,444	-0,198	-0,530	0,676	0,404	-0,259
Dilator muscle	0,156	-0,160	1,076	-1,009	-0,894	0,247
Vocal cord	-0,417	-0,394	-0,159	0,126	0,334	0,130
External constrictor muscle	0,020	0,074	-0,047	0,111	0,025	0,029
Anterior constrictor muscle	0,006	0,065	-0,120	0,167	-0,091	0,030
Posterior constrictor muscle	0,180	0,151	0,129	-0,190	-0,205	-0,047

* $P < 0.05$; ** $P < 0.01$.

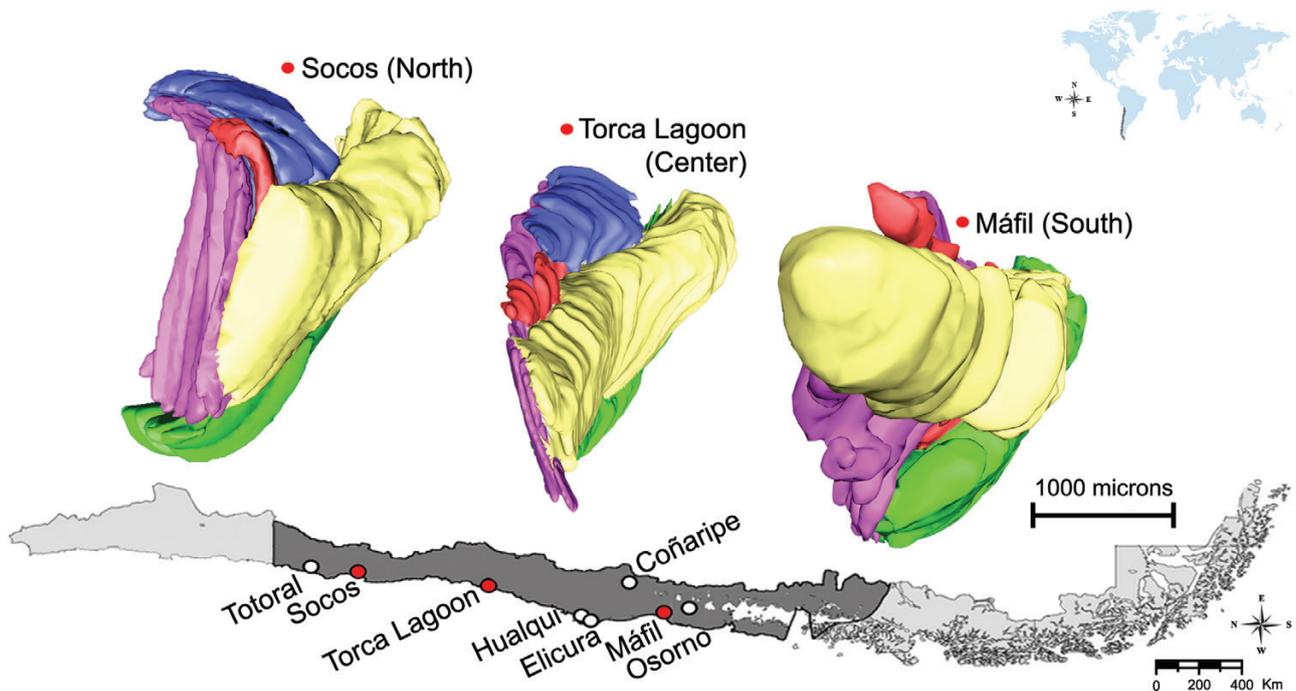


Figure 3. Representative 3D-reconstructions of left halves of larynges of males of *Pleurodema thaul* from three populations along the geographic distribution of this species in Chile. Colours of laryngeal structures as in Figure 1. The 1000 μm scale applies to the three reconstructions. The dark grey zone in the map indicates the geographic distribution of *P. thaul* in Chile.

dominant frequency). Similar relationships between morphological structures and acoustical parameters have been reported in previous studies in other anurans (Martin, 1971; Martin & Gans, 1972; McClelland & Wilczynski, 1989; Wilczynski *et al.*, 1993; McClelland *et al.*, 1996, 1998; Boul & Ryan, 2004).

The fundamental frequency of the call depends on the structure of the vocal cords (Martin, 1971; McClelland & Wilczynski, 1989; Riede & Brown, 2013), and an inverse relationship between fundamental

frequency and the mass of vocal cords and/or body size that generally occurs in frogs (Martin & Gans, 1972; Ryan & Drewes, 1990; Castellano *et al.*, 2000). A recent study has demonstrated that *P. thaul* follows Bergmann's rule, as body size increases with latitude in this species (Rivas *et al.*, 2018). Those results do not agree with the geographic variation in dominant frequency of advertisement calls of this species, as central populations having intermediate size have higher dominant frequency relative to both northern

and southern populations (Velásquez *et al.*, 2013, 2018). Results of the current study show that raw vocal cord volumes do not differ among populations and, thus, no relationship between this dimension and dominant frequency occurs. Moreover, after standardizing vocal cord volume by SVL, the laryngeal anatomical measure is smaller in the southern zone, the populations having the lower dominant frequency. Overall, our results show that vocal cord size is not related to dominant frequency. An incongruence between larynx–body mass–dominant frequency similar to our results in *P. thaul* has been reported in an interspecific study in Neotropical anurans of the genus *Hyla*, in which *H. microcephala* emits a call with higher dominant frequency (~6 kHz) than *H. ebraccata* (~3 kHz) or *H. plebodes* (~3.8 kHz), despite having vocal cords with a larger volume (Wilczynski *et al.*, 1993). However, a caveat for the comparison of our data with those of the Central American frogs is that the dominant frequency in *P. thaul* corresponds to the fundamental frequency of the call spectrum, whereas in the treefrog group these frequencies correspond to higher harmonics that could be accounted for by resonant structures of the vocal apparatus, rather than by the physical structure of the vocal cords.

In addition to the dimensions of the vocal cords, other factors are known to affect the fundamental frequency produced by these structures. The air pressure applied to the larynx (Gridi-Papp, 2008, 2014; Colafrancesco & Gridi-Papp, 2016), vocal cord tension and larynx shapes (McAlister, 1961) have been identified as determinants of the fundamental frequency of cord vibrations. It is possible that these factors could account for incongruences in the relationship between dominant frequency and size of the sound-producing structures. To our knowledge, tension and shapes of larynxes have not been studied extensively in anurans (Suthers *et al.*, 2006), but vocal cord tension has been measured in other vertebrates and shows a direct relationship with frequency (Hast, 1966; Riede, 2010; Klemuk *et al.*, 2011). Future studies measuring these morphofunctional features could shed light on how these variables are involved in differences in the dominant frequencies of advertisement calls of males.

In the current study, some morphometric characteristics of the larynx accounted significantly for the variation of some spectral and temporal characteristics of the advertisement calls emitted by males of *P. thaul*, as shown in Table 1. Among these, the arytenoid cartilage volume was negatively related to the variation of pulse duration and positively related to the variation of pulse rate. These relationships agree with previous studies on call geographic variation, showing that pulse duration is lower in southern than in northern populations and the opposite occurs for pulse rate (Velásquez *et al.*, 2013, 2018). In addition,

the dilator muscle volume was negatively related to the variation in pulse rate and dominant frequency, and positively related to the variation of pulse duration. The relationship found between the dilator muscle and dominant frequency is particularly interesting as it is in correspondence with the geographic variation of this spectral parameter along geographic distribution of *P. thaul* in Chile (Velásquez *et al.*, 2013, 2018).

These results bear similarities with previous studies in males of cricket frogs, *Acris crepitans* Baird, 1854 (McClelland *et al.*, 1996), because in this species, as in *P. thaul*, the arytenoid cartilage volume is positively related to pulse rate and dilator muscle volume is negatively related to pulse rate and dominant frequency of the advertisement call.

Environmental conditions along the distribution of *P. thaul* could play an important role in explaining the relationships between laryngeal structures and acoustic signals, because factors such as desiccation can influence the body size of individuals (Rowe & Dunson, 1995; Márquez-García *et al.*, 2009). *Pleurodema thaul* is distributed across dissimilar biogeographic zones differing in temperatures and precipitations (Hajek & Di Castri, 1975). Correspondingly, body sizes of males also vary according to these environmental variables, namely subjects with larger body size are found in zones with lower minimal temperatures and higher precipitations, and subjects with smaller body size occur in regions having contrasting environmental conditions (Rivas *et al.*, 2018). As such, the size of some laryngeal structures could affect some characteristics of calls.

Interestingly, the differences between volumes of some laryngeal structures (i.e. arytenoid cartilage, dilator muscle and vocal cords), after standardizing data by SVL, indicate that factors other than body size could account for these dissimilarities, as reported for other anurans (Ryan & Wilczynski, 1991). In different vertebrates, morphological characteristics of the sound-emitting organs and acoustic signal characteristics are related to sexual selection processes (e.g. West-Eberhard, 1983, 2003). In *P. thaul*, a peculiar pattern of sexual selection is likely to operate, as females do not discriminate between local and foreign calls (Velásquez *et al.*, 2015), but males do (Velásquez *et al.*, 2014) suggesting that in this species the selectivity of the behavioural responses to acoustic signals depends on male discrimination, rather than on female discrimination. It would be worthwhile to determine how this peculiar mode of selection may relate to the particular relationships between vocal structure and signals reported in this study.

Overall, our study contributes to understanding the geographic variation in the acoustic communication system along the extensive geographic distribution of *P. thaul*. Additional efforts are necessary to unveil

laryngeal functional correlates between structural characteristics and acoustical parameters. A further pending issue is to elucidate if the morphological variation of the sound-producing organs observed in the current study is in correspondence with geographic variation in the receiver organs along the extensive distribution of the study species.

ACKNOWLEDGEMENTS

We are grateful to Marta Mora, Macarena Casarejos and Francisco Dumas for participating in histological procedures. This study was supported by FONDECYT 3120208 and 11140752 to NAV. MLC was supported by the Chilean Millennium Science Initiative (Millennium Institute P09-015-F) and the Chilean National Commission for Scientific and Technological Research (FONDAP 15150012 and Ring Initiative PIA ACT1402).

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