

# The vocal sac of Hylodidae (Amphibia, Anura): Phylogenetic and functional implications of a unique morphology

Agustin J. Elias-Costa<sup>1</sup>  | Rachel Montesinos<sup>2</sup> | Taran Grant<sup>2,3</sup> | Julián Faivovich<sup>1</sup>

<sup>1</sup>División Herpetología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" – CONICET, Av. Angel Gallardo 470, Buenos Aires C1405DJR, Argentina

<sup>2</sup>Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo. Rua do Matão, Travessa 14, 321, Cidade Universitária, CEP 05508-090, São Paulo, SP, Brazil

<sup>3</sup>Museu de Zoologia, Universidade de São Paulo, Av. Nazaré, 481, Ipiranga, CEP 04263-000, São Paulo, SP, Brazil

## Correspondence

Julián Faivovich, División Herpetología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" – CONICET, Av. Angel Gallardo 470, C1405DJR, Buenos Aires, Argentina.  
Email: julian@macn.gov.ar

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## Abstract

Anuran vocal sacs are elastic chambers that recycle exhaled air during vocalizations and are present in males of most species of frogs. Most knowledge of the diversity of vocal sacs relates to external morphology; detailed information on internal anatomy is available for few groups of frogs. Frogs of the family Hylodidae, which is endemic to the Atlantic Forest of Brazil and adjacent Argentina and Paraguay, have three patterns of vocal sac morphology—that is, single, subgular; paired, lateral; and absent. The submandibular musculature and structure of the vocal sac mucosa (the internal wall of the vocal sac) of exemplar species of this family and relatives were studied. In contrast to previous accounts, we found that all species of *Crossodactylus* and *Hylodes* possess paired, lateral vocal sacs, with the internal mucosa of each sac being separate from the contralateral one. Unlike all other frogs for which data are available, the mucosa of the vocal sacs in these genera is not supported externally by the *mm. intermandibularis* and *interhyoideus*. Rather, the vocal sac mucosa projects through the musculature and is free in the submandibular lymphatic sac. The presence of paired, lateral vocal sacs, the internal separation of the sac mucosae, and their projection through the *m. interhyoideus* are synapomorphies of the family. Furthermore, the specific configuration of the *m. interhyoideus* allows asymmetric inflation of paired vocal sacs, a feature only reported in species of these diurnal, stream-dwelling frogs.

## KEYWORDS

multimodal communication, submandibular musculature, unilateral inflation, vocalization

## 1 | INTRODUCTION

Traditionally, the structure of the anuran vocal sac has been a source of morphological information for systematic studies (Liu, 1935; Trewavas, 1933; Tyler, 1971a, 1985; Tyler & Duellman, 1995); however, detailed knowledge of fine-scale structural diversity of vocal sacs in most clades remains limited. As happens with many other character systems, our understanding of vocal sac structure, submandibular musculature, and larynx anatomy is based on a few classic, seminal papers that have been scarcely revisited since their publication.

In its usual usage, the term "vocal sac" is applied to an association of three different structures. The internal lining of the sac is a mucosa that originates as an evagination of the buccal floor. This diverticulum communicates with the buccal cavity via one or two small apertures (the

vocal slits) lateral to the tongue; the vocal slits vary greatly in shape among species (Tyler, 1971a). The mucosa is enveloped by submandibular musculature: by the sheath-like *mm. intermandibularis* and *interhyoideus* ventrally and posteriorly and by the *mm. geniohyoidei* and *m. sternohyoideus* dorsally. The histological properties of this association influence vocal sac inflation and deflation (Jaramillo, Rand, Ibáñez, & Dudley, 1997). The third structure is the gular skin, which is pleated or even forms lateral pockets in some species. When these skin modifications are present, vocal sacs are referred to as being "external"; when the gular skin is unmodified, the sac is referred to as being "internal" (Boulenger, 1882; Liu, 1935).

Vocal sacs are present in males of most species of frogs included in the clade Acosmanura (Cannatella, 2006; Johnston, 2011; Trewavas, 1933; Tyler, 1980). They are absent in most basal frogs [Leiopelmatidae,



**FIGURE 1** Vocal sac diversity in the diurnal, stream-dwelling family Hylodidae. (a, b) *Crossodactylus schmidtii*. (c) *Hylodes asper* foot-flagging while vocalizing. (d) *Hylodes phyllodes*. Notice the difference in shape and position of the vocal sac between species of both genera. In *H. asper* and *H. phyllodes*, vocal sacs are spherical and ventrolateral, whereas in *C. schmidtii* they are kidney-shaped with some dorsal development (i.e., they reach a position posterior to the tympanic membrane). Photos a and b by Vinicius Caldart, c and d by TG

Bombinatoridae, Alytidae, Pipidae—but see Tyler (1974) regarding *Rhinophrynus*] and in isolated groups nested within major clades in which the sac is present plesiomorphically [e.g., Bufonidae (Liu, 1935), Brevicipitidae (Largen & Drewes, 1989); Craugastoridae (Liu, 1935), Dicroglossidae (Boulenger, 1882; Dubois, 1992; Emerson & Berrigan, 1993), Eleutherodactylidae (Joglar, 1989; Liu, 1935); Hemiphractidae (Tyler & Duellman, 1995); Leptodactylidae (Liu, 1935); Limnodynastidae and Myobatrachidae (Tyler, 1972); Megophryidae (Boulenger, 1882, 1908); Hylidae (Faivovich, Baèta, Candiotti, Haddad, & Tyler, 2011; Hedges, 1987; Tyler, 1971a); Hyperoliidae (Drewes, 1984); Petropedetidae (Liu, 1935); Pyxicephalidae (Perret, 1966); Ranidae (Boulenger, 1882; Dubois, 1992; Hayes & Krempels, 1986; Yang, 1991); Rhacophoridae (Inger, 1954; Liu, 1935); Rhinodermatidae (Barrio, 1970); Telmatobiidae (Liu, 1935)].

“Paired” vocal sacs occur in many groups of anurans. However, this term is defined vaguely in the literature and is applied to a wide array of morphologies that include lateral projections of a single, subgular structure [e.g., *Leptotalax fritiniensis* (Dehling & Matsui, 2013: fig. 3), *Pseudis minuta* (Goldberg, Barrasso, Agostini, & Quinzio, 2016: fig. 1b)] and completely independent lateral lobes [e.g., *Trachycephalus typhonius* (Stamberger, Preininger, & Hödl, 2014: fig. 1), *Odorrana tormota* (Zhang et al., 2016: fig. 3)]. Furthermore, given that most knowledge of vocal sac diversity is based on external morphology, the expression “paired sacs” says nothing about the

internal structure of those sacs. Thus, it is likely that a great amount of variation has been overlooked. For instance, the vocal sac mucosa develops bilaterally from the mouth and fuses to form a single, subgular structure (Inger & Greenberg, 1956; Tyler, 1971b). McAlister (1959) reported that these lateral evaginations remain separate in adult male *Spea* (Scaphiopodidae), which possesses externally bilobate sacs. Species of the Atlantic Forest endemic Hylodidae provide an excellent model to study vocal sac anatomy because the species have highly divergent vocal sac morphologies, the internal structures of which are unknown.

Hylodidae currently contains 47 species in the genera *Crossodactylus*, *Hylodes*, and *Megaelasia* (Frost, 2017). Reportedly, some of these have paired, lateral vocal sacs (e.g., Pombal, Prado, & Canedo, 2003) or single, subgular sacs (Pimenta, Caramaschi, & Cruz, 2015), and some lack vocal sacs entirely (Giaretta, Bokermann, & Haddad, 1993). This disparity raises questions about the evolutionary history of the character system that has important implications in the reproductive biology of the frogs. For example, species of *Hylodes* perform visual displays in their reproductive behavior (Haddad & Giaretta, 1999; Narvaes & Rodrigues, 2005; Vogel, Abrunhosa, & Weber, 2004) as do several other distantly related, diurnal species that mate in noisy environments such as mountain streams (Hödl & Amézquita, 2001; Krishna & Krishna, 2006; Preininger, Boeckle, & Hödl, 2009). In particular, male *H. japi* perform complex, multimodal displays that involve a wide range of

stereotyped movements, perhaps the most exceptional of which is the asymmetric inflation of the vocal sacs (de Sá, Zina, & Haddad, 2016). Depending on the social context, males can convey information to conspecifics by inflating only one or both of their paired vocal sacs. This kind of control has not been reported for any other anuran. The anatomical mechanism that allows unilateral inflation is unknown.

We examined the submandibular musculature and vocal sac structure across the diversity of hylodid frogs and related taxa to characterize the morphology of members of Hylodidae and identify synapomorphies that might delimit internal clades in future systematic studies. In addition, we explored the functional basis for the inflation of only one portion of the bilateral vocal sac.

## 2 | MATERIALS AND METHODS

### 2.1 | Taxa examined

We studied the external morphology of 87 specimens of 33 species of the three hylodid genera. These include 31 specimens of 12 of the 14 species of *Crossodactylus*, 51 specimens of 18 of the 26 species of *Hylodes*, and 4 specimens of 2 of the 7 species of *Megaelasia* (Frost, 2017). Thirty-three specimens were dissected to study submandibular musculature and, in males, vocal sac structure. For the remaining species of the family, we relied on descriptions in the literature. The phylogenetic position of Hylodidae has not been stable in recent major analyses and there is no consensus as to its sister taxon. Grant et al. (2006) found it to be sister to Dendrobatidae + Aromobatidae on the basis of molecular and morphological evidence. However, Frost et al. (2006) considered hylodids to be a subfamily of their Cycloramphidae as the sister taxon of Cycloramphinae, species of which are currently distributed among Alsodidae, Batrachylidae, Cycloramphidae, Odontophryinae, and Rhinodermatidae. In more recent molecular analyses, Pyron (2014) reported a weakly supported sister-taxon relationship between Hylodidae and Alsodidae + Batrachylidae and Fouquet et al. (2013) recovered a sister-taxon relationship between Hylodidae and *Alsodes* + *Eupsophus*. This instability compelled us to include a large outgroup sample, including 27 species of 11 anuran families representing all the aforementioned groups. For collection abbreviations see Sabaj (2016).

### 2.2 | Specimens examined

Specimens followed by an asterisk (\*) were studied for external morphology only, and those with a plus sign (+) were also processed histologically. All outgroup specimens are adult males.

**Hylodidae:** *Crossodactylus aeneus* MNRJ 39369 (m), MNRJ 39364 (m)+, MNRJ 47765 (f)\*; *C. boulengeri* MZUSP 111020 (f)\*, MZUSP 111051 (m); *C. cyclospinus* MNRJ 40220 (m), MNRJ 40222 (m)\*; *C. dan-tei* MNRJ 39444 (f)\*, MNRJ 39446 (m); *C. dispar* MZUSP 23578(m)\*, MZUSP 23587 (m); *C. gaudichaudii* MNRJ 74088 (f)\*, MNRJ 74089 (m); *C. grandis* MNRJ 48356 (f)\*, MNRJ 48357 (m)+; *C. lutzorum* MNRJ 4761 (f, paratype), MNRJ 4762 (? , paratype)\*; *C. schmidtii* MACN 42159 (m)\*, MACN 42188 (m)\*, MACN 42415 (f), MACN 48998 (f)+, MACN 49592 (m)+, MACN 42422 (m)+, MACN 49500 (m)+; *C. timbuhy* USMN 200449 (m)\*, USMN 200450 (?)\*; *C. trachystomus* UFMG

5355 (f), USNM 98017 (?)\*, USNM 98018 (f)\*; *C. werneri* MZUSP 69099 (f), MZUSP 69100 (f)\*; *Hylodes amnicola* MZUSP 141746 (m)\*, MZUSP 141747 (m); *H. asper* MZUSP 112639 (m), MZUSP 112641 (m)\*, MZUSP 157462 (f)\*, MZUSP 157499 (m)+, MZUSP 157464 (m)\*; *H. babax* USNM 222553 (m)\*, USNM 239938 (m)\*, USNM 239939 (m)\*; *H. cardosoi* MZUSP 112578 (m), MZUSP 112579 (f)\*; *H. charadra-naetes* MZUSP 112841 (m), MZUSP 112843 (f)\*, USNM 245901 (m, paratype)\*; *H. fredii* MNRJ 35217 (f, paratype)\*, MNRJ 38936 (m, paratype)\*; *H. glaber* AMNH 103902(m)\*, USNM 245940 (m)\*, USNM 245943 (m)\*; *H. heyeri* MZUSP 093246(m)\*, MZUSP 093248 (m), USNM 541344 (m)\*; *H. lateristrigatus* CENAI 8413 (m), USNM 207680 (m)\*, USNM 208557 (m)\*, USNM 208558 (m)\*, *H. magalhaesi* AMNH 74425 (m, paratype)\*, MZUSP 112661 (f)\*, MZUSP 112662 (m), USNM 565123 (m)\*; *H. nasus* AMNH 72456 (m)\*, USNM 164112 (f)\*, USNM 164113 (f)\*; *H. perplicatus* MTR 26735 (m), USNM 101716 (f)\*, USNM 101717 (m)\*; *H. ornatus* AMNH 103914(m)\*, USNM 245929 (f, subadult)\*, MZUSP 60845 (m)\*; *H. phyllodes* USNM 243491 (m, paratype)\*, MACN 17252 (m), MZUSP 133184 (m)+, MZUSP 137532 (m)\*, TG 3603 (m)+; *H. pipilans* MNRJ 35990 (f, paratype)\*, MNRJ 39374 (m, paratype)\*, *H. sazimai* MZUSP 136796 (m)\*, MZUSP 136797 (m); *H. uai* MZUSP 23861 (m, subadult), MZUSP 23863 (m)\*; *H. vanzolini* USNM 222554 (?)\*; *Megaelasia apuana* MTR 26059 (f), UFMG 13536 (m); *M. goeldii* CFBH 23800 (m)\*, CFBH 23801 (m)\*. **Alsodidae:** *Alsodes neuquensis* MACN 48996, *Eupsophus roseus* MACN 49594, *Eupsophus vertebralis* CENAI 3747, *Limnomedusa macroglossa* MACN 40642. **Aromobatidae:** *Mannophryne herminae* CENAI 2617. **Batrachylidae:** *Atelognathus patagonicus* MACN 37883, *Batrachyla leptopus* MACN 48995, *Batrachyla taeniata* MACN 41265. **Bufoinae:** *Atelopus pastuso* ICN 26587, *Melanophryniscus klappenbachi* MACN 49593, *Rhinella arenarum* MACN 43405. **Cycloramphidae:** *Thoropa taophora* CFBH 12734, *Cycloramphus eleutherodactylus* MACN 17041. **Dendrobatidae:** *Ameerega flavopicta* MZUSP 90635, *Hyloxalus subpunctatus* ICN 26928, *Oophaga histrionica* MZUSP 100070. **Hylidae:** *Scinax granulatus* MACN 38218, *Hypsiboas pulchellus* MACN 40485, *Trachycephalus typhonius* MACN 49595. **Leptodactylidae:** *Leptodactylus bufonius* MACN 38173, *Paratelmatobius poecilogaster* CFBH 9879, *Pseudopaludicola falcipes* MACN 37880. **Odontophryinae:** *Odontophrynus achalensis* MACN 2973, *Proceratophrys avelinoi* MACN 36849. **Rhinodermatidae:** *Rhinoderma darwini* CENAI 5370, *Insuetophrynus acarpicus* CENAI 6898. **Telmatobiidae:** *Telmatobius oxycephalus* MACN 49004, *Telmatobius rubigo* MACN 41608.

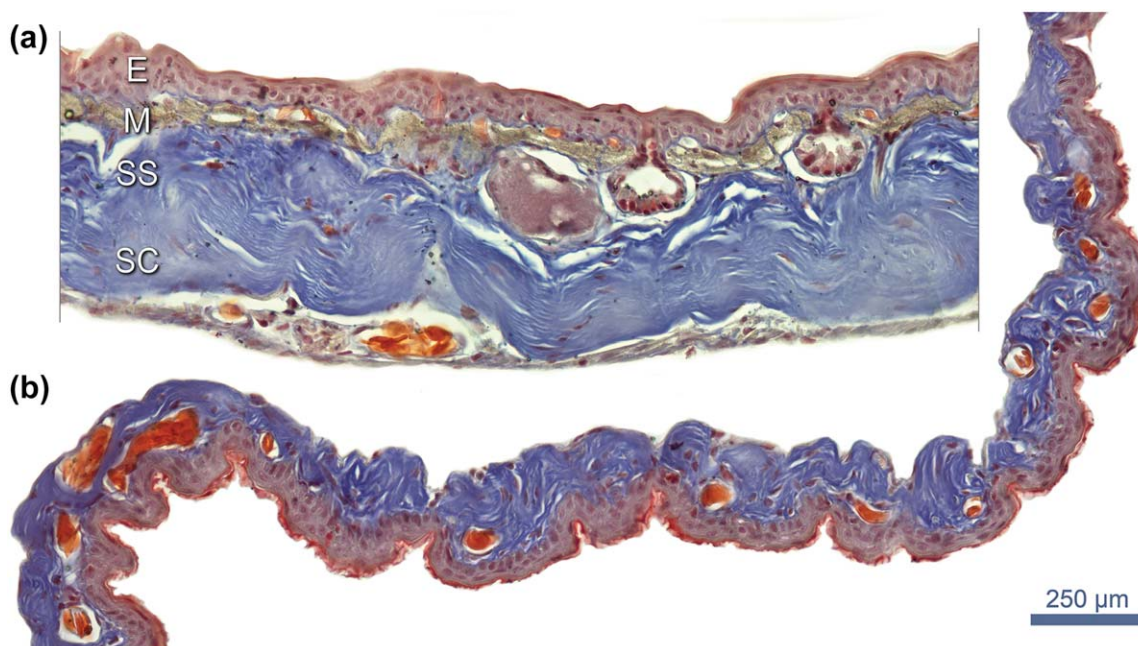
### 2.3 | Dissections

Dissections were performed using a stereoscopic microscope with the aid of the topical application of an iodine/potassium iodide solution (Bock & Shear, 1972). We used the terminology of Tyler (1971a, 1971b) for submandibular musculature and lymphatic septa, and that of Diogo and Ziermann (2014) for pectoral girdle musculature.

### 2.4 | Histological procedures

Specimens were fixed in formalin and stored in 70% ethanol. Samples were decalcified in a formic acid (85% m/v)/Sodium citrate (20% m/v)





**FIGURE 2** Gular skin heterogeneity of *Hylodes asper* (MZUSP 157499) stained with modified Masson's trichrome. (a) Medial gular skin. Note the thickness of the moderately vascularized *stratum compactum* of the dermis and the presence of exocrine serous glands in the *stratum spongiosum*. (b) Expansible skin pouch. Both photos are in the same scale. E, epidermis; M, melanophores; SS, *stratum spongiosum* of the dermis; SC, *stratum compactum* of the dermis

solution for seven days and then washed in tap water (Bancroft & Gamble, 2008) and transferred to 70% ethanol. Skin samples were obtained from the gular region. For Paraplast embedding, samples were dehydrated in increasing concentrations of ethanol for several hours and cleared in toluene. Parasagittal sections (6 µm) were stained with modified Masson's trichrome to describe general morphology and nitric orcein to reveal the presence of elastic fibers (Humason, 1972; Bancroft & Gamble, 2008).

### 3 | RESULTS

Adult male *Crossodactylus*, *Hylodes*, and *Megaelosia apuana* have paired, lateral vocal sacs and paired, elongate vocal slits (Figure 1). Adult male *Megaelosia goeldii* lack vocal sacs. All outgroup species have single, subgular sacs, except for the following: (1) The vocal sac of *Leptodactylus bufonius* is bilobate because the subgular region inflates ventrally and possesses two ventrolateral projections; (2) The vocal sac of *Trachycephalus typhonius* is paired, lateral because the sacs are inflated only laterally and the gular region is unaffected; and (3) *Alsodes neuquensis*, *Atelopus pastuso*, and *Thoropa taophora* lack vocal sacs.

#### 3.1 | Gular skin differentiation

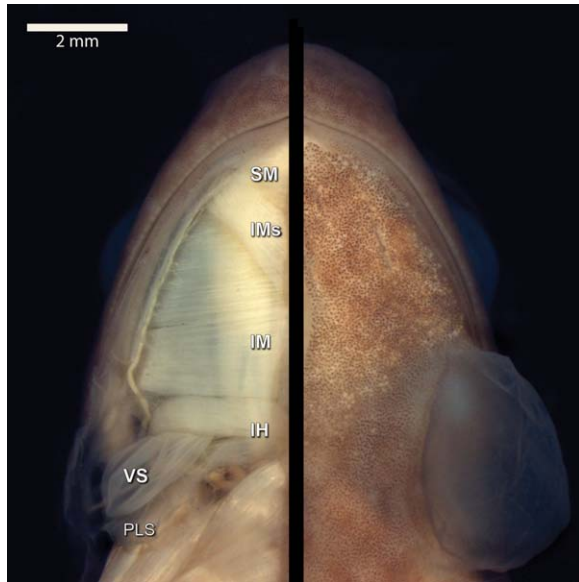
In adult male *Hylodes* and *Megaelosia apuana*, gular skin is differentiated into a pair of ventrolateral pouches that are discrete regions of highly specialized skin associated with each vocal sac. In *H. asper* and *H. phyllodes*, the histology of the pouches differs from the unmodified gular skin between them in the following ways: (1) the skin is half as thick; (2) serous (granular, poison) glands are absent; (3) elastic fiber content

is greater; (4) vascularization is greater; and (5) melanophores are absent (Figure 2). External site-specific skin differentiation is evident in preserved specimens of all studied species of *Hylodes*; the skin pouches always remain expanded (Figure 3, right). In *Crossodactylus*, neither external morphology nor histology revealed any regionalization in adult male *C. aeneus*, *C. grandis*, or *C. schmidtii*. In contrast to *Hylodes*, there is no sexual dimorphism in the gular skin in this genus.

#### 3.2 | Superficial submandibular musculature

The *m. submentalis* in all specimens examined is small and araphic. *Crossodactylus*, *Hylodes* and *M. apuana* have distinct apical supplementary elements of the *m. intermandibularis* (Figure 3), a condition shared with Alsodidae, Batrachylidae, Telmatobiidae, and *Rhinoderma darwinii*. Slender anterolateral supplementary elements are present in Aromobatidae, Dentrobatidae, *Paratelmatobius poecilogaster*, and *Pseudopaludicola falcipes*. Supplementary elements of the *m. intermandibularis* are absent in *Megaelosia goeldii*.

In adult male hylodids, the medial portion of the *m. intermandibularis* is in close contact with the *mm. geniohyoidei* dorsal to it. This arrangement resembles the condition in females, juvenile males, and adult males of species lacking vocal sacs and prevents expansion of the vocal sacs into the medial gular region. Contralateral fibers of the *m. intermandibularis* are separated by a narrow, median aponeurosis along the entire length of the muscle. Posteriorly, this connective tissue continues as, first, the aponeurosis of the *m. interhyoideus* and, second, the epimysia of the *m. procoracohumeralis* and the *m. supracoracoideus* + *m. coracoradialis* (*sensu* Diogo & Ziermann, 2014; *m. episternohumeralis* and *m. coracoradialis*, respectively, in older terminology).



**FIGURE 3** Ventral view of gular skin (right) and submandibular musculature (left) of an adult male *Hyloides phyllodes* (MACN 17252). Note the longitudinal *mm. geniohyoidei* and *m. sternohyoideus* running dorsal to the *m. intermandibularis* and *m. interhyoideus* and the expanded skin pouch in the gular skin, present in *Hyloides* and species of *Megaelosia* that possess a vocal sac. IH, *m. interhyoideus*; IM, *m. intermandibularis*; IMs, apical supplementary element of *m. intermandibularis*; PLS, post-mandibular lymphatic septum; VS, right vocal sac

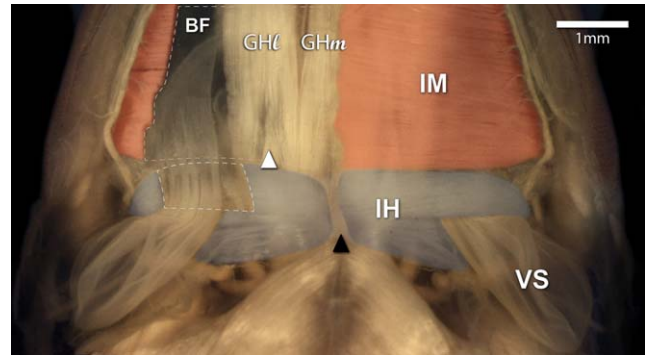
In adult male hylodids that possess a vocal sac, the sac mucosa protrudes through the belly of the *m. interhyoideus* into the submandibular lymphatic sac. The mucosa separates the muscle's anterior (remaining ventral) and posterior fibers (dorsal to the mucosa; Figure 4). Fibers of the *m. interhyoideus* originate on the hyalia and extend transversely to a medial insertion on an aponeurosis that is attached dorsally to the sternum.

In all the outgroup taxa examined, the belly of the *m. interhyoideus* is not pierced. If a vocal sac is present, the vocal sac mucosa always is enveloped by the submandibular muscles and never in direct contact with the submandibular lymphatic sac (Figure 5).

### 3.3 | Vocal sac structure

On each side of the buccal cavity, the mucosa of the mouth floor evaginates posteroventrally to form a tubular pocket of vocal sac mucosa that is independent from the contralateral pocket. The short, narrow vocal slits lie anterolateral to the hyalia in the posterior third of the buccal cavity, and are not occluded by the tongue. Internal separation of the vocal sac mucosa occurs in *Trachycephalus typhonius*, *Eupsophus roseus*, and *Limnomedusa macroglossa*. In all other outgroup taxa examined, the pair of vocal slits connect the buccal cavity with a single, common vocal sac.

Histologically, the vocal sac mucosa is a simple, squamous epithelium associated with a thin *lamina propria* with an extracellular matrix rich in elastic fibers. The vocal sac mucosa is underlain by the *m. intermandibularis* and the anterior portion of the *m. interhyoideus* and



**FIGURE 4** Ventral view of superficial and deeper submandibular musculature of an adult male *Hyloides phyllodes* (MACN 17252). On the left, the *m. intermandibularis* and the anterior fibers of *m. interhyoideus* were removed to reveal the overlying tubular vocal sac mucosa. Note the aponeurosis between contralateral elements of the *m. interhyoideus* (black arrowhead). BF, floor of the buccal cavity; GHl, *m. geniohyoideus lateralis*; GHm, *m. geniohyoideus medialis*; IH (blue), *m. interhyoideus*; IM (red), *m. intermandibularis*; VS, vocal sac; white arrowhead, *m. sternohyoideus*

overlain by the posterior portion of *m. interhyoideus* (Figure 4). Posteriorly, the vocal sac mucosa is free to expand into the submandibular lymphatic sac, which extends from the mandible to the post-mandibular septum, occupying the whole gular region. The post-mandibular septum is oriented transversely, separates the submandibular and pectoral lymphatic sacs, and binds the *m. interhyoideus* (and vocal sacs in hylodids) to the gular skin.

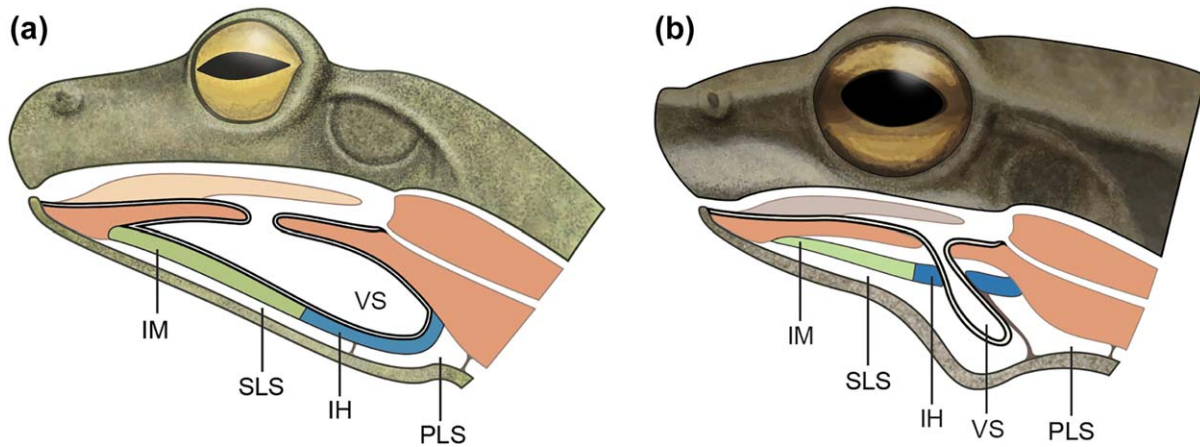
## 3.4 | Variation in Hylodidae

### 3.4.1 | The vocal sac mucosa and the *m. interhyoideus*

In *Hyloides*, the anterior fibers of the *m. interhyoideus* are clearly separated from the protruding portion of the vocal sac mucosa, and the location of the protrusion is conspicuous. Conversely, in *Crossodactylus* and *Megaelosia apuana*, a connective tissue sheath envelopes the *m. interhyoideus* and the projected portion of the vocal sac mucosa ventrally, and the gap in the muscle belly and projection of the mucosa are less evident than in *Hyloides*. Still, the general disposition of the elements is the same in both arrangements because muscle fibers do not line the protruding portion of the mucosa (Figure 6).

### 3.4.2 | Position of the vocal sacs

In *Hyloides* and *M. apuana*, vocal sacs are ventrolateral in position and spherical when inflated (Figure 1c,d). Internally, the portion of the mucosa that protrudes into the submandibular lymphatic sac is small and not further extended laterally (Figure 6). In contrast, in *Crossodactylus*, the free portion of the mucosa is proportionally larger and extends dorsolaterally to occupy the space caudal to the mandibular joint. The dorsolateral development of the sac mucosa of *C. schmidtii* can be observed externally because the vocal sacs are kidney-shaped when inflated (Figure 1a,b).



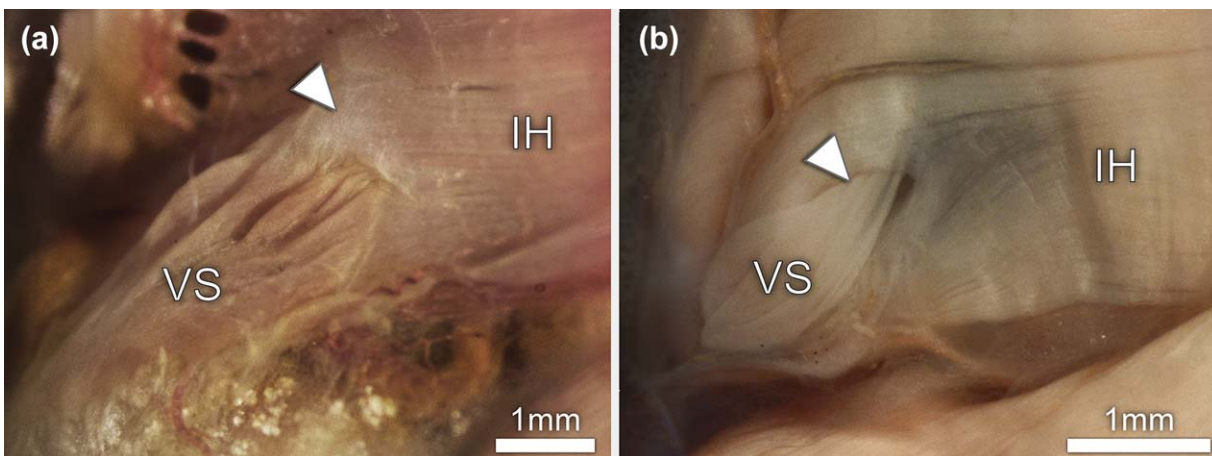
**FIGURE 5** Schematic representation of vocal sacs in (a) a generalized anuran, and (b) species of hylodids in a parasagittal section coincident with the vocal slits. Only structures described in the text were included in the illustration for clarity. All anurans for which the internal structure of the vocal sac is known possess variations of pattern (a) in which the internal mucosa is enveloped by the *mm. intermandibularis* (IM) and *interhyoideus* (IH) and never contacts the lymphatic sacs. In contrast, in hylodids (b), the mucosa pierces the belly of the *m. interhyoideus* and protrudes into the submandibular lymphatic sac (SLS). PLS, pectoral lymphatic sac; VS, lumen of the vocal sac

**3.4.3 | Apical supplementary elements of *m. intermandibularis***

Two patterns of apical supplementary elements occur in *Hylodes* and *Crossodactylus* (Figure 7). In the first, fibers radiate medially from the anterior end of the mandible and insert on a medial raphe. The most posterior fibers are oriented posteromedially, whereas those closest to the *m. submentalis* are transverse and contact the *m. submentalis*. Apical supplementary elements are triangular and completely underlie the fibers of the main element of the *m. intermandibularis*. In the second pattern, all supplementary fibers are parallel and oriented posteromedially. The apical element is V-shaped and fibers of the

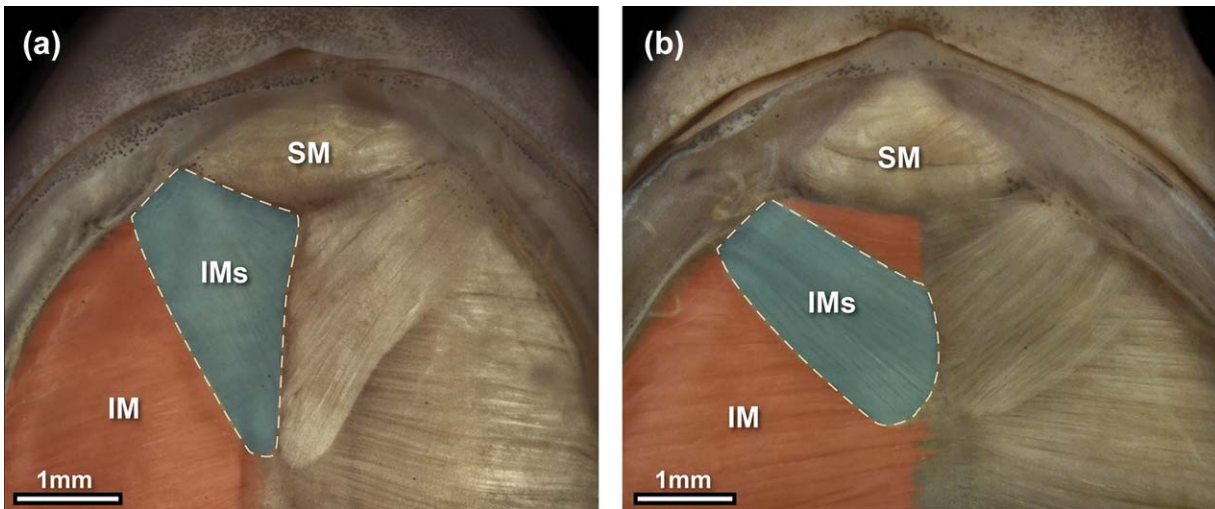
main element are visible in the midline, posterior to the *m. submentalis*.

Apical elements are triangular in *Crossodactylus boulengeri*, *C. cyclopinus*, *C. grandis*, *C. schmidtii*, *C. trachystomus*, *Hylodes asper*, *H. phyllodes*, and *H. uai*. Supplementary elements are V-shaped in *C. dantei*, *C. gaudichaudii*, *H. cardosoi*, *H. charadranaetes*, *H. lateristrigatus*, *H. magalhaesi*, and *M. apuana*. Intermediate states were observed in all other examined specimens. A survey of intraspecific variation might prove necessary to assess the systematic value of this character. Apical supplementary elements of the *m. intermandibularis* are absent in the only specimen of *Megaelosia* available for dissection (*M. apuana*).



**FIGURE 6** Ventral view of right vocal sacs of (a) *Crossodactylus dispar* (MZUSP 23587) and (b) *Hylodes magalhaesi* (MZUSP 112662), with gular skin removed. In both, the projected portion of the vocal sac mucosa is not supported ventrally by fibers of the *m. interhyoideus*, which are oriented transverse to the midline. Arrowheads indicate contact between the vocal sac mucosa and the anterior fibers of the *m. interhyoideus*. In (a), a thin connective tissue sheath covers the gap in the muscle belly. It may appear that the vocal sac mucosa is contained inside a lobe of the *m. interhyoideus* but no fibers of this muscle develop over the mucosa. The same condition is observed in all specimens of the genus examined. In (b), the connective tissue sheath is absent and the passage of the vocal sac mucosa is clearly distinguishable. The same condition occurs in all specimens examined of *Hylodes*. IH, *m. interhyoideus*; VS, vocal sac wall





**FIGURE 7** Variation in the shape of apical supplementary elements of the *m. intermandibularis*. (a) *H. asper* (MZUSP 112639). The muscle is triangular and no fibers of the principal element (IM, red) are visible between supplementary fibers (IMs; blue) and the *m. submental* (SM). (b) *H. cardosoi* (MZUSP 112578). The muscle is V-shaped and transverse fibers of the principal element are visible in the midline

## 4 | DISCUSSION

### 4.1 | Vocal sac diversity in Hyloidea

The internal structure of the paired vocal sacs of hylodids is unique in that the mucosa (the internal wall of the sac) is not entirely enveloped by submandibular musculature. In all previously studied anurans, the diverticula of the buccal floor are supported externally by submandibular muscles, mainly the *m. interhyoideus*. In contrast, the vocal sacs of hylodids are paired diverticula that are free posteriorly in the submandibular lymphatic sac, bound only to the post-mandibular septum.

The belly of each contralateral portion of the *m. interhyoideus* in adult male hylodids is pierced by the projection of the vocal sac mucosa—a condition unknown in other anurans. Ontogenetically, the *m. interhyoideus* fibers separate prior to the protrusion of the vocal sac mucosa into the submandibular lymphatic sac, with the *m. interhyoideus* of juvenile and subadult males having slender, lateral gaps between the fibers that contact the lymphatic septum in the area where the vocal sac mucosa projects through the muscle in adults.

The gular skin of adult male *Hylodes* is highly modified to form conspicuous pouches that house the vocal sac mucosae that protrude through the *m. interhyoideus*. These discrete pouches are half as thick and have more elastic fibers than non-modified gular skin. When air pressure inside the buccal cavity is low, there is no external evidence of this differentiation; however, when the frog vocalizes, the gular skin expands and becomes translucent in these regions (Figure 1c,d).

In contrast, the gular skin of adult male *Crossodactylus* is homogeneous in thickness, vessel and glandular content, pigmentation, and fiber composition of the dermis. Aside from a slight lateral expansion in some specimens, there is no external evidence of internally paired vocal sacs. Bilaterality is only evident externally when the frog is vocalizing. This is probably why most species of *Crossodactylus* have been reported to have single, subgular sacs in the taxonomic literature. Vocal sacs have been reported as absent in *C. aeneus* and *C. trachystomus*

(Müller, 1924) and single, subgular in *C. aeneus*, *C. bokermanni*, *C. cyclospinus*, *C. dantei*, *C. gaudichaudii*, *C. lutzorum*, *C. schmidti*, and *C. trachystomus* (Lynch, 1971; Pimenta, Cruz, & Caramaschi, 2014; Pimenta et al., 2015). Subtle bilobular expansions have been reported only in *C. bouleengeri* (Pimenta, Wachlewski, & Cruz, 2008), *C. caramaschii* (Bastos & Pombal, 1995), and *C. dispar* (Pimenta et al., 2014). Nevertheless, study of internal vocal sac anatomy revealed the presence of paired, lateral vocal sacs in *C. aeneus*, *C. bouleengeri*, *C. cyclospinus*, *C. dantei*, *C. dispar*, *C. gaudichaudii*, *C. grandis*, and *C. schmidti*.

Material of *Megaelosia* is extremely rare in herpetological collections and only one male and one female *M. apuana* and two male *M. goeldii* (which lacks vocal sac) were available for the present study. The adult male *M. apuana* (UFMG 13536) has paired, ventrolateral vocal sacs with distinct skin pouches in the gular skin, V-shaped apical supplementary elements, and a connective tissue sheath ventral to the *m. interhyoideus* and the protruding mucosa. External and internal morphology suggest that the sacs are spherical when inflated, like those of species of *Hylodes*. Of the seven described species of *Megaelosia*, four [*M. apuana* (Pombal et al., 2003), *M. lutzae* (Izecksohn & Gouvêa, 1987, 1985), *M. boticariana*, and *M. massarti* (Giaretta et al., 1993)] are reported to have externally paired vocal sacs similar to those of *Hylodes*. The condition of *M. bocainensis* and *M. jordanensis* remains unknown, as only females are known (Heyer, 1983; Pombal et al., 2003; Verdade & Rodrigues, 2008).

Most of the species of *Hylodes* we examined were reported to have well developed, paired vocal sacs [*H. dactylocinus*, (Pavan, Narvaes, & Rodrigues, 2001); *H. japi* (de Sá, Canedo, Lyra, & Haddad, 2015; de Sá et al., 2016); *H. meridionalis* (Mertens, 1927); *H. otavioi* (Sazima & Bokermann, 1982); *H. perere* (Silva & Benmaman, 2008); *H. regius* (Gouvêa, 1979)]. There is no reference to vocal sacs in the original description of *H. mertensi* and no males are known to date (Bokermann, 1956). The two species of *Crossodactylus* that were unavailable for this study were reported to have weakly developed paired subgular vocal sacs [*C. caramaschii* (Bastos & Pombal, 1995), *C. franciscanus*

(Pimenta et al., 2015)]. Nevertheless, given the broad taxonomic sample of Hylodidae analyzed here, we expect that the internal structure of the vocal sacs of species not examined will be found to resemble the general pattern described above.

All recent phylogenetic analyses support a sister-taxon relationship between *Crossodactylus* and the other hylodid genera, with *Megaelosia* being either sister to, or nested within, *Hylodes* (Frost et al., 2006; Fouquet et al., 2013; Grant et al., 2006; Pyron, 2014). Nevertheless, *M. goeldii* was the only species of *Megaelosia* included in those studies. Thus, the monophyly of the genus has not been tested and the relationships among *M. goeldii*, other species of *Megaelosia*, and *Hylodes* are untested. Small spherical ventrolateral vocal sacs and the presence of distinct pouches in the gular skin are reported for all species of *Hylodes* and four species of *Megaelosia*, absent in *M. goeldii*, and unknown in the remaining two species of the genus. Based on currently known species, the only scenario that would contradict the inference of these two character states as synapomorphies for *Hylodes* + *Megaelosia* would be the placement of *M. goeldii* as sister taxon of all the remaining species of *Hylodes* and *Megaelosia*. In this scenario, the absence of vocal sacs in *M. goeldii*, which makes these characters inapplicable, would result in an ambiguity in the ancestral node of *Hylodes* + *Megaelosia*. The small spherical ventrolateral sacs and the skin pouches would be a synapomorphy for a less inclusive clade containing all *Hylodes* and the remaining species of *Megaelosia*. With any other placement of *M. goeldii*, these states would be synapomorphies for the common ancestor of *Hylodes* + *Megaelosia*. If males of the unexamined species of *Megaelosia* (i.e., *M. bocainiensis* and *M. jordanensis*) are found to lack vocal sacs, then their placement may provide additional interpretations of the evolution of these characters.

The presence of a connective tissue sheath covering the *m. interhyoideus* seems to be plesiomorphic for the family and to have been lost in *Hylodes*. The loss of vocal sacs and apical supplementary elements of the *m. intermandibularis* are autapomorphic for *M. goeldii*.

## 4.2 | Three synapomorphies for Hylodidae

The unique arrangement of submandibular muscles and vocal sac structure in *Crossodactylus*, *Hylodes*, and *Megaelosia* allows us to identify the three morphological synapomorphies for the family Hylodidae described below.

### 4.2.1 | Externally paired, lateral vocal sacs

Proposed by Grant et al. (2006), this synapomorphy is corroborated here with a greatly expanded taxon sampling. The presence of paired vocal sacs sets hylodids apart from all anurans with which they have been related in recent phylogenetic analyses. All species of Alsodidae, Aromobatidae, Batrachylidae, Bufonidae, Cycloramphidae, Dendrobatidae, Odontophrynidae, and Rhinodermatidae have externally single, subgular vocal sacs, when sacs are present (Boulenger, 1882; Burton, 1998; Cei, 1980; Grant et al., 2006; Liu, 1935; Lynch, 1971; Tyler 1971a). Externally paired, lateral vocal sacs are present in all species of *Crossodactylus*, *Hylodes*, and *M. apuana* examined, and are reported for three more species of *Megaelosia*.

### 4.2.2 | Internal disconnection of vocal sac mucosae

The vocal sac mucosae in anurans originate bilaterally and, in most species, fuse along the midline shortly after metamorphosis (Inger & Greenberg, 1956; Tyler, 1975). Nevertheless, as first discovered by McAlister (1959) in *Spea*, both evaginations of the buccal cavity can remain unconnected in adulthood, thereby resulting in internally bilateral vocal sacs. Vocal sacs in *Spea* are externally bilobate (i.e., the subgular region inflates ventrally and possesses two ventrolateral projections), but internal disconnection also occurs in many species with externally paired, lateral vocal sacs (i.e., the gular region is unaffected by the inflation of the sacs, which occurs only laterally), or externally single, subgular sacs, such as *Eupsophus roseus* and *Limnomedusa macroglossa* (Alsodidae, this study). In contrast, externally bilobate and paired, lateral vocal sacs can be connected via a single, common cavity, as in *Leptodactylus fuscus* or *Osteocephalus taurinus*. Based on these observations, we consider external appearance and internal connection of vocal sacs to be independent characters.

The vocal sac mucosae are disconnected internally in all specimens of *Crossodactylus*, *Hylodes*, and *M. apuana* examined, and among outgroup taxa this condition occurs only in the aforementioned alsodids. Optimization of character states in the two alsodid species does not interfere with the recognition of this as a synapomorphy for Hylodidae in any recent phylogenetic hypothesis.

### 4.2.3 | Belly of *m. interhyoideus* pierced by the projection of the vocal sac mucosa

The internal structure of the paired vocal sacs of hylodids differs from the structure of all other anurans described to date. In all species for which the myology and internal anatomy of paired vocal sacs have been described, the mucosa is enveloped by the *m. interhyoideus* (Inger, 1956; Tyler 1971a). Consequently, Hylodidae is unique in having vocal sacs that protrude through the *m. interhyoideus* into the submandibular lymphatic sac.

These newly described synapomorphies supplement morphological synapomorphies that have already been proposed for hylodids; however, it is likely that the status of some will change as knowledge of the phylogenetic relationships between Hylodidae and other hyloid lineages improves. The presence of dermal scutes on the top of the digital discs, T-shaped terminal phalanges, and an oblique lateral stripe are all shared with dendrobatoids (Grant et al., 2006). Therefore, the status of these characters depends on the sister-group relationship of these taxa. In this context, putative synapomorphies of the family proposed by Grant et al. (2006; their character numbers in parentheses) are as follows: origin of preaxial fringe on Finger II (Ch. 13: 0–1); origin of preaxial fringe on Finger III (Ch. 15: 0–1); origin of tarsal fringe (Ch. 30: 0–1; Lynch, 1971); origin of preaxial fringe on Toe I (Ch. 36: 0–1); origin of fringe on postaxial fringe on Toe V (Ch. 45: 0–1); loss of oocyte pigmentation (Ch. 68: 1–0); loss of fibers of the *m. depressor mandibulae* originating from the *annulus tympanicus* (Ch. 74: 1–0); gain of lateral-line stitches (98: 0–1). Haas (2003) further proposed the presence of a distal confluence between both *partes corporeae* of the *cartilago*



*labialis superior* (Ch. 87:1–0). In addition, the breeding behavior of hylodids, whereby males dig underwater chambers in which eggs are deposited probably is another synapomorphy for the family (de Sá et al., 2015; Faria, Silva, & Rodrigues, 1993; Forti & Castanho 2012; Haddad & Giarretta, 1999; Haddad & Prado, 2005; Narvaes & Rodrigues 2005; Weygoldt & Carvalho-e-Silva, 1992).

### 4.3 | Functional implications of the pierced *m. interhyoideus* in hylodids

de Sá et al. (2016) thoroughly described the complex intraspecific communication in *Hylodes japi*, which includes the use of multimodal signaling in intra- and intersexual interactions with acoustic, visual, and tactile stimuli. Male visual displays include movements of the head, body, arms, hands, legs, feet, toes, and vocal sacs. Previous studies reported that vocal sacs can provide visual cues for intraspecific interaction (Gómez et al., 2009; Rosenthal, Rand, & Ryan, 2004), but *H. japi* is the first species known to convey information by inflating only one of their paired sacs. Depending on the context, males can inflate both sacs, only the left one, or only the right one. This degree of control is unprecedented in anurans.

Unilateral inflation of paired sacs in Hylodidae involves a set of morphological requirements. First, both vocal sac mucosae must be internally isolated so that exhaled air is compartmentalized and inflation of one sac does not affect the other. Second, a valve or valve-like structure is required to allow selective closure of only one vocal slit. This could be achieved by the contraction of a muscular sphincter surrounding small, round vocal slits, such as those seen in arthroleptids, ranids, and phrynobatrachids (Drewes, 1984; Perret, 1966). However, no such structure was observed in hylodids, in which vocal slits are elongate and are not surrounded by muscle fibers. Instead, selective inflation appears to be enabled in hylodids by the unique morphology of the *m. interhyoideus*. Fibers of this muscle originate on the hyalia and insert medially on the fascia of the *m. episternohumeralis*, whereas in most anurans, they are connected to one another and do not contact the *m. episternohumeralis*. The arrangement in hylodids provides a firm anchor that prevents the transmission of the contraction on the *mm. interhyoidei* from one side to the other; thus, the contralateral muscles, which are innervated independently by the Cranial Nerve VIIth, are mechanically isolated. Thus, what is customarily termed the “*m. interhyoideus*” actually represents two independent functional units (left and right). This contrasts with the *mm. submentalis* and *intermandibularis*, which lack medial anchors and, thus, form single contractile units. Further, each contralateral *m. interhyoideus* is pierced by the vocal sac that projects into the submandibular lymphatic sac. We hypothesize that contraction of *m. interhyoideus* clamps the entrance of the vocal sac shut, thereby preventing airflow. If only one of the contralateral muscles is contracted, exhaled air would only enter the opposite sac, resulting in unilateral inflation.

To date, single-sac expansion has been documented only in *H. japi* (de Sá et al., 2016) and *H. asper* (Hödl et al., 1997), but the anatomical

modifications hypothesized to achieve it occur in all hylodids that we examined. This suggests that this behavior might also occur in other hylodids as well.

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