THREE-DIMENSIONAL RECONSTRUCTION OF THE HYOBRANCHIAL APPARATUS OF *HYLA NANA* TADPOLES (ANURA: HYLIDAE)

María Florencia Vera Candioti [1] & Alexander Haas [2]

[1] Instituto de Herpetología, Fundación Miguel Lillo. Miguel Lillo 251. (4000) S. M. de Tucumán, Argentina.
f l o r i v c @ h o t m a i l . c o m
[2] Zoologisches Institut und Zoologisches Museum Martin-Luther-King-Platz 3. 20146 Hamburg, Germany.
a l e x a n d e r . h a a s @ u n i - h a m b u r g . d e

R e s u m e n. — En el presente trabajo se estudia el esqueleto hiobranquial y musculatura de la canastilla branquial de larvas de *Hyla nana* a través de reconstrucción tridimensional de secciones histológicas. El esqueleto cartilaginoso se caracteriza por el marcado desarrollo de los ceratohiales y la reducción de la canastilla branquial. La topología muscular es congruente con lo reportado para la mayoría de los hílidos, con características particulares como un *m. subarcualis rectus I* con sólo dos cabezas (dorsal y ventral), y un *m. subarcualis rectus II-IV* discontinuo e insertado anterolateralmente en el ceratobranquial I. Estos caracteres no son frecuentes entre los hílidos, pero presumiblemente los comparten otras especies del grupo de *H. microcephala*.

Palabras clave: esqueleto hiobranquial, musculatura branquial, renacuajos, *Hyla nana,* reconstrucción 3D.

A b s t r a c t. — This study describes the hyobranchial skeleton and branchial musculature of *Hyla nana* tadpoles, employing three-dimensional reconstruction from histological sections. The cartilaginous skeleton is characterized by a marked development of the ceratohyals and a reduction of the branchial basket. The muscular topology is congruent with the pattern found in most hyline hylids, with particular features such as the *m. subarcualis rectus I* with only two heads (dorsal and ventral), and a discontinuous *m. subarcualis rectus II-IV*, inserted anterolaterally in the ceratobranchial I. Such features, infrequent among hylids, are presumably shared by other species within the *H. microcephala* group.

Key words: hyobranchial skeleton, branchial musculature, tadpoles, *Hyla nana,* 3D reconstruction.

INTRODUCCIÓN

Tadpole skeleton and musculature have been described in numerous hylid species (i.a., De Sá, 1988; Fabrezi and Lavilla, 1992; Fabrezi and Vera, 1997; Haas, 1996a; 1996b; 2001; 2003; Haas and Richards, 1998; Lavilla and Fabrezi, 1987; Sokol, 1981). The technique most frequently employed for the analysis of cartilage and bone structures in anurans is clearing and differential staining (Wassersug, 1976; Dingerkus and Uhler, 1977; Taylor and Van Dicke, 1985). These protocols are both highly available and useful, but dissection becomes difficult in the case of complex or small-sized specimens. Sectioning and histological analysis are also frequently used, but they imply loss of the 3D perception and integrity of specimens.

Haas and Fischer (1997) developed a method of morphological reconstruction from serial histological sections, by using commercially available product-design software packages. Specimens are cut following standard histology protocols and then manually reconstructed with software that models the samples three-dimensionally. This interactive technique is a reliable option for precise morphological studies, and with adequate training, the time consumed to prepare a reconstruction is acceptable. Furthermore, virtual reconstructions are not mere visual representations of objects, since they can be measured and analyzed accurately.

3D reconstruction methods are being increasingly used, not only in herpetology, but also in other fields of vertebrate and invertebrate morphology (v.g., Haas and Richards, 1998; Gorb and Beutel, 2000; Adriaens *et al.*, 2001; Sánchez-Villagra *et al.*, 2002).

Hyla nana tadpoles are common inhabitants of lentic ponds in Chaco and Litoral regions from Argentina. The external morphology has been extensively described by Lavilla (1990). The internal morphology, particularly cartilaginous skeleton and cranial musculature, has been also previously studied (Lavilla and Fabrezi, 1992; Vera Candioti *et al.*, 2004). Data on branchial muscles still remained, which constitutes one of the reasons for the selection of this species. Furthermore, the reduced size of the specimens ad their marked differences with the general morphological pattern found in other taxa derive in a particular difficulty in the study of the said muscular group, and consequently, the 3D reconstruction technique becomes very helpful.

This paper presents a morphological study of the hyobranchial skeleton and branchial musculature of *Hyla nana* tadpoles, employing three-dimensional computer-aided reconstructions from serially sectioned specimens.

MATERIAL AND METHODS

Hyla nana larvae in stages 31-36 (according Gosner, 1960) were examined. The study focused on the hyobranchial skeleton and branchial and spinal muscle groups (according to the classification shown in Cannatella, 1999).

Five larvae were collected in Santa Fe City (Ciudad Universitaria, El Pozo, Santa Fe, Argentina), during January 2001. The specimens were fixed in neutral 4% formalin. Part of them was prepared according to standard histological protocols (Böck, 1989). For serial sectioning, specimens were decalcified (Dietrich and Fontaine, 1975), dehydrated, embedded in paraffin, and sectioned at 7 μm. Sectioning was performed on a Micrcom HM360 rotary microtome equipped with the Microm water transfer system. Sections were then stained with Azan trichrome (Böck, 1989) and digitalized with a Nikon Coolpix 990 camera on a Zeiss microscope. The pixel files obtained were loaded into the software package Alias/Wavefront Studio™ 9.5 on a Silicon Graphics Octane workstation as background images (image planes). Contours of selected structures were drawn manually on the background images. The resulting set of contours was used as a scaffold around which a surface was created manually to fit the contours tightly (Haas and Fischer, 1997). The musculature was reconstructed on one side only and no difference to the other side musculature was found in histological sections.

The remaining specimens were prepared according to the clearing and staining protocol of Taylor and Van Dicke (1985). To examine the musculature, some individuals were removed before the digestion step (Lavilla, pers. commun.) and then stained with a lugol solution (Böck and Shear, 1972). Dissections were made employing a stereomicroscope equipped with a camera lucida (Zeiss SV 11). The larvae so treated served as control against which the re-
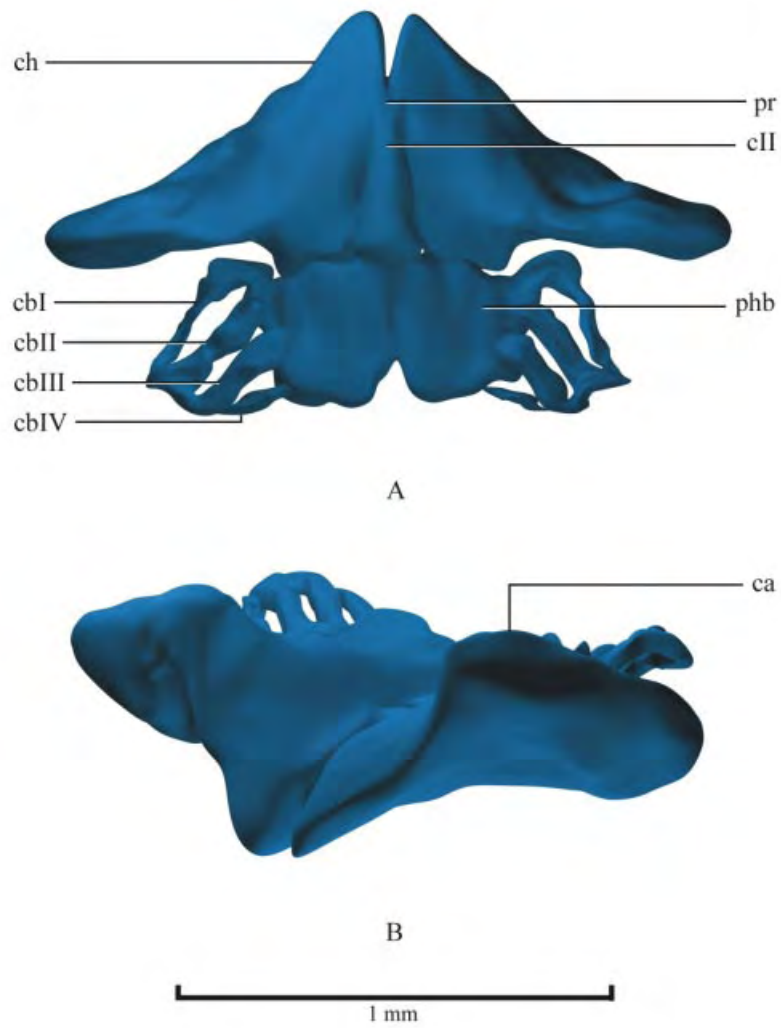
Figure 1. *Hyla nana* Stage 31. Hyobranchial skeleton. A. Ventral view. B. Anterolateral view. Legends: ca, *condylus articularis*; cb (I-IV), ceratobranchial; ch, ceratohyal; cII, *copula II*; phb, *planum hypobranchiale*; pr, *pars reuniens*.

sults obtained with the 3D reconstruction method were compared.

The final virtual three-dimensional model of the *Hyla nana* hyobranchial apparatus was animated with Alias Wavefront Studio software and the resulting image series were composed to a movie with Apple Quicktime 6.1 Pro on an Apple Macintosh G4 workstation.

The terminology used for the following description corresponds to Haas (2003).

RESULTS

The cartilaginous skeleton of *Hyla nana* tadpoles is described in Lavilla and Fabrezi (1992) and Vera Candioti *et al.* (2004). Cartilaginous structures of specimens examined in the present work do not show significant differences as compared to former descriptions (Fig. 1).

Although the cartilaginous branchial basket is reduced in *Hyla nana*, all the branchial and spinal muscles reported for hylids are present (Figs. 2 and 3).

Mm. levatores arcuum branchialium. These four muscles extend from the lateral and posterolateral margin of the branchial basket to the posterior part of the palatoquadrate and otic capsule. In *Hyla nana* all four muscles are distinguishable.

M. levator arcuum branchialium I. Lateral margin of *arcus subocularis*-ceratobranchial I. It is a wide muscle, with insertion surfaces occupying a large area on the ceratobranchial I and palatoquadrate.

M. levator arcuum branchialium II. *Larval processus oticus-commissura terminalis I* and ceratobranchial II. It is separated from the *m. levator arcuum branchialium I* by a narrow gap in its ceratobranchial insertion, but the separation becomes less evident in the insertion into the palatoquadrate.

M. levator arcuum branchialium III. Lateral part of otic capsule-ceratobranchial III.

M. levator arcuum branchialium IV. Posterolateral part of the otic capsule-ceratobranchial IV.

Mm. constrictores branchiales. These are four muscles which extend between the medial edge of their own ceratobranchial and the *commissurae terminales*. In *Hyla nana* three of these four muscles are present.

M. constrictor branchialis II. *Processus branchialis II-commissura terminalis I*.

M. constrictor branchialis III. *Processus branchialis II-commissura terminalis II*.

M. constrictor branchialis IV. Fibers are continuous with those of the anterior part of the *m. subcualis rectus II-IV*. They insert on the *commissura terminalis II* and distal portion of ceratobranchial III.

Mm. subarcuales recti. This muscle group extend from the ceratohyals to the ceratobranchials, and between ceratobranchials. It can be divided into several portions in anuran larvae. The muscles found in *Hyla nana* are as follows:

M. subarcualis rectus I. It is formed by a ventral and a dorsal slip. Both originate in the lateral region of *processus posterior hyalis*. The ventral slip is well developed and runs posteriorly to the *processus branchialis III*, while in the dorsal slip, fibers assume an oblique direction and insert in the lateral proximal part of the ceratobranchial I.

M. subarcualis rectus II-IV. It is formed by two parts, anterior and posterior. The anterior part originates in connective tissue next to the lateral side of the ceratobranchial I, approximately at mid-length. Its fibers are confluent with those of the *m. constrictor branchialis IV* and *m. subarcualis rectus II-IV*, posterior part. The posterior part includes fibers of the anterior part and fibers which originate in the *processus branchialis III*. This slip inserts in the ventromedial side of the ceratobranchial IV.

M. subarcualis obliquus. *Processus urobranchialis-processus branchialis II* and *III*.

M. tympanopharyngeus. Lateral parts of the otic capsule-ceratobranchial IV and *planum hypobranchiale.*

M. rectus cervicis. Peritoneum-*processus branchialis III.*

M. geniohyoideus. Cartilago labialis inferior-planum hypobranchiale.

The attached CD shows a two-minute animation of the 3D reconstruction of the hyobranchial apparatus of *Hyla nana* tadpoles (see the file Readme.doc, also in the CD).

DISCUSSION

As mentioned by Lavilla and Fabrezi (1992) and Vera Candioti *et al.* (2004), the hyobranchial skeleton of *Hyla nana* tadpoles is considerably different from the one described for most hylids. The large relative size of ceratohyals contrasts with the evident reduction in size of the branchial basket. Features that are commonly present in other species show a different configuration or are absent in *H. nana*. The ceratobranchials, for example, are almost straight bars, scarcely chondrified and devoid of *spicula* and lateral projections. Lateral projections from the ceratobranchials are a unique character of Anura, and they give support to the interbranchial septa and filter epithelia (Haas, 2003). *Spicula* and lateral projections are also reduced or absent in carnivorous tadpoles of *Hymenochirus boettgeri, Lepidobatrachus laevis, L. llanensis, Ceratophrys ornata* and *C. cranwelli,* macrophagous larvae such as *Hyla microcephala* and *Anotheca spinosa,* gastromizophorous larvae of *Atelopus tricolor,* suctorial larvae of *Boophis* sp. and endotrophic larvae of *Cycloramphus stejnegeri, Eupsophus calcaratus, Flectonotus goeldii* and *Rhinoderma darwini* (Haas, 1996b; 2003; Haas and Richards, 1998; Lavilla, 1987; 1991; Lavilla and Fabrezi, 1992; Lavilla and de Sá, 2001; Sokol, 1962; Ruibal and Thomas, 1988; Wassersug and Hoff, 1979; pers. obs.).

As is the case with *Hyla microcephala,* and contrary to what has been observed in most hylids, the *copula I* and *processus urobranchialis* are also absent. Finally, *commissurae proximales* between ceratobranchials are absent, a character shared by some hylid species (*H. microcephala, H. cinerea, H. annectans, H. ebraccata, Litoria genimaculata, L. lesueuri,* phyllomedusines; Haas, 2003; Haas and Richards, 1998; pers. obs.).

The configuration of the hyobranchial musculature is not concomitant with cartilaginous skeleton reduction. All the muscles reported for representatives of the Neobatrachia are present, and the difficulty in the observation and identification of each muscle is due to the small size of the individuals, rather than to poor development of the musculature itself. Compared to other species within the Hylidae, some features need to be mentioned.

Personal examination of *Hyla microcephala* tadpoles revealed a pattern of hyobranchial musculature identical to the one described here for *H. nana.* Tadpoles of species of the *H. microcephala* group share several characters in external morphology, cartilaginous skeleton, musculature, buccal apparatus and buccopharyngeal cavity (i.a., Duellman, 1970; 2001; Haas, 1996a; Vera Candioti *et al.*, 2004; Wassersug, 1980). Previous data on hyobranchial musculature of the other species integrating the group are not available, but the similarities might include this particular group of muscles. More extensive taxa samples will show whether or not these shared character states constitute apomorphic character states suitable to define the species group.

The *mm. subarcuales recti* show several peculiarities as compared to other species of the family. The *m. subarcualis rectus I* has two bundles, ventral and dorsal. This is also the case of *Aplastodiscus perviridis, Phrynohyas resinifictrix, Scinax ruber* and *Phyllomedusa* spp. tadpoles (Haas, 2003). The presence
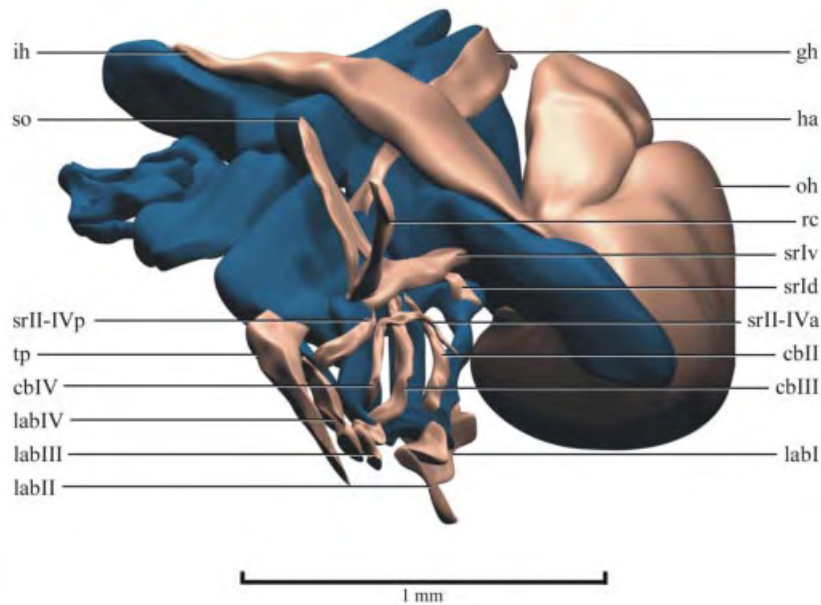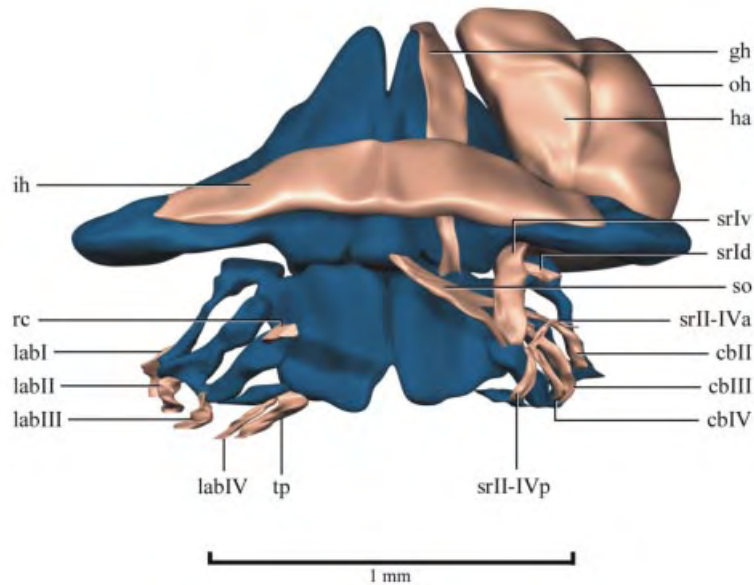
Figure 2 (top). *Hyla nana* Stage 31. Branchial and spinal musculature. Some muscles of the hyoid group are also shown. Ventral view. Legends: cb (II-IV), *constrictor branchialis*; gh, *geniohyoideus*; ha, *hyoangularis*; ih, *interhyoideus*; lab (I-IV), *levator arcuum branchialium*; oh, *orbitohyoideus*; rc, *rectus cervicis*; so, *subarcualis obliquus*; srI (d-v), *subarcualis rectus I* (dorsal head - ventral head); srII-IV (a-p), *subarcualis rectus II-IV* (anterior part - posterior part); tp, *tympanopharyngeus*.

Figure 3 (bottom). *Hyla nana* Stage 31. Branchial and spinal musculature. Some muscles of the hyoid group are also shown. Ventrolateral view. Legends: cb (II-IV), *constrictor branchialis*; gh, *geniohyoideus*; ha, *hyoangularis*; ih, *interhyoideus*; lab (I-IV), *levator arcuum branchialium*; oh, *orbitohyoideus*; rc, *rectus cervicis*; so, *subarcualis obliquus*; srI (d-v), *subarcualis rectus I* (dorsal head - ventral head); srII-IV (a-p), *subarcualis rectus II-IV* (anterior part - posterior part); tp, *tympanopharyngeus*.

of two heads in the *m. subarcualis rectus I*, instead of three, is not a frequent feature among hylids. In fact, among 25 studied species, only eight have this configuration, while the 70% corresponds to the alternative state. This proportion is also maintained considering Anura as a whole (24 over a total of 81 species studied by Haas, 2003).

As to the *m. subarcualis rectus II-IV*, it also shows a rather atypical configuration as compared to other hylids. Similar to pseudines (Haas, 2003; Vera Candioti, 2004), the muscle in *Hyla nana* is discontinuous. Its fibers are interrupted at the level of *processus branchialis III*. In *H. nana*, however, this division is not complete. Some fibers of the anterior part merge with those of the *m. constrictor branchialis IV*, and other fibers join to the posterior part. All of the other hylid species examined have a single muscle that extends between the ceratobranchial IV and the ceratobranchials I or II (Haas, 2003).

The *m. subarcualis obliquus* originates in the ceratobranchials II and III, acquiring a clearly bifurcated shape. This is frequent among hylines (Haas, 2003; pers. obs.). In pelodryadine hylid species such as *Litoria genimaculata*, *L. nannotis*, *L. xanthomera*, *L. rheocola* and *Nyctimystes dayi* the *m. subarcualis obliquus* originates from ceratobranchials II and III and fibrous tissue between them, acquiring a fan shape (Haas and Richards, 1998; Haas, 2003).

The *mm. levatores arcuum branchialium I* and *II* are almost undistinguishable, or even fused in several taxa (Haas, 2003) whereas in *Hyla nana*, even when these muscles are very close to each other, it is still possible to tell them apart. In like manner, the *m. tympanopharyngeus* and *m. levator arcuum branchialium IV* are undistinguishable in several taxa, whereas in *H. nana*, there exists a clear separation. *H. nana* features coincide with those of hylid species, such as *H. ebraccata*, *Scinax ruber*, *S. nasicus* and Phyllomedusi-

nae (Haas; 2003; pers. obs.).

Comparisons of the mentioned skeletal and muscular characters among *Hyla nana* and other hylid tadpoles and non-hylid tadpoles with similar feeding habits are summarized in Table 1.

Some skeletal characters in the hyobranchial apparatus of *Hyla nana,* such as large ceratohyals and hypobranchials, bar-like ceratobranchials with reduced or absent *spicula* and lateral projections, seem to be linked to ecological habits (see also Alcalde and Rosset, 2003; Vera Candioti *et al.*, 2004). They are associated with a type of feeding which does not depend on the filtration capability of the hyobranchial apparatus (macrophagy, endotrophy), or with the generation of great suction forces inside the buccal cavity (macrophagy, suctorial habits). They then appear in both taxonomically related and taxonomically unrelated species which share a similar functioning of those features (other macrophagous hylids, Ceratophryinae, endotrophic "Leptodactylidae", Pipidae, Rhacophoridae, Rhinodermatidae). Other characters, such as the *commissurae proximales* and *copula I*, even when their absence is shared by all the non-hylid macrofages, cannot be accounted for solely on ecological grounds due to their variable distribution within Hylidae and their co-occurrence with different feeding types.

On the other hand, characters in branchial muscles do not seem to be related to feeding habits but to phylogeny, since muscular configuration in *Hyla nana* and *H. microcephala* resembles that of Pseudinae rather than that of other macrophagous tadpoles (pseudines also share other muscular characters with *H. microcephala* group tadpoles; Vera Candioti, 2004). At least in a qualitative study —i.e., without considering muscles weights, type of muscular fibers, etc.— there appear no noticeable differences among the species.

Gradwell (1972) studied the functional morphology of the hyobranchial appa-

		Cartilages					Musculature					
		Spicula	Lateral projections	copula I	processus urobranchialis	commissurae proximales	Sr I	Sr II-IV	So	Lab I and II	Lab IV and Tp	Literature
Ma	*Hyla nana*	absent	absent	absent	absent	absent	2 bundles	discontinuous	bifurcated	small gap	separated	
Ma	*Anotheca spinosa*	absent	absent	absent	absent	II-III present?	?	single	?	?	?	Wassersug & Hoff (1979)
	Aplastodiscus perviridis	present	present	present	present	III present	2 bundles	single	bifurcated	small gap	Tp absent	Haas (2003)
	H. annectans	present	present	absent	present	absent	2/3 bundles	single	single/bifurcated	small gap	distinguishable	Haas (2003)
	H. cinerea	present	present	absent	present	absent	3 bundles	single	bifurcated	small gap	distinguishable	Haas (2003)
	H. ebraccata	present	present	present	present	absent	3 bundles	single	bifurcated	small gap	separated	Haas (2003)
Ma	*H. microcephala*	absent	absent	absent	absent	absent	2 bundles	discontinuous	bifurcated	small gap	separated	pers. obs.
	H. pulchella cordobae	present	present	present	present	III present	3 bundles	single	bifurcated	small gap	distinguishable	Haas (2003)
	Osteocephalus planiceps	present	present	present	present	II present	3 bundles	single	bifurcated	small gap	distinguishable	Haas (2003)
	Phrynohyas resinifictrix	present	present	present	present	II present	2 bundles	single	bifurcated	small gap	distinguishable	Haas (2003)
	S. nasicus	present	present	present	present	absent?	3 bundles	single	single/bifurcated	small gap	separated	Vera Candioti *et al.* (2004); pers. obs
	Scinax ruber	present	present	present	present	II-III present	2 bundles	single	bifurcated	small gap	distinguishable	Haas (2003)
	Smilisca baudini	present	present	present	present	II-III present	3 bundles	single	single/bifurcated	small gap	distinguishable	Haas (2003)
	Lysapsus limellus	present	present	absent	present	II present	3 bundles	discontinuous	single	small gap	separated	Vera Candioti (2004)
	Pseudis minuta	present	present	absent	present	II present	3 bundles	discontinuous	single	small gap	distinguishable	Haas (2003)
	P. paradoxa	present	present	absent	present	II present	3 bundles	discontinuous	single	small gap	distinguishable	Haas (2003)
	Agalychnis callidryas	present	present	present	present	absent	3 bundles	single	single	small gap	distinguishable	Haas (2003)
	Phyllomedusa distincta	present	present	present	present	absent	2 bundles	single	single	small gap	distinguishable	Haas (2003)
	P. vaillanti	present	present	present	present	absent	2 bundles	single	single	small gap	distinguishable	Haas (2003)
	Litoria inermis	present	present	present	present	II present	3 bundles	single	fan-shaped	small gap	distinguishable	Haas (2003)
	L. genimaculata	present	present	present	present	absent	3 bundles	single	fan-shaped	small gap	distinguishable	Haas (2003)
	L. lesueuri	present	present	present	present	absent	3 bundles	single	fan-shaped	small gap	distinguishable	Haas (2003)
	L. nannotis	present	present	absent	present	II present	3 bundles	single	fan-shaped	small gap	distinguishable	Haas (2003)
	L. rheocola	present	present	present	present	absent?	3 bundles	single	fan-shaped	small gap	distinguishable	Haas (2003)
	L. xanthomera	present	present	present	present	II present	3 bundles	single	fan-shaped	small gap	distinguishable	Haas & Richards (1998)
	Nyctimystes dayi	present	present	absent	present	II present	3 bundles	single	fan-shaped	small gap	distinguishable	Haas (2003)
MaC	*Hymenochirus boettgeri*	absent	absent	absent	absent	absent	1 bundles	absent	single	absent?	Tp absent	Sokol (1962)
MaC	*Lepidobatrachus laevis*	absent	absent	absent	absent	absent	2 bundles	single	single	wide gap	distinguishable	Haas (2003)
MaC	*L. llanensis*	absent	absent	absent	present	absent	2 bundles	single	fan-shaped	wide gap	distinguishable	Lavilla & Fabrezi (1992); pers. obs.
MaC	*Ceratophrys cranwelli*	absent	reduced/absent	absent	present	absent	1 bundle?	single	single	wide gap	distinguishable	Lavilla & Fabrezi (1992); pers. obs.
MaC	*C. ornata*	absent	present	absent	present	absent	2 bundles	single	single	wide gap	distinguishable	Haas (2003)

Table 1. Comparison among *Hyla nana* tadpoles and other hylids and non-hylids (only those non-hylids with similar feeding habits were included). Left column show macrophages (Ma) and macrophages carnivores (MaC). Sr I = *subarcualis rectus I*, Sr II-IV = *subarcualis rectus II-IV*, So = *subarcualis obliquus*, Lab I and II = *levator arcuum branchialium I* and *II*, Lab IV and Tp = *levator arcuum branchialium* and *tympanopharyngeous*.

ratus of *Rana catesbeiana* tadpoles, during normal gill irrigation. He described the participation of some of the muscles in pharyngeal and branchial pump movements. The action of some other branchial muscles was established by Sokol (1962). Furthermore, the morphological variation of the hyobranchial apparatus has been correlated with slight modifications in mechanisms of normal gill irrigation (i.a., Gradwell, 1971; 1972; 1973; 1974). Formerly, hyobranchial movements and muscle actions during irrigation and feeding were considered to be essentially the same (De Jongh, 1968). However, electromyographic studies have shown differential participation of certain muscles in such processes (Larson and Reilly, 2003). Thus, a more profound study of branchial musculature would be necessary to look for correlation between branchial muscles and ecological habits (not only feeding but also gill irrigation). Or, as sustained by several authors, the anatomy mainly reflects inherited characters, and morphological designs are flexible enough to not require modifications according specific ecological habits (i.a., Gans *et al.*, 1985; Gans and De Vee, 1987; Abdala and Moro, 1996).

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