



## Research paper

Morphology and development of the hyolaryngeal apparatus of *Pipa arrabali* (Anura: Pipidae)O.G.S. Araújo<sup>a,\*</sup>, L.A. Pugener<sup>b</sup>, C.F.B. Haddad<sup>c</sup>, H.R. Da Silva<sup>a</sup><sup>a</sup> Departamento de Biologia Animal, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Caixa Postal 74524, CEP 23851-970, Brazil<sup>b</sup> Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Uruguay 151, Código Postal 6300, Santa Rosa, La Pampa, Argentina<sup>c</sup> Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, São Paulo, Caixa Postal 199, CEP 13506-970, Brazil

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## ABSTRACT

Two different perspectives guide ontogenetic studies. On one hand, there is a concern with objective processes and the understanding of the underlying mechanisms that governed the appearance of morphological differences between taxa; on the other hand, there are similarities between ontogenetic patterns that are used as phylogenetic characters. One focus of ontogenetic studies is to understand how morphology and timing of development differ among species. Such studies also aim to serve as a guide in proposing hypotheses of homology, especially when extremely modified structures are under scrutiny, such as the hyobranchial apparatus of amphibians. Here, we describe the morphology and development of the hyobranchial apparatus of *Pipa arrabali* (based on the examination of 51 embryos and six newly hatched specimens). Its morphology is compared to that described in the literature for other species. In *P. arrabali*, Copula I is absent, Copula II is present, and the ceratobranchials are simple, without spines or cartilaginous rays. In general, the ontogeny of the hyobranchial apparatus of *P. arrabali* seems to be accelerated when compared with that of other frogs, with some stages being skipped and the absence of some tadpole-specific structures observed in other species of pipids and non-pipids.

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## 1. Introduction

Pipids are obligate aquatic frogs that only venture onto land to migrate between bodies of water (Cannatella and Trueb, 1988a, 1988b; Duellman and Trueb, 1986). They are also well known because of their unusual appearance, which readily distinguishes them from other anurans. For example, their body is dorsoventrally depressed, their feet are fully webbed, adults possess a lateral line system (only larvae have it in most other frogs), and they lack a tongue. Living pipids are found in sub-Saharan Africa [*Hymenochirus* Boulenger (1896) (4 sp.), *Pseudhymenochirus* Chabanaud (1920) (1 sp.), and *Xenopus* Wagler (1827) (22 sp.)], Panama and northern South America [*Pipa Laurenti* (1768) (7 sp.)] (Duellman and Trueb, 1986; Frost, 2016). Neotropical pipids, usually called Surinam Toads or Star-fingered Toads, are clearly distinguishable from their African counterparts by several internal and

external morphological features. For example, the sacrourostylelic region is formed by the fusion of Vertebrae IX, X, and the urostyle (Trueb et al., 2000), and their fingertips bear several lobed, sensory structures (Trueb and Cannatella, 1986). In addition, females brood their eggs in small, enclosed pouches on their dorsum. *Pipa arrabali* Izecksohn (1976), *P. aspera* Müller (1924), *P. pipa* (Linnaeus (1758)), and *P. snethlageae* Müller, 1914 are borne as fully developed toadlets, whereas the three other members of the genus [*P. carvalhoi* (Miranda-Ribeiro, 1937), *P. myersi* Trueb, 1986, and *P. parva* Ruthven and Gaige, 1923] have free-swimming tadpoles that swim away from the mother's back (Trueb and Cannatella, 1986; Trueb and Massemin, 2001).

Although pipids have interested morphologists and systematists for over two centuries, relatively few publications have included descriptions of the skeletal anatomy or larval development of species in this family (e.g., Fermin, 1764; Haas, 1995; Henle, 1839; Higgins, 1920; Parker, 1876; Paterson, 1955; Paterson, 1960; Ridewood, 1896; Roček and Veselý, 1989; Roček, 2003; Sokol, 1975; 1977; Trueb et al., 2000; Yeh, 2002). Moreover, these works have focused primarily on *Pipa pipa* and *P. carvalhoi*. All published observations on pipid morphology—none of which involved the hyolaryngeal skeleton—have been a by-product of systematic or

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comparative osteological studies (e.g., Trueb and Cannatella, 1986; Cannatella and Trueb, 1988; Trueb and Massemin, 2001), except perhaps for Ridewood (1896).

Herein, we examine the hyolaryngeal skeleton of *Pipa arrabali* to (1) provide detailed, baseline descriptive data regarding embryonic morphology and ontogenetic development, (2) offer new insight into the processes by which some of the structures originated, and (3) assess the consequences of development, when a free-living larva is lacking, for the hyobranchium and larynx. The results are discussed in the context of what is currently known for other anurans in general and pipids in particular.

## 2. Materials and methods

We examined 51 embryos of *Pipa arrabali* in different stages of development, plus six recently emerged juveniles and one subadult. The embryos were collected from the dorsum of 17 females (three embryos from each female). The specimens were differentially stained as whole mounts for bone and cartilage using the method described by Taylor and van Dyke (1985). Staging follows Araújo et al. (2016). Observations were performed with the aid of an Olympus SZ stereomicroscope equipped with a camera lucida and a Zeiss stereomicroscope equipped with a camera lucida and a Canon digital photographic camera. Some specimens were disarticulated to facilitate examination, photography, and illustration. All specimens are deposited in the Coleção Herpetológica da Universidade de Brasília (CHUnB). Specimens were fixed in 4% formaldehyde and stored in 70% alcohol.

Terminology of the larval hyobranchial apparatus follows Sokol (1975, 1977) and occasionally Sedra and Michael (1957), and that of the laryngeal apparatus follows Sedra and Michael (1957). The terms for the adult hyoid are those of Ridewood (1896), unless otherwise indicated.

## 3. Results

In larval *Pipa arrabali*, the hyoid skeleton is a dorsoventrally flat structure formed by two pairs of major components, the ceratohyals and the branchial baskets. In addition, there are four elements that connect these components, a single pars reuniens, a single Copula II, and the paired hypobranchial plates. The hyobranchium is a larval structure that during ontogeny gives way to the adult hyoid. The hyoid skeleton consists of a central, cartilaginous bar that bears two large pairs of processes, the hyoid alae and the thyrohyalia. The larynx is a functionally adult structure that starts to form early in development. The laryngeal skeleton consists of a single cricoid and its associated processes, and the paired arytenoids.

The ceratohyals are robust structures that lie anterior and below the otic capsules. These cartilages are the largest components of the larval hyobranchial apparatus and together form a boomerang-shaped long bar that is oriented transversely to the axis of the body. The medial region of each ceratohyal is in an oblique position, with the posterior margin being more dorsal than the anterior margin. The lateral portion of the ceratohyal is almost vertical and the lateral margin coincides with the lateral margin of the otic capsule. In the youngest stage available for examination (Stage II-2) the ceratohyal already bears six processes: anterior and anterolateral processes on the anterior margin, posteromedian and postcondylar processes on the posterior margin, hyoquadrate process dorsally, and retroarticular process on the distal end (Fig. 1A). The anterior process is rounded, with a wide base, and is located on the anteromedial margin of the ceratohyal. The anterolateral process is a minuscule protuberance lateral to the anterior process. The posteromedian process is triangular with a wide base and a blunt apex and is located opposite to the anterior process; it is about

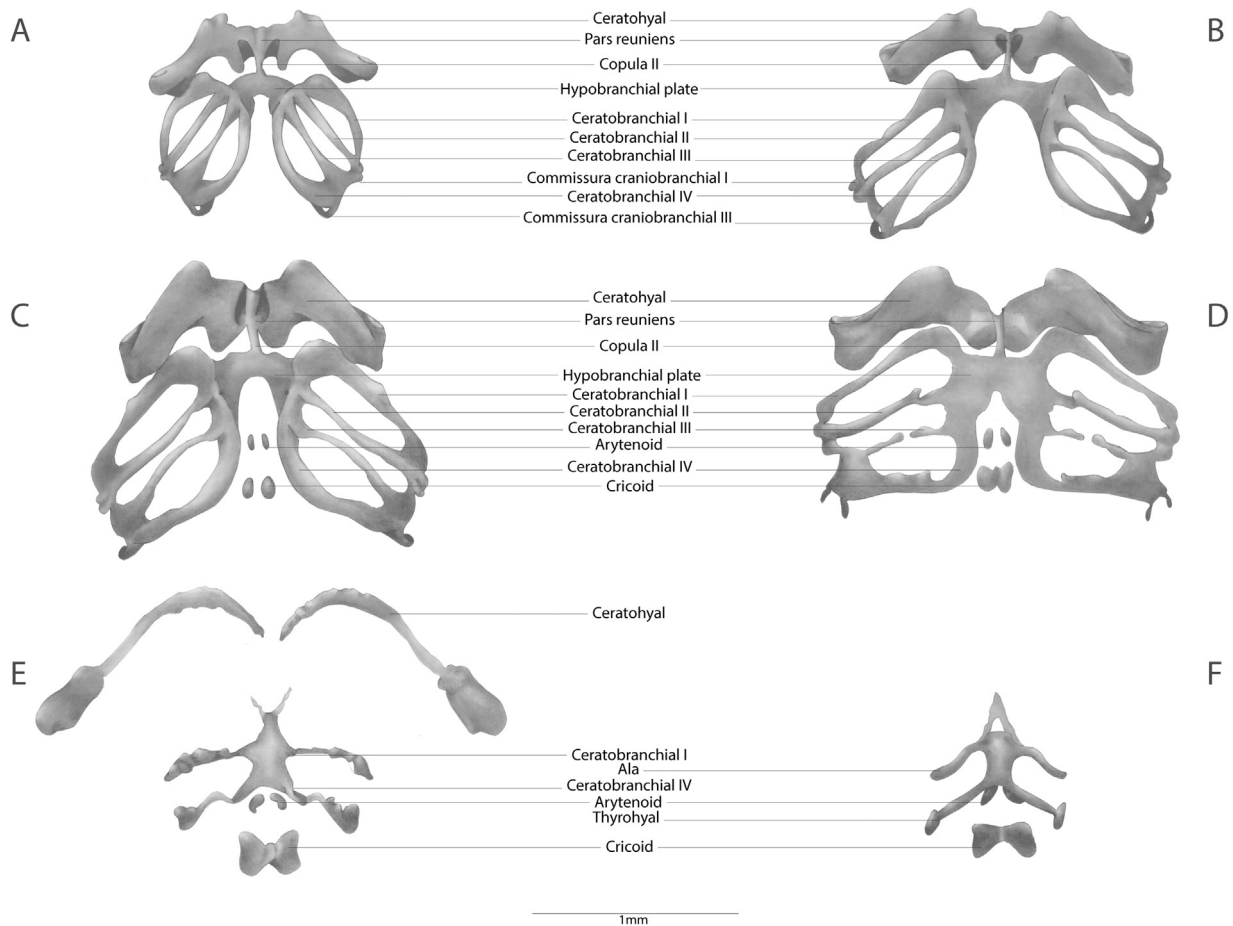
twice the size of the anterior process and comprises approximately 30% of the extension of the ceratohyal. The hyoquadrate process is high, oval, and has a flat dorsal surface that articulates with the condyle at the base of the palatoquadrate cartilage. The retroarticular process is a minute, posteroventrally directed protuberance located lateral to the base of the hyoquadrate process. Opposite to the hyoquadrate process there is a posterolaterally-oriented process with a wide base and a round apex that we assume it to be the postcondylar process of Haas (1997). The hyoquadrate, retroarticular, and postcondylar processes together give the lateral margin of the ceratohyal a roughly rounded shape. Medially, the ceratohyals are interconnected through the pars reuniens. Copula I is absent.

The pars reuniens is folded, forming a canal-like structure that opens dorsally. There is no suture or articulation in the area of confluence between each ceratohyal and the pars reuniens; the area is formed by a thin, continuous cartilage. Continuous with, and posterior to, the pars reuniens, between the posteromedian processes of the ceratohyal, there is a narrow, tubular Copula II, which serves as a connection between the pars reuniens and both hypobranchial plates. The urobranchial process is absent.

The hypobranchial plates are rectangular and lie posterior to the proximal area of the ceratohyals. The anterior margin of each hypobranchial plate is approximately straight and lies posterior to the posteromedian process of the ceratohyal, but without making contact with it. Medially, each hypobranchial plate is synchondrotically fused with the plate of the opposite side. The posteromedial margin is curved and the posterolateral margin is synchondrotically fused with the proximal portions of the ceratobranchials.

The ceratobranchials are long, flat, and thin bars that run in a latero-caudal direction. The combined ceratobranchials (four on each side) form shallow, dorsally concave branchial baskets. The baskets are roughly oval and lie posterior to the ceratohyals, underlying about three-fourths of the length of the otic capsules. Laterally, each basket extends to about the same level of the lateral margin of the ceratohyal. The lateral margin of each basket also coincides with the lateral margin of their respective otic capsule (Fig. 1A). The ceratobranchials are confluent with each other proximally through the commissurae proximales and distally through the commissurae terminales. Proximal Commissurae I, II, and III, and Terminal Commissurae I, II, and III are connect to the Ceratobranchials I and II, II and III, and III and IV, respectively. The proximal commissurae form a continuous ridge and are synchondrotically fused to the hypobranchial plates. Terminal Commissura III is about three times as wide and shorter than the other commissurae; as a result, Ceratobranchials III and IV almost touch each other distally. Cartilaginous rays are absent; in consequence, the margins of the ceratobranchials are smooth and the region gives origin to the Commissura Craniobranchial II, which is Y-shaped and dorsally oriented. Commissura Craniobranchial I originates on the terminal portion of Ceratobranchial II; it is U-shaped with dorsally oriented ends. The commissurae craniobranchiales are united to the crista parotica via ligaments. The ceratobranchials, hypobranchial plates, and commissurae terminales outline three spaces: Gill slit I between Ceratobranchials I and II, Gill slit II between Ceratobranchials II and III, and Gill slit III between Ceratobranchials III and IV. Spiculae are missing.

By Stage II-3 the branchial baskets expand laterally to become about one-third larger than the ceratohyals (Fig. 1B). However, because of the growth of the otic capsules, the lateral margins of the branchial baskets no longer coincide with those of the otic capsules as they did in the previous stage. At Stage II-4 the anterolateral processes are no longer visible, and the cricoid and arytenoids arise as two pairs of areas of cartilaginous condensation between the proximal portions of Ceratobranchials IV (Fig. 1C). In some of the specimens at this stage (e.g., CHUnB 35000A), there seems to be a foramen for the thyroid artery. By late Stage II-4 the cerato-



**Fig. 1.** Camera lucida drawings of the ventral view of the hyolaryngeal apparatus of cleared-and-stained *Pipa arrabali*. A: Stage II-2 (CHUnB 35027-A); B: Stage II-3 (CHUnB 35055-A); C: Stage II-4 (CHUnB 35000-A); D: Stage II-5 (CHUnB 35045-C); E: Stage II-6 (CHUnB 35019-A); F: Stage II-7 (CHUnB 35030-B).

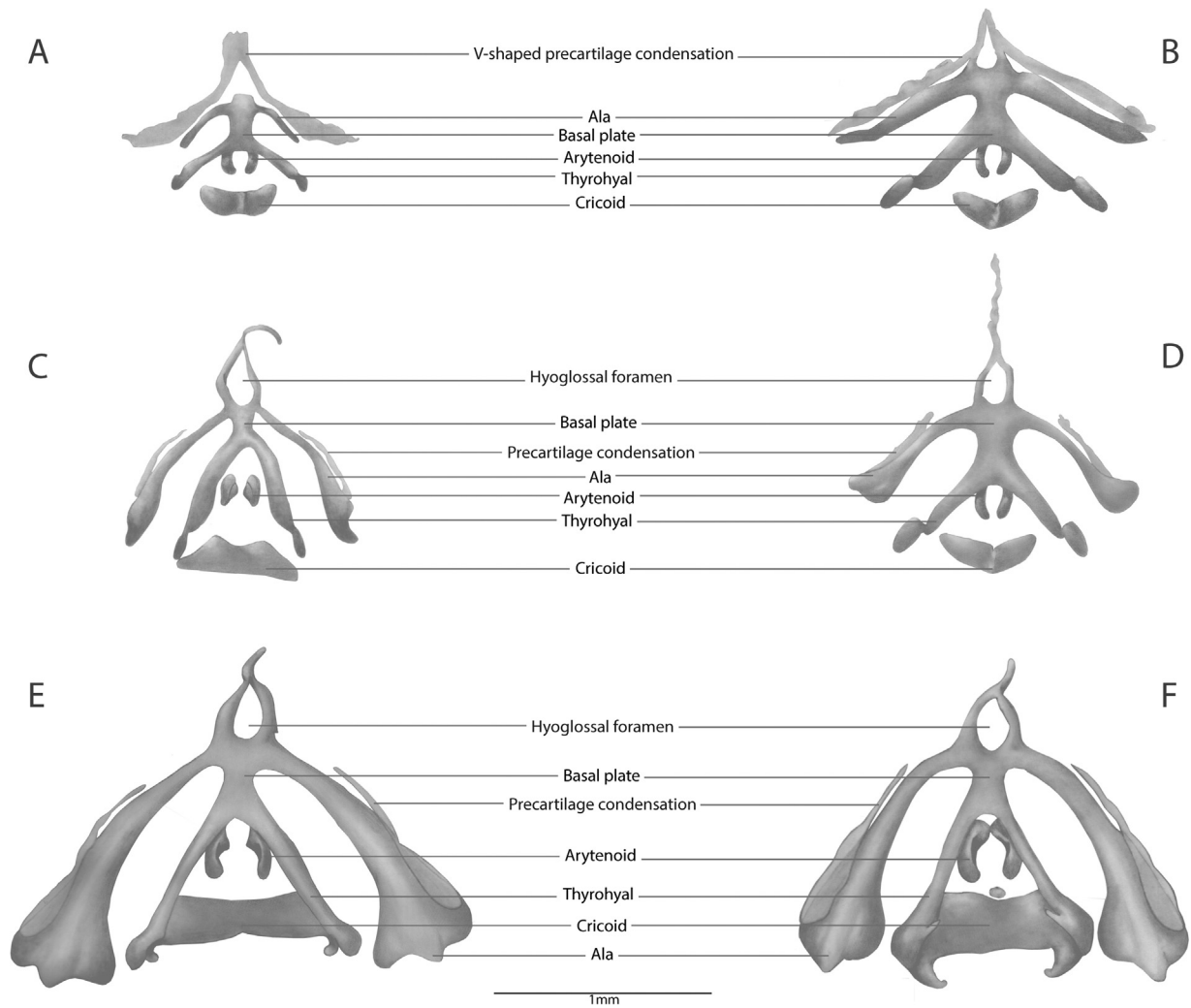
branchials start to lose their original shape and both halves of the cricoid fuse at the midline forming an oval shape.

By Stage II-5 the cartilage of the proximal portion of each ceratohyal is less dense. Also, the ceratobranchials are thinner than in previous stages and have lost the dorsal concavity. Ceratobranchials II and III start to break apart in the medial portion of the bar and the commissurae craniobranchiales are free and distended. The proximal commissurae are flattened and appear at the same level as the hypobranchial plates. The cricoid attains a butterfly-like shape; i.e., two slightly expanded lobes with a constriction in the area of fusion (Fig. 1D).

By Stage II-6 the ceratohyals are highly eroded (Fig. 1E), with only the distal end of each ceratohyal remaining chondrified and still articulating with the palatoquadrate. The medial portion is slender and composed of a low-density tissue that lacks chondrocytes, while the proximal portion has disappeared except for a small piece that remains attached to the anterior end of Copula II. The pars reuniens is no longer visible. The anterior end of Copula II is dorsally curved and free, whereas the posterior portion continues to be fused to the hypobranchial plates. Ceratobranchials II and III have completely disappeared. Ceratobranchials I and IV are now slender and, except for their proximal ends, the bars are composed of partially eroded cartilage. Terminal Commissura III is still present, but already in the process of disintegration. The central portion of the fused hypobranchial plates remains well chondrified; hereafter this structure is called the corpus hyoidei. The arytenoid cartilages remain between the proximal portions of Ceratobranchials IV, but the cricoid becomes somewhat isolated from the rest of the hyolaryngeal skeleton because of the receding branchial baskets.

At Stage II-7 the ceratohyals are no longer visible. Ceratobranchials I and IV have completely disappeared, except for their proximal ends. The vestigial proximal portions of Ceratobranchials I grow posterolaterally to form a pair of rudimentary, vaguely S-shaped hyoid alae. The proximal ends of Ceratobranchials IV also grow posterolaterally to form a pair of straight, cartilaginous thyrohyalia (= posteromedial processes). The distal end of each thyrohyal has a constriction followed by an oval knob. At this stage, the hyoid alae are about one third shorter than the thyrohyalia. The bar-like portion of the corpus hyoidei that connects the hyoid alae and thyrohyalia is called the basal plate. At the anterior margin of the basal plate there is a wide, rounded process that is dorsally concave. This process is the remnant of the posterior end of Copula II, and associated with it is a precartilag condensation in the shape of an inverted V. The cricoid and arytenoids continue to slowly but steadily increase in size (Fig. 1F).

In specimens at early Stage II-9 the V-shaped precartilag condensation has grown and now extends through the entire anterior margin of the basal plate and projects laterally (Fig. 2A and B). The hyoid alae and thyrohyalia are about equal in length. The arytenoid cartilages acquire a bean shaped appearance, with the concavity directed medially, while the anterolateral angles of the cricoid grow anteriorly. By mid-Stage II-9 there is an increase in the overall size of the hyoid alae; they are slightly longer than the thyrohyalia and the lateral end of each one coincides with the lateral terminus of the precartilag condensation. Also, two small, anteriorly directed processes have appeared on the remnant of Copula II. These processes are partially fused to the central portion of the precartilag condensation, thus forming an incipient hyoglossal foramen (Fig. 2C). By



**Fig. 2.** Camera lucida drawings of the ventral view of the hyolaryngeal apparatus of cleared-and-stained *Pipa arrabali*. A: Stage II-9 (CHUnB 35015-A); B: Stage II-9 (CHUnB 35015-B); C: Stage II-9 (CHUnB 35014-A); D: Stage II-9 (CHUnB 35015-C); E: Stage II-11 (CHUnB 35001-B); F: early juvenile (CHUnB 014942-C).

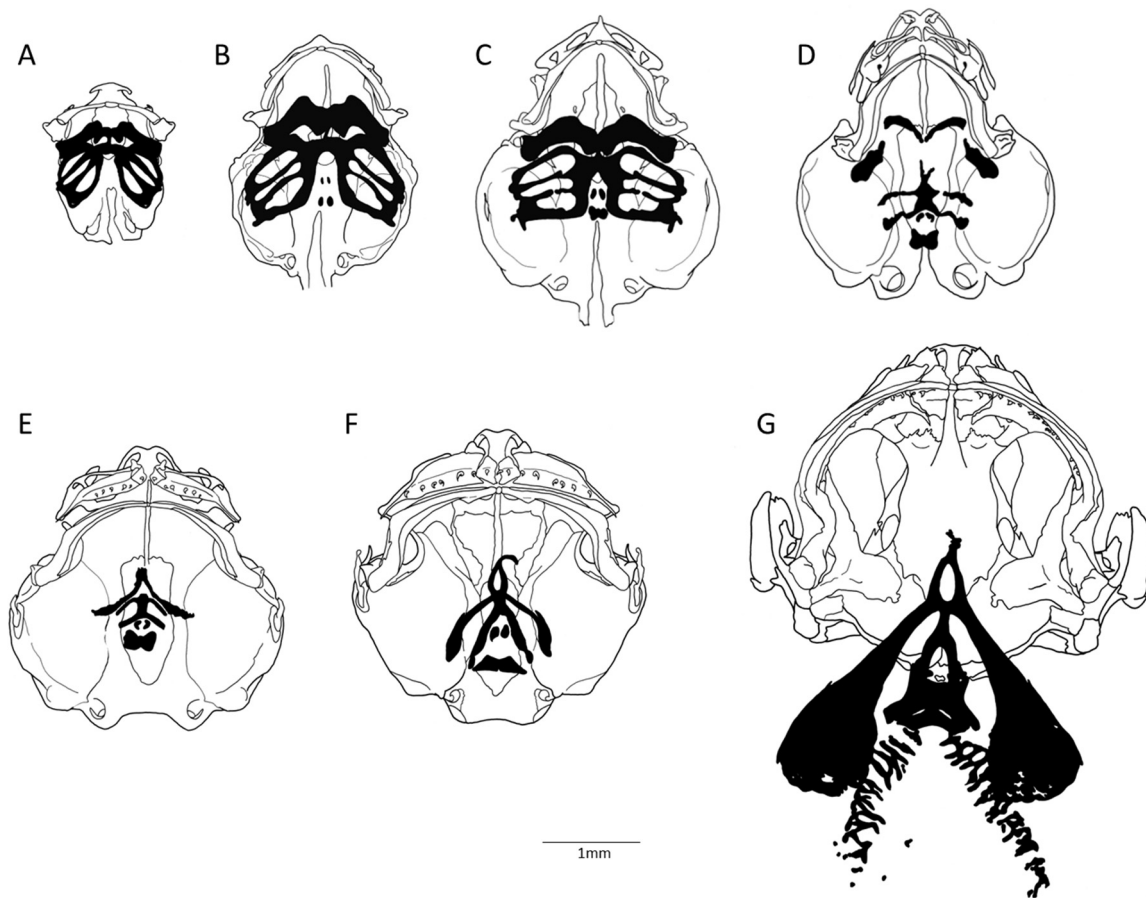
late Stage II-9, the hyoglossal foramen is better defined and encircled by two medially concave rods that fuse anteriorly (2B). Each rod originates from the amalgamation of one of the processes of Copula II and the precartilage condensation, which in this area is partially chondrified by now. The area of union of both rods constitutes the point of origin of a long (about three times the length of the hyoglossal foramen), anteriorly directed process. The precartilage condensation has disappeared from the proximal portion of the hyoid ala but remains attached to the mediolateral margin. Each hyoid ala is distally expanded. In some specimens at late Stage II-9 the rods that encircle the hyoglossal foramen and the process coming off their point of union are completely cartilaginous (Fig. 2D); the latter is a thin, shorter rod (approximately the same length as the hyoglossal foramen) that, depending on the specimen, is curved to the left or to the right. The hyoglossal foramen is teardrop-shaped; its posterior margin is just anterior to the cranial end of the hyoid alae. The precartilage condensation is a strip-like structure present on the mediolateral margin of each hyoid ala; it is difficult to see in some specimens. The distal expansion of the ala is in an oblique direction, because the medial margin is more dorsal than the lateral one, and asymmetrical because the medial margin is longer than the lateral margin. The cricoid is slightly expanded on each posterolateral corner.

In all specimens after Stage II-5, the hyolaryngeal skeleton underlies only a relatively small area between the otic capsules

(Fig. 3) and its morphology remains without major alterations between Stages II-9 and II-10. In Stage II-11 (e.g., CHUnB 35001-B), however, the hyolaryngeal skeleton is about twice as large as in younger specimens (Figs. 2E and 3). Also, each hyoid ala has grown, exceeding now the level of the cricoid, and the distal end is symmetrically expanded with both margins, lateral and medial, more dorsal than the center. Because of the expansion of the medial portion of the distal end of the ala, the former tip of the medial margin is now a blunt process on the posterior margin of the ala. The strip on the anterolateral margin of the ala is slightly expanded distally. The thyrohyals have also grown to surpass the level of the cricoid. The oval knob at the distal end of each thyrohyal, which was first visible in Stage II-7, seems less prominent now because of the increase in width of the constriction. On the medial margin of the thyrohyal, just anterior to the former constriction, there is an incipient process. Also, in this area the thyrohyal shows a slight, lateral curvature. The cricoid is larger than in previous stages; its anterolateral corners are dorsally rounded and the expansions on the posterolateral corners are dorsally curved.

In juveniles (e.g., CHUnB 014942-C), the strip on the anterolateral margin of the hyoid ala has a cotyle on the dorsal surface of its posterior portion that articulates with a condyle on the lateroventral margin of the ala (Fig. 2F). The process on each thyrohyal that had started to form in younger specimens is now well developed and anteroventrally oriented with its lateral curvature more con-





**Fig. 3.** Camera lucida drawings of the hyolaryngeal apparatus (in black) of cleared-and-stained *Pipa arrabali*, showing its position in relation to the skull in ventral view. A: Stage II-2 (CHUnB 35027-A); B: Stage II-4 (CHUnB 35000-A); C: Stage II-5 (CHUnB 35045-C); D: Stage II-6 (CHUnB 35019-A); E: Stage II-9 (CHUnB 35015-A); F: Stage II-9 (CHUnB 35014-A); G: subadult (CFBH 16686).

spicuous (Fig. 2F). A precartilag condensation of the procricoid is visible on the anterior margin of the cricoid cartilage, between the posterior ends of the arytenoids.

In the subadult, the hyoglossal foramen is ovoid (Fig. 4). Some ossification invades the lateral margins of the hyoglossal foramen. The process that comes off the point of union of both bars has irregular margins and is about two-thirds the length of the hyoglossal foramen. The cartilage of the basal plate is almost transparent. The cranial and medial portions of each thyrohyal are ossified whereas the distal portion is cartilaginous and curved laterally just anterior to the small, medially-directed processes; its distal end remains knob-like.

The hyoid alae are large (more than twice the length of the thyrohyalia), flat, and club-shaped (Fig. 4). The proximal area of each ala is partially ossified and the central portion of the expanded area is calcified. The lateral margin of the proximomedial section of each ala is straight and the medial margin is slightly concave. The cartilage in the region of the ala adjacent to the lateral margin, and posterior to the ossification, is thicker than in the region next to the medial margin. The cartilage of the most posterior portion of each ala is extremely thin, almost transparent in some sections and with open spaces in others; because of growth in this area, the blunt process on the posterior margin of the ala of younger specimens is no longer visible. The strip on the anterolateral margin of the ala is ventromedially directed, with a free ventral margin and a dorsal margin entirely fused to the margin of the ala; the cotyle-condyle articulation of younger specimens is still visible but also fused.

The arytenoids have grown anteriorly, with the posterior end of each lying anterior to the anterior margin of the cricoid, and the

anterior end at the level of the contact between the basal plate and the thyrohyals. Also, at the anterior end of each arytenoid the articular facet for the articulation with the other arytenoid is already developed. The cricoid forms a complete ring. In dorsal view, the cricoid is a rectangular piece of cartilage with concave anterior and posterior margins. The anterolateral corners are expanded and dorsally curved. The end of each expansion is located dorsal to the thyrohyal. Medial to each anterolateral corner is an ovoid articular process, which serves for the articulation with the arytenoids. The posterolateral corners are dorsally curved and fused to a V-shaped plate that fits between the distal portions of the thyrohyalia. Anterior to the anterior margin there is an ovoid, cartilaginous procricoid. Cardiac and esophageal processes are absent.

Two sets of bronchial rings extend posterolaterally from the posterolateral corners of the cricoid. These rings are more or less independent from each other and provide support to about two-thirds of the bronchus circumference; the medial area of the bronchus lacks support.

## 4. Discussion

### 4.1. Morphology of the mid-embryonic hyobranchial apparatus

In most anurans, the ceratohyals are bilaterally symmetrical bars of cartilage that underlie the floor of the buccal cavity and establish the main connection between the hyobranchial apparatus and the chondrocranium (Severtsov, 1968; Sokol, 1975; Wassersug and Hoff, 1979). In *Rhinophrynus dorsalis* Duméril and Bibron

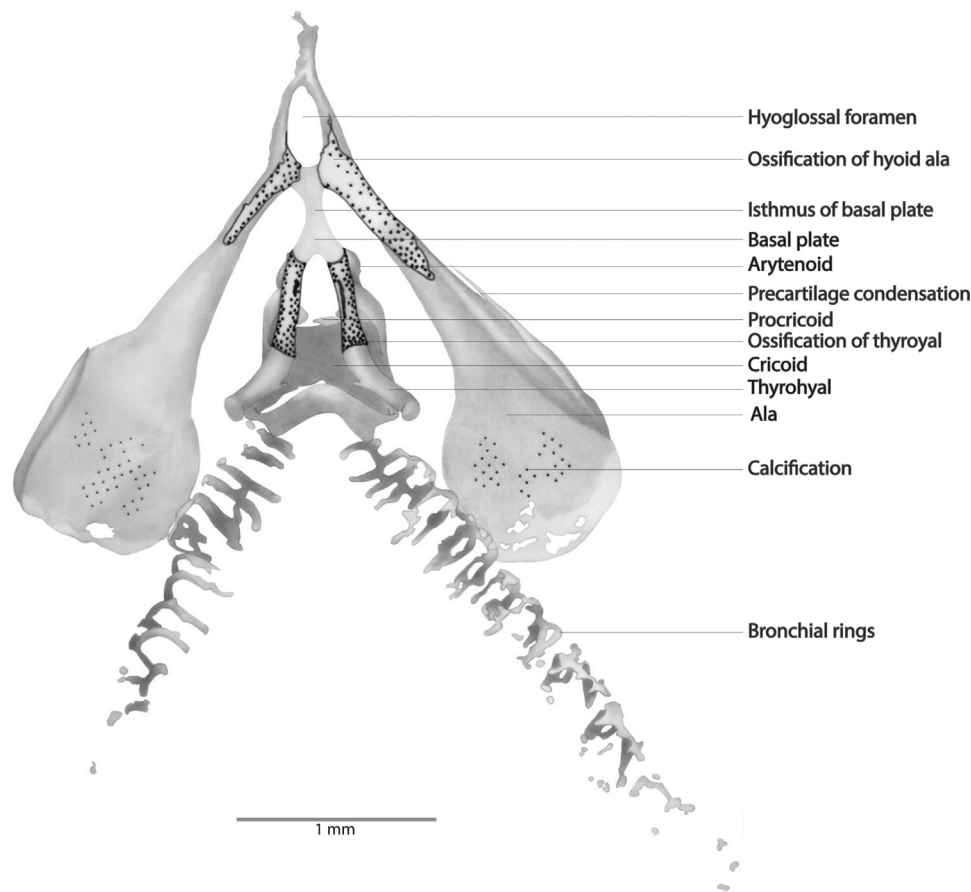


Fig. 4. Camera lucida drawing of the ventral view of the hyolaryngeal apparatus of a subadult cleared-and-stained *Pipa arrabali* (CFBH 16686).

(1841), *Xenopus laevis* (Daudin, 1802), *X. tropicalis* (Gray, 1864), *Pipa carvalhoi*, and *P. parva*, the ceratohyals are robust structures, each with a greatly enlarged anterior process (Sokol, 1975, 1977), whereas the anterolateral process seems to be absent. In contrast, the ceratohyals of *P. arrabali* are delicate and both anterior and anterolateral processes are present, although extremely reduced. Thus, even though the ceratohyals in *P. arrabali* are the largest elements of the hyobranchial apparatus, they underlie only a small portion of the buccal floor (Fig. 3). Alternatively, in other xenoanuran species (except *Hymenochirus*) the ceratohyals underlie the entire buccal floor (Sokol, 1975, 1977).

The ligamentum interhyale, which is located anterior to the pars reuniens of the hyoid arch, may have a chondrified body that has been referred to as the copula anterior, Copula I, or basihyal (Haas, 2003; Sokol 1977; Ridewood, 1896, 1898). This body has varying degrees of chondrification that result in it varying in sizes across taxa. For example, it is particularly large in *Alytes* Wagler, 1830, *Bombina* Oken, 1816, *Discoglossus* Otth, 1837 (Haas, 1997; Maglia and Pugener, 1998; Pugener and Maglia, 1997; Roček, 2003), and *Rhinophrynus* Duméril and Bibron (1841) (Pugener et al., 2003; ; Swart and de Sá, 1999). In other taxa, such as *Ascaphus* Stejneger, 1899, *Pelodytes* Bonaparte (1838), *Rhinella* Fitzinger (1826), *Bufo* Garsault (1764), *Dendrobates* Wagler (1830), *Phyllobates* Bibron in De la Sagra (1840), and *Spea* Cope (1866), Copula I is usually small or absent; when present, it develops in late larval stages and in some cases may even be subject to intraspecific variability (Haas, 1995, 2003). Copula I is absent in *Pipa arrabali*, as it is in other pipids such as *P. carvalhoi*, *P. parva*, *Xenopus laevis*, *X. muelleri* (Peters, 1875), and *X. tropicalis* (Pugener et al., 2003; Roček, 2003). *Hymenochirus* might be the exception within Pipidae because the pars reuniens

has a robust anterior process that could represent a fused Copula I (De Sá and Swart, 1999; Pugener et al., 2003; Sokol, 1969, 1975).

In free-swimming larvae, the pars reuniens usually consist of a flexible, fibrous matrix with sparse groups of small chondrocytes that binds the ceratohyals together (Haas, 1997; Cannatella, 1999). In *Rhinophrynus dorsalis* the pars reuniens seems to be totally chondrified (Swart and de Sá, 1999), whereas other xenoanurans exhibit a variety of conditions for this structure. In *Xenopus* the fibrous matrix seems to be less chondrified (pers. obs.) whereas in *Hymenochirus* the pars reuniens is well chondrified and fused to Copula I (Sokol, 1977). In *Pipa carvalhoi* and *P. parva* the pars reuniens consists of rows of chondrocytes immersed in a collagenous matrix (Sokol, 1977); this condition is relatively similar to that of *Xenopus*. Our observations revealed that the pars reuniens in *P. arrabali* is densely chondrified and indistinguishably fused to the anteromedial margins of the ceratohyals. Although Ridewood (1896) did not discuss this particular structure, based on his figures we think that the same state is present in *P. pipa*. Thus, two aspects of the pars reuniens, in which species of *Pipa*, with and without tadpoles, clearly differ, warrant further investigation: (1) the degree of chondrification and (2) the type of attachment to the ceratohyals.

Copula II connects the ventral ends of the ceratohyal, by means of the pars reuniens, to the hypobranchial plates (Cannatella, 1999; Haas, 1996; Roček, 2003; among others). It has long been assumed that Copula II is absent in *Pipa* (e.g., Haas, 1996; Parker, 1876; Pugener et al., 2003; Roček, 2003; Sokol, 1975, 1977). However, we have found that *Pipa arrabali* does have a Copula II, and, based on an illustration of the hyobranchial apparatus of *P. pipa* by Ridewood (1896), we identified a structure located in the same general position that we think is homologous with Copula II. Therefore, the assumed absence of Copula II in all species of *Pipa* is a misconception.

tion that probably originated from the extrapolation of the state present in *P. carvalhoi* and *P. parva*. These species are unique among anurans because they do indeed lack a Copula II; thus, the hyoid arch is attached to the branchial baskets only by the posteromedian processes of the ceratohyals (Haas, 2003; Roček, 2003; Sokol, 1975, 1977).

Copula II is linked to the branchial baskets by the hypobranchial plate, but this system of connection presents several different configurations (Haas, 1997; Roček, 2003; Sokol, 1975). Copula II separates the two hypobranchial plates in *Ascapheus*, *Alytes*, *Bombina*, and *Discoglossus* (Haas, 1996; Maglia and Pugener, 1998; Ridewood, 1898). In most other taxa, including *Rhinophrynus*, *Xenopus*, *Hymenochirus*, and the species of *Pipa* with free-living tadpoles, the two hypobranchial plates are in contact. However, in *P. arrabali* there is a continuous plate formed by the posterior part of Copula II and the hypobranchial plates. Two possible scenarios may explain this structure; either Copula II extends between both hypobranchial plates separating them throughout their entire medial length or the hypobranchial plates are fused with each other and Copula II is only fused to a portion of the anterior region. In order to have a better understanding of the relationships among these structures further analyses are necessary using, for example, serial histological sections of earlier stages of development.

The urobranchial is a posteriorly directed, transient process located on the ventral surface of Copula II. The larva of *Ascapheus* is the only known anuran tadpole with a bifurcate urobranchial process (Haas, 1997; Pugener et al., 2003). In all other frogs, when present, the urobranchial may be a single, knob-like or elongate structure that does not extend beyond the posterior margin of the hypobranchial plates (e.g., discoglossids), or a worm-like process that extends well beyond the posterior margin of the hypobranchial plates (e.g., some microhylids; Haas, 2003; Pugener et al., 2003). Within pipoids, a long, worm-like urobranchial process is present in *Rhinophrynus dorsalis* (Haas, 2003; Swart and De Sá, 1999) and absent in those *Pipa* species that lack Copula II (Haas, 2003; Pugener et al., 2003; Sokol, 1977). Interestingly, although Copula II is present in *P. arrabali*, the urobranchial process is definitely absent. The occurrence of this structure in other pipids is debatable. Haas (2003), Sedra and Michael (1957) and Sokol (1975, 1977) considered it to be absent whereas Pugener et al. (2003) argued that it might be present as a longitudinal ridge. These contradicting arguments raise an obvious question: is the longitudinal ridge of *Xenopus* and *Hymenochirus* homologous to the urobranchial process of other anurans, or a modification of Copula II to cope with the absence of the urobranchial process? Furthermore, is the urobranchial process really missing in *P. arrabali*, or is its presence so ephemeral that younger stages would be necessary in order to observe it? If the absence of the urobranchial process could be established with certainty in all pipids, then it would represent an important phylogenetic character for the group.

In *Pipa arrabali* each ceratobranchial consists of a slightly curved, thin bar of cartilage with smooth margins. This morphology, which is also observed in *P. pipa* (Parker, 1876), contrasts markedly with the highly complex morphology and overlapping disposition that is characteristic of the ceratobranchials of *Rhinophrynus*, *Xenopus*, and the species of *Pipa* with free-living larvae (Pugener et al., 2003; Sokol, 1977; Swart and de Sá, 1999). Moreover, in these taxa the ventral margins of the ceratobranchials are expanded to form a net of cartilaginous trabeculae that partially occlude the pharyngeal slits (Sokol, 1977). Highly complex, overlapping ceratobranchials may represent a synapomorphy for Xenoanura that has reversed in *P. arrabali* and *P. pipa*. The ceratobranchials of *Hymenochirus*, although reduced to just one pair on each side (De Sá and Swart, 1999; Sokol, 1969), are morphologically similar to those of *P. arrabali* and *P. pipa*. However, this condition may have evolved independently from that of the species of *Pipa* that lack tadpoles.

The size of the branchial baskets represents another conspicuous difference between *Pipa arrabali* and other xenoanurans. The branchial baskets of *P. arrabali* are proportionally smaller (about 40% of the length of the chondrocranium; Fig. 3B) than those of *Xenopus tropicalis* (~85%; Sokol, 1977), *P. carvalhoi* (~66%; Sokol, 1977), and *Rhinophrynus dorsalis* (~85%; pers. obs.). Similarly, the highly derived branchial baskets of *Hymenochirus boettgeri* (Tornier, 1896) are about 70% of the length of the chondrocranium (Sokol, 1977). The tadpoles of *Xenopus* and *Rhinophrynus* are microphagous suspension feeders (Wells, 2007), although *Rhinophrynus* may also feed on large zooplankton (Wassersug, 1973). Similarly, *P. carvalhoi* is a macrophagous suspension feeder (Sokol, 1977) and *Hymenochirus* is an obligate macrophagous carnivore (Wassersug, 1973). The small size of the branchial baskets in *P. arrabali* is probably related to the absence of a free-living larval stage and the absence of feeding during development. These conditions are strikingly similar to those reported for other Neobatrachian species of frogs without free-living tadpoles (e.g., Hanken et al., 1992; Kerney et al., 2007), and may represent a convergent pattern related to this mode of development.

The commissurae proximales are bands of cartilage that connect the four ceratobranchials at their proximal ends, although they may be absent in some species (Haas, 1997). According to Haas (1997), when the proximal commissurae are absent, the proximal ends of the ceratobranchials are usually fused to the hypobranchial plates. In *Pipa arrabali* the Commissura Proximal III is present. In addition, distinct bands of cartilage connect the proximal ends of Ceratobranchials I and II, and II and III. Although these structures are fully fused to the hypobranchial plates, we consider them to be homologous with Commissurae Proximales I and II. Therefore, all three commissurae proximales are present in *P. arrabali*. The commissurae proximales are also present in all other xenoanurans, except *Hymenochirus* (Haas, 1996; Sokol, 1977).

#### 4.2. Morphology of the adult hyolaryngeal apparatus

The adult hyolaryngeal apparatus of Pipidae is markedly different from that of the typical anuran (see Trewavas, 1933). The morphological peculiarities that distinguish it, plus the wealth of differences among taxa within the family, have been an endless source of controversial discussions and studies for over two centuries (e.g., Fermin, 1764; Grönberg, 1894; Henle, 1839; Irisarri et al., 2011; Parker, 1876; Ridewood, 1896; Sedra and Michel, 1957; Trueb and Cannatella, 1982; Trueb, 1992). Some of these peculiar morphologies, namely the presence of a hyoglossal foramen and an enlarged, box-like laryngeal apparatus, constitute synapomorphies of Pipidae (Ridewood, 1896; Sokol, 1969; Cannatella and Trueb, 1988a, 1988b) and are present in *Pipa arrabali*. Likewise, hyalia seem to be absent in all species of *Pipa* (Cannatella and Trueb, 1988a), and probably represent a loss inherited from their common ancestor, whereas the presence of a net like bronchial skeleton is unique to *P. arrabali*.

In most anurans the hyalia are a pair of anterior cartilaginous structures that are a relict of the ceratohyal larval cartilage that develops from the anterolateral corners of the hyoid plate (Gaupp, 1894; Ridewood, 1896, 1898). In *Xenopus* and *Rhinophrynus* the hyalia are present and cartilaginous (Ridewood, 1896; Trueb and Cannatella, 1982; Trueb, 1992), whereas in *Hymenochirus* and *Pseudohymenochirus* they are present but ossified, a feature that is unique among anurans (Irisarri et al., 2011; Ridewood, 1899; Cannatella and Trueb, 1988a, 1988b). Based on their analysis of *Pipa pipa*, Henle (1839) and Ridewood (1896) discussed that although the hyalia are absent, a remnant ceratohyal persists as a piece of cartilage on the adult hyobranchial apparatus. Our observations on *P. arrabali* indicate that no remnants of the ceratohyal persist in the adult (see the results and discussion of the ontogeny of this



structure). Hyalia have been considered absent for the genus *Pipa* in different studies (e.g., Pugener et al., 2003; Cannatella and Trueb, 1988a; Trueb and Messemín, 2000) and our results lend support to this hypothesis. These contradicting observations clearly indicate the need for further ontogenetic studies on the different species of *Pipa*.

The hyoglossal foramen is a fenestra for the passage of the *hyoglossus muscle* located immediately anterior to the basal plate (Ridewood, 1896). It is absent in *Rhinophrynus* and other non-pipid anurans (Trueb and Canatella, 1982; Cannatella and Trueb, 1988a). The hyoglossal foramen is present in *Pipa arrabali* as well as in all other studied pipid taxa, although reduced in *Hymenochirus* and *Pseudohymenochirus* (Cannatella and Trueb, 1988b; Sokol, 1969). Moreover, this feature is considered a synapomorphy of Pipidae (Cannatella and Trueb, 1988a, 1988b; Sokol, 1969).

In anurans, the basal plate is a flat piece of cartilage located anterior to the laryngeal apparatus (Trewavas, 1933). The basal plate of *Xenopus* and *Pipa* is constricted in the middle forming an isthmus (Ridewood, 1896). In some *Pipa* this isthmus may be so narrow that it does not chondrify (Ridewood, 1896; Cannatella and Trueb, 1988a). *Pipa arrabali* has the typical-looking basal plate of other *Pipa* species. In its antero-lateral portion, the basal plate has two wing-like processes that are characteristic of all pipids (Cannatella and Trueb, 1988a, 1988b; Ridewood, 1896, 1899; Trueb and Cannatella, 1982; Trueb, 1992), but in *P. arrabali* these alar processes display some peculiarities that have not been described in other species of *Pipa* nor in other pipids (e.g., areas of calcification, small random fenestrae close to the distal margin of the alae, and “thicker” lateral margin).

The laryngeal apparatus, or voice box, is a triangular, usually cartilaginous capsule (larynx) that is suspended between the posteromedial processes (thyrohyals) (Trewavas, 1933; Duellman and Trueb, 1986). In pipids, the larynx is greatly enlarged compared to other anurans, and consists of a box (showing varying degrees of ossification among species) formed by a large cricoid cartilage and the thyrohyals (which do not take part in the formation of the larynx in non-pipid frogs) (Ridewood, 1896). In *Pipa arrabali*, *P. carvalhoi*, and *P. pipa*, the thyrohyals are associated with the ventral part of the cricoid ring (Grönberg, 1894; Henle, 1839; Ridewood, 1896; Araújo and Pugener, personal observation). The same disposition is found in males of *Xenopus laevis*, whereas in females the thyrohyals are fused to the sides of the laryngeal box (Ridewood, 1896). In *Hymenochirus boettgeri* the thyrohyals are also lateral to, and in close contact with, the laryngeal box, but they are not fused to it (Ridewood, 1899).

In most anurans, the cricoid partially encloses the arytenoid cartilages, which are a pair of shell-like structures that support the glottis and house the vocal cords (Trewavas, 1933; Yager, 1996). It has been suggested that vocal cords are absent in all pipids (Duellman and Trueb, 1986; Ridewood, 1899), even though Ridewood (1896) mentioned their presence (although rudimentary) in females of *Pipa pipa*. In *Hymenochirus boettgeri*, *P. carvalhoi*, *P. pipa*, *Pseudohymenochirus merlini* Chabanaud (1920), *Xenopus borealis* Parker (1936), and *X. laevis* the posterior portions of the arytenoid cartilages form two stout, calcified rods (Irisarri et al., 2011; Rabb, 1960; Ridewood, 1896; Yager, 1996; Araújo and Pugener, personal observation). Ossified arytenoids are absent in young females of *P. pipa* (Breyer and Rudolphi, 1811). In *P. arrabali* the arytenoids are shell-like instead of bar-like, but were lacking in the oldest specimen we examined.

In pipids, the modified arytenoids generate a sharp, metallic click-like sound. The sound is then amplified by the enlarged voice box that serves as an internal vocal sac (Yager, 1992, 1996). The sound production mechanism was described in detail for *Xenopus borealis* (Yager, 1992, 1996), and has also been observed in *Hymenochirus boettgeri* (Rabb and Rabb, 1963), *P. carvalhoi*

(Weygoldt, 1976), *P. pipa* (Rabb, 1960), and *X. laevis* (Müller and Scheer, 1970; Yager, 1996). We are unaware of any studies involving vocalization in *P. arrabali*.

Pipids are unique among anurans by having a bronchial skeleton formed by bronchial rings that provide support for the bronchi (Duellman and Trueb, 1986). In *Pipa arrabali*, the bronchial skeleton forms a loosely connected net that extends for about two-thirds of the lateral portion of the bronchus circumference. In *P. pipa*, the bronchial skeleton consists of loosely connected rings with bifurcated ends that also provide support to the lateral margin of each bronchus; however, they extend for about three quarters of the circumference (Ridewood, 1896). This slight difference could be age-related more than species-related. To the contrary, in *Xenopus laevis* the adult bronchial skeleton consists of a continuous, medial bar of cartilage with rings that extend about half-way around the circumference, but provide support for the lateral margin of the bronchus (Ridewood, 1896). The bronchial skeleton is absent in *Hymenochirus* (Ridewood, 1899).

#### 4.3. Skeletal ontogeny of the hyolaryngeal apparatus

The two most thorough description of the morphology and development of the hyolaryngeal apparatus of *Pipa pipa* (Parker, 1876; Ridewood, 1896) showed several contradictory results. It is possible that these contradictory results are a consequence of different observation techniques, or even just a matter of semantics. Nevertheless, the findings of Ridewood (1896) coincide more closely with our observations on *P. arrabali*, although several differences can still be pointed out. For example, according to Ridewood (1896), the basal plate of the adult *P. pipa* is a residual larval cartilage—formed by the fusion of Copula II and hypobranchial plates—that during development lies between the enlarged thyroid foramina. In *P. arrabali* the basal plate has a similar larval origin, but the thyroid foramina do not increase in size. In this species, erosion of Ceratobranchials II and III and Commissurae Proximales I and II generates a couple of deep, lateral notches that engulf the minute thyroid foramina and creates the lateral margins of the isthmus. In *Xenopus laevis* the basal plate is also a cartilage of larval origin that persists in the adult, whereas the thyroid foramen seems to have a similar fate than that described for *P. arrabali* (based on our interpretation of the illustrations provided by Sedra and Michael, 1957).

In *Pipa arrabali* the ceratohyals are completely eroded and a *de novo*, V-shaped condensation of precartilaginous mesenchyme grows on the anterior margin of the hyobranchial plate and the hyoid alae. As development proceeds, the medial portion of the precartilaginous condensation fuses to a couple of small, anteriorly directed processes of Copula II, giving rise to the anterior and lateral margins of the hyoglossal foramen. Ridewood's (1896) observations on *P. pipa* agree in part with ours; according to him, the hyoglossal foramen forms by the chondrification of a bar that connects the proximal ends of the ceratohyals, a process that seems to be more or less concomitant with the erosion of the medial and distal portions of the ceratohyals. On the other hand, Parker (1876) described the origin of the hyoglossal foramen as an opening that perforates Copula II. In *Xenopus laevis*, the anterior erosion of the hypobranchial plates gives origin to the hyoglossal sinus which, in subsequent stages, is bounded anteriorly by a procartilaginous condensation that later becomes cartilage (Sedra and Michael, 1957). Ridewood (1896, 1899) described the formation of the anterior margin of the hyoglossal foramen in *X. laevis* as a coalescence of the right and left basal plates.

In *Pipa arrabali*, the medial portion of the ceratobranchials is the one that erodes first during ontogenetic development. On the contrary, Ridewood (1896) noted that in *P. pipa* the first elements to disintegrate were the commissurae terminales (beginning with



Terminal Commissura I, then II, and finally III), and that the erosion of the ceratobranchials followed a distal to proximal progression. The condition reported for *Xenopus laevis* is similar to our findings for *P. arrabali*; that is, the initial stages of erosion are more evident in the ceratobranchials than in the commissurae terminales (Sedra and Michael, 1957). Comparisons with non-pipid anurans are difficult because there are so few publications dealing with the skeletal ontogeny of the hyobranchial apparatus; however, those few accounts seem to indicate a great variability in the process of erosion of the branchial baskets. For example, in *Spea multiplicata* (Cope, 1863) erosion of these elements follows an anterior to posterior progression, beginning with the commissurae proximales and the proximal portions of the ceratobranchials (Banbury and Maglia, 2006), whereas in *Ceratophrys cornuta* (Linnaeus, 1758) both the commissurae proximales and terminales erode first (Wild, 1997).

Parker (1976) described the hyoid alae of the adult hyoid skeleton of *Pipa pipa* as being formed by the fusion of Ceratobranchials I and II. Ridewood (1896), on the other hand, suggested that the hyoid alae formed as a secondary outgrowth of cartilage that originated at the anterolateral margins of the basal plate. This account can be confirmed by our observations on *P. arrabali*, although a slight difference is worth noting. Ridewood (1896) described the point of origin of the hyoid ala as being anterior to the level of Ceratobranchial I, whereas our observations clearly indicate that in *P. arrabali* it starts to grow from the vestigial proximal portions of Ceratobranchial I. A similar developmental pattern seems to be present in *Spea multiplicata* (Banbury and Maglia, 2006). To the contrary, in *Xenopus laevis* the hyoid alae are *de novo* structures that develop from the lateral margins of the fused Copula II and the hypobranchial plates (Sedra and Michael, 1957).

In *Pipa arrabali* the proximal ends of Ceratobranchials IV (the only portions that persist) grow posterolaterally to form a pair of straight, cartilaginous thyrohyalia. In *Pipa pipa*, Ridewood's (1896) observations agree with ours, whereas Parker (1876) suggested that the formation of the thyrohyals occurred by fusion of Ceratobranchials III and IV. In *Xenopus laevis*, the thyrohyals emerge as bars of cartilage that grow from the posterior margins of the hypobranchial plates, medially to the eroding branchial baskets (Ridewood, 1896; Sedra and Michael, 1957). In *Spea bombifrons*, Wiens (1989) described the thyrohyals as originating from the posteromedial margins of the hyoid plate. This mode of development is coincident with that described for *Xenopus laevis* (Ridewood, 1896; Sedra and Michael, 1957).

In *Pipa arrabali*, the components of the adult hyoid apparatus are formed *de novo* around the only larva-derived element that persists, the basal plate. The developing hyoid apparatus of *P. arrabali* seems to be the result of past events of pre-displacement and acceleration (*sensu* Alberch et al., 1979). In most of the anurans studied so far, such as *P. carvalhoi* (Araújo and Pugener, personal observation), *Xenopus laevis* (Ridewood, 1896; Sedra and Michael, 1957), *Pelobates* (Roček, 1981), *Rana temporaria* (De Jongh, 1968), and *Spea multiplicata* (Banbury and Maglia, 2006), the structural remodeling of the hyobranchial apparatus is set in motion at the onset of, and in some cases even beyond the termination of, metamorphosis. Our findings indicate that in *P. arrabali* such remodeling occurs earlier in development (by comparing to, for example, the cranial ontogeny of this species – Fig. 3). This may be the result of the lack of a free-swimming larva in *P. arrabali*. In order to go through metamorphosis inside of the brood chambers formed by the dorsal skin of females, the embryos have a small space to develop and grow, and do not feed or swim. It is possible that some of the steps of development may have been deleted and/or there is acceleration in the development relative to other frogs without free-swimming tadpoles (e.g., Callery et al., 2001; Downie et al., 2004; Elinson, 2001; Lynn, 1942).

All species of *Pipa* share an adult hyoid apparatus distinct from those of other anurans (Cannatella and Trueb, 1988a; Parker, 1876; Ridewood, 1896; Trueb, 1996), although the morphology at earlier stages (larval) the hyobranchial is quite similar to that in other anuran tadpoles. This may indicate that either different ontogenetic routes can generate similar adult skeletons, or that the development of juvenile and adult structures are decoupled, in which case the apparent sequence of stages may represent a more complex process where erosion and reassembly may not necessarily explain those events. It is possible that the techniques used to study the ontogenetic process (clear-and-stained whole specimens) have limits in gathering evidence and that another level of investigation, perhaps using a combination of histology, histochemistry, and gene expression, may be necessary to answer this question.

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