

Comparative morphology of pond, stream and phytotelm-dwelling tadpoles of the South American Redbelly Toads (Anura: Bufonidae: *Melanophryniscus*)

DIEGO BALDO^{1*}, FLORENCIA VERA CANDIOTI², BELÉN HAAD², FRANCISCO KOLENC³, CLAUDIO BORTEIRO³, MARTÍN O. PEREYRA⁴, CAROLINE ZANK⁵, PATRICK COLOMBO⁶, MARCOS R. BORNSCHEIN^{7,8}, FLAVIA NETTO SISA⁹, FRANCISCO BRUSQUETTI^{9,10}, CARLOS E. CONTE^{11,12}, PAULO NOGUEIRA-COSTA¹³, PATRICIA ALMEIDA-SANTOS¹⁴ and MARCIO R. PIE^{7,8,11,15}

¹Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-UNaM), Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones, N3300LQF Posadas, Argentina

²CONICET – Instituto de Herpetología, Fundación Miguel Lillo, 4000 Tucumán, Argentina

³Sección Herpetología, Museo Nacional de Historia Natural, 11000 Montevideo, Uruguay

⁴CONICET – División Herpetología, Museo Argentino de Ciencias Naturales ‘Bernardino Rivadavia’, 1405 Buenos Aires, Argentina

⁵LAZA Serviços de Engenharia e Meio Ambiente Sociedade Simple, 91180-380 Porto Alegre, Brazil

⁶Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais, Universidade Federal do Rio Grande, 96203-900 Rio Grande, Brazil

⁷Programa de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas, Universidade Federal do Paraná, 81531-980 Curitiba, Brazil

⁸Mater Natura – Instituto de Estudos Ambientais, 80250-020 Curitiba, Brazil

⁹Instituto de Investigación Biológica del Paraguay, 1429 Asunción, Paraguay

¹⁰Departamento de Zoologia, Instituto de Biociências, UNESP–Universidade Estadual Paulista, 13506-970 Rio Claro, Brazil

¹¹Departamento de Zoologia, Centro Politécnico, Universidade Federal do Paraná, 81531-980 Curitiba, Brazil

¹²Instituto Neotropical: Pesquisa e Conservação, 81531-980 Curitiba, Brazil

¹³Museu Nacional, Departamento de Vertebrados, Universidade Federal do Rio de Janeiro, 20940-040 Rio de Janeiro, Brazil

¹⁴Laboratório de Vertebrados, Departamento de Ecologia, Universidade do Estado do Rio de Janeiro/ UERJ, 20550-013 Rio de Janeiro, Brazil

¹⁵Laboratório de Dinâmica Evolutiva e Sistemas Complexos, Departamento de Zoologia, Centro Politécnico, Universidade Federal do Paraná, Jardim das Américas, 81531-990 Curitiba, Brazil

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We present a comprehensive review of larval morphology in the Neotropical toad genus *Melanophryniscus*. The taxa studied included 23 species with representatives of recognized phenetic groups and different larval ecomorphological guilds: pond, stream, and phytotelm-dwelling tadpoles. Their external morphology variation is congruent with current phenetic arrangement based on adult features, but also reflects the habitat where larvae develop. Lotic tadpoles (i.e. *M. tumifrons* group and *M. krauczuki*) in general exhibit a more depressed body, a longer tail with lower fins, and larger oral discs than lentic forms (i.e. *M. stelzneri* group, *M. moreirae*,

*Corresponding author. E-mail: diegobaldo@gmail.com

M. sanmartini, and *M. langonei*). Despite their peculiar, confined microhabitat, phytotelm larvae do not diverge markedly from non-arboreal species. The distinctive features of all species are the presence of a pineal end organ and the placement of the intestinal reversal point at the left of the abdomen in typical larval stages. The buccal cavity and musculoskeletal anatomy are quite conserved between species, yet some characteristics differ from those of other bufonids. The presence of one pair of subhyoid muscles is apparently an exclusive trait of *Melanophryniscus* among Bufonidae. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **112**, 417–441.

ADDITIONAL KEYWORDS: amphibian – buccal cavity – chondrocranium – cranial muscles – hyobranchial skeleton – lentic tadpoles – lotic tadpoles – phytotelm tadpoles – South America.

INTRODUCTION

Melanophryniscus is a Neotropical genus of small- to medium-sized toads that are characterized by conspicuous aposematic colour patterns. At this time, this taxon is composed of 26 nominal species that are distributed east of the Andes, from central Argentina to Espírito Santo in Brazil (Frost, 2014). These toads inhabit diverse biomes that include large open areas that are dominated by grassland (e.g. pampas and Uruguayan savanna), extensive woodland savannas (e.g. Chaco and Cerrado), and subtropical rainforests (e.g. Atlantic Forest and Yungas). The reproduction of these species is explosive; most of them are diurnal and breed in temporary ponds or temporary streams. Recently, a new reproductive mode was described for the genus, and two species were found to breed in the water that accumulates in leaf axils of *Eryngium* spp. and bromeliads (Langone *et al.*, 2008; Steinbach-Padilha, 2008). Although phytotelm dwelling is not rare in tadpoles, and several anuran families include species with this reproductive mode (Lehtinen, Lannoo & Wassersug, 2004a), there are only a few bufonids known to exhibit this property; the report by Langone *et al.* (2008) is the first for the genus *Melanophryniscus*.

During the last few decades the taxonomic knowledge on the genus has increased significantly, but this situation has not been accompanied by a comprehensive understanding of other areas of their biology. Larval forms have been described formally in nine species (see Appendix S1), but aspects of larval anatomy have been reported for only four of them (Echeverría, 1992; Haas, 2003; Larson, de Sá & Arrieta, 2003). The broad morphological and ecological variation in adult stages foresees a comparable diversity in larval development and biology once more taxa have been revised.

Melanophryniscus has been recovered recurrently as the most basal lineage of the Bufonidae (e.g. Frost *et al.*, 2006; Van Bocxlaer *et al.*, 2010; Pyron & Wiens, 2011; Peloso *et al.*, 2012), and its monophyly, although still not tested rigorously, is suspected based on to

available morphological, biochemical, behavioural, and molecular evidences (McDiarmid, 1971; Larson *et al.* 2003; Daly *et al.*, 2008; Toledo, Sazima & Haddad, 2011; Peloso *et al.*, 2012). Unfortunately, an inclusive phylogenetic analysis to assess internal relationships within the genus is lacking. The intrageneric phenetic grouping followed by different authors recognizes three groups on the basis of morphological characters and colouration patterns (Cruz & Caramaschi, 2003), as follows:

1. The *Melanophryniscus tumifrons* group (sensu Cruz & Caramaschi, 2003; Fig. 1A) currently contains eight species (see Appendix S1 for a full list of species and their authorships), and there is morphological and chromosomal evidence supporting this grouping. Baldo & Basso (2004) proposed the frontal swelling (frontal macrogland) as a putative synapomorphy, and recently Baldo *et al.* (2012) have suggested two additional chromosomal synapomorphies. All known tadpoles of this species group live in temporary streamlets (Prigioni & Langone, 1990; Santos *et al.*, 2010; Dos Santos *et al.*, 2011; Fig. 1B).
2. The *Melanophryniscus stelzneri* group is poorly defined and integrated by species that are characterized by the absence of a frontal swelling, presence of corneous spines on shallow warts or directly on skin, and a conspicuous colour pattern of contrasting spots or blotches on dorsum (sensu Cruz & Caramaschi, 2003; Fig. 1C). While its monophyly has not been tested properly, some cytogenetic characters support this group (Baldo *et al.*, 2012). As defined originally (Cruz & Caramaschi, 2003) and with subsequent additions, the *M. stelzneri* group includes ten species (Appendix S1). All these species have tadpoles that develop in small temporary ponds (Bustos Singer & Gutiérrez, 1997; Lavilla & Vaira, 1997; Cairo, Zalba & Úbeda, 2008; Fig. 1D). *Melanophryniscus krauczuki*, an endemic form from northeastern Argentina and adjacent areas in Paraguay (Baldo & Basso, 2004), was included in the *M. stelzneri*

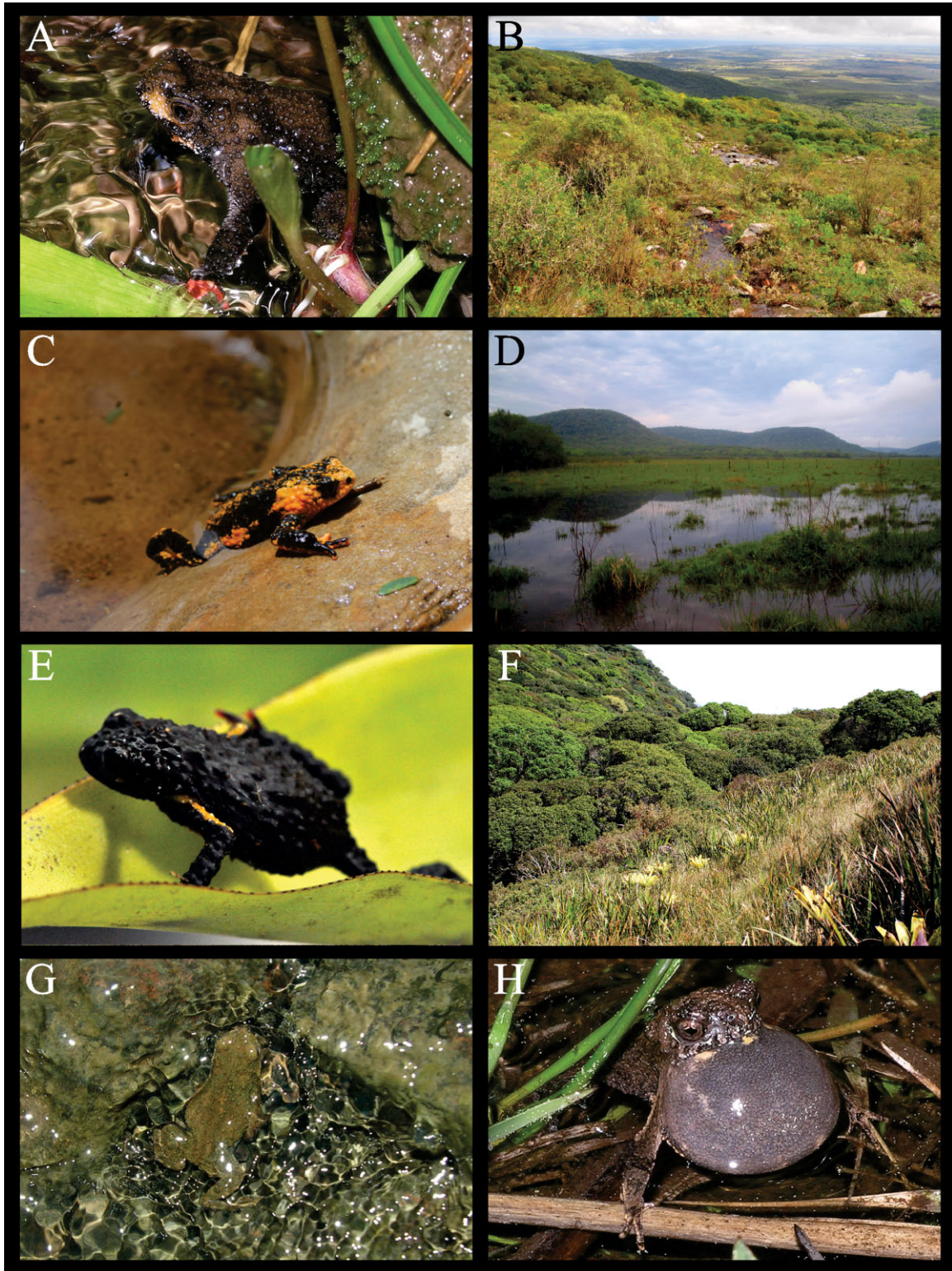


Figure 1. A, male of *Melanophryniscus* sp. 2 aff. *pachyrhynus* in their calling site in a temporary streamlet. B, a temporary streamlet, breeding sites of *M. pachyrhynus*. C, male of *M. rubriventris* in a temporary pond. D, a temporary pond-breeding site of *M. atroluteus*. E, male of *M. alipioi* in a terrestrial bromeliad. F, grassland with terrestrial bromeliads in the Atlantic Rain Forest, breeding habitat of *M. alipioi*. G, amplexant pair of *M. krauczuki* in a temporary streamlet. H, male of *M. sanmartini* calling in a temporary small pond.

group in the original description, but recent chromosomal evidence suggests that it does not belong to this group (Baldo *et al.*, 2012).

3. The *Melanophryniscus moreirae* group (sensu Cruz & Caramaschi, 2003; Fig. 1E) was composed originally of two species from southern Brazil and Uruguay, and later a second Uruguayan species was associated with it (Maneyro, Naya & Baldo, 2008). An unpublished molecular analysis, however (which we will follow here, D. Baldo *et al.*, unpubl. data), recovered a clade that included the nominal pond species *M. moreirae* plus five phytotelm-breeding taxa, three of which are still undescribed (Appendix S1; Fig. 1F).

Finally, larval habitats are variable in those species that are unassigned to groups. Tadpoles of *Melanophryniscus krauczuki* inhabit temporary streamlets that run over basaltic beds (Baldo & Basso, 2004; Fig. 1G), whereas the tadpoles of the two Uruguayan species formerly in the *M. moreirae* group, *M. sanmartini* and *M. langonei*, inhabit small ponds (Maneyro *et al.*, 2008; Fig. 1H).

In this study we provide a comparative description of larvae from 23 species of *Melanophryniscus*, including the external, buccal cavity, and musculoskeletal morphology. Seventeen of these 23 species are formally recognized taxa and, additionally, larval stages of six more potential new species are presented for the first time. Results are discussed based on the abovementioned intrageneric grouping and ecomorphological patterns.

MATERIAL AND METHODS

We revised tadpoles from 23 species of *Melanophryniscus* that include representatives of the three intrageneric groups (six species of the *M. moreirae* group, six of the *M. stelzneri* group, and eight of the *M. tumifrons* group) plus three species still not assigned to groups. Appendix S1 gives a full list of species of the genus, intrageneric groups, species addressed in this work, and nine species whose larval forms remain unknown. Vouchers are housed at the herpetological collections of: Fundación Miguel Lillo, San Miguel de Tucumán, Argentina (FML); Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical, CONICET-UNaM, Posadas, Argentina (LGE); Museo de La Plata, La Plata, Argentina (Diego Baldo Collection, MLP DB); Departamento de Zoología, Universidade Federal do Paraná, Curitiba, Brazil (DZUP); Museu de História Natural Capão da Imbuia, Curitiba, Brazil (MHNCI); Museu de Ciências e Tecnologia of the Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil (MCP); Museu Nacional, Rio de

Janeiro, Brazil (MNRJ); Instituto de Investigación Biológica del Paraguay, Asunción, Paraguay (IIBP-H); Museo Nacional de Historia Natural, Montevideo, Uruguay (MNHN); and Colección de Vertebrados, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay (ZVCB).

Larvae were collected in the field between 1998 and 2012, euthanized with the anaesthetic MS222 and preserved in 10% formalin. Gosner (1960) Stages 29–37 were considered (excepting two tadpoles of *Melanophryniscus vilavelhensis* Stages 38/39) and standard characteristics for tadpole external morphology descriptions were scored (Altig & McDiarmid, 1999a).

Twenty morphometric variables were registered based on descriptions in Lavilla & Scrocchi (1986) and Altig & McDiarmid (1999a): (TL) total length; (BL) body length; (TAL) tail length; (BMW) body maximum width; (BWN) body width at nostrils; (BWE) body width at eyes; (BMH) body maximum height; (MTH) maximum tail height; (TMH) tail muscle height; (RSD) rostro-spiracular distance, measured horizontally from the tip of the snout to the posterior edge of the spiracular tube; (FN) fronto-nasal distance, from the tip of the snout to the anterior edge of nostrils; (EN) eye-nostril distance, from the posterior edge of nares to the anterior edge of eyes; (N) nostril major axis; (IND) internarial distance, measured between the internal edges of nares; (END) extranarial distance, measured between the external edges of nares; (E) eye diameter; (IOD) interorbital distance measured between the external edges of pupils; (OD) oral disc width; (DG) dorsal gap length; and (VG) ventral gap length.

A sample of tadpoles was prepared for internal morphology examination. The buccopharyngeal cavities of one to three specimens per species were dissected and stained with methylene blue in accordance with the method described in Wassersug (1976a). Additionally, specimens of nine species (i.e. *Melanophryniscus alipioi*, *M. atroluteus*, *M. klappenbachi*, *M. krauczuki*, *M. macrogranulosus*, *M. rubriventris*, *M. sanmartini*, and *M. simplex*) were prepared for scanning electron microscopy (SEM) and observed and photographed with JEOL CF35 and JEOL 6480LV microscopes. A second subset of one to five larvae per species was cleared and stained for bone and cartilage observation (modified in accordance with Wassersug, 1976b, using trypsin instead of KOH for tissue digestion). Only in two taxa (i.e. *M. vilavelhensis* and *M. sp. 3* aff. *vilavelhensis*) did the scarcity of specimens or the developmental stage impede anatomical studies. All dissections, descriptions, and drawings were made using a stereomicroscope that was equipped with a camera lucida. Buccal terminology is that proposed by Wassersug (1976a),



Figure 2. External morphology. Dorsal, ventral, and lateral views of tadpoles of eight species of *Melanophryniscus tumifrons* group: A, *M. cambaraensis* St. 36. B, *M. devincenzii* St. 33. C, *M. macrogranulosus* St. 31. D, *M. pachyrhynchus* St. 35. E, *M. simplex* St. 33. F, *M. aff. spectabilis* St. 30. G, *M. sp. 1 aff. devincenzii* St. 35. H, *M. sp. 2 aff. pachyrhynchus* St. 37. Scale bars = 2 mm.

and musculoskeletal nomenclature follows that of Haas (2003), with English names when available.

RESULTS

EXTERNAL MORPHOLOGY

We describe the larval external morphology of 23 species of *Melanophryniscus* altogether, highlighting interspecific variation. We also re-examine species that have been already described in literature (see Appendix S1), and include further descriptions when pertinent. For comparative purposes, we provide photographs and oral disc schemes of all tadpoles analysed: external morphology (Fig. 2: *M. tumifrons* group; Fig. 3: *M. stelzneri* group; Fig. 4: *M. moreirae* group; Fig. 5: species not assigned to group), oral disc illustrations (Fig. 6), and SEM micrographs of a typical oral disc and varied labial teeth (Fig. 7). Appendices S2 and S3 summarize comparative results of registered measurements.

The body shape in *Melanophryniscus* tadpoles in the dorsal view was oval (*M. stelzneri* group, *M. vilavelhensis*, *M. langonei*, and *M. sanmartini*) or slightly oblong (*M. tumifrons* group, remaining species of the *M. moreirae* group, and *M. krauczuki*), with the maximum width behind the eyes or at the abdominal region (intraspecifically variable). The body was slightly depressed, usually with a rounded snout in dorsal view (most species), sometimes truncated (*M. cambaraensis*, *M. macrogranulosus*,

M. simplex, *M. aff. spectabilis*, and *M. krauczuki*), and rounded in lateral view (most species) or slightly truncated (*M. simplex*, *M. aff. spectabilis*, *M. sp. 1 aff. devincenzii*, and species of the *M. moreirae* group). The ventral body contour was flat or somewhat concave at the gular and branchial regions, and convex at the abdominal region.

The eyes were medium to large sized (15–35% of the body width at eye level, higher values in phytotelm species), and dorsal to dorsolateral (interorbital distance about 25–50% of the body width at eyes, lower values in *Melanophryniscus devincenzii*, *M. macrogranulosus*, *M. aff. spectabilis*, and *M. krauczuki*). Nostrils were small (about 10% of the body width at nare level), dorsal to dorsolateral (internarial distance 30–40% of the body width at nares, lower values in species of the *M. stelzneri* group), rounded, visible in dorsal, lateral, and frontal views, with a slightly elevated rim in all species but *M. sp. 3 aff. vilavelhensis*. Nostrils were usually closer to the eyes than to the tip of the snout (most of the species), at half distance (*M. alipioi*, *M. langonei*, *M. moreirae*, and *M. sp. 3 aff. vilavelhensis*) or more close to the snout (*M. cambaraensis*, *M. klappenbachi*, *M. rubriventris*, *M. sanmartini*, *M. stelzneri*, and *M. sp. 2 aff. pachyrhynchus*). The pineal end organ was evident as a pigmentless spot between the anterior edges of the eyes in all species but *M. langonei*, *M. moreirae*, and some specimens of *M. aff. spectabilis*.

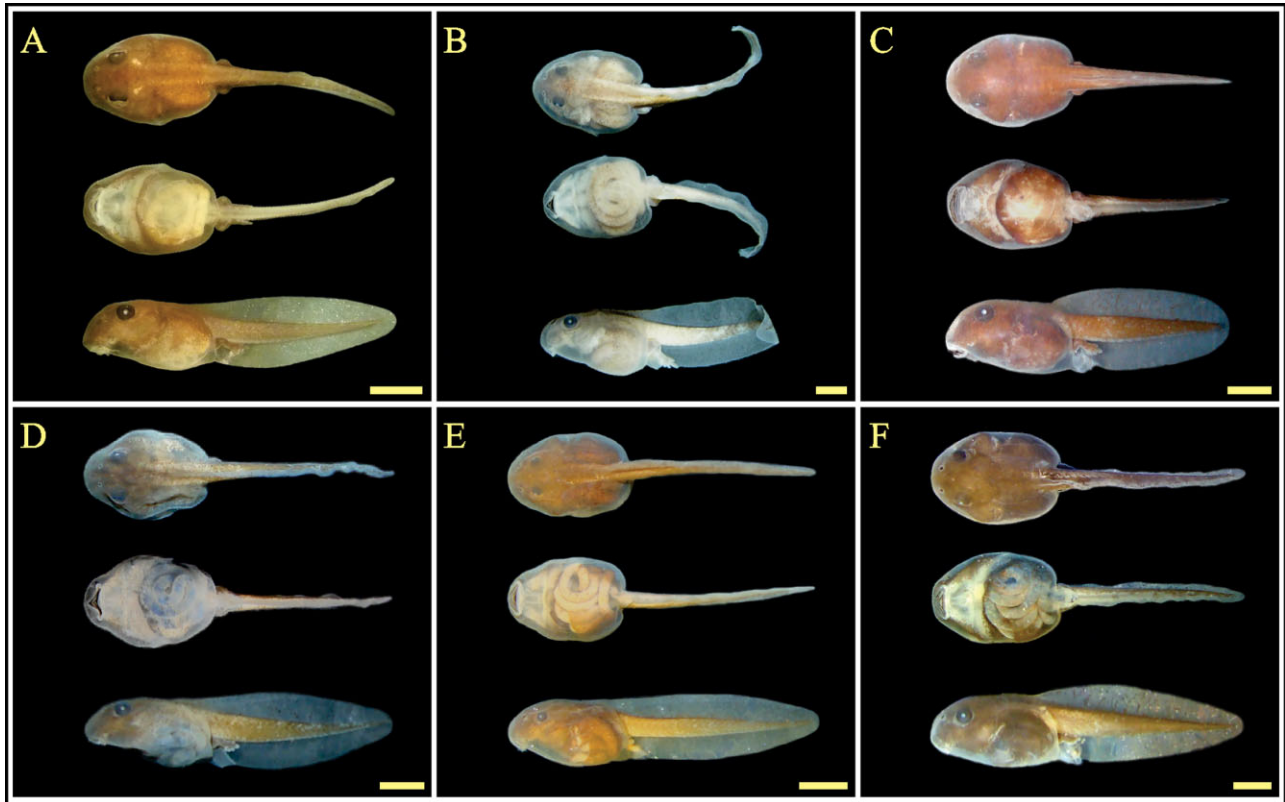


Figure 3. External morphology. Dorsal, ventral, and lateral views of tadpoles of six species of *Melanophryniscus stelzneri* group: A, *M. atroluteus* St. 37. B, *M. klappenbachi* St. 37. C, *M. montevidensis* St. 36. D, *M. paraguayensis* St. 35. E, *M. rubriventris* St. 30. F, *M. stelzneri* St. 34. Scale bars = 2 mm.

The spiracle was sinistral, very short, placed at the posterior half of the body (rostro-spiracular distance about 50–70% of the body length) and directed posterodorsally, with the inner wall free at its end (*Melanophryniscus atroluteus*, *M. devincenzii*, *M. macrogranulosus*, *M. montevidensis*, *M. paraguayensis*, *M. sanmartini*, *M. stelzneri*, *M. sp. 1* aff. *devincenzii*, *M. sp. 2* aff. *pachyrhynus*, and some specimens of *M. pachyrhynus*) or fused to the body wall (remaining species and some specimens of *M. pachyrhynus*). The spiracular opening was oval, with its major axis oriented dorsoventrally and smaller than that of the tube, visible from lateral and dorsal views. The intestinal reversal point ('point de rebroussement', sensu Hourdry & Beaumont, 1985) was placed at the left of the abdomen, slightly more centred in *M. alipioi* and *M. sp. 3* aff. *vilavelhensis*. The vent tube was conical, reached the margin of ventral fin, and ran along its right side; the vent opening was dextral (most species) or medial (*M. klappenbachi*, *M. paraguayensis*, and some specimens of *M. montevidensis*).

The tail was straight and comprised 57–67% of the total length (higher values in *Melanophryniscus*

cambaraensis, *M. devincenzii*, *M. krauczuki*, and *M. sp. 3* aff. *vilavelhensis*), and was of equal height than the body or even taller (most species), but sometimes slightly lower (*M. devincenzii*, *M. krauczuki*, *M. macrogranulosus*, *M. pachyrhynus*, *M. simplex*, and *M. sp. 2* aff. *pachyrhynus*). Caudal musculature presented evident myosepts in the whole tail extension, becoming gradually narrower towards the tail tip, and not reaching it. The dorsal fin originated on the body (most species of the *M. stelzneri* group, and *M. aff. spectabilis*) or at the body–tail junction (*M. atroluteus*, most species of the *M. tumifrons* group, the *M. moreirae* group, and species not assigned to groups). It reached its maximum height at about half its length in species of the *M. stelzneri* group, *M. moreirae*, and *M. aff. spectabilis*, but it was almost uniformly high in the remaining species. In *M. alipioi*, *M. sp. 3* aff. *vilavelhensis*, *M. sp. 4*, and *M. sp. 5* it abruptly became tall and then parallel to the tail axis until the tip. The ventral fin originated on the left of the vent tube and its height was almost uniform. The tip of the tail was rounded, wider in *M. sp. 3* aff. *vilavelhensis* and *M. sp. 4*. Lateral lines were visible with a stereomicroscope at usual



Figure 4. External morphology. Dorsal, ventral, and lateral views of tadpoles of three species of *Melanophryniscus* *moreirae* group: A, *M. alipioi* St. 37. B, *M. moreirae* St. 30. C, *M. vilavelhensis* St. 38, 39. D, *M. sp. 3* aff. *vilavelhensis* St. 30. E, *M. sp. 4* St. 37. F, *M. sp. 5* St. 37–38. Scale bars = 2 mm.

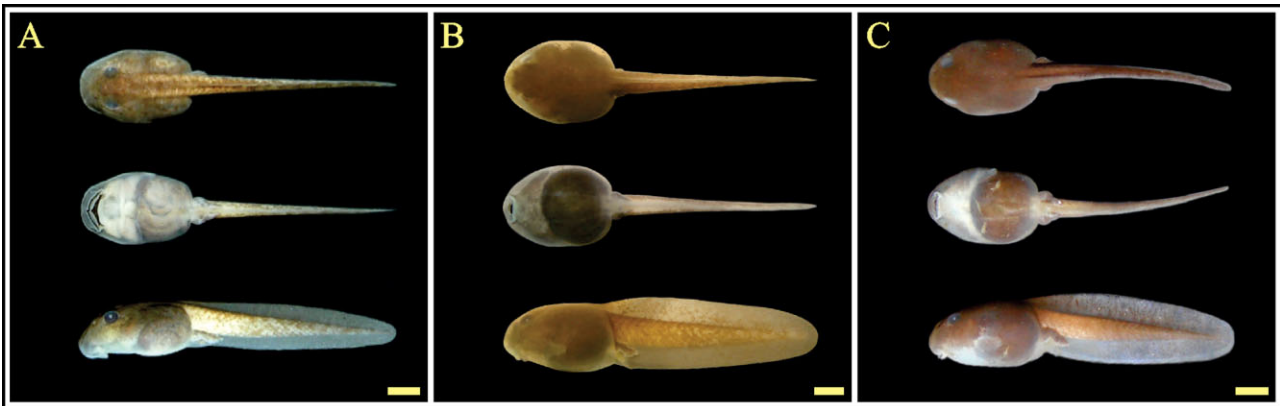


Figure 5. External morphology. Dorsal, ventral, and lateral views of tadpoles of three species of *Melanophryniscus* unassigned to any group: A, *M. krauczuki* St. 36. B, *M. langonei* St. 34. C, *M. sanmartini* St. 34. Scale bars = 2 mm.

magnifications in *M. devincenzii*, *M. montevidensis*, and *M. simplex*.

The oral disc (Figs 6, 7) was emarginate in all species, being anteroventral (most species) or ventral (*Melanophryniscus macrogranulosus*, *M. sp. 3* aff. *vilavelhensis*, and *M. krauczuki*). Its size was variable, there were small (about 22% of the body

length, *M. moreirae* group), medium-sized (23–30% of the body length, *M. stelzneri* group, *M. langonei*, and *M. sanmartini*), and large discs (> 30% of body length, most species of the *M. tumifrons* group and *M. krauczuki*). Marginal papillae were arranged in a single row. The dorsal gap ranged between 70–90% of the oral disc length (higher values in the *M. tumifrons*

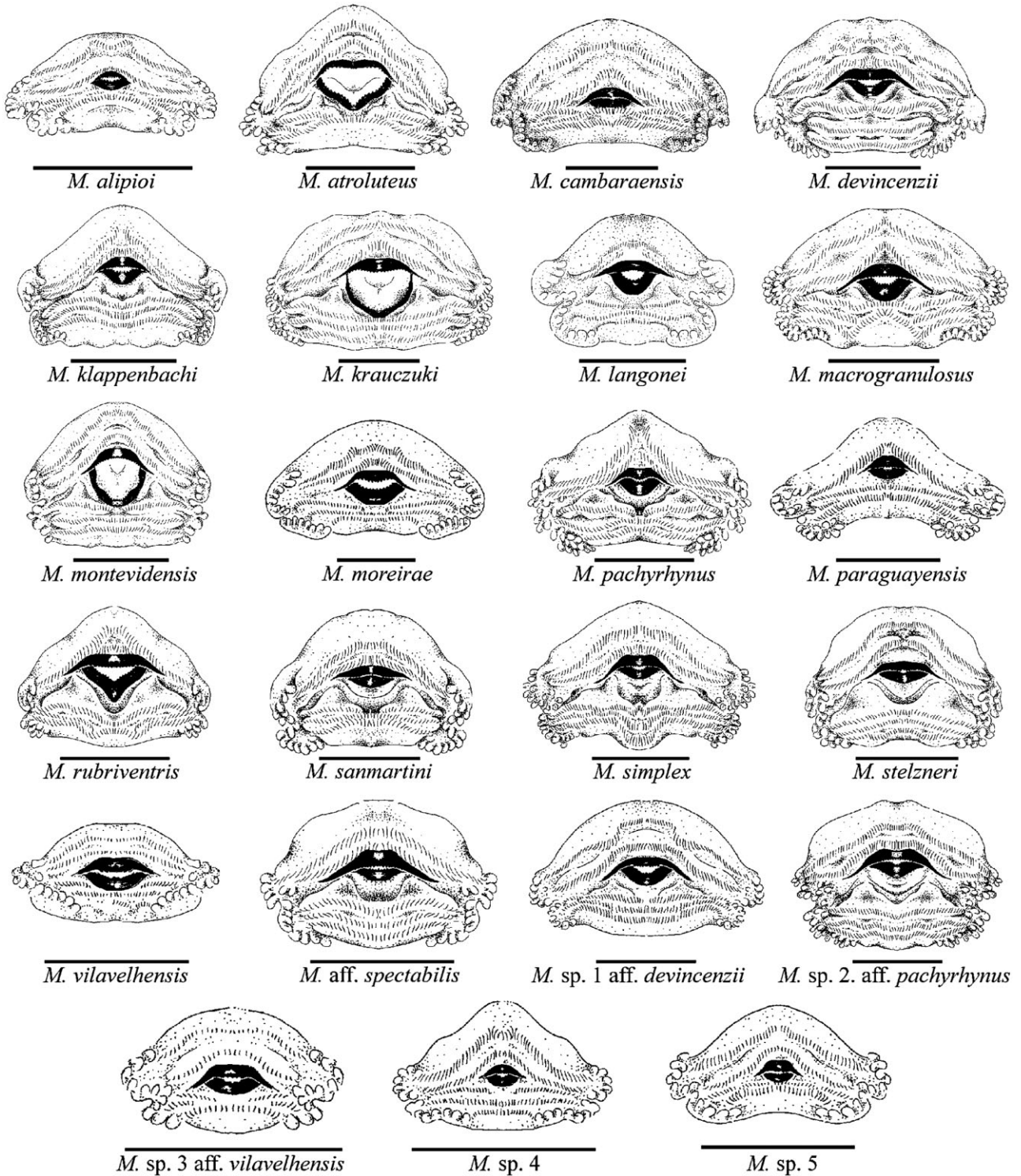


Figure 6. Schematized oral discs in 23 species of *Melanophryniscus*. Scale bars = 1 mm.

group and *M. krauczuki*), and the ventral gap extended for 30–60% of the oral disc length (higher values in the same species). Submarginal papillae were absent at the commissures (*M. klappenbachi*,

M. paraguayensis, *M. sp. 3 aff. vilavelhensis*, *M. sp. 4*, *M. sp. 5*, most specimens of *M. langonei*, *M. sanmartini*, and some specimens of *M. devincenzii*, *M. macrogranulosus*, *M. pachyrhynus*, *M. rubriventris*,

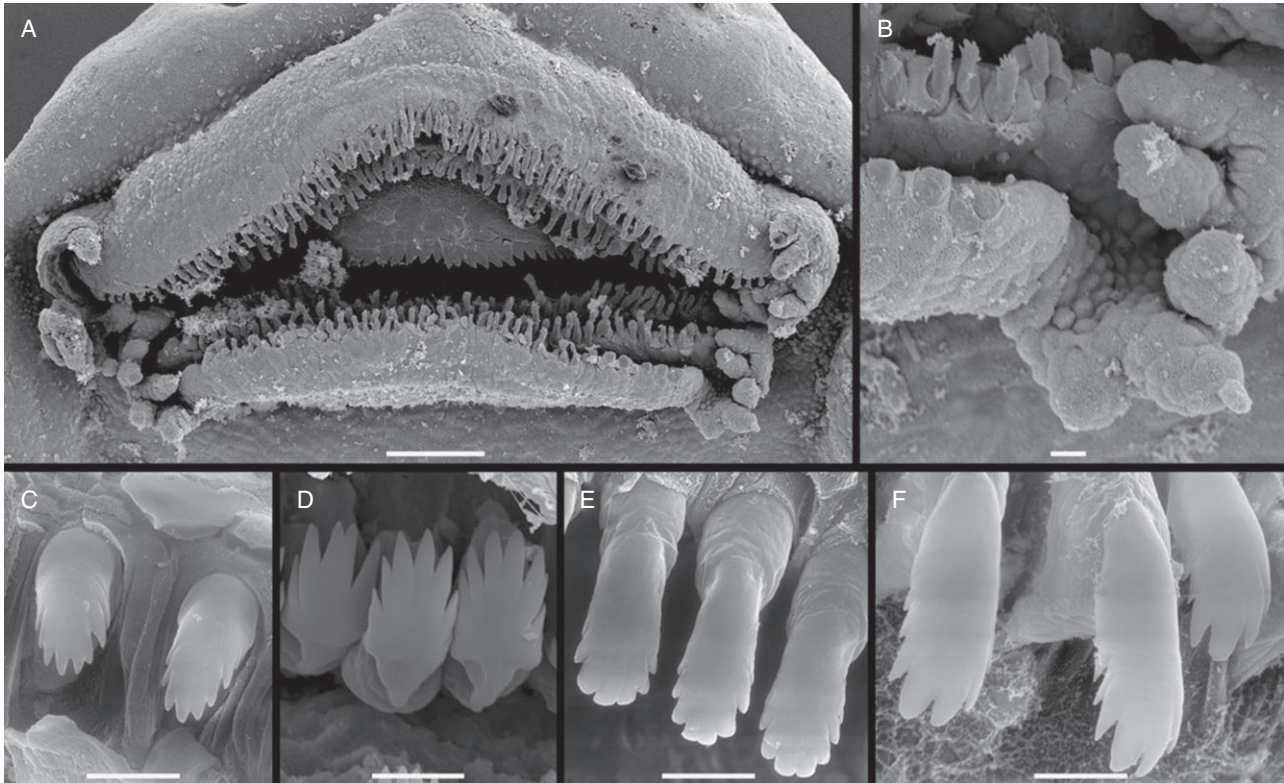


Figure 7. Scanning electron microscopy photographs: A, oral disc of *Melanophryniscus devincenzii* St. 29. B, detail of left commissure showing emerging labial teeth of P3. Labial teeth in: C, A2 of *M. alipioi*. D, P3 of *M. atroluteus*. E, A1 of *M. macrogranulosus*. F, A2 of *M. simplex*. Scale bars = 100 μm (A) and 10 μm (B–F).

and *M. aff. spectabilis*), or scarce ones may be present (1–5, remaining species, most specimens of *M. macrogranulosus*, and some specimens of *M. devincenzii*, *M. langonei*, *M. pachyrhynchus*, *M. rubriventris*, *M. sanmartini*, and *M. aff. spectabilis*). Jaw sheaths were thin and distally serrated; the upper jaw sheath was curve and the lower one was smaller and V shaped. The labial tooth row formula (LTRF) varied in the division of labial tooth rows; observed formulas are: (1) 2/3: *M. atroluteus*, *M. cambaraensis*, *M. klappenbachi*, *M. krauczuki*, *M. macrogranulosus*, *M. paraguayensis*, *M. rubriventris*, *M. simplex*, *M. aff. spectabilis*, *M. sp. 2 aff. pachyrhynchus*, most specimens of *M. montevidensis* [including those described by Garrido-Yrigaray (1989)], some specimens of *M. devincenzii*, specimens of *M. moreirae* described by Starrett (1967), and of *M. stelzneri* described by Fernández (1927) and Bustos Singer & Gutiérrez (1997); (2) 2/3(1): all phytotelm species, *M. langonei*, *M. sp. 1 aff. devincenzii*, all specimens of *M. moreirae* we examined and those described by Bokermann (1967), most of *M. sanmartini* we examined, some specimens of *M. devincenzii*, *M. montevidensis*, *M. pachyrhynchus* [including all described by Prigioni & Langone (1990)], *M. aff. spectabilis*, and *M. simplex*,

and a few individuals of *M. stelzneri* described by Bustos Singer & Gutiérrez (1997); (3) 2(2)/3: specimens of *M. stelzneri* we studied, most specimens of *M. pachyrhynchus*, and some of *M. atroluteus* and *M. cambaraensis*; and (4) 2(2)/3(1): some specimens of *M. atroluteus* and *M. sanmartini*, some specimens of *M. moreirae* described by Starrett (1967). Tooth rows had similar length in most of the species, whereas in all phytotelm species and in specimens of *M. moreirae* that we studied the third lower row (P3) was visibly shorter [specimens of *M. moreirae* described by Starrett (1967), show posterior rows shorter than the anterior rows, and decreasing slightly in size posteriorly]. Individual labial teeth had an oblongated head with eight to 12 marginal cusps and a wide sheath (Fig. 7). Abnormal oral discs are frequent. For instance, in all exemplars of *M. langonei* we examined, many labial teeth, mainly from labial ridges A1 and P3, were lost, and P3 was absent in half the specimens. The single specimen of *M. vilavelhensis* we observed had the P3 fragmented in short segments and reticulated, and one specimen of *M. aff. spectabilis* showed non-medial gaps in A1 and A2. In the literature, specimens of *M. stelzneri* described by Bustos Singer & Gutiérrez (1997) had a LTRF 2/2,

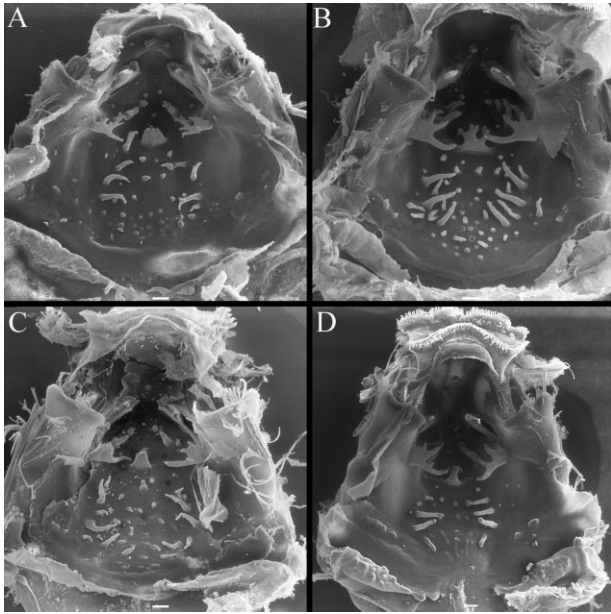


Figure 8. Buccal roofs of: A, *Melanophryniscus klappenbachi*. B, *M. macrogranulosus*. C, *M. rubriventris*. D, *M. simplex*. Scale bars = 100 μ m.

and in specimens of *M. sanmartini* described by Prigioni & Arrieta (1992) the row A1 was absent.

Finally, in *Melanophryniscus alipioi* (Stage 37) and *M. sp. 3* aff. *vilavelhensis* (Stage 30) adhesive glands were observed as two oblongated regions, slightly prominent and pigmented, posterolateral to the oral disc; glands were in regression but still evident in *M. sp. 4* and *M. sp. 5*.

In preserved specimens, the skin was translucent. The dorsum was dark brown, often with golden spots (*Melanophryniscus klappenbachi*); the venter was translucent with scattered melanophores in most of the species, and dark and opaque in *M. langonei* and *M. sanmartini*. Tail musculature was light brown with large unpigmented areas; in *M. alipioi* the tail musculature was pigmented mostly in dorsal myosepts. Fins were transparent, the dorsal fin often has scattered melanophores or dark spots.

BUCCAL CAVITY

Here we describe the buccal cavity of 20 species altogether, commenting on morphological differences when necessary. We could register only some aspects of the buccal cavity of phytotelm *Melanophryniscus sp. 4*, and none of three species, *M. vilavelhensis*, *M. sp. 3* aff. *vilavelhensis*, and *M. sp. 5* due to the scarcity of material.

On the buccal roof (Fig. 8), the pre-narial arena had small pustulations (a wide ridge in *Melanophryniscus*

paraguayensis). The large choanae were arranged obliquely; the anterior margin had small pre-narial papillae and the narial valve was developed. Two, or less commonly three, pairs (in *M. alipioi*, *M. atroluteus*, *M. klappenbachi*, *M. moreirae*, *M. sp. 2* aff. *pachyrhynus*, and *M. aff. spectabilis*) of conical post-narial papillae disposed in an inverted-V shape; the second pair was often bifid (e.g. in *M. atroluteus* and *M. montevidensis*). The lateral ridge papillae were flat, wide, and well developed, with two to four acute tips. The shape of the median ridge was highly variable (Fig. 9), from broad based, semicircular or trapezoidal (e.g. in *M. montevidensis*, *M. moreirae*, *M. rubriventris*, *M. stelzneri*, and *M. sp. 2* aff. *pachyrhynus*) to tall, papilla-like (e.g. in *M. devincenzii*, *M. pachyrhynus*, and *M. simplex*); it often has a pustulate free margin. A small pre-median ridge or papillae appear in *M. sanmartini* and *M. stelzneri*. The buccal roof arena was defined by three to six pairs of long, conical papillae; scarce pustulations appeared on the central region of the arena; additionally, two to four small papillae diverged on both sides from the roof arena, describing a curve that followed the glandular zone. The less papillated arenas were those of *M. klappenbachi*, *M. montevidensis*, and *M. simplex*, whereas those of *M. devincenzii* and *M. macrogranulosus* showed the most profuse papillation. The glandular zone was arranged as a wide U-shaped band on the posterior region of the buccal roof. The dorsal velum was short, medially interrupted, and showed three to four long papillae at the medial region and shorter marginal projections all along (Fig. 9); in *M. moreirae*, *M. langonei*, and *M. sanmartini* the medial gap was very narrow, and in some specimens of *M. sanmartini* the velar margin was undulate without conspicuous projections.

On the buccal floor (Fig. 10), posterior to the lower jaw, there was a single pair of flap-like infralabial papillae, very elaborate, with three to seven points that often overlapped each other on the midline (not in *Melanophryniscus klappenbachi*, *M. langonei*, *M. moreirae*, *M. paraguayensis*, *M. simplex*, *M. stelzneri*, *M. sp. 2* aff. *pachyrhynus*, and *M. aff. spectabilis*); small pustulations appeared on the infrarostral cartilages. The tongue anlage was very wide, and lingual papillae varied in number and shape (Fig. 11). Most specimens had four conical papillae, including the single phytotelm individual *M. sp. 4*; in *M. cambaraensis*, *M. rubriventris*, and in one specimen of *M. alipioi* the two central papillae were long and conical, whereas the two lateral were small pustulations. In contrast, tadpoles of *M. krauczuki* and some specimens of *M. montevidensis* and *M. stelzneri* may have had only two long papillae or none at all. Five small pustulations appeared in some

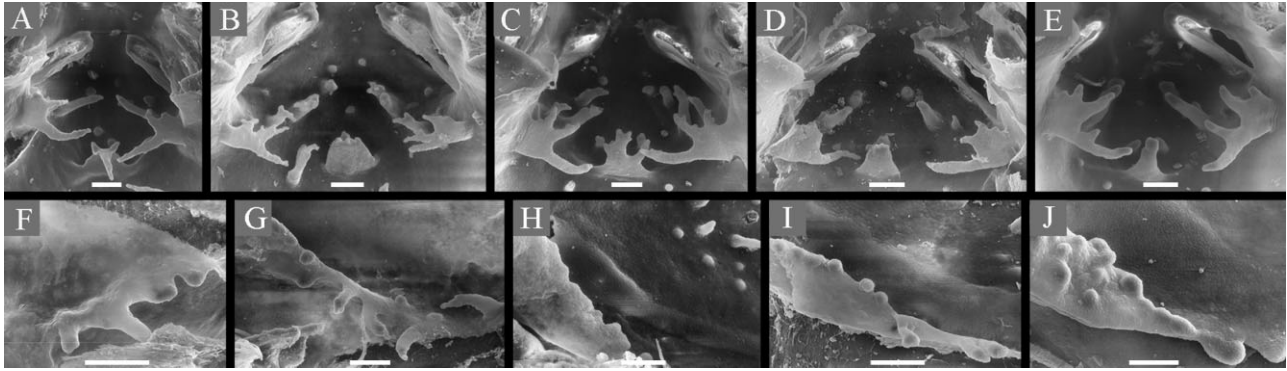


Figure 9. Variable features in buccal roofs of *Melanophryniscus*. Median ridge of: A, *M. alipioi*. B, *M. klappenbachi*. C, *M. macrogranulosus*. D, *M. rubriventris*. E, *M. simplex*. Dorsal velum of: F, *M. alipioi*. G, *M. klappenbachi*. H, *M. macrogranulosus*. I) *M. rubriventris*. J, *M. simplex*. Scale bars = 100 μ m.

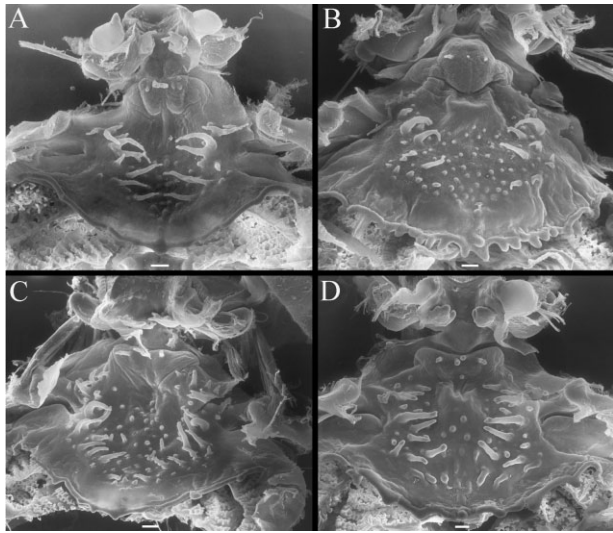


Figure 10. Buccal floors of: A, *Melanophryniscus alipioi*. B, *M. klappenbachi*, C, *M. macrogranulosus*. D, *M. simplex*. Scale bars = 100 μ m.

specimens of *M. sanmartini*; the single specimen of *M. klappenbachi* that we analysed had three lingual papillae, and a second specimen of *M. alipioi* and *M. langonei* had two long central papillae and three small pustulations. The buccal floor arena was oval and delimited by five to 11 pairs of tall, conical buccal floor arena papillae; the papillae nearest the buccal pockets were the largest, often bifid. Several pustulations and low papillae were scattered among the main papillae. As in the buccal roof, two to four small papillae diverged following the ventral velum. Two to four small pre-pocket papillae appeared in most of the species, but were absent in *M. alipioi*, *M. devincenzii*, *M. klappenbachi*, *M. montevidensis*, and *M. stelzneri*. The buccal pockets were elongated and arranged transversely. The ventral velum was semicircular

and supported by spicules; three main marginal projections (particularly developed in *M. atroluteus*, *M. klappenbachi*, *M. sanmartini*, and *M. aff. spectabilis*) appeared on each side over each filter plate, and smaller projections appeared mostly at the median region. In *M. alipioi* and *M. klappenbachi* small pustulations were arranged in a longitudinal line from the floor arena to the edge of the ventral velum. The median notch was absent, and secretory pits occurred on the edge of the velum. The glottis was exposed and placed slightly posteriorly to the ventral velum; in most specimens it was very hard to discern, because lips were not evident, the whole tissue was very soft, and cartilages and muscles of the region were not yet developed.

MUSCULOSKELETAL MORPHOLOGY

The configuration of the cartilaginous skeleton was very similar among species of *Melanophryniscus*. For that reason, we describe features of 20 species together in one section, mentioning relevant differences when pertinent. The articulated skeleton of *M. aff. spectabilis* was pictured as an example in Figure 12, and neurocrania and hyobranchial skeletons of all the studied species are shown in Figures 13 and 14, respectively. As to the three remaining species, the skeleton of phytotelm *M. sp. 5* is described in part but not included in figures because we had a single larva and we preferred to keep the skeleton articulated and with musculature as a reference. Two other phytotelm species, *M. vilavelhensis* and *M. sp. 3 aff. vilavelhensis*, were not cleared and stained and thus not included in descriptions, because of the scarce material available.

The neurocranium of *Melanophryniscus* was low and quadrangular (width about 90% of the length), with its maximum width at the level of the posterior region of the subocular bar; the neurocranium of

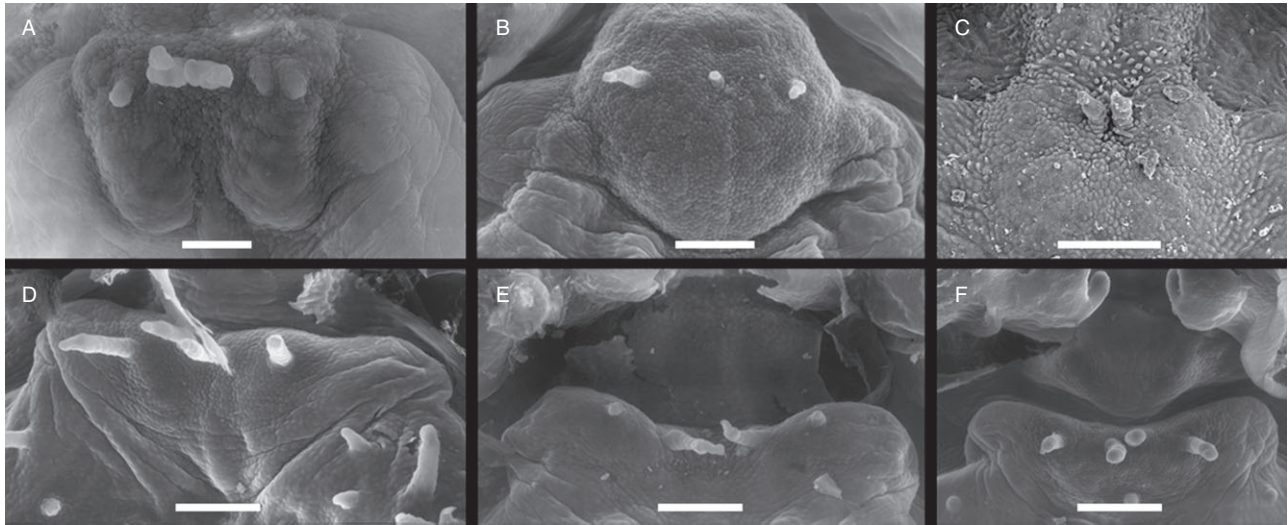


Figure 11. Lingual papillae of: A, *Melanophryniscus alipioi*. B, *M. klappenbachi*. C, *M. krauczuki*. D, *M. macrogranulosus*. E, *M. rubriventris*. F, *M. simplex*. Scale bars = 100 μm .

M. atroluteus, *M. montevidensis*, and *M. rubriventris* was visibly wider than those of the remaining species (almost 98%). The suprarostrals cartilages were formed of a central pars corporis and lateral partes alares, joined dorsally by a cartilaginous connection. The pars corporis was composed of two rectangular bars fused ventrally resulting in a V-shaped structure; each pars alaris was triangular, with a smooth ventral margin and prominent dorsal anterior and posterior processes. The configuration of the trabecular horns varied among species: they were relatively short and divergent in *M. atroluteus*, *M. cambaraensis*, *M. klappenbachi*, *M. moreirae*, *M. pachyrhynchus*, *M. rubriventris*, *M. stelzneri*, and phytotelm larvae (18–23% of the neurocranium length; $\cong 50^\circ$), and long and less divergent in the remaining species ($\cong 25\%$; $\cong 40^\circ$). The horns of *M. krauczuki* were the least divergent among all the species ($\cong 35^\circ$). Trabecular horns were uniformly wide along their length in most of the species, but were distally wider in *M. krauczuki*. A rounded lateral trabecular processes was evident on the lateroventral margins of the horns of all the species but *M. atroluteus* and *M. simplex*. The ethmoid region showed interspecific variation in the extent of development at the analysed stages, and a small nasal septum was evident in *M. atroluteus*, *M. klappenbachi*, *M. krauczuki*, *M. sanmartini*, some specimens of *M. pachyrhynchus* and *M. montevidensis*, as a triangular, acute process posterior to the confluence of the trabecular horns. The lamina orbitonasalis was outlined as a small, triangular projection that grew laterally from the most anterior part of each cartilago orbitalis. It was particularly visible in

some specimens of *M. atroluteus*, *M. devincenzii*, *M. klappenbachi*, *M. krauczuki*, *M. langonei*, *M. macrogranulosus*, *M. pachyrhynchus*, *M. rubriventris*, *M. sanmartini*, *M. simplex*, *M. aff. spectabilis*, *M. sp. 1 aff. devincenzii*, and *M. sp. 2 aff. pachyrhynchus*. The ventral part of the lamina grew anterolaterally to form the antorbital processes, already developed at the stages analysed in all species.

The floor of the chondrocranium was composed of a thin sheet of cartilage occluding a still visible pituitary fenestra. The foramina carotica primaria were visible at the base of the cranial trabeculae, completely surrounded by cartilage in *Melanophryniscus atroluteus*, *M. klappenbachi*, *M. krauczuki*, *M. langonei*, *M. macrogranulosus*, *M. montevidensis*, *M. sanmartini*, *M. sp. 2 aff. pachyrhynchus*, *M. sp. 1 aff. devincenzii*, and phytotelm larvae, and as a small notch at the posterolateral region of the fenestra in the remaining species. The foramina craniopalatina were visible only in *M. atroluteus*, *M. sp. 2 aff. pachyrhynchus*, and in some specimens of *M. montevidensis*; in the rest of the species they are not defined because of the light chondrification of the intertrabecular region. The lateral walls of the chondrocranium are represented by the orbital cartilages, which are low and poorly developed at the studied stages; the optic and oculomotor foramina were visible in the posterior region of each cartilage. The chondrocranium opened dorsally through the frontoparietal fenestra, lined laterally by the thin taeniae tecti marginales. Posteriorly, the tectum synoticum was hardly visible and very thin. Further roofing of the cranial cavity develops later; we observed taeniae tecti medialis and transversalis delimiting parietal fenestrae at metamorphic stages

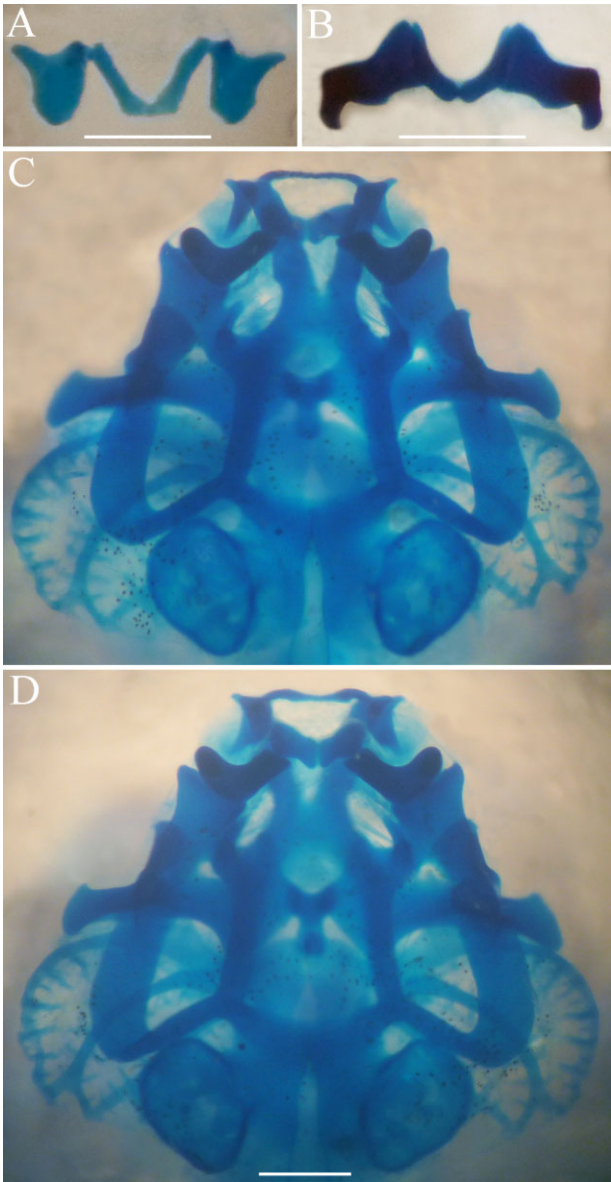


Figure 12. Articulated chondrocranium and hyobranchial skeleton of *Melanophryniscus* aff. *spectabilis*. A, suprarostrals. B, lower jaw cartilages. C, dorsal and D, ventral views of the whole cranium. Scale bars = 500 μm .

(*M. atroluteus* Stage 42, *M. pachyrhynchus* Stage 44, and *M. krauczuki* Stage 46). The otic capsules were large and oval (29–34% of the neurocranium length); in *M. alipioi* and *M. sp. 4* capsules were proportionately larger (36%). The oval window occurred ventrolaterally, and jugular and perilymphatic inferior foramina were seen ventrally; the operculum was not yet developed. A thin crista parotica could be seen in some specimens of *M. devincenzii*, *M. pachyrhynchus*, *M. rubriventris*, and *M. sanmartini*, and a small anterolateral process was evident in *M. macrogranulosus*.

The palatoquadrate was long and narrow, and described an angle of around 18° regarding the longitudinal axis of the cranium; this angle was slightly larger in *Melanophryniscus atroluteus*, *M. rubriventris*, and *M. sp. 1* aff. *devincenzii* ($22\text{--}25^\circ$). The subocular bar was curved, slightly wider posteriorly and had a smooth margin; in phytotelm species it was straighter and uniformly wide along its length. Its posterior region was rounded and in lateral view may be scarcely curved or flat (in *M. atroluteus*, *M. klappenbachi*, *M. langonei*, *M. montevidensis*, *M. rubriventris*, *M. stelzneri*, *M. paraguayensis*, and *M. sp. 1* aff. *devincenzii*), or deeply concave so that an edge was visible from dorsal view (in the remaining species). The palatoquadrate was anteriorly attached to the neurocranium via the wide quadrato cranial commissure; the quadratoethmoid process was well developed in all the species. The pseudopterygoid process was absent. Posteriorly, the palatoquadrate was connected to the neurocranium through a long, narrow ascending process that was attached below the oculomotor foramen (low attachment). The angle the ascending process describes regarding the longitudinal axis of the neurocranium varies between $62\text{--}64^\circ$ (in *M. krauczuki* and *M. simplex*) and $72\text{--}74^\circ$ (e.g. in *M. rubriventris* and *M. sp. 1* aff. *devincenzii*); the largest angles are those of phytotelm species (78°). The otic process was absent in all species. The articular process was short and wide in *M. krauczuki* and *M. pachyrhynchus* (length/width $\cong 0.45$ and 0.56 , respectively), and longer and narrower in the remaining species; the highest values ($\cong 1$) were those of *M. klappenbachi*, *M. sanmartini*, *M. stelzneri*, and *M. aff. spectabilis*. The muscular process also varied among species: it was small and narrow in phytotelm species, and wide, high, and rounded in the remaining species. It curves medially approaching the antorbital process. In ventral view, the hyoquadrate process was well developed in *M. simplex* and *M. macrogranulosus*. Orbital fenestrae were visibly smaller in phytotelm species. The Meckel's cartilages were sigmoid, with retroarticular, dorsomedial and ventromedial processes evident. The infrarostral cartilages were rectangular and articulated between them through an alcianophilic connection.

In the hyobranchial skeleton, the ceratohyals were long and narrow, and they were arranged almost perpendicular to the longitudinal axis of the chondrocranium. The anterior process was high and broad, with a rounded tip. The anterolateral process was thin and medially oriented; it was poorly developed or absent in *M. sanmartini*, *M. langonei*, and in phytotelm *M. alipioi*, and *M. sp. 4*. The articular condyle was prominent and robust, and its anterior tip was seen also from a ventral view. In the posterior margin of the ceratohyal, the posterior process was

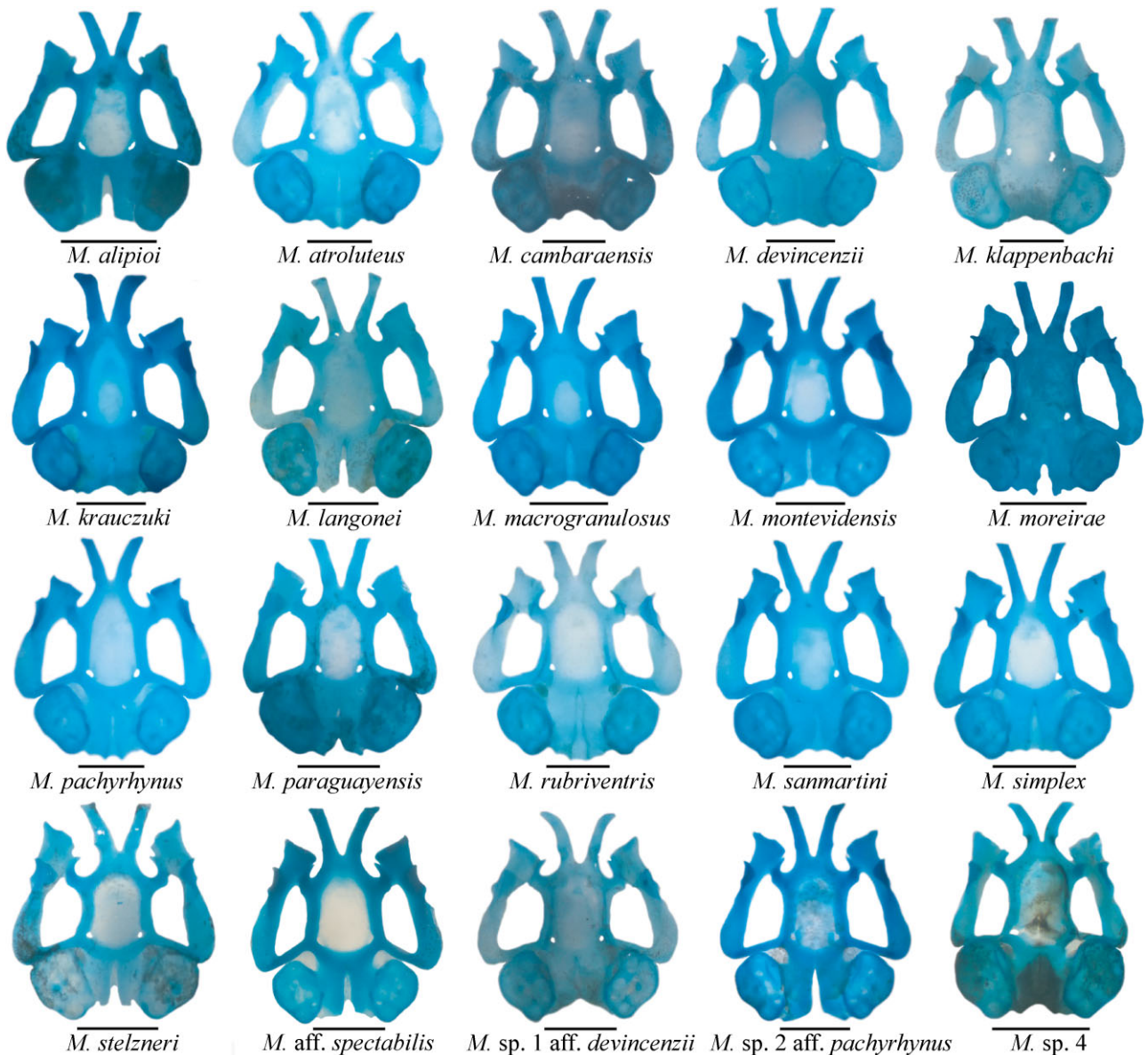


Figure 13. Neurocrania of 20 species of *Melanophryniscus*. The skeletons of the remaining three bromeliad species, *M. vilavelhensis*, *M. sp. 3* aff. *vilavelhensis*, and *M. sp. 5*, were not included because of the scarce material available. Scale bars = 1 mm.

triangular and wide and its tip overlapped the anterior margin of the hypobranchial plate. The ceratohyals were joined medially through a quadrangular pars reuniens and a long, thin basibranchial fused to the former. A short, quadrangular urobranchial process grew from the posterior edge of the basibranchial. The basihyal was present as a small chondrification in *M. atroluteus*, *M. cambaraensis*, *M. klappenbachi*, *M. krauczuki*, *M. langonei*, *M. moreirae*, *M. stelzneri*, *M. aff. spectabilis*, *M. sp. 1* aff. *devincenzii*, and some specimens of *M. montevidensis*. The basibranchial was fused to the hypobranchial

plates; these latter were flat, wide, and articulate medially leaving a posterior triangular notch. In the posterior edge of each hypobranchial plate, a small mass of cartilage (possibly homologous to spiculum IV, according to Larson *et al.*, 2003) was evident (Fig. 15). The branchial basket included four ceratobranchials joined distally by terminal commissures. The ceratobranchiales were thin, curved, with marginal projections evident in ceratobranchial I of all the species, and in ceratobranchials II–IV of *M. cambaraensis*, *M. montevidensis*, *M. pachyrhynchus*, *M. rubriventris*, *M. stelzneri*, and *M. aff. spectabilis*.

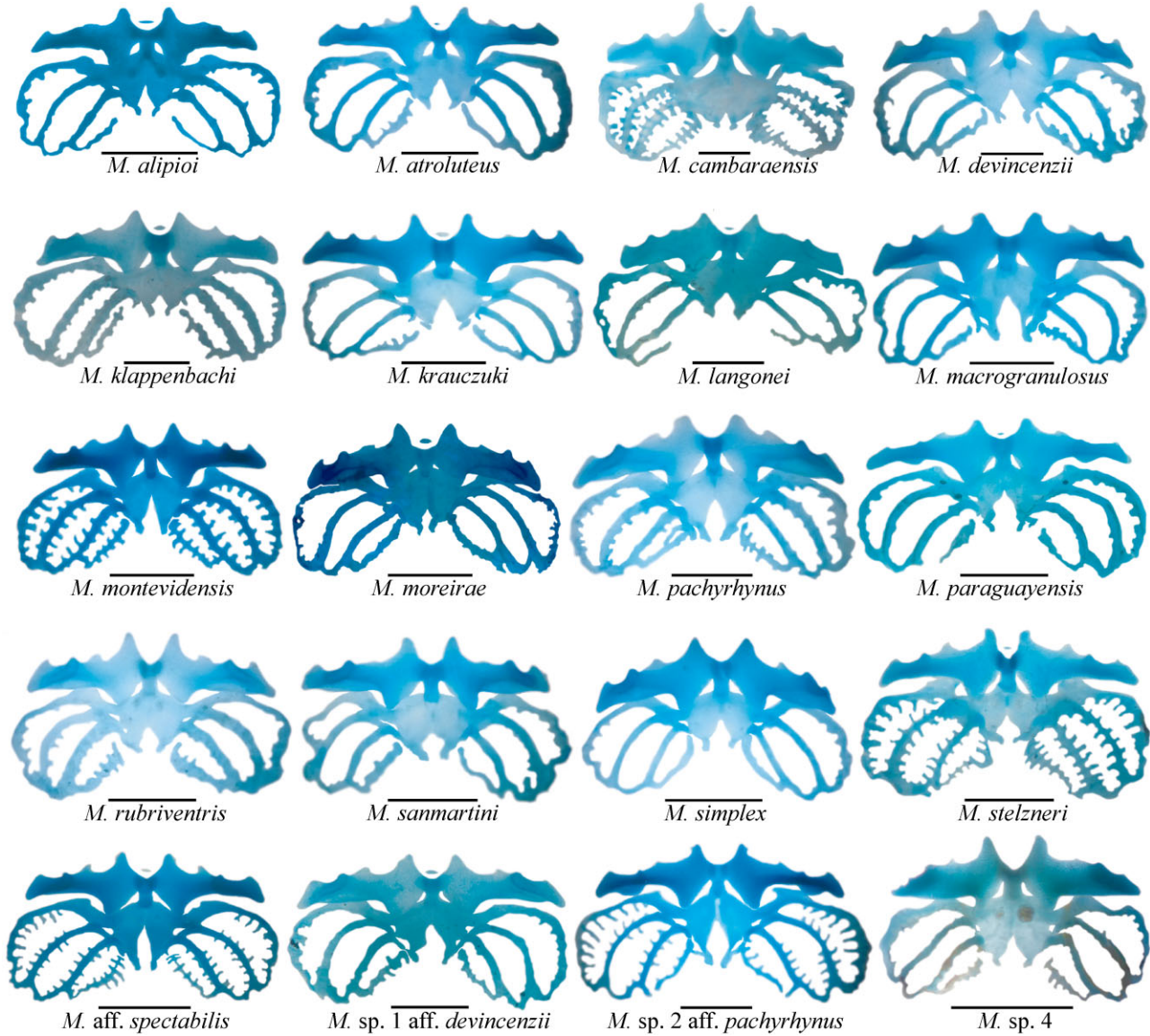


Figure 14. Hyobranchial skeletons of 20 species of *Melanophryniscus*. The skeletons of the remaining three bromeliad species, *M. vilavelhensis*, *M. sp. 3* aff. *vilavelhensis*, and *M. sp. 5*, were not included because of the scarce material available. Scale bars = 1 mm.



Figure 15. Detail of the posterior region of the hypobranchial plate of: A, *Melanophryniscus krauczuki*. B, *M. macrogranulosus*. C, *M. montevidensis*. D, *M. simplex*. E, *M. sp. 1* aff. *devincenzii*. The asterisks indicate the hypobranchial mass possibly homologous to the spicule IV (Larson *et al.*, 2003), and the arrow points out the cartilaginous bridge joining it with the spicule III.

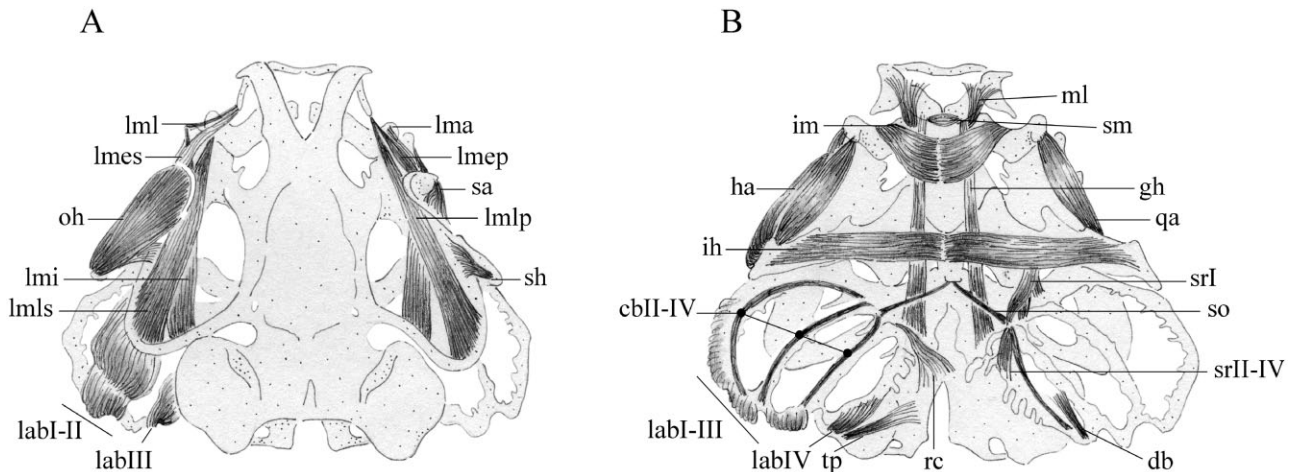


Figure 16. Scheme of musculature in a generalized larva of *Melanophryniscus*. Although all these muscles are bilateral, in most cases they are shown in only one side of the skeleton for image clarity: CBII–IV, constrictor branchialis II–IV; DB, diaphragmatobranchialis; GH, geniohyoideus; IH, interhyoideus; IM, intermandibularis; HA, hyoangularis; LABI–IV, levator arcuum branchialium I–IV; LMA, levator mandibulae articularis; LMEP, levator mandibulae externus profundus; LMES, levator mandibulae externus superficialis; LMI, levator mandibulae internus; LML, levator mandibulae lateralis; LMLP, levator mandibulae longus profundus; LMLS, levator mandibulae longus superficialis; ML, mandibulolabialis inferior; OH, orbitohyoideus; QA, quadratoangularis; RC, rectus cervicis; SA, suspensorioangularis; SH, suspensoriohyoideus; SM, submentalis; SO, subarcualis obliquus; SRI–IV, subarcualis rectus I–IV; TP, tympanopharyngeus.

The ceratobranchial I was continuous with the hypobranchial plate and showed an anterior branchial process oriented medially. The ceratobranchial II were a high branchial process and it was connected with the hypobranchial plate via a cartilaginous bar. The ceratobranchial III showed a small branchial process and it was also connected to the hypobranchial plate by a small mass of cartilage. Finally, the ceratobranchial IV was very thin and was not attached to the hypobranchial plate; it was hardly visible in one specimen of *M. langonei*. Dorsally, the spicules I–III are short, curve, and weakly chondrified; in all species but *M. devincenzii*, *M. simplex*, and *M. sp. 1* aff. *devincenzii* the spicule III was connected to the cartilaginous mass of the posterior edge of the hypobranchial plate through a cartilaginous bridge. Finally, arytenoid cartilages of the glottis were not developed at the stages analysed.

Larval musculature features and interspecific variation are listed in Appendix S4 and schematized in Figure 16. General aspects include the m. levator mandibulae lateralis absent or scarcely developed at the stages analysed, m. submentalis present at larval stages of some species, m. subarcualis rectus II–IV with a lateral slip diverging and invading the branchial septum IV, mm. constrictores branchiales disposed on ceratobranchiales I–III, so that the ceratobranchial IV bears the diverging slip of the m.

s.r. II,IV, mm. levatores arcum branchialium I and II wide and close to each other, mm. interhyoideus posterior and diaphragmatopraecordialis lacking, m. constrictor laryngis absent and m. dilatator laryngis absent or scarcely developed, and ramus V₃ of the trigeminal nerve dorsal to the mm. levatores externus. A distinctive feature in *Melanophryniscus* was the presence of a pair of subhyoid muscles that extended between the articular condyle of the ceratohyal and the skin ventral to the hypobranchial skeleton (Fig. 17); in *M. pachyrhynchus*, a pair of subquadrate muscles extended between the articular process of the palatoquadrate and the ventral skin, with fibers converging to those of the subhyoid muscle. The muscle rectus abdominis had a distinct configuration and its development varied among species. In general, the lateral fibers originated at the mid abdomen, while the medial ones reached far anteriorly at the septum transversum level. In phytotelm species the muscle was scarcely developed: in *M. alipioi* (hind limb at Stage 31), while all cranial muscles were developed (even the sutil mm. levatores mandibulae lateralis and externus superficialis), no differentiated fibers can be observed corresponding to axial musculature; a very thin muscle sheet appears in *M. sp. 4* and *M. sp. 5*, at Stage 37 (Fig. 17). Also, the m. geniohyoideus was starting to divide into mm. mediales and laterales in these two latter species.

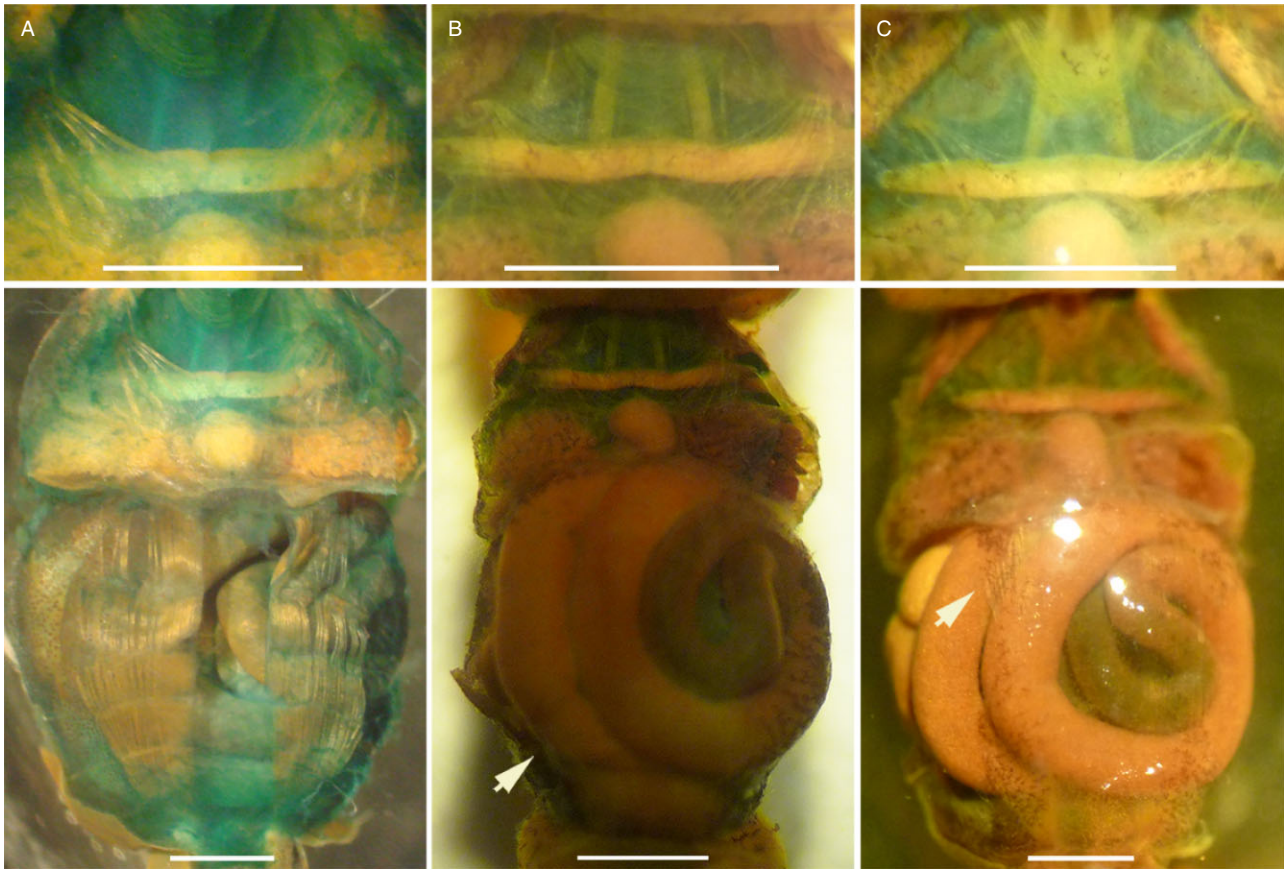


Figure 17. Details of subhyoid (top) and rectus abdominis (bottom) muscles of: A, *Melanophryniscus cambaraensis* St. 36. B, *M. alipioi* St. 31. C, *M. sp. 4* St. 37. Arrow points out the poorly developed m. rectus abdominis of phytotelm species. Scale bars = 1 mm.

DISCUSSION

EXTERNAL MORPHOLOGY

Published descriptions and those presented in this paper show that *Melanophryniscus* tadpoles are very similar to each other, and exhibit morphological characteristics that are typical of bufonid larvae (Altig & McDiarmid, 1999b). Two distinctive characters deserve attention: the pineal end organ and the intestinal reversal point. The pineal end organ, first reported in the genus for *M. krauczuki* (Baldo & Basso, 2004), is evident in most of the species we studied but it was overlooked in other available larval descriptions. Although this structure has been barely mentioned in larval anuran characterizations, there are some reports of its occurrence in *Rhinella* (Bufonidae; Borteiro *et al.*, 2006; Blotto, Pereyra & Baldo, in press) and *Pleurodema* (Leptodactylidae; Kolenc *et al.*, 2009), and thus its presence across Leptodactyliformes should be revisited. The intestinal reversal point at the left of the abdominal cavity was described in *M. rubriventris*, *M. krauczuki*, and

M. atroluteus (Lavilla & Vaira, 1997; Baldo & Basso, 2004; Baldo, Maneyro & Laufer, 2010), and it occurs in all species we studied. In tadpoles of the five phytotelm species the digestive tract is notably shorter; nevertheless the intestinal reversal point is placed at the left of the abdomen, just as in the remaining species. Interestingly, this character state is apparently acquired during ontogeny, because embryos at early stages of examined species (e.g. *M. krauczuki* and young larvae of *M. sp. 3* aff. *vilavelhensis* we described, Fig. 4) have comparatively shorter guts with the intestinal reversal point placed almost at the centre of the abdominal cavity.

Comparative analyses of the body shape (relative warp analysis; Zelditch *et al.*, 2004) and oral disc measurements (multivariate analysis of variance) of 12 of the 23 species in our work (see Haad, Vera Candioti & Baldo, 2011, for method explanations) show that differences observed among *Melanophryniscus* tadpoles are mainly quantitative. Patterns of morphological variation are, in general, consistent with the intrageneric groups and also with

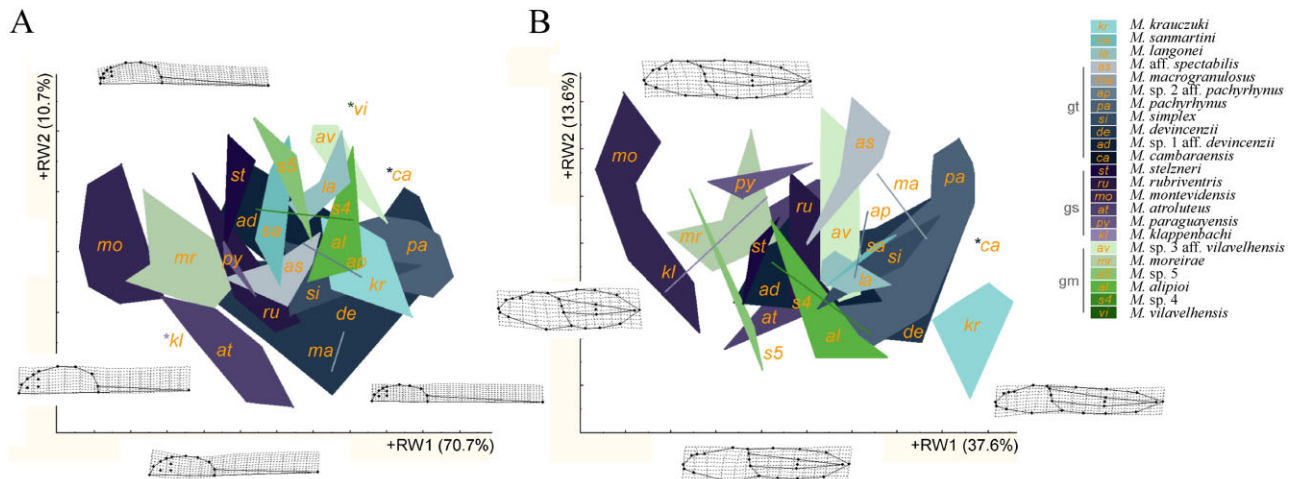


Figure 18. Principal component analysis of shape variation in *Melanophryniscus* in A, dorsal and B, lateral views. Species are coloured according to intrageneric groups: GM, *M. moreirae* group; GT, *M. tumifrons* group; GS, *M. stelzneri* group. Grids show shape change along variation axes RW1 and RW2.

the type of water body in which the larvae develop (i.e. lentic vs. lotic). A more comprehensive ordination analysis that includes the remaining species studied herein (Fig. 18; we also reanalysed oral disc measurements including all species, Appendices S2 and S5) recover the morphological pattern of variation discussed by Haad *et al.* (2011). The main variation in body shape discriminates tadpoles of the *M. stelzneri* and *M. tumifrons* groups. Pond tadpoles of the *M. stelzneri* group have a tall and globose body, short tail with fins higher than the body; these features are more noticeable in *M. klappenbachi* and *M. montevidensis*. Oral discs are similar among species, in average occupying 30% of the body length with wide dorsal and ventral gaps; labial teeth have an oblongated head with 8–12 marginal cusps and a wide sheath. Stream tadpoles of the *M. tumifrons* group have a more depressed body, long tail with lower fins, the dorsal one originating more caudally; these features are more evident in *M. pachyrhynus*, *M. devincenzii*, and *M. cambaraensis*. Oral discs are in general also similar among species of this group, and, in at least two species (i.e. *M. devincenzii* and *M. pachyrhynus*), are significantly larger and with larger gaps than in those of the *M. stelzneri* group; labial teeth are similar to those of the species of the *M. stelzneri* group. Some species of both groups (e.g. *M. rubriventris* and *M. sp. 1 aff. devincenzii*) have intermediate features and overlap in geometric morphospaces. Phytotelm tadpoles are, in general, more similar to those of the *M. tumifrons* group in their body shape, but they differ mainly because they have eyes and nares located rostrally and a more acute snout. They also share a distinctive tail, with fins almost parallel to the caudal axis and a

wide, rounded tip. These phytotelm species possess oral discs that are proportionately smaller than other species and with smaller gaps than those of pond- and stream-dwelling species; labial teeth are comparatively smaller and shorter, with a short head scarcely differentiated from the sheath. Conversely, the pond tadpoles of *M. moreirae*, which are phylogenetically closely related to phytotelm species, resemble species of the *M. stelzneri* group. Among species not assigned to groups, *M. sanmartini* and *M. langonei* arrange close to phytotelm species (in dorsal view) or average shapes (lateral view). Their oral discs are similar to those of lentic tadpoles of the *M. stelzneri* group. Lastly, tadpoles of *M. krauczuki* have a stream-like morphology but in the lateral view they are placed at one end of the maximum variation axis, clearly diverging from all other species. The oral disc of these tadpoles differs from that of most species, as it is proportionately larger and with wider gaps in marginal papillae.

Patterns of body shape change between pond and stream tadpoles of *Melanophryniscus* agree with those described previously for different tadpole ecomorphological guilds (Altig & McDiarmid, 1999a), and with numerous more recent descriptions of lentic and lotic tadpoles (e.g. Matsui *et al.*, 2006; Aguilar, Siu-Ting & Venegas, 2007; Van Buskirk, 2009). As in other lotic tadpoles (e.g. Altig & Johnston, 1989; Lötters *et al.*, 2005), the oral discs of lotic *Melanophryniscus* are, in general, proportionately larger than that of their lentic relatives. Unlike most anuran groups with lotic tadpoles, however, their gaps in marginal papillae are also larger. Regarding arboreal (phytotelm) tadpoles, Lannoo, Townsend & Wassersug (1987) characterized five morphotypes

according to their morphological features and stressed that larvae of several species are elongate and attenuate [tail/body ratio > 1.7; maximum length/maximum diameter > 4.5; Lannoo *et al.* (1987)]. The compilation by Lehtinen *et al.* (2004a) reported seven species of bufonids with arboreal tadpoles: *Rhinella castaneotica*, *Dendrophryniscus brevipollicatus*, *Frostius pernambucensis*, *Nectophryne afra*, *Pelophryne brevipes* [Leong & Teo (2009), also report arboreal tadpoles in *P. signata*], and the highly modified larvae of *Mertensophryne* species. Phytotelm tadpoles of *Melanophryniscus* have a tail/body ratio > 1.7 (especially at early stages), but this value seems to be intermediate for the genus; in fact, lotic tadpoles of the genus are much more streamlined (Appendix S2 and Fig. 18). Furthermore, the oral discs in phytotelm *Melanophryniscus*, although visibly smaller and delicate, are similar in their configuration to those of pond and stream species, yet exhibiting the almost universal bufonid features of LTRF 2/3 and marginal papillae with a ventral gap (Altig & McDiarmid, 1999b; Haas, 2003). Conversely, other phytotelm bufonids have reduced LTRF [e.g. *Mertensophryne* species; Müller, Measey & Malonza (2005)], more evident are those that are endotrophic [e.g. *F. pernambucensis* and *Pelophryne* species: Cruz & Peixoto (1982); Leong & Teo (2009)].

BUCCAL CAVITY

The features of the buccal cavity are similar in all the studied *Melanophryniscus* (Echeverría, 1992; 1998; Larson *et al.*, 2003; present work), with no apparent differences neither between species groups nor microhabitats. Common traits are one pair of flap-like infralabial papillae, two to three pairs of postnarial papillae, the general disposition of the buccal roof and floor arena papillae, the arrangement of the glandular zone, and the configuration of the ventral velum, with median notch absent. Three characteristics show high variability and deserve discussion. The shape of the median ridge can vary from a low, trapezoid structure to a tall, conical, papilla-like configuration. The dorsal velum also varies, and while it shows long medial papillae and smaller projections in the whole margin in most of the species, it is gently undulate with no projections in some specimens of *M. macrogranulosus* and *M. sanmartini*. Finally, the number of lingual papillae is often a highly conserved trait within species and among related taxa. In the majority of bufonids, four lingual papillae are described [e.g. species of *Ansonia*, *Pedostibes*, *Phrynoidis*, *Bufo*, and *Rhinella*; Viertel (1982); Inger (1985); Echeverría (1998)], although some taxa within those genera have less or even no papillae [e.g. some species of *R. veraguensis* group, *A. minuta*; Inger (1985);

Aguayo *et al.* (2009)]. In *Melanophryniscus*, we observed both intra and interspecific variation. In *M. sanmartini*, *M. langonei*, and *M. alipioi*, some specimens show four and others five papillae or pustulations on the tongue anlage; *M. krauczuki* has none or two lingual papillae, and the number four apparently stabilizes within the *M. stelzneri* and *M. tumifrons* groups, with reductions in *M. klappenbachi*, *M. montevidensis*, and *M. stelzneri*.

MUSCULOSKELETAL MORPHOLOGY

The morphology of the skeleton is relatively conserved among species of *Melanophryniscus*. Distinctive traits, described previously in the literature (Haas, 2003; Larson *et al.*, 2003), are the tetrapartite suprarostrals with dorsal anterior and dorsal posterior processes, trabecular horns with lateral trabecular process, low and scarcely developed orbital cartilages, palatoquadrate with quadratoethmoid process and low attachment to the neurocranium, crista parotica reduced or absent (and thus larval otic process also absent), quadrato-orbital commissure absent, and in the hyobranchial skeleton, ceratobranchial IV very thin and not attached to the hypobranchial plate, and spiculum III often connected to the cartilaginous mass (spicule IV) posterior to the hypobranchial plate.

Among these features, the presence of a well developed finger-like dorsal anterior process in the suprarostrals distinguishes the genus from other Bufonidae (Larson *et al.*, 2003). This process is not evident or low and in other bufonids (e.g. Fabrezi & Vera, 1997; Larson, 2004; Kolenc *et al.*, 2013) and wide and square in *Rhinella quechua* and *R. rumbolli* (Aguayo *et al.*, 2009; Haas, Vera Candioti & Baldo, in press). The presence of the quadrato-orbital commissure joining the muscular process and the neurocranium was proposed as a synapomorphy of the clade joining all bufonids but *Melanophryniscus* [character in Frost *et al.* (2006: 297), corrected according to the original definition by Haas (2003), character 78]. On the other hand, four characters were suggested as synapomorphies of Bufonidae by Larson *et al.* (2003): crista parotica reduced or absent, poorly developed orbital cartilage, ceratobranchial IV reduced and not connected to the hypobranchial plate, and larval otic process absent. These characters are also similar in all bufonids described since (e.g. Vera Candioti, 2007; Aguayo *et al.*, 2009). The phylogenetic hypothesis of Anura based on larval characters presented by Haas [(2003), taken up later in Frost *et al.* (2006)] does not include the three first features as defined in those exact terms, and the fourth one (absence of larval otic process) is implied by the two first states of the additive character 66. The single

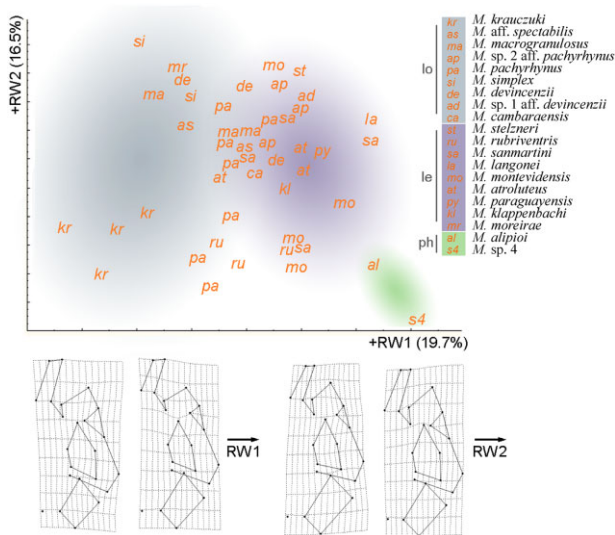


Figure 19. Principal component analysis of shape variation in neurocrania of *Melanophryniscus*. Species are coloured according to the microhabitat where larvae develop: le lentic, lo lotic, ph phytotelm. Grids show shape change along variation axes RW1 and RW2.

character state, coming from the larval skeleton, that optimizes as synapomorphy of Bufonidae is the absence of anterolateral process of the crista parotica (Haas 66.0). Among the species we studied, a triangular anterolateral process of the crista parotica appears in *M. macrogranulosus*.

Another visibly variable feature is the shape of the posterior region of the palatoquadrate. It is almost flat in all species of the *Melanophryniscus stelzneri* group and *M. sp. 1* aff. *devincenzii*, but deeply concave in the remaining species, so that a thick edge is evident in dorsal view. As this position is where the mm. levatores longus (jaw adductors) originate, it could be related to the thickness of these muscles and thus have trophic implications, but neither quantitative nor functional studies have been addressed so far.

The scatterplot in Figure 19 shows an exploratory geometric morphometric analysis of the neurocrania of the studied species, and summarizes the quantitative differences mentioned in the Results section, pertaining to the proportional size of the trabecular horns and otic capsules, the orientation of the ascending process of the palatoquadrate, and the aspect of the articular and muscular processes. Stream larvae of the *Melanophryniscus tumifrons* group and *M. krauczuki* have in general longer trabecular horns than those of lentic tadpoles, wider and shorter articular processes, and ascending processes attaching to the neurocranium floor in a more acute angle. Interestingly, not all aspects of variation patterns are the same when compared to lentic and lotic tadpoles

of the bufonid *Rhinella*. Whereas stream species of this genus have short articular processes and ascending processes attached in an acute angle, just like in lotic *Melanophryniscus*, trabecular horns are instead, longer in pond *Rhinella* species (Haad, *et al.*, in press). The neurocranium of phytotelm tadpoles is the most different, with short trabecular horns, unusually large otic capsules, and an ascending process almost perpendicular to the axis of the neurocranium. The skeletal anatomy of other, non-bufonid phytotelm tadpoles varies between scarcely modified pond-like morphologies [e.g. *Dendrobates tinctorius* and *Phyllobates bicolor*; Haas (1995)] and highly modified configurations often correlated with ecological specializations [e.g. *Hoplophryne* and *Osteopilus* species; Noble (1929); Lannoo *et al.* (1987)].

The musculature is in general typical of Bufonidae [see a summary in Haas (2003)], with m. mandibulolabialis with a single (inferior) slip, m. subarcualis rectus II–IV invading the branchial septum IV, and mm. interhyoideus posterior and diaphragmatopraecordialis absent. Also, as in other bufonids, the mm. levatores arcuum branchialium I and II are very wide and leave a narrow gap between them. Some characteristics, however, differ from those of other genera within the family, and some are even exclusive to the genus. A functional m. levator mandibulae lateralis is absent in larvae of most species of *Melanophryniscus* and in *Atelopus tricolor* (Haas, 2003), however it is present in other studied Bufonidae. The occurrence of a functional m. levator mandibulae lateralis is also the most widespread morphological condition in Hyloides (Haas, 2003), and thus its absence can represent a putative synapomorphy of Bufonidae. The mandibular branch of the trigeminal nerve is dorsal to the externus group of the mm. levatores mandibulae, but it runs between the superficial and profundus slips in all the remaining bufonids, and it represents a synapomorphy of the sister clade of *Melanophryniscus* (Frost *et al.*, 2006).

Some characters show an interesting interspecific variation within the genus. For instance, in all bufonids studied by Haas (2003), excluding *Barbarophryne brongersmai* but including *Melanophryniscus pachyrhynus* (as *M. orejasmirandai*), the m. subarcualis rectus I has three slips inserting respectively on ceratobranchials I, II, and III. In larvae of four species we studied (*M. macrogranulosus*, *M. paraguayensis*, *M. rubriventris*, and *M. sp. 2* aff. *pachyrhynus*) only two slips are identified, and the ventral one often has fibers that reach both branchial processes II and III. In addition, in most species, the configuration of the m. rectus abdominis is similar to that of *Rhinella* tadpoles, with the muscle extending anteriorly to the level of the branchial basket

(e.g. Vera Candioti, 2007; Aguayo *et al.*, 2009). The m. rectus abdominis is involved in feeding, breathing, and locomotion, by supporting the abdomen and aiding in movements of the ceratobranchials (Gradwell, 1972), and it shows a peculiar organization in several stream tadpoles. It inserts anteriorly on the palatoquadrate or Meckel's cartilage in suctorial *Ascaphus truei* (Gradwell, 1973), *Boophis*, *Hyloscirtus*, *Litoria* (Haas & Richards, 1998), and burrowing *Otophryne robusta* (Wassersug & Pyburn, 1987) and *Leptobranchella mjobergi* (Haas, Hertwig & Das, 2006). Similarly, other species have subbranchial or subquadrate musculature continuous with the m. rectus abdominis, so that the effective insertion of this muscle reaches far rostrally as well (e.g. *Heleophryne*; *Telmatobius*; Noble, 1929; Vera Candioti, 2008). Whereas there are no noticeable differences between the configuration of the m. rectus abdominis in species of the *M. stelzneri* and *M. tumifrons* groups, nor regarding the pond tadpoles of *M. moreirae*, the late development of this muscle in phytotelm species is astonishing and decoupled from that of the rest of the musculature. A functional explanation related to the reduction of movement in confined spaces is tempting but, conversely, Noble (1929) suggested that the prominent rectus abdominis–rectus cervicis complex of *Hoplophryne* and *Osteopilus ocellatus* aids their wiggling locomotion in phytotelm tanks, and Carr & Altig (1992) also found developed mm. rectus abdominis in *Bromeliophyla bromeliacea*. An ecomorphological interpretation, at least as related to locomotion, seems then doubtful.

Finally, within Bufonidae the presence of subhyoid musculature is an apparently exclusive trait of *Melanophryniscus*; these muscles are neither present in any *Rhinella* analysed nor in tadpoles of *Dendrophryniscus* (F. Vera Candioti, unpubl. data). They are fairly conspicuous in all species and visible at least until Stage 44 in metamorphic specimens examined. In *M. pachyrhynchus* larvae, a second, subquadrate muscle pair extends between the articular process and the ventral skin. A similar condition is seen in tadpoles of *Telmatobius* (Telmatobiidae) and large hylids (*Bokermannohyla* and *Hypsiboas* spp.; T. Pezzuti, pers. comm.), but an earlier interpretation of these muscles being branchial muscles interhyoideus posterior or diaphragmatopraecordialis (Vera Candioti, 2008) is unlikely (the latter two are absent in all known bufonids). Conversely, paired subhyoid and subquadrate ligaments have been reported to extend from ceratohyals and palatoquadrates to the ventral skin, with a very similar trajectory and fan-shape (e.g. Noble, 1929). These ligaments are evident in *Rhinella* tadpoles, and particularly developed in gastromyzophorous larvae of *R. quechua* and *Atelopus*

species, where they insert on the dorsal skin of the abdominal sucker (Kaplan, 1997; Aguayo *et al.*, 2009). As the disposition of the subquadrate and subhyoid muscles of *Melanophryniscus* mimics the arrangement of subquadrate and subhyoid ligaments of other bufonids, homology between them seems plausible. The replacement of muscles by connective tissue (e.g. tendons) is common for certain structures and groups (e.g. turtle limbs; Abdala, Manzano & Herrel, 2008) and it confers some mechanical advantages related to elastic storage (e.g. Hildebrand, 1985), but further studies are needed to explore its functional implications. As to their function, these muscles could participate in buccal pumping, or could even assist the m. rectus abdominis by extending its effective insertion rostrally.

FINAL REMARKS

Melanophryniscus shows a remarkable diversity of oviposition strategies that include three of the six known types for Bufonidae (summarized by Van Bocxlaer *et al.*, 2010), namely temporary ponds, small temporary streams, and micro-waterbodies (phytotelm). The intrageneric distribution of these reproductive patterns is complex. Breeding in temporary ponds is found in species of the *M. stelzneri* group, *M. moreirae* (*M. moreirae* group), and in two species unassigned to any group (*M. sanmartini* and *M. langonei*). The lotic pattern is present in the *M. tumifrons* group and *M. krauczuki*. Meanwhile, breeding in phytotelms is shared by some species of the *M. moreirae* group and, presumably, *M. setiba* (an atypical, recently described species; Peloso *et al.*, 2012).

The evolutionary origin of phytotelm breeding in anurans is still poorly understood, and phylogenetic information points out different scenarios for different groups. In dendrobatoid frogs, phytotelm breeding evolved independently three times from species that breed in ground level pools or streams (summarized by Grant *et al.*, 2006), and in mantellines of Madagascar it evolved twice from pond-breeding ancestors (Lehtinen, Richards & Nussbaum, 2004b). Moreover, facultative phytotelm-dwelling within these two groups also seems to be a precursor to obligate phytotelm breeding. The phytotelm *Melanophryniscus* species are included in a clade with a pond-breeding species, *M. moreirae* (D. Baldo *et al.*, unpubl. data), but as this reproductive mode is also likely to be present in *M. setiba* (as inferred from the egg size and number, Peloso *et al.*, 2012), further analysis should be made before conclusions are drawn about its origin within this genus.

Arboreality is often associated with trophic specializations, and macrophagy, oophagy, and endotrophic

development are common in phytotelm species (see Table 1 in Lehtinen *et al.*, 2004a). Regarding bufonids, a detritus-based diet is reported in *Rhinella castaneotica*, macrophagy is suspected in *Mertensophryne*, and non-feeding larvae develop in *Dendrophryniscus*, *Frostius*, *Nectophryne*, and *Pelophryne* (e.g. Izecksohn & Cruz, 1972; Cruz & Peixoto, 1982; Peixoto, 1995; Leong & Teo, 2009; and compilations by Altig & McDiarmid, 1999b, and Lehtinen *et al.*, 2004a). The tadpoles of four species of phytotelm *Melanophryniscus* that we studied feed actively. A quick examination of the gut content of tadpoles of *M. alipioi* and *M. sp. 4* showed mainly rotifers, diatoms, euglenophytes, and arthropod and macrophyte remnants; in *M. vilavelhensis* and *M. sp. 5* we did not dissect the digestive tract, but its content can be hinted at by its green-brownish colour in most parts. Some of these tadpoles (even those at advanced stages, St. 38–39) show yolk remnants inside the gut. Larvae of our fifth species, *M. sp. 3* aff. *vilavelhensis*, were all too young (about St. 30) and their guts were filled only with vitellum. This finding could indicate either that active feeding starts later in phytotelm species as compared with exotrophic tadpoles, or that endotrophy is facultative, as it is known in *Incilius periglenes* and *Rhaebo haematiticus* (Crump, 1989; McDiarmid & Altig, 1990). Nevertheless, the rather typical body proportions, the bufonid-like oral disc, and the overall generalized anatomy, plus an omnivorous diet in these phytotelm tadpoles, highlights the morphological and ecological diversity present in this kind of environments, yet indicates that arboreality could be not as specialized as in other groups (Lannoo *et al.*, 1987).

As with other Bufonidae (Van Bocxlaer *et al.*, 2010), *Melanophryniscus* apparently repeatedly colonizes different types of microhabitats and its tadpole morphology is often strongly associated with the environments where they occur. An evolutionary reconstruction in a comprehensive phylogenetic framework, however, is necessary to elucidate the evolutionary history of morphological traits and their consequences for diversification in *Melanophryniscus*. The basal position of *Melanophryniscus* in Bufonidae underscores the importance of comprehensive data to establish the polarity of the transformation of larval characters and reproductive modes in the Bufonidae ingroup node or nodes close to it. These data would be of great value for the understanding of the evolution of this diverse and cosmopolitan toad family.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

- Appendix S1** Genus *Melanophryniscus* composition, including all species described previously and the six previously undescribed species addressed in this study.
- Appendix S2** Measurements taken from tadpoles of 23 species of *Melanophryniscus*.
- Appendix S3** Oral disc measurements of 23 species of *Melanophryniscus*.
- Appendix S4** Larval musculature of *Melanophryniscus*.
- Appendix S5** Bonferroni's tests results after multivariate analysis of covariance on three oral disc measurements with body length as covariate.