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RESEARCH

Larval morphology of *Frostius pernambucensis* (Anura): contribution of larval characters for the systematics of the family Bufonidae and evolution of endotrophic tadpoles

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Abstract

Frostius pernambucensis is a phytotelm-breeding frog with endotrophic larvae. Although the larvae were formally described, no aspect of its internal morphology is known. In this paper, we re-describe the tadpole based on a large sample, describe its internal anatomy (buccopharyngeal cavity and musculo-skeletal system), provide data on natural history, and discuss the evolution of endotrophy and phytotelma colonization. The tadpoles of *F. pernambucensis* are highly modified, with depressed bodies, reduced mouthparts, and long tails. Many character-states described for these tadpoles can be related to its endotrophic development. Consequence of this highly modified phenotype, we propose several novel putative synapomorphies for the genus: (1) labial tooth row formula 1/1; (2) absence of pustulation in the buccal roof and (3) floor; (4) absence of median ridge; (5) absence of lateral ridge papillae; (6) absence of secretory ridges and pores; (7) absence of filter plates; (7) *m. subarcualis rectus* II–IV originating on ceratobranchial III; (8) *m. subarcualis rectus* II–IV inserting on ceratobranchial I; (8) ventral slip of the *m. subarcualis rectus* I inserting on the ceratobranchial III; (9) suprarostral corpora fused to the *cornua trabeculae*; (10) *commissura quadratoorbitalis* absent; (11) cerabranchial II attached to the *planum hypobranchiale*; and (12) ceratobranchial III attached to the *planum hypobranchiale*. Finally, we also propose that the presence of a single pair of infralabial papilla could represent a synapomorphy of bufonids. The colonization of phytotelma seem to have created a selective pression on the development of *F. pernambucenis*, favoring the evolution of endotrophy.

Keywords Buccopharyngeal cavity · Larval cranium · Larval muscle · Synapomorphies · Systematics

Introduction

The species-rich family Bufonidae Gray, 1825 is a nearly cosmopolitan clade that originated in South American in the Upper Cretaceous and rapidly expanded all over the major continents except the Australo-Papuan region, Madagascar, and Antarctic (Pramuk et al. 2008; Van Bocxlaer et al. 2010; Frost 2023). Within the more than 600 known species, a relevant diversity has been reported, including a great spectrum of reproductive modes, from oviparity with lentic or lotic free-living exotrophic tadpoles, endotrophic tadpoles that develop in phytotelmata, up to viviparous species with direct development (Baldo et al. 2014; Wake 2015; Chandramouli et al. 2016; Liedtke et al. 2022).

Originally associated with the genus *Atelopus* Duméril & Bibron, 1841, the bufonid *Frostius pernambucensis* (Bokermann 1962) was described (as *Atelopus pernambucensis*) for a remnant of the Atlantic Forest in the municipality of Recife, state of Pernambuco, Brazil (Bokermann 1962). After two decades of the species discovery, Cruz and Peixoto (1982) described its tadpole from the type locality, as well as information on spawning and oviposition site. The species is a phytotelm-breeding frog with endotrophic larvae (Cruz and Peixoto 1982). The reproductive differences observed between *F. pernambucensis* and the remaining *Atelopus*

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suggested that they belong to different lineages (Cruz and Peixoto 1982). Later, Cannatella (1986), based on data on adult morphology, larvae, and spawning, proposes the genus *Frostius* to accommodate this species. The genus remained monotypic until the recent description of *F. erythrophthalmus* Pimenta & Caramaschi 2007 for the Atlantic Forest of the municipality of Uruçuca, state of Bahia, Brazil, whose tadpole remains unknown (Pimenta and Caramaschi 2007). Currently, *Frostius* is endemic to the Atlantic Rainforests of northeastern Brazil (Frost 2023). The genus is one of the early divergent lineages within bufonids, being the sister to all bufonids but *Melanophryniscus* (Jetz and Pyron 2018).

Larval morphology information proved to be very important and informative in understanding the taxonomy and the phylogenetic relationships of several anurans (e.g., Noble 1929; Orton 1953; Maglia et al. 2001; Haas 2001, 2003; Pugener et al. 2003; Dias et al. 2021, 2023), but data are lacking for several taxa. This is the case of *Frostius pernambucensis*; even though larval morphology was pivotal for the generic classification of the species, since the original tadpole description no further studies were performed, and aspects of its internal anatomy remain unknown. Moreover, the tadpole of *F. pernambucensis* was characterized based on seven individuals in early developmental stages and one egg from the same spawning raised in laboratory, and thus, several important characters were not observed or described. Recently, Dubeux et al. (2020a) provided a brief characterization for the tadpoles of *F. pernambucensis* for state of Alagoas, aiming a generic identification, but not addressing other morphological aspects of the tadpole. The fact that the larvae of *F. pernambucensis* are endotrophic (Cruz and Peixoto 1982) is contrasting with most of bufonids, making it a very intriguing piece in the puzzled evolutionary history of the family.

Given the above, a more detailed approach is needed to understand the evolution of the unique tadpoles of *Frostius pernambucensis*. Herein, we present a complete redescription of external morphology and the first description of internal anatomy of tadpole of this species. Additionally, we discuss the importance of larval characters in the systematics of bufonids and address the evolution of endotrophic tadpoles.

Materials and methods

Tadpoles were collected in Atlantic Forest remnants in the state of Pernambuco and Alagoas (Fig. 1). The three first lots (MHNUFAL 14572, 16192, 16196) are from *Estação Ecológica de Murici*, municipality of Murici, state of Alagoas, Brazil, 167 km south-west from the type locality of species (35° 52′ 41.50" W; 9° 12′ 54.74" S, 462 m a.s.l.; Fig. 1). Tadpoles were collected in July 2015, June, and July 2021, respectively. The other two lots (MHNUFAL 17359, 17360) are from *Reserva Particular do Patrimônio Natural Eco Fazenda Morim*, municipality of São José da Coroa Grande, state of Pernambuco, Brazil, 99 km south from the type locality of species (8° 52′ 10.46" S, 35° 12′ 28.57" W; 92 m a.s.l.), and were collected in May 2022.

The analyzed specimens are deposited in the herpetological collection of the Museu de História Natural of the Universidade Federal de Alagoas (MHNUFAL). Additionally, we also analyzed the original series of larval description of the species (EI 7253; Cruz and Peixoto 1982).

External morphology

Measurements were made in 17 tadpoles in stages 37 to 41 (Gosner 1960). Five of these tadpoles (stage 38) were used for qualitative description (MHNUFAL 16192, 16196). Measurements followed Altig and McDiarmid (1999): body length (BL), maximum tail height (MTH), tail length (TaL), tail muscle height (TMH), tail muscle width (TMW), and total length (TL); Lavilla and Scrocchi (1986): body width at eye level (BWE), body width at nostril level (BWN), extranarial distance (EnD), extraorbital distance (EoD), eye diameter (ED), intranarial distance (InD), intraorbital distance (IoD), maximum body height (MBH), maximum body width (MBW), narial diameter (ND), oral disc width (ODW), snout-spiracle distance (SSD), and spiracle-posterior body distance (SPD); and Grosjean (2005): dorsal fin height (DFH) and ventral fin height (VFH).

All measurements were taken using an ocular micrometer installed on a Leica® MZ6 stereomicroscope, except for TL, which was measured with digital calipers (0.1 mm accuracy). Terminology follows Altig and McDiarmid (1999). For color descriptions, we used the terminology proposed by Köhler (2012) with their corresponding color codes.

Buccopharyngeal cavity

To study buccopharyngeal anatomy, we dissected two tadpoles in Gosner stages 30 and 31 according to Wassersug (1976) and, after inspection using a stereoscopic microscope, we submitted them to the protocol of Alcalde and Blotto (2006) for scanning electron microscopy (SEM). Terminology of buccopharyngeal features follows Wassersug (1976, 1980).

Musculo-skeletal system

To study musculo-skeletal system, six tadpoles in Gosner stages 33 and 41 were processed according to the clearing and double staining protocol of Dingerkus and Uhler (1977); for two individuals, the procedure was interrupted after the alcian blue step, and specimens were manually dissected for

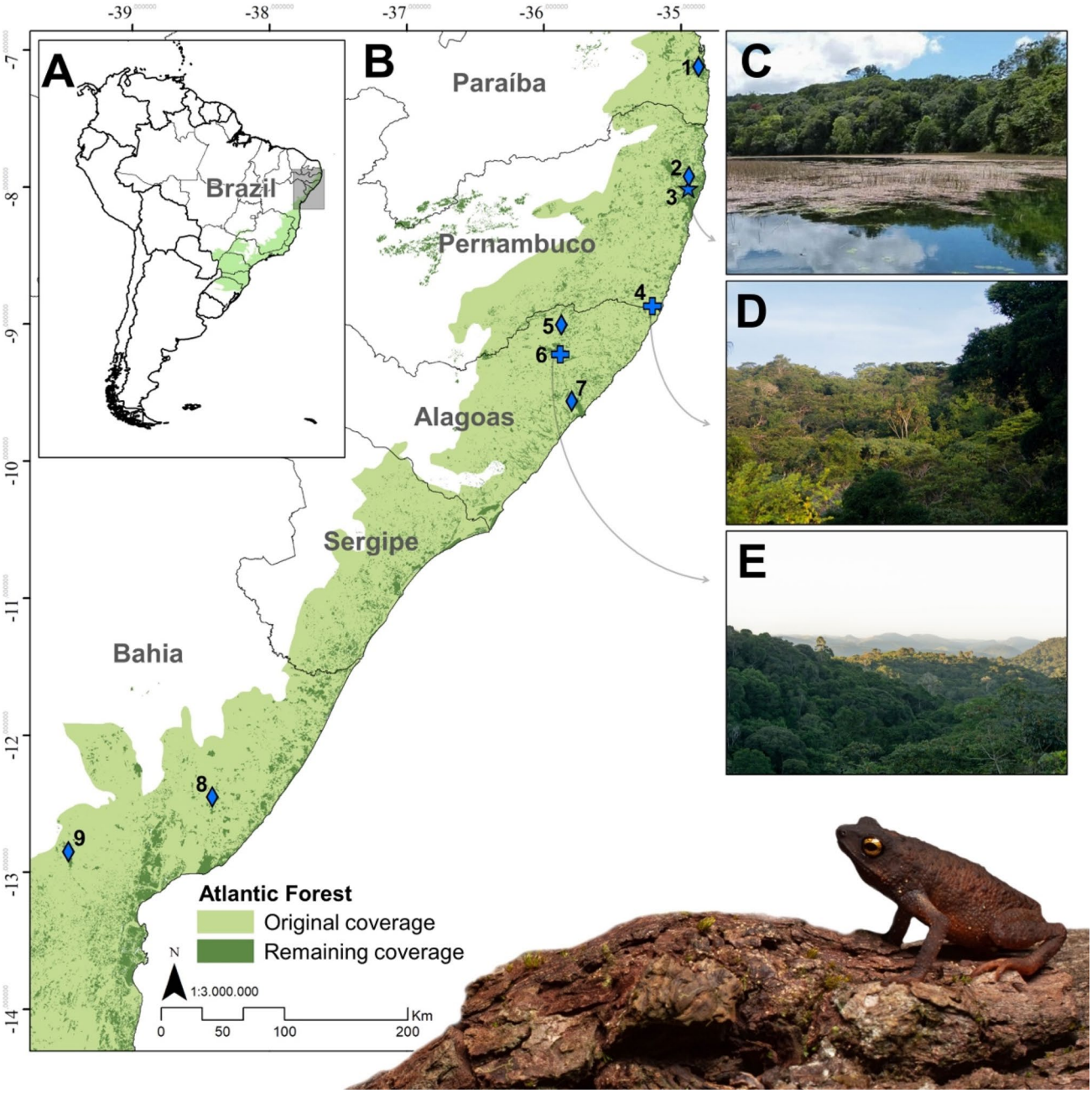

Fig. 1 Geographic distribution of the *Frostius pernambucensis* highlighting the areas in which the tadpoles analyzed in this study were collected (cross and star). **A** Inset map: South America, highlighting the distribution of the Atlantic Forest (green) and the magnified area showed in A (gray rectangle). **B** Known localities of the species (diamonds) and its type locality (star). **C** Panoramic view of *Parque Estadual Dois Irmãos*, municipality of Recife, state of Pernambuco. **D** Panoramic view of *Reserva Particular do Patrimônio Natural Eco Fazenda Morim*, municipality of São José da Coroa Grande, state of Pernambuco. **E** Panoramic view of *Estação Ecológica de Murici*, municipality of Murici, state of Alagoas. List of locations and references: 1 = Paraíba: João Pessoa (Pimenta and Caramaschi 2007); 2 = Pernambuco: Paulista (Barbosa et al. 2017); 3 = Pernambuco: Recife (Bokermann 1962); 4 = Pernambuco: São José da Coroa Grande (*present study*); 5 = Alagoas: Ibateguara (Pimenta and Caramaschi 2007); 6 = Alagoas: Murici (Pimenta and Caramaschi 2007); 7 = Alagoas: Maceió (Dubeux et al. 2020b); 8 = Bahia: Varzedo (Juncá and Borges 2002); 9 = Bahia: São Sebastião do Passé (Freitas et al. 2019)

inspection of cranial muscles. We employed lugol solution to aid in the identification of muscles. The protocol was carried out until the end for the remaining individuals aiming to study the larval cranium. Terminology for the musculoskeletal elements is that of Haas (1995, 2001, 2003).

Phylogenetic relationships and character optimization

Besides *Frostius pernambucensis*, we examined tadpoles of other representatives of several genera of Neotropical bufonids (see complete list of examined material in the Appendix 1) to investigate character evolution within the family; our sampling was complemented with information available in the literature. We based our taxon sampling in the phylogenetic hypothesis of Jetz and Pyron (2018) which has a dense taxonomic sampling, although *F. pernambucensis* is not included in it. Besides Cannatella`s (1986) hypothesis, *F. pernambucensis* were never included in another phylogenetic hypothesis and the monophyly of the genus have never been tested. Nevertheless, based on adult morphology and behavior, we assumed the monophyly of the genus and the sister relationship of *F. pernambucensis* and *F. erythrophthalmus*—diagnostic characters, such as the iris coloration have been demonstrated polymorphic within *F. erythrophthalmus*, and all call parameters evaluated so far are overlapping between the two species (Juncá et al. 2012) rendering both species phenotypically very similar—for comparisons and optimizations purposes. We do not claim that we have an exhaustive sampling of bufonids, for that would be beyond of the scope of this paper; we recognize the extensive variation of bufonids, and we claim for further studies with deeper taxon and character sampling. Our analysis represents an initial step toward a better understanding of the evolution of bufonid larvae. Finally, the lack of data for several basal lineages, such as *Oreophrynella*, prevent an unambiguous optimization of some characters.

We delimited 25 transformation series (Hennig 1960; Grant and Kluge 2004) to describe the variation observed (Appendix 2), focusing on character-states of *Frostius pernambucensis*. The character matrix was constructed in Mesquite V. 3.51 (Maddison & Maddison 2018). These characters were optimized using parsimony as a criterion (Fitch 1971) into the topology of Jetz and Pyron (2018) using T.N.T.v. 1.5 (Goloboff & Catalano 2016). This tree was pruned to reflect the relationships among taxa for which characters were scored, focusing on the generic-level relationships; notwithstanding, we kept taxa for which no data are available to highlight the parts of the bufonids tree of life that require more studies on larval morphology. Most of the characters in our matrix are invariable within each genus, and most of the unique states are restricted to *Frostius pernambucensis*. We complemented our observations with information available in the literature. Finally, it is worthy to stress that our analysis is exploratory intended to identify some interesting states and major evolutionary patters in *Frostius* evolution; future studies including a more detail taxonomic sampling and phylogenetic analysis are required to test our propositions.

Natural history

We provide comments on the natural history and breeding behavior of *Frostius pernambucensis* based on field observations. Additionally, we briefly comment on the egg clutch based on field and laboratory observations (MHNUFAL 17359; collected at Reserva Particular do Patrimônio Natural Eco Fazenda Morim).

Results

External morphology

Total length 17.21 ± 1.06 mm (15.65–18.33 mm; $n = 5$; stage 38; Fig. 2). Body ovoid in dorsal and ventral views (MBW/BL = 0.52–0.58), depressed and globular in lateral view (MBH/MBW = 0.76–0.86). Ventral contour of body sloping, slightly concave in peribranchial region and convex in abdominal region. Body length about 1/4 of total length (BL/TL = 0.26–0.32); maximum body width on middle of body and maximum body height on its end portion. Snout oval in dorsal view and truncate in lateral view, slightly sloping. Eyes located dorsally and directed laterally, representing nearly 32% of intraorbital distance (ED/IoD = 0.30–0.37) and 24% of maximum body width (ED/MBW = 0.23–0.27). Nostrils dorsal and directed frontolaterally, with external opening reniform (Fig. 2F), and a small cutaneous projection (apophysis) on its inner margin; each nostril represents about 14% of intranarial distance (ND/InD = 0.14), 4% of maximum body width (ND/MBW = 0.04) and located closer the eyes than the tip of snout. Oral disc equivalent to 36% of maximum body width (ODW/MBW = 0.34–0.38), ventral, arched-shape, lips modified in a thick dermal fold laterally interrupted (Fig. 2D). Marginal papillae present, short and blump; submarginal papillae absent. Labial tooth row formula (LTRF) 1/1; A1 arched, P1 U-shaped, and shorter in length than A1; large and sparse labial teeth, 24–26 labial teeth in A1 and 8–10 in P1. Labial teeth composed of a basal sheath and convex head with poorly developed cusps (Fig. 3). Jaw sheaths with only the terminal portion pigmented; upper jaw sheath arc-shaped and finely serrated, lower jaw sheath open U-shaped and finely serrated, more robust than the upper jaw. Spiracle sinistral (Fig. 2E), located below the body midline, opening on middle third of body (SPD/SSD = 0.63–0.71), directed posterodorsally, forming an angle of approximately 45° with the longitudinal body axis, visible from ventral view. Inner wall free from body and external wall slightly shorter than inner wall. Vent tube with medial opening (Fig. 2G and H), longer than wide, with both walls attached directly to the ventral fin, except the tip, which is free. Tail length approximately 72% of total length (TaL/TL = 67.92–74.09), higher at the middle of tail;

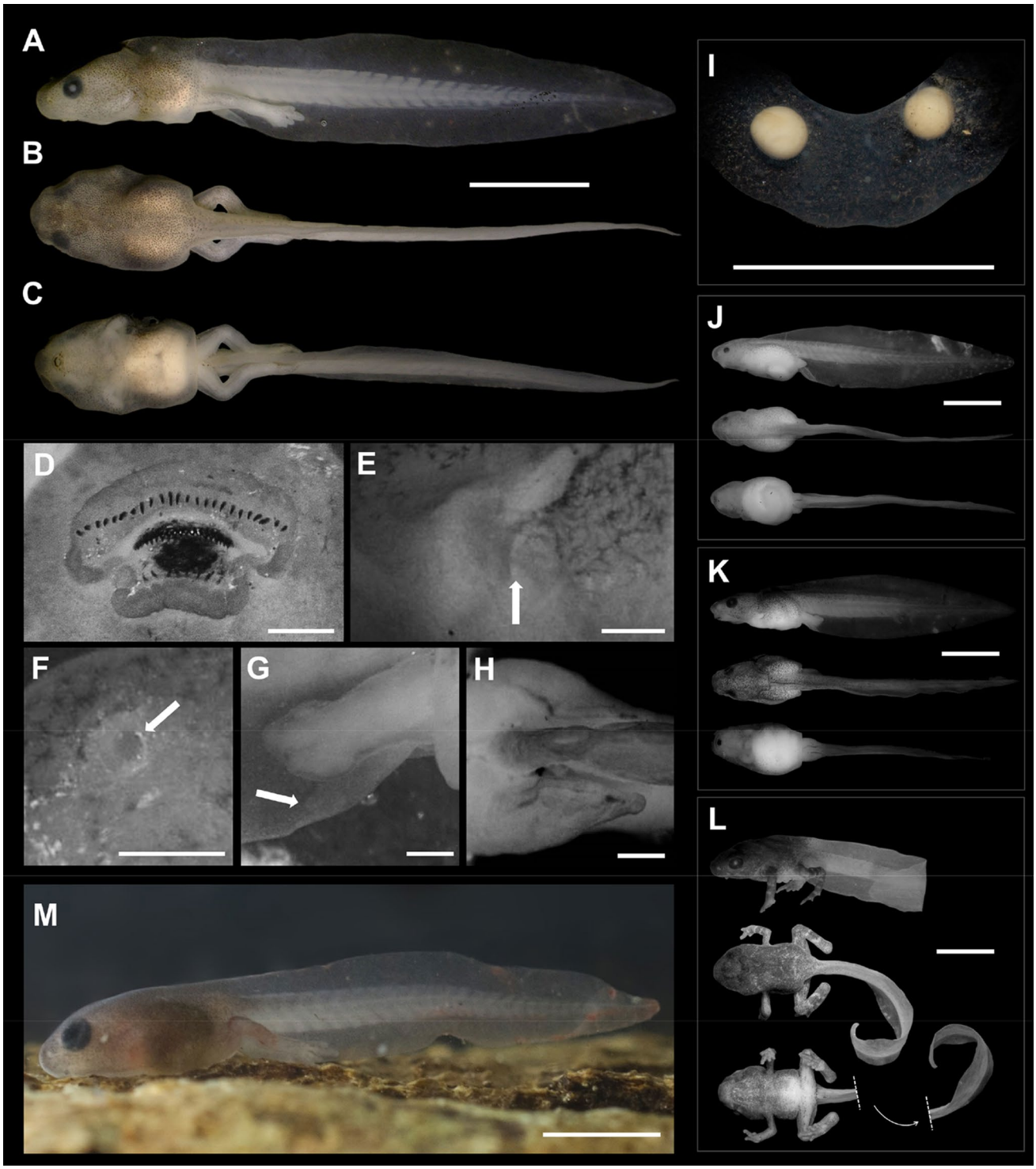

Fig. 2 Tadpole of *Frostius pernambucensis*, stage 36 (MHNUFAL 16196), in **A** lateral, **B** dorsal, and **C** ventral views. **D** Detail of the oral disc. **E** Detail of the spiracle. **F** Detail of the narial aperture. Detail of the vent tube in **G** lateral (right side) and **H** ventral views. Details of the eggs of *F. pernambucensis* (**I**). Lateral, dorsal, and ventral views of tadpoles of *F. pernambucensis* in stages (**J**) 28, (K) 32 and (L) 42 (MHNUFAL 16196). **M** Tadpole of *F. pernambucensis*, stage 37 (MHNUFAL 16196), in life. Scale bars = 3 mm (**A**–**C**, **I**–**L**), and 0.3 mm (**D**–**H**)

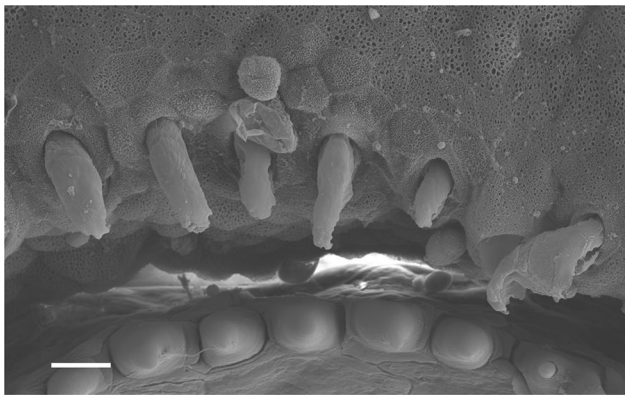

Fig. 3 Labial teeth of *Frostius pernambucensis* (EI 7253) at stage 30. Scale bar = 10 µm

maximum height corresponding to half the height of body (MBH/MTH = 0.74–0.85). Tail tip acute. Maximum tail musculature height less than 1/3 of the maximum height of tail (TMH/MTH = 0.33–0.37), becoming progressively thinner posteriorly and extending to tail tip. Myosepta partially visible from half of its length. Dorsal fin height 32% of tail height (DFH/MTH = 0.30–0.37) and slightly higher than ventral fin (VFH/DFH = 0.30–0.33). Dorsal fin beginning at the end of body, with contour parallel to caudal musculature in almost all its length, slightly arched in its second half, maximum higher at the beginning of the last third. Ventral fin beginning at the base of tail, with origin concealed by vent tube and contour parallel to the caudal musculature; maximum height at first third of tail. Measurements are presented in Table 1.

Coloration

In life, body Drab-Gray (256), covered by numerous Fawn Color (258) dots (Fig. 2L). Due to its translucency, in dorsal view, it is possible to observe Tawny Olive (17) color of the calf on lateral margins of the second half of body, as well as Vandyke Brown (281) color in inner part of the eyeballs. In ventral view, Fawn Color (258) points restricted to lateroventral surfaces, becoming less evident in the medial portion of belly. Belly Pale Buff (1), and evident calf in its posterior portion. In lateral view, posterior portion of body darker, Fawn Color (258). Translucent fins color Light Sky Blue (191) and caudal musculature opaque, colors similar to fins. Myosepta partially visible. In preservative, color pattern is maintained, background becomes Pale Horn Color (11) and dots distributed on the body become Dark Brownish Olive (127) (Fig. 2A–C).

Table 1 Measurements (in mm) of *Frostius pernambucensis* tadpoles

Stages	37	38 (n = 5)	39 (n = 3)	40 (n = 3)	41 (n = 5)
TL	16.18	17.21 ± 1.06 (15.65–18.33)	16.67 ± 1.47 (15.04–17.9)	16.47 ± 0.98 (15.39–17.3)	15.81 ± 1.17 (14.52–17.7)
BL	4.98	4.8 ± 0.13 (4.69–5.02)	4.92 ± 0.31 (4.56–5.11)	4.6 ± 0.56 (4.09–5.2)	4.86 ± 0.21 (4.56–5.08)
TaL	11.2	12.41 ± 1.15 (10.63–13.58)	11.75 ± 1.17 (10.48–12.8)	12.37 ± 0.38 (12.1–12.64)	10.96 ± 0.99 (9.96–12.62)
MBH	2.44	2.16 ± 0.12 (2.05–2.34)	2.06 ± 0.01 (2.05–2.06)	2.2 ± 0.05 (2.15–2.25)	2.04 ± 0.12 (1.92–2.16)
MBW	2.81	2.69 ± 0.05 (2.61–2.73)	2.59 ± 0.15 (2.42–2.68)	2.68 ± 0.12 (2.57–2.8)	2.68 ± 0.19 (2.42–2.85)
TMW	0.92	0.9 ± 0.05 (0.82–0.92)	0.89 ± 0.06 (0.82–0.92)	0.85 ± 0.06 (0.82–0.92)	0.82 ± 0.00 (0.82–0.82)
DFH	0.92	0.9 ± 0.09 (0.82–1.02)	0.92 ± 0.00 (0.92–0.92)	0.85 ± 0.06 (0.82–0.92)	0.88 ± 0.09 (0.82–1.02)
TMH	0.92	1.00 ± 0.05 (0.92–1.02)	0.99 ± 0.06 (0.92–1.02)	0.95 ± 0.06 (0.92–1.02)	0.94 ± 0.05 (0.92–1.02)
VFH	0.92	0.88 ± 0.06 (0.82–0.92)	0.82 ± 0.00 (0.82–0.82)	0.82 ± 0.00 (0.82–0.82)	0.84 ± 0.05 (0.82–0.92)
BWN	1.02	0.88 ± 0.09 (0.72–0.92)	0.99 ± 0.06 (0.92–1.02)	0.92 ± 0.00 (0.92–0.92)	0.94 ± 0.05 (0.92–1.02)
BWE	1.94	2.00 ± 0.06 (1.94–2.04)	2.04 ± 0 (2.04–2.04)	2.04 ± 0.00 (2.04–2.04)	1.98 ± 0.06 (1.94–2.04)
MTH	2.76	2.78 ± 0.05 (2.76–2.86)	2.72 ± 0.06 (2.66–2.76)	2.62 ± 0.12 (2.55–2.76)	2.66 ± 0.1 (2.55–2.76)
EnD	1.02	0.88 ± 0.09 (0.72–0.92)	0.99 ± 0.06 (0.92–1.02)	0.92 ± 0.00 (0.92–0.92)	0.94 ± 0.05 (0.92–1.02)
InD	0.72	0.72 ± 0.00 (0.72–0.72)	0.72 ± 0.00 (0.72–0.72)	0.75 ± 0.06 (0.72–0.82)	0.69 ± 0.05 (0.61–0.72)
EoD	1.94	2 ± 0.06 (1.94–2.04)	2.04 ± 0.00 (2.04–2.04)	2.04 ± 0.00 (2.04–2.04)	1.98 ± 0.06 (1.94–2.04)
IoD	0.92	0.96 ± 0.06 (0.92–1.02)	0.99 ± 0.06 (0.92–1.02)	0.89 ± 0.12 (0.82–1.02)	0.9 ± 0.09 (0.82–1.02)
ED	0.72	0.65 ± 0.06 (0.61–0.72)	0.68 ± 0.06 (0.61–0.72)	0.61 ± 0.00 (0.61–0.61)	0.65 ± 0.06 (0.61–0.72)
ND	0.10	0.1 ± 0.00 (0.1–0.1)	0.1 ± 0.00 (0.1–0.1)	0.1 ± 0.00 (0.1–0.1)	0.1 ± 0.00 (0.1–0.1)
ODW	1.02	0.98 ± 0.06 (0.92–1.02)	0.99 ± 0.06 (0.92–1.02)	0.95 ± 0.06 (0.92–1.02)	0.98 ± 0.09 (0.92–1.12)
SSD	3.04	2.82 ± 0.15 (2.71–3.08)	2.91 ± 0.26 (2.62–3.07)	2.73 ± 0.56 (2.15–3.26)	2.98 ± 0.18 (2.82–3.24)
SPD	1.94	1.98 ± 0.06 (1.94–2.04)	2.01 ± 0.06 (1.94–2.04)	1.87 ± 0.12 (1.74–1.94)	1.88 ± 0.12 (1.74–2.04)

Data presented as mean ± SD (range)

Stages according to Gosner (1960)

Variation

Tadpoles show little variation in relation to morphological characteristics analyzed. However, when compared to more initial stages of development, a progressive change in body's coloration in life is observed, ranging from a clear translucent tone in Sulphur Yellow (80) and very evident calf in initial stages to a dark tone in Dark Lavender (203) and calf almost imperceptible in metamorphic stages. The eyes also develop becoming larger as development progresses. It is worth noting that tadpoles at stage 26 already have a total length similar to those in final stages of development (~ 16 mm); all growth is post-metamorphic (Fig. 2I–K; Table 1).

Buccopharyngeal cavity (Fig. 4)

Buccal roof

Buccal roof elliptical (Fig. 4A). Obtuse, medial, short papilla present in the prenarial arena (Fig. 4C). Internal nares elliptical, sagittally oriented in an angle of 60°; posterior valve free (Fig. 4D). Pre and postnarial papillae absent. Median ridge absent. Buccal roof arena absent. Buccal roof devoid

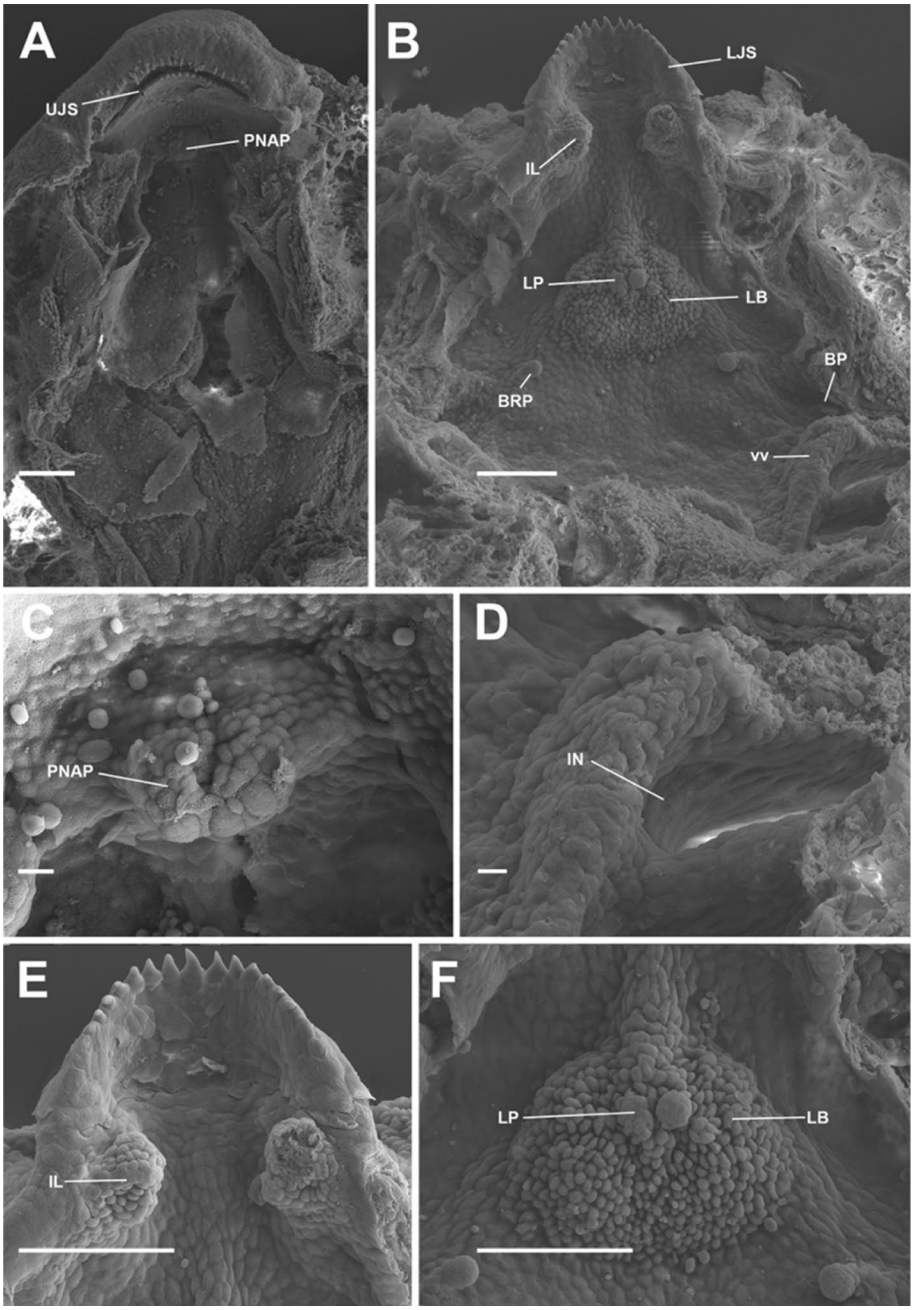

Fig. 4 Buccopharyngeal cavity of a tadpole of *Frostius pernambucensis* (EI 7253) at stage 30. **A** Buccal floor, **B** buccal roof, and **C** details of the prenarial arena papilla, **D** internal nares, **E** infralabial papillae, and **F** lingual papillae. *BP* buccal pocket, *BRP* buccal roof papilla, *IL* infralabial papillae, *IN* internal nares, *LB* lingual bud, *LJS* lower jaw sheath, *LP* lingual papillae, *PNAP* prenarial, *UJS* upper jaw sheath. Scale bars = 100 µc (**A**, **B**, **E**, **F**), and 20 µm (**C**, **D**)

of pustulation and papillae. Dorsal velum poorly developed, V-shaped, medially interrupted.

Buccal floor

Buccal floor triangular (Fig. 4B). Single pair of short, blunt, infralabial papillae (Fig. 4E). Lingual bud rounded; three short, round, lingual papillae (Fig. 4F). Buccal floor arena absent; single buccal floor papillae present above the buccal pocket line. Prepocket pustulation and papillae present. Buccal pockets deep, perforated, oblique, slit-shaped. Ventral velum present, reduced, inconspicuous; spicular support inconspicuous; secretory pits absent; secretory ridges absent; marginal projections absent; medial notch absent. Glottis fully exposed. Branchial basket reduced, semi-circular; filter cavities reduced; filter plates absent.

Table 2 Patterns of origin and insertion of larval muscles of *Frostius pernambucensis*

Muscle	Origin	Insertion
Mandibular group, n. trigeminus (c.n. V) innerved		
Levator mandibulae longus superficialis	External posterior margin subocular arc	Dorsomedial Meckel`s cartilage
Levator mandibulae longus profundus	External margin (curvature) subocular arc	External margin of the suprarostral alae
Levator mandibulae externus	Inner *processus muscularis* (superior)	Suprarostral alae
Levator mandibulae articularis	Inner *processus muscularis* (inferior)	Dorsal Meckel`s cartilage
Levator mandibulae laterais	–	–
Submentalis (intermandibularis anterior)	–	–
Intermandibularis	Median aponeurosis	Ventromedial Meckel`s cartilage
Mandibulolabialis	–	–
Levator mandibulae internus	Ascending process	Meckel`s cartilage
Hyoid group, n. facialis (c.n. VII)		
Hyoangularis	Dorsal ceratohyal	Retroarticular process of Meckel`s cartilage
Quadratoangularis	Ventral palatoquadrate	Retroarticular process of Meckel`s cartilage
Suspensorioangularis	Ventral palatoquadrate	Retroarticular process of Meckel`s cartilage
Orbitohyoideus	*Processus muscularis*	Lateral edge of ceratohyal
Suspensoriohyoideus	Posterior descending margin of *processus muscularis* and subocular arc	Lateral process of ceratohyal
Interhyoideus	Median aponeurosis	Ventral ceratohyal
Branchial group, n. Glossopharyngeus (c.n. IX) and vagus (c.n. X)		
Levator arcuum branchialium I	Lateroventral otic capsule	Ceratobranchial I
Levator arcuum branchialium II	Lateroventral otic capsule	Ceratobranchial II
Levator arcuum branchialium III	Lateroventral otic capsule	Ceratobranchial III
Levator arcuum branchialium IV	Lateroventral otic capsule	Ceratobranchial III
Tympanopharyngeus	Lateroventral otic capsule	Ceratobranchial III
Dilator laryngis	–	–
Constrictor branchialis I	–	–
Constrictor branchialis II	*Processus branchialis* II	*Commissura terminalis* I
Constrictor branchialis III	*Processus branchialis* II	*Commissura terminalis* II
Constrictor branchialis IV	–	–
Subarcualis rectus I	Posterior lateral base of ceratohyal	*Processus branchialis* II (two slips) and ceratobranchial I
Subarcualis rectus II-IV	Ceratobranchial III	Ceratobranchial I
Subarcualis obliquus II	*Processus urobranchialis*	Ceratobranchial II
Diaphragmatobranchialis	–	–
Spinal group, spinal nerve innervation		
Geniohyoideus	Hypobranchial plate	Infrarostral cartilage
Rectus abdominis	Peritoneum (diaphragm)	Abdominal wall
Rectus cervices	Peritoneum (diaphragm)	*Processus branchialis* II

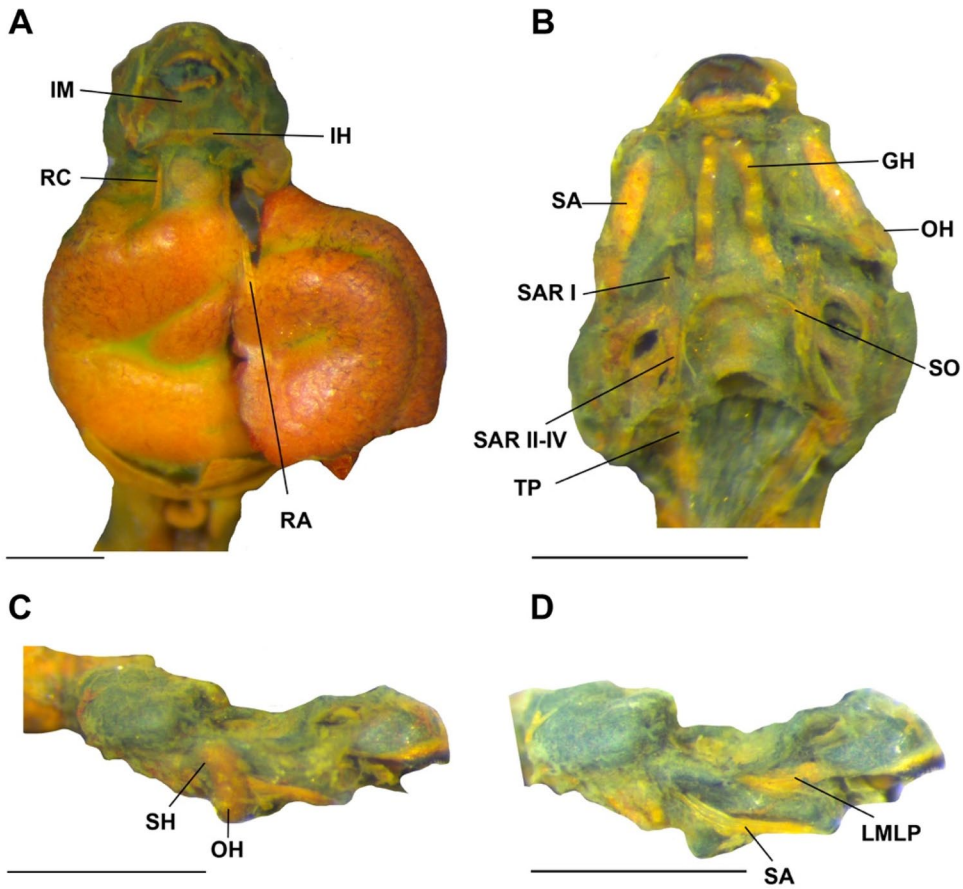

Fig. 5 Larval musculature of *Frostius pernambucensis* at stage 33 (MHNUFAL 16196). **A**–**B** Ventral view and **C–D** lateral view. *GH* geniohyoideus, *IH* interhyoideus, *IM* intermandibularis, *LMLP* levator mandibulae longus profundus, *OH* orbitohyoideus, *RA* rectus abdominis, *RC* rectus cervicis, *SA* suspensorioangularis, *SH* suspensoriohyoideus, *SAR I* subarcualis recuts I, *SAR II–IV* subarcualis rectus II–IV, *SO* subarcualis obliquus, *TP* tympanopharyngeus. Scale bars = 1.0 mm

Musculature

We observed 22 muscles in the larvae of *Frostius pernambucensis* (Table 2; Fig. 5). Some muscles typically observed in other tadpoles, such as *levator mandibulae lateralis* (Haas 2003), were not observed; we stress the lack of this muscle is likely to be an observational problem rather than the loss of the muscle in the species. We suggest that future studies incorporating other techniques, such as histology and immunohistochemistry, could aid in the detection of these small muscles. Nevertheless, it is interesting noting the shift of some muscles, such as the *levator arcuum branchialium* IV, that inserts on the ceratobranchial III, the *subarcualis rectus* I that has two slips inserting on the *processus branchialis* II, and the *suspensioangularis* originating ventrally in the palatoquadrate.

Larval cranium

Neurocranium triangular; greatest width at otic capsule level (Fig. 6). Suprarostral cartilage (Fig. 6C) formed by two elements, suprarostral alae and corpus completely fused, although the line of fusion seems to be recognizable; dorsal posterior process of the suprarostral ala present, suprarostral corpora fused distally. Suprarostral corpus fused to the *cornua trabeculae*. Ethmoidal region short; *cornua trabeculae* short, parallel to each other and fused terminally with the suprarostral corpus. Basicranial fenestra open. Frontoparietal fenestra large, rectangular. Orbital cartilage (Fig. 6d) poorly developed, not reaching the otic capsule (*foramen prooticum* open). *Capsula auditiva* robust, large, rhomboidal in dorsal view, representing about 40% of chondrocranium length. Palatoquadrate thin in lateral view (Fig. 6C), attached to neurocranium through a wide anterior *commissura quadratocranialis* and an almost perpendicular *processus ascendens* posteriorly; *processus ascendens* with a high (sensu Haas 2003) attachment. *Processus muscularis quadrati* triangular, poorly developed, and curved dorsomedially. *Commissura quadratoorbitalis* absent. In the lower jaw (Fig. 6E), Meckel's cartilage sigmoid, transversely oriented, almost perpendicular to the chondrocranium longitudinal axis. Infrarostral cartilages rectangular in frontal view, curved, joined at the symphysis.

Ceratohyals (Fig. 6F) short, flat, and subtriangular; anterior margin with poorly developed anterior and anterolateral processes, posterior processes triangular. Ceratohyals confluently joined by a chondrified *pars reuniens*. Basibranchial rectangular, with rounded *processus urobranchialis* present;

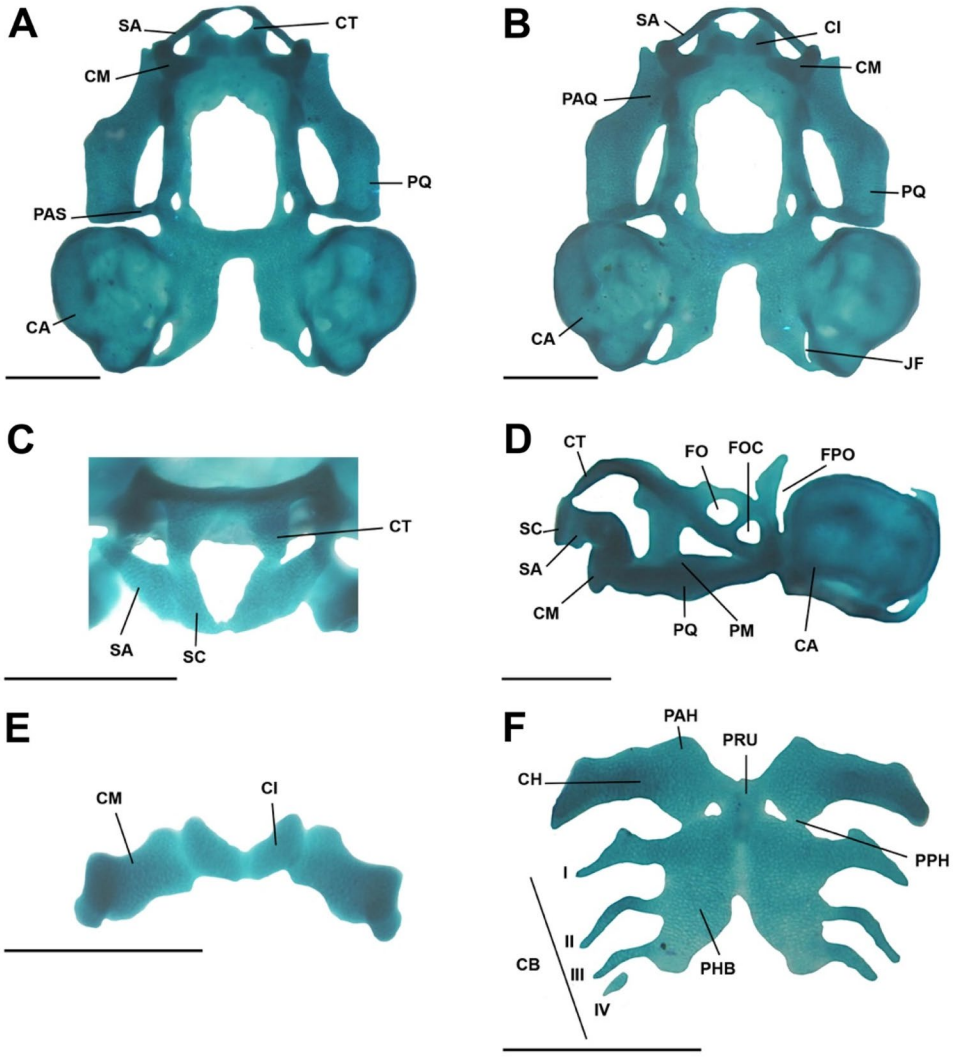

Fig. 6 Larval cranial skeleton of *Frostius pernambucensis* at stage 41 (MHNUFAL 16196). **A** Dorsal view; **B** ventral view; **C** detail of the suprarostral cartilage; **D** lateral view; **E** detail of the lower jaw; **F** hyobranchial apparatus. *CA* capsula auditiva, *CB* ceratobranchial, *CI* cartilago infrarostralis, *CM* cartilago Meckeli, *CT* cornua trabeculae, *FO* foramen opticum, *FOC* foramen oculomotorium, *FPO* foramen prooticum, *PAH* processus anterior hyalis, *PAQ* pars articularis quadrati, *PAS* processus ascendens, *PHB* planum hypobranchiale, *PM* processus muscularis, *PRU* pars reuniens, *SA* suprarostral ala, *SC* suprarostral corpus. Scale bars = 0.5 mm

basihyalis absent. Hypobranchial plates long, rectangular, free from each other. Branchial basket with four curved ceratobranchials, lacking lateral projections; ceratobranchials I, II, and III attached to the hypobranchial plate; ceratobranchial IV reduced, free. Ceratobranchial I with a triangular anterior *processus branchialis*. Spicules absent.

Natural history notes

Spawns and tadpoles of *Frostius pernambucensis* were observed both in hollow trees ($n = 7$) and in bromeliads ($n = 2$) containing accumulated rainwater. All reproductive sites were inside the forest, at least 200 m from the nearest edge, varying between 60 and 150 cm in height (Fig. 7). Males of *F. pernambucensis* were observed close to or inside the reproductive sites in almost all visits, many times in vocalization activity (during the night or late afternoon and on rainy days) and showed parental care behaviors during the collection of larvae.

All clutches found had a large number of eggs and/or tadpoles (~ 20 to 50 individuals), even in reproductive sites with reduced space, such as in bromeliads (Fig. 7D and E; see a video of the density of tadpoles in the same bromeliad in Supplementary Material 1). The clutches (Fig. 2I) consist of gelatinous string of yellowed eggs; the entire string measured 4.4 cm on average (4.3–4.5 ± 0.08; $n = 5$), accommodating eggs of 2.0 mm (1.8–2.3 ± 0.13; $n = 10$).

Some clutches had tadpoles in early (newly hatched) and advanced (above stage 38) stages of development, indicating that the same reproductive site was used simultaneously more than once. The reuse of the same reproductive site consecutive times during the reproductive period was observed, with different spawns observed in June, July, and August 2021. During a visit to a tree hollow whose tadpoles were in an advanced stage of development, the behavior of cannibalism was observed, where some individuals feeding on two dead tadpoles, which already had most of the tail devoured.

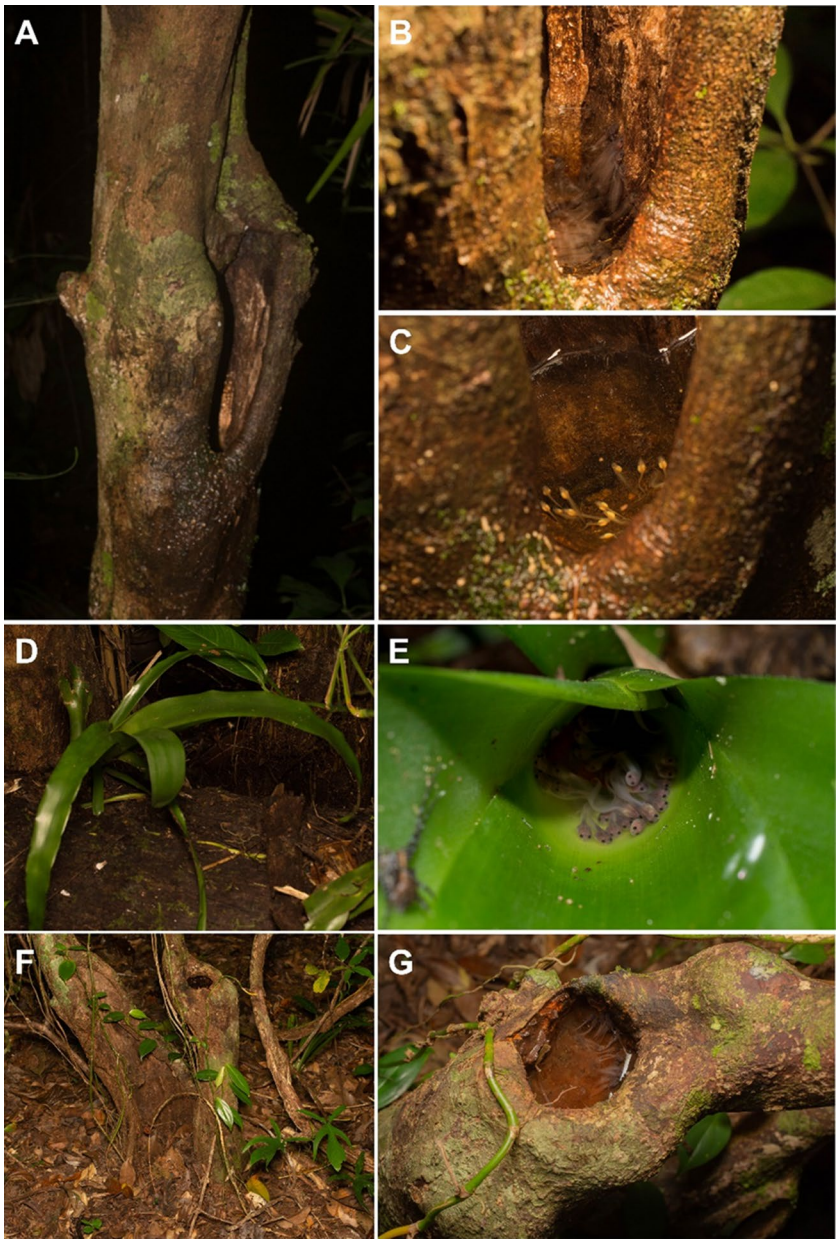

Fig. 7 Breeding sites of *Frostius pernambucensis* in Estação Ecológica de Murici, municipality of Murici, Alagoas state. **A–C** Different spawning developing in the same tree hollow with water accumulated at about 150 cm in height. **D–E** Spawn developing in the central cup of an epiphytic bromeliad with water accumulated at about 100 cm in height (see a video in Supplementary Material 1). **F–G** Spawn developing in tree hollow with water accumulated at about 60 cm in height

Discussion

Larval morphology and the systematics of bufonids

More than 65% of known genera of bufonids have information on larval morphology, showing high phenotypic and ecological diversity (e.g., Duellman and Lynch 1969; Lamotte and Xavier 1972; Müller et al. 2005; Haas et al. 2009; Meegaskumbura et al. 2015; Viertel and Channing 2017; Müller 2019; Romero-Carvajal et al. 2023), with many larval characters presenting interesting evolutionary history and phylogenetic significance (Channing 1978; Haas 2003; Frost et al. 2006; Hirschfeld et al. 2012). Very few larval external features of *Frostius pernambucensis* support the notion that the species is part of the family Bufonidae, such as the eyes placed dorsally, the medial vent tube, and the sinistral spiracle (located in general below the medial line). On the other hand, many features are unique to *F. pernambucensis* tadpoles, probably related to its phytotelmata habits and endotrophic development (discussed below), suggesting putative synapomorphies—although the lack of information for its putative sister species, *F. erythrophthalmus*, and some other lineages of bufonids, precludes unambiguous optimizations.

The majority of bufonids tadpoles are characterized by a tail of moderated length that ends in a rounded tip. Moreover, the oral disc is in general emarginated, with marginal papillae absents dorsally and ventrally (putative synapomorphy of the family; Haas 2003) and a standard

LTRF 2/3 (e.g., McDiarmid and Altig 1990; Inchaustegui et al. 2014; Vera Candioti et al. 2020). The long tail (representing ¾ of the total length) and the acute tail tip are two features that seems singular to *F. pernambucensis,* standing out from most other bufonids. However, the simplified aspect of the oral disc is the one that deserve the most attention. The marginal papillae are reduced, the lips are modified in a thick dermal fold interrupted laterally, the labial teeth are reduced to two rows (one anterior and one posterior) with few and sparce teeth, and jaw sheaths are almost inconspicuous with only the terminal portion pigmented. Among bufonids, endotrophic larvae are rare, also being reported for *Blythophryne*, *Pelophryne,* and *Altiphrynoides* (Grandison 1978; Müller 2019). Endotrophy evolved independently in those taxa, and some character-states associated with lack of feeding, such as the reduction of mouthparts, are convergent in those lineages.

The oral discs of these taxa present unique character-states; *Blythophryne beryet* has marginal papillae (with dorsal gap) and a well-developed jaw sheath, but no labial teeth (LTRF 0/0) (Chandramouli et al. 2016); while *Pelophryne* spp. have the oral disc with no observable marginal papillae and a LTRF 1/0 (Inger 1960; Malkmus et al. 2002; Leong and Teo 2009). The LTRF 1/1 and the presence of a modified lip in a thick dermal fold are autapomorphic conditions of *F. pernambucensis* and could represent a synapomorphy of *Frostius*, pending data on *F. erythrophthalmus*.

Data on buccopharyngeal cavity are available for several lineages of bufonids: *Anaxyrus* (Wassersug and Rosenberg 1979); *Altiphrynoides* (Müller 2019); *Ansonia* (Inger 1985); *Bufo* (Viertel 1982); *Ingerophrynus* (Inger 1985); *Melanophryniscus* (Baldo et al. 2014); *Osornophryne* (Romero-Carvajal et al. 2023); *Poyntonophrynus* (Lambris 1994); *Rentapia* (Inger 1985); *Rhinella* (e.g., Vera Candioti 2007; Oliveira et al. 2013); *Schismaderma* (Lambris 1994; Viertel and Channing 2017); *Sclerophrys* (Lambris 1994); and *Vandijkophrynus* (Lambris 1994). Although some features may be missing in some taxa, the almost complete absence of elements in the buccal roof and buccal floor is unique to the tadpoles of *F. penambucensis*. However, the lack of information for several lineages of bufonids (e.g., *Oreophrynella*) prevents an unambiguous optimization of these characters. We address, though, some interesting patterns that must be elucidated in the future.

Frostius pernambucensis has a single pair of infralabial papillae, the same as other bufonids for which the buccopharyngeal cavity is known (e.g., Oliveira et al. 2013; Müller 2019; Vera Candioti et al. 2020; this study), including the most basal genus of the family, *Melanophryniscus* (Baldo et al. 2014), contrasting with the four papillae (two pairs) observed in Bufonidae`s sister taxa Odontophrynidae (e.g., Nascimento et al. 2013; Dias et al. 2014, 2019). Other closely related lineages, such as centrolenids or leptodactylids (mostly), also present two pairs of infralabial papilla (e.g., Wassersug and Heyer 1988; Vera Candioti et al. 2007; Rada et al. 2019; Dias et al. 2020; Nascimento et al. 2020). Thus, we suggest that the presence of a single pair of infralabial papillae is a putative synapomorphy of Bufonidae.

We observed three, rounded, poorly developed lingual papillae in *F. pernambucensis*. Most bufonids present four lingual papillae with few exceptions: lingual papillae are absent in members of the *Rinella veraguensis* species group (Cadle and Altig 1991; Aguayo et al. 2009), and in *Ansonia* tadpoles (Inger 1985), *R. rumboli* may have 3 or 4 lingual papillae (Haad et al. 2014), and *Ingerophrynus divergens* present a single pair (Inger 1985). Three lingual papillae seem to be a unique feature of *Frostius*, although the occurrence of polymorphic condition in other bufonids, such as *R. rumboli*, cast a shadow in that interpretation; further specimens of *Frostius* need to be examined regarding their buccopharyngeal cavity to determine the occurrence or not of polymorphism in this character. It is worthy to note that, despite the number of papillae, the reduction in its size has never been reported in any other bufonid as far as we know.

The reduction in the number of buccal floor and roof papillae and pustulation is another characteristic of *Frostius pernambucensis*—single pair of buccal floor arena papillae and no pustulation at all—in comparison with other bufonids. Buccal roof papillae are absent in the larvae of *Ansonia* (Inger 1985), but all other bufonids with their buccopharyngeal cavity described present some papillae and pustulations on the buccal roof and floor. Also, postnarial papillae are missing in *Frostius*. We thus suggest that the absence of pustulations on the roof and floor, as well as the absence of papillae in the buccal roof and postnarial papillae could represent synapomorphies for the genus *Frostius*. Other elements are missing the buccopharyngeal cavity of *Frostius* tadpoles: median ridge, lateral ridge papillae, secretory ridges and secretory pores, and filter plates, that should be better explored in other bufonid genera, but could represent putative synapomorphies for the genus.

The musculo-skeletal system of *Frostius pernambucensis* is also highly modified, with interesting evolutionary implications. Haas (2003) suggested some larval synapomorphies derived from the muscles and cartilages for the family Bufonidae: (1) the absence of *m. interhyoideus posterior*; (2) the absence of the *diaphragmatopraecordialis*; (3) a slip of the *subarcualis rectus* II–IV reaches far laterally into interbranchial septum IV; and 4) the absence of *processus anterolateralis*. *Frostius pernambucensis* is in accordance with three of these synapomorphies, but differs regarding the *subarcualis rectus* II–IV, which originates in the ceratobranchial III and reaches the ceratobranchial I, both conditions are putative synapomorphies for the genus.

Many bufonids have the *m. subarcualis rectus* I divided into two slips inserting on the ceratobranchials II and III (e.g., Haas 2003; Vera Candioti 2007). In *Frostius pernambucensis*, the two slips are also present, but both are inserted in the ceratobranchial III; the changing in the pattern of insertion of the dorsal slip is a putative synapomorphy of *Frostius*. Also, the insertion of the *levator arcuum branchialium* IV and that of the *tympanopharyngeus* are modified in *F. pernambucensis*; in this species, all the mentioned muscles are inserting on the ceratobranchial III, in contrast with all other bufonids, in which these muscles insert on the ceratobranchial IV.

Regarding the larval cranium, *Frostius pernambucensis* also has several interesting transformations. Suprarostral corpus and alae completely fused it is not commonly observed among bufonids, but it was reported for the gastromyzophorous larvae of *Atelopus* (Lavilla and de Sá 2001). *Amazophrynella*, *Dendrophryniscus*, and *Melanophryniscus* do not present such fusion (Baldo et al. 2014; P.H.D. pers. obs.), but the lack of data for other basal lineages creates a difficulty in optimizing this character state. Notwithstanding, the fusion on the suprarostral with the *cornua trabeculae* had never been reported in bufonids—*Gastrophryne carolinensis* larvae have these cartilages fused (Trueb et al. 2011), but this is not closely related species—which represents an additional putative synapomorphy of *Frostius*.

The *commissura quadratoorbitalis* is present in the majority of bufonids (Haas 2003), but gives its presence in other closely related taxa, such as Odontophrynidae (Dias et al. 2019); it could represent a synapomorphy of a more inclusive clade. Such *commissura* was lost in *F. pernambucensis*, representing another putative synapomorphy for the genus.

Finally, the ceratobranchials II and III attached to the hypobranchial plates, the absence of *commissura terminalis* uniting the ceratobranchial, and the free ceratobranchial IV are also putative synapomorphies of the genus, given that these conditions are not observed in other members of the family (e.g., Haas 2003; Aguayo et al. 2009; Haad et al. 2014).

Living in phytotelmata and the evolution of endotrophy

Phytotelmata, such as bromeliads tanks, bamboo stumps, tree holes, nut shells, and palm leaves, can be defined as water filled plants or parts of plants (Thienemann 1934). This habitat can be drastically different from other water bodies in its physicochemical properties, presenting low dissolved oxygen, low pH, and elevated viscosity (Laessle 1961; Maguire. 1971; Richardson 1999). Another characteristic of phytotelmata is that the food chain and the primary productivity are very particular (Richardson et al. 2000; Kitching 2001) with reduced availability of food resources, and thus, organisms inhabiting those habitats may face distinguished selective pressures. Frogs have colonized phytotelmata several times (Lehtinen et al. 2004) and tadpoles developing in that environment have often evolved differentiated trophic strategies; although many phytotelm dwellers are detritivorous and filterers (e.g., Caldwell 1993; Dias and Pie 2021), several species are predators (carnivory), oophagous, or endotrophic (Lannoo et al. 1987; Lehtinen et al. 2004). Within bufonids, phytotelm usage for development has been reported in several taxa (e.g., Caldwell 1993; Langone et al. 2008; Malkmus and Dehling 2008), and the tadpoles of some species inhabiting these plants are endotrophic (e.g., Leong and Teo 2009; Chandramouli et al. 2016).

Most of unique character-states in *Frostius pernambucensis* are related to the reduction or loss of elements in oral disc, buccopharyngeal cavity, musculature, and larval cranium. Such pattern can be directly related to the endotrophic development of these tadpoles. Endotrophic tadpoles usually lack mouthparts, pigmentation, tail fins, and spiracle (e.g., Kaiser and Altig 1994; Caldwell and Lima 2003; Randrianiaina et al. 2011; Etter et al. 2021), although a variety of combination of traits losses can be observed in different lineages (Altig and Johnston 1989).

The buccopharyngeal cavity of several endotrophic larvae follow similar pattern, with the reduction or loss of features. Wassersug and Duellman (1984) investigated the phenotypic variation in buccopharyngeal cavity of several hemiphractid tadpoles. Their findings regarding the endotrophic forms are similar to our results with *Frostius pernambucensis*, including the loss or reduction of several papillae and other buccal features.

Similarly, the musculo-skeletal system is quite modified in endotrophic forms. We reported some differences regarding the pattern of origin and insertion of some muscles (e.g., *levator arcuum branchialium*), although the general pattern of origin and insertion of muscles in quite similar to that of exotrophic tadpoles. This is also true for other endotrophic tadpoles for which larval muscles are known—*Eupsophus emiliopugini* (Vera Candioti et al. 2011a, b) and *Fritziana goeldi* (Haas 1996).

Larval cranium morphology data are available for few endotrophic tadpoles: *Cycloramphus stejnegeri* (Lavilla 1991), *Eupsophus calcaratus* (Vera Candioti et al. 2005), *E. emiliopugini* (Vera Candioti et al. 2011a, b), *E. nahuelbutensis* (Nuñez and Úbeda 2009), *E. queulensis* (Cárdenas-Rojas et al. 2007), *Rhinoderma darwinii* (Lavilla 1987), and *Fritziana goeldi* (Haas 1996). Some character-states present in most of these larvae, as well as in *Frostius pernambucensis*, are the short *processus muscularis quadrati*, large *capsula auditiva*, short *cornua trabecula*, tall orbital cartilage, and reduced hyobranchial apparatus. With exception of the enlargement of *capsula auditiva*, these character-states can

be directly correlated with the lack of feeding activity in these larvae.

Two no exclusive mechanisms have been evoked to explain the evolution of endotrophy: (1) the loss of some specific tadpole characters and (2) the acceleration in the development of some adult characters (Elinson 2001). Empirical evidence seems to support these ideas; data suggest that endotrophy is associated with the remodeling and/or loss of cartilaginous elements typical of tadpoles, as the suprarostral cartilages and palatoquadrate, and with changes in the onset of some skeletal elements (Hanken et al. 1992; Yeh 2002; Kerney et al. 2007). The fusion between the suprarostral and *cornua trabeculae* provides additional evidence to the idea of truncation of development. The suprarostral cartilage is likely evolved by the addition of an articulation in the existing trabecular cartilages (Sevensson and Haas 2005; Lukas and Olsson 2018) during development; the fact that these cartilages are not completely separated could indicate that tadpole`s development was truncated.

Changes in development is also valid for buccopharyngeal cavity; Wassersug and Duellman (1984) examined the ontogeny of buccopharyngeal cavity in the exotrophic larvae of *Gastrotheca riobambae* and observed that early stage embryos (Gosner 23) lacked most of buccal features and resembled the cavity of endotrophic forms and concluded (Wassersug and Duellman 1984:35) that endotrophic forms may have arisen by a simple truncation or acceleration of the "tadpole developmental program". As far as we know, there is no information on the buccopharyngeal cavity of bufonids in early developmental stages (24-), but the external morphology of *F. pernambucensis* resembles that of the embryos of other bufonids, with poorly developed oral discs and mouthparts and poorly pigmented bodies (Vera Candioti et al. 2016).

The real processes involved in the evolution of endotrophy are still poorly understood. Altig and Crother (2006) hypothesized that endotrophic forms evolved from developmental reppartening via changes in some gene family responsible for the development of tadpole-specific traits (see also Altig 2006). Although there are no molecular and developmental studies addressing this issue, the fact that the loss of exotrophic tadpoles and the evolution of endotrophic forms occurred independently and repeatedly in some lineages (besides bufonids), such as aromobatids (e.g., Vacher et al. 2017) and hemiphractids (e.g., Castroviejo Fischer et al. 2015), suggests that some regulatory program can be altered relatively easily. Further studies are necessary to test these hypotheses.

Finally, endotrophy also had an impact in the ecological interactions and space use in *Frostius pernambucenis* larvae. We observed several individuals in the same phytotelm (up to 50 tadpoles at different developmental stages). Usually, phytotelm breeders tend to lay few eggs per site; for example, in dart-poison frogs (Dendrobatidae) the number of eggs in the clutches and tadpoles deposited per site tend to be small (Caldwell and Araújo 1998; Lehtinen et al 2004; Duarte-Marin et al. 2020). Large number of tadpoles inhabiting the same phytotelm seems to be correlated with oophagy (Schiesari et al. 2003), and we suggest that the large number of *F. pernambucenis* tadpoles found in a single site could be related to endotrophy, giving that feeding resources would not be a limiting factor for special usage. Further studies are necessary to test this hypothesis.

Remarks and conclusions

The endotrophic tadpoles of *Frostius pernambucenis* are highly modified in comparison with exotrophic larvae of other bufonids, leading us to propose several novel synapomorphies for the genus. Notwithstanding, data are scarce for many lineages of bufonids (especially for early divergent clades), and a more comprehensive study will be necessary to test our hypotheses.

The colonization of phytotelma, an environment relatively poor of food resources, could have created a selective pression on the development of *F. pernambucenis*, favoring the evolution of endotrophy, although further evidence is necessary to test this hypothesis. The evolution of non-feeding larvae is associated with the loss and/or remodeling of several elements of the anatomy of these tadpoles. This fact suggests that *F. pernambucensis* might be an excellent model for future evo-devo studies.

Appendix 1 Additional tadpoles studied

Taxa	External morphology	Buccopharyngeal cavity	Musculo-skeletal system
Adenomus kelaartii	Haas et al. (1997)		
Amazophrynella minuta	This study	This study	This study
Anaxyrus americanus	This study	–	This study
Ansonia hanitschi	This study	This study	This study
Atelopus nanay	This study	This study	–
Barbarophryne brongersmai	This study	This study	–
Bufo bufo	This study	Viertel (1982)	–

Taxa	External morphology	Buccopharyngeal cavity	Musculo-skeletal system
Capensibufo tradouwi	Channing et al. (2012)	–	–
Dendrophryniscus brevipollicatus	This study	This study	This study
Duttaphrynus melanostictus	This study	This study	This study
Frostius pernambucensis	This study	This study	This study
Incilius coniferus	This study	This study	This study
Ingerophrynus divergens	Inger (1985)	Inger (1985)	–
Melanophryniscus klappenbachi	Baldo et al. (2014)	Baldo et al. (2014)	Baldo et al. (2014)
Mertensophryne taitana	Müller et al (2005)	–	–
Nannophryne variegata	This study	–	–
Osornophryne occidentalis	Romero-Carvajal et al. (2023)	Romero-Carvajal et al. (2023)	–
Peltophryne peltocephala	This study	This study	This study
Poyntonophrynus fenoulheti		Lambris (1994)	
Rentapia hosii	Inger (1985)	Inger (1985)	
Rhaebo glaberrimus	This study	–	This study
Rhinella marina	This study	This study	This study
Schismaderma carens	Viertel and Channing (2017)	Lambris (1994; Viertel and Channing (2017)	–
Sclerophrys maculata	This study	This study	–
Vandijkophrynus gariepensis	Channing et al. (2012)	Lambris (1994)	–
Werneria mertensiana	This study	–	–
Outgroup			
Macrogenioglottus alipioi	This study	This study	This study
Odontophrynus cultripes	This study	This study	This study
Proceratophrys appendiculata	This study	This study	This study

Acronyms: *AMNH* American Museum of Natural History, *CFBH* Celio F.B. Haddad herpetological collection, housed at Universidade Estadual Paulista, UNESP, Rio Claro, *ICN* Instituto de Ciencias Naturales, Universidad Nacional de Colombia, *INPA* Instituto Nacional de Pesquisa da Amazônia, *UCR* Herpetological Collection, Universidade de Costa Rica, *UNIRIO* Herpetological Collection, Universidade Federal do Estado do Rio de Janeiro, *MHNUFAL* Museu de História Natural, Universidade Federal de Alagoas

Amazophrynella minuta (Melin, 1941): ICN 54915.

Anaxyrus americanus (Holbrook, 1836): AMNH 153168.

Atelopus nanay Coloma, 2002: QCAZ 3672.

Barbarophryne brongersmai (Hoogmoed, 1972): ZMH 12162.

Dendrophryniscus brevipollicatus Jiménez de la Espada, 1870: UNIRIO 3394.

Duttaphrynus melanostictus (Schneider, 1799): ZMH 13591.

Incilius coniferus (Cope, 1862): UCR 18999.

Macrogenioglottus alipioi MHNUFAL 10811.

Melanophryniscus spectabilis Caramaschi & Cruz, 2002: CFBH 3719.

Nannophryne variegata Günther, 1870: AMNH 81404.

Odontophrynus cultripes UFMG937.

Peltophryne peltocephala (Tschudi, 1838): AMNH 38451.

Proceratophrys appendiculata UNIRIO4036.

Rhaebo glaberrimus (Günther, 1869): ICN 49629.

Rhinella marina (Linnaeus, 1758): ICN 53853.

Sclerophrys maculata (Hallowell, 1854): ZMH 11955.

Werneria mertensiana Amiet, 1976: ZMB 79695.

Appendix 2: Character evolution

For the analysis of phenotypic evolution, we sample taxa based on Jetz and Pyron (2018) phylogenetic hypothesis. Characters were scored based on personal observation and/or literature information. We assumed the monophyly of *Frostius* and the sister relationship between *F. erythrophthalmus* and *F. pernambucensis* (see Materials and methods). We recognize that in the absence of data for the larvae of *F. erythrophthalmus,* our synapomorphies propositions are only putative, pending further investigation.

Phenotypic characters

Character 1: Number of tooth rows on the upper lip: one (0); two (1)

The typical tadpole has flexible, dermal ridges on both upper and lower lip; on top of those ridges, there are keratinous teeth, or keratodonts (Altig and McDiarmid 1999). The number and organization of these tooth row can vary drastically, reaching up to 17 superior and 21 inferior tooth rows (Altig and Johnston 1989). In bufonids, however, most species present the same number of rows in the upper (2) and in the lower (3) lips. Given that the number of rows in each lip seem to evolve independently (Altig 2006), we opted for coding each lip separately.

Taxonomic distribution and optimization: The examined larvae of *Frostius pernambucensis* possess a unique condition, with a single row in each lip. Variation of the 2/3 is also observed in other bufonids, such as 0/0 in *Blythophryne beryet* (Chandramouli et al. 2016) and 1/0 in *Pelophryne* spp (Inger 1960; Malkmus et al. 2002; Leong and Teo 2009); these taxa, however, were not present in the phylogenetic hypothesis used by us. Our optimization suggests that the presence of a single row in each lip is a synapomorphy of *Frostius* (Fgi. 8A).

Character 2: Number of tooth rows on the lower lip: one (0); three (1)

See Character 1

Taxonomic distribution and optimization: Our optimization suggests that the presence of a single row in each lip is a synapomorphy of *Frostius* (Fig. 8B).

Character 3: Lip morphology: "normal" (0); dermal fold (1); expanded (2)

The "normal" lips of tadpoles consist of a dermal structure bearing marginal papillae and the tooth ridges, usually larger than the upper labia, free on its edges. Although both marginal (and submarginal) papillae, as well as the ridges can vary significantly, the lips per se are more or less uniform in several taxa. Interesting variation have been reported, as the reduction of lips in microhylids (e.g., Haas 2003) and in endotrophic larvae (e.g., Randrianiania et al. 2011), or the enlarged, funnel-like structure of umbelliform tadpoles (e.g., Dias et al. 2019, 2021). In the examined bufonids, we observed besides the "normal" configuration, an enlarged lip in suctorial and/gastromyzophorous forms, and a tick dermal fold, as in *Frostius*.

Taxonomic distribution and optimization: while and enlarged lip evolved independently at least three times in bufonids—*Atelopus*, *Ansonia*, and *Weneria*—the modification into a dermal fold occurred just in *Frostius*, and therefore could be an additional synapomorphy for the genus (Fig. 8C).

Character 4: Number of infralabial papillae: one pair (0); two pairs (1)

The infralabial papillae are located in the buccal floor, right after buccal opening (Wassersug 1976); several functions have been suggested for those papillae (Wassersug 1980), but no conclusive evidence has been presented. Within bufonids and their closely related taxa, two main conditions have been reported in the literature: a single pair in bufonids (e.g., Vera Candioti et al. 2020), or two pairs in odontophrydis (e.g., Nascimento et al. 2013; Dias et al. 2014)—with some variation in specific taxa (e.g., Dias et al. 2019; Dias 2020).

Taxonomic distribution and optimization: *Frostius pernambucensis*, as well as all bufonids presented a single pair of infralabial papillae, a condition that optimized as a synapomorphy of Bufonidae (Fig. 8D).

Character 5: Number of lingual papillae: two (0); three (1); four (2)

Lingual papillae are located on the lingual bud of tadpoles (Wassersug 1976). Considerable variation has been reported in the number and arrangements of these papillae (e.g., Wassersug and Heyer 1988).

Taxonomic distribution and optimization: the optimization of this character proved ambiguous; *Frostius pernambucensis*, as well as *Melanophryniscus klappenbachi* presented three lingual papillae, contrasting with the most of other bufonids, which have four papillae. Additionally, the absence of these papillae in *Osornophryne* (Romero-Carvajal et al. 2023) and the lack of data for *Oreophrynella*, precluded the optimization of this characters (Fig. 9A). It is important to note that variation exists within *Melanophryniscus* (Baldo et al. 2014), so further studies are required. Also, *Amazophrynella minuta* had a unique condition among bufonids—two lingual papillae; this genus also should be investigated further.

Fig. 8 Parsimonious optimization of characters 1 (**A**), 2 (**B**), 3 (**C**), and 4 (**D**). Grey represents unknown condition

Fig. 9 Parsimonious optimization of characters 5 (**A**), 6 (**B**), 7 (**C**), and 8 (**D**). Grey represents unknown condition

Character 6: Papillae in the buccal roof: absent (0); present (1)

Both buccal roof and floor are, usually, covered with papillae of different size, shape, and morphology (Wassersug 1976, 1980). These papillae can be distributed in different regions, such as the pre-pocket area or delimiting the roof and floor arenas. In rare cases, such as in endotrophic (e.g., Wassersug and Duellman 1984) or macrophagous (e.g., Vera Candioti et al. 2021) larvae, these papillae are absent. Many bufonids lacked papillae in the buccal roof.

Taxonomic distribution and optimization: state 0 was present in several lineages, including *Frostius*, *Atelopus*, *Osornophryne*, *Ansonia*, and *Amazophrynella*. Besides that, this condition is unknown for *Oreophrynella*. This distribution of states rendered the optimization of this character ambiguous (Fig. 9B).

Character 7: Pustulations in the buccal roof: absent (0); present (1)

As for the buccal roof papillae, may taxa have pustulations in the buccal floor and roof; these pustulations can be scattered in the buccal floor, as in many taxa (e.g., Vera Candioti 2007), or form a dense field of pustulations, as in many stream dwellers (e.g., Dias et al. 2014; Montesinos et al. 2022).

Taxonomic distribution and optimization: the distribution and optimization of this character is quite similar to that of character 6, with exception of *Amazophrynella*, that presented pustulations (Fig. 9C).

Character 8: Pustulations in the buccal floor: absent (0); present (1)

See characters 7 for description and distribution (Fig. 9D).

Characters 9: Postnarial papillae: absent (0); present (1)

Postnarial papillae are located immediately posteriorly to narial opening and can vary in number, arrangements, and morphology (e.g., Wassersug and Heyer 1988; Dias et al. 2021). Very rarely, it is absent from tadpoles.

Taxonomic distribution and optimization: within bufonids, postnarial papillae were absent in *Frostius*, *Osornophryne*, and *Amazophrynella*. The optimization of this character suggests that the lack of these papillae could be a synapomorphy of *Frostius* (Fig. 10A). Also, if its absence is confirmed in *Oreophrynella*, it could be a synapomorphy for *Osornophryne* + *Oreophrynella*. Finally, it deserves further inspection in other *Amazophrynella* species.

Character 10: Median ridge: absent (0); present (1)

The median ridge stands at the end of the postnarial arena (Wassersug 1976, 1980); this feature usually has a triangular shape, but variation has been reported regarding its morphology (e.g., Dias et al. 2019). A putative function of the median ridge is to divide the water flow into left and right due to its medial location (Wassersug 1980). This structure has been reported absent in endotrophic larvae (e.g., Wassersug and Duellman 1984).

Taxonomic distribution and optimization: in our dataset, only two species lacked a median ridge within bufonids: *Frostius pernambucensis* and *Osornophryne occidentalis* and the optimization suggests that such absence could be a synapomorphy for *Frostius* (Fig. 10B).

Character 11 Lateral ridge papillae: absent (0); present (1)

The lateral ridge papillae are present in "type IV" tadpoles and can vary in size, shape, and morphology (e.g., Chou and Lin 1997). They are located laterally on the buccal roof, facing the buccal roof (Wassersug 1976).

Taxonomic distribution and optimization: similar to character 10 (Fig. 10C), but lateral ridge papillae are also absent in *Ansonia*.

Character 12: Secretory ridges: absent (0); present (1)

Secretory ridges are responsible for the production of mucus and pivotal for the functioning of the food traps of buccopharyngeal cavity (Kenny 1969; Wassersug 1972; Wassersug and Rosenberg 1979). They are located on the ventral surface of ventral velum and are characterized by an irregular, concentric pattern of ridges. Secretory ridges morphology usually varies accordingly to taxonomy and putative diet (Wassersug and Rosenberg 1979) and seem to be reduced or absent in macrophagous and endotrophic larvae.

Taxonomic distribution and optimization: within bufonids, the secretory ridges are absent in *Frostius pernambucensis* and *Osornophryne occidentalis*. Whereas the lack of data for *Oreophrynella* impacts the optimization regarding *Osornophryne*, the current optimization suggests that

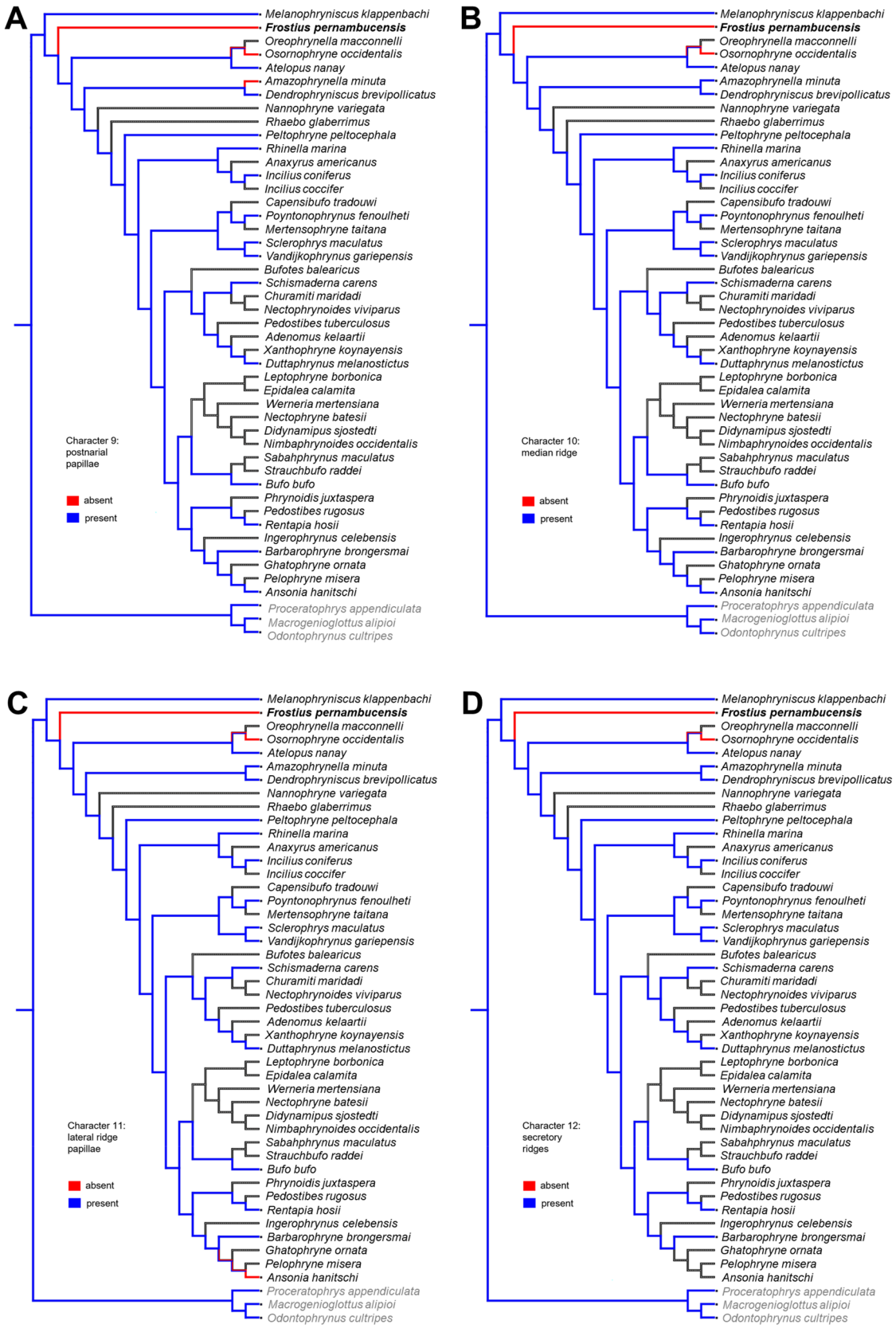

Fig. 10 Parsimonious optimization of characters 9 (**A**), 10 (**B**), 11 (**C**), and 12 (**D**). Grey represents unknown condition

it could represent an additional synapomorphy of *Frostius* (Fig. 10D).

Character 13: Filter plates: absent (0); present (1)

The filter plates are the substrate for the gill filters, another essential structure involved in the food entrapment system of anurans (Kenny 1969; Wassersug 1972). A few studies dealt with these features in detail, but some evolutionary patters were identified; Wassersug (1980), for instance, suggested that there is a correlation between the number of filter rows and the capacity of filtering of a given tadpole. Macrophagous and/or endotrophic larvae have been reported with reduced filter plates (e.g., Wassersug and Duellmam 1984).

Taxonomic distribution and optimization: optimization of this character is similar to that of the secretory ridges and the absence of filter plates is a putative synapomorphy of *Frostius* (Fig. 11A).

Character 14: Muscle *subarcualis rectus* II–IV, origin: ceratobranchial IV (0); ceratobranchial III (1)

This small muscle usually runs from the ceratobranchil IV to the *processus branchialis* II of the ceratobranchial II (Haas 1997, 2003). Notwithstanding, it can be split in different portions, as in some hylids (e.g., Haas 2003; Dias et al. 2023), or having some fibers invading the interbranchial septum IV (e.g., Haas 2003; Vera Candioti et al. 2011a, b). In *Frostius*, a unique condition is present, and the muscle originates at the ceratobranchial III.

Taxonomic distribution and optimization: state 1 is present solely in *Frostius* (autapomorphic condition), and could be a synapomorphy for the genus, pending confirmation in *F. erythrophthalmus* (Fig. 11B).

Character 15: Muscle *subarcualis rectus* II–IV, insertion: ceratobranchial I (0); ceratobranchial II (1)

See character 14.

Taxonomic distribution and optimization: *Frostius pernambucensis* also have a unique condition for the insertion of the m. *subarcualis rectus* II–IV (Fig. 11C).

Character 16: Muscle *subarcualis rectus* I ventral a` slip, insertion: ceratobranchial II (0); ceratobranchial III (1)

Anurans may present up to three slips of the m. *subarcualis rectus* I; generally the dorsal slip inserts on the ceratobranchial I, and the two ventral slips (a and `a) on the ceratobranchials II and III, respectively (Haas 1997, 2003). Deviations from this pattern have been reported in many lineages (Haas 2003). We observed in *Frostius pernambucensis* that both ventral slips of the m. *subarcualis rectus* I are inserted in the ceratobranchial II.

Taxonomic distribution and optimization: state 0 is restricted to *Frostius* and it is a putative synapomorphy of the genus (Fig. 11D).

Character 17: Muscle *levator arcuum branchialium* IV, insertion: ceratobranchial IV (0); ceratobranchial III (1)

Anurans may present up to three *levator arcuum branchialium*. The levators I–IV usually inserts on the respective ceratobranchials (Haas 2003). Usually, there is some variation in the insertion pattern of the fourth levator, which can reach a distal or proximal portion of the ceratobranchial IV. In *Frostius*, there is a unique condition in which the levator IV inserts on the ceratobranchial III.

Taxonomic distribution and optimization: state 1 is restricted to *Frostius* and it is a putative synapomorphy of the genus (Fig. 12A).

Character 18: Muscle *tympanopharyngeus*, insertion: ceratobranchial IV (0); ceratobranchial III (1)

The *tympanopharyngeus* is closely associated with the *levator arcuum branchialium* IV, indistinguishable for it in some case (Haas 2003). In *Frostius*, there is a unique condition in which the *tympanopharyngeus* inserts on the ceratobranchial III.

Taxonomic distribution and optimization: state 1 is restricted to *Frostius* and it is a putative synapomorphy of the genus (Fig. 12B).

Fig. 11 Parsimonious optimization of characters 13 (**A**), 14 (**B**), 15 (**C**), and 16 (**D**). Grey represents unknown condition

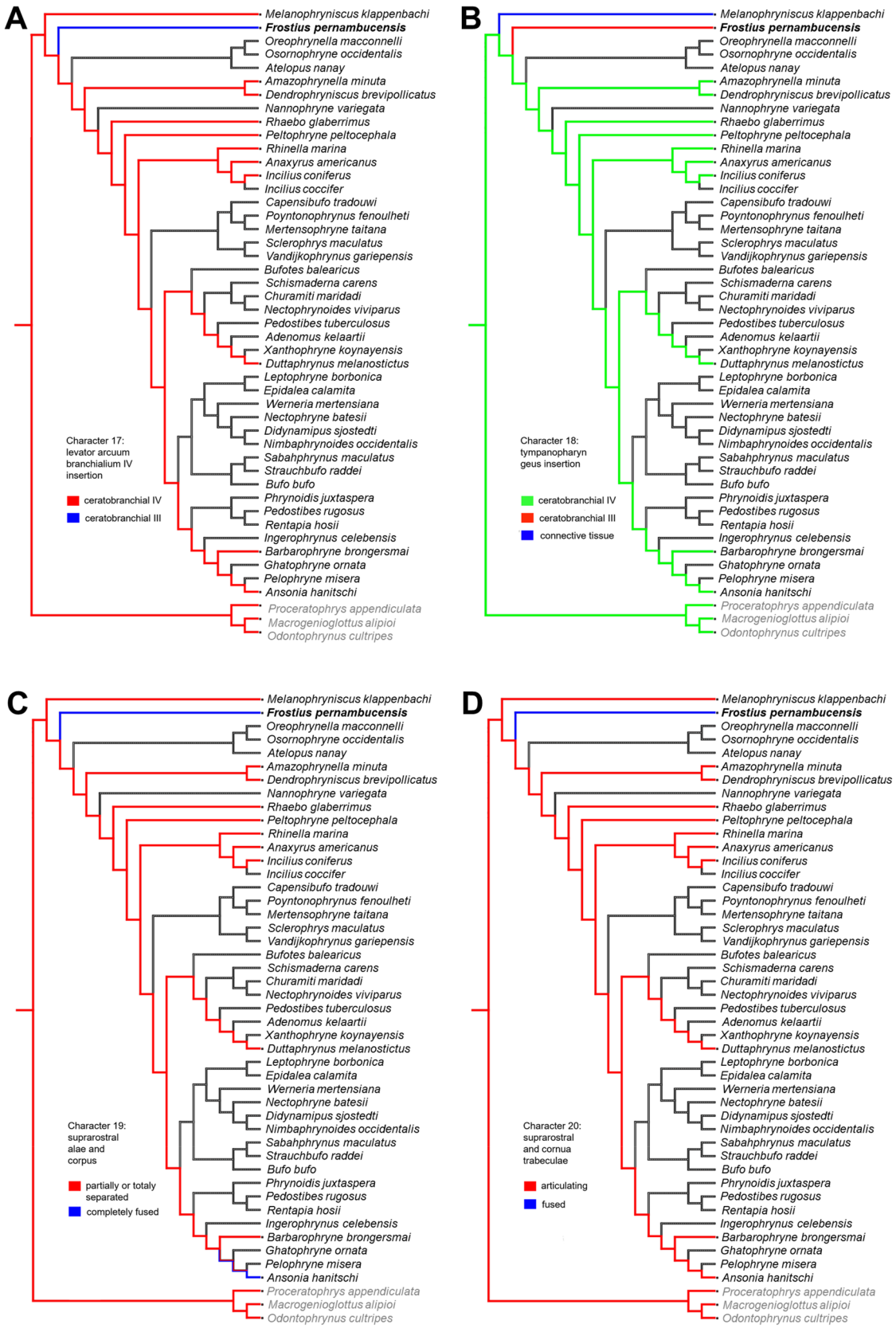

Fig. 12 Parsimonious optimization of characters 17 (**A**), 18 (**B**), 19 (**C**), and 20 (**D**). Grey represents unknown condition

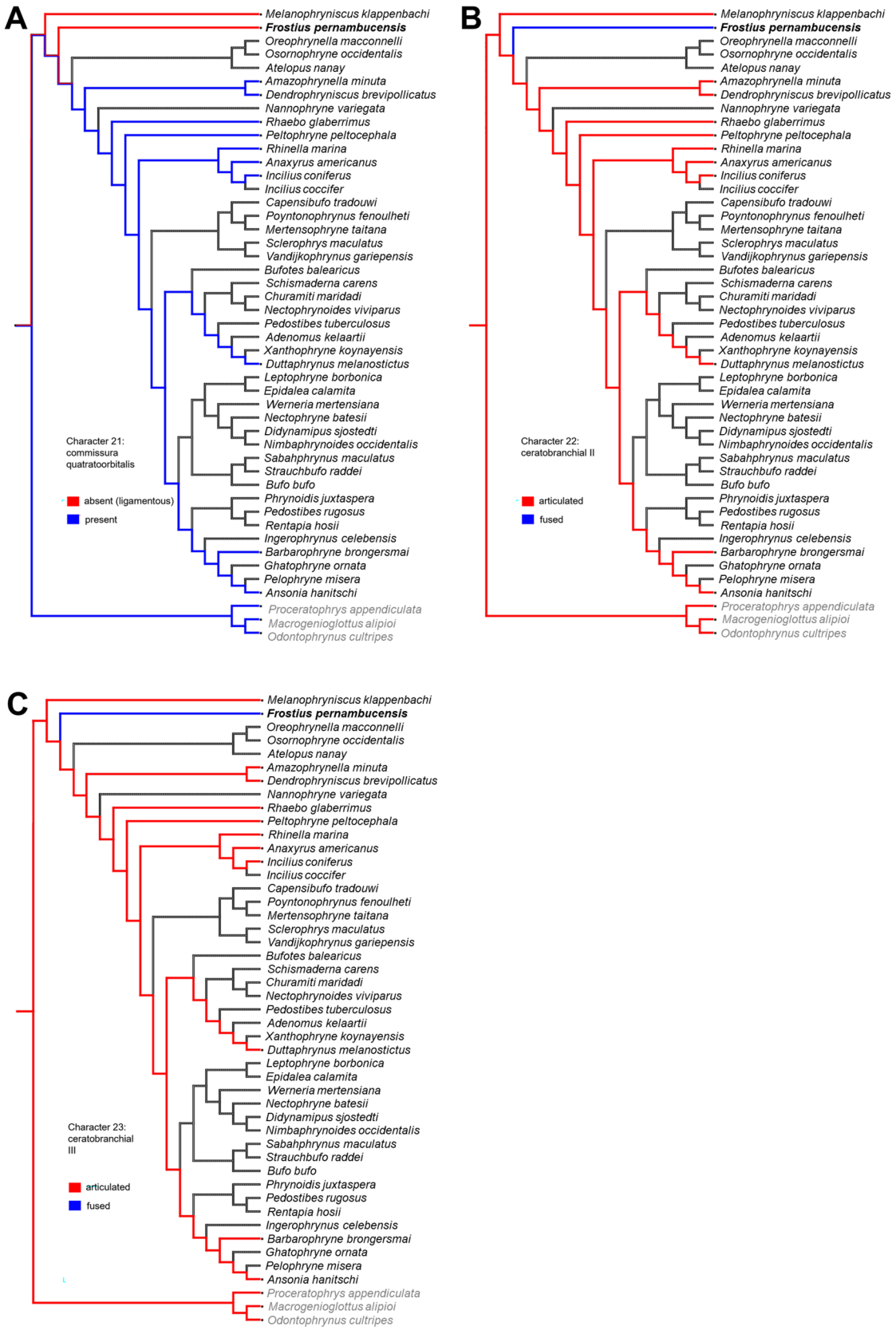

Fig. 13 Parsimonious optimization of characters 21 (**A**), 22 (**B**), and 23 (**C**). Grey represents unknown condition

Character 19: Suprarostral alae and corpus: partially or completely separated (0); fused (1)

The suprarostral cartilages provide support for the upper jaw sheath. Usually, these cartilages have a tri- or tetrapartite condition, with the alae completely free or partially free from the corpora, which are very often continuously distally (Haas 1995, 2003). Different variation has been reported in that configuration (e.g., Dias et al. 2021), but a completely fused corpora is not common (e.g., Vera Candioti 2005; Dias et al. 2019). *Frostius pernambucensis*, as well as the suctorial larvae of *Ansonia* present a single-element corpora.

Taxonomic distribution and optimization: optimization of this character suggests that state 1 is a putative synapomorphy for *Frostius* (Fig. 12C).

Character 20: Suprarostral cartilage and *cornua trabeculae*: articulating (0); fused (1)

Suprarostral cartilages and the *cornua trabeculae* are articulating elements in the anuran larvae (Haas 1995, 2003). In *Frostius*, there is a stripe of cartilage fusing these two elements synchodroticaly. This condition has never been reported or observed in any other bufonid as far as we know.

Taxonomic distribution and optimization: optimization of this character suggests that state 1 is a putative synapomorphy for *Frostius* (Fig. 12D).

Character 21: *Commissura quadratoorbitalis*: absent (ligamentous) (0); present (1)

The *commissura quadratoorbitalis* is a cartilaginous connection between the *processus muscularis* and the *processus anthorbitalis* (Haas 1995). In several taxa, this cartilaginous bridge is not formed, and such a connection is made of ligamentous (Haas 2003). In most bufonids, the *commissura* is present (Haas 2003), although it has been previously reported absent, as in *Melanophryniscus* (Baldo et al. 2014). The *commissura*, however, is also present in bufonid`s sister taxa, Odontophrynidae (e.g., Nascimento et al. 2013; Dias 2020).

Taxonomic distribution and optimization: optimization of this character is ambiguous (Fig. 13A). It seems that it was lost early in the evolution of bufonids and regained in less inclusive clades. The current available evidence does not allow a test for this hypothesis.

Character 22: Ceratobranchial II and *planum hypobranchiale*: articulating (0); fused (1)

Anurans usually have four ceratobranchials, of which the first is fused to the planum hypobranchiale, whereas the others are articulating with it (Haas 1995, 2003). In *Frostius*, the ceratobranchials II and III (see character 23) are fused to the *planum hypobranchiale*, a unique condition among bufonids.

Taxonomic distribution and optimization: optimization of this character suggests that state 1 is a putative synapomorphy for *Frostius* (Fig. 13B).

Character 23: Ceratobranchial III and *planum hypobranchiale*: articulating (0); fused (1)

See character 22.

Taxonomic distribution and optimization: optimization of this character suggests that state 1 is a putative synapomorphy for *Frostius* (Fig. 13C).

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Data availability All relevant data are within the manuscript.

Declarations

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