



Embryonic morphology in two species of the *Physalaemus signifer* clade (Anura: Leptodactylidae)

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We studied the embryonic morphology of *Physalaemus camacan* and *P. signifer*, two small foam-nesting frogs endemic to the Atlantic Forest. We analysed the development of transient embryonic structures and of the larval oral disc. These embryos have features typical of most congeneric species, such as the kyphotic dorsal curvature, three pairs of gills and the configuration of hatching and adhesive glands. Main differences regarding embryos of the *P. cuvieri* clade are the larger size and yolk provision at tailbud stage, less developed external gills and an apparently novel pattern of oral marginal papilla ontogeny. While some shifts could be correlated with variant modes of oviposition, others appear to be developmental modifications not related with ecomorphological aspects.

Keywords: adhesive glands, external gills, hatching gland, Leiuperinae, oral disc.

INTRODUCTION

The Neotropical genus *Physalaemus* (Fitzinger, 1826) currently includes 50 species (Frost, 2021) grouped into two major clades (*sensu* Lourenço et al., 2015): *P. signifer* clade (with *P. nattereri* and phenetic species groups of *P. signifer* and *P. deimaticus*) and *P. cuvieri* clade (with *P. aguirrei*, *P. cicada* and the species groups of *P. biligonigerus*, *P. cuvieri*, *P. gracilis*, *P. henselii* and *P. olfersii*). These species reproduce in a wide variety of environments, like rainforests to seasonal habitats (Ceil, 1980; Heyer et al., 1990), and have a broad geographic distribution across northern and central Argentina, eastern Bolivia, Paraguay, Uruguay, Brazil, the Guianas, lowlands of southern Venezuela, and llanos of south-eastern Colombia (Frost, 2021). Species of *Physalaemus* generally deposit eggs in foam nests and tadpoles develop in puddles (Lynch, 1971). The foam nests are interpreted as an adaptation to environments with sparse rainfall, high temperature, and intense solar radiation, as they are suggested to provide protection for these and many other factors (Heyer, 1969; Duellman & Trueb, 1986; Méndez-Narváez et al., 2015).

Frogs of the *P. signifer* clade are endemic to the Atlantic Forest and breed in or close to small puddles inside the forest (Pupin et al., 2010). While most species in the genus build foam nests on the water surface, several species of this clade inhabiting forested environments

exhibit a tendency toward terrestrial reproduction (Pupin et al., 2010). This has been reported in *P. atlanticus*, *P. bokermanni*, *P. caete*, *P. crombiei*, *P. erythros*, *P. signifer* and *P. spiniger*, which may build their foam nests directly on the humid forest floor, tree holes, axils of bromeliads, or between leaves on the floor (reviewed in Pupin et al., 2010, 2018). *P. signifer* is distributed in the Brazilian States of Bahia, Espírito Santo, Rio de Janeiro and São Paulo; these frogs build foam nests on the forest floor near small waterbodies (Wogel et al., 2002). *P. camacan* has apparently a more restricted distribution, and it is only reported in localities of Bahia; the species is known to reproduce in small shallow ponds inside forest patches (Pimenta et al., 2005). During field-work in breeding areas, we found vocalising males and foam nests among leaves on humid soil, making this the first report of terrestrial nests for this species.

The early ontogeny in frogs has acquired a renewed interest, since comparative studies have shown the wide morphological and heterochronic variation potentially informative for interpretations about species diversification and evolution (e.g., Nokhbatolfgoghahai et al., 2005; Vera Candiotti et al., 2016). In *Physalaemus*, the embryonic morphology has been explored comparatively in previous studies. Vera Candiotti et al. (2011) studied the development of the oral disc, and later, Grosso et al. (2019) studied the embryonic morphology and heterochronic development

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in twelve species of the genus. These studies included only species from the *P. cuvieri* clade with oviposition in aquatic foam nests; therefore, information on the *P. signifer* clade is needed to complete the comparative panorama of the genus. In this study, we explore the early ontogeny in *P. camacan* and *P. signifer*, two species representative of the *P. signifer* clade with terrestrial foam nests. We describe morphological and developmental diversity in embryonic and larval characters, compare with information available for species of the *P. cuvieri* clade, and discuss our results in the context of early development in aquatic and terrestrial environments.

MATERIALS & METHODS

We analysed embryonic series of *P. camacan* and *P. signifer*, obtained from clutches and from amplexant adults collected in the field. Embryos are deposited in the amphibian collection of the Universidade Federal do Sul da Bahia (UFSB517, *P. camacan*: municipality of Itabuna, Campus from the Universidade Estadual de Santa Cruz – UESC, State of Bahia, Brazil, 14°47'46.7" S, 39°10'19.7" W; UFSB515 and UFSB516, *P. signifer*: municipality of Porto Seguro, State of Bahia, Brazil, 16°23'19.2" S, 39°10'07.5" W). In all cases foam nests were found on humid soil and placed among leaves. Vocalising males and amplexant pairs of each species were found near the nests in both locations, and no other congeneric frogs were active at that moment. Additionally, some embryos were reared until older larval stages to confirm species identity. Clutches were moved and maintained in containers with puddle water and under natural conditions of light and temperature. The specimen manipulation was carried out following the recommendations in the Guidelines for Ethical Conduct in the CEUA-UFBA protocol (43/2017). Embryos were euthanised every 6–8 h by immersion in water with lidocaine, and then preserved in 8 % formalin. We focused on the period between tailbud stage and emergence of hind limbs and complete development of the oral disc (from Stage 17 to 18 to 26; Gosner, 1960: here abbreviated as GS). We studied a total of 98 embryos of *P. signifer* (two clutches) and 49 embryos of *P. camacan* (one clutch); while the ontogenetic series of *P. signifer* is complete, the series of *P. camacan* unfortunately lacks the earliest stages (the first available embryos already had the operculum differentiated at the gill base). Specimens were examined and photographed with a stereomicroscope Leica EZ4E. Methylene blue solution was used to contrast structures such as gills, adhesive glands, and oral papillae (Wassersug, 1976). Additionally, nine embryos of *P. signifer* were dehydrated using serial dilutions of ethanol and coated with gold to perform scanning with a Zeiss Supra 55VP electron microscope at Centro Integral de Microscopía Electrónica–CIME– (CONICET, Tucumán). The images were obtained mainly from a ventral view because of the arrangement of most morphological structures. The characterisation of transient embryonic structures follows Nokhbatolfighahai & Downie (2005, 2007, 2008) and Nokhbatolfighahai et al. (2005). The oral disc development was described following Thibaudeau & Altig (1988) and

Vera Candiotti et al. (2011). The definitive configuration of the oral disc was determined by comparison with the original tadpole description (e.g., Weber & Carvalho-e-Silva, 2001; Pimenta et al., 2005). In addition, we followed Grosso et al. (2019) to register the embryo body length and area, yolk area and the extent of dorsal curvature at tailbud stage (this latter measured in lateral view, as the angle subtended by the body from a dorsal midpoint), and the length of the primary filament of first gill pair as an indicative of gill development. Measurements were taken from photographs in lateral view, using the Leica Application Suite software (V4.4.0) and areas were estimated using the software Image J.

RESULTS

The following description represents the main developmental changes in early development for both focused species and are mostly based on *P. signifer* (Figs. 1-5 and 7B); differences regarding *P. camacan* (Figs. 6 and 7A) were highlighted whenever necessary. Gosner (1960) stages are estimated from embryo general aspect and consigned when possible. Measurements are summarised in Table 1; to facilitate intrageneric comparisons, values from Grosso et al. (2019) for species in the *P. cuvieri* clade were included in Table 1.

At tailbud stage (GS17-18; Fig. 1A), embryos are about 2 mm long, unpigmented, and markedly kyphotic (average dorsal curvature ca. 97°), curved over a large subspherical yolk mass that occupies ca. 50 % of the body surface area. Later, the first gill pair buds are visible on both sides of the cephalic region (GS19; Fig. 1B). Type-C adhesive glands (*sensu* Nokhbatolfighahai et al., 2005) differentiate and are visible as two bumps posterolateral to the stomodeum. The second pair of gills differentiate (GS19-20; Fig. 1C) and both pairs start to branch (GS20-21; Fig. 1D,E). The hatching gland is evident as revealed by ultrastructural analysis in *P. signifer*. Hatching cells are arranged in a T-shaped area, frontally and along a long dorsal line (Fig. 2A); individual cells are scattered in a discontinuous patch (Fig. 2B) and show rather short microvilli (Fig. 2C). When the tail reaches the body length, a short third gill pair develops (Fig. 2A). At this point, a slight pigmentation appears, with the first melanophores occurring dorsally on the cephalic region and the proximal tail. Gills at full development reach only the first third of the body length (Fig. 3A). Gills are branched and ciliated; the first pair is the longest and branches into 6-8 (*P. signifer*; Fig. 3A,B) or 5 (*P. camacan*; Fig. 6A,E) filaments, with the primary filament being the longest (0.43 and 0.55 mm in both species, respectively). The second pair has 5-6 (*P. signifer*) or 4-5 (*P. camacan*) filaments, and the third pair remains very short, non or scarcely branched, and almost covered by the operculum margin. Adhesive glands are conical, prominent, with small secretory cells arranged at the central region (Fig. 3A). As development progress, the operculum differentiates at the gill base (GS22-23; Figs. 1F,G, 3A and 6A), later fuse medially (Figs. 1H, 3B, and C) (before gills reach full development in *P. camacan*; Fig. 6B), and gills begin regression. The right gill is concealed

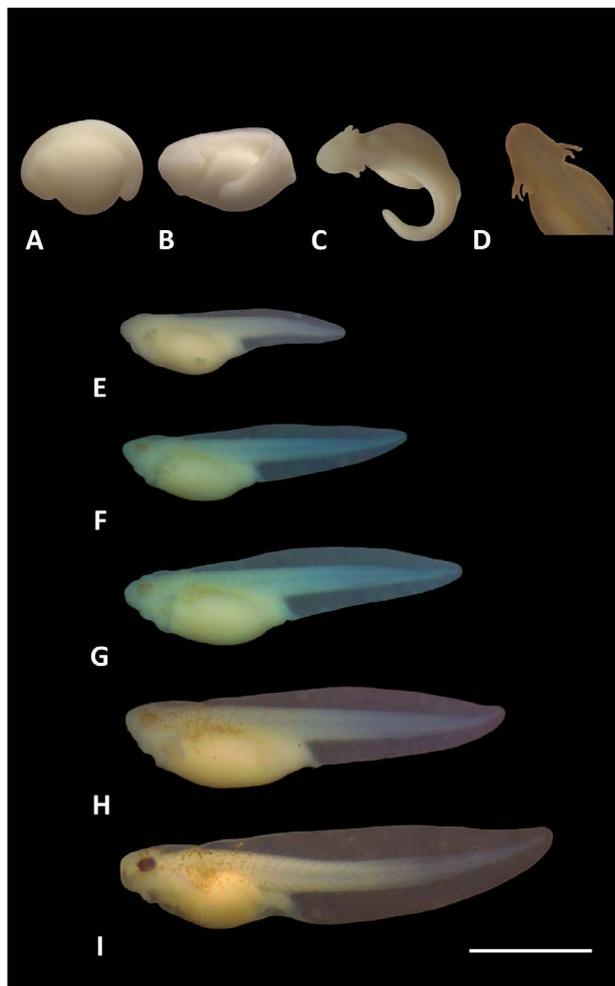


Figure 1. Developmental series of *Physalaemus signifer*. **(A)** Embryo at tailbud stage. **(B)** Differentiation of the first gill pair. **(C)** Differentiation of the second gill pair. **(D–E)** First and second gill pairs branched. **(F)** Operculum at the gill base. **(G)** Gills at full development. **(H)** Operculum medially fused. **(I)** Right gill concealed. Scale bar = 2 mm.

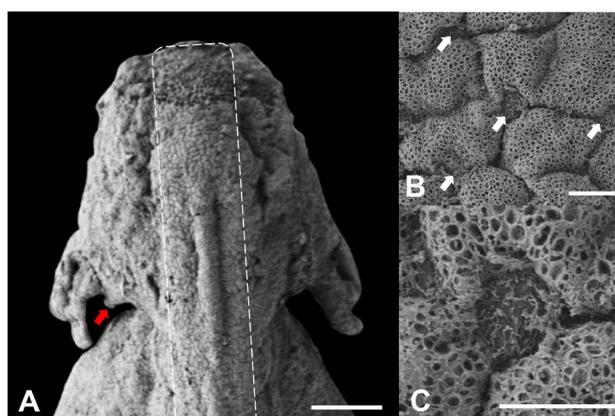


Figure 2. Hatching gland in embryos of *Physalaemus signifer*. **(A)** Dorsal view showing gland arrangement (dotted area). **(B)** Distribution of secretory (white arrows) and epidermal cells. **(C)** Individual secretory cell with microvilli. Note the short third gill pair almost covered by the developing operculum (red arrow). Scale bars = 200 μm **(A)** and 5 μm **(B, C)**.

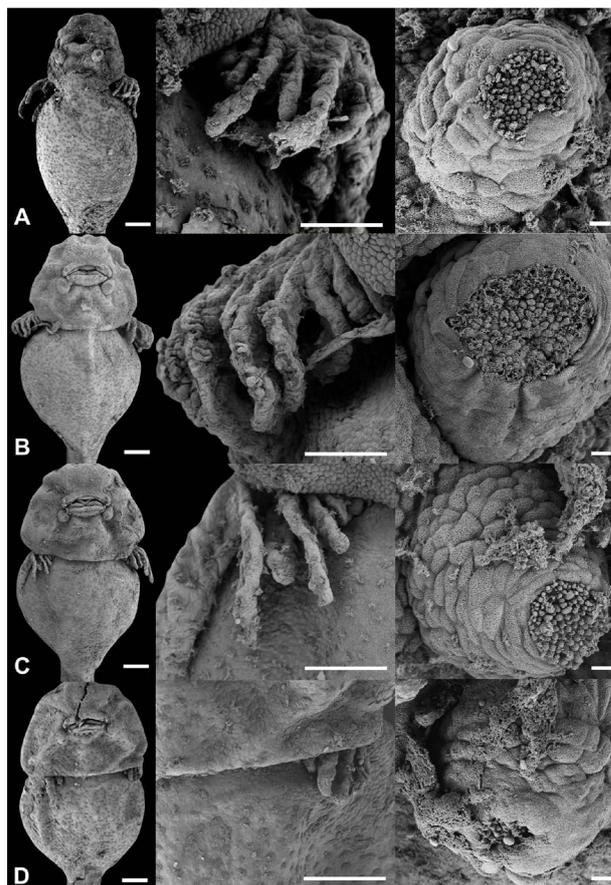


Figure 3. External gill and adhesive gland development in *Physalaemus signifer*. **(A)** Embryo with operculum at the gill base, showing details of the left gill and left adhesive gland. **(B)** Embryo with operculum medially fused, and details of right gill and left gland. **(C)** Embryo with operculum medially fused and gills starting regression, and details of right gill and right gland. **(D)** Concealment of the right gill, and details of left gill and left gland. Scale bars = 200 μm (left column), 100 μm (middle column) and 10 μm (right column).

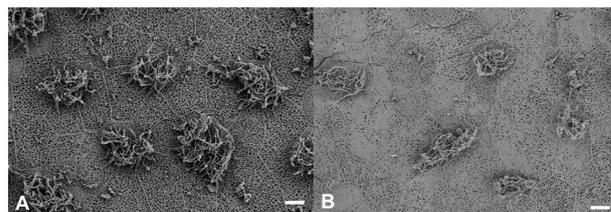


Figure 4. Body ciliation in embryos of *Physalaemus signifer*. **(A)** Maximum density of ciliated cells in the abdominal region of a specimen with operculum at the gill base. **(B)** Ciliation regressing from right gill concealment. Scale bars = 5 μm .

by the operculum (GS24; Figs. 1I and 3D), and finally the left gill regresses and the spiracle is formed (GS25; Fig. 6C,D). Limb buds differentiate before (*P. signifer*) or shortly after (*P. camacan*) the complete formation of the spiracle. Adhesive glands become less prominent throughout this lapse, and the secretory region starts to regress concomitant with the right gill regression (Fig. 3D). Body

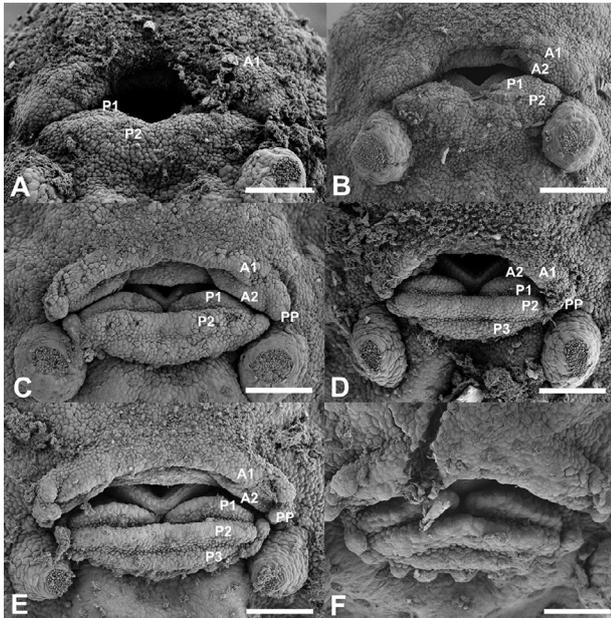


Figure 5. Development of the oral disc in *Physalaemus signifer*. **(A)** Embryo with fully developed gills, showing rows A1, P1 and incipient P2. **(B)** Embryo with operculum at the gill base showing differentiated row A2. **(C)** Embryo with operculum medially fused and developing marginal papillae (PP). **(D)** Embryo with operculum medially fused and row P3. **(E)** Embryo with regressing gills and marginal papillae progressing medially. **(F)** Embryo with right gill concealed and complete marginal papillae. Scale bars = 100 μ m.

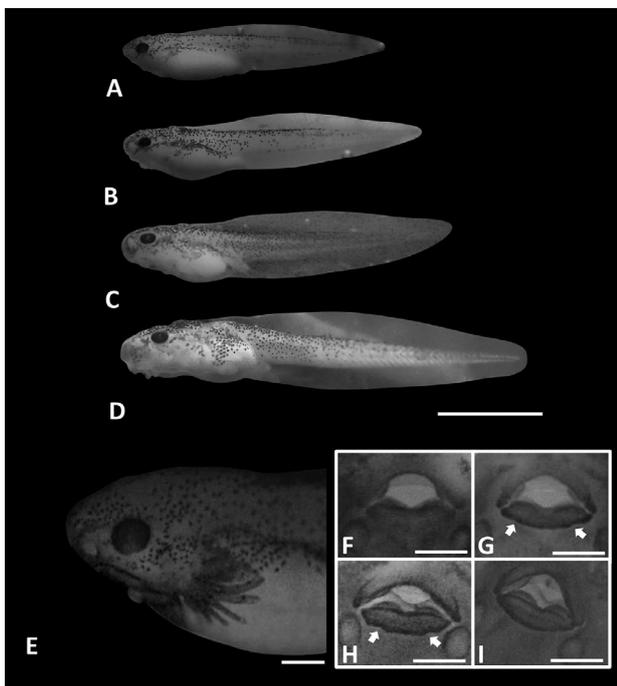


Figure 6. Developmental series of *Physalaemus camacan*. **(A)** Embryo with operculum at the gill base. **(B)** Operculum medially fused. **(C)** Gills concealed. **(D)** Hind limbs at Gosner Stage 26. **(E)** Detail of the gills in the specimen figured in **(A)**. **(F–I)** Development of the oral disc, from specimens with operculum at the gill base to gills concealed. Note the lower lip with small ventrolateral indentations (white arrows). Scale bars = 2 mm **(A–D)**, and 0.5 mm **(E–I)**.

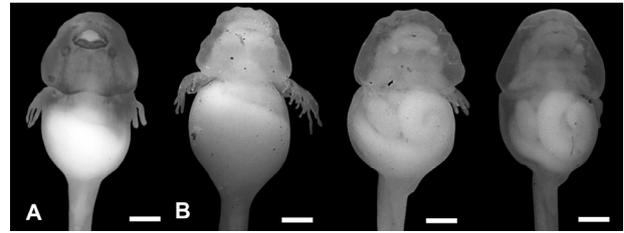


Figure 7. Development of the digestive tract. **(A)** *Physalaemus camacan*, showing the beginning of coiling in a specimen with operculum medially fused. **(B)** *Physalaemus signifer*, showing coiling progression from the stage of medially fused operculum to left gill regression. Scale bars = 0.5 mm.

ciliation also changes during the embryonic ontogeny: from large, densely arranged ciliated cells of embryos with fully developed gills (Fig. 4A), cells become smaller and sparsely disposed during gill occlusion (Fig. 4B). The development of the oral disc begins concomitant with the differentiation of the operculum, when a curved upper lip and a slightly indented lower lip are evident. Later, labial tooth ridges begin differentiation, starting with rows A1, P1, P2 (Fig. 5A) and followed by A2 (Figs. 5B and 6F). The marginal papillae appear at commissures (Fig. 5C) and row P3 differentiates as a transverse ridge distal to P2 (Fig. 5D). Marginal papillae development progresses medially (Fig. 5E), until large, rounded, widely spaced papillae surround the whole lower lip (Figs. 5F and 6I). Ventrolateral gaps are apparently not defined, but the lower lip in embryos of *P. camacan* shows two shallow, transient indentations that could be comparable (Fig. 6G,H). Keratinisation of the jaw sheaths and labial ridges (i.e. serrations and labial teeth) completes after the soft mouthparts are formed. The larval oral disc shows a C3 pattern (*sensu* Vera Candiotti et al., 2011), consisting of a LTRF 2(2)/3(1) and complete lower marginal papillae. Finally, in the digestive tract, the first coils develop shortly after gills are fully developed and row P3 is differentiated in the oral disc; yolk persists after the spiracle is formed (Fig.7).

DISCUSSION

Embryonic morphology is, in general, conserved in species of *Physalaemus* (see Vera Candiotti et al. 2011; Grosso et al. 2019), and, with some slight differences, the close similarity between embryos of *P. camacan* and *P. signifer* and regarding congeneric species was expected. Grosso et al. (2019) recovered some putative morphological and heterochronic synapomorphies for embryonic *Physalaemus*, but since no representatives of the *P. signifer* clade are included in that analysis, the authors highlighted that those features could indeed define the *P. cuvieri* clade. Small size (less than 2 mm), deeply kyphotic dorsal curvature, and lack of pigmentation at tailbud stage are common to most species of *Physalaemus* including the two in this study; they are shared with embryos of *Engystomops* and some widespread among other leiupeirines. Also, like most congeneric species, *P. camacan* and *P. signifer* develop three pairs of gills. After inclusion of these species in a

Table 1. Measurements of embryos at tailbud stage and gill aspects for species of *Physalaemus*. Absolute values are given as average \pm standard deviation (excepting the first gill filament where only the longest is consigned), and those corresponding to species from the *P. cuvieri* clade are taken from Grosso et al. (2019). Tailbud stage (**TB**): Body length (**BL**), Body area (**BA**), Yolk area (**YA**), Yolk proportion (**YP**), Dorsal curvature (**DC**); Gill at full development (**GFD**): First gill filament (**FGF**); number of specimens (**N**). Cells with (-) indicate that embryos were not available for those measurements.

Species	N	TB					GFD	
		BL (mm)	BA (mm ²)	YA (mm ²)	YP (%)	DC (°)	N	FGF (mm)
<i>P. signifer</i>	4	2.01 \pm 0.07	1.06 \pm 0.07	1.12 \pm 0.08	51	96.9 \pm 0.01	6	0.43
<i>P. camacan</i>	-	-	-	-	-	-	3	0.55
<i>P. aff. albonotatus</i>	8	1.53 \pm 0.1	1.13 \pm 0.19	0.48 \pm 0.05	42.5	66 \pm 5.06	12	0.73
<i>P. albifrons</i>	2	1.40 \pm 0.04	1.12 \pm 0.1	0.38 \pm 0.01	33.7	46 \pm 5.66	8	0.79
<i>P. albonotatus</i>	7	1.50 \pm 0.06	1.16 \pm 0.04	0.44 \pm 0.08	38	62 \pm 2.98	14	0.91
<i>P. biligonigerus</i>	5	1.65 \pm 0.06	1.3 \pm 0.14	0.52 \pm 0.13	40	75 \pm 2.88	8	0.68
<i>P. carrizorum</i>	3	1.90 \pm 0.06	1.94 \pm 0.08	0.91 \pm 0.07	46.8	80 \pm 2	7	0.97
<i>P. cicada</i>	-	-	-	-	-	-	5	0.84
<i>P. cuvieri</i>	3	1.55 \pm 0.12	1.86 \pm 0.04	0.77 \pm 0.15	41.5	63 \pm 4.5	6	0.72
<i>P. fernandezae</i>	-	-	-	-	-	-	5	0.30
<i>P. gracilis</i>	-	-	-	-	-	-	8	0.75
<i>P. henselii</i>	-	-	-	-	-	-	3	0.25
<i>P. riograndensis</i>	3	1.36 \pm 0.14	0.96 \pm 0.17	0.46 \pm 0.03	48	64 \pm 2.31	15	1.04
<i>P. santafecinus</i>	3	1.43 \pm 0.09	1.21 \pm 0.1	0.57 \pm 0.03	47	71 \pm 2.52	10	0.69

phylogenetic analysis, this feature could maintain the status as a putative synapomorphy of *Physalaemus*, with an instance of reduction to two pairs in the *P. henselii* species group. Regarding adhesive glands, Grosso et al. (2019) recovered the morphogenetic type C as the plesiomorphic state for Leiuperinae, and accordingly, glands in *P. camacan* and *P. signifer* have the same morphology and development as those described for most *Physalaemus* and *Pseudopaludicola*. Likewise, the configuration of the hatching gland and the general morphology of hatching gland cells are like those of other *Physalaemus*. Finally, hind-limb development follows a similar pattern as in *Engystomops* + *Physalaemus* clade, with limb buds differentiating almost simultaneously with spiracle formation.

Combined with these features overall conserved at the generic level, some traits appear to be distinctive for species of the *P. signifer* clade. Species studied are almost identical in development, with some slight variations in

differentiation of hind-limb buds (earlier in *P. signifer*) and medial fusion of the operculum (earlier in *P. camacan*). Main differences of these species regarding most members of the sister clade *P. cuvieri* are related to egg/embryo size, gill development and the ontogeny of the oral marginal papillae. Some of these transformations could be correlated with the different modes of oviposition, but others appear to be developmental modifications not related with ecomorphological aspects.

Previous reports highlight differences in clutch and egg sizes of species of the *P. signifer* clade as compared with congeneric species: while egg number is significantly higher in species with aquatic oviposition, egg size is larger in species with terrestrial nests (Pupin et al., 2010). Large eggs usually develop into large embryos with increased yolk provision (Salthe & Duellman, 1973). Although our small sampling prevents us from definitive conclusions, preliminary observations indicate that early embryos of *P. signifer* clade are larger and provisioned

with proportionately more yolk than known embryos of the *P. cuvieri* clade (Table 1). Persistence of yolk in the developing digestive tract is also longer than in other *Physalaemus* (Grosso et al., 2019). Yolk supply may ensure survival of embryos within the nests, and it likely represents an advantage for hatchlings in terrestrial nests that depend on being flooded or washed away to water bodies for further development (Salthe & Duellman, 1973; Pupin et al., 2010, 2018). A similar correlation between embryo size, yolk proportion, and persistence of hatchlings within the nests is reported for embryos of the *Leptodactylus fuscus* species group that develop in nests in underground galleries (Downie, 1984; Grosso et al., 2017). Interestingly, size differences between embryos of *P. signifer* and *P. cuvieri* clades persist at older embryonic stages (GS 24–26; Oliveira et al., unpubl. data) but reverse in larval and postmetamorphic periods, rendering the tadpoles and adults of *P. signifer* and *P. camacan* among the smallest in the genus (Weber & Carvalho-e-Silva, 2001; Pimenta et al., 2005). The initial investment in body growth at the expense of yolk provision, along with a likely later beginning of active feeding suspected from the yolk persistence in the digestive tract, could explain these differences in proportional size-increase from embryos to tadpoles to adults in species of *Physalaemus*. From a functional perspective, large size and yolk provision could be only essential for these embryos at first feeding in their aquatic environments, in a context of interspecific competition or uncertain availability of food resources.

Except for species of the *P. henselii* group, gills are in general well developed in *Physalaemus* (Grosso et al., 2019). Within the genus, gills are larger and more branched in embryos of *P. cicada* and species of the *P. cuvieri* group, and a relation with breeding in warm, xeric environments has been suggested for *P. cicada* and other Leiuperinae (Grosso et al., 2019). Gill size and branching is comparatively smaller in *P. camacan* and *P. signifer* here studied (Table 1) but wider sampling is needed before making generalisations at clade level and correlations with oviposition sites.

The development of the oral disc was studied in several species of the *P. cuvieri* clade, and ontogenetic patterns were summarised by Vera Candiotti et al. (2011) and Grosso et al. (2019). Our study confirms that the first lower tooth ridge (P1) is the earliest to differentiate on the lower lip of species of the *P. signifer* clade, as occurs in all other known species of *Physalaemus* and *Pseudopaludicola*, but unlike *Pleurodema* (row P2 develops first). Nevertheless, development of the lower marginal papillae indicates that the scenario could be more complex than that synthesised by the cited previous studies. According to those contributions, *Physalaemus* and *Pseudopaludicola* are characterised by the occurrence (transient or maintained in larval stages) of ventrolateral gaps in the lower marginal papillae. Additionally, the five different configurations of the oral disc known for *Physalaemus* would result from common ontogenetic trajectories ending at different states, or from trajectories that differ initially in the formation of

a ventral gap (see Figs. 4 and 10 in the cited papers). The oral disc development of the species here studied was expected to fit in one of two main trajectories, likely that leading to the oral configuration of *P. gracilis* with a similar larval oral disc (labial tooth row formula 2/3 plus complete marginal papillae, i.e. the C3 configuration *sensu* Vera Candiotti et al., 2011). However, observations in both *P. camacan* and *P. signifer* apparently reveal a different pattern: although the small indentations of the lower lip margin could represent some variant of gaps, we never observed proper marginal papillae developing initially on the mental region, thus ventrolateral gaps cannot be undoubtedly defined as present as in other species described in that trajectory. If this novel trajectory was confirmed for the *P. signifer* clade (with more resolution in developmental series and including additional species), this would imply that the most widespread oral configuration in *Physalaemus*, the C3 configuration, develops according varied pathways that so far follow: i) a complex, recapitulatory way that includes only ventrolateral gaps as in closely related species (in *P. gracilis* of *P. gracilis* group); ii) an alternative way that includes ventral and ventrolateral gaps (in *P. carrizorum* of *P. gracilis* group); iii) a combined way that joins the development of all gaps with an early filling of ventral gap (in some specimens of *P. cicada*; for these first three, please see Fig. 10 in Grosso et al., 2019); and iv) a novel, “telescoped” version of some of these trajectories, where papillae appear to progress medially, but the mental region still exhibits some vestigial structures defining incipient ventrolateral gaps (in *P. camacan* and *P. signifer* of *P. signifer* clade). Clearly, a more exhaustive sampling of species with C3 oral discs and detailed studies of how they develop are needed to assess this subject.

In contrast to uncertain conditions characteristic of temporary ponds in open seasonal areas, more predictable environments such as ponds inside the Atlantic rainforest may favour the evolution of varied reproductive strategies (Haddad & Prado, 2005; Pupin et al., 2018). In this context, embryonic morphology and physiology could be also highly adaptive to face requirements of alternative microhabitats, and transient embryonic and larval features exhibit transformations correlated with survival and resource acquisition. Our studies in early ontogeny of species of *Physalaemus* are framed in this scenario, but further investigations, especially from ecological and experimental perspectives, are encouraged to deepen this subject.

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Authors' Contribution

Marianna Isabella Rosa Rodrigues de Oliveira: Conceptualisation; formal analysis; investigation; methodology; project administration; writing-original draft; writing review & editing. Jimena Grosso: Methodology; investigation; writing review & editing. Marcelo Felgueiras Napoli: Supervision; writing review & editing. Luiz Norberto Weber: Supervision; conceptualisation; formal analysis; methodology; resources; supervision; writing original draft. Florencia Vera Candiotti: Supervision; conceptualisation; formal analysis; investigation; methodology; supervision; writing-original draft; writing review & editing.

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