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Embryonic morphology in five species of *Hypsiboas* (Anura: Hylidae)

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Research concerning the early development of anuran tadpoles has sparked new interest, ever since comparative studies revealed structural and temporal variations of embryonic stages within different taxonomic groups. In this paper we studied the early ontogeny of five species of the hylid genus *Hypsiboas*: *H. curupi*, *H. pulchellus*, *H. riojanus* and *H.* sp. from the *H. pulchellus* group, and *H. faber* from the homonymous group. We analyse the development of typical larval structures (oral disc), and of embryonic transient structures (external gills, ciliated cells, hatching glands and adhesive glands). The diversity in structural patterns is mainly related to the number and size of external gills, size of the adhesive glands, ciliation density and number of labial tooth ridges. In turn, heterochronic shifts mostly concern the time of adhesive gland division, and the regression of the hatching gland and ciliated cells. In some cases these variations appear to be related with oviposition sites and environments where embryos and larvae develop. *Hypsiboas faber* embryos, which develop in small nests outside water bodies, exhibit the largest hatching gland and large, densely ciliated and highly branched external gills as a possible response to low oxygen environments. The large and persistent adhesive glands of *H. curupi* and *H.* sp. gr. *pulchellus* might be related to the development of embryos and larvae in small streams. Within the same intrageneric group, certain embryonic traits of *H. pulchellus* (e.g., tooth row formula 2/3, minute external gills, low body ciliation) appear to be paedomorphic regarding ancestral ontogenies, but the ecological/functional correlation (if any) of these features is uncertain.

Key words: adhesive glands, ciliation, external gills, hatching gland, oral disc

INTRODUCTION

The idea that different vertebrate groups undergo similar developmental states with only a late surge of distinctive characters during ontogeny has impregnated developmental biology in the XIX century (e.g., Von Baer, 1828; De Beer, 1851; Haeckel, 1874). However, comparative studies, mainly published in the last decades, revealed the occurrence of significant variations among embryonic stages of different vertebrate groups (e.g., Richardson, 1995; Richardson et al., 1997). In anurans, early ontogeny involves the development of exomorphological characters typical of the larval period (e.g., oral disc and lateral lines), and the appearance of embryonic transient features which disappear around hatching (Altig & McDiarmid, 1999). These structures may vary interspecifically, both in their morphological patterns and in developmental timing. Heterochronic changes are a well-established source of evolutionary change (Klingenberg, 1998), and the diversity of larval and embryonic structures entails an intrinsic interest because of the early mechanisms that cause it and of its high evolutionary potential.

Embryonic structures include external gills, epidermic ciliated cells and hatching and adhesive glands. Two or three pairs of external gills develop along with the larval gills on the ventral and ventrolateral surface of the gill arches; while the latter persists up until metamorphosis, transient gills are covered by the operculum usually soon after hatching (Viertel, 1991; Brunelli et al., 2004). The ciliated epidermic cells arise during neurula stage and disappear after hatching (e.g., Morgan, 1897), and they likely play various roles; e.g., gas exchange, pre and post-hatching movements, and sensory functions (Altig & McDiarmid, 1999). The hatching gland is a concentration of cells with microvilli on the anterior region of the head and body, which releases proteolytic enzymes that break egg membranes during hatching, disappearing once larval life begins (Altig & McDiarmid, 1999). Finally, the adhesive glands are generally paired structures located posterolaterally to the oral disc, which produce mucus that provides embryos of adherence to surfaces both inside and outside the egg. In

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the last decade, interspecific variations in morphogenetic patterns of transient structures have been studied in about twenty anuran species, in relation to taxonomy and ecomorphological groups (Nokhbatolfoghahai & Downie, 2005; 2007; 2008; Nokhbatolfoghahai et al., 2005; 2006; 2015). Among larval structures, the oral apparatus exhibits a wide morphological and functional variation across different lineages. Studies on the early development of the oral disc involve mainly species with a generalised configuration; i.e., with labial teeth in two anterior and three posterior rows (labial tooth row formula – LTRF– 2/3). Nevertheless, observations in species with different oral arrangements revealed variations in the morphology and developmental sequence of mouth parts (e.g., the formation of ventrolateral gaps and changes in differentiation order of tooth ridges; Hall et al., 1997; Vera Candioti et al., 2011). Embryonic and larval structures have a well-known role during embryo and tadpole development (e.g., hatching, gas exchange, feeding), for which variation can be expected when comparing species with varied oviposition and developmental modes.

In this work, we studied the early ontogeny in five tree frog species of the Neotropical genus *Hypsiboas* Wagler, 1830 (Hylidae: Hylinae: Cophomantini), namely *H. faber*, *H. pulchellus*, *H. riojanus*, *H. curupi*, and a currently unnamed species similar to the latter, referred here as *H.* sp. gr. *pulchellus*. These species belong to *H. faber* and *H. pulchellus* groups, which are two closely related, derived clades within the genus (Faivovich et al., 2005). However, ovipositional modes and larval morphology greatly differ between them: *H. faber* (similarly to most of the species of the group) lay the eggs in a nest built by males with sand, clay or mud, at the edges of temporary ponds or streams (e.g., Kluge, 1979; Martins, 1993); while species of the *H. pulchellus* group breed in ponds, puddles or small streams, attaching their eggs to submerged plants (e.g., Carrizo, 1991; Garcia et al., 2007). Larval oral discs of *Hypsiboas* exhibit several configurations, including a variety of labial tooth row formulae: 2/3–2/4 and rarely 3/4, 3/5, 4/7, 5/8, and 6/9 (see Kolenc et al., 2008). Species in our study ranged from a LTRF 2/3 to 3/5. While information about embryonic structures of three congeneric species is available (Nokhbatolfoghahai and collaborators op. cit.), there is no data to date regarding oral disc ontogeny in *Hypsiboas*, and the development of tooth ridges in different oral arrangements other than the basic 2/3 pattern is a poorly explored subject.

The main purpose of our study is thus to compare the ontogenetic trajectories of *Hypsiboas* embryos, accounting for (1) structural variations in external gills, adhesive and hatching glands, body ciliation, and oral discs, and (2) temporal variations in the developmental sequence of the mentioned structures.

MATERIALS AND METHODS

We worked with embryos of *Hypsiboas curupi*, *H.* sp. gr. *pulchellus*, *H. pulchellus*, *H. riojanus* (*H. pulchellus* group) and *H. faber* (*H. faber* group; Faivovich et al., 2005). Clutches of each species were obtained in the field from amplectant adults (Online Appendix 1), transported to the laboratory, and maintained in containers with dechlorinated water. Sub-samples of each clutch were fixed in 10% formalin at the time of collection, and every 6–8 hours for about a week. We examined embryos of the developmental series (*n*=28–1192; see Online Appendix 1) searching for relevant morphological changes. We focused mainly on the lapse between tailbud and limb emergence stages (Gosner, 1960 stages 18 to 25), extending our observations whenever necessary. Additional definition of certain stages was mandatory in some cases, since developmental events were not discriminant in the studied sample. Stages 18 to 20 were redefined based on the ratio tail length / tail height at the base as follows: St. 18: tail shorter than tail height; St. 19: tail shorter than twice the height at the base; St. 20: tail length twice the height at the base. Stages 21 and 22 were distinguished exclusively by the ratio tail length / body length as follows: St. 21: tail as long as the body; St. 22: tail longer than the body.

Specimens were examined, photographed and schematised with a stereomicroscope equipped with a camera lucida, and methylene blue was employed to stain and contrast structures such as gills, adhesive glands, and oral papillae (Wassersug, 1976). Some specimens (8–10 per species) were prepared for scanning electron microscopy following Fiorito de López & Echeverría (1984). Oral disc development was described following Thibaudeau & Altig (1988) and Vera Candioti et al. (2011). For the characterisation of embryonic structures, we followed Nokhbatolfoghahai & Downie (2005; 2007; 2008) and Nokhbatolfoghahai et al. (2006). Quantification of ciliation density was performed following Nokhbatolfoghahai et al. (2005), with some modifications introduced (detailed in Online Appendix 2). A table summarising the sequence of relevant developmental events was made for each species.

In addition to the identification of different morphological patterns, the sequence of developmental events was also registered (sequence heterochrony; Smith, 2001). Landmark events were defined for the ontogeny of each structure described (e.g., gill buds, gill branching, gills at full development), and a chronological table combining all character events was obtained for each species.

RESULTS

We describe the development of *Hypsiboas faber* (developmental series is summarised in Fig. 1), and differences are synthesised for the other four species whenever relevant (Figs. 5–11). Table 1 summarises event sequences in all species, and Online Appendices 2 and 3 show comparative ciliation patterns.

Hypsiboas faber

After tailbud stage, embryos are slightly lordotic with curved tails and branchial arches appearing as two small bumps on both sides of the anterior region (Fig. 1). The hatching gland is noticeable as a dorsal pigmented line up to approximately half of the body, and the adhesive glands are posterior to the stomodeum and are V-shaped, with rounded, prominent halves and a deep groove between them. The stomodeum is closed and a shallow groove is visible between it and the nostrils. The abdomen is

Table 1. Developmental events sequence in five species of *Hypsiboas*. Synchronic events are noted with the same number, while those not directly observed are indicated with (?). Coloured events correspond to operculum development (Gosner stages 23, 24, and 25: orange scale), oral disc with definitive LTRF (green), and differentiation of hind limbs (yellow) to highlight relevant interspecific changes in the sequence.

H. faber	*H. riojanus*	*H. pulchellus*	*H. curupi* and *H.* sp. gr. *pulchellus*
adhesive gland as V-groove	adhesive gland as V-groove	adhesive gland as V-groove (?)	adhesive gland as V-groove
gill buds	gill buds	gill buds (?)	adhesive glands separated
hatching gland at full development	hatching gland at full development	hatching gland at full development (?)	hatching gland at full development
gill branching	gill branching	1. densest body ciliation	gill buds
adhesive glands at full development	densest body ciliation	1. operculum at the gill base	gill branching
adhesive glands separated	operculum at the gill base	1. adhesive glands at full development	hatching gland not visible
operculum at the gill base	hatching gland not visible	gill branching	operculum at the gill base
LTRF 1/1	1. gills at full development	adhesive glands separated	densest body ciliation
densest body ciliation	1. adhesive glands at full development	operculum medially fused	gills at full development
hatching gland not visible	operculum medially fused	LTRF 1/1	operculum medially fused
gills at full development	LTRF 1/1	gills at full development	LTRF 1/1
operculum medially fused	adhesive glands separated	1. marginal papillae in upper lip and commissures	1. right gill covered
1. LTRF 2/2	2. LTRF 2/2 (?)	1. body ciliation in regression	1. LTRF 2/2
1. LTRF 2/3	2. LTRF 2/3	1. right gill covered	1. LTRF 2/3
1. marginal papillae in upper lip and commissures	3. marginal papillae in upper lip and commissures	1. LTRF 2/2	1. adhesive glands at full development
2. first labial teeth	3. right gill covered	LTRF 2/3	left gill covered
2. marginal papillae complete	first labial teeth (?)	hatching gland not visible	spiracle formed
right gill covered	body ciliation in regression	left gill covered	marginal papillae in upper lip and commissures
body ciliation in regression	left gill covered	spiracle formed	marginal papillae complete
left gill covered	spiracle formed	marginal papillae complete	first labial teeth (?)
spiracle formed	marginal papillae complete	first labial teeth (?)	labial teeth on P3
labial teeth on P3 (?)	labial teeth on P3	labial teeth on P3	LTRF 2/4
LTRF 2/4	adhesive glands not visible	adhesive glands not visible	body ciliation in regression
adhesive glands not visible	commissural papillae with teeth	hind limbs	adhesive glands not visible
commissural papillae with teeth	hind limbs		LTRF 3/5 (fragmented rows)
hind limbs			commissural papillae with teeth
			2. hind limbs
			2. LTRF 3/5 (complete rows)

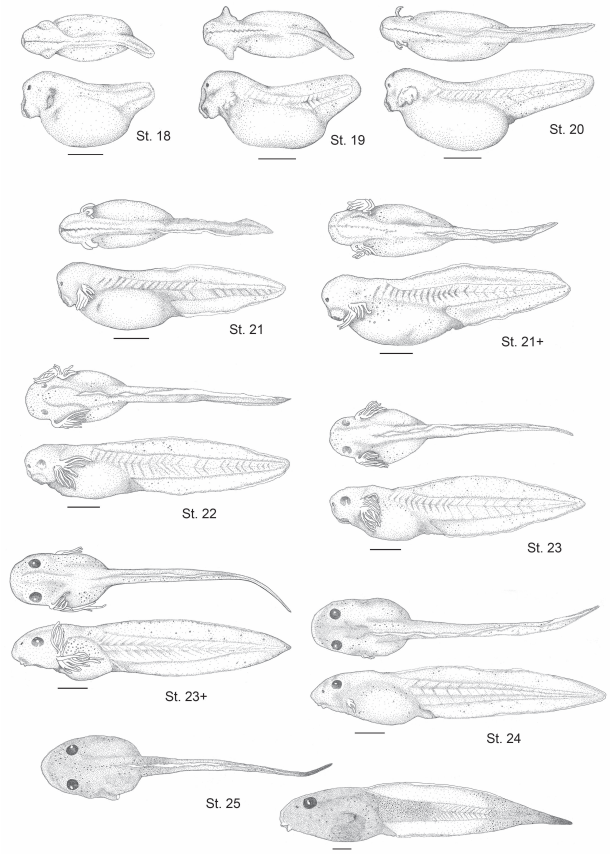

Fig. 1. *Hypsiboas faber*, developmental series (St. 18–25) of embryos in dorsal and lateral views. Bar=1 mm.

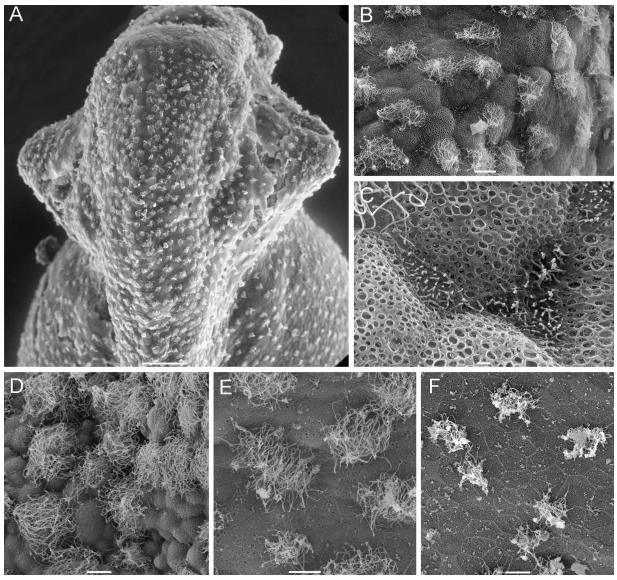

Fig. 2. *Hypsiboas faber*. Hatching gland at St. 19: (A) Dorsal view showing gland extension, (B) Detail of secretory and epidermic ciliated cells, (C) Detail of secretory cells with apical microvilli. Ciliation: (D) St. 21, (E) St. 23+++, (F) St. 25+. Bars=10 µm, except (A) 100 µm and (C) 1 µm.

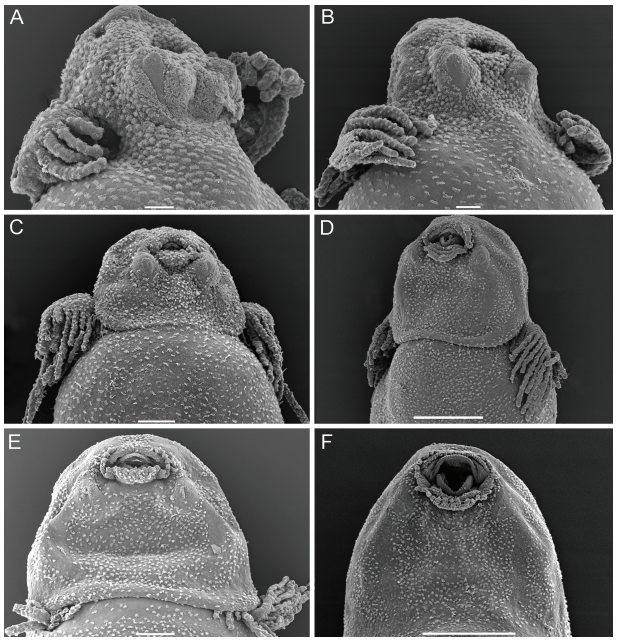

Fig. 3. *Hypsiboas faber*, adhesive gland and external gill development: (A) St. 21, (B) St. 22, (C) St. 23, (D) St. 23+, (E) St. 23+++, (F) St. 25. Bars=100 µm (A, B), 200 µm (C), 500 µm (D, E, F).

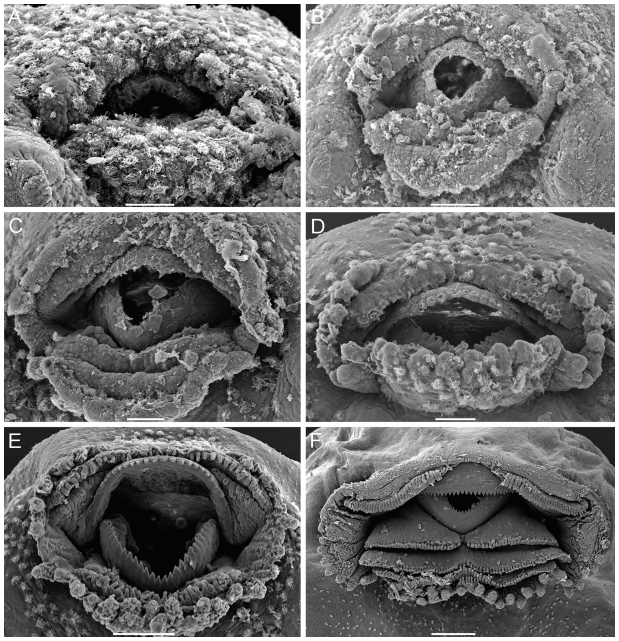

Fig. 4. *Hypsiboas faber*, oral disc development until reaching larval LTRF 2/4: (A) St. 23, (B) St. 23+, (C) St. 23++, (D) St. 23+++, (E) St. 25, (F) St. 25+. Bars=50 µm (A–D), 100 µm (E), 200 µm (F).

rounded, full of yolk. As the tail lengthens, pigmentation appears, and the first pair of gills is evident as a curved branched bar; while the second pair of gills emerges as a short bulge. The hatching gland (Fig. 2A-C) is visible at SEM as a thin, non-ciliated dorsal line; secretory cells are concentrated in the transverse frontal region and along a zigzag pattern in the dorsal midline; irregular elongated cells bearing short microvilli appear irregularly arranged, often contiguous. Ciliation is profuse in most body regions, and becomes sparser later; ciliated cells are large, with long cilia (Fig. 2D-E). In the tail, myomeres are distinguished in the first third, and the fins are opaque with a rounded tip. As the tail length reaches twice the height of the base, body pigmentation intensifies and the second gill pair ramifies. By the time the tail is as long as the body, embryos are heavily pigmented and densely ciliated, mainly on the anterior ventral head and oral region. The adhesive gland (Fig. 3A) reaches its full development (9.5% of the body length) but is still a single V-shaped structure, which later divides into two separated glands connected by a non-ciliated and shallow depression. Each gland is prominent; with mostly ciliated supporting cells and a drop-shaped secretory central region with small cells showing apical microvilli. In the tail, myomeres are visible in the whole length and fins are translucent with marginal chromatophores; the curved dorsal fin originates behind the external gills and the ventral fin is straight and lower. Later, the tail becomes longer than the body and the cornea becomes transparent. At this point, ciliation acquires its maximum density, and becomes more evident on the anterior ventral head, the stomodeum and adhesive glands. External gills (Fig. 3B-C) grow as to surpass the mid-abdomen, with the first and second pair of gills exhibiting extensive, long secondary filaments; the third pair appears as a short bar and ramifies later. The operculum covers the base of each gill. The oral disc begins to develop at this point, and a curved upper lip, a lower lip with a medial notch, and the upper jaw become very noticeable (Fig. 4A). The tail is longer than the body and fins are transparent. The heart is still evident as a protuberance posterior to the adhesive glands. As the operculum progresses medially (Fig. 3C), eyes start to pigment, to develop completely before the right gill is concealed. Ciliation becomes sparse on the abdomen and adhesive glands, but remains dense in the oral region and gills. External gills become fully developed, with long filaments which surpass the mid-abdomen; the third pair remains scarcely developed (about 1/5 of the first pair length). In the oral disc, rows A1 and P1 differentiate. Later, the operculum grows and fuses medially, its posterior free margin becoming thick, and gills start to regress (Fig. 3D). Ciliated cells undergo a gradual regression which involves the loss of cilia from the periphery to the center, especially in those regions where lateral lines later develop. Adhesive glands are visible as two pigmented rounded structures and the hatching gland on the anterior region of the head disappears. By this time, a fleshy projection appears on the medial margin of each nostril. In the oral disc (Fig. 4B), A2, P2 and P3 develop, 2–3 papillae outline on the commissural region, and jaw sheaths begin to keratinise; later, labial teeth emerge on row A1 and lower marginal papillae are complete (Fig. 4C-D). The coiled gut begins to form when the operculum is medially fused. Next, the operculum covers the right gill (Fig. 3E). The hatching gland is at this point no longer evident and the adhesive glands appear as distinct pigmented regions with an oval secretory zone. As the spiracle develops (Fig. 3F), an important increase in body length (of about 100%) takes place. Embryos have an almost transparent skin with scattered melanophores, and the eyes and internal organs are visible. In the oral disc, five rows bear labial teeth and marginal papillae are arranged in a single, alternate series (Fig. 4E). Later on, the disc is fully developed with a medial, very short P4 which grows laterally up to one third of the length of P3; papillae of the mental region are sparsely arranged, in some cases leaving a small ventral gap. Afterwards, adhesive glands become undistinguishable and sub marginal commissural papillae with teeth differentiate in the oral disc (Fig. 4F). As the spiracular tube becomes longer and separates from the body wall, the tail acquires its definitive colour pattern, with a sharp, pigmented tip.

Hypsiboas pulchellus group

Hypsiboas riojanus

At tailbud stage (Fig. 5), embryos are straight and exhibit a developed hatching gland, visible at SEM images as a non-ciliated, T-shaped region from the frontal region to half the body; secretory cells concentrate at its anterior edge, disposed in a continuous patch and are pentagonal or hexagonal with short, sparse microvilli (Fig. 6A-B). With a tail shorter than twice the height of their base, embryos exhibit the densest ciliation (Fig. 7A), mainly on the adhesive glands; ciliated cells are hexagonal or polygonal in the ventral surface of the head, adhesive glands and gills, and larger and elongated on the ventral trunk. Ciliation starts to regress (Fig. 7B) after the differentiation of the operculum; regression occurs gradually on the gills, glands, anterior head and ventral trunk, but persists on the oral region. As the tail lengthens, the adhesive glands reach their full development (14% of the body length) (Fig. 8A). Fully developed gills reach about 1/3 of the body length (Fig. 8B). When the tail length surpasses that of the body and the operculum is medially fused, the adhesive glands separate into two oblong structures posterolateral to the disc (Fig. 8B). The hatching gland is barely evident on the frontal region and the oral disc starts to develop prominent lips. When the right gill is covered by the operculum (Fig. 8C), ciliation reduces on the oral region, while the adhesive glands are still visible as protuberant rounded regions. In the oral disc, row A2 develops, ventral and commissural papillae begin to develop and jaw sheaths are keratinised (Fig. 9A). The spiracle forms and embryos are translucent with sparse chromatophores on the dorsal and ventral regions, especially concentrated on the caudal myomeres; larval colour pattern begins to outline, with a dark dorsal line on the caudal musculature (Fig. 5). Adhesive glands are slightly prominent and still bear differentiated cells; they disappear in older specimens. In the oral disc, developed labial teeth are observed in rows A1, P1 and P2, whereas in A2 and P3 the first teeth start to emerge (Fig. 9B); later, row A1 presents the typical deep notch and marginal

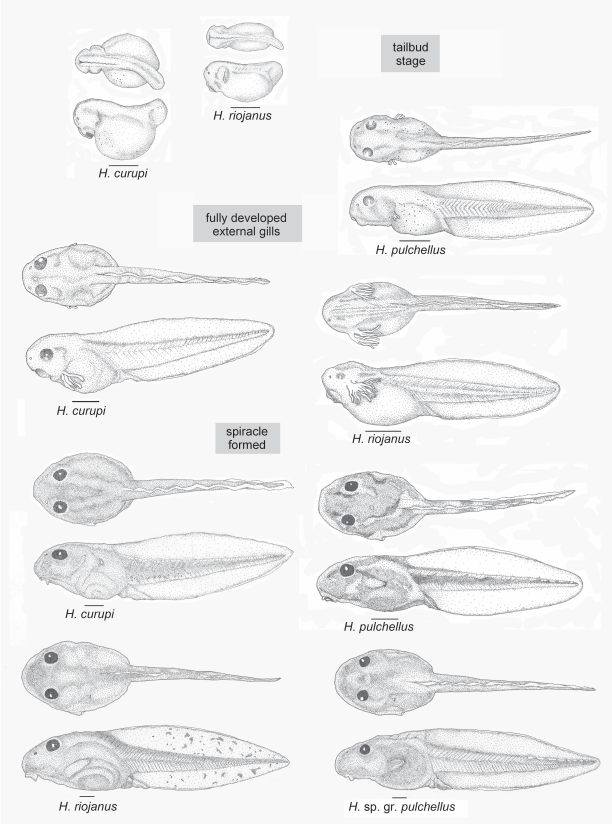

Fig. 5. Comparative drawings of embryos of the *Hypsiboas pulchellus* group (*H. riojanus*, *H. pulchellus*, *H. curupi*, and *H.* sp. gr. *pulchellus*) showing specimens at tailbud stage, external gills at full development, and tadpoles with spiracle formed (St. 25). Bars=1 mm.

papillae complete (Fig. 9C). Larger specimens show submarginal commissural papillae with teeth.

Hypsiboas pulchellus

The youngest embryos of this series were at St. 20, and show a barely evident T-shaped hatching gland. Secretory cells are sparse, usually elongated or fusiform, with short microvilli (Fig. 6C-D). Ciliation is very sparse, with scattered ciliated cells restricted to the stomodeum region and to the adhesive glands; cells are small to medium, irregular, elongated and transversely arranged (Fig. 7C); ciliation starts to regress (Fig. 7D) when the operculum covers the right gill. External gills are very small (Fig. 8D) and the operculum is visible at the base before the gill branches. The adhesive gland is fully developed at this point (17% of the body length), and constitutes a single U-shaped medial structure, with prominent edges bearing supporting ciliated cells, and a deep central groove with secretory cells. Afterwards, adhesive glands divide. The oral disc begins to develop at this stage, with thick lips and keratinised jaw sheaths. Gills reach full development once the operculum is medially fused (Fig. 8E). When the operculum covers the right gill, changes occur in the oral disc: marginal papillae and labial teeth on A1 differentiate and the lower labium exhibits an indented P1, a distinct P2 and commissural papillae (Figs. 8F and 9D); later at this stage, labial teeth emerge on row P1, marginal papillae appear on the mental region, and row P3 is distinguished as a short medial region. Embryos with spiracle formed (Fig. 5) are 50% larger than in the previous stage and their pigmentation intensifies, mainly on the abdominal region. The oral disc shows labial teeth on the anterior and posterior rows P1 and P2, and complete marginal papillae (Fig. 9E); row P3 is a short, medial ridge in which teeth develop later (Fig. 9F). The tail tip is rounded.

Hypsiboas curupi and *H.* sp. gr. *pulchellus*

Embryos and tadpoles of these two species are very similar, for which we describe their ontogenetic trajectories together.

At tailbud stage, *Hypsiboas curupi* embryos are slightly lordotic (Fig. 5) and show mild ciliation concentrated mainly on the stomodeum and gland regions. The hatching gland of *H.* sp. gr. *pulchellus* shows elliptic or triangular cells disposed contiguously in a characteristic, flower-like

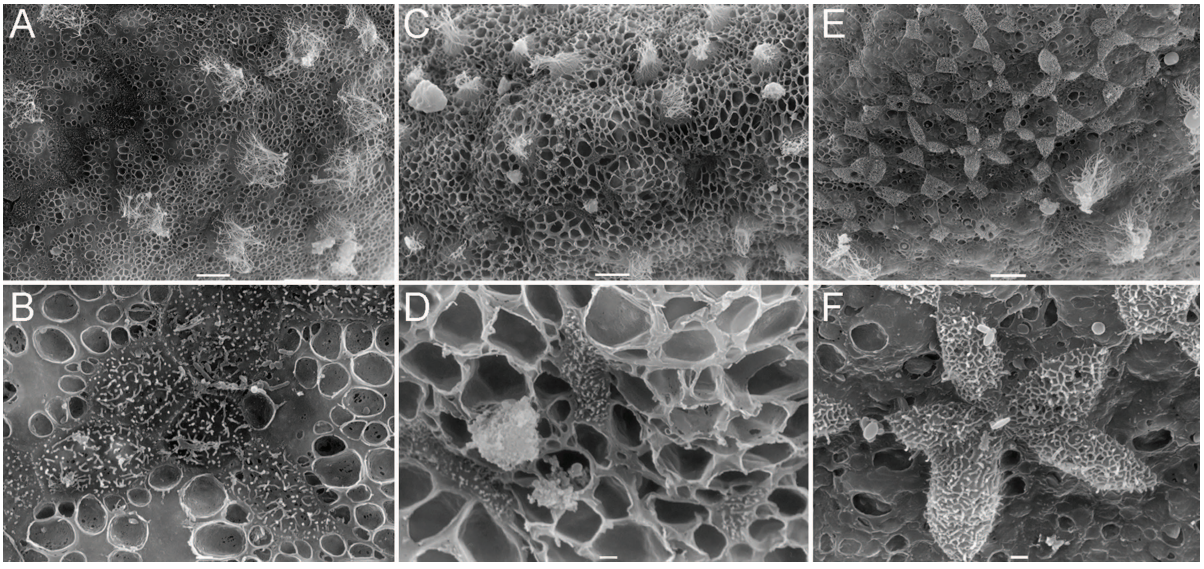

Fig. 6. Hatching gland in three species of *Hypsiboas pulchellus* group, showing the general arrangement (top) and a detail of the secretory cells (down): (A,B) *H. riojanus* St. 18, (C, D) *H. pulchellus* St. 20, (E, F) *H.* sp. gr. *pulchellus* St. 20. Bars=10 µm (A, C, E) and 1 µm (B, D, F).

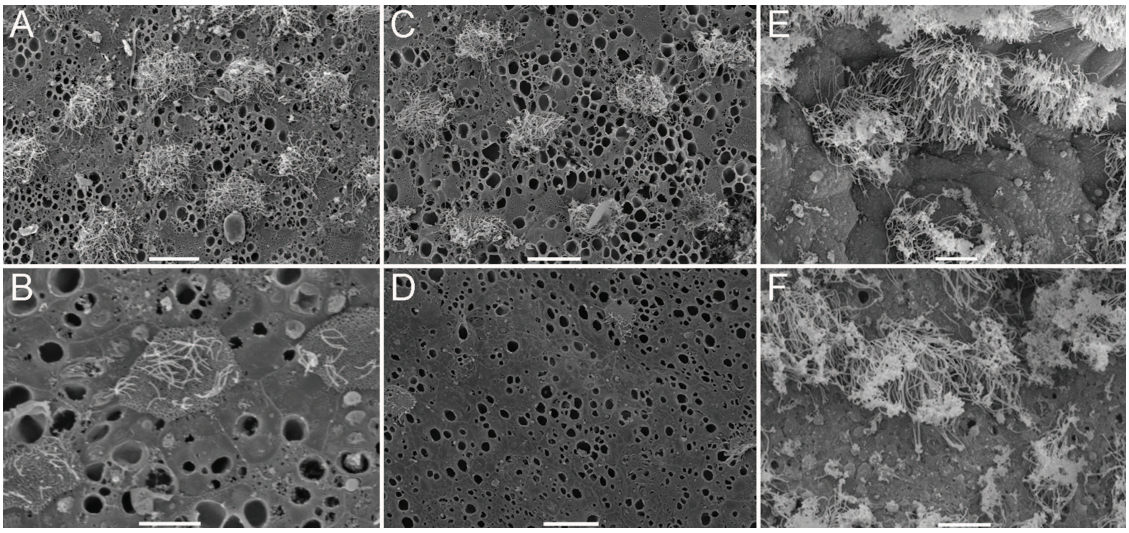

Fig. 7. Ciliation pattern in three species of *Hypsiboas pulchellus* group: (A,B) *H. riojanus* St. 19 and St 23++; (C,D) *H. pulchellus* St. 20 and St. 24; (E,F) *H. curupi* St. 20 and St. 24. Bars=10 µm except A and D–F (20 µm).

arrangement (Fig. 6E-F). As the tail lengthens, ciliation becomes profuse in *H. curupi* embryos, especially in the head region; ciliated cells are elongated and polygonal, larger than non-ciliated ones, with very long cilia (Fig. 7E-F). Adhesive glands are already separated at this stage, and appear as rounded and prominent, closely spaced paired structures (Fig. 8G). When the tail reaches the length of the body and the operculum covers the base of each gill, the oral disc starts to develop, showing a smooth upper labium, a medially indented lower labium, and keratinised jaw sheaths (Fig. 10A). In *H. curupi*, ciliation starts to regress gradually on the gills, glands, anterior head and ventral trunk, but persists on the oral region. The hatching gland becomes no longer visible. External gills are fully developed, although relatively short (less than 20% of the body length; Fig. 8H). As the operculum covers the right gill, ciliation in the head of *H.* sp. gr. *pulchellus* embryos decreases; the eyes become completely pigmented and

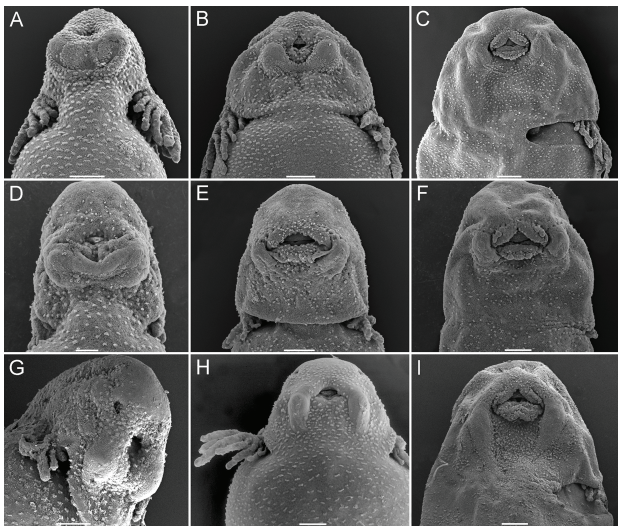

Fig. 8. Adhesive gland and external gill development: *Hypsiboas riojanus*: (A) St. 21-22, (B) St. 23+, (C) St. 24. *Hypsiboas pulchellus*: (D) St. 22, (E) St. 23++, (F) St. 24+. *Hypsiboas curupi*, and *H.* sp. gr. *pulchellus*: (G) St. 20 *H. curupi*, (H) St. 23 *H.* sp. gr. *pulchellus*, (I) St. 24 *H. curupi*. Bars=200 µm.

the pineal end organ becomes evident as a non-pigmented spot between them. Adhesive glands are fully developed (almost 25% of the body length), with secretory cells arranged in an elongated central region (Figs. 8I and 10B). Tooth ridges A1 and A2 are outlined in the oral disc; the lower labium shows an indented P1, and rows P2 and P3 appear as elongated transverse ridges. Marginal papillae develop later on the upper labium and commissures. The intestine is coiled and it still contains yolk in *H. curupi.* The oral disc formation occurs almost entirely once the spiracle is differentiated. Ventral marginal papillae emerge and tooth ridges lengthen (Fig. 10C-D). Later, labial teeth develop on the anterior rows, P1 and P2, and they start to emerge on the medial region of P3 (Fig. 10E); row P4 is differentiated and submarginal commissural papillae emerge, some of them bearing teeth (Fig. 10F). At the same time, regression of the adhesive glands begins, first affecting the secretory zone in a rostro-caudal direction; later, glands become non-prominent, pigmented spots until their disappearance. Then, two new tooth ridges A"0" and P5 emerge distally on both labia of the oral disc (Fig. 11A-C). The three rows additional to the initial labial formula 2/3 (A"0", P4 and P5) develop from papillae and short ridges near the marginal papillae, which bear teeth and then fuse to form whole ridges (about St. 28; Fig. 11E-G). Likewise, submarginal commissural papillae are fused into short ridges with teeth, forming accessory rows orthogonally to the main ridges (Figs. 11D and 11H).

DISCUSSION

Variations of embryonic and larval structures of Hypsiboas

Results show wide variations both in the morphology and development of the studied structures. The diversity in structural patterns is mainly related with the number and size of external gills, size of the adhesive glands, ciliation density, and number of labial tooth ridges. Heterochronic shifts mostly concern the time of adhesive gland division, and the regression of the hatching gland and ciliated cells. We summarise variations per structure in the paragraphs below.

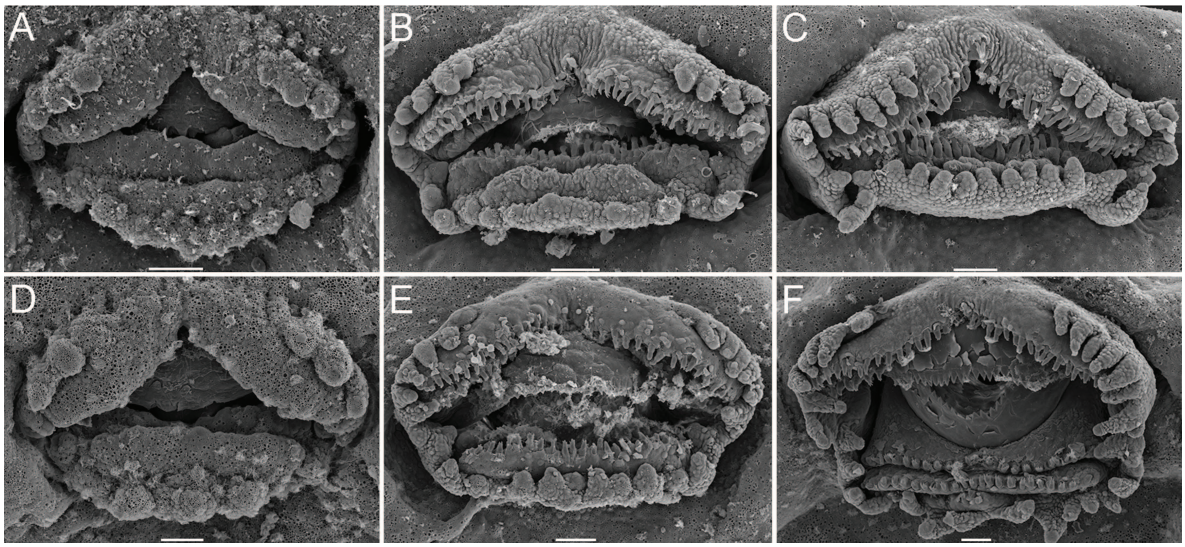

Fig. 9. Oral disc development in *Hypsiboas riojanus*: (A) St. 24, (B) St. 25, (C) St. 25+; and *Hypsiboas pulchellus*: (D) St. 24+, (E) St. 25+, (F) St. 25++. Bars=50 µm.

Hatching gland

Nokhbatolfoghahai & Downie (2007) summarised structural variations in the arrangement of hatching glands in anurans, describing a Y or T-shaped frontal distribution of the secretory cells, and variable extensions along the dorsal midline. In hylids in particular (*Hypsiboas crepitans* and *H. geographicus*), the dorsal extension is a narrow, zigzag line of cells. Among the species we analysed, *H. faber* gland shows the longest and more persisting dorsal line, unlike that of *H. pulchellus*, with a short and straight arrangement. Hatching gland cells are polygonal or oval secretory cells with micro ridges or microvilli (Yoshizaki & Katagiri, 1975; Nokhbatolfoghahai & Downie, 2007), and when fully developed, they are adjacent to each other, forming patches or continuous lines. *Hypsiboas faber* shows a pattern of elongated, oval cells with short and sparse microvilli, unlike *H. crepitans* (which belongs to the same intrageneric group) whose microvilli are dense and large. Within *H. pulchellus* group, the peculiar arrangement of *H. curupi* contrasts with the few dispersed hatching gland cells of the closely related *H. pulchellus*.

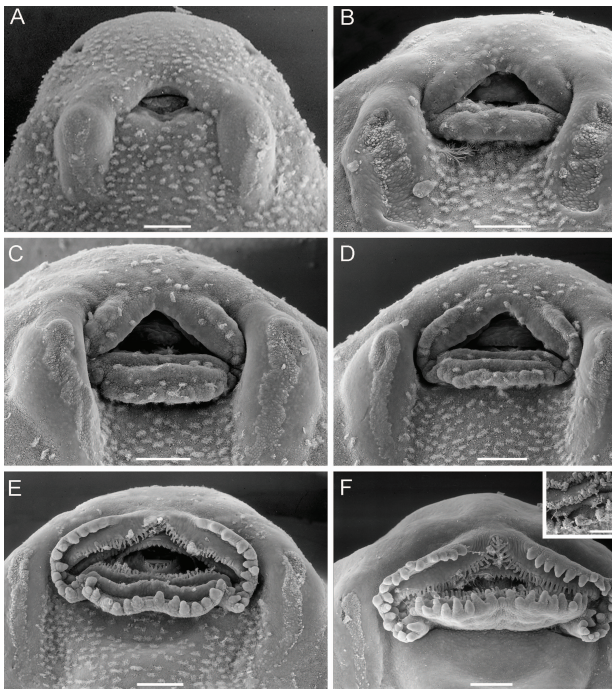

Fig. 10. Oral disc development in *Hypsiboas* sp. gr. *pulchellus*: (A) St. 23, (B) St. 24, (C) St. 25, (D) St. 25+, (E) St. 25++, (F) St. 25+++; inset: detail of developing, fragmented P4 (arrow) in *H. curupi*. Bars=100 µm.

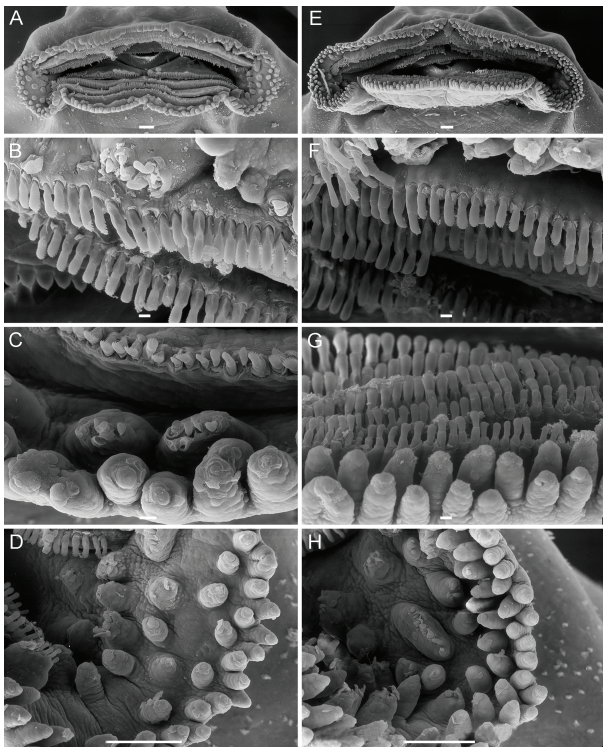

Fig. 11. Oral disc development in *Hypsiboas* sp. gr. *pulchellus*, showing formation of supernumerary and accessory tooth rows: Left: St. 25, showing: (A) early 3/5 disc, (B) A1, A2 and fragmented A "0", (C) fragmented P5, (D) commissural papillae with teeth. Right: St. 28, showing: (E) definitive 3/5 disc, (F) A1, A2, and complete A"0", (G) complete P5, (H) short, lateral commissural accessory rows with teeth. Bars=100 µm (A, D, E, H) and 10 µm (B, C, F, G).

The ontogenetic trajectories include changes in the configuration of the hatching gland, related to both the overall arrangement and the characteristics of the individual cells. Among hylids, previous reports indicate that glands start to develop before the tailbud stage, and regression occurs before the differentiation of the spiracle, when the cells become smaller and interspersed with non-secretory cells (Nokhbatolfoghahai & Downie, 2007). Although we could not observe the sequence of histological ontogenetic changes of the gland, observations under the stereomicroscope indicate that its full development is achieved before the gills begin to ramify, and becomes no longer visible when gills are fully developed (Table 1). In *Hypsiboas pulchellus* embryos, the gland is evident until the right gill is covered by the operculum (Table 1). The elongated, thin and sparse cells with few microvilli observed in this species likely correspond to a regressing gland.

Adhesive glands

Nokhbatolfoghahai & Downie (2005) defined five morphogenetic patterns of anuran adhesive glands, and their observations showed that interspecific morphological variations are associated to taxonomic and ecomorphological groups. Among hylines, these authors report gland morphogenetic Type A and Type C. Type A pattern develops from an initial single medial structure invaginated by a V-shaped groove which later displays an M-shape and finally divides in two circular or oval structures posterolateral to the oral disc. This pattern has been observed in all the *Hypsiboas* species studied to date (Cophomantini tribe), lophiohylini *Trachycephalus typhonius* and *Argenteohyla siemersi*, and apparently in the hylini *Hyla intermedia* and *Pseudacris regilla* (Eakin, 1963; Pennati et al., 2000; Nokhbatolfoghahai & Downie, 2005; this study; FVC unpubl. data). Type C pattern, in contrast, is typical of the tribe Dendropsophini (Nokhbatolfoghahai & Downie, 2005; FVC unpubl. data). Interspecific variations in *Hypsiboas* glands are mainly related to their relative size and ciliation pattern. Glands of *H. curupi* and *H.* sp. gr. *pulchellus* are the largest, whereas those of *H. faber* are comparatively smaller. Regarding ciliation, *H. faber*, *H. riojanus*, and the three species of *Hypsiboas* revised by Nokhbatolfoghahai & Downie (2005) show a dense pattern, whereas *H. pulchellus* exhibit sparse ciliated cells near the central groove.

The glands develop around neurulation and split by Gosner stages 20–22, to disappear when the spiracle differentiates or later (Nokhbatolfoghahai & Downie, 2005). In *Hypsiboas riojanus*, division occurs once the opercular fold is medially fused, and earlier in the other species (even coexisting with gill buds in *H.* sp. gr. *pulchellus*) (Table 1). The achievement of full development (i.e., the largest size before the secretory region start to regress) is also variable: in *H. curupi* and *H.* sp. gr. *pulchellus* full development occurs after the operculum covers the right gill, later than in other species, in which it occurs around the differentiation of the operculum at the gill bases (Table 1). In the former two species, regression is relatively delayed, in relation to a late differentiation of the oral disc. Tissue regression occurs in a caudal-rostral direction, and old specimens still have secretory cells in the anterior-most portion of a barely prominent gland. Gland ciliation also undergoes changes during the ontogeny, reaching the highest density between Gosner stages 19 and 23, except for *H. pulchellus*, which invariably presents sparse ciliation.

External gills

A previous comparative study showed important structural and heterochronic differences in external gill development, often correlated with delayed hatching and incubation in oxygen-poor environments (Nokhbatolfoghahai & Downie, 2008). Embryos of *Hypsiboas faber*, *H. boans* and *H. crepitans* have three pair of gills, of which the third one is very small and develops later. The remaining known embryos exhibit only the first two pairs. Gills also differ in their development extent, varying from small, simple gills with few filaments as in *H. geographicus* to large, and profusely branched gills as in *H. boans*, *H. crepitans*, *H. rosenbergi* and *H. faber* (Noble, 1927; Donoso-Barros & León-Ochoa, 1972; Kluge, 1981; Nokhbatolfoghahai & Downie, 2008; this study). Species in the *H. pulchellus* group exhibit wide variation, from mid-size gills and numerous filaments in *H. riojanus* to very small and ephemeral gills in *H. pulchellus.* Ciliation is also variable among species. Nokhbatolfoghahai & Downie (2008) reported intermediate gill ciliation for *H. boans* and *H. geographicus* (*H. semilineatus* group). Our observations show an overall dense ciliation pattern in the *H. faber* group, whereas ciliation varies from intermediate (*H. riojanus*) to very sparse (*H. pulchellus*) in the *H. pulchellus* group.

Developmental trajectories display some variation in the sequence of events. External gills appear after tailbud stage as bulging paired buds, located on both sides of the anterior region. Full development occurs before the medial fusion of the operculum, almost synchronically with the beginning of differentiation of the oral disc. Exceptionally, full development in *Hypsiboas pulchellus* occurs with an already fused operculum, and *H. faber* embryos reach fully developed gills when the oral disc is already evident (Table 1). The regression of gills generally co-occurs with the decrease in body ciliation, except for *H.* sp. gr. *pulchellus*, in which ciliated cell regression starts later, once the spiracle is formed (Table 1).

Ciliation pattern

A broad variation in body ciliation has been reported among hylids, ranging from *Hypsiboas*, with the densest patterns, to species of *Dendropsophus* with the lowest levels of ciliation (Nokhbatolfoghahai et al., 2005). Within genera, there are also interspecific differences regarding distribution and persistence of cilia after hatching. Nokhbatolfoghahai et al. (2005; 2006) concluded that ciliation patterns also vary individually, with no clear phylogenetic or ecological correlates.

In *Hypsiboas*, dense patterns are observed around the nostrils, on the external gills and on the ventral region of the head (Nokhbatolfoghahai et al., 2005; 2006; this study); the highest ciliation period takes place between

stages 19 and 23 (Appendices 2 and 3). Density levels (as the average of the six body regions measured) show a high variation, from very dense or dense in *H. faber* and *H. riojanus*, to intermediate in *H. curupi*, and very scattered in *H. pulchellus*. Regression of the ciliated surface may initiate at Gosner stages 23, 24, or 25. Early localised ciliation regression is related to the development of the lateral line system (Nokhbatolfoghahai et al., 2005), where bands free of ciliated cells can be seen in the integument. Total regression of ciliated cells on the body surface is apparently highly variable, and is not directly related to hatching nor to gill functionality (Nokhbatolfoghahai et al., 2005). In this study, none of the species showed a complete regression until advanced instances of St. 25, when the spiracle was already formed.

Oral disc

Among hylids, the development of mouthparts was studied in the hylini *Hyla chrysoscelis*. In this species, the acquisition of the labial tooth row formula 2/3 involves a sequential formation of the stomodeum, upper and lower labia, marginal papillae, upper and lower jaw sheaths, labial rows, submarginal papillae and finally, keratinised labial teeth (Thibaudeau & Altig, 1988; Altig & McDiarmid, 1999).

Comparisons of ontogenetic trajectories of oral discs in the studied species revealed interesting variations regarding the mentioned sequence (Table 1). First, different oral configurations seem to be obtained from similar (overlapping) trajectories which end at different states. The LTRF 2/3 is achieved after a generally very similar sequence of changes between species. The disc begins to form before (*Hypsiboas faber*), or after (rest of the species) the operculum medial fusion. After a 1/1 disc, the following rows (A2, P2 and P3) appear almost simultaneously (except in *H. pulchellus*) and before spiracle formation. Once the 2/3 disc is acquired, the first papillae differentiate (except in *H. pulchellus,* whose papillae are present with a lower formula), and the first teeth emerge almost simultaneously in rows A1, A2, P1 and P2. Row P3 is the last to bear teeth in this formula, and this occurs invariably after the complete differentiation of the marginal papillae and the spiracle. The oral disc of *H. pulchellus* is defined around St. 25, and remains almost unchanged during the larval period (occasionally, commissural papillae with teeth are formed; Kolenc et al., 2008). Instead, *H. riojanus* larvae maintain a 2/3 disc (e.g., Lavilla, 1984) or later develop a fourth, short and fragmented row P4 (Kolenc et al., 2008), displaying high inter- and intrapopulational variation. The rest of the species, *H. faber*, *H. curupi*, and *H.* sp. gr. *pulchellus*, always develop further rows in one or both labia. Altig & Johnston (1989) schematised the development of labial rows in species with formulae larger than 2/3, and postulated that after the acquisition of the initial formula 2/3, further rows are added following four patterns, one of them departing distally on both labia. In *H. faber* embryos, once labial teeth emerge on row P3, P4 differentiates distally on the lower lip. In *H. curupi* and *H.* sp. gr. *pulchellus*, row P4 and later, rows A"0" and P5 appear distally on both labia, in relation to the marginal papillae. An occasional A"0" is reported in tadpoles of *H. riojanus*, *H. marianitae*, and *H. gladiator* (Kolenc et al., 2008). A similar mechanism of supernumerary row development has been hypothesised for the far larger formulae 17/19 in the related genus *Hyloscirtus* (Sánchez, 2010).

Several species of the *Hypsiboas pulchellus* and *H. faber* groups (reviewed in Kolenc et al., 2008), and species of *Hyloscirtus* (Sánchez, 2010) share the presence of short accessory rows with teeth. In *H. curupi*, *H.* sp. gr. *pulchellus*, and *H. faber*, certain commissural papillae with teeth may join, resulting in additional rows which are parallel to the oral disc margin and perpendicular to the main rows. The ontogeny of supernumerary and accessory rows supports the hypothesis by Altig (2006), which proposes that the oral tissue is multipotent and can thus differentiate into papillae, ridges, or labial teeth.

Marginal papillae can either develop quickly (St. 23 in *Hypsiboas faber* or St. 24–25 in *H.* sp. gr. *pulchellus*), or gradually (St. 23 to 25 in *H. riojanus* and *H. pulchellus*). Papillae formation occurs from the lateral to the medial region in *H. riojanus* and *H.* sp. gr. *pulchellus,* and along the entire margin in *H. faber*. In specimens of *H. pulchellus*, papillae at the commissures and the mental region are initially differentiated, delimiting two small ventrolateral gaps which are later completed with papillae. This pattern was already mentioned by Thibaudeau & Altig (1988) for some specimens of *Hyla chrysoselis.* In certain specimens of *H. faber* and other species of the group, and in some individuals of *H. raniceps* (reviewed in Kolenc et al., 2008), a small ventral gap may appear on marginal papillae. As already mentioned, in all cases the margin completes its differentiation after LTRF 2/3 is achieved. Despite variations in larval configuration, the oral discs of *Hypsiboas* are already outlined (i.e., definitive LTRF and marginal papillae) at stage 25, before hind limb differentiation. This indicates differential growth rates of the oral apparatus between these species, suggesting a certain restriction in the developmental sequence of characters as different as oral disc and limbs. However, in other non-related species with LTRF larger than 2/3, the development of the oral discs differs considerably, in terms of the developmental sequence of the initial 2/3 LTRF, the addition of supernumerary rows, and the stage at which the definitive formula is acquired (Hall et al., 1997; FVC unpubl. data).

Labial tooth row formulae larger than 2/3 have been proposed as a tentative synapomorphy for Cophomantini. Also, an evolutionary trend towards the simplification and reduction of oral disc size and LTRF in more derived clades (such as *Hypsiboas*) is interpreted (Faivovich et al., 2005). Kolenc et al. (2008) found a similar pattern within *Hypsiboas*, and this diversification could be related to the truncation of heterochronic processes involving oral disc development. In basal configurations with large LTRFs, the interruption of development would result in discs with LTRF 2/4 (e.g., discs of *H. faber* and *H. punctatus* groups, and of Andean species of the *H. pulchellus* group) and with LTRF 2/3 (e.g., discs of some species of the *H. pulchellus* group). At a subtler level, the configuration of rows P3 and P4 indicates different degrees of disc development. At

larval stages, row P4 may be entire (e.g., *H. melanopleura*; Lehr et al., 2011) or segmented, retaining its earlier morphology (probably the case of several species revised by Kolenc et al., 2008). In turn, row P3 may vary in length, being reduced to about 1/2 or 1/3 of the length of P2 (e.g., *H. pulchellus* and *H. caingua*). Finally, in *H. raniceps* (*H. albopunctatus* group), the shape of the larval labial teeth corresponds to the shape of the first emerging teeth of other closely related species (e.g., St. 25 *H. pulchellus*, Fig. 12F; i.e., short teeth with barely marked head and scarce distal cusps; Vera Candioti & Altig, 2010), a pattern already reported in other groups (e.g., Vera Candioti et al., 2011).

Ecomorphological considerations

Morphological and ontogenetic variations in some structures can be related to oviposition sites and environments where embryos and larvae develop. For example, environments with low oxygen provision might determine morphological and behavioural responses in hatching and gas exchange (e.g., *Agalychnis*; Warkentin, 2000; 2007). Among *Hypsiboas* species*, H. faber* embryos which develop in small nests outside water bodies exhibit the largest hatching gland, probably in relation to early hatching (at around St. 15, as indicated by the absence of extraembryonic membranes). *Hypsiboas faber* and other species of the group (*H. crepitans* and *H. rosenbergi*) exhibit also more developed external gills in terms of size, ramification, ciliation and motility, which results in a greater exchange surface compared to that of other forms (Noble, 1927; Kluge, 1981; Martins & Haddad, 1988; Nokhbatolfoghahai & Downie, 2008). Likewise, body ciliation is suspected to have an important respiratory role (Kessel et al., 1974), and accordingly, *H. faber* embryos showed the highest ciliation density.

On the other hand, tadpoles of lotic water bodies often have morphological attributes related to the avoidance or resistance to flowing water (e.g., Altig & McDiarmid, 1999). For instance, Nodzenski & Inger (1990) observed that oral structures of suctorial larvae of *Ansonia* (Bufonidae), and gastromyzophorous tadpoles of *Meristogenys* (Ranidae, including the abdominal sucker as well in this case) metamorphose later in relation to other developmental events, so that the adhesion mechanism is guaranteed at older stages. The large and persistent adhesive glands of *Hypsiboas curupi* and *H.* sp. gr. *pulchellus* could be related to embryos and larvae development in small streams. Further observations, mainly involving behaviour on specimens in situ, are thus crucial to discuss the heterochronic processes involved in morphologies associated to different ecological constraints. Lastly, embryonic morphology of *H. pulchellus* is puzzling, since some of its embryonic traits appear to be paedomorphic regarding ancestral ontogenies (e.g., the LTRF 2/3, the scarcely developed external gills and low body ciliation). Within our sample, *H. pulchellus* is the only species which also breeds in large, permanent ponds (Kwet et al., 2004), but the ecological/functional correlation (if any) of the above mentioned features remains uncertain.

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