

External ontogenetic changes of larval structures in *Elachistocleis bicolor* (Anura: Microhylidae: Gastrophryninae)

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Abstract: Although the external larval morphology of many species of Microhylidae has been characterized, ontogenetic studies in the family are scarce and partial. In the present study, we describe the development of the oral funnel, external gills, external nares, operculum, spiracle, and adhesive organs of *Elachistocleis bicolor* with the aim to contribute to future ontogenetic, evolutionary, and phylogenetic studies. The development of *E. bicolor* is similar to that of other species with free-swimming tadpoles as *Microhyla ornata*, *Dermatonotus muelleri* and *Uperodon globulosus*. The ontogeny of the oral apparatus shows only the early stages of the pattern most common in anurans. After the invagination of the stomodeum, the anterior and posterior labia are defined, but the keratinized structures are not formed. The conical adhesive glands are similar to those of *Physalaemus* and *Dendropsophus* tadpoles, but in *E. bicolor*, these develop in contact with the posterior labium. The external nares remain closed during most of the larval phase. During ontogeny, the external gills are simultaneously covered by a transverse operculum fold, which later advances towards the posteromedial ventral region, forming the spiracle, while in Type IV larvae the operculum folds close from right to left, covering the right gill first. The distinct larval characters of Microhylidae appear between the last embryonic stages and the first larval stages. Therefore, the consideration of these stages can be of great value in future studies of anuran development in phylogenetic and evolutionary contexts. Ontogenetic studies in microhylid tadpoles are fundamental to identify heterochronic processes, to interpret the variability of larval structures, and to establish or reinforce hypotheses about the origin of larval structures.

Key words: anurans; development; spiracle; oral funnel; external gills; nares

Introduction

The ontogeny of an individual consists of a series of stages whose number could be considered infinite. However, operationally, it is necessary to quantify them in discrete stages, both to obtain a clear perception of the processes involved and to be able to make valid comparisons among different taxa (Lavilla & Rougés 1992). In anurans, staging tables based mainly on morphological and physiological characters, are a condensed way to describe ontogenetic changes (Hall et al. 1997).

The family Microhylidae, widely distributed in the world, contains species with different reproductive modes, including direct development. In the case of species with exotrophic tadpoles, larvae exhibit distinctive morphological features, and are named Type II tadpoles (Orton 1953). The external larval morphology has been characterized in many species (e.g., Rada de Martínez 1981; Williams & Gudynas 1987; Donnelly et al. 1990; Lavilla 1992; Kwet & Di-Bernardo 1998; Wogel et al. 2004; Nascimento & Skuk 2006; Rossa Feres & Nomura 2006; Vera Candioti 2006; Grosjean et al. 2007; Langone et al. 2007; Lehr et al. 2007; Rodrigues et al. 2008; Mac Culloch et al. 2008; Bowatte & Meegaskum-

bura 2011; Magalhaes et al. 2012; Pereyra et al. 2013). However, ontogenetic studies in Microhylidae are scarce and partial, and highly contrast with what is known about species with Orton's Type IV tadpoles (Marinelli et al. 1985; Takahama et al. 1987; Thibaudeau & Altig 1988; Fiorito de López & Echeverría 1989; Hall et al. 1997; Zaracho et al. 2003; Nokhbatolfoghahai & Downie 2005a, 2008; Altig 2006; Salica et al. 2011).

For Microhylidae, a development table with 29 stages based on external morphology changes has been proposed for *Chiasmocleis antenori* (Walker, 1973), a species whose larval life is adapted to bromeliads (Krügel & Richter 1995). Also, different development tables have been proposed for *Microhyla ornata* (Duméril et Bibron, 1841) (Padhye & Ghatge 1989; Baorong et al. 1996; Shaolong et al. 1996; Shimizu & Ota 2003; Xu & Xu 2012). In addition, the normal development of *M. ornata* and *Uperodon globulosus* (Günther, 1864) has been described and compared following the stages of Gosner (1960) (Narzary & Bordoloi 2013). Variations during larval development (stages 26–46), including information on external and internal morphology, have been described for *Dermatonotus muelleri* (Boettger, 1885) (Fabrezi et al.

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2012). The development of the operculum, the presence of external gills, tail structure, gut development and timing of forelimb emergence have been researched in Australopapuan microhylid species with direct development (Townsend & Stewart 1985, Anstis et al. 2011).

Although microhylids present considerable ontogenetic diversity, the initial steps of embryogenesis show the standard pattern of cleavage in amphibians (Desnitskiy 2012). Thibaudeau & Altig (1988) and Altig (2006) carried out an inclusive and general review of the origin and evolution of the oral apparatus in anuran tadpoles, including those of Microhylidae, whereas Donnelly et al. (1990) reported the morphological variations in free-swimming microhylid larvae. Nokhbatolfoghahai & Downie (2005a) described the narial development of *Elachistocleis ovalis* (Schneider, 1799) and also analyzed the development and morphology of the adhesive gland and external gills of the same species (Nokhbatolfoghahai & Downie 2005b, 2008). These studies revealed five patterns of adhesive gland morphogenesis and an important diversity at the anatomical and cellular levels in the extent and length of the external gills.

Previous works in *Elachistocleis bicolor* (Guérin-Meneville, 1838) tadpoles have described the external larval morphology (Williams & Gudynas 1987), the chondrocranium and hiobranquial apparatus (Lavilla & Langone 1995), the internal oral morphology (Echeverría & Lavilla 2000), the shape variation in the paired oral flaps (Laufer et al. 2013), and ontogenetic changes in the spiracle and vent tube orientation during late larval stages (Lavilla & Langone 1991).

In this work, we describe the development of the oral funnel, external gills, external nares, operculum, spiracle, and adhesive organs of *E. bicolor*. These data may contribute to future ontogenetic, evolutionary, and phylogenetic studies.

Material and methods

Embryos and tadpoles of *Elachistocleis bicolor* were collected in Corrientes, Argentina (27°28'04" S; 58°46'52" W), between October 2009 and March of 2010. They were collected from small temporary ponds of 30 cm of maximum water depth, formed after copious rains. These ponds were in a grassy area subject to human activity or intervention.

Embryos and tadpoles were kept in plastic bowls according to Lavilla & Rougés (1992). Larvae were placed in bowls with water of the same pond where they were collected, also including some aquatic plants. The water was renewed periodically. Embryos and tadpoles were fixed periodically in 10% formalin every 2 hours for embryonic stages and every 3 hours for the larval stage (until stage 26) and every 24, 48 or 72 hours up to metamorphosis. Embryos and tadpoles were analyzed by stereoscopic microscope and Scanning Electron Microscope (SEM). For this, the specimens were washed with 0.1 M phosphate-buffer solution series, treated with a graded acetone series, then critical-point-dried and coated with gold. Specimens were examined using a Jeol JSM-5800 LV SEM. Embryos and tadpoles were deposited in the herpetological collection of the Universidad Nacional del Nordeste (Corrientes): UNNEC 11045–11067. Anatomical terminology follows Altig & McDiarmid (1999)

and stages of development are based on the development table of *Microhyla ornata* (Shimizu & Ota 2003) since they better agree with the development of structures such as operculum and spiracle in Orton's Type II larvae.

Results

Development of the oral funnel

The development of the oral funnel initiates at stage 18, with the invagination of the stomodeum in the anteroventral region, between the adhesive organs and the olfactory pits (Fig. 1A). At stage 22, the stomodeal invagination becomes deeper and presents a continuous posterior, longitudinal groove (Fig. 1B). By stage 23, the stomodeum opening is circular in shape (Fig. 1C), and by stage 24 it becomes semicircular and is placed in terminal position. In this last stage, a thick, anterior flange whose posterior ends are continuous with the adhesive glands, appears (Fig. 1D). This structure corresponds to the oral anterior labium bud and has epidermal ciliated cells. At stages 25–26, a posterior flange, which corresponds to the posterior labium bulge, develops (Figs 1E, F). From stage 27 to 32, the anterior labium develops two lateral thickenings, corresponding to the primordia of the labial flaps (Figs 1G, H). During subsequent stages, the oral funnel grows in size and the only noticeable change is the shape of the labial flaps. Although the change is gradual, the labial flaps are mainly short and semicircular between stages 27 and 33. Between stages 34 to 41, they are circular and expanded, and the medial margins are slightly superimposed. The margin of the labial flaps is smooth, except in some larvae that presented slightly serrated margins between larval stages 28–40. This condition was observed in 18% of the tadpoles examined ($n = 56$ tadpoles). The posterior labium divides in anterior and posterior folds. From stage 27, the anterior fold becomes U-shaped and forms a funnel, while the posterior fold remains as lateral folds in contact with the base of this funnel (Figs 1G, H).

By stage 32, the oral funnel is completely formed and shows no significant changes until the metamorphic climax, when it modifies into the adult mouth. By stage 42, the labial flaps of the anterior labium begin to regress progressively and become no longer visible at stage 43 (Figs 2A–C). By stage 43, the posterior labium spreads and loses the appearance of funnel (Figs 2E–G). Then, the margin of the lower jaw becomes semicircular, similar to the adult mouth.

Development of adhesive glands

The adhesive glands in *E. bicolor* develop from stage 18, as two oval, pigmented, and slightly elevated areas posterior and lateral to the stomodeum (Fig. 1A). At stage 22, they have conical shape, and are associated with the ends of the anterior labium (Fig. 1B). Their apices show a glandular central area that produces the adhesive mucus used by the larva to fix to the substrate. The conical shape is maintained until stage 27 (Fig. 1C), when it starts regressing, and at stage 29 it

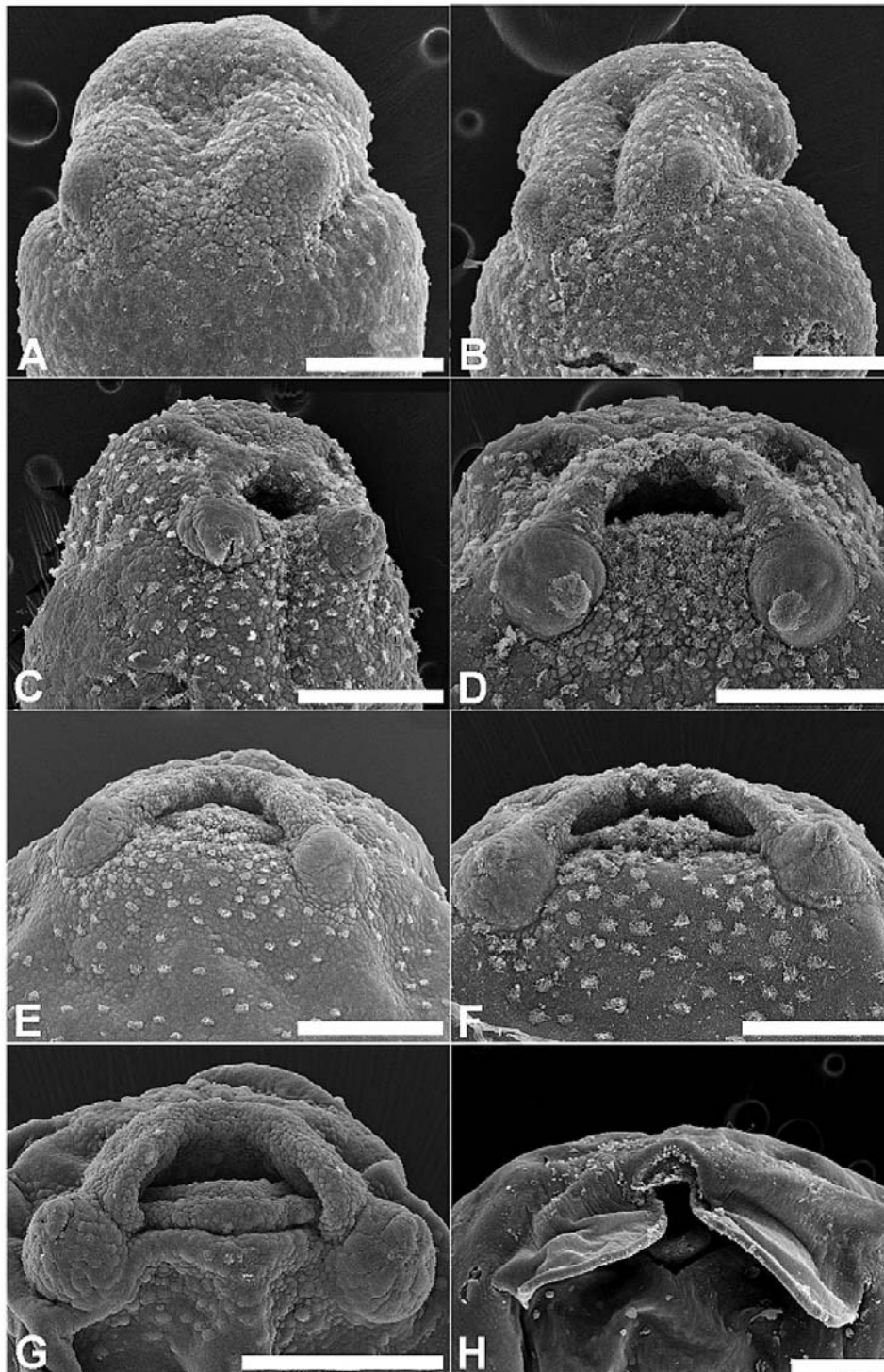


Fig. 1. Scanning electron micrographs of the funnel oral development of *Elachistocleis bicolor* during the early ontogeny. A – stage 18; B – stage 22; C – stage 23; D – stage 24; E – stage 25; F – stage 26; G – stage 27; H – stage 32. Scales 2 mm.

is only visible as dark spots on the region where it was previously placed.

Development of external gills

At stage 19, external gills develop as semispherical bulges on each side of the head, posterior to the adhesive glands and optic vesicles (Fig. 3A). By stage 22, each bulge is subdivided by two vertical grooves,

which define the anterior, medial, and posterior folds. By stage 23, each fold develops digitiform projections, which originate the gill filaments (Figs 3 B–C). The first projections appear on the anterior fold, and then on the medial and the posterior ones. The gill filaments attain their fullest length by stage 24 (Fig. 3E). By stage 26, the external gills are covered by the operculum, being externally invisible.

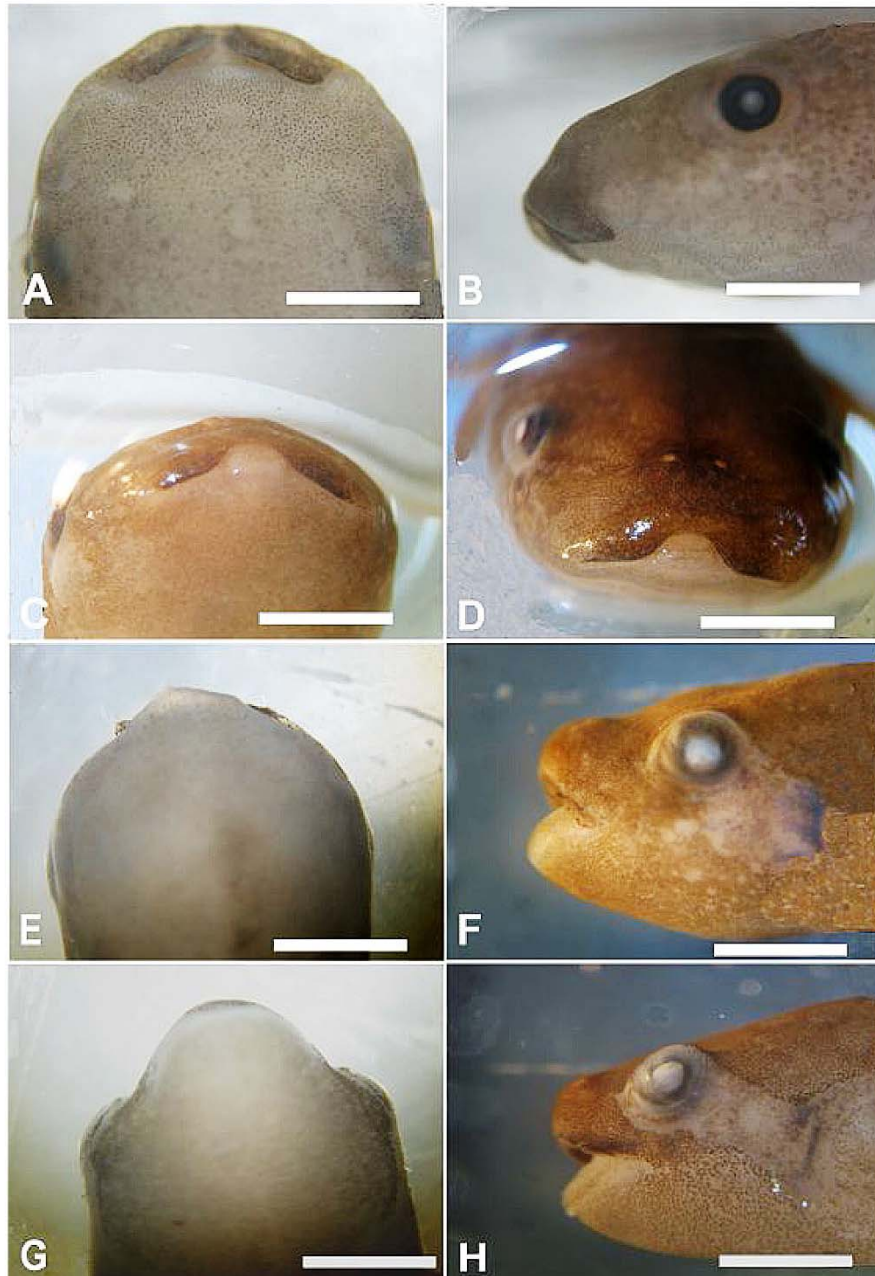


Fig. 2. Oral funnel transformations during the metamorphosis in *Elachistocleis bicolor*. A,B – early stage 42 in ventral and lateral views; C, D – late stage 42 in ventral and frontal views; E, F – early stage 43 in ventral and lateral views; G, H – late stage 43 in ventral and lateral views. Scales 2 mm.

Development of the operculum and spiracle

The development of the operculum starts at stage 24 with the formation of a pair of skin folds, anterolateral to the external gills (Figs 3D–F). These folds grow simultaneously and cover the base of the gill filaments. Then, they extend on the ventral region, forming a V-shaped fold (Fig. 4A, B). This fold subsequently becomes transversally straight and covers half of the external gills (Figs 4C, D). By stage 26, the external gills are completely covered (Figs 4E, F), and two lateroventral folds are fused with the anterior transverse one, delimiting the spiracular opening on the ventromedial region of the body. Then, the operculum extends to the posterior region and forms the spiracular tube

(Fig. 4G), which extends surpassing the vent tube, so that the spiracular opening is oriented toward the left side of the tadpole (stage 28). By stage 41 (at the beginning of metamorphosis), the spiracular tube starts a process of regression. Initially, the spiracular tube locates in the medial axis between the hindlimbs; at stage 43, the spiracular tube is short and its opening is located on the ventral half of the body. By stage 44, only a circular scar between the forelimbs is observed as remains of the spiracular tube. These changes resemble the inverse process observed during their formation.

Development of external nares

The external nares in *E. bicolor* develop at stage

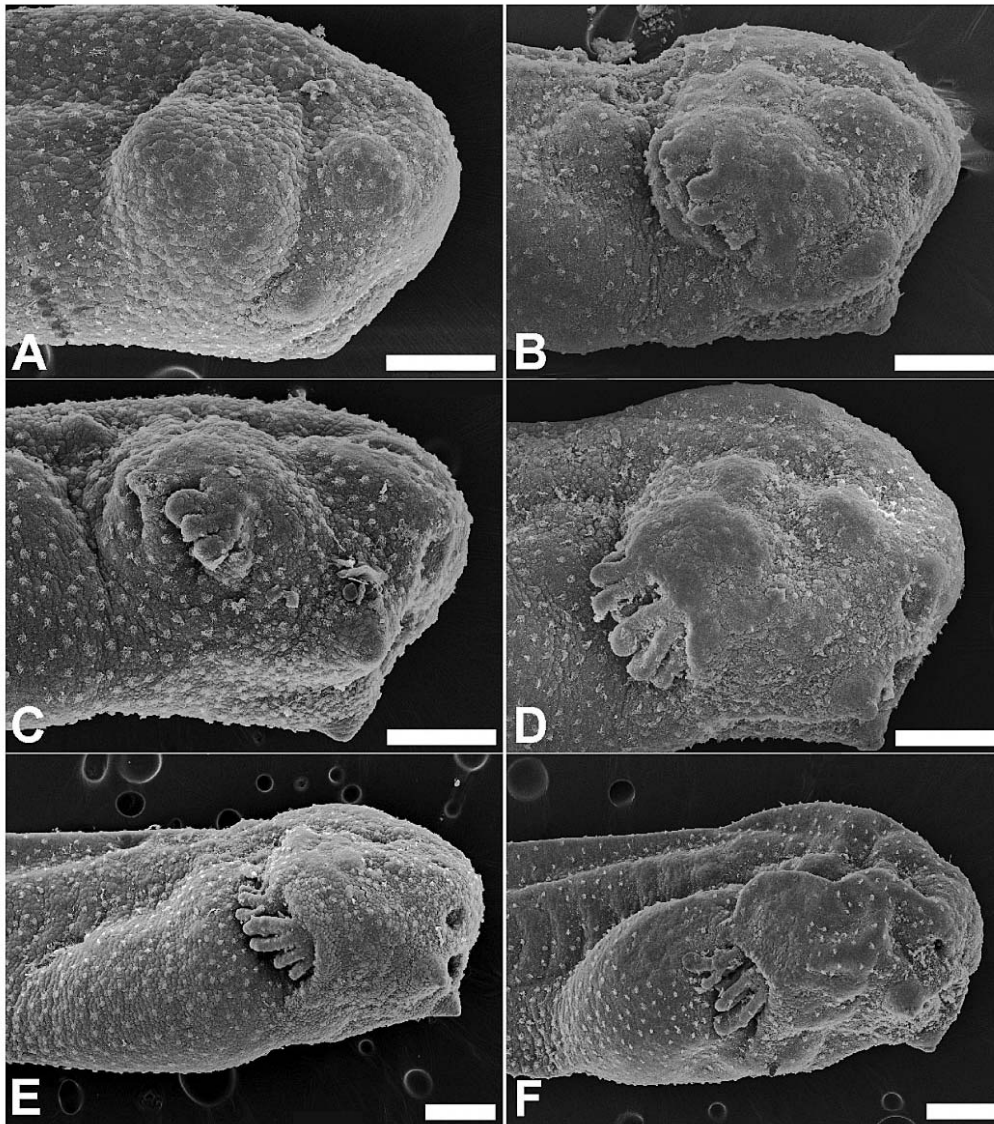


Fig. 3. Scanning electron micrographs of the external gills development of *Elachistocleis bicolor*. A – stage 19; B – early stage 23; C – late stage 23; D – early stage 24, E – late stage 24; F, stage 25. Scales 2 mm.

19 as two small, flattened areas, on the anterior region of the head and dorsal to the stomodeal invagination. From stage 22 to stage 26, they remain as a pair of distinct circular pits. During stages 27–40, the pits are indistinct, although their location is indicated by two small dark stains in live specimens. Through pre-metamorphic stages, the stains of the external nares change their location. At the beginning, they are on the dorsal region, nearer the snout than the eyes, and look like two small, dark spots. Later, the spots approach each other and are located approximately halfway between the eyes and the snout. At the beginning of metamorphosis, the dark spots are displaced forward, approaching each other and placed on paired elevations in the anterior edge of the snout. At stage 41, the external nares are clearly open. In juveniles, the external nares are placed on the tip of the snout, with clear borders, surrounded by more pigmented areas than the contiguous skin.

Discussion

The development of *Elachistocleis bicolor* is similar to that of other species with free-swimming tadpoles as *Microhyla ornata*, *Dermatonotus muelleri* and *Uperodon globulosus* (Shimizu & Ota 2003; Fabrezi et al. 2012; Narzary & Bordoloi 2013). Information about the development of the oral funnel in Microhylidae is fundamental to interpret the origin and evolution of the oral apparatus of anuran tadpoles (Altig 2006). Thibaudeau & Altig (1988) proposed a common development pattern of ontogenetic development and atrophy of the oral apparatus based on six anuran species with four different types of mouth. The pattern is as follows: stomodeal invagination, oral pad development, jaw sheath delimitation, oral disc formation, jaw sheath keratinization, tooth row ridge development, and formation of marginal papillae in rapid sequence with considerable temporal overlap, and lastly, tooth keratinization, although all features may not be fully formed before

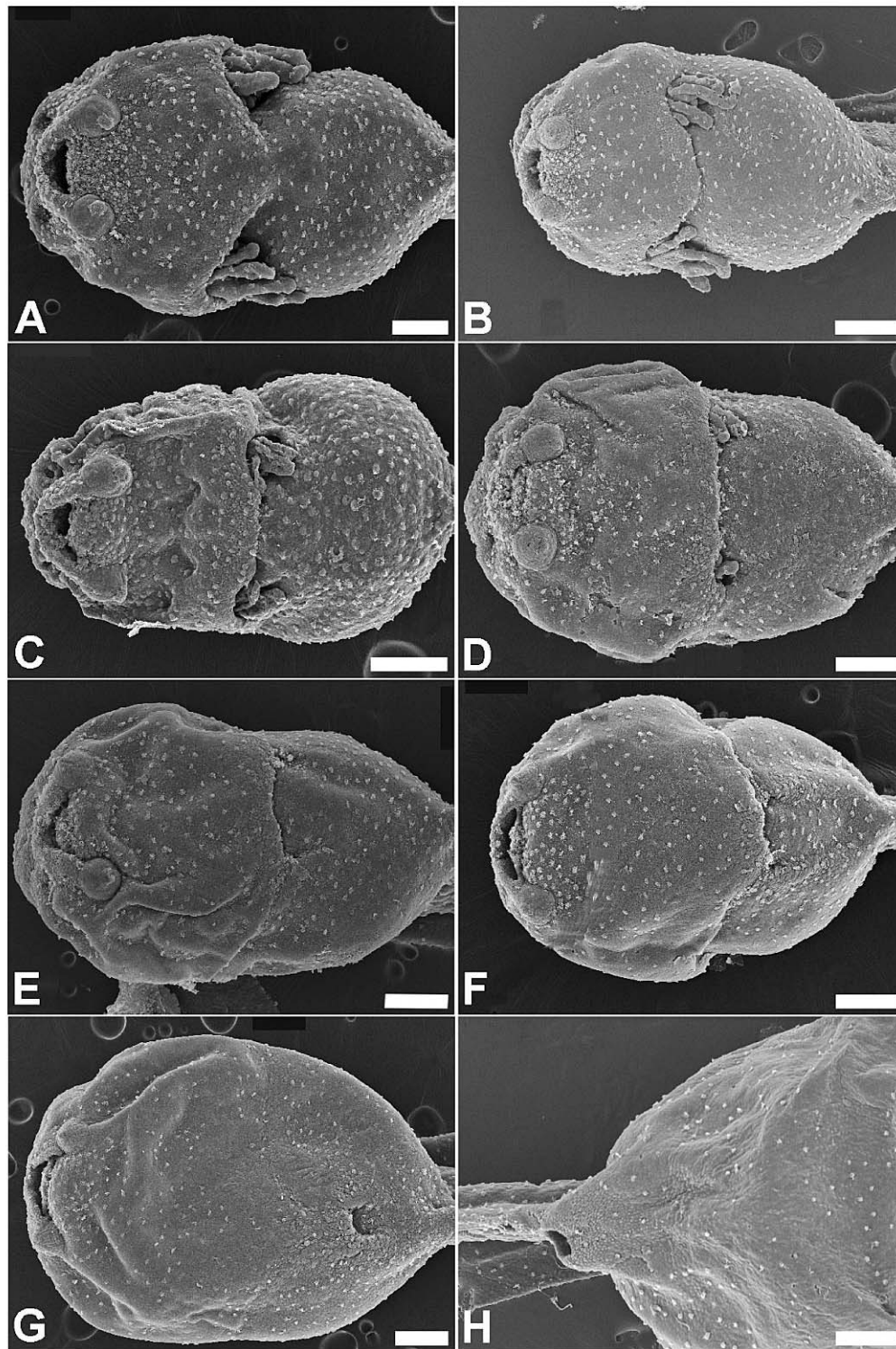


Fig. 4. Scanning electron micrographs of the operculum and spiracle development of *Elachistocleis bicolor*. A, D – stage 25; E – stage 26; F – stage 27; G, H – stage 28. Scales 2 mm.

the next structure appears. *Elachistocleis bicolor* shows only the early stages of this developmental pattern. After the invagination of the stomodeum, the anterior and posterior labia are defined, but the keratinized structures are not formed. The anterior labium with two labial flaps and a medial notch is shared between *E. bicolor* and other microhylids, as *D. muelleri*, *Elachis-*

toleis ovalis, *E. surinamensis* (Daudin, 1802), *E. cesarii* (Miranda-Ribeiro, 1920), *E. erythrogaster* Kwet and Di-Bernardo, 1999, *E. haroi* Pereyra, Akmentins, Laufer et Vaira, 2013, *E. panamensis* (Dunn, Trapido et Evans, 1948), *Gastrophryne carolinensis* Holbrook, 1835, *G. elegans* (Boulenger, 1882), *G. olivacea* Hallowell, 1856, *Hypopachus ustus* (Cope, 1886), *H. pictiven-*

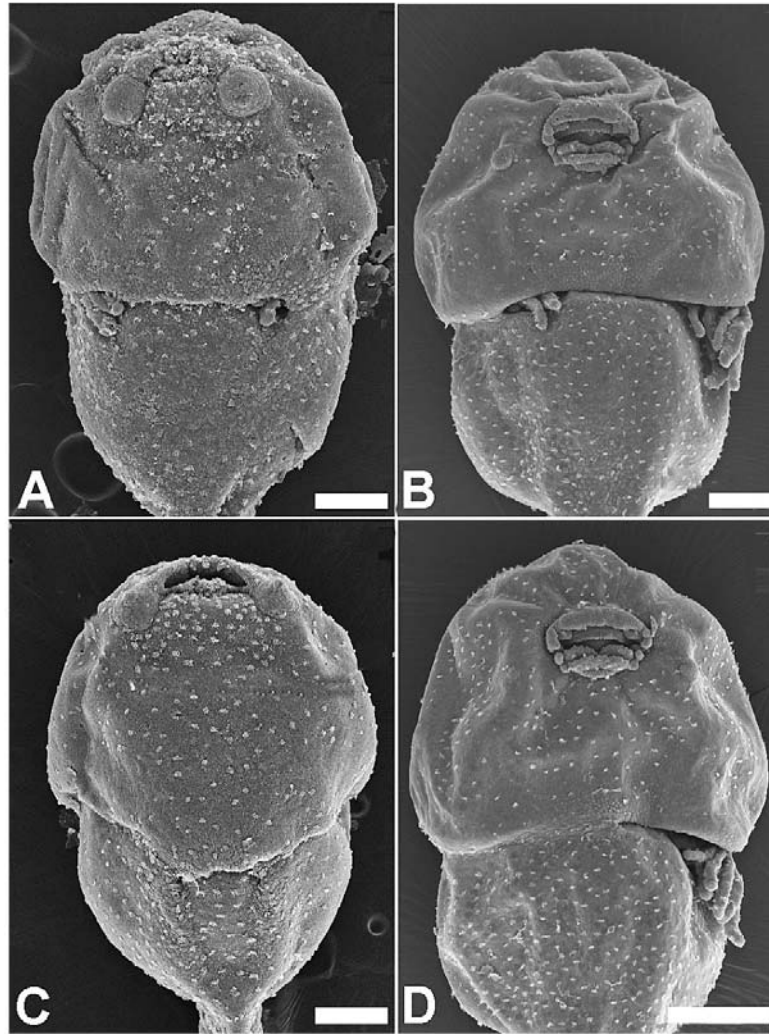


Fig. 5. Scanning electron micrographs of the development of the operculum in Type II (A, C) and Type IV (B, D) tadpoles. To the left, *Elachistocleis bicolor* tadpoles; to the right, *Physalaemus albonotatus* tadpoles. Upper row: stage 23; lower row: stage 24 (stages equivalent to Gosner, 1960). Scales 2mm.

tris (Cope, 1886), *Chiasmocleis leucosticta* (Boulenger, 1888), *C. carvalhoi* (Cope, 1886), and *Microhyla ornata* (Donnelly et al. 1990; Lavilla 1992; Kwet & Di-Bernardo 1998; Shimizu & Ota 2003; Wogel et al. 2004; Vera Candioti 2006; Langone et al. 2007; Fabrezi et al. 2012; Pereyra et al. 2013).

The labial flaps of microhylids are considered homologous with the oral labia of Orton's (1953) Types III and IV tadpoles, and with the lateral labial folds of *Rhinophrynus* (Orton's Type I), because they all originate from an oral pad delimited at the beginning of the development of the oral structures (Thibaudeau & Altig 1988). Additionally, vestigial labial crests have been described in microhylid larvae with reduced oral disks and without labial teeth as *Ctenophryne* sp. and *Otophryne robusta* Boulenger In Lankester, 1900 (Thibaudeau & Altig 1988; Donnelly et al. 1990), although this character could be a convergence since according to the latest comprehensive phylogeny of Microhylidae (de Sá et al. 2012), *Otophryne* is placed closer to *Kalophrynus* from Southeastern Asia than to other new world microhylids. The lack of keratinized structures in the ontogeny of the

oral funnel observed in *E. bicolor* and other microhylid species is considered a truncation of the development (Altig 2006). Heterochronic alterations during development are probably common genetic mechanisms of modification (Thibaudeau & Altig 1988). The presence of integumentary ciliated cells on the presumptive face of the disc, as observed in *E. bicolor*, indicates that the disc face is homologous with the general surfaces surrounding the mouth (Altig 2006). During metamorphosis, the oral funnel changes, originating the adult mouth. In *E. bicolor*, transformations are similar to those described for *D. muelleri*, such as the regression of the oral flaps and the modification the oral funnel to originate the lower jaw (Fabrezi et al. 2012).

The adhesive glands in *E. bicolor* appear early during embryonic development. Five distinct patterns of development have been reported in anurans (Nokhbatolfighahai and Downie 2005b). According to this classification, the conical adhesive organs with a broad base and more or less tapering tip of *E. bicolor* correspond to Type "C". This type of adhesive glands has been reported in *Physalaemus biligonigerus* (Cope, 1961),

P. albonotatus (Steindachner, 1864), *Dendropsophus microcephalus* (Cope, 1886), *D. minutus* (Peters, 1872) and *D. minusculus* (Rivero, 1971) (Zaracho et al. 2003; Nokhbatolfoghahai & Downie 2005b). However, while in these species these glands are developed separately from the oral disk, in *E. bicolor* they are in contact with the ends of the anterior labium during their ontogeny.

The external gills are transitory embryonic structures, and are considerably diverse in their morphology at anatomical and ultrastructural levels (Nokhbatolfoghahai & Downie 2008). In microhylids (as *E. ovalis* and *E. bicolor*), the number and length of filaments are lower than in bufonids and *Xenopus laevis* Daudin, 1802 (Nokhbatolfoghahai & Downie 2008). Microhylid species of the genera *Cophixalus* and *Oreophryne* with direct development have no external gills, but a diminutive bulge is visible on gill arches. However, in live, no circulating blood is observed in this area (Anstis et al. 2011).

In Type IV larvae, the operculum folds close from right to left, covering the right gill first (Del Conte & Sirlin 1951; Gosner 1960; Lavilla & Rouges 1992; Hall et al. 1997; Zaracho et al. 2003). In contrast, in *E. bicolor*, a symmetrical process is observed, where both external gills are covered simultaneously by the transverse operculum fold, and two lateral folds that unite with the transversal one appear on the lateroventral region of the body (Figs 5B, D). These joined structures advance towards the posteromedial ventral region to form the spiracle (Figs 5A, C). A similar pattern has been described in free-swimming, non-feeding tadpoles of *Chiasmocleis antenori* that develop in water-filled bromeliads, although in this species, the external and internal gills are not developed (Krügel & Richter, 1995). The position of the spiracle in *E. bicolor* coincides with the description by Williams & Gudynas (1987). This position is also shared with that in *E. surinamensis*, *E. ovalis* and *E. erythrogaster* (Griffith & Carvalho 1965; Kenny 1969; Rada de Martinez 1981; Kwet & Di-Bernardo 1998). Other microhylids, as *Ctenophryne carpih* (Lehr, Rodríguez et Córdoba, 2002), have a short spiracular tube, whose opening is located in the half line below the abdomen; this position is shared with *C. aequatorialis* (Peracca, 1904), but in this latter species also the ventral spiracle has a medial subdivision anterior to the gut, forming tubes each of which connect with a gill (Lehr et al. 2007). The shape and extension of the spiracle in *Chiasmocleis leucosticta* (Boulenger, 1888) is similar to that in *E. bicolor*, but the opening position is medial and only visible in ventral view (Langone et al. 2007). In *D. muelleri*, the spiracle is posteromedial in position and opens slightly anterior and ventral to the vent tube (Fabrezi et al. 2012).

In microhylids with direct development of the genera *Austrochaperina*, *Cophixalus* and *Oreophryne*, the operculum is not discernible during embryonic development, unlike that described in other non-microhylid species with direct development as *Eleutherodactylus coqui* Thomas, 1966, *Eupsophus emiliopugini* Formas, 1989 and myobatrachids (Callery & Elinson 2000; Vera

Candiotti et al. 2011; Anstis et al. 2007; Anstis 2008). The apparent absence of opercular folds in tadpoles of species with direct development suggests a deletion during ontogeny (Callery & Elinson 2000; Anstis et al. 2011).

A medially oriented vent tube is the typical microhylid condition (Inger 1967). In *E. bicolor*, the vent tube was observed in medial position in Gosner's stage 38–39 tadpoles (Williams & Gudynas 1987), while Lavilla & Langone (1991) mentioned that the vent tube opening migrated from left to right during stages 34–39, changing from a non-microhylid to a typical microhylid position. In *E. bicolor*, we observed that the vent tube opening is oriented toward the left during most of the development.

The fact that the external nares are closed during most of the tadpole life of microhylids (Wassersug 1989; Altig & McDiarmid 1999; Nokhbatolfoghahai & Downie 2005a) has been proposed as an additional diagnostic character to define Orton's Type II tadpoles of Microhylidae (Wassersug 1989). In *E. bicolor*, the external nares are closed externally at the beginning of independent feeding, to be re-opened at stage 41. The unperforated external nares, the divided ventral velum, the structure of branchial food traps and the expanded prenarial region of the buccal roof in microhylid tadpoles have been associated with the efficiency of their buccal pumps in free living tadpoles and suspension feeding (Wassersug 1980, 1989).

The distinct larval characters of Microhylidae appear during the last embryonic stages and first larval stages. Therefore, the consideration of these stages can be of great value in future studies of anuran development in ecological, phylogenetic and evolutionary contexts. Also, the study of the ontogeny of larval structures such as the oral funnel in microhylid tadpoles and in other species with oral funnels with or without vestigial, keratinized structures, are key to identify heterochronic processes, to interpret the variability of larval structures, and to establish or reinforce hypotheses about the origin of larval structures.

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