



Early development of the glass frogs *Hyalinobatrachium fleischmanni* and *Espadarana callistomma* (Anura: Centrolenidae) from cleavage to tadpole hatching

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Abstract.—We report the characteristics of embryonic development from cleavage to tadpole hatching in two species of glass frogs, *Hyalinobatrachium fleischmanni* and *Espadarana callistomma* (Anura: Centrolenidae). This analysis of embryonic development in centrolenid frogs enhances comparative studies of frog early development and contributes baseline information for the conservation and management of Ecuadorian frogs. These frogs reproduced in captivity and their embryos were fixed for developmental analysis. The morphology of embryos was evaluated in whole mounts, bisections, thick sections, and fluorescent staining of cell nuclei. Egg clutches contained an average of 23 and 35 eggs for *H. fleischmanni* and *E. callistomma*, respectively. The eggs of both frogs measured approximately 2.1 mm in diameter. The eggs of *H. fleischmanni* were uniformly pale green. In contrast, the animal hemisphere of *E. callistomma* eggs was dark brown and the vegetal hemisphere was light brown. The developmental time of *H. fleischmanni* and *E. callistomma* under laboratory conditions was 6 and 12 days, respectively from the 32-cell stage until tadpole hatching. Differences in environmental conditions may be associated with the time differences of early development observed in these frogs. The development of glass frogs from egg deposition to tadpole hatching was staged into 25 standard stages according to the generalized table of frog development. Archenteron elongation began in the early gastrula and notochord elongation began in mid to late gastrula, as in *X. laevis*. Development of the gastrocoel roof plate (grp) was precocious in the two centrolenid frogs. The grp was detected in the late gastrula of both species; whereas the grp was detected in neurula stages of *X. laevis*. The presence of the grp in embryos of these frogs suggests that the mechanisms of left-right asymmetry, found in *X. laevis* and other amphibians, may be shared by these centrolenid frogs. The development of *H. fleischmanni* and *E. callistomma* resembles the pattern found in frogs with rapid development such as the aquatic eggs of *X. laevis* and the development in floating foam-nests in the genus *Engystomops* (Leptodactylidae). Differences in egg pigmentation were particularly significant in connection with the divergent reproductive strategies of these glass frogs.

Key words. Developmental time, egg pigmentation, embryonic development, gastrulation, gastrocoel roof plate, neurula

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Introduction

Centrolenid frogs are commonly known as glass frogs because the internal organs of the adult are visible through the transparent ventral body wall. This transparent region varies in size among species (Fig. 1A–B, D–E) (Cisneros-Heredia and McDiarmid 2007). Glass frogs are endemic to the tropical regions of South America from Venezuela to northern Argentina and south-eastern Brazil (AmphibiaWeb 2014) and are particularly diverse in the cloud forests of Colombia and Ecuador (Delia et al. 2010; Guayasamin and Trueb 2007; Ospina-Sarria et al. 2010). These arboreal frogs deposit their eggs in gelatinous masses on the upper or lower surface of plant leaves bordering stream banks. After hatching, the tadpoles drop into the underlying streams. Tadpoles are fossorial and live in the substrate along the shoreline (Delia et al. 2010; Duellman and Trueb 1986).

We studied the early development of the glass frogs *Hyalinobatrachium fleischmanni* and *Espadarana callistomma* (Anura: Centrolenidae) to compare their development with frogs that exemplify different reproductive modes and to contribute to the knowledge of frogs from Ecuador. Development of these centrolenid frogs was compared with the embryogenesis of Túngara frogs, *Engystomops* (Leptodactylidae). Túngara frogs construct foam nests that float in the water (Romero-Carvajal et al. 2009). In addition, this comparison was extended to the terrestrial embryos of poison arrow frogs (Dendrobatidae), embryos of the Marsupial frog, *Gastrotheca riobambae* (Hemiphractidae), and the aquatic embryos of *Xenopus laevis* (Pipidae) and *Ceratophrys stolzmanni* (Ceratophryidae) (Elinson and del Pino 2012; Nieuwkoop and Faber 1994; del Pino et al. 2004) (Table 1). The analysis of *H. fleischmanni* and *E. callistomma* early development was feasible because of the recent successful reproduction of centrolenid frogs in captivity at the Balsa de los Sapos, Centre of Amphibian Investigation and Conservation (CICA), Pontificia Universidad Católica del Ecuador (PUCE).

Hyalinobatrachium fleischmanni (Fig. 1A–C) occurs from southern Mexico to northern South America, including Ecuador. The egg clutches consist of 20–40 pale-green eggs, attached to the underside of plant leaves (Fig. 1C). Parental care of the egg clutch is provided by the male to maintain the needed humidity. The male prevents predation by katydids, wasps, ants, and other insects by kicking with his limbs at the predatory insect (Delia et al. 2010; Greer and Wells 1980; Savage 2002).

Espadarana callistomma (Guayasamin and Trueb 2007) (Fig. 1D–F) occurs in the lowlands of northeastern Ecuador and southern Colombia (Guayasamin and Trueb 2007; Ospina-Sarria et al. 2010). Darkly pigmented eggs are deposited on the upper surface of plant leaves (Guayasamin and Trueb 2007) (Fig. 1F). Egg predation by insects has not been reported for this species.

The left-right asymmetric location of organs, such as the liver and the heart is established in the *X. laevis* gastrocoel roof plate (grp) of the neurula by fluid-flow towards the left side, guided by the clockwise rotation of cilia (Blum et al. 2009; Schweickert et al. 2010). The rotation of cilia in the frog grp, or in equivalent structures of other vertebrates, determines the asymmetric expression of the gene *Nodal* in the grp left side (Blum et al. 2014b). The grp of *X. laevis* derives from the superficial prospective mesoderm of the early gastrula that becomes internalized during gastrulation, and ends up in the dorsal roof of the primitive gut. The grp can be detected by the presence of exposed mesoderm corresponding to the notochord and some paraxial mesoderm in the dorsal roof of the primitive gut, and it is bordered by the lateral endodermal crests (lec). As development advances, the lec close to the midline and the primitive gut cavity becomes totally lined with endoderm (Blum et al. 2009). The left-right asymmetry is determined by fluid flow guided by cilia rotation in the grp of frogs and other vertebrates. However, a comparable structure to the grp has not been reported for the chick and pig, and the left-right symmetry breakage in these vertebrates may depend on a modified mechanism (Blum et al. 2014a, b; Sáenz-Ponce et al. 2012b). We analyzed the presence of the grp in the gastrula and neurula of glass frogs to provide additional comparison.

We characterized the embryos of these glass frogs from cleavage to hatching of the tadpoles. We found that in glass frogs, gastrulation overlapped with body elongation, as in frogs with rapid embryonic development. The grp was detected in the late gastrula of both species of glass frogs. Its presence suggests that the mechanisms of left-right asymmetry, found in *X. laevis* and other amphibians, may be shared by these centrolenid frogs. The reproductive mode of these glass frogs is associated with rapid development. The strategy of egg deposition in the underside or upper surface of leaves is associated with differences in developmental time and pigmentation of embryos and tadpoles.

Materials and Methods

Locality of collection and staging of embryos. *Hyalinobatrachium fleischmanni* and *Espadarana callistomma* were collected from Esmeraldas Province, San Lorenzo, Durango, along the banks of the Río Durango and its tributaries in northwest Ecuador. The altitude of this site is 243 m above sea level, and the geographic coordinates are W 78.62405, N 1.04186. Frogs of both species were collected on 04 October 2009 by Elicio Tapia and Santiago García. The adults successfully reproduced at the Balsa de los Sapos, Centre of Amphibian Investigation and Conservation (CICA), School of Biological Sciences, Pontificia Universidad Católica del Ecuador

H. fleischmanni

E. callistomma

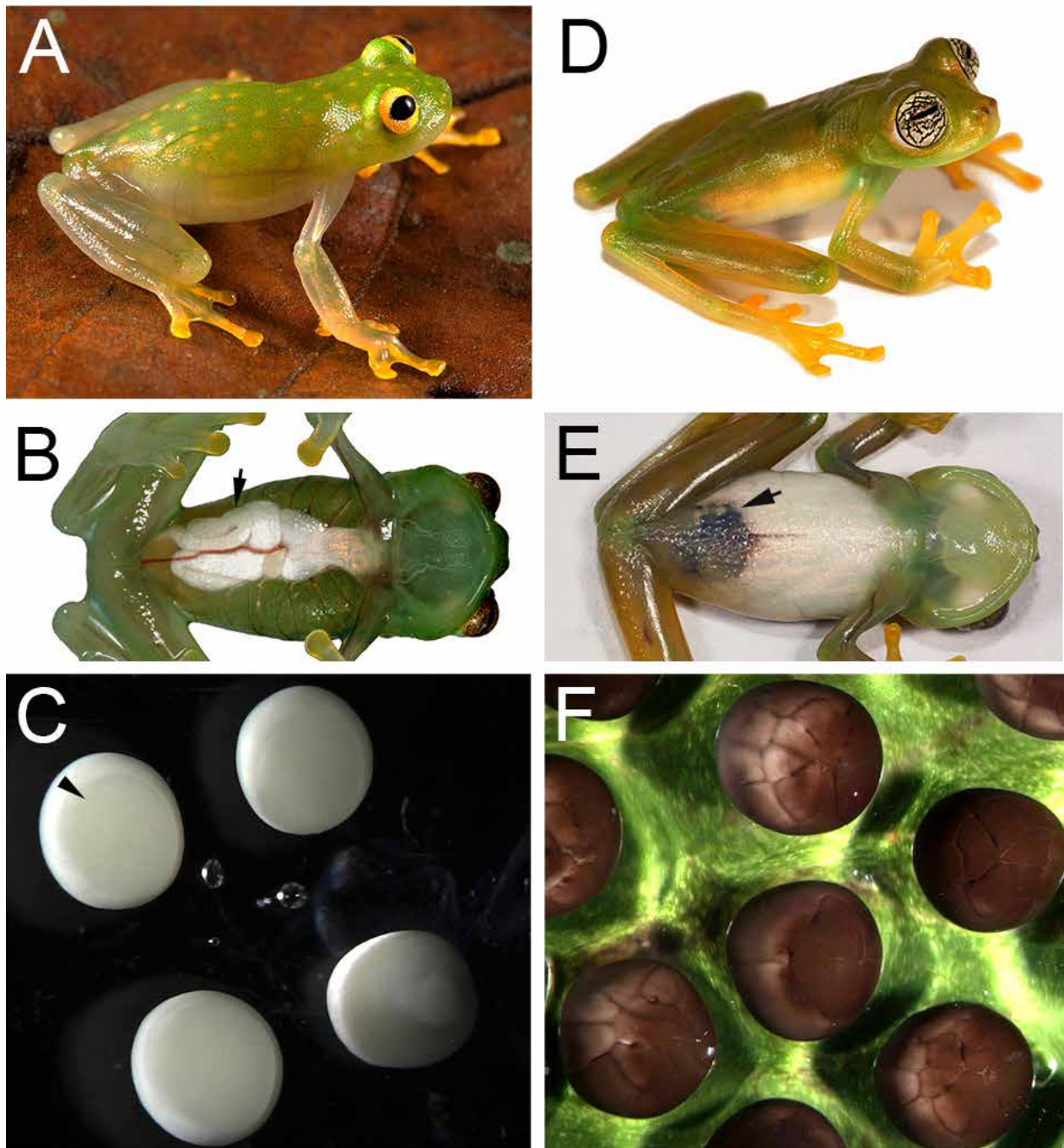


Fig. 1. The adults and egg clutches of glass frogs. **(A–C)** *Hyalinobatrachium fleischmanni*. **(A)** Lateral view of an adult male. **(B)** Ventral view of an adult male. The arrow indicates the border of the transparent body wall. The intestine and a blood vessel are visible. **(C)** Partial view of an egg clutch at the gastrula stage. The embryos are uniformly pale and the blastocoel roof is translucent (arrowhead). **(D–F)** *Espadarana callistomma*. **(D)** Lateral view of an adult female. **(E)** Ventral view of an adult female. The arrow signals the pigmented oocytes visible through the transparent body wall. The size of the transparent region is smaller than in *H. fleischmanni* shown in B. **(F)** Partial view of an egg clutch. The embryos were at stages 5–6 (Table 2). Photographs of adult frogs by Santiago Ron (A–B, D–E).

(PUCE). The permit 016-IC-FAU-DNBAP-MA from the Ministry of the Environment, Ecuador, allowed the collection and maintenance of these frogs at Balsa de Sapos. Egg clutches were donated to the Laboratory of Developmental Biology for analysis of embryonic development. This study was based on the analysis of embryos derived from seven egg clutches of *H. fleischmanni* and four egg clutches of *E. callistomma*.

The number of eggs of each egg clutch was recorded and the embryos were cultured in humid chambers at room temperature, as described for embryos of the dendrobatid frog, *E. machalilla* (del Pino et al. 2004). At various intervals, some embryos were moved to a Petri dish filled with 15% Steinberg's solution (del Pino et al. 2004) and the egg-jelly was manually removed to study embryogenesis. Embryos were staged according to the general table of frog development (Gosner 1960). Egg diameter was measured in fixed embryos with the measuring tool of the program, Axiovision (Carl Zeiss, Oberkochen, Germany).

Fixation, staining and analysis of embryonic development. Embryos were fixed in Smith's fixative (del Pino et al. 2004). The procedures for the bisection of embryos, vibratome sectioning, cell nuclei staining with the fluorescent dye Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA), and the staining of cell boundaries with silver nitrate were previously described (Moya et al. 2007; del Pino et al. 2004). Sections were mounted in glycerol, and were examined with a Stemi SV6 stereo microscope (Carl Zeiss, Oberkochen, Germany) or with fluorescent optics using a Z1 Axio Observer microscope (Carl Zeiss, Oberkochen, Germany). Embryos were photographed with AxioCam cameras, attached to microscopes, and the image capture program, Axiovision (Carl Zeiss, Oberkochen, Germany). The images were edited with Adobe Photoshop CS6.

Results and Discussion

Internal organs of adult glass frogs can be observed through their transparent belly; however the size of the transparent window varies in the different genera of centrolenid frogs as detected for *H. fleischmanni* and *E. callistomma* (Fig. 1A–B, D–E) (Cisneros-Heredia and McDiarmid 2007). In contrast with adults, the eggs of these frogs were opaque (Fig. 1C, F). We also detected significant pigmentation differences as the *H. fleischmanni* eggs and embryos were pale-green and those of *E. callistomma* were dark brown (Fig. 1C, F). Egg pigmentation is a distinctive character of the different genera of Centrolenidae; moreover some species deposit their eggs in the upperside and others in the underside of leaves. However, some species show no particular preference for the upper or underside of leaves for the deposition of their eggs (Cisneros-Heredia and McDiarmid 2007).

Clutch size, egg pigmentation and developmental time. The number of eggs ranged from 14–30 eggs, with a mean of 23 eggs per clutch in *H. fleischmanni*, and 32–39 eggs, with a mean of 35 eggs per clutch in *E. callistomma*. The eggs of both species measured about 2.1 mm in diameter (Table 1). The embryos of *H. fleischmanni* were uniformly pale-green (Figs. 1C; 2A–L; 3A–D). In contrast, the animal hemisphere of *E. callistomma* embryos was dark brown, and the vegetal hemisphere was pale-brown (Figs. 1F; 4A–L; 5A–F).

Dark pigmentation of the animal hemisphere of the egg may provide protection against solar UV radiation and may capture solar heat required to accelerate early development of frog embryos exposed to solar radiation in moist or aquatic environments. In contrast, there is lack of dark pigment in frog eggs and embryos that develop in secluded places (Duellman and Trueb 1986; Elinson and del Pino 2012). We propose that *H. fleischmanni* embryos do not require dark pigmentation because the underside of plant leaves may provide protection against solar radiation. In contrast, the presence of dark pigment in eggs and embryos of *E. callistomma* may be needed, as the egg clutches are directly exposed to UV solar radiation on the upper surface of plant leaves.

The differences in pigmentation were detectable in eggs and embryos until tadpole hatching (Figs. 1C, F; 2–5). At hatching, the tadpoles of *H. fleischmanni* were pale green with little dark pigmentation on the dorsum; whereas *E. callistomma* tadpoles had a brown color (Figs. 3C–D; 5F). The fossorial free-living tadpoles of *H. fleischmanni* remained nearly unpigmented had elongated bodies, and narrow tail fins to enable digging in the sandy stream bottoms. The eyes were reduced in size and were covered by skin characters likely associated with the fossorial habits of *H. fleischmanni* tadpoles (Delia et al. 2010; Duellman and Trueb 1986; Savage 2002). The characteristics of the *E. callistomma* free-living tadpoles are unknown. The differences in tadpole pigmentation at hatching suggest that the larval stages of these two centrolenids may occur in dissimilar aquatic environments.

The differences in egg pigmentation observed in *H. fleischmanni* and *E. callistomma* may depend on different expression levels of the gene Shroom2 during oogenesis. Shroom2, an actin-binding protein, controls pigment granule localization in the animal cortex of *X. laevis* oocytes (Lee et al. 2009). The oocytes of *Engystomops pustulosus* (Leptodactylidae) contain small amounts of Shroom2 protein and are white in color. However, *Engystomops* embryos have dark pigment granules around nuclei of blastomeres (Lee et al. 2009; Romero-Carvajal et al. 2009). Embryos of *H. fleischmanni* are pale and do not have dark pigment around the nuclei of blastomeres; whereas, in *E. callistomma* embryos dark pigment was observed on the cell surface of animal pole blastomeres, as well as around blastomere nuclei.

Embryos of *H. fleischmanni* and *E. callistomma* were maintained under identical laboratory conditions with a

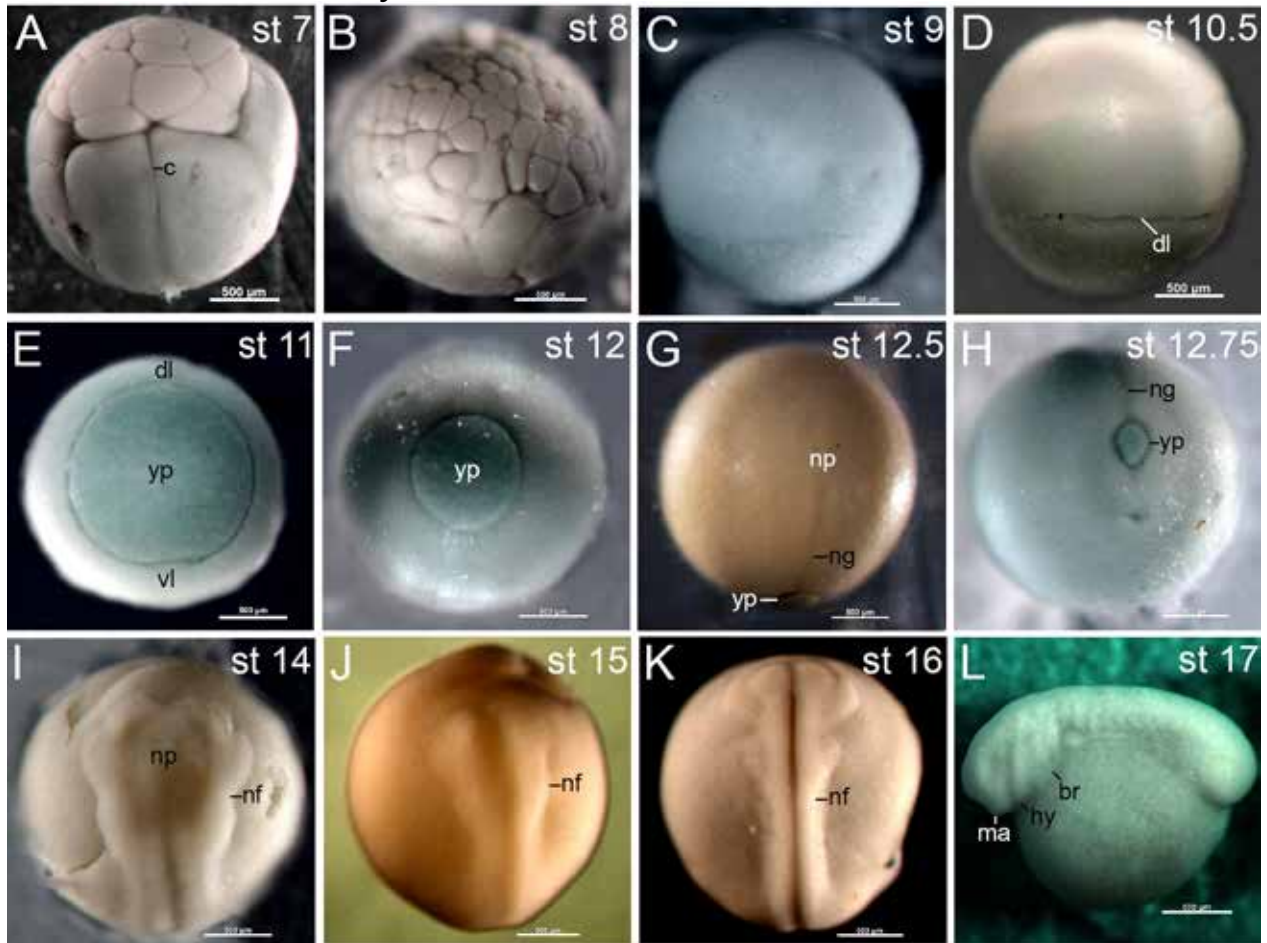
Hyalinobatrachium fleischmanni

Fig. 2. External morphology of *Hyalinobatrachium fleischmanni* embryos from cleavage to the tail bud stage. (A) Stage 7: Thirty-two cell stage. Animal micromeres were much smaller than the vegetal macromeres. (B) Stage 8: Mid cleavage. (C) Stage 9: Blastula. (D) Stage 10.5: Early gastrula. The dorsal blastopore lip can be seen in the dorsal subequatorial region. (E) Stage 11: Mid gastrula. The yolk plug was large. (F) Stage 12: Late gastrula. (G) Stage 12.5: Late gastrula with a small yolk plug. Neural groove and neural plate were visible in embryos of this stage. (H) Stage 12.75: Late gastrula. The neural groove was visible. The yolk plug was small. (I) Stage 14: Early neural fold. (J) Stage 15: Mid-neural fold. The neural folds were more elevated. (K) Stage 16. Closure of the neural tube. The neural folds were near each other. (L) Stage 17. Tail bud stage. The branchial arches were visible. In this and the following figures, numbers in the top right-hand corner give the developmental stage. br, branchial arch; c, cleavage furrow; dl, dorsal blastopore lip; hy, hyoid arch; ma, mandibular arch; nf, neural fold; ng, neural groove; np, neural plate; vl, ventral blastopore lip; yp, yolk plug.

temperature fluctuation of 18–23 °C. However, developmental time diverged greatly between these frogs, as embryos of *H. fleischmanni* required six days and those of *E. callistomma* required 12 days from the 32-cell stage until tadpole hatching. However in nature, great variation in developmental time was observed in *H. fleischmanni*, as egg clutches required 8–21 days from oviposition to tadpole hatching (Greer and Wells 1980). In our laboratory, development of *H. fleischmanni* and *E. callistomma* was slower than in the floating foam-nests of *Engystomops* (Leptodactylidae), and faster than in the terrestrial nests of Dendrobatidae. In two species of *Engystomops*, development from egg deposition until hatching required only three days whereas 19–21 days were required for the same developmental processes by six species of den-

drobatid frogs (del Pino et al. 2004, 2007; Romero-Carvajal et al. 2009) (Table 1).

Reproductive strategies. We propose that rapid development may be favored in *H. fleischmanni* in comparison with *E. callistomma* because eggs deposited on the underside of plant leaves are at a greater risk of desiccation in comparison with eggs deposited on the upperside of leaves (Delia et al. 2010; Savage 2002). Moreover, rapid development may be required in all centrolenids, including frogs of the genus *Espadarana*, to overcome predation from a number of insect families and other arthropods (Cabanzo-Olarte et al. 2013; Duellman and Trueb 1986; Villa 1977; Vockenhuber et al. 2008). The deposition of eggs on the underside of plant leaves and

Table 1. Comparison of reproductive and developmental characteristics of glass frogs.

Family and Species	Reproduction	Clutch size and (egg diameter, mm)	Gastrulation time (hrs) ^a	Presence of the grp in the neurula	Onset of noto-chord elongation	Refs ^d
Rapid Development						1
Centrolenidae						
<i>Hyalinobatrachium fleischmanni</i>	Leaves underside	23 (2.1)	24	Yes	mid gastrula ^b	2
<i>Espadarana callistomma</i>	Leaves upperside	35 (2.1)	23	Yes	mid gastrula ^b	2
Leptodactylidae						
<i>Engystomops randi</i>	Floating foam-nest	110 (1.1)	12.5	Yes	mid gastrula ^b	3, 4
<i>Engystomops coloradorum</i>	Floating foam-nest	130 (1.3)	12.5	Unknown	mid gastrula ^b	3
Ceratophryidae						
<i>Ceratophrys stolzmanni</i>	Aquatic	664 (2.2)	5	Yes	mid gastrula ^b	4, 5
Pipidae						
<i>Xenopus laevis</i>	Aquatic	1000 (1.2)	6	Yes	mid gastrula ^b	6, 7
Slow Development						1
Dendrobatidae						
<i>Epipedobates machalilla</i>	Terrestrial nest	15 (1.6)	65	Yes	After gastrulation ^c	4, 8
<i>Epipedobates tricolor</i>	Terrestrial nest	13 (2.0)	36	Yes	After gastrulation ^c	8, 9
Hemiphractidae						
<i>Gastrotheca riobambae</i>	Egg brooding	128 (3.0)	168	Yes	After gastrulation ^c	1, 4

^aTime from stages 10–13. Embryo culture temperatures for: *X. laevis* 23 °C, and 18–23 °C for other frogs.; ^bStage 11; ^cStage 13; ^dReferences: 1, (del Pino et al. 2007); 2, This work; 3, (Romero-Carvajal et al. 2009); 4, (Sáenz-Ponce et al. 2012b); 5, (Ortiz, 2013); 6, (Nieuwkoop and Faber 1994); 7, (Blum, et al. 2009); 8, (del Pino et al. 2004); 9, (Sáenz-Ponce et al. 2012a).

predation of eggs and embryos by wasps, ants, katydids and other arthropods are likely determining factors in favor of rapid development in *H. fleischmanni*.

Aquatic eggs and embryos characterize the basal mode of frog reproduction, as exemplified by *X. laevis* and *Ceratophrys stolzmanni* (Table 1). These frogs release a large number of small eggs in the water. However, frogs have invaded different environments for reproduction due to competition for water resources, predation, and the dangers of desiccation. Accordingly, clutch size, egg size and developmental time vary among species (Table 1) (Duellman and Trueb 1986). The dissimilar developmental times of *H. fleischmanni* and *E. callistomma* may relate to their egg deposition sites and to different predation pressure on eggs and embryos. Egg deposition in the upperside or underside of leaves associated with differences in egg pigmentation and developmental time, as observed in centrolenid frogs, are different reproductive modes that deserve further investigation.

Development of *H. fleischmanni* and *E. callistomma*.

The characteristics of development are detailed in Table 2, and illustrated in Figs. 2–13. It was of interest to document the characteristics of development of these glass frogs, given the observed differences in embryonic pigmentation and developmental time. The development from early cleavage to tadpole hatching of *H. fleischmanni* and *E. callistomma* was characterized according to the generalized table of frog development (Gosner 1960)

(Table 2). Embryos of *H. fleischmanni* from fertilization to the sixteen cell stage were not available.

Micrographs of the external morphology of embryos illustrate the developmental stages of both species, and clearly demonstrate the pigmentation differences among species (Figs. 1C, F; 2A–L; 3A–D; 4A–L; 5A–F). The internal morphology of embryos from cleavage until the completion of neurulation follows the typical frog pattern, as outlined in the generalized table of development (Gosner 1960) (Figs. 6–13). The most notable differences are the overlap between gastrulation and the onset of neural development, and the lack of pigment in embryos of *H. fleischmanni* in comparison with embryos of *E. callistomma*. In both species cleavage was holoblastic (Figs. 6A–D; 7A–D), and the blastocoel roof was reduced to two-cells in thickness during gastrulation. At gastrulation, a conspicuous dorsal blastopore lip developed in the subequatorial dorsal region (Figs. 8A–F; 9A–E). The onset of neurulation began before completion of blastopore closure (Figs. 10A–D; 11A–F).

Developmental time, gastrulation and body elongation.

Our comparative analysis includes frog species with rapid and slow development (Table 1). Embryonic development occurs rapidly in frog species with aquatic reproductive modes. The analyzed frogs with rapid development and embryos suspended on the vegetation included *H. fleischmanni*, *E. callistomma* (Centrolenidae). Frogs with aquatic eggs and embryos included *X. laevis*

Early development of *Hyalinobatrachium fleischmanni* and *Espadarana callistomma*

Table 2. Characteristics of development of the glass frogs *Hyalinobatrachium fleischmanni* and *Espadarana callistomma*.

G	Morphology observed in Centrolenid frogs ¹
1	Fertilization (not available).
2	Gray crescent (not available).
3	Two cell stage (not available).
4	Four cell stage. The first two cleavage furrows passed from the animal to the vegetal pole. This stage was available only for <i>E. callistomma</i> (not shown).
5	Eight cell stage. The third cleavage furrow was latitudinal in some embryos and longitudinal in others. This stage was available only for <i>E. callistomma</i> (not shown).
6	Sixteen cell stage. Cleavage became asynchronous after the eight cell stage, and embryos with variable numbers of blastomeres were observed. This stage was available only for <i>E. callistomma</i> (not shown).
7	Thirty-two cell stage. Cleavage in both species was holoblastic, and the animal micromeres were much smaller than the vegetal macromeres, as observed for other frogs. (Figs. 2A; 4A; 6A–B; 7A–B).
8	Mid cleavage. Development of the blastocoel began during cleavage, as shown for <i>H. fleischmanni</i> . (Figs. 2B; 6C–D).
9	Blastula. The blastocoel roof was thick and consisted of several cell layers (Figs. 2C; 4B; 7C–D).
10	Early gastrula. A conspicuous blastopore groove was observed on the dorsal subequatorial region of the embryo, and there were bottle cells marking cell ingression at the blastopore groove as shown for both species (Figs. 4C; 8A; 9A–B). In slightly more advance embryos, the dorsal blastopore lip was detected in the dorsal subequatorial region, as shown for <i>H. fleischmanni</i> (Figs. 2D; 8B).
11	Mid gastrula. The blastopore lip surrounded a large yolk plug in embryos of both frogs (Figs. 2E; 4D; 8C). Internally, the archenteron was elongated, without inflation (Figs. 8D; 9C). The blastocoel roof was translucent (Fig. 1C) and consisted of two-cell layers (not shown).
12	Late gastrula and development of the neural plate (Figs. 2F; 4E; 8E). The neural groove and the neural plate were visible in gastrula stage embryos with a small yolk plug (stage 12.5) (Figs. 2G; 4F; 11 A). The archenteron was elongated in an anterior direction and it was inflated, and the blastocoel was reduced in size. The cleft of Brachet, that separates the ectoderm from the endomesoderm, was visible in the roof of the primitive gut (Figs. 8F; 9D–F; 10A; 11 B–C). The notochord was detected in stage 12.5 embryos, as shown for <i>H. fleischmanni</i> (Fig. 10B). In stage 12.75, the neural plate was visible in both species (Figs. 2H; 4G; 11 D). The yolk plug was small, the archenteron was fully inflated, and the germ layers were visible (Fig. 10C–D; 11 E–F). A triangular dorsal structure, considered to be the gastrophilic roof plate (grp), was located in the roof of the primitive gut, and was exposed to the cavity of the gastrophilic (Fig. 12C). The grp included the ventral surface of the notochord and paraxial mesoderm, and was bordered by the lateral endodermal crests (lec). The grp is illustrated for <i>E. callistomma</i> (Figs. 12D).
13	The closed blastopore and the neural plate. The yolk plug was totally retracted, the blastopore was at the slit blastopore stage, and the neural plate was visible (Figs. 4H; 12A). The grp was located in the roof of the primitive gut, and it was bordered by the lec, shown in whole mount for <i>H. fleischmanni</i> (Fig. 12B).
14	Early neural fold stage. The neural folds were slightly elevated (Figs. 2I; 4I; 13A). The grp included the ventral surface of the notochord, and somites, and it was bordered by the lec, shown for <i>E. callistomma</i> (Fig. 12 E–F). The neural ectoderm, paraxial mesoderm, notochord, and endoderm were visible (Fig. 13B).
15	Mid neural fold stage. The neural folds were elevated (Figs. 2J; 4J; 13C). In cross sections, the neural ectoderm, notochord, paraxial mesoderm and endoderm were visible, as shown for <i>H. fleischmanni</i> (Fig. 13 D).
16	Closure of the neural tube. The neural folds were closed (Figs. 2K; 4K; 13 E). In cross sections, the neural tube was visible dorsal to the notochord. The somites were visible on each side. The endoderm completely lined the archenteron, as shown for <i>E. callistomma</i> (Figs. 13 F).
17	Tail bud stage. The tail bud and the head region protruded beyond the yolky endoderm. The branchial arches were visible (Figs. 2L; 4L).
18	Muscular activity. The branchial arches protruded on the sides of the head. The tail became elongated. This stage is only shown for <i>E. callistomma</i> (Fig. 5A).
19	Heart beat. The heart beated, and the gill buds were visible. This stage is only shown for <i>E. callistomma</i> (Figs. 5B, C).
20	Circulation to the external gills. There were two gill pairs, each with two small branches. This stage is not shown.
21	The gills were larger, the first pair gill had two branches for both species and the second pair gill was unbranched in <i>H. fleischmanni</i> (Figs. 3A; 5D).
22	Tail fin circulation. Not observed.
23	The external gills reached their full size. There were five gill branches in the first pair and four branches in the second pair of external gills in embryos of <i>H. fleischmanni</i> . The opercular fold was developing. There were four gill branches in the first pair and three branches in the second pair of external gills of <i>E. callistomma</i> embryos (Figs. 3B–5E).
24	Larval stage. Not observed
25	Tadpole at hatching. Only a small portion of the external gills protruded from the opercular aperture in the hatching tadpoles. The eyes were very small. (Figs. 3C–D; 5F).

¹ The development of the Centrolenid frogs (*C.*, *H. fleischmanni* and *E. callistomma*, was compared with the general staging table for frogs (G) (Gosner, 1960).

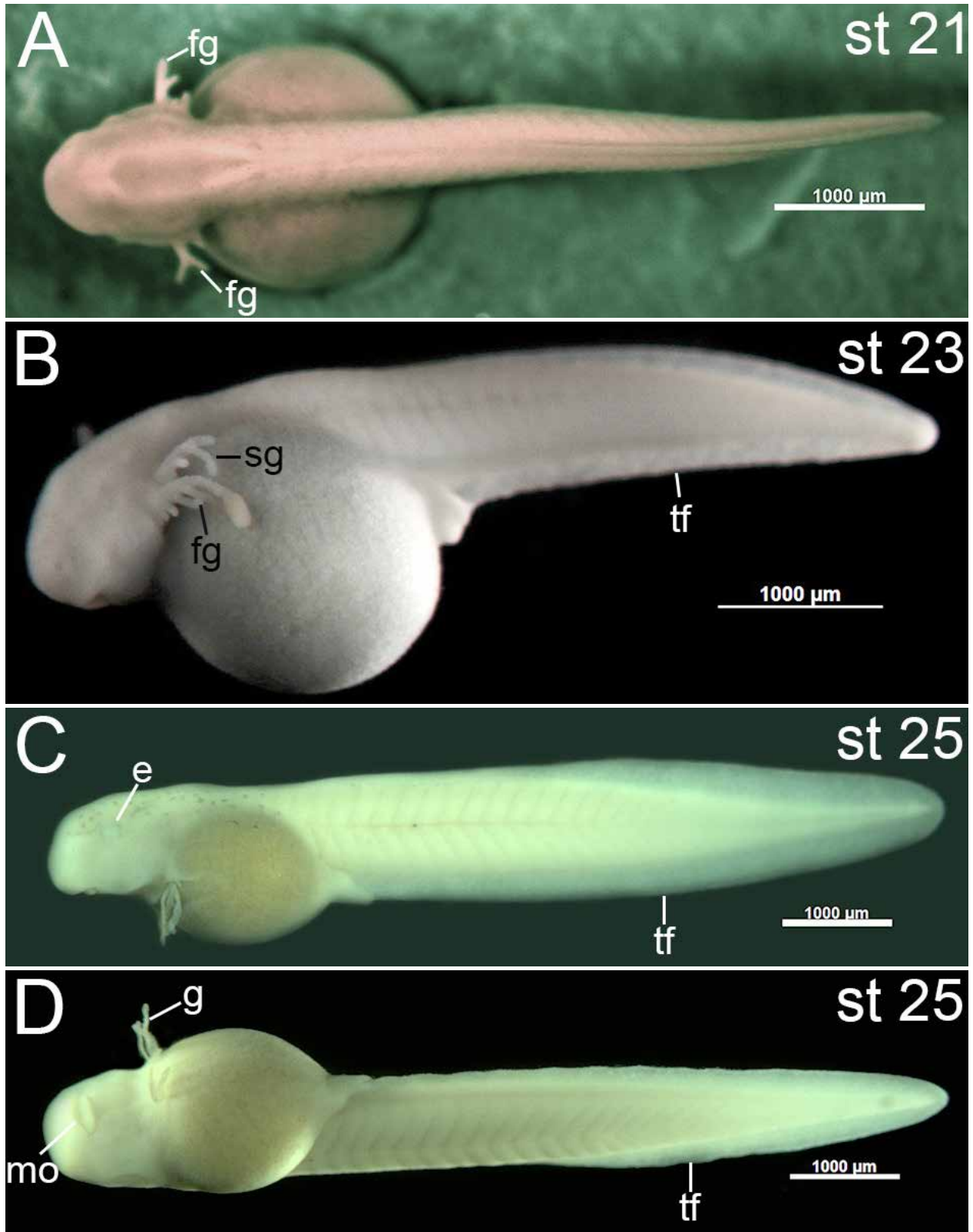
Hyalinobatrachium fleischmanni

Fig. 3. External morphology of *Hyalinobatrachium fleischmanni* embryos from the development of the gills stage to hatching. **(A)** Stage 21: The gills were large, and each gill pair had two branches. **(B)** Stage 23: Full development of the external gills. There were five gill branches in the first pair and four branches in the second pair of gills. **(C)** Stage 25: Lateral view of a tadpole at hatching. The eyes were very small. **(D)** Stage 25: Ventral view of a tadpole at hatching. Only a small portion of the external gills protruded from the opercular aperture. The pink color of the embryo in A was an artifact of fixation. e, eye; fg, first pair gills; g, gills; mo, mouth; sg, second pair gills; tf, tail fin.

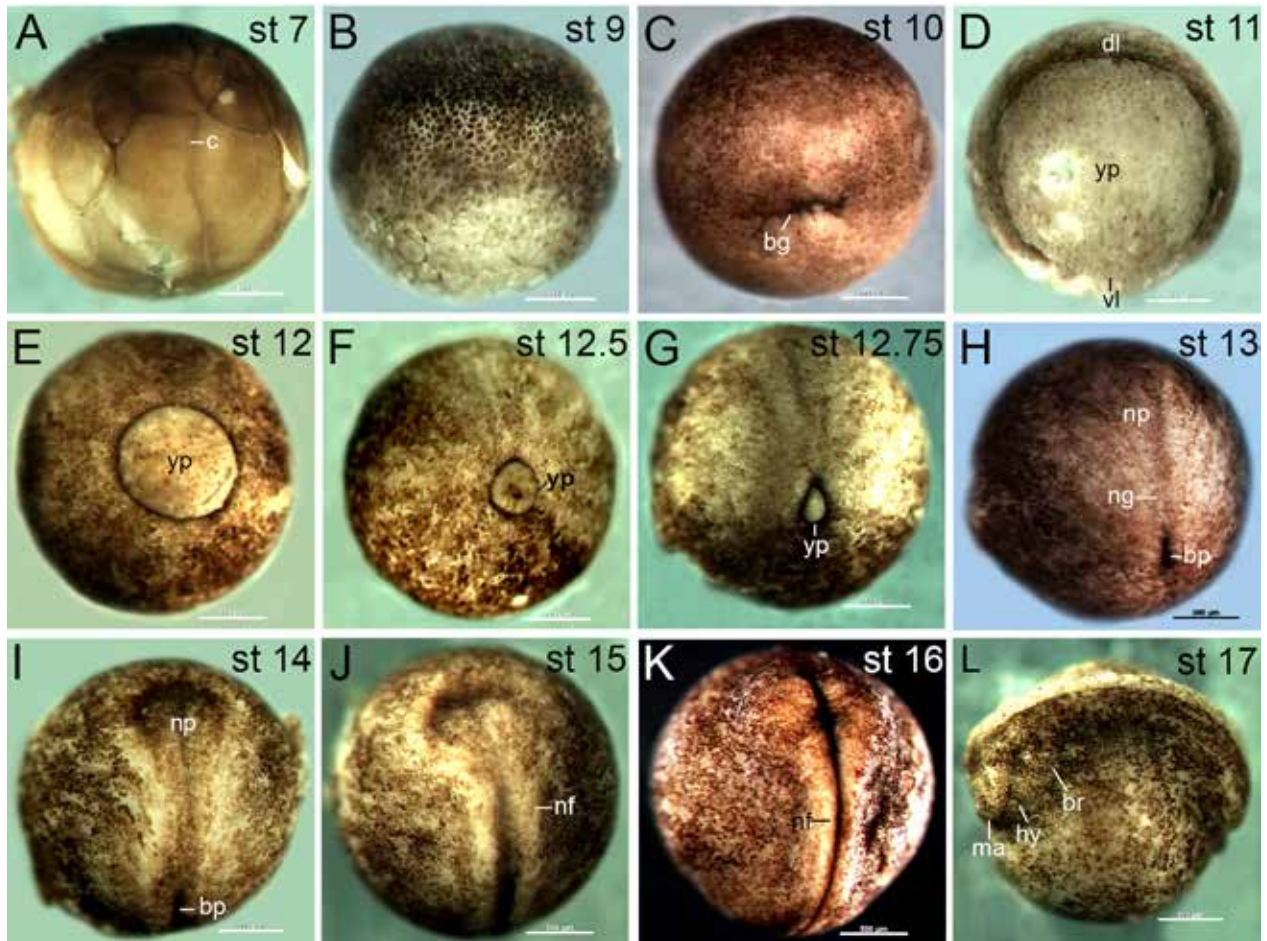
E. callistomma

Fig. 4. External morphology of *Espadarana callistomma* embryos from cleavage to the tail bud stage. **(A)** Stage 7: Thirty-two cell stage. Animal micromeres were much smaller than the vegetal macromeres. **(B)** Stage 9: Blastula. **(C)** Stage 10: Early gastrula. A conspicuous blastopore groove was observed on the dorsal subequatorial region of the embryo. **(D)** Stage 11: Mid gastrula. The blastopore lip surrounded a large yolk plug. **(E)** Stage 12: Late gastrula. **(F)** Stage 12.5: Late gastrula with a small yolk plug. **(G)** Stage 12.75: Late gastrula with a very small yolk plug. The neural plate was visible. **(H)** Stage 13: The neural plate was visible. The yolk plug was totally retracted and the blastopore was at the slit blastopore stage. **(I)** Stage 14: Early neural fold. The neural folds were visible. **(J)** Stage 15: Mid neural fold. The neural folds were elevated. **(K)** Stage 16: Closure of the neural tube. The neural folds were closed. **(L)** Stage 17: Tail bud stage. The branchial arches were visible. bg, blastopore groove; br, branchial arch; bp, closed blastopore; c, cleavage furrow; dl, dorsal blastopore lip; hy, hyoid arch; ma, mandibular arch; nf, neural fold; ng, neural groove; np, neural plate; vl, ventral blastopore lip; yp, yolk plug.

(Pipidae), and *Ceratophrys stolzmanni* (Ceratophryidae), and frogs with embryos placed in floating foam-nests were *Engystomops randi* and *Engystomops coloradum* (Leptodactylidae) (Table 1). In contrast, embryonic development was much slower in embryos of frogs with terrestrial adaptations. Frogs with slow development included the Marsupial frog *Gastrotheca riobambae* (Hemiphractidae) that broods its embryos in a dorsal pouch of the mother and the dendrobatid frogs *Epipedobates machalilla* and *Epipedobates tricolor* (Dendrobatidae) that deposit their eggs in terrestrial nests (Table 1) (del Pino et al. 2007; Elinson and del Pino 2012).

Gastrulation characteristics vary among frogs according to their developmental speed. Gastrulation and body elongation, as detected by the onset of notochord elongation, overlapped in embryos of *X. laevis*, *C. stolzmanni*,

E. randi, and *E. coloradum*, frogs with rapid development (Table 1). Similarly, elongation of the notochord overlapped with gastrulation in the rapidly developing embryos of the centrolenid frogs *H. fleischmanni* and *E. callistomma* (Figs. 8D, F; 9C–F; 10A–D; 11B–C, E–F; 12D; Table 2). In contrast, gastrulation movements occurred before the onset of notochord elongation in the slowly developing dendrobatids *E. machalilla* and *E. tricolor*, and in the Marsupial frog, *G. riobambae*. Egg size is larger in these slowly developing frogs in comparison with the rapidly developing species (Table 1), (Elinson and del Pino 2012; del Pino et al. 2007).

The modular nature of gastrulation allows the separation of dorsal convergence and extension, the mechanism that triggers elongation of the notochord and the body, from gastrulation in the slowly developing frogs, and

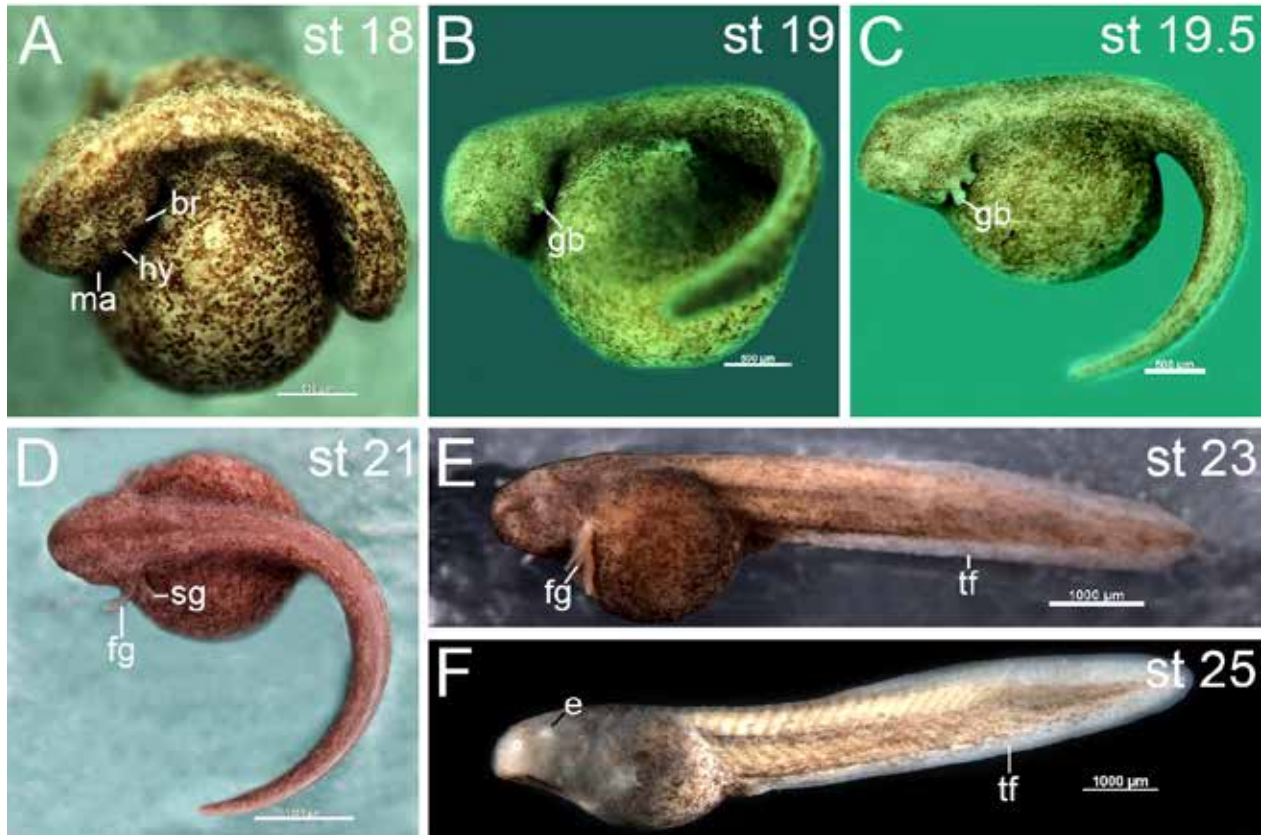
E. callistomma

Fig. 5. External views of *Espadarana callistomma* embryos from the stage of muscular activity to hatching stages. (A) Stage 18: Muscular activity. The branchial arches protruded on the sides of the head. (B) Stage 19: Heartbeat. The gill buds were visible. (C) Stage 19.5: Two gill pairs were visible, each with two small branches. (D) Stage 21: The gills were larger, and each gill pair had two branches. (E) Stage 23: Full development of the external gills. There were four gill branches in the first pair and three branches in the second pair of gills. (F) Stage 25: Tadpole at hatching. The eyes were very small. br, branchial arch; e, eye; fg, first gill pair; gb, gill bud; hy, hyoid arch; ma, mandibular arch; sg, second gill pair; tf, tail fin.

the overlap of these two processes in rapidly developing frog species (Elinson and del Pino 2012). Overlap of gastrulation and body elongation is associated with rapid development in the unstable conditions of the reproductive modes that involve aquatic reproduction of *X. laevis* and *C. stolzmanni*, floating foam-nest development in *Engystomops*, and suspension of eggs on the vegetation, in the case of centrolenids frogs (Table 1), (Elinson and del Pino 2012). The distinct modes of gastrulation likely relate to the reproductive mode of frogs, rather than to phylogenetic relationships.

The gastrocoel roof plate (grp) and left-right asymmetry. It was of interest to determine whether frogs with different reproductive modes, and different onset of notochord elongation share the pattern of left-right asymmetry determination by cilia driven fluid flow towards the left side in the grp, described for *X. laevis* (Blum et al. 2014b; Sáenz-Ponce et al. 2012b). The question is particularly important because the mechanism of symmetry breakage by cilia driven fluid flow in the grp or equivalent structures is universal among vertebrates with

exception of the chick and the pig (Blum et al. 2014a,b). In all frogs analyzed, the gastrocoel roof plate (grp) had a triangular shape and was detected in the dorsal lining of the primitive gut of the late gastrula and neurula, as detected in *H. fleischmanni* and *E. callistomma* embryos (Fig. 12A–F; Table 1). As in *X. laevis* and other frogs, the grp of *H. fleischmanni* and *E. callistomma* embryos consisted of the ventral surface of the posterior notochord and paraxial mesoderm, and it was bordered by the lateral endodermal crests (lec), illustrated for *E. callistomma*, (Figs. 12D–E). However, in a more rostral region, only the notochord was exposed to the cavity of the primitive gut because the paraxial mesoderm was already covered by the closing lec (Fig. 12F). The major difference detected among frogs was the presence of the grp already in the late gastrula of the centrolenid frogs, as shown for *E. callistomma* (Fig. 12D), whereas the grp developed in the neurula of *X. laevis* (Blum et al. 2014b). The precocious onset of grp formation may relate to the overlap of neurulation and gastrulation in centrolenid frogs, another example of the modular nature frog gastrulation.

H. fleischmanni

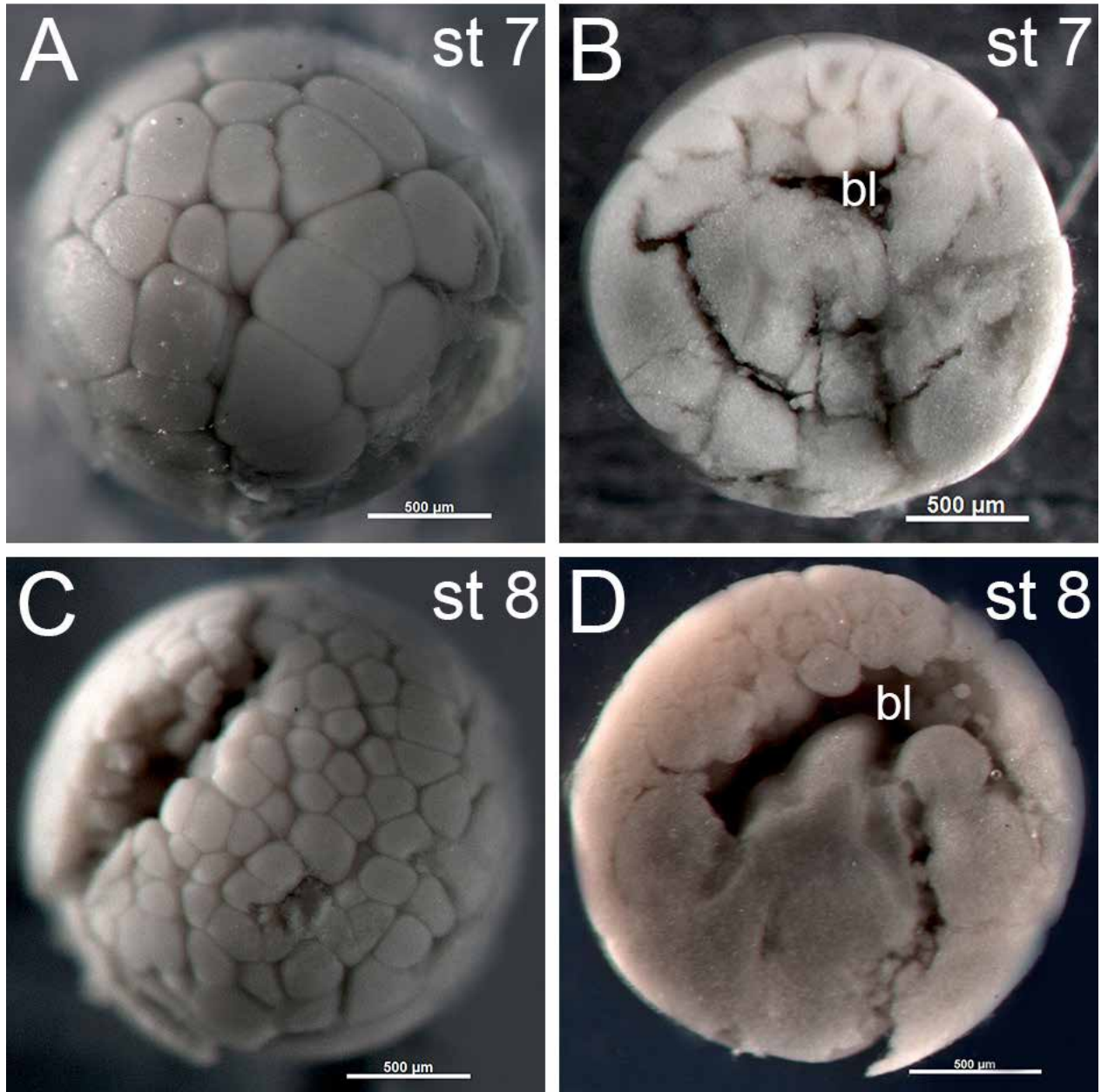


Fig. 6. Cleavage in *Hyalinobatrachium fleischmanni*. (A) Stage 7: Animal view of a 32-cell embryo. (B) Stage 7: The blastocoel of a 32-cell embryo, observed in a sagittal bisection. (C) Stage 8: Animal view of an embryo at mid-cleavage. (D) Stage 8: The blastocoel of a mid-cleavage embryo, observed in a sagittal bisection. bl, blastocoel.

The grp was detected in the neurula of eight frog species with a wide range of reproductive adaptations, and belonging to six different frog families, (Table 1) (Sáenz-Ponce et al. 2012a, b). The presence of the grp in this wide range of frogs suggests that determination of left-right asymmetry may follow mechanisms similar to those described for *X. laevis*. Moreover, cilia were detected in the grp epithelium that lines the dorsal roof of the primitive gut of these various frogs (Sáenz-Ponce et al. 2012a, b). The presence of cilia in the grp in centrolenid frogs was not analyzed.

Conclusions. The reproductive and developmental strategies of the two centrolenid frogs, analyzed in this work, differ from each other. The eggs of *E. callistomma*, deposited on the upper sides of plant leaves, contain dark pigment, and take twice as long to reach the hatching stage in comparison with *H. fleischmanni* embryos. In contrast, the *H. fleischmanni* development on the underside of plant leaves is accompanied by the lack of dark pigment in the egg and embryos and reduced developmental time. As in other frogs with rapid development, there was overlap between gastrulation and body elongation.

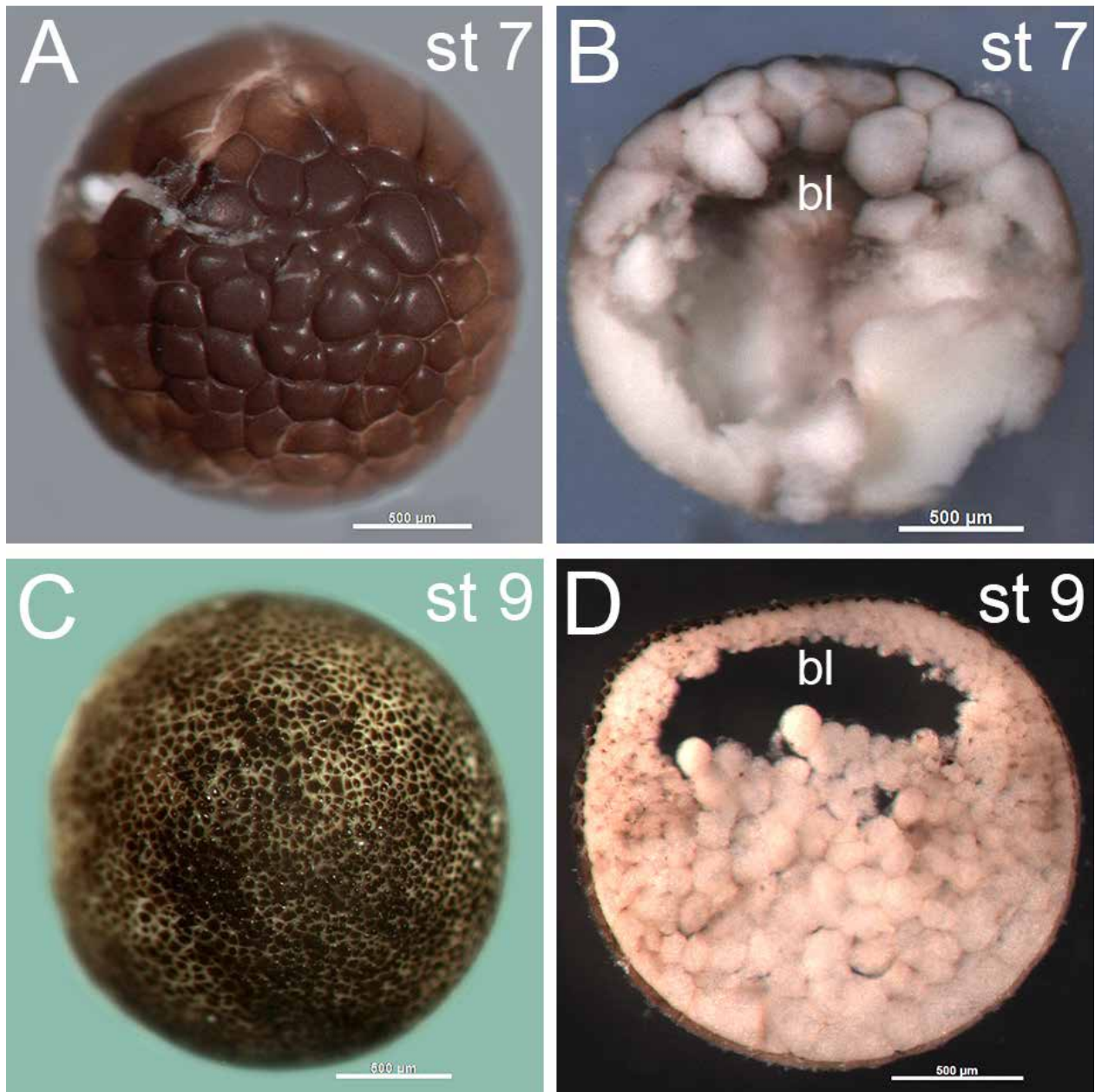
E. callistomma

Fig. 7. Cleavage in *Espadarana callistomma*. (A) Stage 7: Animal view of a 32-cell embryo. (B) Stage 7: The blastocoel of a 32-cell embryo, observed in a sagittal bisection. (C) Stage 9: Animal view of a blastula. (D) Stage 9: The blastocoel of a blastula, observed in a sagittal section. The blastocoel roof consisted of several cell layers. bl, blastocoel.

tion. Moreover, the process of neurulation already started during gastrulation, and the grp became visible in the late gastrula. Presence of the grp in embryos of these centrolenid frogs suggests that the mechanisms of left-right asymmetry is likely similar with the cilia-driven pattern of the *X. laevis* grp.

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Hyalinobatrachium fleischmanni

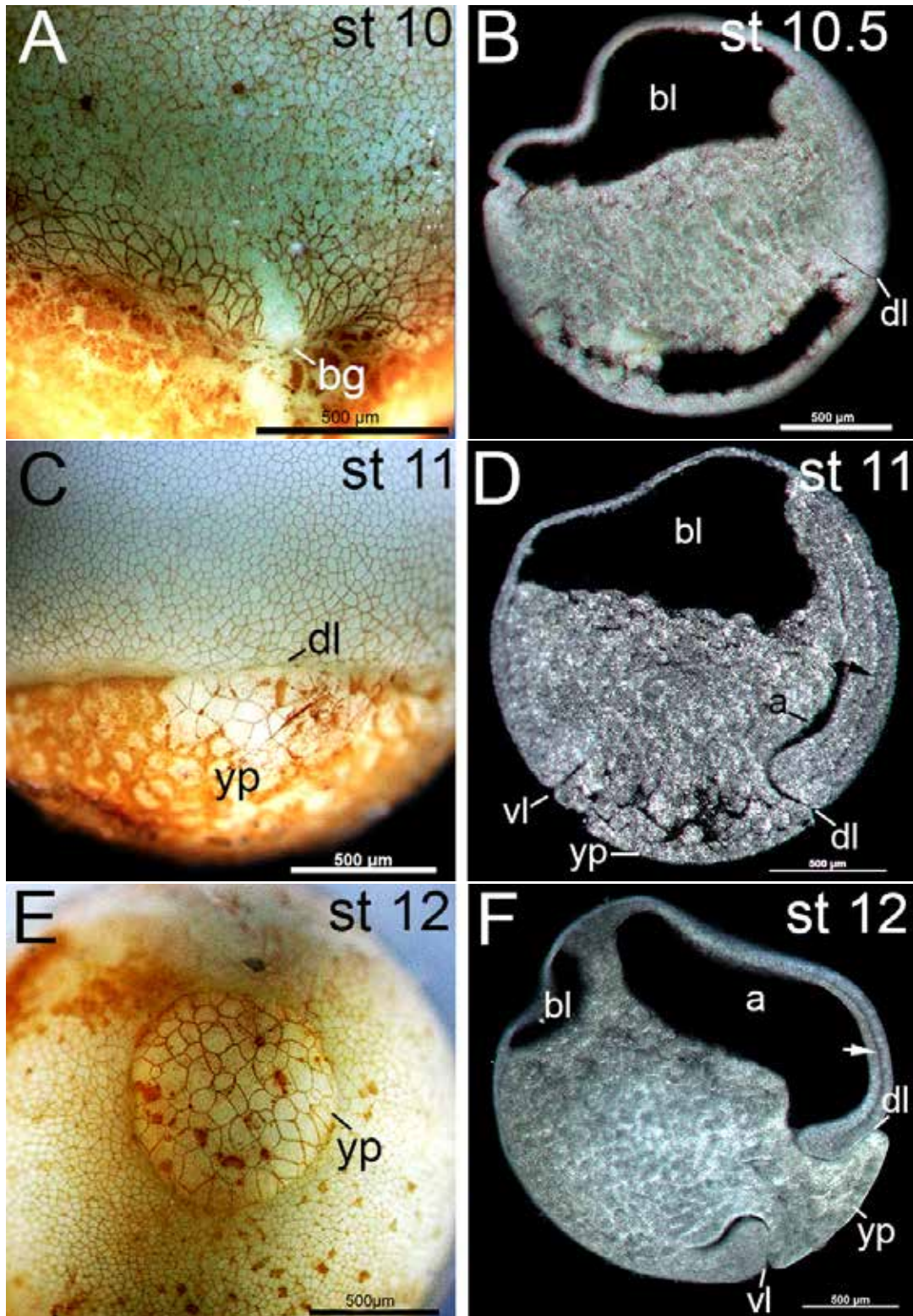


Fig. 8. Gastrulation of *Hyalinobatrachium fleischmanni* (Stages 10–12). Embryos in A, C, E were stained for cell borders. **(A)** Stage 10: Early gastrula. Dorsal subequatorial region. The dorsal blastopore groove was visible between the small cells of the animal region with clearly delineated borders, and the vegetal cells, whose borders were not as clear. **(B)** Stage 10.5: Sagittal section of an early gastrula. The dorsal blastopore lip was visible. **(C)** Stage 11: Mid gastrula. Higher magnification of the dorsal blastopore lip region. There was difference in size of animal and vegetal cells. **(D)** Stage 11: Sagittal section of a mid gastrula. The archenteron was elongated, and the blastocoel roof was reduced to about two cell layers. **(E)** Stage 12: Late gastrula. Higher magnification of the yolk plug region. **(F)** Stage 12: Sagittal section of late gastrula. The arrow indicates the cleft of Brachet. a, archenteron; bg, blastopore groove; bl, blastocoel; dl, dorsal blastopore lip; vl, ventral blastopore lip; yp, yolk plug.

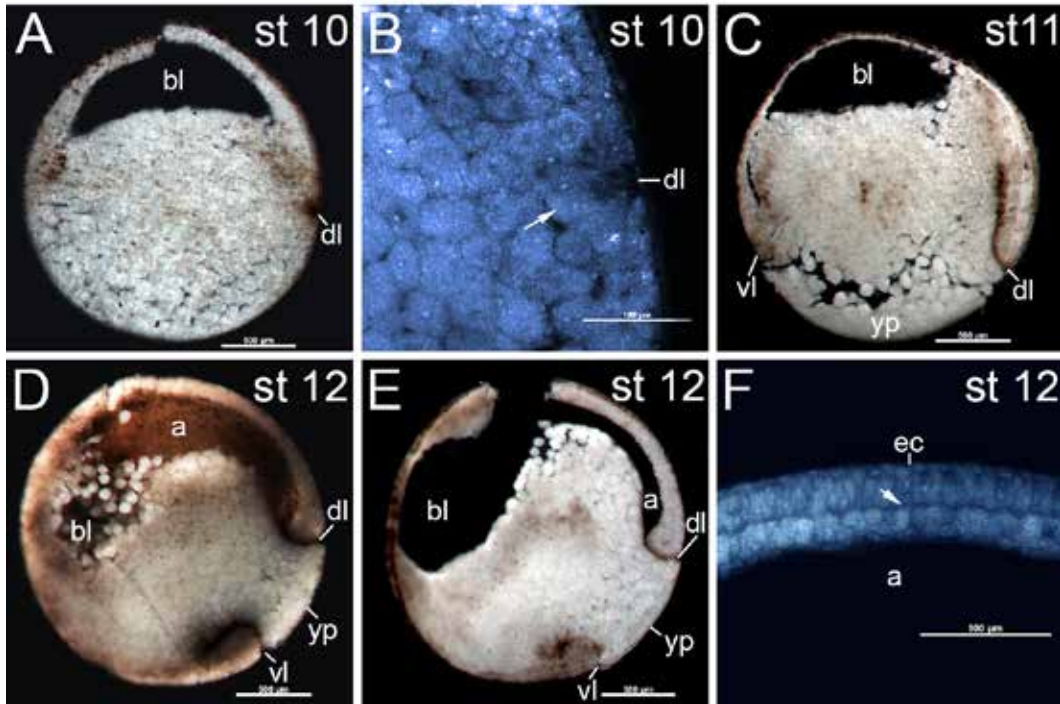
E. callistomma

Fig. 9. Gastrulation of *Espadarana callistomma* (Stages 10–12). (A) Stage 10: Sagittal section of an early gastrula. The dorsal blastopore groove was visible. (B) Stage 10. Higher magnification of the embryo in A, stained for cell nuclei. The arrow indicates a bottle cell of the blastopore groove area. (C) Stage 11: Sagittal section of mid gastrula. (D) Stage 12: Sagittal bisection of late gastrula. (E) Stage 12: Sagittal section of the late gastrula shown in D. The single cavity is an artifact of sectioning, it corresponds to the blastocoel and archenteron, as shown in D. (F) Higher magnification of the archenteron roof from the embryo in E, stained for cell nuclei. The arrow indicates the cleft of Brachet. a, archenteron; bl, blastocoel; dl, dorsal blastopore lip; ec, ectoderm; vl, ventral blastopore lip; yp, yolk plug.

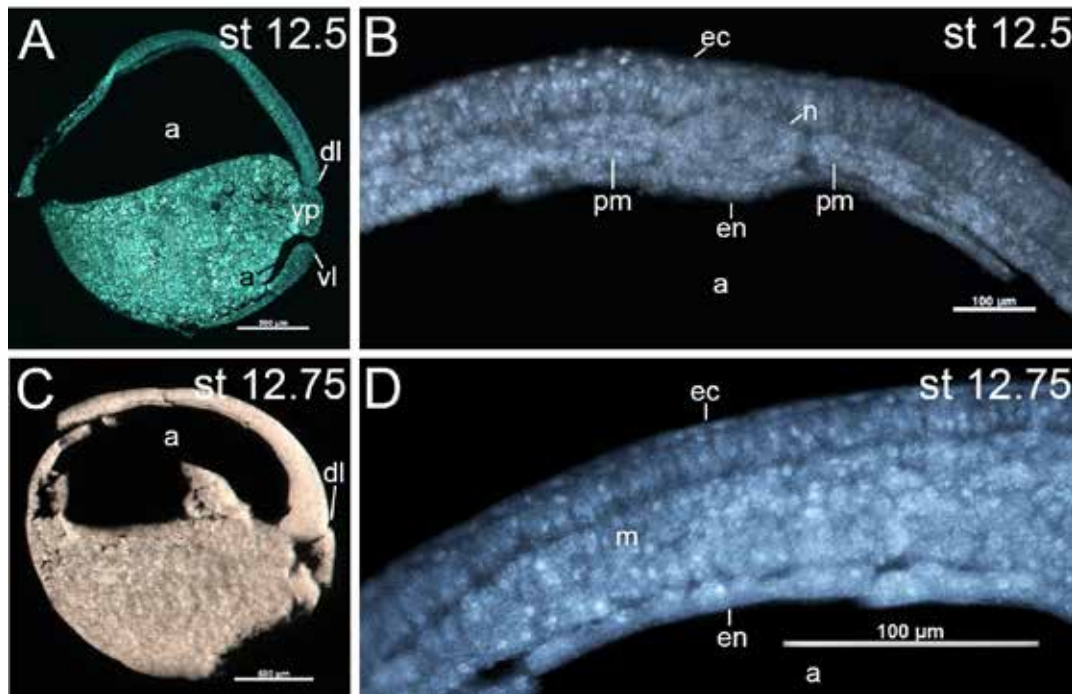
H. fleischmanni

Fig. 10. Gastrulation of *Hyalinobatrachium fleischmanni* (Stages 12.5–12.75). (A) Stage 12.5: Sagittal section of a late gastrula. (B) Stage 12.5: Cross section through the rostral region of a late gastrula, stained for cell nuclei. The endoderm covered the notochord in this rostral section. (C) Stage 12.75: Sagittal section of a late gastrula. (D) Stage 12.75: Higher magnification of the archenteron roof from the embryo in C, stained for cell nuclei. The three germ layers were visible. a, archenteron; dl, dorsal blastopore lip; ec, ectoderm; en, endoderm; m, mesoderm; pm, paraxial mesoderm; vl, ventral blastopore lip; yp, yolk plug.

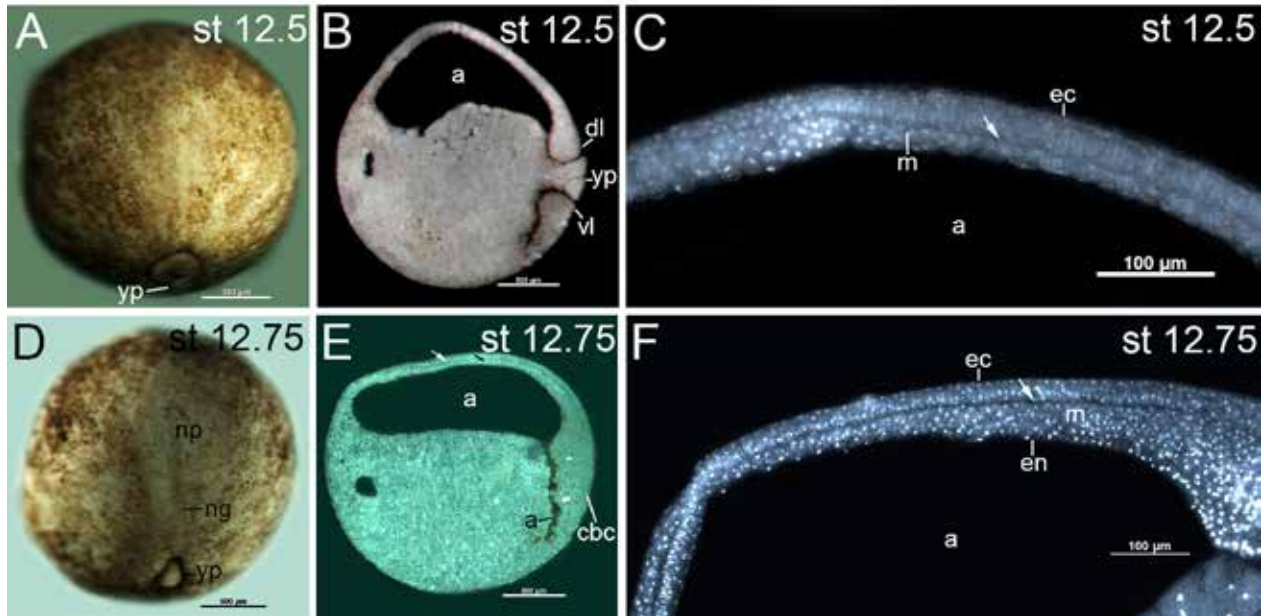
E. callistomma

Fig. 11. Gastrulation of *Espadarana callistomma* (Stages 12.5–12.75). **(A)** Stage 12.5: Late gastrula with a small yolk plug. **(B)** Stage 12.5: Sagittal section of a late gastrula. **(C)** Stage 12.5: Sagittal section of the archenteron roof through the rostral region of a late gastrula, stained for cell nuclei. **(D)** Stage 12.75: Late gastrula. The neural plate was visible. **(E)** Stage 12.75: Parasagittal section of a late gastrula. **(F)** Stage 12.75: Higher magnification of the archenteron roof from the embryo in E, stained for cell nuclei. The arrows in C, E and F indicate the cleft of Brachet. a, archenteron; cbc, circumblastoporal collar; dl, dorsal blastopore lip; ec, ectoderm; en, endoderm; m, mesoderm; ng, neural groove; np, neural plate; vl, ventral blastopore lip; yp, yolk plug.

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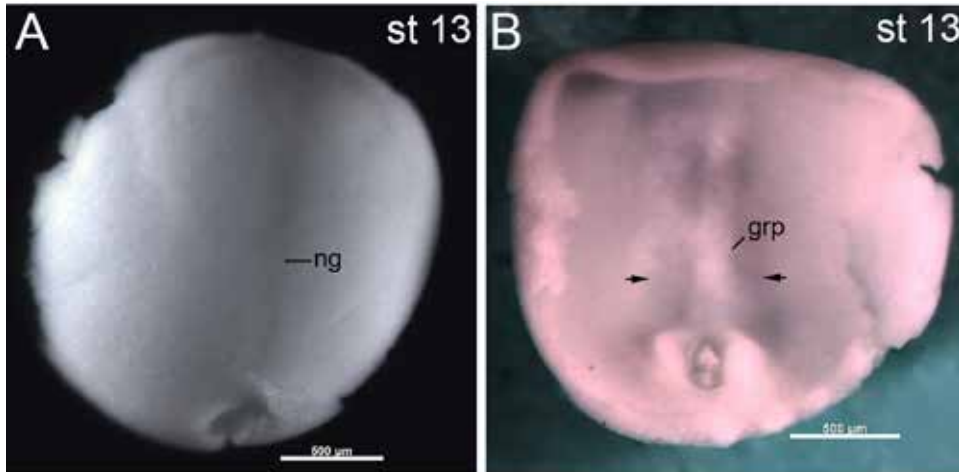
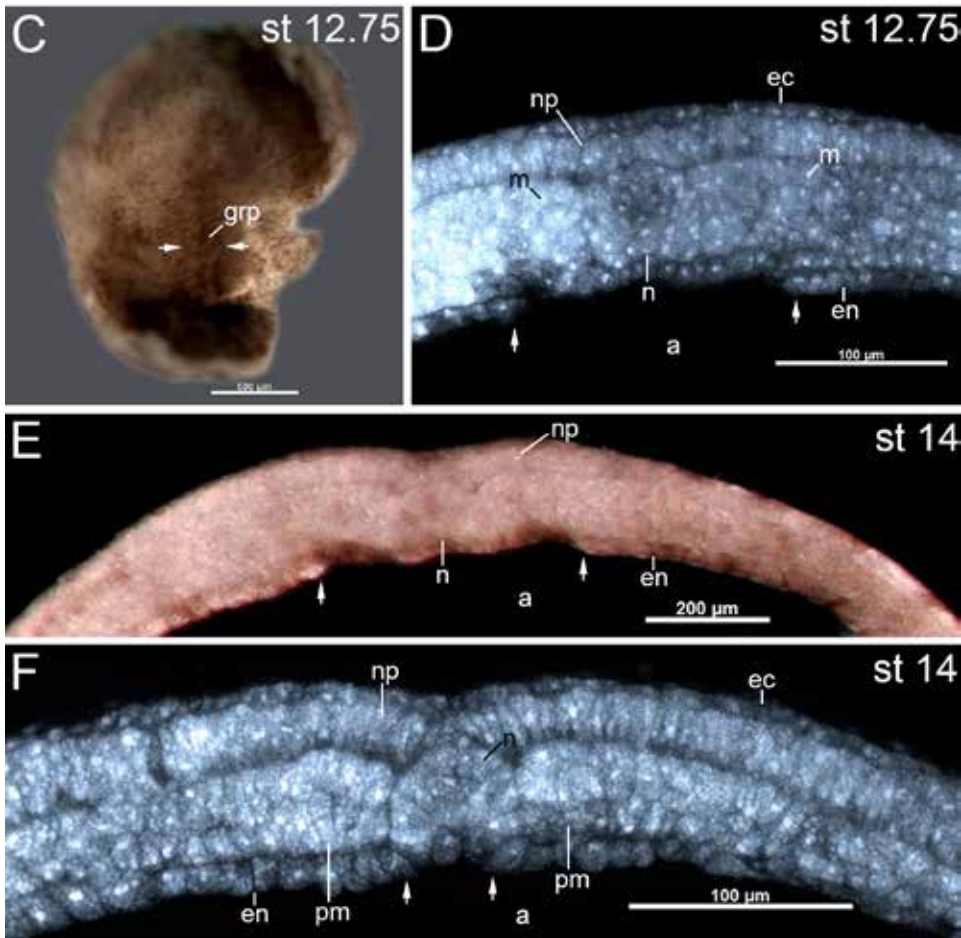
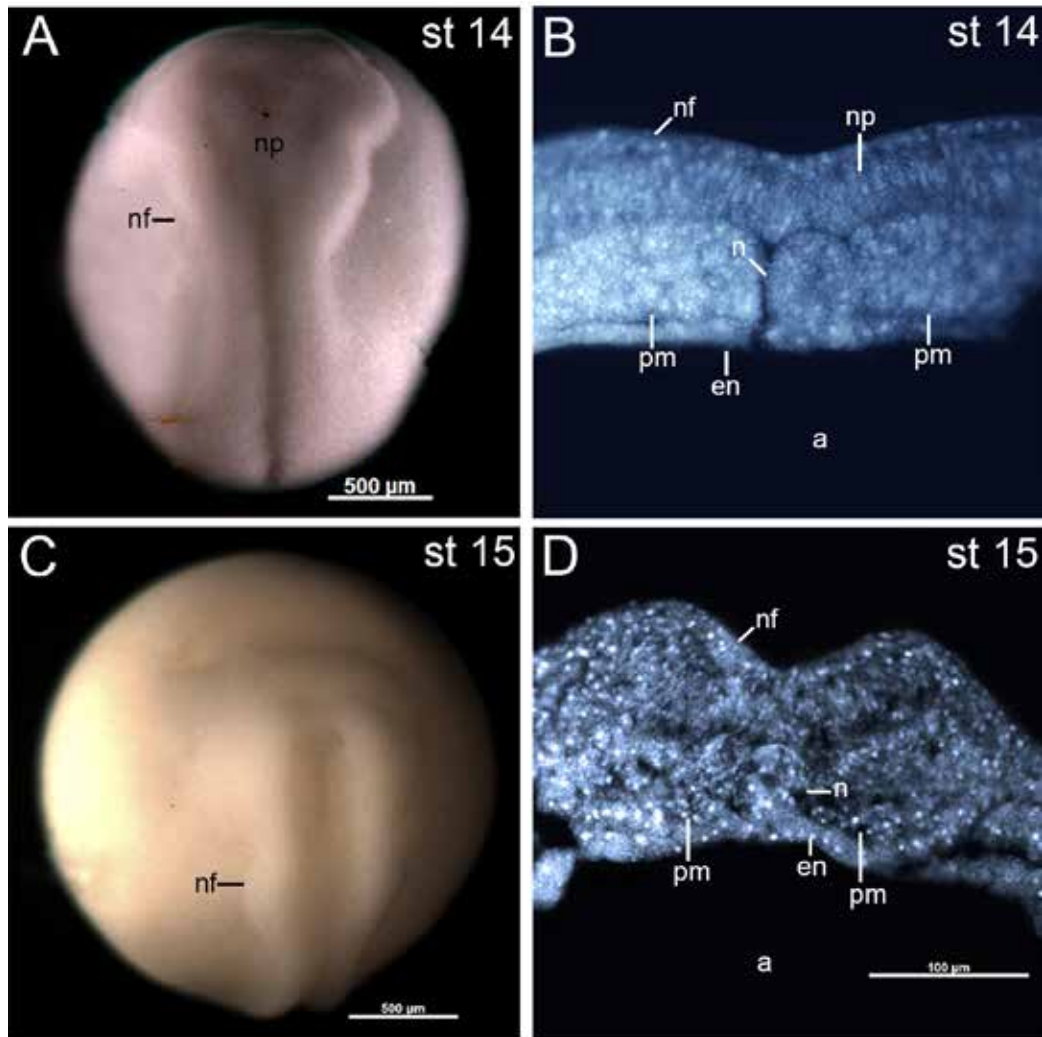
H. fleischmanni*E. callistomma*

Fig. 12. The gastrocoel roof plate (grp) in embryos of *Hyalinobatrachium fleischmanni* and *Espadarana callistomma*. **(A)** Stage 13: External view of the neural plate of *H. fleischmanni*. **(B)** Stage 13: Internal view of the neural plate region of *H. fleischmanni*. The grp was visible in the midline. Arrows indicate the lateral endodermal crests (lec) at the border of the grp in B–F. **(C)** Stage 12.75: Internal view of the neural plate region of *E. callistomma*. The grp was visible in the midline. **(D)** Stage 12.75: Cross section through the caudal region of a late gastrula of *E. callistomma*, stained for cell nuclei. The grp was exposed in the midline and bordered by the lec. The grp consisted of the ventral surface of the notochord and paraxial mesoderm. **(E)** Stage 14: Early neural fold of *E. callistomma*. Cross section through the caudal region. The grp was exposed in the midline and bordered by the lec. The grp consisted of the ventral surface of the notochord and paraxial mesoderm, as in stage 12.75 embryos (shown in D). **(F)** Stage 14: Early neural fold of *E. callistomma*. Cross section through the rostral region, stained for cell nuclei. The grp included only the ventral surface of the notochord, due likely to the rostral closure of the lec. Only the notochord was exposed in the midline, and bordered by the lec. a, archenteron; ec, ectoderm; en; endoderm; grp, gastrocoel roof plate; m, mesoderm; n, notochord; np, neural plate; pm, paraxial mesoderm; ng, neural groove.

H. fleischmanni



E. callistomma

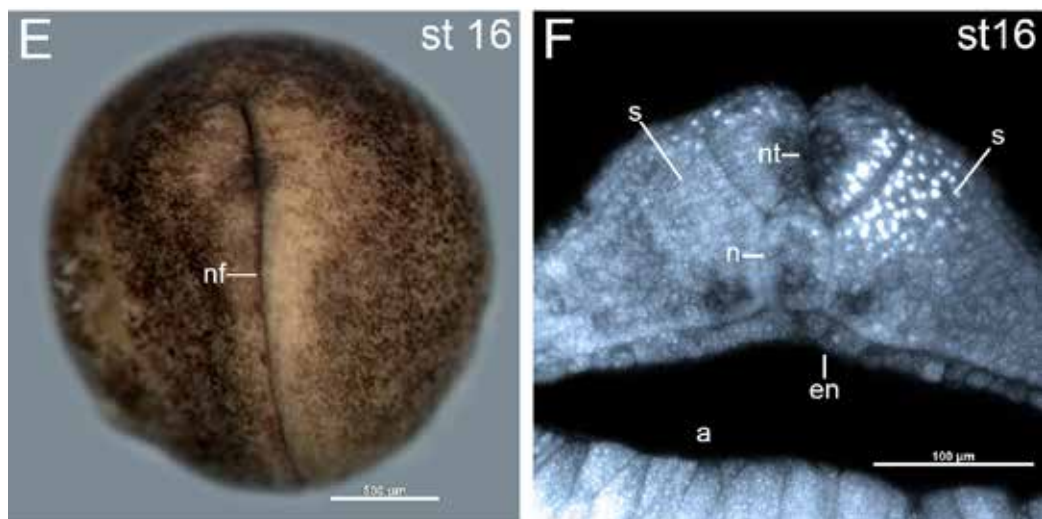


Fig. 13. Neurulation of *Hyalinobatrachium fleischmanni* and *Espadarana callistomma*. **(A)** Stage 14. Early neural fold stage of *H. fleischmanni*. **(B)** Stage 14: Cross section through the rostral region of the embryo in A, stained for cell nuclei. The notochord is totally covered by endoderm. **(C)** Stage 15: Mid neural fold of *H. fleischmanni*. **(D)** Stage 15: Cross section through the rostral region of the embryo in C, stained for cell nuclei. **(E)** Stage 16: Mid neural fold stage of *E. callistomma*. **(F)** Stage 16: Cross section through the rostral region of *E. callistomma*, stained for cell nuclei. The neural folds were closed. a, archenteron; en, endoderm; n, notochord; nf, neural fold; np, neural plate; nt, neural tube; pm, paraxial mesoderm; s, somite.

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