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Development and gastrulation in *Hyloxalus vertebralis* and *Dendrobates auratus* (Anura: Dendrobatidae)

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***Abstract.*—We document the embryonic development of *Hyloxalus vertebralis*, a frog species of the Ecuadorian highlands, declared as Critically Endangered by the International Union for the Conservation of Nature (IUCN) due to significant declines in its populations. Our work may be of value for conservation and management of this endangered frog, especially as it is being bred in captivity to ensure against extinction. We were able to analyze and compare the development of *H. vertebralis* with *Dendrobates auratus* (Dendrobatidae), and other frogs, because of the successful reproduction in captivity of Ecuadorian frogs at the Balsa de los Sapos, Centre of Amphibian Investigation and Conservation (CICA), of the Pontificia Universidad Católica del Ecuador, in Quito. Embryos were fixed, and the external and internal morphology was described from whole mounts, and serial sections. Cellular morphology was analyzed by staining nuclei. Embryos of *H. vertebralis* and *D. auratus* developed from eggs that were 2.6 and 3.5 mm in diameter, respectively. In spite of the large size of their eggs, the morphology of *H. vertebralis* embryos from cleavage to hatching was similar to the morphology of *Epipedobates machalilla* (Dendrobatidae) embryos. The comparison of gastrulation morphology was extended to six additional species of Dendrobatidae (*E. machalilla*, *Epipedobates anthonyi*, *Epipedobates tricolor*, *H. vertebralis*, *Ameerega bilinguis*, *D. auratus*), and to *Xenopus laevis* (Pipidae), and *Gastrotheca riobambae* (Hemiphractidae). We found that elongation of the notochord occurs after blastopore closure in the six species of dendrobatid frogs, as in *G. riobambae*; whereas gastrulation and notochord elongation overlap during *X. laevis* development. We propose that the separation of gastrulation from notochord elongation may relate to slower development patterns, probably associated with the terrestrial reproductive strategies of dendrobatid frogs and marsupial frogs. This analysis contributes to the knowledge of frog embryology and gastrulation, and provides developmental information that may be useful for the conservation and management of *H. vertebralis*.**

Key words. *Ameerega bilinguis*, *Epipedobates machalilla*, *Epipedobates anthonyi*, *Epipedobates tricolor*, notochord, neurula

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Introduction

We analyzed the embryonic development of *Hyloxalus vertebralis* and *Dendrobates auratus* from cleavage to tadpole hatching and compared these patterns with the development of *Epipedobates machalilla* (Dendrobatidae) and *Xenopus laevis* (Pipidae), frogs with well-studied development. These comparisons were then extended to embryos of other Ecuadorian, neotropical frogs (del Pino et al. 2004, 2007; Moya et al. 2007; Nieuwkoop and Faber 1994). Our aim was to extend the analysis of frog embryonic development to additional species and to provide information that may be useful for the conservation and management of *H. vertebralis*, an endangered frog. Most of the *H. vertebralis* population has disappeared, possibly due to chytridiomycosis infection and habitat destruction. For these reasons, the International Union for Conservation of Nature (IUCN) declared *H. verte-*

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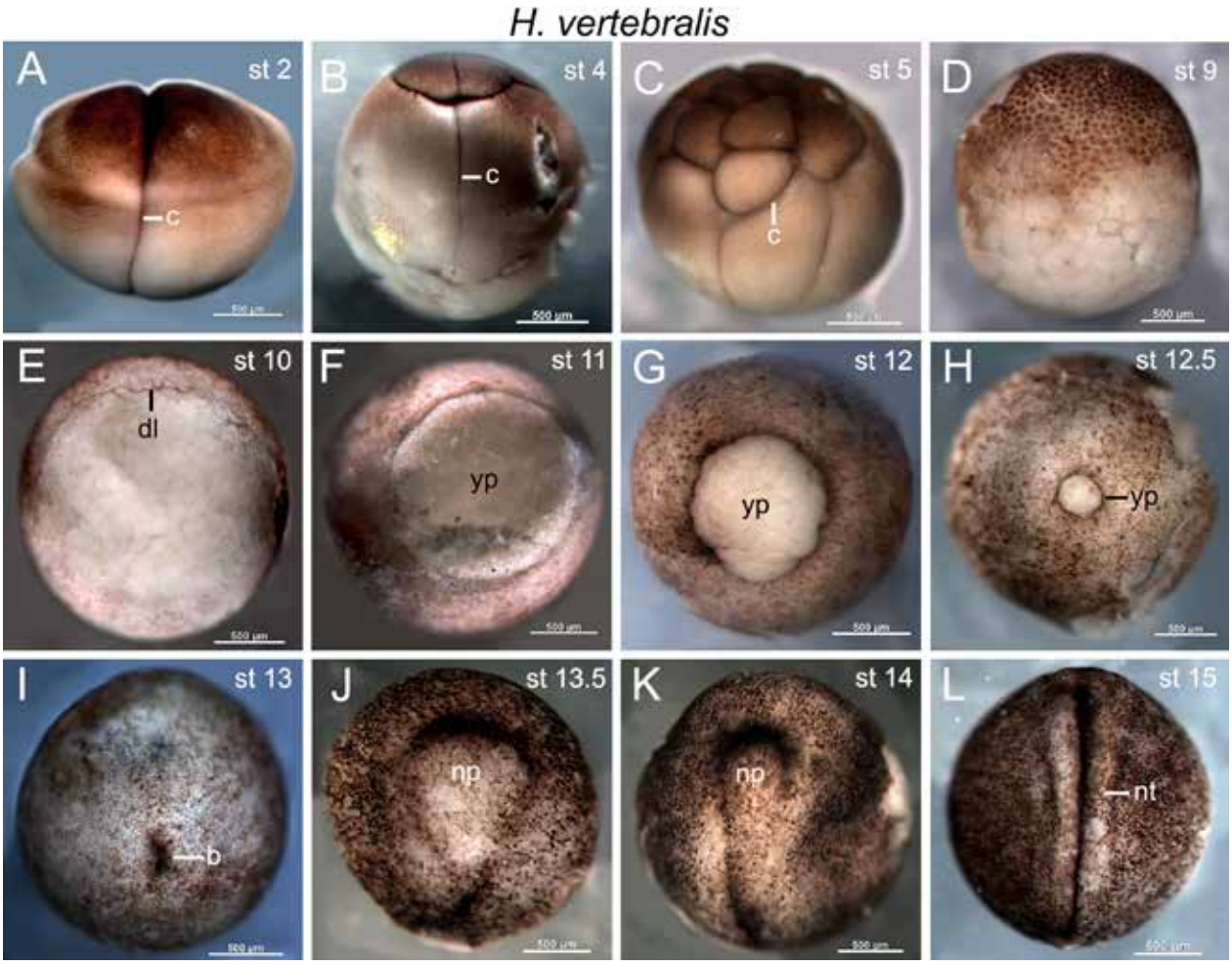

Fig. 1. External views of *H. vertebralis* embryos from cleavage to the neurula stage. **(A)** Stage 2: Two-cell stage. **(B)** Stage 4: Eight-cell stage. **(C)** Stage 5: Sixteen-cell stage. **(D)** Stage 9: Advanced blastula. **(E)** Stage 10: Early gastrula. **(F)** Stage 11: Mid-gastrula. **(G)** Stage 12: Late-gastrula. **(H)** Stage 12.5: Late-gastrula with a small yolk plug. **(I)** Stage 13: Slit-blastopore stage. **(J)** Stage 13.5: Advanced slit-blastopore stage. The neural plate was visible. **(K)** Stage 14: Early neural fold stage. **(L)** Stage 15: Mid-neural fold stage. In this and the following figures, the developmental stage (st) is given in top right-hand corner of each image; b, blastopore; c, cleavage furrow; dl, dorsal blastopore lip; np, neural plate; nt, neural tube; yp, yolk plug.

bralis as Critically Endangered (Coloma et al. 2004). It is currently being bred in captivity to guard against extinction.

Hyloxalus vertebralis occurs at elevations of 1,770–3,500 m above sea level in the inter-Andean valleys of Ecuador. In addition, it occurs on the eastern and western slopes of the Andes in central and southern Ecuador, respectively (Coloma 1995). Its habitat is the cloud forest and it has also been found in ponds, open areas, and streams. The nests consist of 5–12 eggs that are placed on the ground (Coloma 1995). After the tadpoles hatch, the males transport them to streams for further development (Coloma 1995).

Dendrobates auratus is distributed from southeastern Nicaragua to northwestern Colombia (Solís et al. 2004). This species does not occur in Ecuador. These frogs deposit their eggs in terrestrial nests, and embryonic development occurs inside the egg capsules until tadpole hatches in the leaf litter. Brood care is performed by the male. After hatching, tadpoles are transported individually by the male to small seasonal pools (Solís et al. 2004). Eggs of *D. auratus* are the largest among the dendrobatids and measure 3.5 mm in diameter (del Pino et al. 2007; Hervas and del Pino 2013).

Dendrobatid frogs are of great developmental interest because of their great variation in egg size (Table 1), and their modified pattern of gastrulation. Notochord elongation occurs after gastrulation in *E. machalilla*, and *Epipedobates anthonyi*, as in the Marsupial frog, *Gastrotheca riobambae* (Hemiphractidae); whereas, the onset of notochord elongation is a feature of the *Xenopus laevis* mid-gastrula (Benítez and del Pino 2002; Keller and Shook 2004; del Pino et al. 2007; Moya et al. 2007; Montenegro-Larrea and del Pino 2011; Elinson and del Pino 2012). For this reason, we compared the gastrulation characteristics of *Epipedobates anthonyi*, *Epipedobates tricolor*, *H. vertebralis*, *Ameerega bilinguis*, and *D. auratus* with *E. machalilla* (Dendrobatidae). In a previous study, *Ameerega bilinguis* was identified as *Epipedobates ingeri* (del Pino et al. 2007). This analysis

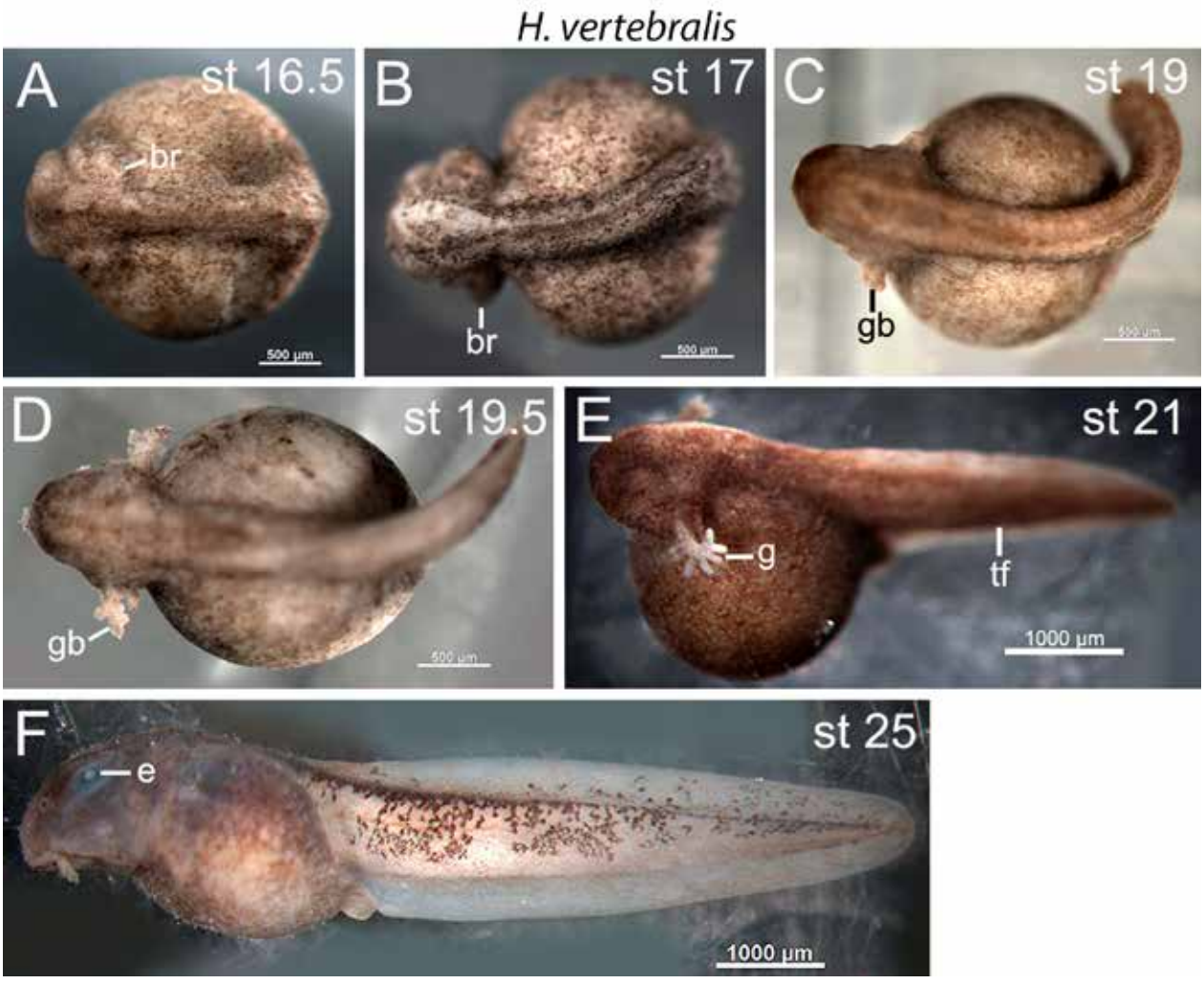

Fig. 2. External views of *H. vertebralis* embryos from closure of the neural tube to hatching. **(A)** Stage 16.5: Closure of the neural tube. **(B)** Stage 17: Tail bud stage. The brachial arches protruded on the sides of the head. **(C)** Stage 19: Embryo at the muscular response stage. **(D)** Stage 19.5: Gill buds of the two external gill pairs were visible. **(E)** Stage 21: Development of the external gills. There were seven branches in the first gill pair and the second gill pair was unbranched. **(F)** Stage 25: Embryo at hatching. br, branchial arch; e, eye; g, gills; gb, gill bud; tf, tail fin.

Table 1. Gastrulation in dendrobatid frogs in comparison with *X. laevis* (Pipidae) and *G. riobambae* (Hemiphractidae).

Family and Species	Eggs per clutch (No. of clutches)	Egg diameter (mm)	Gastrulation time (hrs)[a]	Archenteron elongation	Onset of notochord elongation	References[g]
Pipidae						
Xenopus laevis	--	1.3	5	Early gastrula[b]	Mid gastrula[f]	1
Dendrobatidae						
Epipedobates machalilla	15 (72)	1.6	65	Late gastrula[c]	After gastrulation[e]	2
Epipedobates anthonyi	18 (30)	2.0	36	Late gastrula[c]	After gastrulation[e]	3
Epipedobates tricolor	13 (34)	2.0	36	Late gastrula[c]	After gastrulation[e]	2
Hyloxalus vertebralis	13 (39)	2.6	39	Late gastrula[c]	After gastrulation[e]	4
Ameerega bilinguis	10 (04)	3.0	55	Late gastrula[d]	After gastrulation[e]	4
Dendrobates auratus	05 (42)	3.5	72	Late gastrula[d]	After gastrulation[e]	1
Hemiphractidae						
Gastrotheca riobambae	87	3.0	168	After gastrulation[e]	After gastrulation[e]	5

[a]Time from stages 10–13. Embryo culture temperatures for: *X. laevis* 23 °C, and 18–21 °C for other frogs; [b]Stage10; [c]Stage 12.5; [d]Stage 12; [e]Stage 13; [f]Stage 11; [g]References: 1, (del Pino et al. 2007); 2, (del Pino et al. 2004); 3, (Montenegro-Larrea and del Pino 2011); 4, This work; 5, (del Pino 1996; Moya et al. 2007).

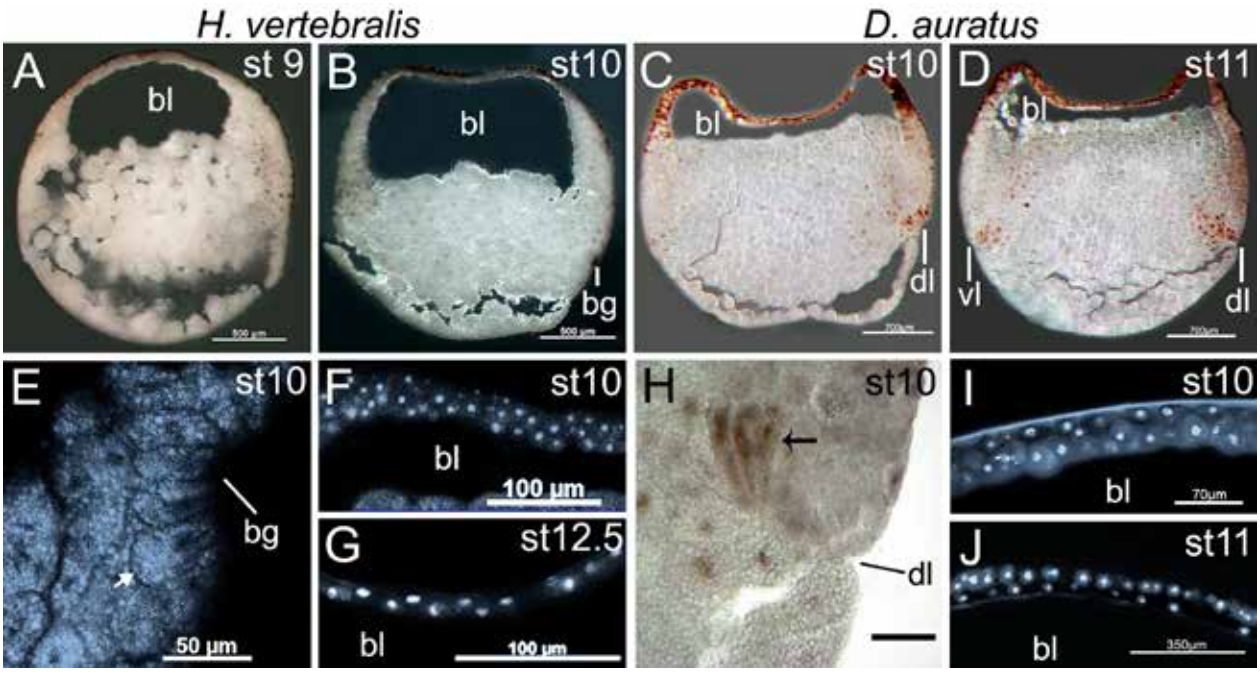

Fig. 3. Internal morphology of the *H. vertebralis* and *D. auratus* early gastrula. Micrographs of *H. vertebralis* embryos are shown in A, B, E–G, and micrographs of *D. auratus* embryos are shown in C, D, H–J. Sections shown in E–G, I–J were stained for cell nuclei. **(A)** Stage 9: Sagittal section of an advanced blastula. **(B)** Stage 10: Sagittal section of an early gastrula. **(C)** Stage 10: Sagittal section of an early gastrula. **(D)** Stage 11: Sagittal section of the mid-gastrula. **(E)** Stage 10: Higher magnification of the dorsal blastopore groove from the embryo in B. The arrow signals a bottle cell. **(F)** Stage 10: The blastocoel roof of an early gastrula. It was two-three cells in thickness. **(G)** Stage 12.5: One cell layer in the blastocoel roof of a late-gastrula. **(H)** Stage 10: Higher magnification from the embryo in C. The arrow signals a bottle cell. **(I)** Stage 10: The blastocoel roof of an early gastrula of two cells in thickness. **(J)** Stage 11: The blastocoel roof of mid-gastrula with one-two cells in thickness. bl, blastocoel; bg, blastopore groove; dl, dorsal blastopore lip; vl, ventral blastopore lip.

of gastrulation in several dendrobatids expands previous studies (del Pino et al. 2007; Montenegro-Larrea and del Pino 2011). The gastrulation pattern of these dendrobatids is similar to the pattern of *E. machalilla*, with the notochord elongation after completion of gastrulation (del Pino et al. 2004, 2007; Moya et al. 2007).

We report the features of development from cleavage to tadpole hatching of *H. vertebralis* and from gastrula to tadpole hatching of *D. auratus*. This study expands the report on the mode of myogenesis, neurulation, and internal features of embryos of these two dendrobatids (Hervas and del Pino 2013). In spite of the large size of their eggs, the external and internal morphology from cleavage until tadpole hatching of *H. vertebralis*, and *D. auratus* is similar to that of *E. machalilla* (del Pino et al. 2004 2007; Hervas and del Pino 2013). Moreover, myogenesis occurs by cell interdigitation, as in embryos of other dendrobatid frogs (del Pino et al. 2007; Hervas and del Pino 2013).

Materials and Methods

Collection sites

Adults of *Hyloxalus vertebralis* were collected by Fernando Dueñas and Ítalo Tapia on 10 September 2008. The locality of collection was Azuay Province, Sevilla de Oro, in southern Ecuador at an altitude 2,418 m above sea level. The geographic coordinates of this site are W 78.60097, S 2.63605. The permit 016-IC-FAU-DNBAP-MA from the Ministry of the Environment, Ecuador, allowed the collection and maintenance of frogs at Pontificia Universidad Católica del Ecuador (PUCE). The Atlanta Zoo donated adults of *Dendrobates auratus* to the PUCE. Adults of both species reproduced successfully at the Balsa de los Sapos, Centre of Amphibian Investigation and Conservation (CICA) of PUCE. Egg clutches were donated to the laboratory of developmental biology for embryonic analysis.

Analysis of embryonic development

The number of eggs per egg clutch was recorded. Embryos were analyzed from cleavage until tadpole hatching, and were staged according to the *E. machalilla* table of stages (del Pino et al. 2004). Embryos were cultured in humid chambers at room temperature (18–23 °C). Procedures for fixation of embryos in Smith's fixative, vibratome sectioning, and the staining of sections for cell nuclei with the fluorescent dye Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) were previously described (del Pino et al. 2004; Moya et al. 2007). Sections were mounted in glycerol, and microscopically examined with normal light using a Stemi SV6 stereomicroscope (Carl Zeiss, Oberkochen, Germany) or with fluorescent optics using a Z1 Axio Observer microscope (Carl Zeiss,

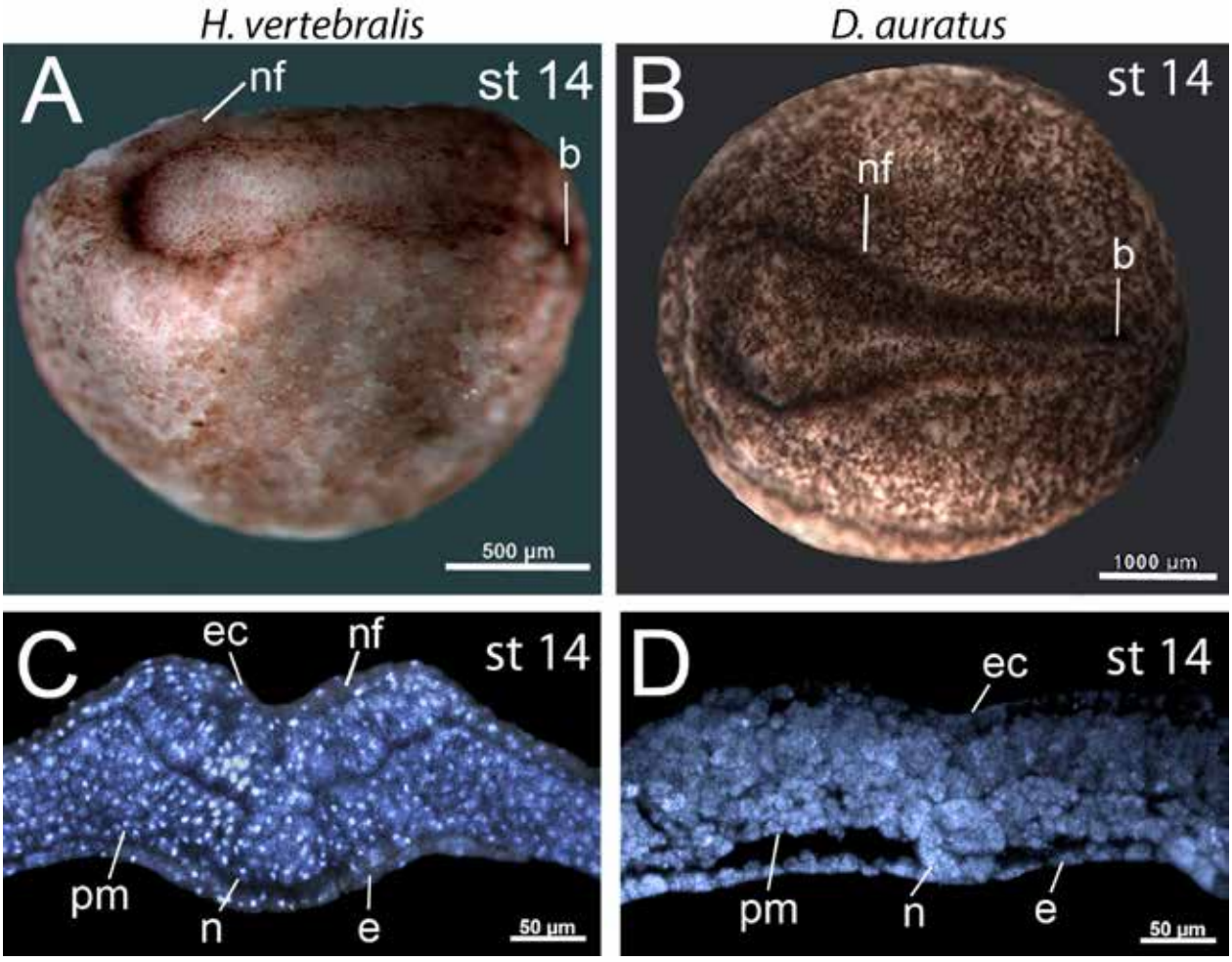

Fig. 4. Stage 14: Early neural fold stage of *H. vertebralis* and *D. auratus* embryos. Micrographs of *H. vertebralis* embryos are shown in A, C, and micrographs of *D. auratus* embryos are shown in B, D. Sections shown in C–D were stained for cell nuclei. **(A)** Lateral view of a neural fold stage embryo. **(B)** Dorsal view of a neural fold stage embryo. The neural plate was visible in embryos of the two species. **(C)** Cross section through the region of the trunk (Reproduced from Hervas and del Pino, 2013). **(D)** Cross section through the caudal region of an embryo. The notochord was visible in C and D. b, blastopore; e, endoderm; ec, ectoderm; n, notochord; nf, neural fold; pm, paraxial mesoderm.

Oberkochen, Germany). Embryos were photographed with Axiocam cameras and the image capture program Axiovision (Carl Zeiss, Oberkochen, Germany). The images were edited with Adobe Photoshop CS6. Egg diameter was measured in fixed embryos with the measuring tool of the program Axiovision (Carl Zeiss, Oberkochen, Germany).

Results and Discussion

Clutch size and developmental time

The number of eggs ranged from 2–25 eggs, with a mean of 13 eggs per clutch in *H. vertebralis*, and 2–8 eggs, with a mean of five eggs per clutch in *D. auratus* (Table 1). The eggs of *H. vertebralis* and *D. auratus* measured about 2.6 and 3.5 mm in diameter, respectively (Table 1). The diameter of *H. vertebralis* eggs was previously reported to be of about three mm (Coloma 1995). The animal hemisphere of embryos was dark brown and the vegetal hemisphere was pale-yellow in embryos of both frogs (Fig. 1A–D; not shown for *D. auratus*). Egg clutches of *H. vertebralis* required 18 days from the two-cell stage to tadpole hatching under laboratory conditions; whereas 19–21 days were required from fertilization to tadpole hatching by the six species of dendrobatid frogs (del Pino et al. 2004, 2007; Hervas and del Pino 2013). The similarity of developmental times suggests that parental care allows slow development in all of the species of dendrobatid frogs examined in comparison with *X. laevis*.

Embryonic development of *H. vertebralis* and *D. auratus*

The development from early cleavage until tadpole hatching of *H. vertebralis* and *D. auratus* was divided into 25 stages, according to the staging criteria for *E. machalilla* (del Pino et al. 2004) given in Table 2. Micrographs of the external and internal morphology of *H. vertebralis*

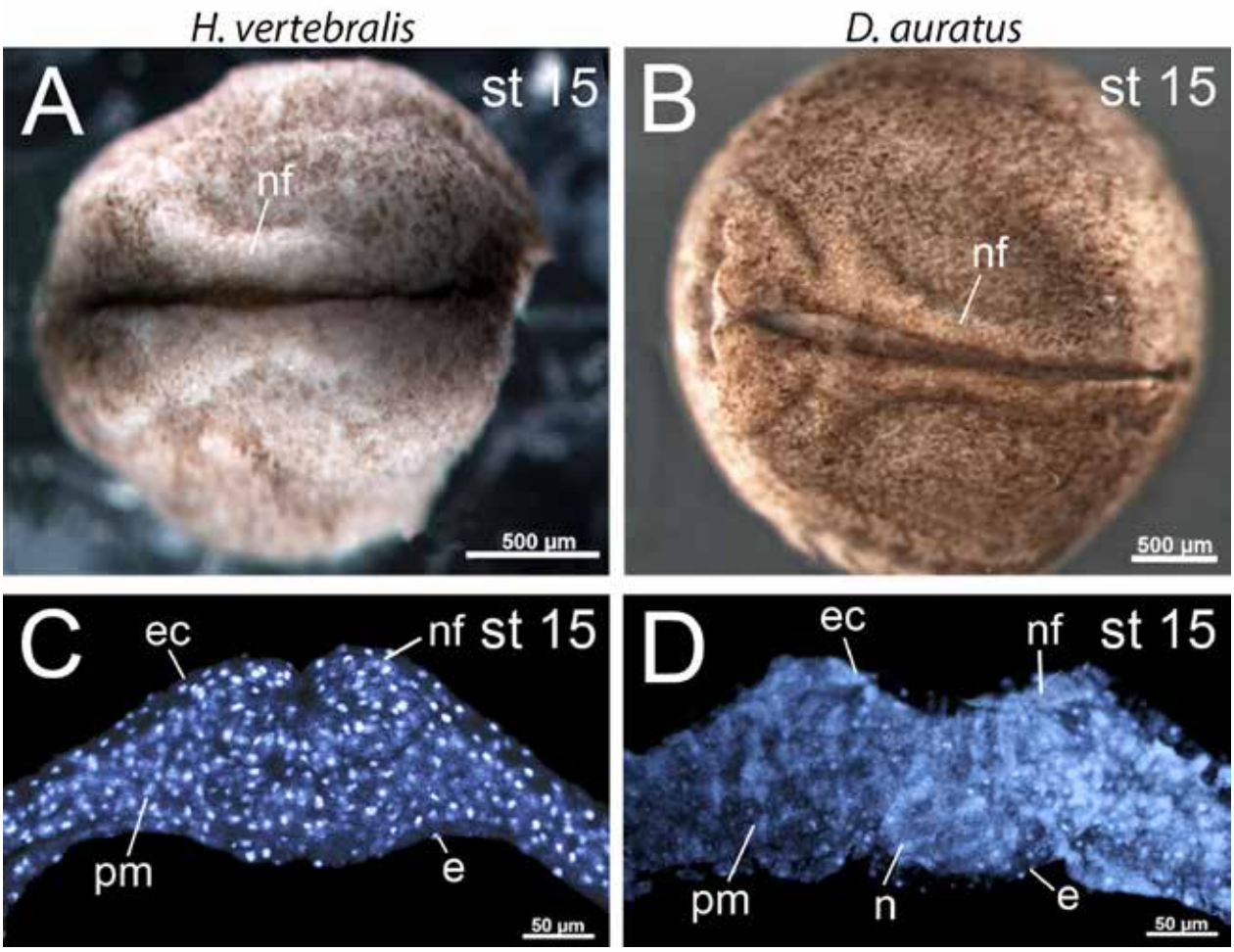

Fig. 5. Stage 15: Mid-neural fold stage of embryos of *H. vertebralis* and *D. auratus*. Micrographs of *H. vertebralis* embryos are shown in A, C, and micrographs of *D. auratus* embryos are shown in B, D. Sections shown in C–D were stained for cell nuclei. **(A–B)** Dorsal views of embryos. The neural tube was open in embryos of both species. **(C)** Cross section through the caudal region. The notochord was not detected in this caudal region (Reproduced from Hervas and del Pino, 2013). **(D)** Cross section through the trunk region. The notochord was visible. e, endoderm; ec, ectoderm; n, notochord; nf neural fold; pm, paraxial mesoderm.

and *D. auratus* embryos illustrate these developmental stages (Figs. 1–12).Cleavage of *H. vertebralis* was holoblastic as in *E. machalilla* (del Pino et al. 2004) (Fig. 1A–C). Cleavage of *D. auratus* was not observed. The *H. vertebralis* blastula consisted of small, pigmented cells in the animal hemisphere; whereas, cells of the vegetal hemisphere were larger. The blastocoel developed during cleavage, and was large in blastula and gastrula stage embryos (Fig. 3A–D). The blastocoel roof, of the two species, was several cell diameters in thickness at stage 10 (Fig. 3F and I), and it was reduced to one cell thickness in the late-gastrula stage of *H. vertebralis* (stage 12.5; Fig. 3G). Similarly the thickness of the blastocoel roof was reduced to one or two cells in thickness in the early gastrula of *D. auratus* (stage 11; Fig. 3J). Thickness of the blastocoel roof in the late-gastrula was not documented for this frog species. In *E. machalilla*, expansion of the blastocoel was accompanied by reduction in its thickness until it was a monolayer of cells in the late-gastrula (del Pino et al. 2004).

The onset of gastrulation in *H. vertebralis* and *D. auratus* was marked by the presence of the dorsal blastopore lip in a sub-equatorial location (Fig. 1E). A field of bottle cells was observed at the blastopore groove (Figs. 3E, H), as in *X. laevis* and *E. machalilla* (Hardin and Keller 1988; Moya et al. 2007). The gastrula developed a conspicuous yolk plug that became smaller during gastrulation, until it was totally retracted by the end of gastrulation (Fig. 1E–I). The closed blastopore looked like a small slit in stage 13 embryos (Fig. 1I), as in *E. machalilla* and other frogs (del Pino et al. 2004). Internally, a small dorsal archenteron developed, which did not elongate until stage 13 in *H. vertebralis* (Fig. 12J–L), as in *E. machalilla* (del Pino et al. 2004); whereas in *D. auratus*, the archenteron was already large and inflated at stage 12 (Fig. 12P) (del Pino et al. 2007).

The neural plate developed in late stage 13 (Fig. 1J). In stage 14, the neural folds were elevated (Figs. 1K; 4A–B). The notochord was observed underneath the neural plate of stage 14 embryos (Fig. 4C–D). The neu-

Table 2. Stages of development of *H. vertebralis* and *D. auratus* in comparison with the *E. machalilla* table of development.

Stage[1]			Characteristics of embryos
D	X	G	
1	1	1	Fertilization (not shown).
1	-	2	Gray crescent (not shown).
2	2	3	Two-cell stage (Fig. 1A). This stage was observed only for *H. vertebralis*.
3	3	4	Four-cell stage (not shown).
4	4	5	Eight-cell stage (Fig. 1B). This stage was observed only for *H. vertebralis*.
5	5	6	Sixteen-cell stage (Fig. 1C). This stage was observed only for *H. vertebralis*.
6	6	7	Thirty-two cell stage (not shown).
7	7	8	Large-cell blastula (not shown).
8	8	-	Medium-cell blastula (not shown).
9	9	9	Advanced blastula (Fig. 1D; 3A).
10	10	10	Early gastrula. The dorsal blastopore lip was formed (Fig. 1E), had a subequatorial location (Figs. 3B, C), and there were bottle cells making the onset of cell ingression at the blastopore, as shown for both species (Figs. 3E, H).The blastocoel was a large cavity, and its roof was several cells in thickness. The thickness of the blastocoel roof was reduced to a single cell in the late gastrula (Figs. 3F, G, I, J).
11	11	11	Mid-gastrula with a yolk plug that measured about 1/2 of the embryo's diameter (Fig. 1F). Internally, the ventral blastopore lip was formed as shown for *D. auratus* (Fig. 3D).
12	12	12	Late gastrula with a yolk plug that was 1/3 of the embryo's diameter or smaller (Figs. 1G). The archenteron of *H. vertebralis* was smaller than *D. auratus* (Figs. 12J, K, P, Q).
13	13	13	Slit blastopore stage (Fig. 1I). Internally, the archenteron was elongated. A large circumblastoporal collar was visible (Figs. 12L; R). The neural plate became visible in the late stage 13 (Fig. 1J).
14	14	14	Early neural fold stage. Images of *H. vertebralis* (Fig. 1K; 4A, C), and of *D. auratus* (Figs. 4B, D). In the trunk region of both species, the neural plate, notochord, and mesoderm were visible (Figs. 4C, D).
15	16	15	Mid-neural fold stage. The neural folds approached each other. Images of *H. vertebralis* (Fig. 1L; 5A, C), and of *D. auratus* (Figs. 5B, D). The neural folds were elevated and touched each other in the trunk region (*H. vertebralis*, Fig. 5C); but were open in the cephalic region (*D. auratus*, Fig. 5D).
16	20	16	Closure of the neural tube in *H. vertebralis* (Fig. 2A; 6A, C), and *D. auratus* (Figs. 6B, D). Closure of the neural tube was complete in both species.
17	24	17	Tail bud stage. The tail bud and the head region protruded beyond the yolky endoderm in *H. vertebralis* (Fig. 2B; 7); not shown for *D. auratus*. The epidermis, neural tube, notochord, somites, and endoderm were visible in the trunk region (Fig. 7B, C).
18	26	18	Muscular activity. The branchial arches protruded on the sides of the head. The eye vesicles were small (not shown). Images of *H. vertebralis* (Figs. 8A, C, E), and of *D. auratus* (Figs. 8B, D, F). The epidermis, neural tube, notochord, rows of somites, and endoderm were visible in the trunk region of both species (Figs. 8C–F).
19	33	19	Heart beat and external gill buds. The gill buds of the two pairs of external gills were visible. Images of *H. vertebralis* (Figs. 2C, D; 9A, C, E), and of *D. auratus* (Figs. 9B, D, F). The dorsal fin was visible (Fig. 9C), the notochord was vacuolated (Fig. 9C), and the pronephros was detectable (Fig. 9D). The number of somites increased (Figs. 9E, F).
20	40	20	Circulation to the external gills. The first gill pair had four or more branches. Images of *H. vertebralis* (Fig. 10A). Internally, the otocysts, brain, notochord, and somites were observed, as shown for *H. vertebralis* (Fig. 10C).
21	41	21	Development of the external gills. The first pair of external gills had seven branches in *H. vertebralis*. The second pair of external gills was small and unbranched (Fig. 2E). In *D. auratus*, the first gill pair had six branches and the second gill pair developed two branches. The tail became elongated (not shown).
22	41	22	The external gills enlarged and eye pigment. The eyes contained pigment. Images of *H. vertebralis* (Fig. 10B). Internally, the otocysts, eye, internal gills, notochord, and somites were observed, as shown for *H. vertebralis* (Fig. 10D).
23	43	23	The external gills reached their full size (Fig. 11). The first pair of external gills had eight and nine branches in *H. vertebralis* and *D. auratus*, respectively. The second gill pair was unbranched in *H. vertebralis* and had two branches in *D. auratus* (Figs. 11A, B). The opercular fold was visible. The eyes and the body were pigmented. Internally the epidermis, eye, otocysts, and somites were detected. Images of *H. vertebralis* (Figs. 11A, C) and of *D. auratus* (Figs. 11B, D).
24	44	24	The external gills were visible only on the left side. The operculum was closed on the right side (not shown).
25	45	25	The spiracle was formed. The embryos hatched and had the appearance of a tadpole. Internally, the brain, otocysts, somites, and yolky endoderm were observed. Images of *H. vertebralis* (Fig. 2F, 11E–G).

[1]D, stages of the dendrobatid frogs, *H. vertebralis* and *D. auratus*, according to the *E. machalilla* standard stages of development (del Pino et al. 2004); X, normal stages of *X. laevis* development (Nieuwkoop and Faber 1994); G, the generalized table of frog development (Gosner 1960).

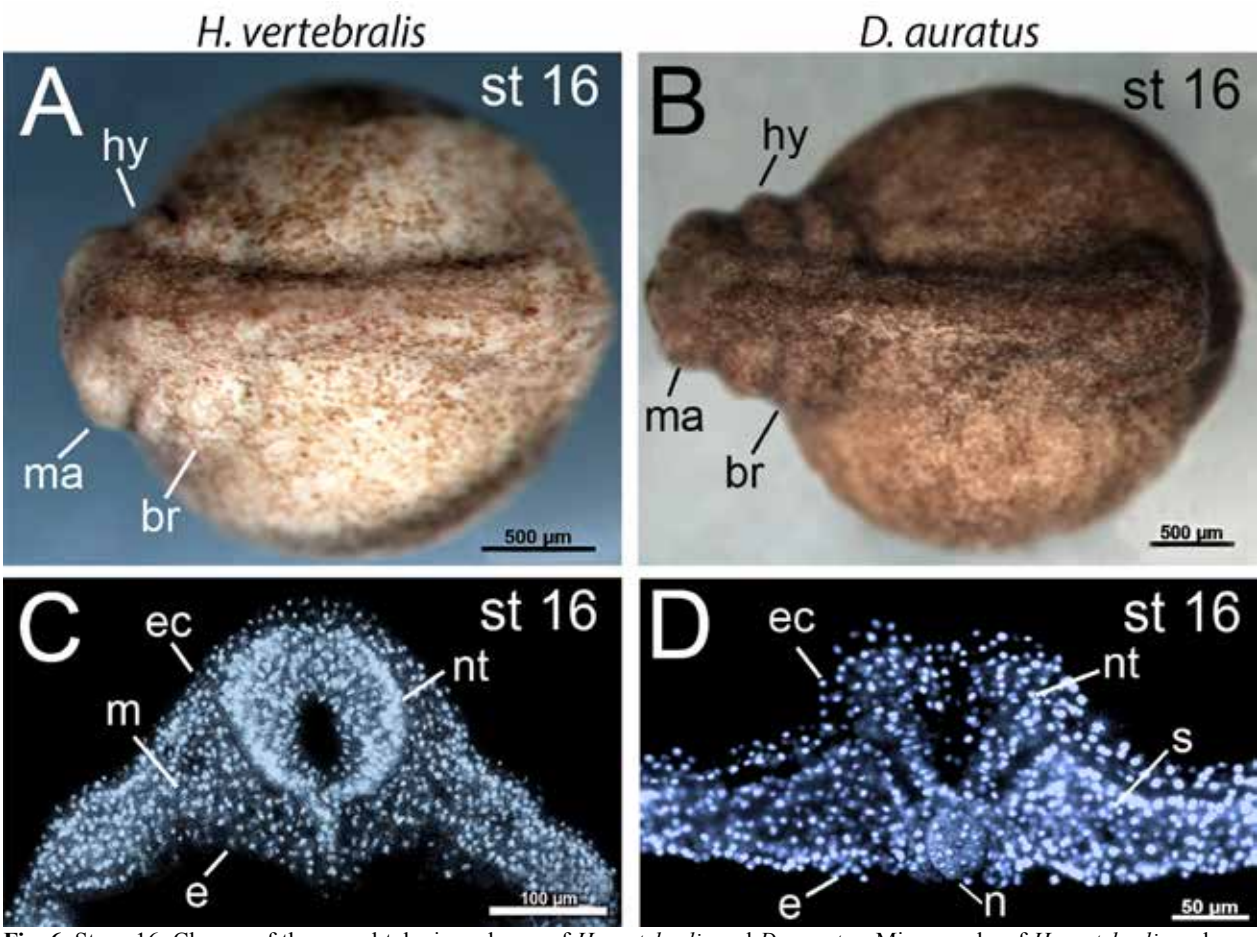

Fig. 6. Stage 16: Closure of the neural tube in embryos of *H. vertebralis* and *D. auratus*. Micrographs of *H. vertebralis* embryos are shown in A, C, and micrographs of *D. auratus* embryos are shown in B, D. Sections shown in C–D were stained for cell nuclei. **(A–B)** Dorsal views of embryos. The neural tube was closed. The branchial arches were visible in the head region. **(C)** Cross section through the rostral region, anterior to the notochord. The neural tube was completely closed. (Reproduced from Hervas and del Pino, 2013). **(D)** Cross sections through the trunk region of an embryo. The somites were visible. br, branchial arch; e, endoderm; ec, ectoderm; hy, hyoid arch; ma, mandibular arch; m, mesoderm; n notochord; nt, neural tube; s, somite.

ral folds became closed along the midline during stages 15–16 (Figs.1L; 2A; 5; 6). The external and internal characteristics of the neurula from stages 14–16 of *H. vertebralis* and *D. auratus* were compared (Figs. 4–6) and were found to be similar to *E. machalilla* embryos (del Pino et al. 2004).

The tail bud embryos (stage 17) of *H. vertebralis* were examined in their external and internal morphology (Figs. 2B; 7). The body became elongated and the head and tail regions protruded over the large yolky endoderm. The branchial arches were visible (Fig. 7A), and the brain and neural tube were detected. Embryos of this stage contained numerous somites (Fig. 7B, C). Embryos of stage 18 were characterized by muscular activity, and the embryos were longer. Buds of the external gills were detected in the head region (Fig. 8A, B). Somites, the notochord and neural tube were detected in the trunk region (Fig. 8C, D). A row of somites was detected on each side of the notochord (Fig. 8E, F). Myogenesis in both species occurred by cell interdigitation, as in other dendrobatid frogs and in the Marsupial frog, *G. riobambae*; whereas, cell rotation is the pattern for *X. laevis* myogenesis (Gatherer and del Pino 1992; Hervas and del Pino 2013). Gill buds were larger in stage 19 embryos (Figs. 2C–D; 9), and the external gills were fully developed in embryos of stage 22–23. The first gill pair of *H. vertebralis* developed eight branches, and the second pair was unbranched; whereas, embryos of *D. auratus* developed nine and two branches in the first and second gill pairs, respectively (Figs. 10; 11A, B). The number of gill branches in the first and second pair of external gills varies among species of Dendrobatidae (del Pino et al. 2004). The tail became longer in embryos of stages 18–25, the brain, spinal cord, somites, and internal organs developed and the embryos gradually acquired the tadpole shape in both species (Figs. 2C–F, 8–11). The processes of neurulation, somitogenesis, and internal embryo morphology of *H. vertebralis* and *D. auratus* were similar to the patterns described for other species of dendrobatid frogs (del Pino et al. 2004, 2007). Embryos of *H. vertebralis* hatched at stage 25 (Figs. 2F, 11E–G). The mouth had darkly pigmented teeth (Fig. 11F), the body had dark pigment, and the embryo had the appearance of a tadpole (Figs. 2F, 11E, G).

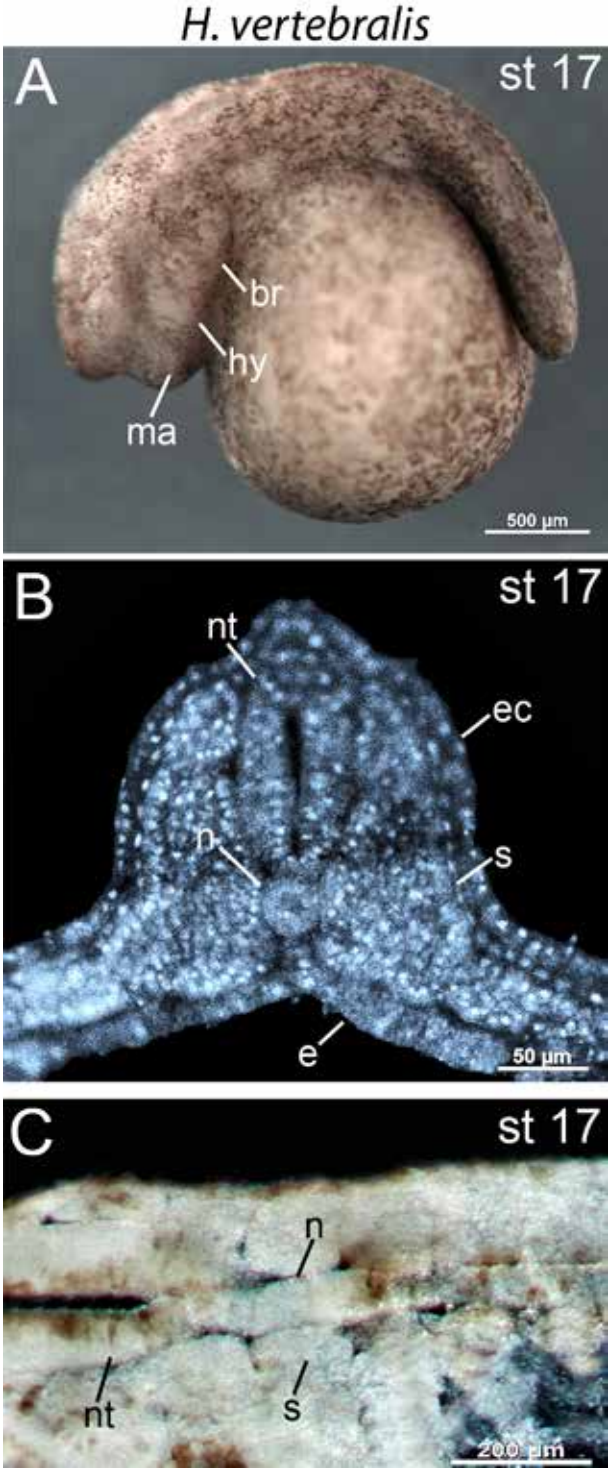

Fig. 7. Stage 17: Tail bud stage of *H. vertebralis* embryos. **(A)** Lateral view of an embryo. **(B)** Cross section through the trunk region of the embryo in A. **(C)** Horizontal section at the level of the notochord and somites with the rostral region towards the left. A row of somites was detected on each side of the notochord. A portion of the neural tube was detected in the rostral region of the section. br, branchial arch; e, endoderm; ec, ectoderm; hy, hyoid arch; ma, mandibular arch; n notochord; nt, neural tube; s, somite.

Comparative analysis of gastrulation

Gastrulation is characterized by common morphogenetic events that occur in all of the analyzed frog species. Formation of the dorsal blastopore lip, its development to enclose a yolk plug, and the process of internalization of cells at the blastopore lip by the movements of involution are among these common morphogenetic processes (Elinson and del Pino 2012). Other developmental events, however, may be dissociated from gastrulation in some frog species. In particular, dorsal convergence and extension and the onset of notochord elongation are separated from gastrulation in the Marsupial frog, *G. riobambae*, and in dendrobatid frogs; whereas, these events occur simultaneously with gastrulation in *X. laevis* and in *Engystomops* (Leptodactylidae) (Table 1) (del Pino 1996; Benítez and del Pino 2002; Keller and Shook 2004; Moya et al. 2007; Elinson and del Pino 2012).

The simultaneous occurrence of gastrulation and onset of notochord elongation may be related to the reproductive adaptation of frogs for rapid development under unstable environmental conditions such as the aquatic environment in which embryos of *X. laevis* develop, or the development in floating foam nests in species of *Engystomops* (Elinson and del Pino 2012). Embryos of these frogs require from 5 hours to 12.5 hours from the onset of gastrulation to blastopore closure (Stage 10–13) (Nieuwkoop and Faber 1994; Romero-Carvajal et al. 2009). Elongation of the notochord and gastrulation occur simultaneously in embryos of *X. laevis*, *Engystomops coloradorum*, and *Engystomops randi* (Leptodactylidae) (Keller and Shook 2004; Romero-Carvajal et al. 2009; Venegas-Ferrín et al. 2010). Early elongation of the notochord may be required for embryos to rapidly acquire the elongated tadpole shape in the unstable conditions of their reproductive environments.

The most divergent mode of gastrulation was detected in embryos of the Marsupial frog, *G. riobambae*. Gastrulation results in the formation of an embryonic disk from which the body of the embryo develops (del Pino and Elinson 1983). Cells that involute during gastrulation accumulate in the blastopore lip, and after blastopore closure give rise to an embryonic disk of small cells, visible on the surface. Internally, the small cells that involuted during gastrulation accumulated in the embryonic disk and in its internal circumblastoporal collar (Moya et al. 2007). Formation of the embryonic disk of *G. riobambae* is associated with delayed onset of notochord elongation that only starts once the blastopore is closed (del Pino 1996). Embryos of the Marsupial frog, *G. riobambae* develop slowly, and take a total of 168 hours from the onset of gastrulation to its completion (Table 1).

As in *G. riobambae*, cells that involuted during gastrulation became accumulated in a large circumblastoporal collar in embryos of dendrobatid frogs, with sepa-

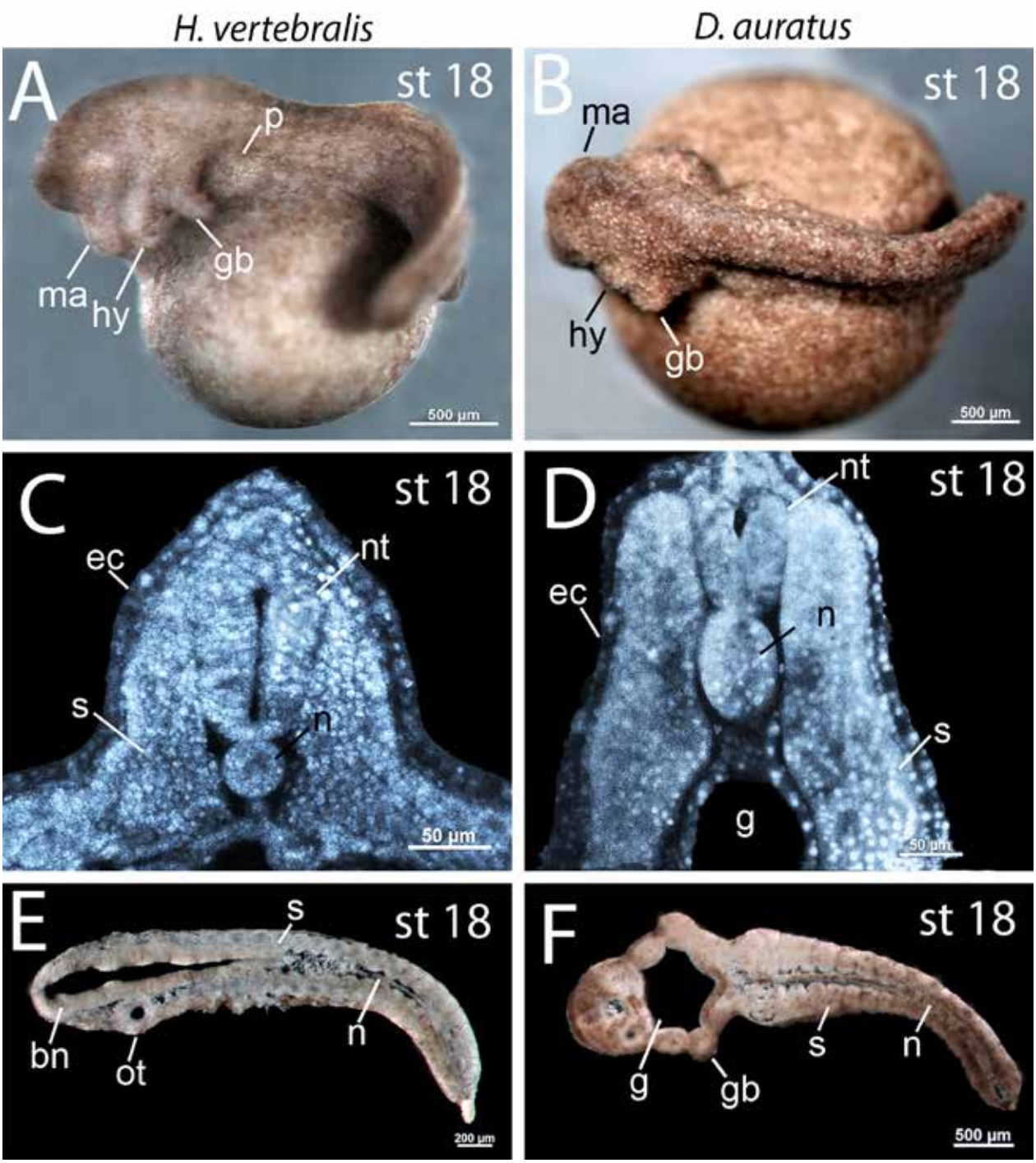

Fig. 8. Stage 18: Muscular activity stage of *H. vertebralis* and *D. auratus* embryos. Micrographs of *H. vertebralis* embryos are shown in A, C, E, and micrographs of *D. auratus* embryos are shown in B, D, F. Sections shown in C–D were stained for cell nuclei. **(A)** Lateral view of an embryo. **(B)** Dorsal view of an embryo. The gill buds were visible on each side of the head in embryos of both species. **(C–D)** Cross sections through the trunk. The cavity in D corresponds to the gut. **(E–F)** Horizontal sections. A row of numerous somites was detected on each side of the notochord. The brain and the otocysts were visible in E, and the gut was visible in F. bn, brain; ec, ectoderm; g, gut; gb, gill bud; hy, hyoid arch; ma, mandibular arch; n notochord; nt, neural tube; ot, otocyst; p, pronephros; s, somite.

ration of the morphogenetic events of gastrulation and the onset of notochord elongation. However, dendrobatid frogs do not develop an embryonic disk (Elinson and del Pino 2012). Egg size varied from 1.6 to 3.5 mm in diameter among dendrobatid frogs (Table 1), and their development was slow. Embryos of dendrobatid frogs require 36–72 hours from the onset of gastrulation to its completion (Stage 10–13; Table 1). We analyzed the characteristics of the gastrula in dendrobatid embryos derived from eggs of different diameters (Table 1; Fig. 12). Protection of embryos in the terrestrial nests of dendrobatids or inside a pouch of the mother in *G. riobambae* may al-

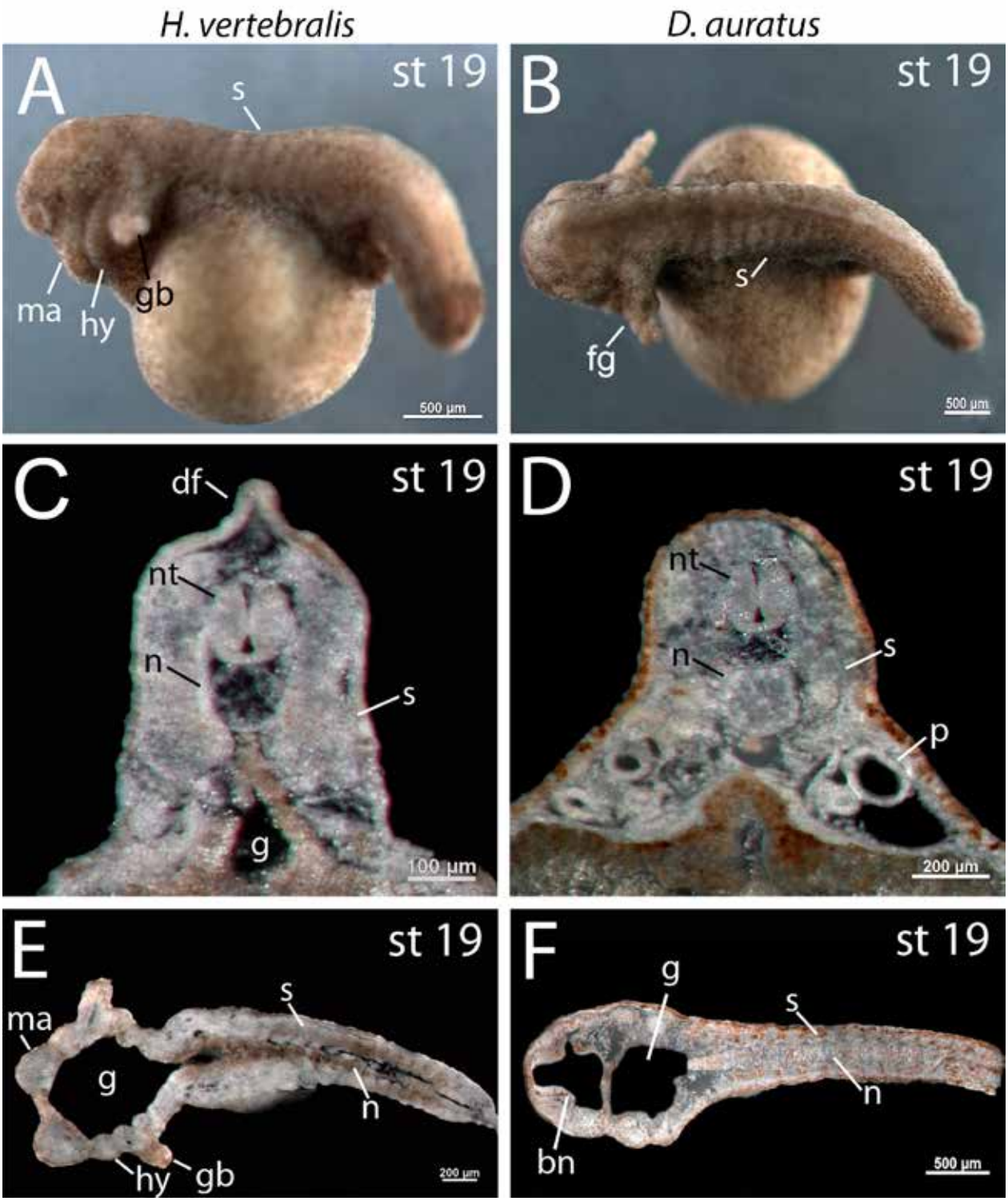

Fig. 9. Stage 19: Muscular response stage of *H. vertebralis* and *D. auratus* embryos. Micrographs of *H. vertebralis* embryos are shown in A, C, E, and micrographs of *D. auratus* embryos are shown in B, D, F. **(A)** Lateral view of an embryo. **(B)** Dorsal view of an embryo. The developing gills were visible. **(C–D)** Cross sections through the trunk. The dorsal fin was visible in C, and the pronephros in D. **(E)** Horizontal section at the level of the gut. **(F)** Horizontal section at the level of the brain and the gut. bn, brain; df, dorsal fin; fg, first gill pair; g, gut; gb, gill bud; hy, hyoid arch; ma, mandibular arch; n notochord; nt, neural tube; p, pronephros; s, somite.

low slow development and the separation of gastrulation from notochord elongation (Elinson and del Pino 2012).

Details of the morphology of the *H. vertebralis* and *D. auratus* gastrula are illustrated in Fig. 1E–I, Fig. 3B–J, and Fig. 12J–L, P–R. The archenteron roof remained relatively thin during gastrulation in *H. vertebralis* and *D. auratus* in comparison with stage 13 embryos of *X. laevis* (Fig. 12C, J–L, P–R). Elongation and inflation of the archenteron varied greatly among dendrobatids. The archenteron remained small during gastrulation and be-

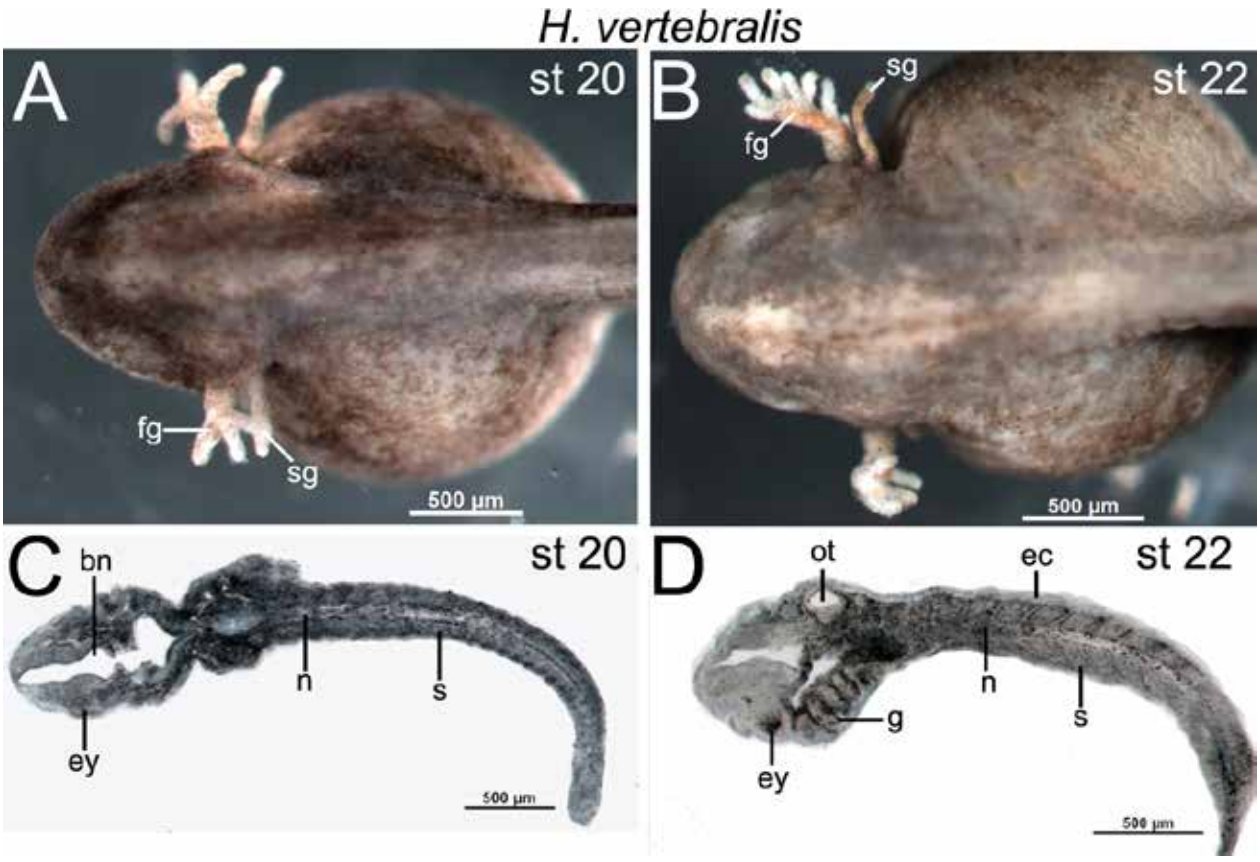

Fig. 10. Stages 20–22: External gill development in *H. vertebralis*. **(A)** Stage 20: Circulation to the external gills. Three branches were visible in the first gill pair. The second gill pair was unbranched. **(B)** Stage 22: The external gills enlarged. Seven branches were visible in the first gill pair. The second gill pair was unbranched. **(C)** Stage 20: Horizontal section at the level of the brain. **(D)** Stage 22: Horizontal section at the level of the internal gills. bn, brain; ey, eye; ec, ectoderm; fg, first gill pair; g, internal gill; nt, notochord; ot, otocyst; s, somite; sg, second gill pair.

came elongated and inflated after gastrulation in *H. vertebralis* (Fig. 12J–L); whereas, at stage 12, the archenteron was already elongated in the very large embryos of *A. bilinguis* and *D. auratus* (Fig. 12 M, P). Moreover inflation of the archenteron was already detected in stage 12 embryos of *D. auratus* (Fig. 12 P). In other species of dendrobatids, we detected variation in the level of archenteron elongation and inflation (Fig. 12D–R). We concluded that in *A. bilinguis*, and *D. auratus*, dendrobatids with very large eggs, the elongation of the archenteron begins earlier in comparison with embryos of dendrobatid frogs with smaller eggs such *E. machalilla* (Table 1) (del Pino et al. 2007).

In spite of the differences detected in the onset of archenteron elongation, the cells that involuted during gastrulation became accumulated in a large circumblastoporal collar at stage 13 in all of the dendrobatid frogs analyzed, as previously reported for *E. machalilla*, and shown for *E. anthonyi*, *E. tricolor*, *H. vertebralis*, *A. bilinguis*, and *D. auratus*, (Fig. 12F, I, L, O, R) (Moya et al. 2007). Notochord elongation is dissociated from gastrulation in these frogs (Benítez and del Pino 2002; del Pino et al. 2007; Moya et al. 2007; Venegas-Ferrín et al. 2010; Montenegro-Larrea and del Pino 2011).

The comparative analysis of gastrulation indicates that in spite of the great variation in egg size and onset of archenteron elongation and inflation, the Dendrobatidae species examined develop a large circumblastoporal collar as a result of gastrulation (Fig. 12D–R; Table 1) (del Pino et al. 2007; Moya et al. 2007; Montenegro-Larrea and del Pino 2011). Moreover, notochord elongation is delayed until after blastopore closure as in *G. riobambae*. In spite of their large circumblastoporal collar, dendrobatid frog embryos did not develop an embryonic disk.

Conclusions

Development of the dendrobatid frogs, *H. vertebralis* and *D. auratus*, shared the developmental characteristics described for *E. machalilla* (del Pino et al. 2004). Gastrulation and notochord elongation occurred as separate morphogenetic events in these frogs in comparison with additional species of Dendrobatidae. Development in a somewhat stable terrestrial environment may be associated with the separation of these developmental events and with comparatively slow development. The developmental analysis of *H. vertebralis* and other frogs contributes to a better knowledge of their biology and may contribute to the conservation and reproductive management of endangered frogs.

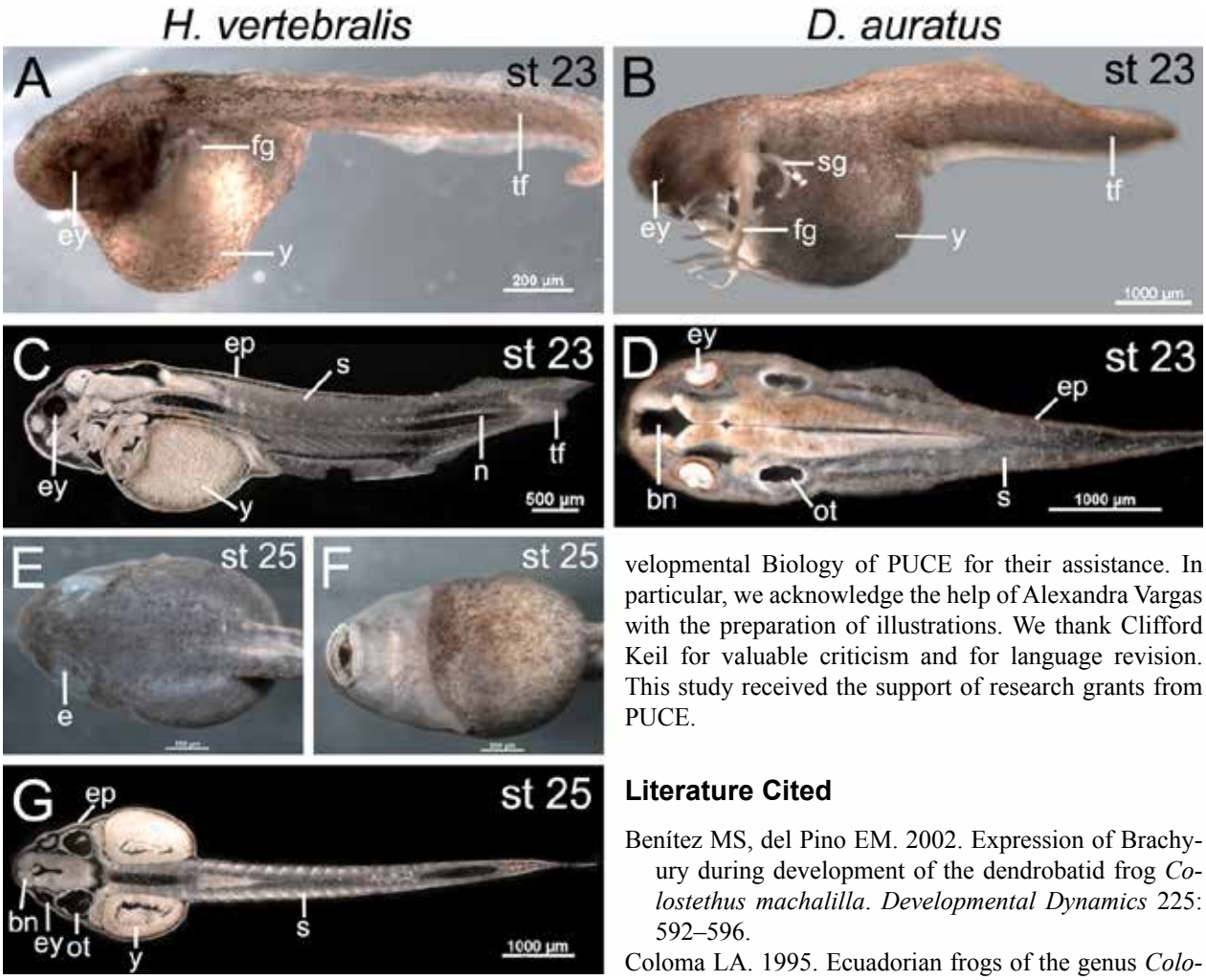

Fig. 11. Stages 23–25: Complete development of the external gills to tadpole hatching in embryos of *H. vertebralis* and *D. auratus*. Micrographs of *H. vertebralis* embryos are shown in A, C, E, F, G, and micrographs of *D. auratus* embryos are shown in B, D. **(A)** Stage 23 of *H. vertebralis*: Full development of external gills. The first gill pair of the external gills had eight branches, which at this stage were fully extended. The second gill pair of external gills was unbranched. **(B)** Stage 23 of *D. auratus*: The first gill pair of the external gills had nine branches, which at this stage were fully extended. The second pair of external gills was smaller and had two branches. In embryos of both species the eyes and the body were pigmented. The tail was elongated. **(C)** Stage 23: Sagittal section. The section was done through the embrionary brain and somites. The eyes, notochord, and tail fin were observed. **(D)** Stage 23: Horizontal section at the level of somites. The eyes and otocysts were visible. **(E)** Stage 25: Head of a tadpole at hatching in dorsal view. The eyes were visible. **(F)** Stage 25: Ventral view of the head of the tadpole shown in A. The spiracle was visible. **(G)** Stage 25: Horizontal section of a tadpole at hatching at the level of the otocysts. The eyes, otocysts, and somites were visible. bn, brain; ey, eye; ep, epidermis, fg, first gill pair; n, notochord; ot, otocyst; sg, second gill pair; s, somite; tf, tail fin; y, yolky endoderm.

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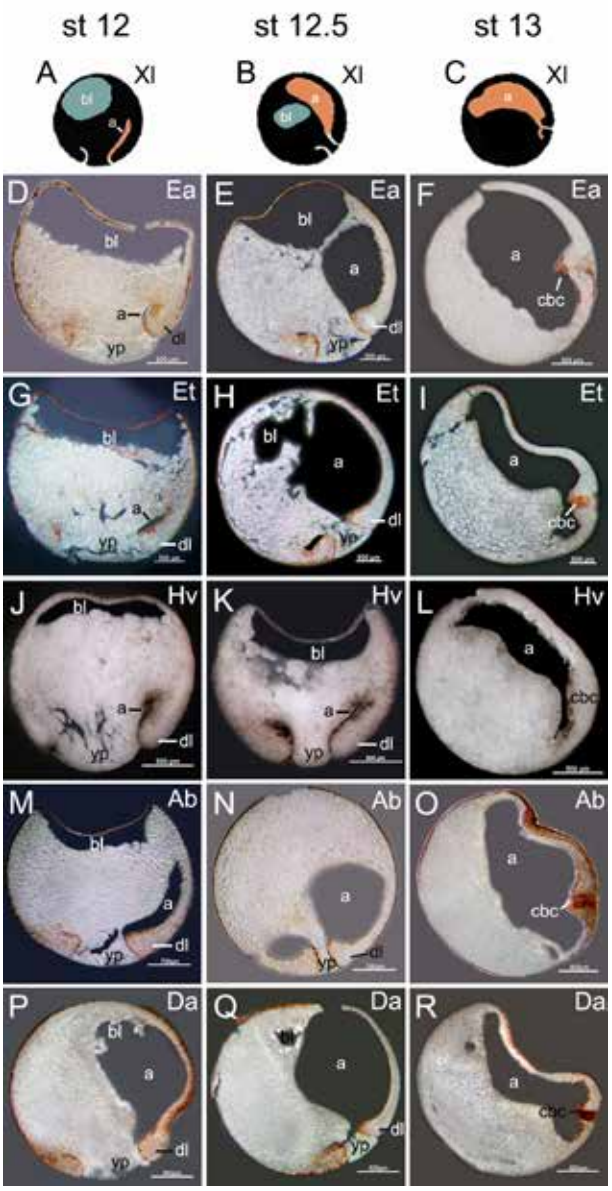

Fig. 12. Gastrulation of dendrobatid frogs in comparison with *X. laevis*. To facilitate the comparison, the stages (st) are given as column headings: Stage 12, late-gastrula; Stage 12.5, advanced late-gastrula; Stage 13, slit blastopore stage. The letters in the upper right-hand corner indicate the species: Ab, *Ameerega bilinguis*; Da, *Dendrobates auratus*; Ea, *Epipedobates anthonyi*; Et, *Epipedobates tricolor*; Hv, *Hyloxalus vertebralis*; Xl, *Xenopus laevis*. **(A–C)** Diagrams of *X. laevis* embryos. **(D–R)** Sagittal sections of gastrulae. **(D–F)** Embryos of *E. anthonyi*. (The section in E was reproduced from Montenegro–Larrea and del Pino, 2011). **(G–I)** Embryos of *E. tricolor*. **(J–L)** Embryos of *H. vertebralis*. **(M–O)** Embryos of *A. bilinguis*. **(P–R)** Embryos of *D. auratus*. a, archenteron; bl, blastocoel; cbc; circumblastoporal collar; dl, dorsal blastopore lip; yp, yolk plug.

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