Variation in the schedules of somite and neural development in frogs

Natalia Sáenz-Ponce[a], Christian Mitgutsch[b], and Eugenia M. del Pino[a,1]

[a]Escuela de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Quito 17, Ecuador; and [b]Laboratory for Evolutionary Morphology, RIKEN Center for Developmental Biology, Kobe 650-0047 Hyogo, Japan

Contributed by Eugenia M. del Pino, November 7, 2012 (sent for review September 3, 2012)

The timing of notochord, somite, and neural development was analyzed in the embryos of six different frog species, which have been divided into two groups, according to their developmental speed. Rapid developing species investigated were *Xenopus laevis* (Pipidae), *Engystomops coloradorum*, and *Engystomops randi* (Leiuperidae). The slow developers were *Epipedobates machalilla* and *Epipedobates tricolor* (Dendrobatidae) and *Gastrotheca riobambae* (Hemiphractidae). Blastopore closure, notochord formation, somite development, neural tube closure, and the formation of cranial neural crest cell-streams were detected by light and scanning electron microscopy and by immuno-histochemical detection of somite and neural crest marker proteins. The data were analyzed using event pairing to determine common developmental aspects and their relationship to life-history traits. In embryos of rapidly developing frogs, elongation of the notochord occurred earlier relative to the time point of blastopore closure in comparison with slowly developing species. The development of cranial neural crest cell-streams relative to somite formation is accelerated in rapidly developing frogs, and it is delayed in slowly developing frogs. The timing of neural tube closure seemed to be temporally uncoupled with somite formation. We propose that these changes are achieved through differential timing of developmental modules that begin with the elongation of the notochord during gastrulation in the rapidly developing species. The differences might be related to the necessity of developing a free-living tadpole quickly in rapid developers.

The transformation of the spherical egg into the elongated body of the larva and adult is a major event in amphibian development. In *Xenopus laevis*, body elongation begins in the mid-gastrula with the formation and elongation of the notochord (1). The process of body elongation in *X. laevis* is guided by convergent extension (2). Convergent extension is the process by which the presumptive notochord and neural plate lengthen and narrow owing to the morphogenetic movements of mediolateral cell intercalation (2), and it involves the genetic control of planar cell polarity pathway genes in mesoderm and neural tissues (1, 3–7). Planar cell polarity signaling mediated by *dishevelled* and *strabismus* (also called *lpp1*, *ltap*, *stb1*, *stbm*, and *vangl2*) is required for elongation of the notochord and neural plate and for the initiation of neural tube closure (3, 6, 8, 9). However, different components of the Wnt signaling network are involved in *X. laevis* convergent extension of the mesoderm and in the closure of the neural tube (3). Besides *X. laevis*, the molecular control of convergent extension has been investigated in the zebrafish (9). In addition, the onset of convergent extension, detected by the expression of the proteins brachyury and lhx1 (previously known as Lim1) in the notochord, is known for several species of anurans (10–12). We investigated the variation in the number of somites and in the advancement of neural development in frogs that differ in their reproductive strategies, developmental rate, and in the timing of notochord elongation (Table 1). *X. laevis* deposits numerous eggs in the water, and the túngara frogs *Engystomops coloradorum* and *Engystomops randi* lay their eggs in foam-nests that float in the water (13). In contrast, the poison-arrow frogs *Epipedobates machalilla* and *Epipedobates tricolor* deposit fewer eggs in terrestrial nests (14, 15). In the case of the marsupial frog *Gastrotheca riobambae*, the developing embryos are transported in a pouch of integument by the mother (14, 15). The analyzed frogs belong to four different families and have notable differences in egg size (Table 1). Frog species that require hours to advance from fertilization to the end of gastrulation are considered to develop rapidly, whereas those that take days for the same process are considered to develop slowly (Table 1). Differences in rates of development were also detected in advanced embryos of these frogs (13, 15, 16). In the rapidly developing embryos of *E. coloradorum* and *E. randi*, elongation of the notochord was detected at the mid-gastrula stage, as in the embryos of *X. laevis* (12, 15, 17). In contrast, in the slowly developing embryos of *E. machalilla*, *E. tricolor*, and *G. riobambae*, elongation of the notochord and consequently of the body begins only after the process of gastrulation is completed and the blastopore is closed (15, 17, 18) (Table 1). The late onset of notochord elongation in the embryos of slowly developing frogs is a demonstration of the modularity of frog gastrulation processes (8, 10). In particular, convergent extension is a module that can be superimposed or separated from other gastrulation morphogenetic movements in the embryogenesis of different frogs (14, 15, 18). Elongation of the notochord occurred earlier relative to the time point of blastopore closure in rapidly developing embryos, in contrast with slowly developing species. Moreover, the development of cranial neural crest cell-streams is accelerated relative to somite formation in rapidly developing frogs, and it is delayed in slowly developing frogs. Acceleration of body elongation and of cranial neural crest cell-stream development occurs in embryos of frog species that require the rapid development of free-living tadpoles.

Results

Analysis of Embryonic Diversity. We removed the epidermis of frog embryos to detect the somites and neural characteristics, as shown for embryos of several species (Figs. 1 *A* and *B* and 2 *A, B*, and *E*). Epidermis removal was not necessary only for the translucent and thin embryonic disk of *G. riobambae* embryos. Scanning electron microscopy was available for *X. laevis* embryos (Figs. 1 *A* and *B* and 2*A*). For other species we used light microscopy after removal of the epidermis (Fig. 2 *B* and *E*). In addition we analyzed embryos after immunostaining against sarcomeric meromyosin and antigen 2G9 to detect the somites and neural crest cell-streams, respectively (Fig. 2 *C*, *D*, and *F*). Immunostaining against sarcomeric meromyosin, however, gave faint evidence of the somites in early embryos, and the expression became strong only in tailbud embryos, as previously reported (15) (Fig. 2*C*). The streams of cranial neural crest are named according to refs. 13, 15, and 19. The specimens displayed either three streams of cranial neural crest (Figs. 1 *A* and *B* and 2 *A*, *B*, and *E*) or four streams once the

Author contributions: C.M. and E.M.d.P. designed research; N.S.-P., C.M., and E.M.d.P. performed research; N.S.-P. and E.M.d.P. analyzed data; and E.M.d.P. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: edelpino@puce.edu.ec.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1219307110/-/DCSupplemental.

Table 1. Developmental characteristics of the frogs

Family and species	Reproductive mode	Egg size (mm)	Developmental time*	Notochord elongation	Embryos analyzed
Rapid development					
Pipidae					
Xenopus laevis	Aquatic	1.2	14 h	Mid-gastrula	14
Leiuperidae					
Engystomops randi	Foam nest	1.1	24 h	Mid-gastrula	32
Engystomops coloradorum	Foam nest	1.3	24 h	Mid-gastrula	26
Slow development					
Dendrobatidae					
Epipedobates machalilla	Terrestrial nest	1.6	4 d	After gastrulation	26
Epipedobates tricolor	Terrestrial nest	2.0	4 d	After gastrulation	18
Hemiphractidae					
Gastrotheca riobambae	Maternal pouch	3.0	14 d	After gastrulation	25

*Time from fertilization to the end of gastrulation, according to ref. 15.

branchial stream of cranial neural crest became divided into two streams (Fig. 2 *D* and *F*).

Diversity in Developmental Timing. Morphological variation, caused through shifts in developmental timing of embryonic structures, was studied by event pairing (20). All of the variable characters encountered are given in Table 2. A similar state of characters 1–3 and 6–9 was shared by all rapidly developing species, whereas a different state of these characters was shared by the slowly developing species (Table 2). None of the event pairs investigated shows a transition from a nonsimultaneous to the opposite nonsimultaneous state when comparing the rapidly vs. slowly developing frogs, with the exception of event pair 1 (notochord elongation and blastopore closure). Species-specific variation was observed in characters 5 and 10 of *X. laevis* and *G. riobambae* (Table 2). Simultaneous states characterizing either group are depicted in Fig. S1.

The event pairs that showed variation among frogs are as follows. (*i*) Notochord elongation and blastopore closure. Elongation of the notochord began before closure of the blastopore in rapidly developing frogs, whereas it started once the blastopore was closed in the slowly developing species (Table 2, character 1). (*ii*) Number of cranial neural crest cell-streams and somite numbers. Three streams of cranial neural crest were detected simultaneously with one to three somites in embryos of fast-developing species and with four to five somites in embryos of slowly developing frogs (Table 2, characters 2 and 3; Fig. S1). This means that rapid developers show an acceleration in cranial neural crest development relative to somite formation. In contrast, cranial neural crest development is delayed relative to somite formation in slow developers. This is also evident at slightly later stages, where most rapidly developing frogs achieve four cranial neural crest cell-streams at a less advanced stage of somite development than in the majority of slowly developing species (Table 2, characters 4 and 5; Fig. S1). The two exceptions are *X. laevis* and *G. riobambae*. (*iii*) Closure of the neural tube (detected as "neural folds in contact") and somite numbers. Neural tube closure was not coordinated with the advancement of somite development. In fact, neural tube closure was detected in embryos with lower somite counts in slowly developing in comparison with rapidly developing frogs (Table 2, characters 6 and 7; Fig. S1). The variation in neural tube closure is exemplified by the comparison of frog embryos with a similar number of somites. For example, the neural tube was open in the four-somite embryo of the rapidly developing frog *E. randi* (Fig. 2*B*). In contrast, the neural tube was closed in the four-somite embryo of the slowly developing frog *E. tricolor* (Fig. 2*E*). (*iv*) Number of cranial neural crest cell-streams and closure of the neural tube (detected as "neural folds in contact"). Event pairing of cranial neural crest cell-streams with neural tube closure indicated that the migration of cranial neural crest cell-streams is uncoupled from the closure of the neural tube (Table 2, characters 8–10; Fig. S1). This analysis reveals that

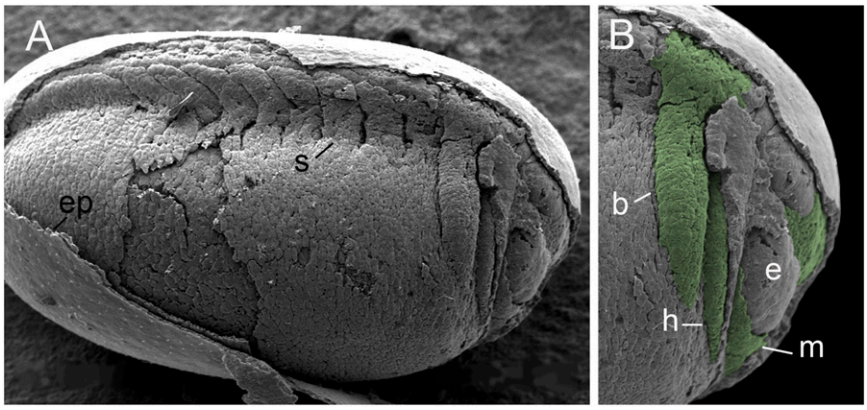

Fig. 1. Somites and neural structures in embryos of *X. laevis*. (*A*) Scanning electron micrograph of a *X. laevis* embryo with the epidermis partially removed. The neural folds are in contact. The embryo has eight somites and three streams of cranial neural crest. The head is oriented toward the right, and the dorsal side is toward the top. (*B*) Pseudo-colored electron micrograph of the embryo shown in *A* at higher magnification to highlight the streams of cranial neural crest. The optic vesicle divides the mandibular stream in two portions. b, branchial stream of cranial neural crest; e, optic vesicle; ep, epidermis; h, hyoid stream of cranial neural crest; m, mandibular stream of cranial neural crest; s, somite.

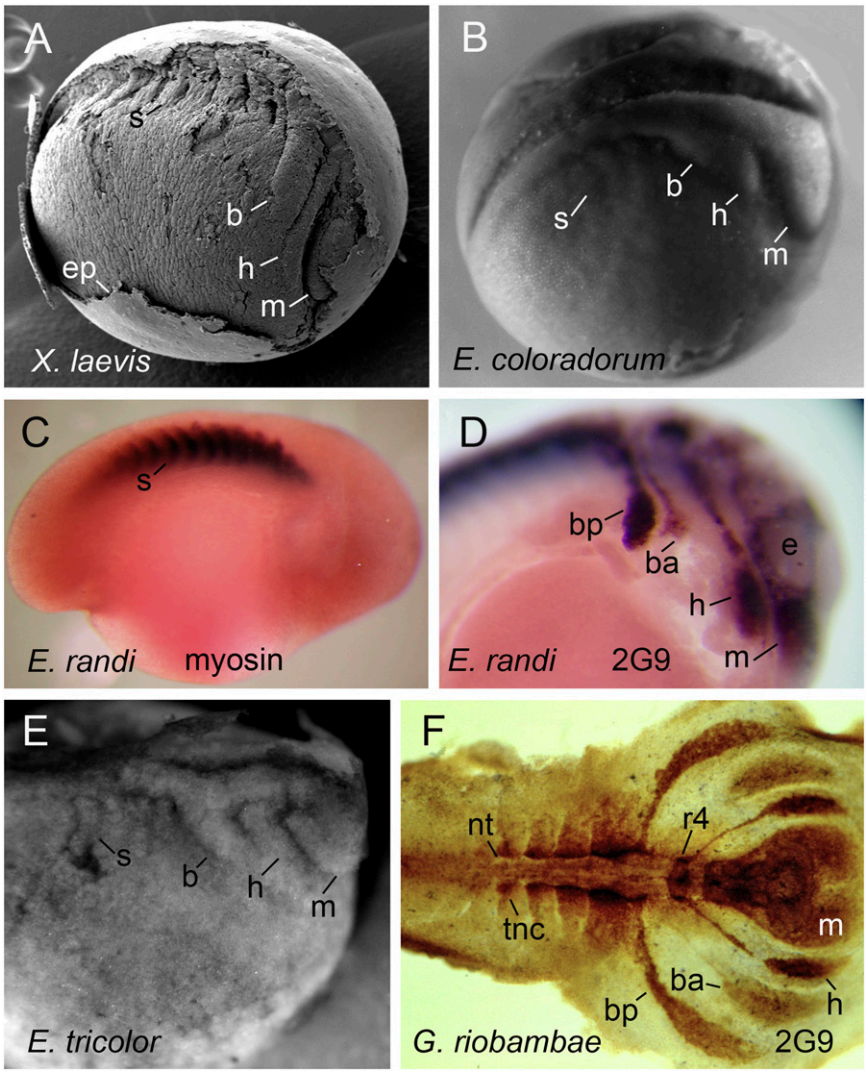

Fig. 2. Somite and neural characteristics of frog embryos. In all images the head is oriented toward the right. The dorsal side is toward the top in *A*–*E*. The epidermis of embryos in *A*, *B*, and *E* was partially removed. (*A*–*D*) Embryos of rapidly developing frogs. (*A*) Scanning electron micrograph of a *X. laevis* neurula with seven somites and closed neural folds. Three streams of cranial neural crest are visible. (*B*) Light micrograph of an *E. coloradorum* embryo. This embryo had open neural folds, four somites, and three streams of cranial neural crest. (*C*) Tailbud embryo of *E. randi* immunostained for sarcomeric meromyosin, labeled as myosin in the image. The 12 most rostral somites gave a positive signal. Sarcomeric meromyosin was not detected in the recently formed somites of the caudal region and in the presomitic mesoderm. (*D*) The cranial neural crest streams of a tailbud embryo of *E. randi* immunostained for antigen 2G9. The four streams of cranial neural crest gave a 2G9-positive signal. Another micrograph of this embryo was published in ref. 15. (*E* and *F*) Embryos of slowly developing frogs. (*E*) Light micrograph of an *E. tricolor* embryo. This embryo had four somites and three streams of cranial neural crest, as in the embryo of *E. coloradorum* shown in *B*. In contrast, the neural folds are in contact in the slowly developing embryo of *E. tricolor* and open in the rapidly developing embryo of *E. coloradorum*. (*F*) Dorsal view of a *G. riobambae* embryo with five to six somites that was removed from the yolky endoderm and immunostained for antigen 2G9. In this embryo, the neural folds were closed, and four streams of cranial neural crest were visible. The cranial and trunk neural crest, the neural tube, rhombomeres 1–2, and rhombomere 4 are 2G9-positive as described by ref. 43. ba, branchial anterior stream of cranial neural crest; bp, branchial posterior stream of cranial neural crest; nt, neural tube; r4, rhombomere 4; tnc, trunk neural crest; other abbreviations as in Fig. 1.

embryos of rapidly developing frogs developed the notochord and cranial neural crest cell-streams more rapidly than those of slowly developing frogs. In contrast, closure of the neural tube was accelerated in embryos of slowly developing frogs compared with the rapidly developing species (Fig. S1).

Discussion

Rapid embryonic development has been demonstrated in species from several anuran groups, such as the Pipidae, Scaphiopodidae, and Leiuperidae (15, 21). This suggests that this life-history trait has evolved several times independently, possibly in connection with particular ecological conditions or specialized reproductive modes. In other craniate taxa, it has been shown that such life-history traits can be related to changes in the timing of embryonic development, one example being the accelerated anterior development in the altricial offspring of marsupials (22), or affect the embryonic morphology, as shown by the comparatively weakly developed branchial neural crest cell-streams in the embryo of the direct-developing frog *Eleutherodactylus coqui* (Terrarana) (23). Vice versa, interspecific differences in developmental timing or embryonic morphology cannot always easily be interpreted in relation to evolutionary changes in adult morphology or in different life-history traits (24–27). Embryonic diversity regarding neural and somite development has been previously reported for several frog species (24, 25, 28, 29). However, possible connections between developmental rate and the onset of notochord

Table 2. Variable event pairs in embryos of rapidly and slowly developing frogs

			Rapidly developing frogs†			Slowly developing frogs†		
Character*	Event A	Event B	Xl	Er	Ec	Em	Et	Gr
	Notochord	Blastopore						
1	Visible	Closed	0	0	0	2	2	2
	Neural crest streams	No. of somites						
2	Three	1–3	1	1	1	2	2	2
3	Three	4–5	0	0	0	1	1	1
4	Four	6–9	2	1	1	2	2	1
5	Four	10–13	1	0	0	1	1	0
	Neural folds	No. of somites						
6	In contact	4–5	2	2	2	1	1	1
7	In contact	6–9	1	1	1	0	0	0
	Neural crest streams	Neural folds						
8	Three	Open	1	1	1	2	2	2
9	Three	In contact	0	0	0	1	1	1
10	Four	In contact	2	1	1	2	2	2

*0, event A occurs before B;1, events A and B occur simultaneously; 2, event A occurs after B. Nonvariable events are not shown.
†Ec, *E. coloradorum*; Em, *E. machalilla*; Er, *E. randi*; Et, *E. tricolor*; Gr, *G. riobambae*; Xl, *X. laevis*.

elongation with somite and neural development have not been analyzed to date.

This study demonstrates that rapidly developing frog species elongate the notochord early relative to the time point of blastopore closure, and it also shows acceleration in the development of cranial neural crest cell-streams relative to somite formation in comparison with slowly developing species (Table 2 and Fig. S1). However, in the direct-developing frog, *E. coqui* (Terrarana), elongation of the notochord is delayed, although its development is very rapid (30). This frog differs from the other rapidly developing frogs in that the embryo derives from a larger egg (14).

Large egg size also occurs in the slowly developing frogs analyzed in this work, whereas the rapidly developing species have smaller eggs (Table 1). Connections between developmental peculiarities, embryogenesis, and yolk content have been demonstrated for a number of anuran species (31–35). However, differences in developmental timing are not necessarily connected to, or caused by, variation in the yolk contents of eggs, as demonstrated for closely related species of *Bombina* and *Discoglossus* (24).

There are differences in the timing of head and trunk organizer development among frogs. Head development begins in the gastrula in both the rapidly and slowly developing frogs that were analyzed. In fact, the prechordal plate (head organizer) appears during gastrulation in the slowly developing embryos of *E. machalilla*, and the notochord (trunk organizer) appears after gastrulation, whereas in the rapidly developing embryos of *X. laevis* and *E. randi* the two organizers develop in the mid-gastrula (12, 14, 17).

The timing of neural tube closure seemed to be temporally uncoupled with the formation of somites in the analyzed frogs (Fig. 2, Table 2, and Fig. S1). The different molecular control of *X. laevis* convergent extension in the mesoderm and in neural tube closure (3) gives support to the notion of relative independence between mesoderm and neural developmental modules. The developmental significance of the acceleration and retardation of neural tube closure in regard to somite counts in frogs remains unknown.

Acceleration of body elongation, detected by the early onset of notochord, and of head development, indicated by the acceleration of cranial neural crest cell-stream development in relation to somite formation, may be connected with the accelerated development of the tadpole in the rapidly developing biphasic species. The biphasic development with a larval stage is considered primitive among living anurans, with direct development arising several times (35). The removal of the tadpole from the frog life cycle in the different frog lineages with direct development led to the suggestion that the original tadpole evolved as an insertion in the ancestor leading to the modern anurans (31). However, the fossil record does not allow the conclusive determination of whether the ancestral amphibian developmental mode was biphasic or direct (31, 35), and in fact the possibility of reevolution of a biphasic life cycle from direct-developing ancestors has been suggested for both anuran and caudate species (36, 37).

The developmental differences observed in this study do not give clues in regard to the features of ancestral anuran development. Moreover, the observed differences in developmental timing are unrelated with the phylogenetic relationships of the various species, (Pipidae + (Hemiphractidae + (Leiuperidae + Dendrobatidae))) (38). Instead it seems that the different timing of development of the notochord and streams of cranial neural crest, in comparison with blastopore closure and the development of somites, is associated with the reproductive adaptations of the various frogs and might be related to the necessity of developing a free-living tadpole quickly in rapid developers.

The variation in the timing of notochord, development of cranial neural crest cell-streams, and neural tube closure in different frogs is made possible by the modularity of developmental events, in particular the separation of convergent extension from gastrulation (14, 15, 18, 39). This plasticity of frog development allows delay or acceleration in the development of free-living tadpoles. Moreover, it allows for the reduction or elimination of the tadpole, as observed in direct-developing hemiphractid frogs and *E. coqui,* respectively (14).

Materials and Methods

Frogs and Embryos. Reproductive and developmental characteristics of the frogs and the number of analyzed embryos are given in Table 1. Adults and embryos were maintained and handled as previously described (13, 15, 18, 24). The collection localities are given in refs. 13 and 15. The authorization 016-IC-FAU-DNBAP-MA from the Ministry of the Environment, Ecuador allowed the collection of frogs from Ecuador. Embryos of *X. laevis* come from laboratory-bred animals at the University of Jena, Germany. Additional embryos of *X. laevis* were donated by several investigators. Procedures for the maintenance and handling of frogs and embryos were approved by the Faculty of Exact and Natural Sciences of the Pontificia Universidad Católica del Ecuador.

Embryo Fixation Removal of the Epidermis and Staining. Fixation of embryos for light and scanning electron microscopy was according to published protocols (13, 18, 25). Somites and neural characteristics of embryos were analyzed after partial removal of the epidermis (25). Removal of the epidermis was done in fixed embryos after incubation in 100% glycerol for approximately 15 min to facilitate manual removal of the epidermis. In

embryos of *G. riobambae*, the epidermis was not removed; instead, the thin embryonic disk was dissected from the endoderm and prepared in whole mount. The white embryos of *E. coloradorum* and *E. randi* were stained with 0.5% crystal violet after removal of the epidermis to reveal somites and neural structures. The cranial neural crest cell-streams of *X. laevis* embryos were analyzed according to refs. 40 and 41.

Whole-Mount Immunostaining. Embryos were immunostained in whole mount with a mAb against antigen 2G9 (42) and sarcomeric meromyosin (mAb MF-20; Developmental Studies Hybridoma Bank) as previously described (13, 43). Antigen 2G9 is an uncharacterized and abundant neural protein, first detected in *X. laevis* (42). Several immunostained embryos come from previous work (13, 15, 43).

Scanning Electron Microscopy and Light Microscopy. Scanning electron microscopy of *X. laevis* embryos was done with a Philips ESEM XL30 scanning electron microscope according to ref. 25. Embryos of other frogs were analyzed and photographed with a Stemi SV 6 stereo light microscope (Zeiss).

Developmental Events and Analysis of Developmental Timing. The specimens were ordered according to their somite counts, and the sequences of developmental events were tabulated for each species, as described in ref. 25. The tables of developmental stages for *X. laevis*, *Engystomops*, *E. tricolor*, and *G. riobambae* (13, 16, 41, 44) were not used because embryo morphology and the stages of development differ among species. We applied the event-pairing method to detect time variation of developmental event characters (20) between rapidly and slowly developing frogs (Table 1). For each species we coded the character states for each pair of events, consisting of events A and B, as "0" when event A occurs before B, "1" for the simultaneous occurrence of events A and B, and "2" when event A occurs after B, according to ref. 20. The term "character" indicates a pair of developmental events (20). The following 10 developmental events were examined: *Gastrula:* blastopore closed; notochord visible. End of gastrulation was detected by the closure of the blastopore. *Neural tube*: the neural folds are open, indicating elevated neural folds that do not touch; the neural folds are in contact, indicating that the neural folds touch each other at least at one point. *Cranial neural crest cell-streams*: three streams are visible (the mandibular, hyoid, and branchial streams); four streams are visible (mandibular, hyoid, branchial anterior, and branchial posterior streams). *Somites*: 1 to 3 somites are visible; 4 to 5 somites are visible; 6 to 9 somites are visible; 10 to 13 somites are visible.

ACKNOWLEDGMENTS. We thank Elizabeth A. Jones (University of Warwick, United Kingdom) for the donation of antiantigen 2G9; Santiago R. Ron and other members of the Amphibian Division, Museum of Zoology, Pontificia Universidad Católica del Ecuador and its conservation program "Balsa de los sapos," for the donation of embryos of various frogs; Andrés Romero-Carvajal and other members of the E.M.d.P. laboratory for help with immune detection and assistance in the care of frogs; and Igor B. Dawid, Lennart Olsson, and Gerhard Schlosser for the critical reading of the manuscript. This project was supported by the Paläontologisches Institut und Museum der Universität Zürich, Zurich, Switzerland, particularly Marcelo R. Sánchez-Villagra, and the Laboratory for Evolutionary Morphology, RIKEN Center for Developmental Biology, Kobe, Japan. C.M. was supported by the Institutional Program for Young Researcher Overseas Visits by the Japan Society for the Promotion of Sciences. E.M.d.P. received grants from the Pontificia Universidad Católica del Ecuador in 2011 and 2012.

1. Keller R, Shook D (2004) *Gastrulation from Cells to Embryo*, ed Stern CD (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp 171–203.
2. Keller R, Davidson LA, Shook DR (2003) How we are shaped: The biomechanics of gastrulation. *Differentiation* 71(3):171–205.
3. Wallingford JB, Harland RM (2001) *Xenopus* Dishevelled signaling regulates both neural and mesodermal convergent extension: Parallel forces elongating the body axis. *Development* 128(13):2581–2592.
4. Wallingford JB, Fraser SE, Harland RM (2002) Convergent extension: The molecular control of polarized cell movement during embryonic development. *Dev Cell* 2(6):695–706.
5. Goodrich LV, Strutt D (2011) Principles of planar polarity in animal development. *Development* 138(10):1877–1892.
6. Goto T, Keller R (2002) The planar cell polarity gene *strabismus* regulates convergence and extension and neural fold closure in *Xenopus*. *Dev Biol* 247(1):165–181.
7. Skoglund P, Keller R (2010) Integration of planar cell polarity and ECM signaling in elongation of the vertebrate body plan. *Curr Opin Cell Biol* 22(5):589–596.
8. Copp AJ, Greene NDE, Murdoch JN (2003) Dishevelled: Linking convergent extension with neural tube closure. *Trends Neurosci* 26(9):453–455.
9. Ueno N, Greene NDE (2003) Planar cell polarity genes and neural tube closure. *Birth Defects Res C Embryo Today* 69(4):318–324.
10. del Pino EM (1996) The expression of Brachyury (T) during gastrulation in the marsupial frog *Gastrotheca riobambae*. *Dev Biol* 177(1):64–72.
11. Benítez M-S, del Pino EM (2002) Expression of Brachyury during development of the dendrobatid frog *Colostethus machalilla*. *Dev Dyn* 225(4):592–596.
12. Venegas-Ferrín M, Sudou N, Taira M, del Pino EM (2010) Comparison of Lim1 expression in embryos of frogs with different modes of reproduction. *Int J Dev Biol* 54(1):195–202.
13. Romero-Carvajal A, et al. (2009) Embryogenesis and laboratory maintenance of the foam-nesting túngara frogs, genus *Engystomops* (= *Physalaemus*). *Dev Dyn* 238(6): 1444–1454.
14. Elinson RP, del Pino EM (2012) Developmental diversity of amphibians. *WIREs Dev Biol* 1:345–369.
15. del Pino EM, et al. (2007) A comparative analysis of frog early development. *Proc Natl Acad Sci USA* 104(29):11882–11888.
16. del Pino EM, et al. (2004) Development of the dendrobatid frog *Colostethus machalilla*. *Int J Dev Biol* 48(7):663–670.
17. Taira M, Jamrich M, Good PJ, Dawid IB (1992) The LIM domain-containing homeo box gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev* 6(3):356–366.
18. Moya IM, Alarcón I, del Pino EM (2007) Gastrulation of *Gastrotheca riobambae* in comparison with other frogs. *Dev Biol* 304(2):467–478.
19. Theveneau E, Mayor R (2012) Neural crest migration: Interplay between chemorepellents, chemoattractants, contact inhibition, epithelial-mesenchymal transition, and collective cell migration. *WIREs Dev Biol* 1:435–445.
20. Smith KK (1997) Comparative patterns of craniofacial development in eutherian and metatherian mammals. *Evolution* 51:1663–1678.
21. Zweifel RG (1968) Reproductive biology of anurans of the arid South-West, with emphasis on adaptation of embryos to temperature. *Bull Am Mus Nat Hist* 140:1–64.
22. Weisbecker V, Goswami A, Wroe S, Sánchez-Villagra MR (2008) Ossification heterochrony in the therian postcranial skeleton and the marsupial-placental dichotomy. *Evolution* 62(8):2027–2041.
23. Moury JD, Hanken J (1995) Early cranial neural crest migration in the direct-developing frog, *Eleutherodactylus coqui*. *Acta Anat (Basel)* 153(4):243–253.
24. Mitgutsch C, Olsson L, Haas A (2009) Early embryogenesis in discoglossoid frogs: a study of heterochrony at different taxonomic levels. *J Zoological Syst Evol Res* 47:248–257.
25. Mitgutsch C, Piekarski N, Olsson L, Haas A (2008) Heterochronic shifts during early cranial neural crest cell migration in two ranid frogs. *Acta Zool* 78:69–78.
26. Werneburg I, Sánchez-Villagra MR (2011) The early development of the Echidna, *Tachyglossus aculeatus* (Mammalia: Monotremata) and the Grundmuster of mammalian development. *Acta Zool* 92:75–88.
27. Richardson MK (1999) Vertebrate evolution: The developmental origins of adult variation. *Bioessays* 21(7):604–613.
28. Smetanick MT, de Sá RO, Radice GP (2000) Do timing and pattern of myogenesis correlate with life history mode in anurans? *J Herpetol* 34:637–642.
29. Radice GP, Neff AW, Shim YHEE, Brustis JJ, Malacinski GM (1989) Developmental histories in amphibian myogenesis. *Int J Dev Biol* 33(3):325–343.
30. Ninomiya H, Zhang Q, Elinson RP (2001) Mesoderm formation in *Eleutherodactylus coqui*: Body patterning in a frog with a large egg. *Dev Biol* 236(1):109–123.
31. Callery EM, Fang H, Elinson RP (2001) Frogs without polliwogs: Evolution of anuran direct development. *Bioessays* 23(3):233–241.
32. Elinson RP, Beckham Y (2002) Development in frogs with large eggs and the origin of amniotes. *Zoology (Jena)* 105(2):105–117.
33. Chipman AD (2002) Variation, plasticity and modularity in anuran development. *Zoology (Jena)* 105(2):97–104.
34. Arendt D, Nübler-Jung K (1999) Rearranging gastrulation in the name of yolk: Evolution of gastrulation in yolk-rich amniote eggs. *Mech Dev* 81(1-2):3–22.
35. Gomez-Mestre I, Pyron RA, Wiens JJ (2012) Phylogenetic analyses reveal unexpected patterns in the evolution of reproductive modes in frogs. *Evolution*, 10.1111/j.1558-5646.2012.01715.x.
36. Wiens JJ, Kuczynski CA, Duellman WE, Reeder TW (2007) Loss and re-evolution of complex life cycles in marsupial frogs: Does ancestral trait reconstruction mislead? *Evolution* 61(8):1886–1899.
37. Chippindale PT, Bonett RM, Baldwin AS, Wiens JJ (2004) Phylogenetic evidence for a major reversal of life-history evolution in plethodontid salamanders. *Evolution* 58(12):2809–2822.
38. Vitt LJ, Caldwell JP (2009) *Herpetology* (Elsevier, Amsterdam), 3rd Ed.
39. Ewald AJ, Peyrot SM, Tyszka JM, Fraser SE, Wallingford JB (2004) Regional requirements for Dishevelled signaling during *Xenopus* gastrulation: Separable effects on blastopore closure, mesendoderm internalization and archenteron formation. *Development* 131(24):6195–6209.
40. Sadaghiani B, Thiébaud CH (1987) Neural crest development in the *Xenopus laevis* embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev Biol* 124(1):91–110.
41. Nieuwkoop PD, Faber J (1994) *Normal Table of Xenopus laevis (Daudin)* (Garland Publishing, New York).
42. Jones EA, Woodland HR (1989) Spatial aspects of neural induction in *Xenopus laevis*. *Development* 107(4):785–791.
43. del Pino EM, Medina A (1998) Neural development in the marsupial frog *Gastrotheca riobambae*. *Int J Dev Biol* 42(5):723–731.
44. del Pino EM, Escobar B (1981) Embryonic stages of *Gastrotheca riobambae* (Fowler) during maternal incubation and comparison of development with that of other egg-brooding hylid frogs. *J Morphol* 167(3):277–295.