
REVIEWS

Evolutionary Transformations of Ontogenesis in Anuran Amphibians

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Abstract—A review of the recent published data on ontogenesis of direct developing and marsupial frogs. The development of these representatives of anuran amphibians seems to be evolutionary advanced and considerably differs from the development of species traditionally used in amphibian embryology.

Key words: anuran amphibians, evolution of development, *Eleutherodactylus coqui*, *Gastrotheca riobambae*, *Xenopus laevis*.

The overwhelming majority of Anura species used for studying the early ontogenesis occurs in moderate latitudes of Europe, Asia, and North America. These animals lay eggs, as a rule, in water; swimming tadpoles develop from embryos, which then undergo metamorphosis (Dabagyan and Sleptsova, 1975, 1991; Reiss, 2002). A similar type of ontogenesis is also characteristic for the South African clawed toad *Xenopus laevis*, which became during the recent decades one of favorite model objects in developmental biology studies (Dettlaff and Rudneva, 1975, 1991). However, the early development of many anurans occurring in the tropics proceeds in a different way. For example, the embryos develop in a pouch, from which tadpoles pass to water. In some species, the development is direct, without the stage of tadpole, when miniature frogs hatch from the eggs laid onto the ground (for reviews see del Pino, 1989; Elinson, 1990; Elinson *et al.*, 1990; Duellman, 1992). Apparently, Elinson *et al.* (1990) were quite right in their statement that if developmental biology started with the studies of tropical species, our views on the main features of early ontogenesis of anurans would be radically different.

Studies of the patterns of embryogenesis in frogs with direct development began approximately 15 to 20 years ago. Here, we present a review of the relevant literature. In addition, the results of studies on these species and *X. laevis* were compared.

The stage of free-living tadpole is absent in more than 800 anurans, i.e., ca. 20% of all described species. It has been acknowledged that, within the order Anura, direct development appeared independently more than 10 times and it is evolutionarily more advanced than the biphasic life cycle including the larval stage (Duellman, 1985, 1992; Hanken, 1989; Elinson, 1990). However, such a frequent elimination of the tadpole stage suggests that initially, this larval stage appeared as an

“insert” in the evolutionary lineage that led to the development of anuran amphibians (Elinson, 1990; Callery *et al.*, 2001).

Among the Anura lacking the tadpole stage in their ontogenesis, the most detailed studies have recently been carried out on the coqui frog *Eleutherodactylus coqui*, a typical representative of the Puerto Rico Island fauna. Note that the genus *Eleutherodactylus* includes about 500 species occurring in humid tropical forests of Central America, all of them, apparently, with direct development (Callery *et al.*, 2001). However, some representatives of the genus *Eleutherodactylus* are at the brink of disappearance (Fogarty and Vilella, 2001). For example, the last findings of *E. eneidae*, *E. jasperi*, and *E. karlschmidti* were reported more than 20 years ago. Thus, these species, like many others, can hardly ever serve for developmental biology studies.

In *E. coqui*, fertilization is internal and polyspermic, unlike the external and monospermic fertilization in most anuran amphibians, and takes place 1 to 3 h before egg laying. Ovulation appears to require the corresponding sensory signals during the period of mating, since all attempts to stimulate ovulation by hormonal injections failed (Elinson *et al.*, 1990). Under the ideal laboratory conditions, each female lays approximately 40 eggs, 3.5–4.0 in diameter, rich in yolk and practically unpigmented, every five to eight weeks. Let us remember that the mature egg of *X. laevis* is 1.3 mm in diameter and its volume amounts to only 5% of that of *E. coqui* egg. In a humid chamber at 25°C, miniature frogs, ca. 6 mm in size, hatch from the eggs within ca. 17 days of incubation (Elinson *et al.*, 1990). The body size of adult coqui frog is 30–35 mm.

In nature, development takes place on the ground or in nests located at a height of 1–2 m above the ground. The male sits on the batch of eggs for ca. three weeks

and protects them against drying and predators, including conspecific males (Stewart and Pough, 1983). In the natural populations, the coqui frogs appear to be able to reproduce the whole year round, but their reproductive activity is lower during the period of rains than during the dry season (Fogarty and Vilella, 2002).

The general course of embryogenesis in *E. coqui* was described in several papers (Elinson, 1987, 1990; Hanken *et al.*, 1992, 1997; Elinson and Fang, 1998; Callery and Elinson, 2000a; Olsson *et al.*, 2002). The order of the early cleavage furrow differs from that in *Xenopus* and many other anuran amphibians. For example, the third cleavage division in *E. coqui* embryos, like the two preceding divisions, is longitudinal (meridional) and morphological differentiation into animal and vegetative blastomeres takes place only in 16-cell embryos, i.e., after the fourth cleavage division. The resulting eight small animal blastomeres account for only 1% of the total egg volume. They formed later a very thin semitransparent blastocoel roof, give rise to ectoderm and many derivatives of mesoderm. Since the eggs are devoid of pigment, the prospective dorsal and abdominal sides of the embryo are indistinguishable until the early gastrula stage, when the dorsal blastopore lip is formed in the equatorial zone. Let us remind that in *Xenopus* and *Rana*, the dorsal blastopore lip is formed in the vegetal half of the embryo. The development of larval structures, characteristic for Anura with the "classical" (biphasic) type of development, is suppressed in *E. coqui*. The cement gland (sucker), lateral line organs, larval jaws, and spirally coiled larval intestine do not develop, while the development of gills and operculum is markedly suppressed. The only evident larval structure is the tail rudiment, which serves for gas exchange, rather than for movement, and which degenerates within two days after hatching.

On the contrary, some structures are formed in *E. coqui* at a higher rate. For example, limb buds appear soon after the neural tube closure (Hanken *et al.*, 2001), while in *R. temporaria* and *X. laevis*, they are formed only at the stage of feeding tadpole (Dabagyan and Sleptsova, 1975, 1991; Dettlaff and Rudneva, 1975, 1991). According to the recent data (Schlosser, 2003), the spinal cord develops in *E. coqui* also at a higher rate, due to a more intense cell proliferation, than in Anura with biphasic type of ontogenesis. These facts suggest that the appearance of direct development in anuran amphibians is related not only to the loss of larval phase, but also to a complex of embryonic transformations.

Let us consider now some experimental embryological and molecular biological data. The first work dealing with inductive interactions in development of *E. coqui* was published less than 10 years ago (Fang and Elinson, 1996). It was shown that after transplantation of the region of animal pole of the *X. laevis* blastula into presumptive head region of the *E. coqui* gastrula, the transplanted material formed the cement gland in

the anterior region of head. On the contrary, when fragments of blastocoel roof of the *E. coqui* embryos were transplanted in the presumptive head region of *R. pipiens* blastula or gastrula, the transplanted material never formed the cement gland cells. Note that in the last experimental variants, the embryos of *R. pipiens*, another species of frogs with biphasic type of ontogenesis and similar to *E. coqui* rate of embryogenesis, were used, rather than *X. laevis* embryos characterized by a very high development rate.

Thus, the presumptive head region of *E. coqui* embryos appears to generate signals essential for the cement gland formation but the ectoderm of *E. coqui* is not capable of responding to these inductive signals. In other words, when direct development appears, the competence to the formation of specific tadpole structures, such as cement gland, is lost. This suggestion was also confirmed in the corresponding *in vitro* experiments: in both variants of interspecific (heterospecific) recombination of the reacting and inducing tissues from the *X. laevis* and *E. coqui* embryos (Fang and Elinson, 1996).

In *X. laevis*, the homeobox gene *otx2* directly controls the cement gland formation, but it is expressed not only in the presumptive rudiment of this gland, but also in the anterior region of neural plate (Pannese *et al.*, 1995; Gammill and Sive, 1997). It has recently been shown (Fang and Elinson, 1999) that the gene *Ecotx2*, identified in *E. coqui* and a cloned homolog of the *X. laevis* gene *otx2*, is capable of expression and induction of cement gland in the *X. laevis* ectoderm, while in the *E. coqui* embryo, the gene *Ecotx2* is normally expressed only in the anterior neuroectoderm.

Comparative studies of development of the lateral line organs in amphibians (Schlosser *et al.*, 1999; Schlosser, 2002) have shown that the gene *NeuroD*, which is transiently expressed at the early stages of neurogenesis in *X. laevis* and *E. coqui*, is later expressed also in the lateral line placodes in *X. laevis*. The experiments with heterospecific transplantation of ectoderm between the *E. coqui* and *Ambystoma mexicanum* embryos suggested that the lateral line organs in *E. coqui* were not formed as a result of the loss of competence to the corresponding inducing signals that were generated in the *E. coqui* embryos. This noninduced region of ectoderm of *E. coqui* is later characterized by the absence of *NeuroD* expression (Schlosser *et al.*, 1999).

Thus, the experimental data that account for the absence of cement gland and lateral line organs in the *E. coqui* embryos agree quite well with each other: in both cases, the ectoderm is devoid of specific competence (it cannot respond to the corresponding inducing signals) and later lacks the expression of tissue specific genes.

The gene *noggin*, its transcripts, and the corresponding protein play an important role in embryonic induction and differentiation in *X. laevis* (Smith *et al.*, 1993;

Knecht *et al.*, 1995). The ectopic expression of *noggin* RNA after its microinjection in the ventral region of *X. laevis* embryos induces only the formation of a partial secondary axis, without head structures. However, the expression of *noggin* RNA from *X. laevis* after its injection in the *E. coqui* embryos induces the complete secondary axis, including the head (Fang *et al.*, 2000). These significant differences in the response of two frog species to exogenous *noggin* RNA of *X. laevis* suggest that the dorsal determinants located in the narrow region of vegetal cortex in *X. laevis* are distributed much wider in larger *E. coqui* eggs. The dorsal axis is specified in embryogenesis of *E. coqui* later than in *X. laevis* (Elinson and Ninomiya, 2003). Comparative analysis of specific developmental features of these two species (Ninomiya *et al.*, 2001) has shown that in *E. coqui*, mesoderm is formed closer to the animal pole and closer to the embryo's surface than in *X. laevis*. This difference can also be explained by an evolutionary increase of egg size and yolk content in *E. coqui*.

Despite the radical changes in the type of development, metamorphosis still persists in ontogenesis of *E. coqui*, which takes place before hatching and is controlled by the thyroid hormone (Callery and Elinson, 2000b). This was shown in the experiments with methimazole, an inhibitor of thyroid hormone synthesis. If the treatment was performed before the thyroid gland maturation in the embryo, the metamorphic changes of head, limbs, tail, skin, muscle, and cartilage were inhibited. However, the *E. coqui* embryos blocked by methimazole could undergo metamorphosis after additional treatment with thyroid hormone.

Thus, the published data suggest that the coqui frog *E. coqui* became a promising model object for developmental biology studies in several laboratories. New studies can be expected on the embryos of this anuran species. The studies of cell cycles in embryogenesis would be of special interest. So far, the changes in the intensity of cell proliferation in the *E. coqui* embryos were followed only during the spinal cord development (Schlosser, 2003).

Other anuran species with "nonstandard" types of ontogenesis have been less extensively studied as concerns embryogenesis. Among marsupial tree frogs of the tropic South America, *Gastrotheca riobambae* was used in some studies, which occurs in the mountain valleys of Ecuador at a height of 2500–3200 m above sea level (del Pino, 1989; Elinson *et al.*, 1990). The mating of these frogs takes place on the ground, fertilization is external. The batch contains 130 eggs, on average, with a diameter of ca. 3 mm. The eggs are rich in yolk and very weakly pigmented. Under the high mountain conditions (lowered atmospheric pressure), the embryos develop in a pouch on the mother's back for 3 to 3.5 months at a temperature in the terrarium of 17–23°C. The resulting tadpoles are then liberated from the pouch into water. Metamorphosis takes place within several months, while the tadpoles feed and grow from

18–20 to 70 mm. Note that when these frogs are brought to Europe, their embryogenesis under the normal atmospheric pressure proceeds faster by a factor of 1.5–2 but the mortality rate is ca. 50% (Elinson *et al.*, 1990).

Thus, the studies on embryology of *G. riobambae* are mostly carried out at the laboratory of E. del Pino at the Catholic University of Ecuador in Quito located at a height of 2800 m above sea level (del Pino and Escobar, 1981; del Pino and Elinson, 1983; del Pino and Loo-Vela, 1990; del Pino, 1996; del Pino and Medina, 1998). The survival rate of embryos in the laboratory amounts to 99.4% and the reproduction of frogs can be stimulated by progesterone at any season. The early embryogenesis of *G. riobambae* is characterized by a very slow rate: the duration of one cell cycle during the period of cleavage at 17°C is 15–20 h. The furrows of the three first cleavage divisions are meridional and gradually pass over the egg from the animal pole to the vegetal one (del Pino and Loo-Vela, 1990). Desynchronization of divisions, appearance of nucleoli, and related activation of rRNA synthesis take place already at the 8-cell stage. Let us remember that in *X. laevis*, the period of synchronous cleavage divisions includes ca. 10 cell cycles, with the duration of 45 min at 17°C (Detlaff and Rudneva, 1975, 1991).

The early embryogenesis resembles in some features the early development of mammals: a very slow rate of cleavage, very early desynchronization of cleavage divisions, and RNA synthesis during the period of cleavage (see Gilbert, 1991). The embryos of marsupial frogs resemble also the mammalian embryos in that they receive water from the maternal organism and gas exchange is also realized with the help of mother (del Pino and Escobar, 1981; del Pino, 1989). However, the early embryogenesis of marsupial frogs differs from the early development of placental mammals in a large size of eggs and great endogenous reserve of yolk in the embryos, which is used for their feeding (there is no transfer of nutrients from the mother to the embryos).

In *G. riobambae*, the stages of middle blastula, early gastrula, and early neurula occur approximately within 4, 7, and 14 days after fertilization, respectively (del Pino and Escobar, 1981; Elinson *et al.*, 1990). The blastula of *G. riobambae* differs from the typical amphibian blastulae only in size. It consists of small animal blastomeres (micromeres), which form the semitransparent roof of a vast blastocoel, and large yolk-rich vegetal macromeres. However, gastrulation, which represents a combination of epiboly and invagination, proceeds in an unusual way (del Pino and Elinson, 1983; del Pino, 1996). The analysis of gastrulation is facilitated by the fact that the embryos of *G. riobambae* can successfully develop in saline, outside the pouch. By the end of midgastrula stage, blastocoel almost completely disappears in the animal hemisphere due to the expansion of large yolk-rich cells. In the vegetal hemisphere, small cells of closing blastopore lips

form the germ disc surrounding the short archenteron. As the archenteron elongates, the germ disc is also enlarged and forms the archenteron roof. In the very end of gastrulation, the embryo overturns under the influence of gravity and the germ disc comes upwards and later gives rise to neural ridges and all other embryonic structures which are, thus, located on the yolk mass surface, like in reptiles and birds. It is still unknown whether in *G. riobambae* the dorsal organizer functions before or after the blastopore closure (del Pino, 1996).

G. plumbea is another representative of marsupial frogs of the genus *Gastrotheca*, for which there is certain information concerning embryogenesis (Elinson *et al.*, 1990). It occurs in humid tropical forests of Ecuador at a height of 1300–2350 m above sea level and is characterized by large eggs (4–5 mm in diameter) and direct development. After ca. 120 days of incubation in the mother's pouch, 25–30 miniature frogs are born. The stage of free living tadpole is absent. Despite the differences in ontogenesis of *G. plumbea* and *G. riobambae*, an embryonic disc is also formed during gastrulation in *G. plumbea* (del Pino and Elinson, 1983).

It is of interest to consider oogenesis of the South American marsupial frogs. The oogenesis of *Flectonotus pygmaeus*, *G. fissipes*, and some other species is characterized by the presence of a transient multinuclear phase, while in *G. riobambae*, *G. plumbea*, and some other species, oocytes are mononuclear at all developmental stages (del Pino and Humphries, 1978; Elinson *et al.*, 1990). More detailed studies were carried out on *F. pygmaeus* (Macgregor and del Pino, 1982). Its early oocytes contain 2000 nuclei each, on average, and each nucleus amplifies its own rDNA, while the level of amplification varies in different nuclei. During vitellogenesis, only one nucleus is preserved in the oocyte, while all others degenerate. As a result of such "multinuclear" oogenesis, the mature eggs of *G. pygmaeus* (3 mm in diameter) are provided with a very great reserve of rDNA. Del Pino *et al.* (1986) analyzed oogenesis in *G. riobambae* as well and showed that the level of amplification of ribosomal genes and amount of stored rDNA were insignificant, as compared to either *X. laevis*, or *F. pygmaeus*.

Note that under the same conditions of terrarium, embryonic and larval development of *F. pygmaeus* proceeds at a much faster rate than in *G. riobambae*. *F. pygmaeus* bears 5–11 (more often 6–7) embryos in its pouch for 20–30 days. Thereafter, tadpoles pass to the water, which are so advanced in their development that do not feed and undergo metamorphosis within one to three weeks (del Pino and Escobar, 1981; del Pino, 1989; Elinson *et al.*, 1990). Thus, the comparison of ontogenetic features of these two marsupial frogs suggests that the multinuclearity of oocytes and accumulation of a great reserve of rRNA in the eggs provides for a significant acceleration of embryogenesis in *F. pyg-*

maeus. In addition, it may well be that in this species, desynchronization of cell divisions and activation of rRNA synthesis take place at a later stage of embryogenesis than in *G. riobambae*. However, the chronology of embryogenesis, as well as cleavage and gastrulation of *F. pygmaeus* were not specially studied. Unfortunately, this marsupial frog from the tropical forests of Venezuela is a very rare species, which limits the possibilities of embryological research on this species.

The yellow-spotted reed frog *Hyperolius puncticulatus* may become a promising object of developmental biology studies. It occurs in Ruanda, Uganda, and Tanzania. Its eggs insignificantly exceed in size (1.5–1.8 mm) those of *X. laevis* but are very rich in yolk. The first data on morphology and chronology of embryogenesis of *H. puncticulatus* have recently been published (Chipman *et al.*, 1999, 2000; Chipman, 2002). Apparently, the type of development of *Hyperolius*, which includes the tadpole stage, can be considered intermediate between the "classical" type of development of eggs with a relatively low yolk content, as in most Anura, and extreme type of development of eggs with the highest yolk content and germ disc formation, characteristic of the genus *Gastrotheca*.

Let us state in conclusion that analysis of the literature on comparative embryology of frogs with direct development and of marsupial frogs shows a highly variable ontogenesis in Anura. The recent experimental embryological studies on tropical species of anuran amphibians, above all, on *E. coqui*, disclose certain mechanisms of evolutionary transformations of ontogenesis of the frogs with large and yolk-rich eggs. The authors working in this field (Ninomiya *et al.*, 2001; Elinson and Beckham, 2002) propose that these transformations of early development in frogs are parallel to the events that led to the appearance of large yolk-rich eggs of higher vertebrates (Amniota) more than 350 million years ago. Hence, further studies of early embryogenesis of *E. coqui* will also be helpful for testing hypotheses on the origin of amniotes.

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