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The pattern of early cleavage of the marsupial frog *Gastrotheca riobambae*

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Summary

Comparison of early development of the marsupial frog *Gastrotheca riobambae* with *Xenopus laevis* suggests that the cleavage pattern of *Xenopus* and the tight coupling of events at the midblastula transition are features of the accelerated development of small amphibian eggs with aquatic reproduction, rather than generalized features of amphibian development. The large eggs of the marsupial frog *Gastrotheca riobambae* not only display an atypical holoblastic pattern of cleavage and a very slow rate of development, but the events of the midblastula transition are uncoupled, suggesting that amphibians may have a diversity of developmental patterns. Early cleavage of the egg, which measures about 3 mm in diameter, occurs mostly by meridional and vertical furrows. The first cleavage cycle takes about 16 to 20 h and about four days may be required to reach the midblastula stage. Cleavage becomes asynchronous at about the third cleavage cycle evidenced by the formation of cleavage furrows. However, during cleavage (up to 342-cells), the majority of the nuclei divide synchronously and only 15 to 40 % of the nuclei of a given embryo have a different cleavage schedule. At the 8-cell stage, nucleoli become visible (approximately 24 h after amplexus), signaling that transcription of rRNA has started at this early stage. Cell motility was detected in three- to four-day old embryos and seems to be associated with changes in cell shape and with expansion of the blastocoel at this stage.

Key words: cleavage, frog development, midblastula transition, marsupial frogs, *Gastrotheca*, *Xenopus*.

Introduction

The pattern of early development is known for a limited number of frog species, the majority of which reproduce in the water and spawn eggs of about 1 to 2 mm in diameter. Among frogs, there are many reproductive strategies and, associated with them, the size of the egg and the schedule of early developmental events may vary. However, for frogs with unusual reproductive modes, the characteristics of early development remain for the most part unknown (reviewed by del Pino, 1989). This study aims at identifying and characterizing developmental changes during the period of cleavage in the marsupial frog *Gastrotheca riobambae*.

Significant deviations of the reproductive mode and of the size of the egg occur among the marsupial frogs (Hylidae). Eggs of *G. riobambae*, for example, measure 3 mm in diameter and other members of this genus such as *Gastrotheca cornuta* produce enormous eggs of about 10 to 12 mm in diameter, which are the largest eggs so far described for the anurans (del Pino and Escobar, 1981). A further deviation is that development in marsupial frogs does not occur in the water but inside a pouch located on the back of the mother. The conditions of the pouch are different from fresh water as can be inferred from the information available (reviewed by del Pino, 1989). The developmental strategies of *G. riobambae* include limited amplification of the ribosomal genes during oogenesis. In addition, the amount of oocyte rRNA is apparently lower than in *X. laevis* (del Pino *et al.* 1986). Such deviations of the process of oogenesis are followed by a modified scheme of development which includes an extremely slow developmental rate and changes in the patterns of cleavage and gastrulation (del Pino and Elinson, 1983; Elinson and del Pino, 1985). Among other species of egg brooding frogs, such as *Flectonotus pygmaeus*, there are additional modifications of oogenesis and of the developmental pattern. Early oocytes of *F. pygmaeus* are multinucleated with about 2000 nuclei instead of one germinal vesicle (del Pino and Humphries, 1978; Macgregor and del Pino, 1982) and early development occurs faster than in *G. riobambae* (del Pino, 1989).

Materials and methods

Embryos and their culture

Early embryos of *Gastrotheca riobambae* were obtained from 10 spontaneous matings in the laboratory. To obtain a sequence of early developmental stages, only a few embryos were removed at any one time, and the remaining were left in the pouch for further development. The pattern of early

cleavage could be followed in embryos immersed in modified Barth saline (MBS, according to Gurdon, 1968). The age of the embryos was calculated in regard to the time when the frogs separate after amplexus and the stages of development were determined according to del Pino and Escobar (1981). The study is based on the analysis of approximately 350 embryos. Frogs and embryos were kept at room temperature (17°C to 18°C).

Observation of fixed embryos and histological procedures

The cleavage pattern of *G. riobambae* embryos was studied in fixed material. Embryos were fixed in Smith's fixative for periods of 24 h to several days (Rugh, 1965), washed with distilled water, and stored in 4% formalin until the time of observation. Embryos swell in this fixative making the cleavage furrows distinct. However, at the one-cell stage, embryos explode during fixation if they are not protected by the thin envelope of jelly. For each embryo, the cleavage pattern was recorded by drawing the animal and vegetal views, some embryos were photographed.

To analyze the phase of the cell cycle for each blastomere as well as the number of nuclei per embryo, embryos were embedded in Paraplast+ and cut into 5 μm thick sections. Sectioned embryos were double stained with the Schiff reagent using Brilliant-Cresyl-Blue as colorant (according to P. Hausen, personal communication). The Schiff reagent was used in the Feulgen reaction to identify the nuclei as well as in the Periodic-acid-Schiff method (PAS) to stain the cell borders. Double staining facilitated the identification of the nuclei as well as of the individual blastomeres in the yolky embryos of this frog. The Schiff reagent was prepared by dissolving 4.5 g of Brilliant-Cresyl-Blue in 600 ml of a solution that contains 75 ml 1 N HCl and 3.7 g $Na_2S_2O_5$ brought to a final volume of 600 ml with distilled water. This reagent was filtered before use.

The onset of cell movements

Living embryos were ruptured with watchmaker forceps in 1 ml MBS or in modified Ringer solution (MMR) containing 5% Ficoll (Kimelman *et al.* 1987) inside a tissue culture plate of 1.5 ml capacity. The perivitelline space in embryos of this frog is very small and we have been unable to remove manually the thin layer of jelly and the fertilization envelope without wounding the embryo. The contents of the ruptured embryo spread over the bottom of the well and individual blastomeres could be identified with the stereo-microscope at the periphery of the embryo. Sketches of the shape of blastomeres in regard to their location were done at regular intervals. Some blastomeres were transferred to a depression slide and their movements were observed at higher magnification. The study is based on the analysis of 16 embryos from two females.

The onset of embryonic transcription

Embryos of *G. riobambae* at the one-cell stage were microinjected with 0.1 to 0.5 μCi of [^{3}H]uridine with a specific activity of 29 Ci mmol^{-1} (Amersham) in 100 to 500 nl of MBS and allowed to develop inside a humid chamber. Eggs were wounded by micro-injection and lost yolk, but continued to cleave for 24 h or more. To observe the incorporation of [^{3}H]uridine at later stages, embryos were incubated in a 10 μl drop of MBS containing 1 to 5 μCi of [^{3}H]uridine. Afterwards, embryos were fixed, sectioned and processed for autoradiography using the Ilford Nuclear Research Emulsion type L4. After exposures of 3 to 6 days at 4°C, slides were developed and double stained with the Schiff reagent. As controls, some sections of each embryo were treated with 0.5 to 2 mg ml^{-1} of pancreatic ribonuclease for 4 to 24 h at 37°C. Additional controls included embryos treated with MBS and embryos treated with [^{3}H]thymidine. The concentrations of [^{3}H]thymidine were the same as those of [^{3}H]uridine in the parallel experiments. Micro-injection and incubation in the radioactive precursors were performed by the end of the first cell cycle (about 12 h after the end of amplexus). By this time, the polar bodies have been extruded and, in some embryos, the first cleavage furrow was forming. Embryos manipulated earlier during the first cell cycle did not develop. The study is based on the analysis of 19 embryos from four females (data not shown).

Table 1. *Age and number of blastomeres in embryos from the brood of a* G. riobambae *female*

Time after amplexus (hours)	Number of blastomeres*	Number of embryos analyzed
0.35–13.18	1	49
19.43	2–16	17
42.68	43–73	6
69.18	184–388	5
Total of embryos analyzed:		77

* Includes completed blastomeres as well as those whose furrows are being formed.

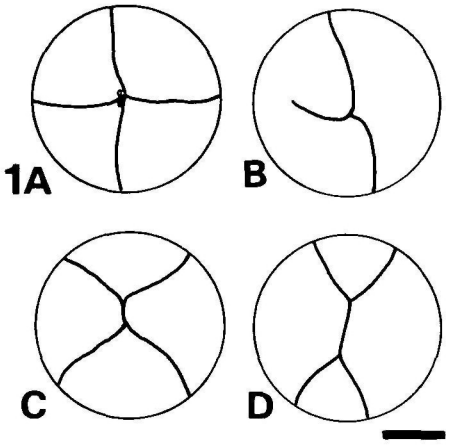
Results

The cleavage pattern

The conditions for *in vitro* fertilization of the *G. riobambae* eggs are unknown and the embryos for this study were obtained from spontaneous matings in the laboratory. Amplexus allows the successful fertilization of eggs, but the time of fertilization is uncertain, because embryos taken from the pouch at a given time display several developmental stages (Table 1). At the time of amplexus, the male may deposit semen over the female's back and each egg may become fertilized during the journey from the cloaca to the pouch. Although the transfer of each individual egg takes only a few seconds, the movement of an average of 128 eggs to the pouch lasts from 6–8 h. Differences in the time of fertilization of individual eggs may allow for the observed differences in the age of embryos.

In the following description of the cleavage pattern, the planes of cleavage are described according to Nelsen (1953) as being meridional, vertical, equatorial or latitudinal. A meridional plane of cleavage tends to pass in a direction that would bisect both poles of the egg passing through the egg's median axis, while the vertical plane of cleavage passes through one side of the median axis of the egg. The equatorial plane bisects the egg at right angles to the median axis at equal distance from the animal and vegetal poles. The latitudinal plane also bisects the egg at right angles to the median axis, but in contrast to the equatorial plane, it is displaced from the median region towards one of the poles (Nelsen, 1953).

The cleavage furrows divide the large and pale eggs



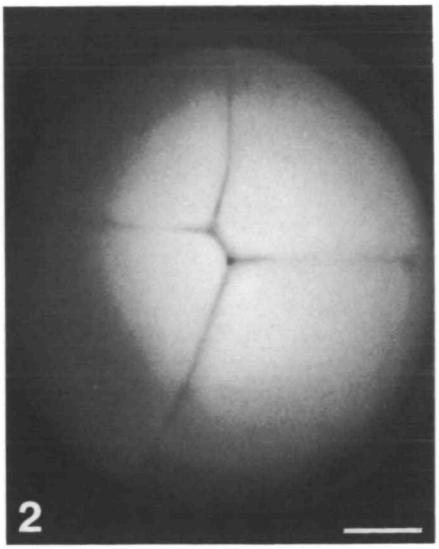


Fig. 1. Drawings of the 4-cell embryo of *G. riobambae* (Stage 3). (A–C) View from the animal pole. (D) View from the vegetal pole. The first two cleavage furrows intersect at the animal pole in some embryos (A), while in many others, these two furrows meet at a distance from the animal pole (C). In some embryos, the second cleavage furrows are asynchronous, as shown in B. In the vegetal hemisphere, the first two cleavage furrows meet at a great distance from the vegetal pole (D). Bar, 1 mm.

Fig. 2. Photograph of a stage 3 embryo of *G. riobambae* viewed from the animal pole, after fixation in Smith's fixative. This embryo has the same pattern shown in Fig. 1C. The first two cleavage furrows do not intersect at the animal pole in the majority of the embryos, producing blastomeres of different sizes. Bar, 0.5 mm.

of *G. riobambae* completely, but the pattern of cleavage is different from frogs with aquatic eggs of 2 mm in diameter or less such as the eggs of *Rana* or *X. laevis* (see Nieuwkoop and Faber, 1967; Shumway, 1940; and Nelsen, 1953, for comparison). In most cases, the first cleavage furrow is meridional as in the mentioned anurans. However, there are frequent cases in which this furrow is vertical and one of the blastomeres is larger than the other. Embryos of *G. riobambae* may require 16 to 20 h from the time of amplexus to the completion of first cleavage (stage 2).

The second cleavage furrows of the *Gastrotheca* egg begin to form in the animal hemisphere once first cleavage has been completed (Stage 3). As in the case of *X. laevis*, these furrows are meridional (Fig. 1A) in most eggs. In addition in many cases, the second cleavage furrows are vertical and form at some distance from the median axis of the egg (Figs 1C; 2). In the vegetal hemisphere, the second cleavage furrows meet the first one at greater distance from the median axis than in the animal region (Fig. 1D). Although the formation of these furrows is synchronous in most embryos, there are cases in which one of the blastomeres cleaves first, producing a transient three-blastomere embryo (Fig. 1B). The second division of the *G. riobambae* egg produces 4 blastomeres of unequal size (Figs 1, 2) and may occur 16 to 21 h after amplexus.

The third cleavage furrows (stage 4) form once the second cleavage furrows are completed. These furrows are vertical instead of latitudinal and, at this stage, each egg seems to follow a unique pattern of cleavage (Figs 3, 4). The formation of the third cleavage furrows occurs after approximately 24 h from the end of amplexus. There are cases in which these furrows do not appear in synchrony and embryos with higher or with lower numbers of blastomeres than the expected number of 8 blastomeres are found among the embryos of a brood (Fig. 3E–F).

In embryos of *X. laevis* latitudinal and meridional furrows alternate during early cleavage. The third cleavage plane is latitudinal and divides the egg into 4 animal blastomeres of smaller size and 4 larger vegetal blastomeres (Nieuwkoop and Faber, 1967) (Fig. 3A). However, there are cases in which the third cleavage furrow has been replaced by two vertical furrows, a situation that resembles the pattern of cleavage of the *G. riobambae* eggs. The cleavage pattern of *X. laevis* is synchronous. At the 16-cell stage (Fig. 5A) cleavage produces 8 animal and 8 vegetal blastomeres and the following cleavage cycle gives rise to 32 blastomeres (Fig. 6A). In contrast, embryos of *G. riobambae* display a variety of patterns and cell numbers during early cleavage (Figs 5B–I; 6B–C; 7). After the third cleavage cycle, the blastomeres of a *Gastrotheca* embryo divide at different speeds. In addition, the pattern of cleavage differs among embryos (Figs 5, 6, 7).

In contrast to *Xenopus*, the third cleavage furrows of a *Gastrotheca* embryo may still be incomplete before new division begins in some blastomeres (Fig. 3B–I). Such cleavage pattern generates some fully formed blastomeres, while others are at different stages of furrow formation. For example, the embryo shown in Fig. 3E has 5 complete blastomeres and 5 additional blastomeres in the process of cleavage adding to a total

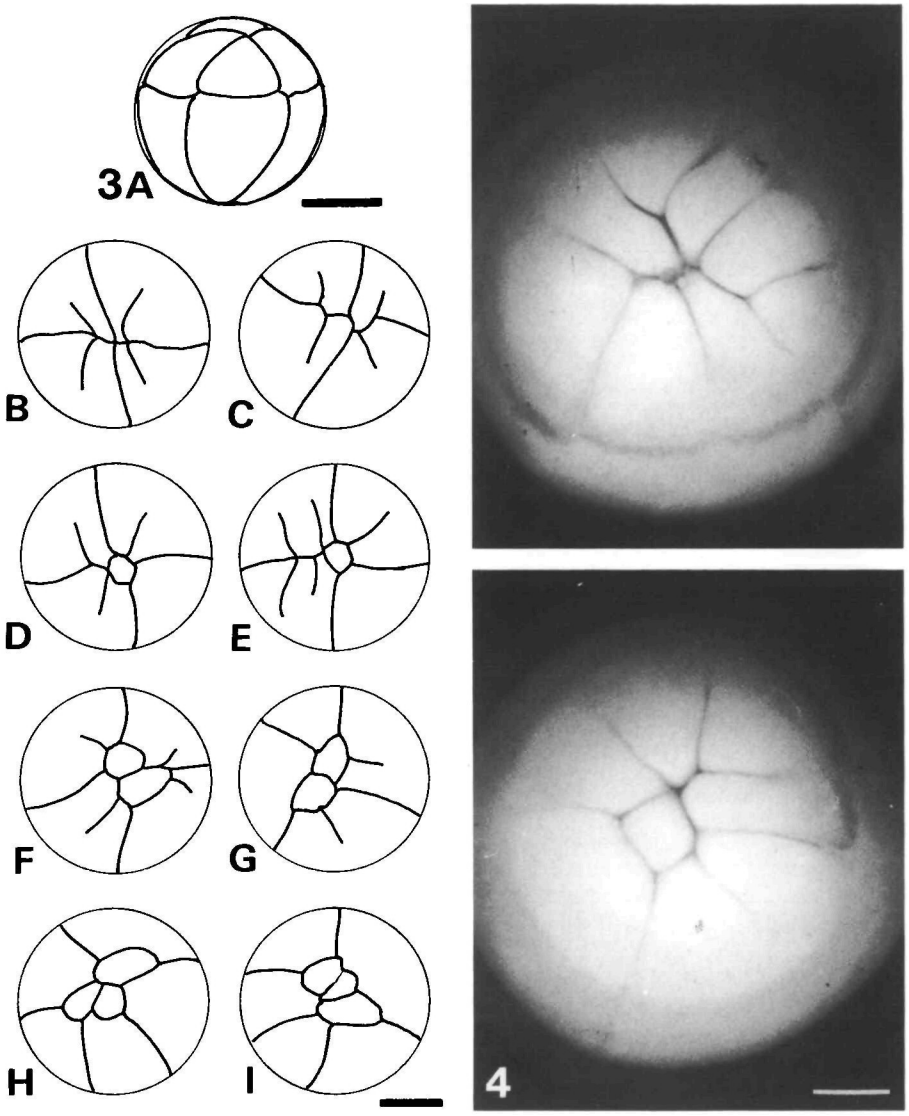


Fig. 3. The 8-cell embryos of *G. riobambae* (stage 4) and of *X. laevis*. (A) 8-cell embryo of *X. laevis* redrawn from Nieuwkoop and Faber (1967), lateral view. Bar, 0.5 mm. (B–I) Embryos of *G. riobambae* viewed from the animal pole. Bar, 1 mm. The third cleavage furrow in *X. laevis* is latitudinal and divides the egg into 4 animal and 4 vegetal blastomeres (A). In *G. riobambae*, in contrast, the third cleavage furrow has been replaced by vertical furrows and each embryo seems to follow a different pattern of cleavage (B–I). In the vegetal region, the first two cleavage furrows meet at a distance from the vegetal pole as shown in Fig. 1D. The embryos shown in E–F contain 10 blastomeres, including the totally cleaved blastomeres and those undergoing cleavage.

Fig. 4. Photographs of stage 4 embryos of *G. riobambae* viewed from the animal pole, after fixation in Smith's fixative. The third cleavage furrows are vertical and divide the eggs with irregular patterns. The vegetal view was comparable to the pattern shown in Fig. 1D. Bar, 0.5 mm.

of 10 cells for this embryo. These deviations in the formation of cleavage furrows suggest asynchrony of division in the embryos of *G. riobambae*. During early cleavage, the majority of cleavage furrows are vertical and originate in the animal region. This pattern of cleavage produces smaller and more numerous blastomeres in the animal hemisphere (Figs 5B,D,F,H; 6B; 7) than in the vegetal region (Figs 5C,E,G,I; 6C). The majority of the nuclei are displaced towards the surface of the animal region, as seen in histological sections. The vegetal and the internal regions of the embryo slowly become fragmented into smaller blastomeres as cleavage proceeds, but the cleavage planes that produce internal blastomeres have not been analyzed. A few binucleate blastomeres were detected mostly in the vegetal region of the embryo, possibly because the cleavage furrows were still incomplete.

The speed and the synchrony of early cleavage

Cleavage of the large eggs of *G. riobambae* is extremely slow (Fig. 8). Embryos of *X. laevis*, in contrast, cleave rapidly and regularly every half hour or so (Fig. 8), based on the data of Nieuwkoop and Faber (1967). Temperature influences the rate of development and the comparison of Fig. 8 may, to some extent, misrepresent the differences, since development of *G. riobambae* was studied at 17–18 °C, while development of *X. laevis* embryos corresponds to 20–21 °C. The *G. riobambae* embryos not only cleave slowly, but the synchrony of cleavage is lost early in development as evidenced by the cleavage pattern (Figs 1–7) and by the scattering of the data in Fig. 8. Cleavage can become asynchronous as early as at the time of formation of the second cleavage furrows (Fig. 1B). Cleavage asynchrony becomes conspicuous during subsequent stages as shown in Figs 3–7. Among the embryos of one female, a diversity of cell numbers were found instead of the powers of 2, expected from synchronous cleavage (Table 1). In addition, only one of 40 embryos from the broods of different females, had 8 complete blastomeres at stage 4. In most embryos, by the time that the third cleavage furrows were completed, several furrows were already being formed,

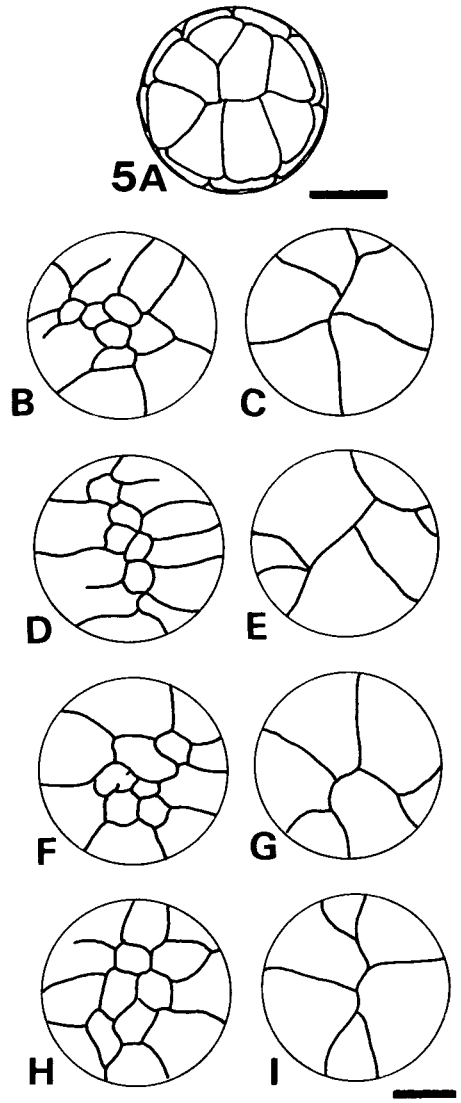


Fig. 5. The 16-cell embryo of *X. laevis* and early stage 5 embryos of *G. riobambae*. (A) 16-cell embryo of *X. laevis* redrawn from Nieuwkoop and Faber (1967), viewed from the animal pole. Bar, 0.5 mm. (B–I) Embryos of *G. riobambae*. Bar, 1 mm. The animal view (B,D,F,H) and the vegetal view (C,E,G,I) of four embryos is given. The pattern of cleavage varies among embryos.

producing embryos with unusual numbers of blastomeres (Fig. 3E,F). By stage 5, only one of 23 embryos studied had the expected number of 16 fully formed cells, others had lower or higher numbers of blastomeres (Fig. 5B–I). Similarly, the number of blastomeres at more advanced stages of cleavage did not coincide with the expected values for synchronous cleavage (Figs 6B; 7, 8), suggesting that embryos of *G. riobambae* cleave asynchronously. In addition in living embryos cultured *in vitro*, the formation of new blastomeres was irregular and asynchronous (Table 2). The observation of the living embryos was restricted to the early stages because the furrows become difficult to distinguish at more advanced stages of cleavage.

The phases of the cell cycle were analyzed in 14

Table 2. *Cleavage pattern in living embryos*

Embryo number	Time (hours)	Number of complete blastomeres	Total number of blastomeres†
1	0.0*	2	4
	4.0	2	4
	5.5	6	8
	8.5	10	14
2	0.0*	2	4
	4.0	2	4
	5.5	7	8
	8.0	10	14
3	0.0*	5	6
	4.0	9	14
	6.5	11	16
4	0.0*	5	11
	0.5	10	13
	1.5	20	22
	3.0	23	25
	4.0	26	29
5	0.0*	9	10
	4.5	11	12
	6.5	11	13
6	0.0*	10	10
	0.5	13	15
	2.0	15	16
7	0.0*	12	16
	1.0	15	20

† Number of blastomeres totally formed plus those that are incompletely cleaved.
* Beginning of observations.

embryos that were cut into serial sections (Table 3). For most embryos, the majority of the nuclei were found in interphase or in prophase, suggesting that these stages occupy most of the cell cycle. In one embryo of stage 5, all nuclei were found in mitosis, which signals tight synchrony in the cleavage schedule of this embryo (Table 3). However, for the remaining embryos of stage 5, blastomeres were found in two major phases, interphase and mitosis. 51–85 % of the cells were found in one of these phases, suggesting that most of the cells were cleaving synchronously. However, the remaining 15–49 % of the cells had a different cleavage schedule (Table 3). Therefore, although the bulk of blastomeres maintains synchrony of cleavage, a small population of blastomeres seems to cleave asynchronously in the early embryos of *G. riobambae*.

Cell movements during early development

Cell dissociation of cleaving stage embryos (stage 5) of *G. riobambae* occurs spontaneously after wounding the embryo in MBS. Uninjured cleavage stage embryos of *X. laevis*, in contrast, require a raise in the pH of the medium (Kimelman *et al.* 1987) to become dissociated. Because of differences in the procedures, no conclusions can be made in regard to the attachment between early blastomeres of the *G. riobambae* embryo. Large and small blastomeres from early embryos of *G. riobambae* are spherical and appear motionless. However, 78 h after amplexus, blastomeres

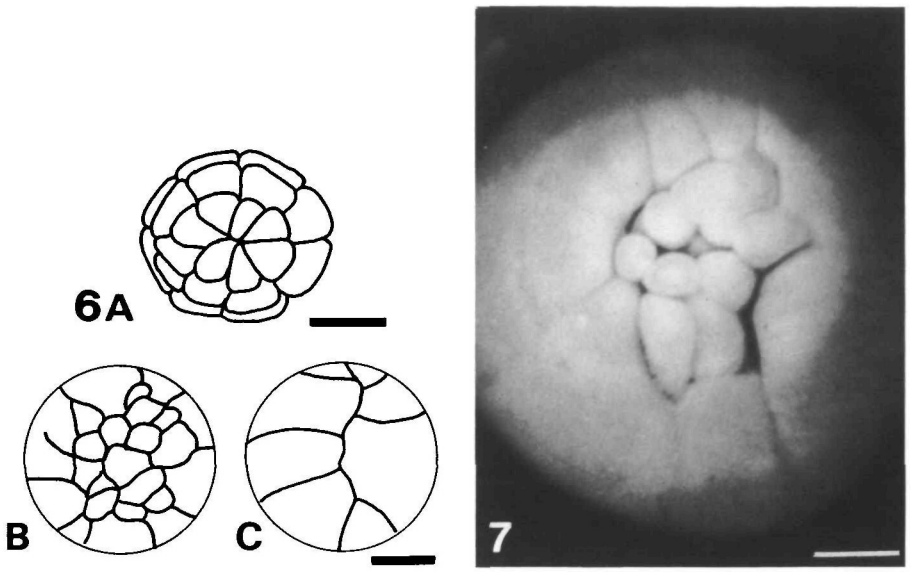


Fig. 6. The 32-cell embryo of *X. laevis* and an early stage 5 embryo of *G. riobambae*. (A) 32-cell embryo of *X. laevis* redrawn from Nieuwkoop and Faber (1967), viewed from the animal pole. Bar, 0.5 mm. (B–C) An embryo of *G. riobambae* viewed from the animal and vegetal pole, respectively. Bar, 1 mm. This embryo contains about 28 blastomeres.

Fig. 7. Photograph of an embryo of *G. riobambae* with 19 blastomeres viewed from the animal pole, after fixation in Smith's fixative. Bar, 0.5 mm.

begin to acquire non-spherical shapes and have numerous small and large protrusions that display random movements. Some protrusions are resorbed, while others enlarge in the course of 1 to 4 h allowing changes in the shape of the cells and translocation of the blastomeres. Cells were observed at intervals of 15 to 60 min and sketches of cell shape and position in regard to neighboring cells and to landmarks of the dish were required to identify the individual blastomeres. The extent of cell translocation before a cell divided cannot be determined from these observations. These movements may be comparable to the translocation and formation of protrusions described by Kimelman *et al.* (1987) for dissociated cells of *X. laevis* embryos at the midblastula transition.

The onset of motility in embryos of *G. riobambae* coincides with changes in the shape of cells at the midblastula stage and with expansion of the blastocoel.

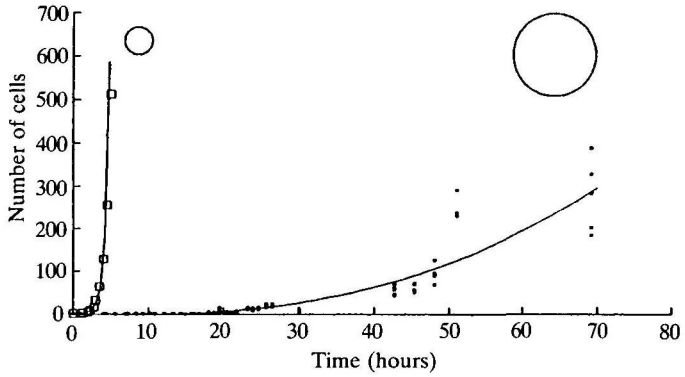


Fig. 8. Comparison of the speed of early development of *X. laevis* (□) and *G. riobambae* (●). The values for *X. laevis* were obtained from Nieuwkoop and Faber (1967). The small egg of *X. laevis* cleaves rapidly and in synchrony. The large egg of *G. riobambae* measures 3 mm in diameter and its volume amounts to about 16 times that of the egg *X. laevis*. Circles indicate the differences in the diameter of eggs.

The blastocoel begins to form during the cleavage stages, such as in *X. laevis*, but in embryos of *G. riobambae* the blastocoel expands considerably. The fully formed blastocoel occupies at least the animal third of the embryo, and its gradual increase in size, produces a recognizable increase in the diameter of the embryo (del Pino and Escobar, 1981; Elinson and del Pino, 1985). At the midblastula stage (3–4 days of development), a slightly translucent area, which corresponds to the blastocoel, is visible at the animal pole. The translucent roof enlarges significantly during the next two days, as determined by the increase in the size of vital staining marks (Elinson and del Pino, 1985), and it becomes transparent as the single sheet of cells lining the roof of the blastula becomes thin and transparent (stage 6).

The onset of transcription in blastomeres

We could not detect the incorporation of [^{3}H]uridine or of [^{3}H]thymidine in embryos of *G. riobambae*. Therefore, the onset of embryonic transcription by the blastomeres of *Gastrotheca* remains unknown. However, since, the presence of nucleoli and nucleolar size correlate with the presence and rates of rRNA synthesis in blastomeres (Brown, 1966), we searched for the presence of nucleoli in the early embryos of *G. riobambae*. Nucleoli become a feature of the blastomeres at the 8- cell stage at about 24 h of development (Fig. 9). Nucleoli enlarge by the blastula stage, 6 days after amplexus, suggesting a higher rate of rRNA transcription at this stage. In some embryos, there were 2 nucleoli per nucleus, while in other embryos, only one nucleolus could be found in each blastomere. The differences in the number of nucleoli derives from the chromosomal location of the nucleolar organizer region (NOR) in the genome. The NOR is associated with the X-chromosome in *G. riobambae* (Schmid *et al.* 1986) and, therefore, female frogs have two NORs while in the genome of the male there is only one NOR.

Table 3. *Stages of the cell cycle in blastomeres of* G. riobambae *embryos*

Embryo†		Number of	Percentage of nuclei in:				
Number	Stage	nuclei*	Interphase	Prophase	Metaphase	Anaphase	Telophase
1	3	3	0	100	0	0	0
2	4	7	86	14	0	0	0
3	4	13	77	23	0	0	0
4	5	20	65	35	0	0	0
5	5	25	60	40	0	0	0
6	5	35	57	43	0	0	0
7	5	43	49	33	19	0	0
8	5	84	71	19	4	4	2
9	5	97	0	4	58	36	2
10	5	115	21	76	3	1	0
11	5	122	32	59	9	0	0
12	5	288	34	25	16	7	18
13	5	331	15	73	13	0	0
14	5	342	34	60	6	0	0

† Embryonic stages according to del Pino and Escobar (1981).
* Refers to the number of nuclei found in serial sections. Nuclei in metaphase and anaphase were scored as one nucleus.

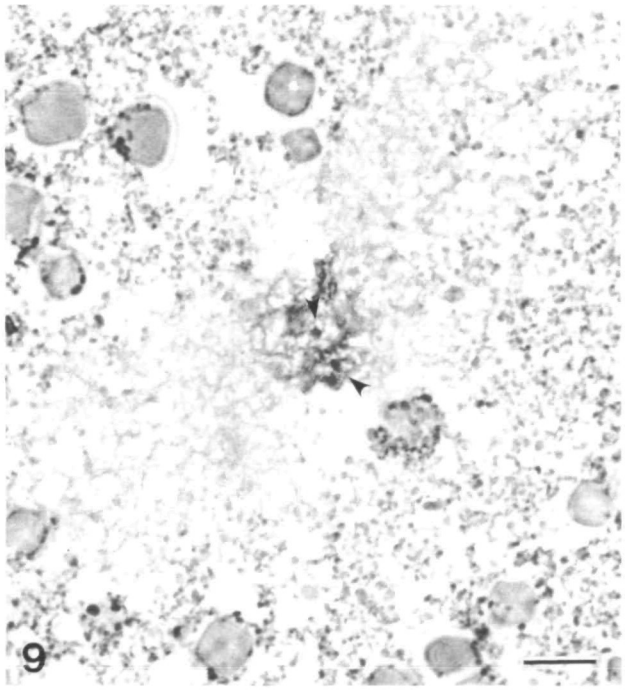


Fig. 9. Histological section through the nucleus of a blastomere of an 8-cell embryo of *G. riobambae*. Arrows signal two small round objects considered to be nucleoli. Nucleoli become conspicuous by the blastula stage, about 6 days after amplexus. Presence of nucleoli by the 8-cell stage indicates that the synthesis of rRNA begins early in the blastomeres of this frog. Bar, 10 μm.

Discussion

The characteristics of early cleavage of the marsupial frog *G. riobambae* differ significantly from those of *X. laevis* and suggest that amphibians may have several developmental strategies for the construction of an embryo. The rapid development exemplified by *X. laevis* is one of them and it is the best-studied pattern. In this case, the egg undergoes a period of 12 rounds of synchronous and rapid cleavage cycles without embryonic transcription. The midblastula transition consists of a slow down of the cleavage rate associated with the onset of embryonic transcription and with cell movements (Gerhart, 1980; Kimelman *et al.* 1987; Newport and Kirschner, 1982*a*,*b*). The tight coupling of events of the midblastula transition may characterize those amphibian embryos that undergo a fast rate of embryonic development. In an aquatic environment, fast embryonic development may be advantageous, since a tadpole may be less exposed to predation than the immobile eggs of a frog. However, in certain amphibians adapted to terrestrial life, the environmental demands are different, such as in the case of *G. riobambae*, and embryos have a different schedule for the changes that normally occur at the midblastula stage in *X. laevis*.

Accelerated early development, which depends on the reserves of the egg, characterizes the development of most animals (see Davidson, 1986 for a review). In consequence, the development of *X. laevis* follows a basic trend of animal development. However, there are animals that have evolved other strategies. For example, the mammals, whose development occurs at slow speed, embryonic transcription begins during early cleavage and cleavage is asynchronous (reviewed by Davidson, 1986). Slow development seems to be independent of egg size and seems to result from modifications of the developmental program of the egg (reviewed by del Pino, 1989). It occurs in animals, such as the mammals and the marsupial frogs, that protect their embryos from the external environment. Other organisms, such as the birds and reptiles develop rapidly but had to contend with large egg volume to accommodate all of the nourishment required to produce a new animal. The increase in egg size alters not only the pattern of cleavage, but the gastrulation movements and the location of developmental information within the egg, changes that are associated to

the evolution of diverse reproductive strategies (Elinson, 1989).

Early development of *G. riobambae* displays modifications associated with large eggs and with slow developmental rate. Cleavage of the *G. riobambae* egg can be defined as atypical holoblastic of the transitional type, according to Nelsen (1953), and it is comparable to that of other amphibian species with large eggs, such as the frog *Eleutherodactylus portoricensis* (Gitlin, 1944) and the large eggs of the urodele *Necturus maculosus* (of 5 to 6 mm in diameter) (reviewed by Nelsen, 1953). In addition, the cleavage pattern of *G. riobambae* (Figs 3–7) superficially resembles the pattern found in other vertebrates with large eggs such as the lungfish, *Lepidosiren paradoxa*, the sturgeon, *Acipenser sturio* (reviewed by Nelsen, 1953), and the bony fish, *Amia calva* (Ballard, 1986; Nelsen, 1953). *Gastrotheca* cleavage is also similar to the pattern seen in the blastoderm of the chick embryo (Eyal-Giladi and Kochav, 1976; Romanoff, 1960), although, cleavage of the *G. riobambae* egg is holoblastic. These similarities suggest that when animal eggs exceed a certain size, possibly the 2 mm in diameter, latitudinal cleavages planes no longer occur during the first rounds of division and the meridional cleavages are restricted to the first and possibly the second cleavage furrows. It may be that, as the volume of the egg increases, the mitotic spindles of the blastomeres can no longer orient themselves at right angles to the previous ones, producing atypical vertical cleavage planes.

In *G. riobambae*, the slow developmental rate (Fig. 8), the asynchronous cleavage pattern (Tables 2, 3) and the long period of interphase (Table 3), suggest the presence of the G_1 phase in early blastomeres and, possibly, the early onset of transcription in embryonic nuclei. The onset of transcription is unknown for embryos of this frog. However, the appearance of nucleoli in embryonic cell nuclei (Fig. 9) indicates that the synthesis of rRNA has already started in embryos as early as the 8-cell stage, at about 24 h of development. In embryos of *Xenopus*, *Rana* and the newt, *Notophthalmus*, nucleoli first appear at the gastrula stage, while in mammalian embryos, nucleoli have been observed during early cleavage stages (Brown, 1966). The presence of nucleoli in the early blastomeres of *G. riobambae* correlates with the low amplification of the ribosomal genes and the smaller amounts of rRNA in the oocytes (del Pino *et al.* 1986) as well as with the slow rate of early development. Transcription and cell motility are activated at the midblastula stage in *Xenopus* (Newport and Kirschner, 1982*a*; Kimelman *et al.* 1987), while in *G. riobambae*, cell motility seems to accompany changes in cell shape and the enlargement of blastocoel at the midblastula stage. These observations suggest that the molecular organization of the egg, laid down during oogenesis, affects not only the speed of development, but the schedule of developmental events.

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References

Ballard, W. W. (1986). Stages and rates of normal development in the holostean fish *Amia calva*. *J. exp. Zool.* **238**, 337–354.

Brown, D. D. (1966). The nucleolus and synthesis of ribosomal RNA during oogenesis and embryogenesis of *Xenopus laevis*. In *International symposium on the nucleolus, its structure and function* (eds. W. S. Vincent and O. L. Miller). Department of Health, Education and Welfare, Bethesda, Maryland. National Cancer Institute Monograph **23**, 297–307.

Davidson, E. H. (1986). *Gene Activity in Early Development.* Orlando, Florida: Academic Press Inc.

del Pino, E. M. (1989). Modifications of oogenesis and development in marsupial frogs. *Development* **107**, 169–188.

del Pino, E. M. and Elinson, R. P. (1983). A novel development pattern for frogs: gastrulation produces an embryonic disk. *Nature* **306**, 589–591.

del Pino, E. M. and 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. Morph.* **167**, 277–296.

del Pino, E. M. and Humphries, A. A., Jr (1978). Multiple nuclei during early oogenesis in *Flectonotus pygmaeus* and other marsupial frogs. *Biol. Bull. mar. biol. Lab., Woods Hole* **154**, 198–212.

del Pino, E. M., Steinbeisser, H., Hofmann, A., Dreyer, C., Campos, M. and Trendelenburg, M. F. (1986). Oogenesis in the egg-brooding frog *Gastrotheca riobambae* produces large oocytes with fewer nucleoli and low RNA content in comparison to *Xenopus laevis*. *Differentiation* **32**, 24–33.

Elinson, R. P. (1989). Egg evolution. In *Complex Organismal Functions: Integration and Evolution in Vertebrates* (ed. D. B. Wake and G. Roth), pp. 251–262. New York: John Wiley and Sons.

Elinson, R. P. and del Pino, E. M. (1985). Cleavage and gastrulation in the egg-brooding, marsupial frog, *Gastrotheca riobambae*. *J. Embryol exp. Morph.* **90**, 223–232.

Eyal-Giladi, H. and Kochav, S. (1976). From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. *Devl Biol.* **49**, 321–337.

Gerhart, J. C. (1980). Mechanisms regulating pattern formation in the amphibian egg and early embryo. In *Biological Regulation and Development*, vol. **2** (ed. R. F. Goldberger), pp. 133–293. New York: Plenum Press.

Gitlin, D. (1944). The development of *Eleutherodactylus portoricensis*. *Copeia* **1944**, 91–98.

Gurdon, J. B. (1968). Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. exp. Morph.* **20**, 401–414.

Kimelman, D., Kirschner, M. and Scherson, T. (1987). The events of the midblastula transition in *Xenopus* are regulated by changes in the cell cycle. *Cell* **48**, 399–407.

Macgregor, H. C. and del Pino, E. M. (1982). Ribosomal gene amplification in multinucleate oocytes of the egg brooding hylid frog *Flectonotus pygmaeus*. *Chromosoma (Berl.)* **85**, 475–488.

Nelsen, O. E. (1953). *Comparative Embryology of the Vertebrates.* New York: The Blakeston Co.

Newport, J. and Kirschner, M. (1982*a*). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675–686.

Newport, J. and Kirschner, M. (1982*b*). A major developmental

transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* **30**, 687–696.
NIEUWKOOP, P. D. AND FABER, J. (1967). *Normal Table of* Xenopus laevis *(Daudin)*. North Holland Publishing Co.
ROMANOFF, A. L. (1960). *The Avian Embryo. Structural and Functional Development.* New York: The Macmillan Co.
RUGH, R. (1965). *Experimental Embryology.* Minneapolis, Minnesota: Burgess Publishing Co.
SCHMID, M., SIMS, S. H., HAAF, T. AND MACGREGOR, H. C. (1986). Chromosome banding in Amphibia. X. 18S and 28S ribosomal RNA genes nucleolus organizers and nucleoli in *Gastrotheca riobambae*. *Chromosoma (Berl.)* **94**, 139–145.
SHUMWAY, W. (1940). Stages in the normal development of Rana pipiens. *I. External form. Anat. Rec.* **78**, 139–147.

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