J. Embryol. exp. Morph. **90**, 223–232 (1985)
Printed in Great Britain © *The Company of Biologists Limited 1985*

Cleavage and gastrulation in the egg-brooding, marsupial frog, *Gastrotheca riobambae*

RICHARD P. ELINSON
Department of Zoology, University of Toronto, 25 Harbord Street, Toronto M5S 1A1, Canada
AND EUGENIA M. DEL PINO
Departamento de Biología, Pontificia Universidad Católica del Ecuador, Apartado 2184, Quito, Ecuador

SUMMARY

The marsupial frog *Gastrotheca riobambae* has several reproductive adaptations, most prominent of which is the incubation of the embryo in a pouch on the mother's back. We have followed cleavage and gastrulation by microscopical observation and by vital staining, and have found several alterations in these processes which may reflect the reproductive adaptations. The large, yolky egg has a cap of yolk-poor cytoplasm at the animal pole which is incorporated into a translucent blastocoel roof consisting of a single cell layer. The epithelium of the yolk sac is derived from the roof. The inconspicuous blastoporal lips form near the vegetal pole from cells of the marginal region. Gastrulation movements include the epibolic stretching of the surface towards the blastopore and a contraction of the vegetal surface. The blastoporal lips close over a small archenteron, and the cells of the lips become the embryonic disc, a discrete group of small cells which give rise to most of the embryo's body. The great size difference between animal and vegetal blastomeres during cleavage, the single-celled blastocoel roof, the dissociation in time between archenteron formation and its expansion, the embryonic disc and the slow development distinguish *G. riobambae* embryos from those of other frogs. The importance of the marginal region which produces the embryonic disc and the unimportance of the most animal region whose fate is primarily yolk sac emphasizes the role of the marginal region in amphibian development.

INTRODUCTION

Our concepts of anuran amphibian development are based on the careful study of embryos from a few species. These include a variety of frogs and toads, in particular several *Rana* and *Bufo* species and *Xenopus laevis*. While these animals are not closely related, they all have relatively small eggs (1·3–1·8 mm in diameter) and aquatic development. These shared ecological characters would be expected to contribute to the similarity of embryogenesis in these species.

There are, however, a number of anurans which have terrestrial development (Lamotte & Lescure, 1977) and very large eggs (Salthe &Duellman, 1973; del Pino & Escobar, 1981), and embryos of these animals might differ in interesting ways from the commonly studied anurans. Embryonic development in *Gastrotheca riobambae*, which broods its embryos in a pouch on the female's back, is different from that of aquatic reproducing frogs. The most remarkable change corresponds

Key words: gastrulation, *Gastrotheca riobambae*, cleavage, marsupial frog.

to the formation of an embryonic disc as a result of gastrulation and to the subsequent development of the embryo's body from this disc (del Pino & Elinson, 1983). Development from a disc of cells is common among other vertebrates such as birds and reptiles, but not among frogs. In this report we analyse early cleavage of *G. riobambae* and the formation of the embryonic disc.

MATERIALS AND METHODS

Animals and embryos

Gastrotheca riobambae were collected in Ecuador at several localities in the interandean valley of the Province of Pichincha. A few frogs came from the Province of Imbabura. Frogs were maintained as described previously (del Pino & Escobar, 1981). Females with embryos in their pouch were obtained in three ways. Some females were pregnant when captured, others mated spontaneously in the laboratory, while still others were encouraged to mate by the injection of both the female and the male with human chorionic gonadotropin (del Pino & Escobar, 1981). In order to work on living embryos, embryos were removed from the mother's pouch and placed in a minimal amount of modified Barth's solution (Gurdon, 1976) under vaseline oil. Normal development usually continued *in vitro* for six days or more allowing us to perform a series of vital staining experiments. Embryos were staged according to del Pino & Escobar (1981). This study is based on embryos from 24 frogs.

Vital staining

Pieces of agar containing Nile blue sulphate were prepared by dissolving the stain in 1·5 % agar with boiling. The liquid was dried on a glass plate, and the film was cut into small pieces for use. A *G. riobambae* embryo was taken from the pouch, its outer jelly was removed by rolling the embryo on a moist paper towel, and the embryo was placed in a humid chamber. Using a piece of dried agar like a brush, spots of stain were applied to the surface of the remaining jelly. After a few minutes, the embryo's surface was stained, and the stain left in the jelly was picked away. The stained embryo was cultured as described above.

Fixation and specimen preparation

Embryos were fixed and prepared by several procedures. Because of their pale colour, it is very difficult to see changes of the surface of the living embryo before and during gastrulation. In order to visualize the surface, we followed Kageyama's silver procedure (1980) which stains the cell boundaries.

For internal examination, embryos were fixed with Smith's fixative (Smith, 1912*a*) or in 3 % glutaraldehyde in 0·1 M-phosphate buffer, pH 7·3, embedded according to Sentein (1976), and sectioned at 9 μm. The sections were stained by several procedures with the best being staining with 2 % Safranin O in aniline water and counterstaining with 0·5 % light green in 95 % ethanol. Safranin O stains yolk platelets reddish–purple allowing the yolk distribution to be seen, but with a red filter on the light source, the yolk is not visible and the nuclei and cell borders are clear. Glutaraldehyde-fixed embryos were also postfixed in 1 % OsO_4, embedded in Epon, and used for thick plastic sections.

RESULTS

Cleavage and blastula formation

The egg of *G. riobambae* is 2·1 to 3·6 mm in diameter, and is surrounded by a thin layer of jelly. The egg is pale yellow; however, most eggs have a whiter animal region while the eggs of some frogs have a yellower spot at the vegetal pole. When

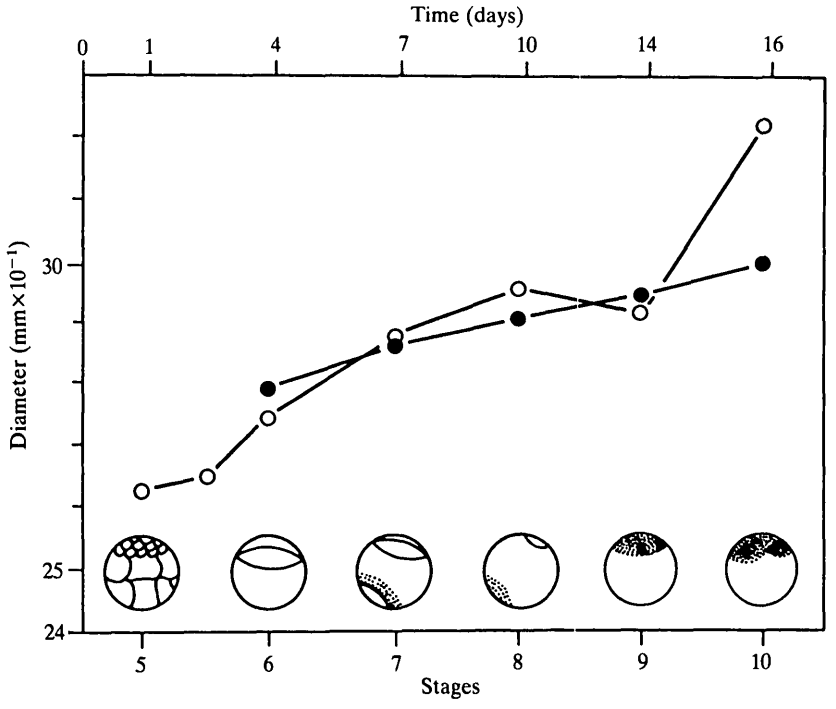
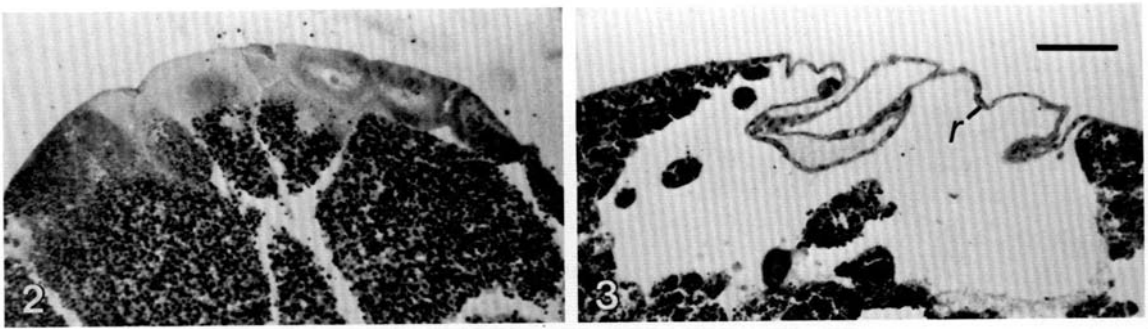


Fig. 1. Increase in diameter with development in *G. riobambae*. Embryos taken directly from the pouch (●——●) are compared to those cultured *in vitro* (○——○). Each point is the mean of 12–34 measurements. Young embryos, when placed in modified Barth's solution, swell and burst after about one day. When cultured in a minimal amount of modified Barth's solution under vaseline oil, however, embryos remain viable for a week or longer. Under these conditions, the increase in volume of the embryos is similar to that of embryos in the pouch. The diagrams illustrate the appearance of the embryos at each stage. The disc (stippled) develops from the blastoporal lips, and most of the embryo's body develops from the stippled disc. With the expansion of the archenteron, the whole embryo rotates with respect to gravity between stages 8 and 9.

eggs are glutaraldehyde-fixed, a cap of white cytoplasm at the animal pole can be easily distinguished from the rest of the yellow egg. In sections, this cap of cytoplasm has few yolk platelets while the rest of egg cytoplasm is rich in platelets.

Development is very slow with cleavage and blastula formation taking about a week (Fig. 1). Cleavage furrows divide the egg completely, and the animal cap cleaves more rapidly than the vegetal region. Unlike other frogs, the third cleavage furrows are vertical rather than horizontal (del Pino & Escobar, 1981) and in eggs of some females, cleavage appears completely irregular at this time (stage 4). As a result of cleavage, the animal cap is divided into small blastomeres (stage 5), and these small cells incorporate the white, yolk-poor cytoplasm (Fig. 2). There is a narrow band of medium-sized, pale-yellow cells adjacent to the small, white blastomeres while the remaining three-quarters of the egg surface consists of large cells. These large blastomeres have a flat appearance. In contrast,



Figs 2–3. Development of the animal cap.

Fig. 2. A section through a stage-5 embryo shows a cap of cells with yolk-poor cytoplasm.

Fig. 3. A section through the blastocoel roof (*r*) of a stage-8 embryo shows that it consists of a single layer of yolk-poor cells. The dark spots in the roof, which collapsed during fixation, are nuclei. Bar = 0·2 mm.

blastomeres of the animal region have a rounded appearance and bulge out from the surface of the embryo.

The blastocoel develops in the animal region, and with its appearance the embryo undergoes a corresponding increase in size (Fig. 1). The embryo continues to expand through gastrulation and axis formation. The small, white cells at the animal pole gradually become translucent (stage 6), as the blastocoel roof develops into a single layer of yolk-poor epithelial cells (Fig. 3). Silver staining of embryos becomes possible at stage 6, and three regions can be distinguished: the blastocoel roof, the marginal region, and the vegetal region (Fig. 4). The translucent cells of the blastocoel roof exhibit distinct silver staining of their borders (Fig. 5). The cells of the marginal region are medium sized and irregular in shape. Their cell boundaries are faintly stained with silver, and the cell surfaces bulge out (Fig. 6). Finally the large cells of the vegetal region are completely stained with silver (Figs 4, 6). As the embryos approach gastrulation, the vegetal blastomeres are concentrated around the vegetal pole, the marginal region surrounds them and is displaced vegetally, while the cells of the blastocoel roof extend laterally below the blastocoel floor.

Figs 4–9. Embryos of *G. riobambae* stages 6–8. Bar = 0·3 mm.

Fig. 4. This silver-stained stage-6 embryo shows the translucent blastocoel roof (*r*), the marginal region (*m*), and the vegetal region (*v*).

Fig. 5. The borders of cells of the blastocoel roof at stage 6 stain darkly with silver.

Fig. 6. Marginal region cells at stage 6 stain poorly with silver while vegetal cells have silver-staining on their surfaces.

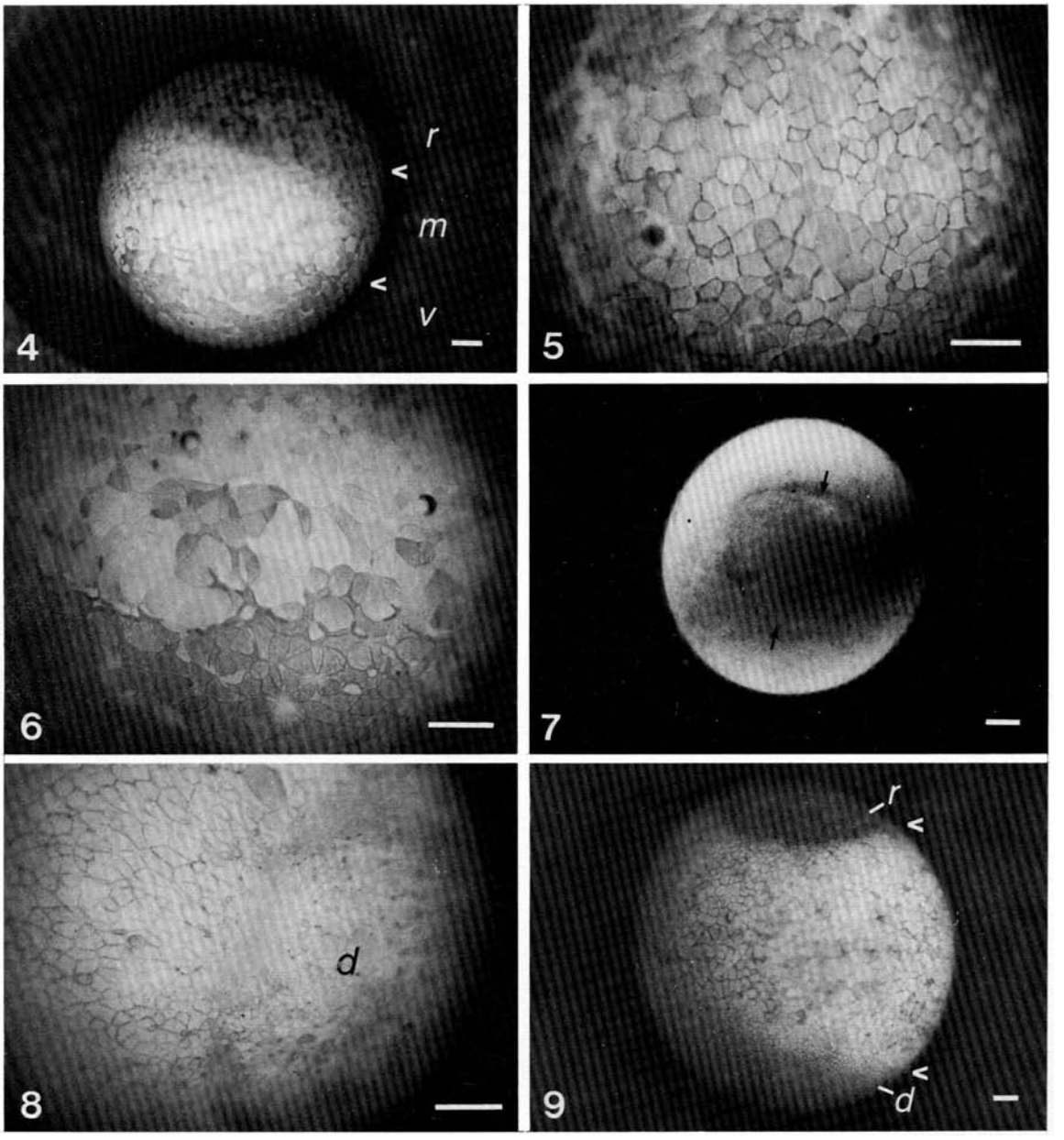
Fig. 7. Glutaraldehyde-fixation of a stage-7 embryo reveals the blastoporal lip (arrow). The lip, when first formed, is a faint invagination near the vegetal pole.

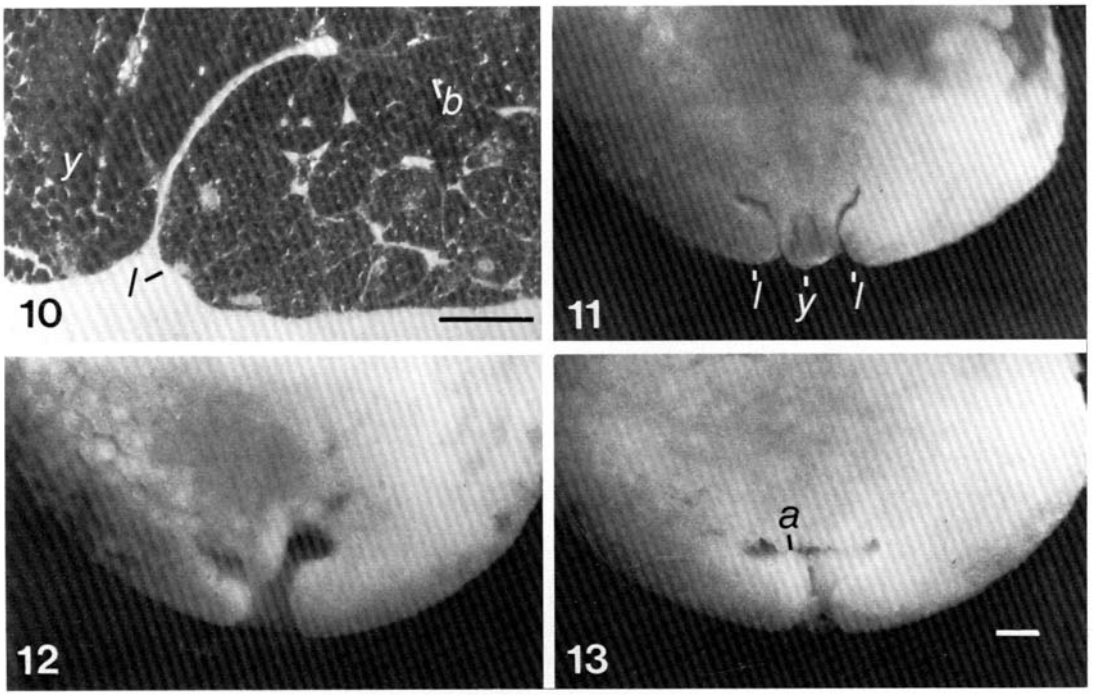
Fig. 8. A silver-stained stage-8 embryo shows the stretching of cells towards the forming embryonic disc (*d*).

Fig. 9. A silver-stained stage-8 embryo has a fully-formed embryonic disc (*d*) while cells continue to migrate along the translucent blastocoel roof (*r*).

Gastrulation

Gastrulation, like cleavage, is very slow, taking about one week (Fig. 1). Stage 7 corresponds to the beginning of gastrulation, but the light colour of the embryos and the shallowness of the invagination makes observation of the blastoporal lip difficult. The onset of gastrulation is more easily determined on living embryos by the internal migration of yolky cells from the edge of the blastocoel floor up along





Figs 10–13. Closure of the blastopore.

Fig. 10. A plastic section of the blastoporal lip (*l*) and the yolk plug (*y*) shows a typical bottle cell (*b*). Bar = 0·1 mm.

Fig. 11. The blastoporal lips and the yolk plug are seen in this bisected embryo.

Fig. 12. The yolk plug withdraws to the interior.

Fig. 13. The blastoporal lips close over a slit-like archenteron (*a*). Bar = 0·2 mm.

the blastocoel roof, an observation made possible by the translucency of the roof. The cells appear to migrate as a coherent sheet, but cell adhesion may be weak since spaces are sometimes present between cells and loose cells can be found in the blastocoel fluid, especially of embryos *in vitro*.

The blastoporal lip is easier to see on fixed embryos (Fig. 7) and is initially found at the marginal–vegetal boundary close to the vegetal pole (about 90 % of the way along the animal–vegetal axis). The early lip may be irregular in shape or may not form a complete circle. We do not know whether this appearance reflects embryonic polarity, but it is clear that a prominent dorsal lip, as seen in other amphibian embryos, is not present. Bottle cells are located at the invagination (Fig. 10). An archenteron forms as the blastopore closes, and the yolk plug is withdrawn to the interior (Figs 11–13). The archenteron is initially a slit-like cavity centred around the closing blastopore, and debris is often left on the surface marking the site of blastopore closure (Fig. 13). Archenteron formation is dissociated in time from its expansion (Table 1). Archenteron expansion occurs two to four days after its formation (Table 1), and the embryo rotates so its body is up (stage 9, Fig. 1).

Relative surface changes during early development

To follow the changes in the surface of the embryo, spots of Nile blue sulphate were placed at the animal pole, at the vegetal pole, or on the lateral surface. The diameter and fate of the marks was recorded. Since the time from fertilization until the end of gastrulation is nearly two weeks (Fig. 1) and since markings do not persist for so long, it was necessary to analyse the changes only from one stage to the next. Between stages 5 and 6, the greatest increase in the size of the spots occurs at the animal pole (Fig. 14). This expansion is correlated with the appearance of the blastocoel and the thinning of the blastocoel roof until it consists of a translucent monolayer of cells. Expansion of the animal region continues from stages 6 to 8, but to a lesser extent. Between stages 6 and 8, spots placed in the lateral region expand (Fig. 14). This expansion corresponds to the epibolic movements which lead to the covering of the vegetal cells by animal ones. With silver staining, we could see that cells bordering the marginal zone on the animal side became elongated (Fig. 8). Concomitant with the lateral stretching of the embryo's surface, spots placed in the vegetal region decrease in size from stage 6 to 7 (Fig. 14). Vegetal cells of the yolk plug at stage 7 are elongated with their long axis perpendicular to the embryonic surface. These observations suggest that a change in vegetal cell shape leads to a reduction of the vegetal surface area. This contraction of vegetal surface would contribute to the disappearance of the vegetal cells from the surface.

The embryonic disc

We had previously described the presence of the embryonic disc (Fig. 9), a discrete group of small cells from which arises most of the embryo's body (del Pino & Elinson, 1983). The present observations indicate that the disc forms from

Table 1. *Archenteron formation and the thickness of the embryonic disc*

	Development		Archenteron size (μm)	Disc thickness	
♀	Day*	Stage		Maximum (μm)	No. of cells
EP 507	12	8	860	360	10–15
	12	8	820	330	11–13
	12	8	640	430	12–14
	14	9	1850	140	5
	14	9	1680	160	4–5
	15	9	1350	130	–
EP 505	11	8	810	470	8–11
	12	8	910	460	12
	13	8	1040	340	–
	15	9	1540	130	5
	15	9	1940	160	4–6
	16	10	1810	60–90	4

*The time of fertilization was extrapolated from the stage when the embryos were first observed. For EP 507, the first embryos observed were 16–32 cells and were probably one day old. For EP 505, the first embryos were in stage 6 and were probably about a week old.

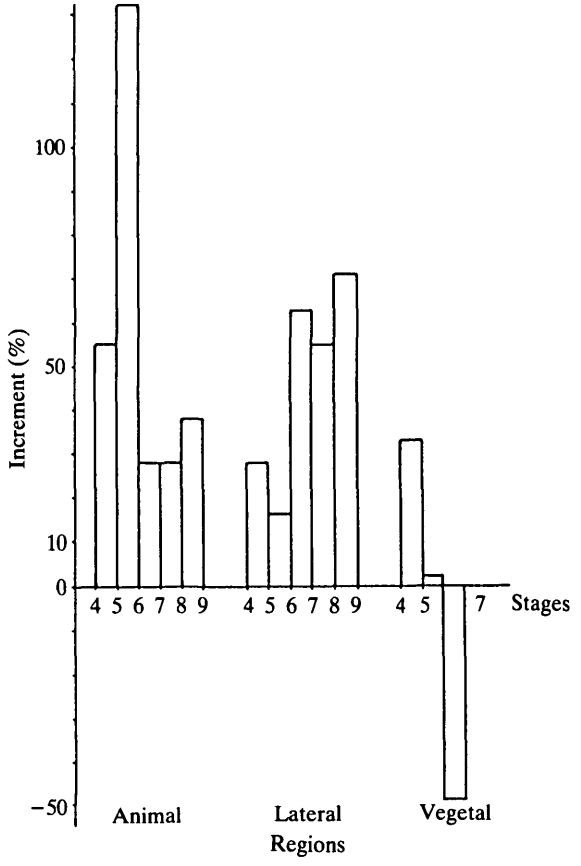


Fig. 14. Change in size of vital dye marks on the surface of the embryo.

the blastoporal lips (Fig. 13) which in turn arose from cells of the marginal region (Fig. 6). Vital staining indicated that the disc expanded by stretching rather than by addition of neighbouring cells (del Pino & Elinson, 1983). This finding is supported by measurement of the archenteron, the embryonic disc, and the later archenteric roof (Table 1). As the archenteron enlarges, the disc thins to become the archenteric roof, and there is a corresponding decrease in the number of cells in cross section. The archenteric roof becomes about four-cells thick except at the site of the blastopore where a small knot of cells persists. Whether this knot is simply the point of fusion of the blastoporal lips or whether any special movements occur there has not been determined.

DISCUSSION

The *G. riobambae* egg begins development with a cap of yolk-poor cytoplasm at the animal pole and completes gastrulation with the formation of the embryonic disc which gives rise to most of the embryo's body. The animal cap cytoplasm is

reminiscent of the small area of yolk-poor cytoplasm of the avian egg, and the embryonic disc is reminiscent of the avian blastodisc. The avian blastodisc, however, is derived from the yolk-poor cytoplasm, a situation quite different from *G. riobambae*. The observations of the embryonic surface and of gastrulation movements in *G. riobambae* suggest that the embryonic disc is derived from cells in the marginal region while the yolk-poor cells derived from the animal cap form primarily the blastocoel roof and later the surface of the yolk sac. Thus, the animal cap contributes very little to the body of the *G. riobambae* embryo. At first glance, the importance of the marginal region and the unimportance of the animal cap in the formation of the embryo's body is surprising. It is, however, similar to the situation in other amphibians where the information for the dorsoventral axis and for mesodermal structures resides in the marginal and vegetal regions (Slack, 1983; Gimlich & Gerhart, 1984; Gurdon, Brennan, Fairman & Mohun, 1984). Cells of the animal region simply form epidermis unless instructed to do otherwise by cells of the other regions.

Since the *G. riobambae* embryonic disc and the avian blastodisc are not homologous, we can ask why such a similar form is found in these animals. One reason is that the large egg size presents difficulties for gas exchange to the interior of the egg. As a result, the rapid events of embryogenesis, including axis and organ formation, all take place at the surface of the embryo in both birds and *G. riobambae*. Besides this environmental consideration, the formation of an embryonic disc may help to overcome a different diffusion problem. The interactions required for the spatial organization of embryos occur at close range and may be limited by diffusion (Crick, 1970; Forman & Slack, 1980; Cooke, 1982). One advantage of disc formation is that it would allow specification of the body plan to occur in just a small area of a large egg.

Cleavage and gastrulation in *G. riobambae* differ from these processes in commonly studied amphibians in several significant ways. The pattern of cleavage becomes irregular after a few divisions, and the blastocoel roof of the blastula is a single-cell thick. The blastopore closes before there is significant anterior extension of the archenteron, and the embryonic disc forms. In addition, cleavage and gastrulation take an unusually long time as compared to other frogs. Surprisingly, *G. riobambae* as well as other amphibians with large eggs have holoblastic cleavage (Apoda: Svensson, 1938; Anura: Chibon, 1960; Wernz, 1966; Urodela: Ishikawa, 1908; Smith, 1912*b*) unlike the meroblastic cleavage found for reptiles and birds. Since it is probably difficult for cleavage furrows to divide a yolky, 3–7 mm egg completely, there must be some importance in breaking the yolk mass into smaller cells. One possibility is suggested by the shrinkage of the vegetal dye marks and the presence of elongated vegetal cells during gastrulation. A reduction in vegetal surface area, which is indicated by these observations and seen as well in *X. laevis* (Keller, 1978), may contribute to the removal of yolky, vegetal cells from the embryonic surface during gastrulation. The division of the most vegetal part of the egg would be necessary for this morphogenetic change.

We thank Carmen de Albuja, Wilson Carlosama, Rafael Narváez, and Sonia Sandoval for collecting *G. riobambae* and Judy Thurston and Peter Pasceri for histological assistance. This work was supported by a grant from the UNDP/UNESCO program RLA/78/024 to E. M. del Pino and by an International Collaborative Research Grant and an Operating Grant from NSERC, Canada, to R. P. Elinson.

REFERENCES

CHIBON, P. (1960). Développement au laboratoire d'*Eleutherodactylus martinicensis* Tschudi, batracien anoure à développement direct. *Bull. Soc. Zool. Fr.* **85**, 412–418.

CRICK, F. (1970). Diffusion in embryogenesis. *Nature* **225**, 420–422.

COOKE, J (1982). The relation between scale and the completeness of pattern in vertebrate embryogenesis: models and experiments. *Am. Zool.* **22**, 91–104.

DEL PINO, E. M. & ELINSON, R. P. (1983). A novel development pattern for frogs: gastrulation produces an embryonic disk. *Nature* **306**, 589–591.

DEL PINO, E. M. & ESCOBAR, B. (1981). Embryonic stages of *Gastrotheca riobambae* (Fowler) during maternal incubation and comparison of development with that of other hylid frogs. *J. Morph.* **167**, 277–295.

FORMAN, D. & SLACK, J. M. W. (1980). Determination and cellular commitment in the embryonic amphibian mesoderm. *Nature* **286**, 492–494.

GIMLICH, R. L. & GERHART, J. G. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Devl Biol.* **104**, 117–130.

GURDON, J. B. (1976). Injected nuclei in frog oocytes: fate, enlargement, and chromatin dispersal. *J. Embryol. exp. Morph.* **36**, 523–540.

GURDON, J. B., BRENNAN, S., FAIRMAN, S. & MOHUN, T. J. (1984). Transcription of muscle-specific actin genes in early *Xenopus* development: nuclear transplantation and cell dissociation. *Cell* **38**, 691–700.

ISHIKAWA, CH. (1908). Ueber den Riesensalamander Japans. *Mitt. dt. Ges. Nat. -U. Volkerk. Ostaciens* **11**, 259–280.

KAGEYAMA, T. (1980). Cellular basis of epiboly of the enveloping layer in the embryo of medaka, *Oryzias latipes*. I. Cell architecture revealed by silver staining method. *Devl. Growth Differ.* **22**, 659–668.

KELLER. R. E. (1978). Time-lapse cinematographic analysis of superficial cell behavior during and prior to gastrulation in *Xenopus laevis*. *J. Morph.* **157**, 223–248.

LAMOTTE, M. & LESCURE, J. (1977). Tendances adaptives à l'affranchissement du milieu aquatique chez les amphibiens anoures. *Terre Vie* **31**, 225–311.

SALTHE, S. N. & DUELLMAN, W. E. (1973). Quantitative constraints associated with reproductive mode in anurans. In *Evolutionary Biology of the Anurans*. (ed. J. L. Vial) pp. 225–249. Columbia, Missouri: University of Missouri Press.

SENTEIN, P. (1976). Methods for fixing, sectioning, & staining eggs for cytological study. *Microsc. acta* **78**, 427–438.

SLACK, J. M. W. (1983). *From Egg to Embryo*. Cambridge University Press.

SMITH, B. G. (1912*a*). The embryology of *Cryptobranchus allegheniensis*, including comparisons with some other vertebrates. I. Introduction; the history of the egg before cleavage. *J. Morph.* **23**, 61–157.

SMITH, B. G. (1912*b*). The embryology of *Cryptobranchus allegheniensis* including comparisons with some other vertebrates. II. General embryonic and larval development, with special reference to external features. *J. Morph.* **23**, 455–580.

SVENSSON. G. O. (1938). Zur Kenntnis der Furchung bei den Gymnophionen. *Acta Zoologica* **19**, 191–207.

WERNZ, J. G. (1966). Pre-hatching stages of the tailed frog, *Ascaphus truei* (Stejneger). M. Sc. Thesis, Oregon State University.

(Accepted 20 June 1985)