Modifications of oogenesis and development in marsupial frogs

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Introduction

Early development in animals is guided by the RNA reserves of the egg (see Davidson, 1986 for a review), and therefore variations in eggs might correlate with changes in their patterns of early development. Frogs are organisms especially suitable for analyzing the relationship of oogenesis to development, because many frog species have evolved distinctive changes to avoid or diminish their period of aquatic dependence (Lamotte and Lescure, 1977; Duellman and Trueb, 1986). The analysis of the developmental adaptations that accompany the modes of frog reproduction, however, is hindered by the fact that the best investigated frogs, such as Xenopus laevis or Rana pipiens, have aquatic reproduction and similar modes of oogenesis and early development, although the former is in a family considered to be primitive (Pipidae), with adult specializations for an extreme aquatic life-style, whereas the latter is in an advanced family (Ranidae), with more pronounced terrestrial adaptations in the adult (Table 1). The modes of reproduction found in the 21 living families of frogs is shown in Table 1. The traditional order of anuran classification has been maintained in Table 1, but the higher taxa are not given. In an ancient and diverse group, such as the anurans, there might be many cases of similarity due to derived features, and the current knowledge of many characters and their evolutionary change does not allow the reconstruction of the anuran phylogeny (Duellman and Trueb, 1986).

In this review, we compare oogenesis and the early developmental strategies of certain egg-brooding tree frogs with X. laevis. Egg-brooding tree frogs belong to the family Hylidae and include 7 genera (Table 2). In egg-brooding tree frogs without pouches, eggs are exposed to the external environment, while in egg-brooding tree frogs with pouches, called marsupial frogs, eggs are totally protected inside a closed pouch, with the exception of some species of *Fritziana*, in which the pouch remains open during incubation (del Pino, 1980; Duellman and Gray, 1983). Comparison of morphological and reproductive characteristics, as well as the immunological comparison of albumins suggest that egg-brooding tree frogs without pouches represent older lineages than marsupial frogs (Duellman and

Hoogmoed, 1984; Scalan et al. 1980; Wassersug and Duellman, 1984). The lineages leading to egg-brooding tree frogs without pouches (*Stefania*, *Hemiphractus* and *Cryptobatrachus*) last shared a common ancestor with the marsupial frogs genus *Gastrotheca* some 40–60 million years ago. Within the genus *Gastrotheca*, the high Andean lineages, such as *Gastrotheca riobambae*, are recent and have the most complex reproductive adaptations. The groups of high Andean species evolved within the last 2–10 million years. Marsupial frogs of the genus *Flectonotus* do not show immunological cross-reactivity with other egg-brooding marsupial frogs (Scalan et al. 1980).

Most egg-brooding tree frogs live in the humid forests of tropical South America and Panama and their limited availability restricts investigation. In spite of this limitation, we have been able to observe developmental aspects in nearly 40 of the 60 described species by the analysis of museum-preserved specimens (del Pino, 1980; del Pino and Escobar, 1981; del Pino and Humphries, 1978). Methods used for the developmental study of marsupial frogs were explained by Elinson et al. (1989). Our work has centered on the adaptations of the marsupial frog Gastrotheca riobambae, a frog that produces advanced tadpoles and occurs with relative abundance in the northern inter-Andean region of Ecuador (Duellman and Hillis, 1987). In addition, we have studied certain aspects of oogenesis and development in *Flectonotus pygmaeus*, a marsupial frog from Venezuela that gives birth to advanced tadpoles (Duellman and Maness, 1980). For other species, only limited comparisons were possible. The unusual modifications of egg-brooding tree frogs demonstrate that amphibian oogenesis and early development possess great flexibility to adapt to the variety of environments in which frogs have deposited their eggs during the course of evolution.

Reproductive characteristics of marsupial frogs

The major differences between the developments of *Xenopus* and marsupial frogs derives from the different relationship of their eggs to the environment. Eggs of *Xenopus* become impermeable to external compounds at the time of oocyte maturation. As a consequence,

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	Number of			
Family†	Genera	Species	Reproductive modes‡	
Leiopelmatidae (Ascaphus)	2	4	2, 17	
Discoglossidae (Alytes, Bombina, Discoglossus)	5	14	1, 24	
Rhinophrynidae	1	1	1	
Pipidae (<i>Pipa</i> , Xenopus)	4	26	1, 10, 11	
Pelobatidae	9	83	1, 2	
Pelodytidae	1	2	1	
Myobatrachidae	20	99	1, 2, 7, 8, 9, 12, 13, 16, 17, 21, 22	
Heleophrynidae	1	3	2, 23	
Sooglossidae	2	3	16, 17	
Leptodactylidae (Eleutherodactylus Leptodactylus)	51	710	1, 2, 4, 5, 8, 13, 15, 17, 20, 21, 22, 28	
Bufonidae (Bufo, Nectophrynoides)	25	335	1, 2, 4, 5, 14, 15, 17, 28, 29	
Brachycephalidae	2	2	17	
Rhinodermatidae	1	2	14, 16	
Pseudidae	2	4	1	
Hylidae (<i>Hyla</i> , egg-brooding frogs§)	37	630	1, 2, 3, 4, 18, 25, 26, 27	
Centrolenidae	2	65	18	
Dendrobatidae	4	117	14	
Ranidae (Rana)	47	667	1, 2, 12, 13, 14, 15, 17, 18, 20	
Hyperoliidae	14	206	1, 12, 13, 18, 19, 22	
Rhacophoridae	10	186	2, 8, 17, 18, 19, 21, 22, 23	
Microhylidae	61	279	1, 6, 15, 17, 18	
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Table 1. Living anurans and their reproductive modes*

* According to Duellman & Trueb (1986).

[†]Genera of frogs used for developmental studies, and those mentioned in this article are given in parenthesis.

[‡]Numbers correspond to 29 reproductive modes as follows (according to Duellman & Trueb, 1986):

§ Genera of egg-brooding tree frogs are given in Table 2.

I. Eggs aquatic

Total

- A. Eggs deposited in water with development of feeding tadpoles (occurrence of nonfeeding tadpoles is indicated) 1. In standing water
 - 2. In running water
 - 3. Eggs and early larval stages in natural or constructed basins; subsequent to flooding, tadpoles in ponds or streams

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- 4. In water in tree holes or aerial plants
- 5. Eggs and nonfeeding tadpoles in water-filled depressions
- 6. Eggs and nonfeeding tadpoles in water in tree holes or aerial plants
- 7. Eggs swallowed by female; egg deposition site unknown. Eggs and tadpoles complete development in stomach
- B. Eggs in foam nest with development of feeding tadpoles
 - 8. In pond
 - 9. Foam nest in pool and tadpoles in stream
- C. Eggs imbedded in dorsum of aquatic female
 - 10. Eggs hatch into feeding tadpoles in ponds
- 11. Eggs hatch into froglets

II. Eggs terrestrial or arboreal

- D. Eggs on ground or in burrows with development of feeding tadpoles (occurrence of nonfeeding tadpoles or froglets is indicated)
 - 12. Eggs and early tadpoles in excavated nest; subsequent to flooding, tadpoles in ponds or streams
 - 13. Eggs on ground or rock above water or in depression or excavated nest; upon hatching, tadpoles move to water
 - 14. Eggs hatch into tadpoles that are carried to water by adult
 - 15. Eggs hatch into nonfeeding tadpoles that complete their development in nest
 - 16. Eggs hatch into nonfeeding tadpoles that complete their development on dorsum or in pouch of adult
 - 17. Eggs hatch into froglets
- E. Eggs arboreal with development of tadpoles or froglets

 - Tadpoles drop into ponds or streams
 Tadpoles drop into water-filled cavities in trees
 - 20. Eggs hatch into froglets
- F. Eggs in foam nest in burrow (occurrence of arboreal nest is indicated)
 - 21. Subsequent to flooding, feeding tadpoles in ponds or streams
 - 22. Nonfeeding tadpoles complete development in nest
 - 23. Nest arboreal; hatching tadpoles drop into ponds or streams
- G. Eggs carried by adult
 - 24. On the legs of male; feeding tadpoles in ponds
 - 25. In dorsal pouch of female; feeding tadpoles in ponds
 - 26. On dorsum or in dorsal pouch of female; nonfeeding tadpoles in bromeliads
 - 27. On dorsum or in dorsal pouch of female; direct development into froglets

III. Eggs retained in oviducts

- H. 28. Ovoviviparous
- 29. Viviparous Ι.

	Number of species				
Total†	With direct development	With multinucleate oogenesis	Egg diameter (mm)	Number of eggs	
I	Frogs without pou	ıch			
3 (2)	2	0	4	26-28	
5 (5)	5	1	7-10	10-18	
7 (4)	3	1	9	6-15	
	Marsupial frogs	7			
1 (1)	1	1	-	-	
2 (2)	2‡	2	3	3–7	
3 (3)	0	0	3-4	10-15	
40 (29)	21	9	2.5-10	6-140	
61 (46)	34	14			
	Total† 3 (2) 5 (5) 7 (4) 1 (1) 2 (2) 3 (3) 40 (29) 61 (46)	Number of specia With direct Total† With direct Frogs without pour 3 (2) 2 5 (5) 5 7 (4) 3 Marsupial frogs 1 (1) 1 2 (2) 2‡ 3 (3) 0 40 (29) 21 61 (46) 34	$\begin{tabular}{ c c c c } \hline & & & & & & & & & & & & & & & & & & $	$\begin{tabular}{ c c c c } \hline & & & & & & & & & & & & & & & & & & $	$\begin{tabular}{ c c c c c c } \hline & & & & & & & & & & & & & & & & & & $

 Table 2. Characteristics of egg-brooding tree frogs (Hylidae)*

* See Elinson et al. (1989) for references to the geographic distribution and systematics of these frogs.

† The number of species examined is given in parenthesis.

‡ Young are born as very advanced tadpoles that do not feed.

early development in water depends solely on the egg's reserves of yolk and nutrients. In contrast in marsupial frogs, the mother incubates her eggs and eggs are never exposed to the osmotic shock of fresh water. After maturation, eggs of G. riobambae retain permeability to compounds of low molecular weight, such as amino acids and sugars, suggesting that the mother may contribute nutrients to her embryos during incubation (Merizalde-de Albuja, 1983).

Fertilization, incubation and birth

Fertilization in Flectonotus pygmaeus and all of the species of Gastrotheca that have been investigated (del Pino and Escobar, 1981; Duellman and Maness, 1980) is external, as in Xenopus, but the process is totally terrestrial. In consequence, the requirements for fertilization and early development of the egg differ completely from the freshwater conditions of Xenopus. In X. laevis, saline solutions of low ionic strength are required for sperm motility and normal development (Wolf and Hedrick, 1971), while Gastrotheca sperm and embryos swell and die in solutions of low ionic strength. In contrast, sperm of Xenopus do not move, and embryos exogastrulate, while sperm of G. riobambae survive, and embryos (up to stage 19 of del Pino and Escobar, 1981) develop normally in amphibian physiological saline solutions (del Pino et al. 1975).

The process of embryonic incubation in *Gastrotheca* is accompanied by complicated behavior that differs totally from *Xenopus* and other frogs. In *G. riobambae*, amplexus occurs on land and it lasts for 24 to 48 h before egg-laying. During amplexus, the male introduces his feet inside the dorsal pouch of the female, and as each egg leaves the female's cloaca, he catches it with his heels and toes and moves it to the pouch avoiding contact of the egg with the ground. Transport of the clutch takes between 6 and 8 h and fertilization probably occurs during the egg's journey to the pouch. After an incubation period of 100 days, the female *G. riobambae* gives birth to a mean of 128 tadpoles (del

Pino and Escobar, 1981). At the time of birth, the mother immerses the lower part of her body in the water and introduces the long toes of the hind legs inside the pouch to remove tadpoles, while with her front legs she holds on to the walls of the water container. Birth has been documented for *Flectonotus pygmaeus* and several species of *Gastrotheca* (del Pino and Escobar, 1981; Duellman and Maness, 1980). The majority of egg-brooding tree frogs give birth to small frogs, and only *Fritziana, Flectonotus* and a few species of *Gastrotheca* produce tadpoles (Table 2).

Development is slow and synchronous in the pouch, lasting about 3 to 4 months in some species of Gastrotheca (del Pino and Escobar, 1981). Tadpoles of G. riobambae, which at birth measure 18 to 20 mm in total length, may require 4 months or more of aquatic life to reach metamorphosis, although in the laboratory metamorphosis can be accelerated (reviewed in Elinson et al. 1989). The newly metamorphosed frogs, in turn, require between 8 months to one year to become adults. Therefore, the entire developmental process from fertilization to the adult stage takes from 15 months to two years in G. riobambae (Elinson et al. 1989). The total developmental time is unknown for other egg-brooding tree frogs, but it might be shorter in some species. In F. pygmaeus, for example, incubation in the pouch lasts only 24 days to produce an advanced tadpole with welldeveloped hind legs (stage 41 of Gosner, 1960) and metamorphosis is completed between 11 and 15 days after birth (Duellman and Maness, 1980). The time to sexual maturity is unknown for *Flectonotus*.

The pouch and maternoembryonic relationship

The pouch of marsupial frogs is located under the dorsal skin of the female and it opens at the pouch aperture, which is found anterior to the cloaca. In some species of *Fritziana*, the pouch consists of lateral infoldings of the dorsal skin, that form a shallow basin. The embryos, therefore, are exposed to the environment during incubation. In other species of *Fritziana* and in *Flecto*-

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notus, embryos are completely covered inside a pouch that consists of two lateral compartments. In these cases, the pouch aperture runs along the entire back of the female, while the pouch aperture of Gastrotheca and Amphignathodon is small and restricted to the posterior portion of the back. In lowland species of Gastrotheca, such as in G. ovifera, the pouch consists of two chambers, while in high Andean species, the two lateral compartments of the pouch have been joined into one. The diverse morphology of the pouch found among living species of marsupial frogs, suggests that during evolution, the pouch originated from infoldings of the dorsal skin of the female (del Pino, 1980). Ontogeny and the histology of the pouch in G. riobambae also suggest that the pouch originates from the dorsal skin. In juvenile females, the skin above the cloaca becomes thick and gives rise to lateral infoldings that originate the pouch (Jones et al. 1973). Once formed, the pouch becomes a permanent structure of the adult female. In the non-incubatory condition the histology of the pouch resembles frog skin, however, during incubation, the pouch becomes enlarged, attenuated, and forms highly vascularized chambers for each egg. Pouch chambers may function in maternoembryonic exchanges during the prolonged period of embryonic incubation (del Pino et al. 1975).

In G. riobambae, the pouch aperture has an inverted U- or V-shape and its borders can be open or closed according to the reproductive state of the female. It is open in frogs that are not in a reproductive state and closes when the ovaries are large and the female is ready for reproduction. At the time of amplexus, the male distends the pouch aperture with his feet for the transport of eggs. Afterwards, the pouch aperture constricts again and it remains closed during the period of incubation, however, access to the pouch for the removal of embryos is possible with a blunt probe. The pouch opens by the time of birth and will remain open until a new batch of oocytes has grown in the ovary.

The pouch protects the embryos and, far from being a simple and passive sheltering structure, it is under the control of reproductive hormones that trigger its formation and reproductive changes. Formation of the pouch in juvenile females can be induced by the administration of 17β -estradiol (Jones *et al.* 1973). In the adult, closure of the pouch occurs after the administration of human Chorionic Gonadotropin (hCG) or progesterone. hCG produces closure only in frogs with large ovaries, while progesterone always produces closure of the pouch, regardless of the size of oocytes (del Pino, 1983).

The maintenance of embryonic incubation seems to be also under hormonal control, since the pouch of G. *riobambae* responds to exogenous progesterone not only with closure, but with the formation of embryonic chambers and with incubation (del Pino, 1983). It is therefore possible, that the postovulatory follicles may contribute progesterone for the maintenance of embryonic incubation. However, postovulatory follicles in frogs with aquatic reproduction normally last for less than one week and do not seem to have a hormonal role (Redshaw, 1972). In *G. riobambae*, in contrast, postovulatory follicles last for about 30 days and ovariectomy performed during that period results in abortion (del Pino and Sánchez, 1977).

As in the case of *Xenopus*, progesterone triggers the breakdown of the germinal vesicle (GVBD) in oocytes of *G. riobambae. In vitro*, the large oocytes of *Gastrotheca* take between 12 and 18 h to undergo GVBD in contrast to the 6 to 8 h in *Xenopus* (de Albuja *et al.* 1983). We found that, after exposure to progesterone, oocytes from frogs with closed pouches undergo germinal vesicle breakdown more rapidly than those of frogs with open pouches. Similarly when the pouch is closed, ovulation can be induced by smaller doses of hCG (de Albuja *et al.* 1983; del Pino, 1983). These findings suggest that endogenous gonadotropins probably stimulate steroid production in ovarian follicles, which in turn influence GVBD of full-grown oocytes, and closure of the pouch.

Development and function of bell gills

Prominent external gills characterize embryos of urodeles and caecilians, while in anuran embryos, only the internal gills are well developed, with the exception of the egg-brooding tree frogs, in which a peculiar type of external gills, named bell gills (Noble, 1927), as well as the internal gills, are well developed. Other anuran species with terrestrial reproduction do not use the external gills for exchanges with the environment. Instead in some species, the abdominal walls become highly vascularized, while in embryos of *Eleutherodactylus* and *Nectophrynoides* (whose terrestrial adaptations are mentioned later in this article), the tail becomes greatly expanded and vascularized for gas and other exchanges (reviewed by Duellman and Trueb, 1986).

Bell gills are highly vascularized disk-shaped structures, that derive from the branchial arches and envelop the embryo totally or partially. In Gastrotheca, Stefania and Hemiphractus bell gills cover the entire embryo forming a cavity which is filled with fluid in which the embryo floats (Fig. 6A), while in Cryptobatrachus, Flectonotus, and Fritziana, bell gills are small and cover only the head region of the embryo (del Pino and Escobar, 1981). By the time of birth, circulation to the bell gills stops, bell gills are resorbed and a new set of secondary gills becomes functional in the tadpoles, while in newborn frogs, bell gills are severed from the body at the time of birth and the frog begins to breath air, however, rudimentary versions of the internal gills are found during embryonic development (del Pino and Escobar, 1981; del Pino et al. 1975).

In marsupial frogs, the bell gills are separated from the vascularized chambers of the pouch only by a thin layer of egg jelly of 10 μ m in thickness and the fertilization envelope. The intimate connection that develops between bell gills and the pouch suggests that certain exchanges might occur between mother and embryos. A rough estimate of the maternal contribution to the embryos is given by the analysis of embryonic weights throughout incubation. In embryos of *G. riobambae*,

the dry weight remains constant, while the wet weight increases threefold, suggesting that the mother provides at least water and gas exchanges to her embryos during the period of incubation (del Pino and Escobar, 1981; del Pino et al. 1975). In addition, the mother may contribute with nutrients. The permeability of the eggs to compounds of low molecular weight, after maturation, gives support to this supposition (Merizalde-de Albuja, 1983). Furthermore, respiration must burn up foodstuffs over the long period of incubation, and would lead to weight loss without the contribution of the mother. Besides these aspects, not all of the yolk is used up during incubation, as may be expected in the absence of a maternal contribution of nutrients. The newborn tadpoles (or froglets in other species) still contain yolk in the intestine and do not need to feed immediately after birth. Besides the aspects of nutrition, the mother might transport and eliminate embryonic nitrogenous waste products during incubation. Although we do not know the nature of the exchanges that occur in the pouch of G. riobambae, the maternoembryonic relationships of this and other marsupial frogs, as well as the hormonal control of embryonic incubation resemble the association between mother and embryos in placental mammals.

Modifications of oogenesis

Embryonic incubation in marsupial frogs is associated not only with reproductive changes in the mother and embryonic development, but also with extraordinary modifications in the process of oogenesis. In many eggbrooding tree frogs, as in most vertebrates, each oocyte contains one germinal vesicle (oocyte nucleus). However, in 14 species (of 36 species investigated) previtellogenic oocytes contain from 4 to 3000 germinal vesicles (Table 2), the numerous germinal vesicles gradually disappear until only one is left in the oocyte. We called this peculiar phenomenon multinucleate oogenesis, and the situation in which there is only one germinal vesicle throughout oogenesis, mononucleate oogenesis (del Pino and Humphries, 1978). All egg-brooding tree frogs with multinucleate oocytes produce large eggs (of about 10 mm in diameter) and incubate embryos to the frog stage, except for the tiny *Flectonotus* that produces eggs of 3 mm in diameter and incubation ends at an advanced tadpole stage. However, large eggs and incubation to a frog stage occur also among egg-brooding species with mononucleated oocytes (del Pino and Escobar, 1981; del Pino and Humphries, 1978). Besides the eggbrooding tree frogs, oocytes of the tailed frog Ascaphus truei, are multinucleated, with 8 nuclei per oocyte (Macgregor and Kezer, 1970).

There are several differences between egg-brooding tree frogs and Ascaphus. Egg-brooding tree frogs belong to an advanced anuran family, the Hylidae, while Ascaphus belongs to the family Leiopelmatidae, the most primitive family of living frogs (Duellman and Trueb, 1986). In addition, there are differences in the life history of these frogs, Ascaphus deposits its eggs in

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Table 3.	C-values, chromosome numbers and				
amplifie	ed rDNA in species with mono- and				
multinucleate oogenesis					

Frog	C-value $(\times 10^{-12} g)$	Extra chromosomal rDNA/nucleu $(\times 10^{-12} g)$	Haploid s chromosome . number
Fr	ogs with mono	nucleate oogene.	sis
Xenopus laevis ^a Gastrotheca	3-1	30 ັ	18 ^b
riobambae	4.6°	≪30 ^d	13°
- Fi	rogs with multir	ucleate oogenes	ris
Flectonotus		• •	• •
pygmaeus'	1.7	2.9	14
Ascaphus truei ^g	4.1	<5	23 ⁿ
^a Brown & Dawi ^b Wickbom (1945	d (1968). 5); Tymowska &	k Fischberg (197	73).
^d Based on qualit	cgregor, unpubl tative estimates	ished. of del Pino <i>et d</i>	ıl. (1986).

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^f Macgregor & del Pino (1982).

⁸ Macgregor & Kezer (1970).

^h Green et al. (1980).

mountain streams (Duellman and Trueb, 1986), while egg-brooding tree frogs incubate their embryos. The C-value of species with mono- and multinucleate oocytes is higher than the C-value of X. laevis, except for F. pygmaeus, a frog that has a low C-value (Table 3). In all species with multinucleate oogenesis, the increased number of nuclei raises the gene dosage in their large oocytes.

Regardless of the mode of oogenesis, egg-brooding tree frogs produce just a few eggs of large size in every reproductive season (Fig. 1). The need to store sufficient nutrients in the egg, to sustain the entire process of development, has resulted in the production of the largest anuran eggs, ranging from 2.5 to 12 mm in diameter (del Pino and Escobar, 1981; Salthe and Duellman, 1973). In addition, the number of eggs is small ranging from 3 to 140 eggs, according to species (Table 2). Oogenesis in X. laevis, in contrast, is geared towards the production of thousands of small eggs to insure that a few will reach the frog stage. The absence of a prolonged, free-living larval stage with inevitable high predation, makes the production of small numbers of embryos a viable reproductive strategy in eggbrooding tree frogs.

Oogenesis in egg-brooding tree frogs is synchronous. This means that in every reproductive season, only one batch of oocytes grows in the ovary, while other oocytes remain small (Fig. 1). When the ovary contains fullgrown oocytes (stage 6), and during the period of embryonic incubation, oocytes of stages 4 and 5 are absent. At the time of incubation, the ovary contains only small oocytes of stages 1 to 3 (up to 1.1 mm in diameter). These oocytes remain small during incubation and will resume growth only after birth (del Pino et al. 1986). Oogenesis of X. laevis, in contrast, is asynchronous in the laboratory, which means that



Fig. 1. Living oocytes of X. laevis (left) and G. riobambae (right). Oogenesis in Xenopus is asynchronous and oocytes of various sizes are found simultaneously in the ovary, while in G. riobambae, only one batch of oocytes develops in every reproductive season, a new batch of oocytes will grow up only after birth. Gastrotheca oocytes are uniformly colored and the animal pole can be distinguished only in full-grown oocytes by the presence of the translucent germinal vesicle (gv). Bar, $500 \,\mu$ m.

oocytes of all stages occur simultaneously in the ovary (Fig. 1). This type of oogenesis is advantageous in a constantly favorable laboratory environment, where the female is always ready to produce eggs. However, X. laevis might be seasonal in its natural environment.

Mononucleate oogenesis of G. riobambae

Oocytes of G. riobambae and of many other species of egg-brooding tree frogs (Table 2) belong to the mononucleate type, but are totally different from X. laevis. A comparison with the stages of Xenopus oogenesis (Dumont, 1972) is given by del Pino et al. (1986) and it is summarized in Table 4. Full-grown oocytes of G. riobambae measure 3.3 mm in diameter and are very large when compared to X. laevis, corresponding to about 16 times the volume of Xenopus stage 6 oocytes, of 1.3 mm in diameter (Fig. 1). The large size of oocytes is due to yolk reserves. Yolk platelet distribution has an animalvegetal gradient, but platelets are larger than in X. laevis. Yolk platelets of G. riobambae, measure 12 to 14 μ m, while in oocytes of X. laevis large platelets reach only 8 to 12 μ m in length. In G. riobambae, oocytes are uniformly yellow and the animal pole cannot be identified on the basis of pigmentation. However, in fullgrown oocytes, the animal pole can be recognized by the presence of the germinal vesicle, which can be seen externally as a translucent spot (Fig. 1). In oocytes of G. riobambae, the germinal vesicle reaches 500 to $600\,\mu\text{m}$ in diameter and is somewhat larger than in Xenopus (del Pino et al. 1986). The large size of the germinal vesicle in both species might be important

with respect to the storage of nuclear RNA and proteins.

A major difference between oocytes of X. laevis and G. riobambae corresponds to the time and extent of ribosomal gene amplification. Oocytes of X. laevis amplify the ribosomal genes at stage 1, during pachytene of the first meiotic prophase. The large quantity of rDNA produced can be seen as a mass, called the nuclear cap, that occupies nearly half of the nucleus in young oocytes (Brown and Dawid, 1968; Gall, 1968, 1969; Van Gansen and Schram, 1972). During the lampbrush phase, the nuclear cap is replaced by numerous nucleoli of round shape (Fig. 2). In contrast, the level of ribosomal gene amplification is low in oocytes of G. riobambae as demonstrated by in situ hybridization to rDNA (del Pino et al. 1986). The time of ribosomal gene amplification is unknown for Gastrotheca oocytes. Although oocytes of this frog do not produce a nuclear cap (Fig. 3A), during the lampbrush phase (stage 2) a few large bodies, that probably correspond to masses of amplified ribosomal genes, can be seen inside the germinal vesicle (Fig. 3B). These large bodies are replaced by small nucleoli of round shape at later stages (Fig. 3C). Although we do not know whether location of the Nucleolar Organizer Regions (NORs) influences ribosomal gene amplification in oocytes, it is of interest to note that the single NOR of the G. riobambae karyotype is located in the short arm of the X chromosome (Schmid et al. 1986).

The low number of amplified ribosomal genes of the G. riobambae oocyte become incorporated into a small number of nucleoli. Oocytes of G. riobambae contain

Stage	G. riobambae	X. laevis
1	Diameter, $<150 \mu m$	Diameter, $<300\mu\text{m}$
	Translucent cytoplasm	Translucent cytoplasm
	Absence of yolk platelets	Absence of yolk platelets
	Absence of nuclear cap	Nuclear cap present
2	Diameter, 150–400 µm	Diameter, $300-450 \mu m$
	Cytoplasm white-translucent	Cytoplasm white-translucent
	Formation of yolk platelets	Formation of yolk platelets
	Lampbrush chromosomes visible	Lampbrush chromosomes visible
	Nucleoli are few and large	Nucleoli are numerous, small, and round
3	Diameter, $400-1100 \mu\text{m}$	Diameter, $450-600 \mu m$
	Oocyte is yellow and opaque	Oocyte has dark pigment
	Nucleoli are numerous	Nucleoli are more numerous
	In large oocytes, nucleoli cluster near chromosomes forming a karyosphere†	No karyosphere formation
4	Diameter, 1100-2100 µm	Diameter, 600–1000 µm
	No distinction of animal pole	Dark pigment occurs at animal pole
	Further clustering of nucleoli in the karyosphere	No karyosphere formation
	No GVBD after treatment with progesterone in vitro	No GVBD after treatment with progesterone in vitro
5	Diameter, $>2100 \mu m$	Diameter, 1000–1200 µm
	No external distinction between animal and vegetal poles	Pigment distinguishes animal from vegetal poles
	GVBD occurs after treatment with progesterone	GVBD occurs after treatment with
6	Diameter $>2100 \mu m$	Diameter, 1200–1300 µm
0	Location of nucleus beneath the surface distinguishes	Equatorial band separates animal from vegetal
	GVBD occurs after treatment with progesterone	GVBD occurs after treatment with
	in vitro	progesterone in witho

Table 4. Comparison of the stages of oogenesis in G. riobambae and X. laevis*

* According to del Pino et al. † Gruzova & Parfenov (1977).



Fig. 2. Nomarski interference optical section through an oocyte (stage 2) of X. laevis. The germinal vesicle contains abundant nucleoli, consequence of the high amplification of rDNA. Bar, 20 µm.

less than 300 nucleoli (del Pino et al. 1986), while Xenopus oocytes, whose ribosomal gene amplification is considerably higher, contain 1000 to 1500 nucleoli (Figs 2, 3C). The structures that were identified as nucleoli in Gastrotheca oocytes contain the nucleolar protein ribocharin (Hügle et al. 1985b), as detected by indirect immunofluorescent microscopy with anti-ribocharin (del Pino et al. 1986). When oocytes of G. riobambae measure about 1 mm in diameter, nucleoli and chromosomes cluster in the center of the germinal vesicle in a structure called the karyosphere (Fig. 3C). The karyosphere is characteristic of amphibian oocytes that have reached a resting phase at the completion of oocyte growth (Gruzova and Parfenov, 1977). The early formation of a karyosphere in oocytes of G. riobambae suggests that the germinal vesicle might become quiescent when the oocyte is relatively small.

The low amplification of the ribosomal genes, might be related to the different profile of rRNA accumulation in oocytes of G. riobambae. Full-grown oocytes of G. riobambae contain less rRNA than stage 2 oocytes of Xenopus, of 0.4 mm in diameter. However, fullgrown oocytes of G. riobambae contain a very large quantity of RNA species of smaller size than oocytes of X. laevis (del Pino et al. 1986). The RNA content of Gastrotheca oocytes could not be accurately quantified due to the presence of yolk contaminants, which were difficult to eliminate without loss of RNA (del Pino et al. 1986).



Given the differences in the RNA reserves of oocytes (del Pino *et al.* 1986), it was of interest to compare oocyte proteins of G. *riobambae* with X. *laevis*. Mono-

clonal antibodies directed against 37 oocyte proteins of X. *laevis* (Stick and Dreyer, 1989) were cross-reacted to oocytes of G. *riobambae*. Cross-reaction was detected

Fig. 3. Comparison of the mononucleated oocytes of G. riobambae with the multinucleated oocytes of F. pygmaeus. (A-C) G. riobambae oocytes; (D-F) Oocytes of F. pygmaeus. The bar shown in C applies to all figures at the indicated magnifications. (A) Young oocyte of *G. riobambae* (oocyte stage 1) from a tadpole (Gosner, 1960, stage 37) showing the absence of the amplified rDNA cap. Bar, 20 µm. Reprinted from del Pino et al. (1986). (B) Germinal vesicle from a G. riobambae oocyte of approximately 160 μ m in diameter (oocyte stage 2) taken from a tadpole (Gosner stage 40). The germinal vesicle contains lampbrush chromosomes and several large bodies of irregular shape that might correspond to masses of amplified rDNA. Bar, 20 µm. (C) Nomarski interference contrast section through the germinal vesicle of a G. riobambae full-grown oocyte of 3.2 mm in diameter (oocyte stage 6). Small nucleoli of round shape are clustered around the chromosomes forming a karyosphere. Bar, $50 \,\mu m$.

by indirect immunofluorescence microscopy as described by Dreyer *et al.* (1982), modified by Wedlich *et al.* (1985). Anti-ribocharin (Hügle *et al.* 1985*b*) was tested on frozen sections of ovary as indicated by Hügle *et al.* (1985*a*). Only 11 antibodies (30%) cross-reacted to *Gastrotheca* oocytes. The remaining reacted negatively or gave an unspecific cross-reaction (Table 5). The immunological comparison indicates that the same abundant nuclear proteins, such as nucleoplasmin, N4, ribocharin and protein xlgv7, are found in the germinal vesicle of oocytes of both frogs.

Multinucleate oogenesis in marsupial frogs

The direct contribution of accessory cells for the construction of an oocyte has been clearly demonstrated in arthropods, such as *Drosophila* (reviewed by Davidson, 1986), and is less known for the vertebrates.

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(D) Histological section through two young oocytes of F. pygmaeus (of about 250 μ m in diameter) showing numerous germinal vesicles of uniform size. Oocyte to the right is slightly bigger and has larger germinal vesicles. However, all germinal vesicles lack the amplified rDNA cap. Bar, 15 µm. (E) Germinal vesicles from an oocyte of F. pygmaeus of approximately $800 \,\mu\text{m}$ in diameter. Oocytes of this size have germinal vesicles of several diameters, as shown here. Large and small germinal vesicles contain several bodies of irregular shape that might correspond to masses of amplified rDNA, while lampbrush chromosomes are found only in germinal vesicles of large size. Bar, 15 μ m. (F) Histological section through the single germinal vesicle of a Flectonotus oocyte of 1.5 mm in diameter. This last germinal vesicle contains small nucleoli of round shape that are clustered around the chromosomes forming a karyosphere. Bar, $40 \,\mu$ m. Note the similarities between nuclei from oocytes of Flectonotus and Gastrotheca.

Certain reptiles have cytoplasmic bridges connecting the oocyte to specialized cells of the follicular envelope. However, the extent to which these accessory cells participate in oocyte formation, remains unknown (reviewed by Macgregor, 1982). Among amphibians, the tailed frog of North America, Ascaphus truei (Macgregor and Kezer, 1970), and some species of eggbrooding tree frogs (del Pino and Humphries, 1978) have multinucleated oocytes. The study of multinucleate oogenesis is restricted by the availability of the frog species concerned. Our studies were done on several living specimens of Flectonotus pygmaeus. In addition, for Amphignathodon guentheri and three species of Gastrotheca, we could observe one living frog of each species. For other egg-brooding tree frogs with multinucleate oocytes, only preserved museum specimens were available (del Pino and Humphries, 1978).

In F. pygmaeus the multinucleate condition derives

 Table 5. Cross-reaction of monoclonal antibodies against germinal vesicle proteins, from X. laevis, with oocytes of G. riobambae

Antibodies against Xenopus oocytes	Number of antibodies	Reaction with G. riobambae	Antibodies*
 Anti-N4†	1	GV	b7-2H4
Anti-nucleoplasmin ⁺	2	GV & C	b7-1A9; b7-1D1
Anti-ribocharin‡	1	Nu	
Anti-protein x1gv7§	1	GV & N	37-1A9
Against other GV proteins	5	GV & N	35-1A7; 41-2D12; 45-1G5; b1-3F5; b4-3E9
0	1	N & C of follicle	b7-1B4
Against other oocyte proteins	16	Negative	16-3C1; 32-4A1; 32-5B6; 45-2C10; 46-2F4; 46-4E1; 46-4H4; 47-7D8; b2-2B10; b3-3F1; b4-3H9; b4-4E6; b4-5F11; b4-5H3; b6-6A2; b6-6E7
Against other oocyte proteins	10	Unspecific	31-1A2; 37-1B2; 45-5E5W; 45-6F11; b3-2D2; b7-1G10; b7-5D3; c1-3A11; c3-6B12; c3-4G9
Total number of antibodies: Cross reaction: Negative and unspecific reaction	37 30 % on: 70 %		

* Antibodies were kindly donated by P. Hausen and C. Dreyer.

† According to Dreyer (1987).

‡ According to Hügle et al. (1985b). Antibody was kindly donated by U. Scheer and B. Hügle.

§ According to Miller et al. (1989).

Abbreviations: C, cytoplasm; GV, germinal vesicle; N, nuclei of somatic cells; Nu, nucleoli.



Fig. 4. Number and diameter of large germinal vesicles of the multinucleated oocytes of *F. pygmaeus*. The multinucleated period is restricted to the previtellogenic stages. Data from del Pino and Humphries (1978).

from mitotic divisions of oogonia within an ovarian cyst, followed by cell fusion to form the multinucleate oocyte (Fig. 3D). The number of germinal vesicles varies among oocytes and individuals and ranges from 1000 to nearly 3000 (del Pino and Humphries, 1978). Each one of the many nuclei that contributes to make an oocyte is a meiotic nucleus with the normal meiotic chromosomal arrangement and the 4C DNA value (Macgregor and del Pino, 1982). In oocytes less than $200 \,\mu\text{m}$ in diameter, nuclear size is uniform but as the oocyte grows, germinal vesicles located toward the periphery of the oocyte are markedly enlarged (Fig. 3E). In germinal vesicles of large size, chromosomes show the lampbrush configuration, characteristic of amphibian oocytes. The multiple germinal vesicles are all active in RNA synthesis, as evidenced by the incorporation of [³H]uridine (del Pino and Humphries, 1978). In comparison to the mononucleate oocytes of X. laevis, the thousands of nuclei of a Flectonotus oocyte increase the gene dosage significantly, presumably allowing more RNA to be made and stored in the oocyte.

Most nuclei of a multinucleate oocyte become small and disappear at the beginning of vitellogenesis, when oocytes measure 700 to 900 μ m in diameter (Fig. 4). The remaining germinal vesicles enlarge further before degenerating, probably due to redistribution of a constant amount of stored nucleoproteins. Eventually only one nucleus remains as the germinal vesicle, with a final diameter of about 500 μ m, as in G. riobambae (Fig. 4). During the multinucleate period, there are no morphological characteristics that identify the nucleus that will remain as the final germinal vesicle. When oocytes contain a single germinal vesicle, this nucleus occupies a central position and, during oocyte growth, it moves towards the animal pole. Oocytes contain a single germinal vesicle during the major growth phase, from 1 to 3mm in diameter, when oocytes accumulate yolk protein reserves by absorbing this liver protein from the blood (Fig. 4).

In spite of the large number of nuclei that contribute to make a Flectonotus oocvte, ribosomal genes become amplified in each one of the numerous germinal vesicles, as seen by in situ hybridization to rDNA (Macgregor and del Pino, 1982). Nuclei of Flectonotus oocytes do not develop a nuclear cap (Fig. 3D). However, during the multinucleate phase, each nucleus contains lampbrush chromosomes and some large structures of irregular shape (Fig. 3E), considered to be nucleoli by del Pino and Humphries (1978). These bodies stain darkly with hematoxylin, and may correspond to masses of amplified rDNA. Subsequently, these bodies are replaced by numerous small, round nucleoli. In the final germinal vesicle of the oocyte, nucleoli become arranged in a karyosphere, as in the case of G. riobambae oocytes (Fig. 3C, F).

During the multinucleate phase of *Flectonotus* oocytes, ribosomal gene amplification of each nucleus is restricted, varies among nuclei, and is independent of the final size of the germinal vesicle, giving an average of $2 \cdot 4 \times 10^5$ rDNA genes per nucleus or about 1/10 the amplification level of the single germinal vesicle of *Xenopus* oocytes (Macgregor and del Pino, 1982). However, when the 2500 or more nuclei of an oocyte are considered, the total amplification of a *Flectonotus* oocyte is 280 times higher than in *X. laevis*, and represents the highest level of rDNA amplification so far encountered in frog oocytes (Macgregor and del Pino, 1982).

Oocytes of Flectonotus fitzgeraldi, the only other species of Flectonotus (Duellman and Gray, 1983), resemble oocytes of F. pygmaeus with regards to the high number of nuclei and in the arrangement of nuclei inside the oocyte (unpublished observations). Multinucleate oocytes of other species of egg-brooding tree frogs contain fewer nuclei. Only 4 to 500 nuclei were counted in each oocyte, according to species (del Pino and Humphries, 1978). These frogs include Gastrotheca dendronastes, G. ovifera, G. walkeri and Amphignathodon guentheri, frogs for which living material was analyzed, as well as eight additional species, for which only museum specimens were available (the names of the various species is given by Elinson et al. 1989). In oocytes of these frogs, the central region does not contain nuclei, although sometimes it includes small nuclei that are degenerating. Large nuclei become arranged in one or two concentric layers toward the periphery. As in Flectonotus, each nucleus contains few nucleoli and develops lampbrush chromosomes. Oocyte growth is accompanied by degradation of nuclei, until only one germinal vesicle remains during vitellogenesis (del Pino and Humphries, 1978).

Multinucleate oogenesis of the tailed frog Ascaphus truei

The tailed frog *Ascaphus truei* was the first anuran species in which multinucleate oocytes were detected (Macgregor and Kezer, 1970). This frog, which inhabits the humid forests of north-western United States,

British Columbia and Canada, contains oocytes that are 8-nucleated, although many ovaries contain a few oocytes with sixteen nuclei (Kezer, personal communication). The 8-nucleated condition results from three nuclear oogonial divisions without cytokinesis. In F. pygmaeus and other egg-brooding tree frogs, in contrast, the multinucleated condition results from fusion of oogonia within an ovarian cyst (del Pino and Humphries, 1978). As in the case of F. pygmaeus, the multiple germinal vesicles of an Ascaphus oocyte are meiotic nuclei, amplify the ribosomal genes to a lower extent than in Xenopus, contain a full set of lampbrush chromosomes, and are active in RNA synthesis. In contrast to Flectonotus, within each oocyte of Ascaphus, all nuclei reach an equivalent level of ribosomal gene amplification. The amplification level corresponds to a mean of 3.1×10^5 rDNA genes per germinal vesicle, or about 1/8 of the amplification level of *Xenopus* oocytes. Similarly, all nuclei of an oocyte contain comparable numbers of nucleoli, ranging from 50 to 399 in different oocytes (Macgregor and Kezer, 1970). Ascaphus oocytes are multinucleated until oocytes measure 2 to 2.5 mm in diameter, thereafter, nuclei degenerate, and only one germinal vesicle remains during vitellogenesis, until oocytes reach their final diameter of 4 mm. As in the case of Flectonotus, there are no visible traits that identify the final germinal vesicle among the several nuclei that contribute to make an oocyte (Macgregor and Kezer, 1970).

rDNA amplification, RNA and protein accumulation in oocytes

Patterns of accumulation of RNA and proteins in the oocytes of egg-brooding tree frogs and in Ascaphus may differ from X. laevis. However, with the exception of the studies reviewed in this article, other aspects of oogenesis in egg-brooding tree frogs and in Ascaphus remain unknown. In multinucleated oocytes, the presence of many nuclei increases the gene dosage and may allow the storage of larger amounts of RNA and proteins than in the mononucleated oocytes of X. *laevis*, which only have a 4C DNA content throughout oogenesis. Not only multinucleated oocytes but also the large mononucleated oocytes of G. riobambae may differ from X. laevis. Oocytes of G. riobambae store only small amounts of rRNA and larger quantities of RNA molecules of small size in comparison to oocytes of X. laevis (del Pino et al. 1986).

Ribosomal gene amplification of the single germinal vesicle of the Xenopus oocyte is high and produces between 30 to 35 pg of extrachromosomal rDNA, equivalent to 2.5×10^6 ribosomal genes (Perkowska et al. 1968). In addition, reduction of the ribosomal genes of the genome to one half, in individuals heterozygous for the anucleolate mutation θ -nu, does not result in a decrease of rDNA amplification (Perkowska et al. 1968). As a consequence, eggs of heterozygous females for the θ -nu mutation contain a large supply of ribosomes and θ -nu homozygous embryos survive to the tadpole stage, although no rRNA synthesis occurs in embryonic cells (Brown and Gurdon, 1964). Similarly,

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amplification of ribosomal genes is high in other amphibians with aquatic reproduction. *Siredon mexicanum*, *Necturus maculosus*, and *Triturus viridescens*, urodele amphibians with widely different numbers of rDNA copies in their somatic genome, accumulate equivalent amounts of extra rDNA and contain about the same number of nucleoli per germinal vesicle as in *Xenopus* oocytes (Brown and Dawid, 1968).

Oocytes of Ascaphus and marsupial frogs (with mono- and multinucleate oogenesis), in contrast, are surprising in the low level of rDNA amplification that occurs in each germinal vesicle (Table 3). In multinucleate oocytes of Ascaphus and Flectonotus, the contribution of many nuclei raises the level of ribosomal gene amplification, allowing the storage of a large quantity of rRNA. However, the amount of rRNA accumulated is unknown. In contrast, amplification of the ribosomal genes remains low in mononucleate oocytes of G. riobambae. The low number of ribosomal genes, in turn, transcribe a small amount of rRNA, an unusual feature for frog oocytes (del Pino et al. 1986).

Similarly, the accumulation of 5S ribosomal RNA (5S RNA) in multinucleate oocytes may differ from X. laevis; however, this aspect has not been investigated in multinucleated oocytes. The 5S RNA genes are not amplified in oocytes of Xenopus (see Davidson, 1986 for a review). The haploid genome has 20 000 copies of oocyte 5S RNA genes, and 400 copies of somatic 5S RNA genes (Brown and Fedoroff, 1978). Transcription of 5S RNA genes depends on transcription factor TFIIIA, which binds to an internal control region of the gene and directs the RNA polymerase III to initiate transcription (Bogenhagen et al. 1980, 1982; Sakonju et al. 1980). The transcription factor, TFIIIA, is one of the abundant proteins of young Xenopus oocytes; however, in contrast to 5S RNA genes, the Xenopus haploid genome contains only one or a few copies of the TFIIIA gene. To provide the oocyte with a large amount of this transcription factor, approximately 5 million copies of TFIIIA RNA are transcribed early in oogenesis (Ginsberg et al. 1984).

If the genome of *Flectonotus* had the same number of 5S RNA genes and TFIIIA genes as in X. laevis, a multinucleated oocyte of *Flectonotus* with 2000 nuclei would be endowed with 3 200 000 copies of the somatic type, 160 000 000 copies of the oocyte type of 5S RNA genes, and with 8000 copies of the gene for TFIIIA, since each nucleus is meiotic and contains a 4C DNA complement. This large dosage of genes may allow accumulation of vast quantities of 5S RNA, and of the protein TFIIIA, compensating for the dilution resulting from the large size of oocytes. In contrast, oocytes of G. riobambae may accumulate 5S RNA and TFIIIA in amounts comparable to those of Xenopus oocytes, since the large oocytes of G. riobambae contain only one germinal vesicle.

Before the onset of vitellogenesis, *Xenopus* oocytes accumulate not only 5S RNA, but many other types of RNA and proteins (see Davidson, 1986 for a review). In previtellogenic oocytes, tRNA (reviewed by Davidson, 1986), snRNA (Forbes *et al.* 1984), histone mRNA (reviewed by Woodland *et al.* 1983), and poly $(A)^+$ RNAs (Golden *et al.* 1980) are the main products of transcription. Similarly many proteins are stored early in oogenesis, such as snRNA-binding proteins (Fritz *et al.* 1984; Zeller *et al.* 1983) and histones (reviewed by Woodland *et al.* 1983), among others (see Stick and Dreyer, 1989 for a review).

The patterns of accumulation of RNA and proteins in multinucleated oocytes is unknown, however, morphological characteristics of previtellogenic oocytes suggest vast accumulation of transcription products and proteins, as the multinucleate condition of oocytes coincides with the previtellogenic period (Fig. 4). Since the many nuclei are active in RNA synthesis (del Pino and Humphries, 1978), a large accumulation of RNA might be possible. In addition, the considerable increase in nuclear volume of the many nuclei with the concomitant increase in oocyte size, suggests the storage of large amounts nuclear-specific RNA molecules and proteins in the numerous oocyte nuclei. In addition, previtellogenic oocytes of Flectonotus are larger than those of X. laevis. Translucent oocytes of Flectonotus measure about 500 µm (del Pino and Humphries, 1978). In contrast, translucent oocvtes of X, laevis measure $300-350 \,\mu\text{m}$ in diameter and at this stage the accumulation of poly $(A)^+$ RNAs is completed (Golden et al. 1980). The mononucleated oocytes of G. riobambae remain translucent until they reach a diameter of about 400 μ m (del Pino et al. 1986), a size slightly larger than that of the previtellogenic oocytes of X. laevis.

Modifications of early development

Marsupial frogs give the first known example of drastic modifications of amphibian oogenesis followed by alterations of the pattern and speed of early development (del Pino and Elinson, 1983; del Pino and Escobar, 1981; Elinson and del Pino, 1985). The large size of the egg, due to the enormous reserves of yolk, does not provide the sole basis for the observed modifications, since the large eggs of other frogs follow a developmental pattern similar to *Xenopus*. For example, in *Eleutherodactylus coqui*, a frog with eggs of 3 mm in diameter (the same diameter of *G. riobambae* eggs), the speed of early development and gastrulation follow a normal amphibian pattern, in spite of the large size of the egg (del Pino and Elinson, 1983; Elinson *et al.* 1989).

The pattern of early development in Gastrotheca

Development has been highly conserved among frogs and it is therefore remarkable how much early embryos of marsupial frogs differ from X. laevis. In spite of the large size of eggs, cleavage of the G. riobambae egg is total, but the pattern has been greatly modified. In G. riobambae, the usual third cleavage of a horizontal furrow is replaced by two vertical furrows (Elinson and del Pino, 1985). Often the third cleavage furrows are irregular and asynchronous, giving rise to intermediate situations with five or six blastomeres. Animal blastomeres cleave more rapidly than vegetal blastomeres and, given the asynchrony of the segmentation pattern, each egg seems to cleave differently. In consequence, there is no general pattern of cleavage that can be described (del Pino and Escobar, 1981). Blastula formation is also modified in embryos of *G. riobambae*. The large blastocoel is displaced to the animal pole and a single layer of transparent cells constitutes the roof (Fig. 5A). This transparent roof allows observation, in the living embryo, of some of the internal cell movements during gastrulation. In contrast, cleavage is synchronous during the first 12 cycles in *X. laevis*, the roof of the blastula consists of two cell layers and the pattern of early development reported by Nieuwkoop and Faber (1967) is common to most eggs.

Additional modifications of early development include changes in the timing and pattern of gastrulation. In *G. riobambae*, the movements of gastrulation follow a basic amphibian pattern and give rise to a blastopore (Fig. 5B), as in *X. laevis* (del Pino and Elinson, 1983; Elinson and del Pino, 1985). However, there is a modification in the timing of archenteron formation and its expansion. In *Gastrotheca*, the process of gastrulation takes about a week, the archenteron is formed first (Figs 5C, 7A) and, after two days, it expands (Fig. 6B). As a result, only a portion of the embryo is involved in archenteron formation. The roof of the tiny archenteron consists of cells that are distinctly smaller

Fig. 5. Blastula and gastrula formation in embryos of G. riobambae. (A) Blastula (stage 6), stained with silver nitrate to demonstrate cell borders, as explained by Elinson & del Pino (1985). Three regions are distinguished after staining: The transparent blastocoel roof (r), the marginal (m), and the vegetal (v) regions. The blastocoel roof (r)consists of a single layer of epithelial cells that expand to cover the embryo. The cell boundaries of the roof stain strongly, while in the marginal region (m), the cell borders are faintly stained. In the vegetal region (v), the entire surface of cells are stained with silver nitrate. The lips of the blastopore will form toward the vegetal pole at the boundary between the marginal and vegetal regions. (B) Gastrula (stage 7), fixed in Smith's fixative. The view is from the vegetal hemisphere, showing the blastoporal lip (b). (C) Gastrula (stage 8), stained with silver nitrate to reveal the cell borders. The closed blastopore (b) is visible at the bottom and surrounding it, there is a disk of small cells (d). The body of the embryo will derive from the small cells of this embryonic disk. This embryo still contains a large blastocoel. The transparency of the blastocoel roof (r) allows observation of the internal movement of peripheral yolky cells, that gradually move upwards over the internal surface of the blastocoel roof. (D) Higher magnification of the boundary between the embryonic disk (d) that surrounds the closing blastopore and the remaining epithelial cells that cover the embryo (stage 8). Note the differences in cell size.(E) Cross-section through an embryo (stage 8) comparable to (C). The blastocoel roof (r) has collapsed after fixation. At the bottom the blastoporal lips, which consist of small cells, have closed over the middle of a tiny archenteron (a). (F) Higher magnification showing the lips of the blastopore and the archenteron (a) at the time of its formation. Bar, 500 μ m for A-C and E; 200 μ m for D and F.

than the remaining cells of the embryo. On the surface, these small cells construct a disk around the closing blastopore (Fig. 5C, D), and the body of the embryo

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derives from this embryonic disk (Fig. 6A-D) (del Pino and Elinson, 1983; Elinson and del Pino, 1985). In contrast, embryos of reptiles and birds develop from a





Fig. 6. Scanning electron micrographs of *G. riobambae* embryos (stage 17) showing the development of the bell gills and the formation of the body from the embryonic disk, above the archenteron. (A) Whole embryo spread over the yolk. The masses of tissue on either side of the head correspond to the developing bell gills (bg) that have collapsed to the sides. In the living condition, bell gills cover the head region at this stage. Later, these gills expand to envelop the embryo completely and function as an extraembryonic membrane during the prolonged period of embryonic incubation. Bar, 150 μ m. (B) Cross section through an embryo showing the large archenteron (a), after its expansion and the formation of the body on top of it. The cell layers that cover the archenteron derive from the embryos showing the notochord (n), neural tube (nt) and somites (s) arranged in a plane, comparable to the development of chick embryos. Bar, $30 \,\mu$ m. (D) Towards the sides of the expanded embryonic disk, the three cell layers, ectoderm (ec), mesoderm (me) and endoderm (en) are clearly seen above the large archenteron. Note the cellular nature of the archenteron floor. The large yolk mass of the *G. riobambae* egg becomes divided into cells during cleavage, in contrast to the chick embryo. Bar, 100 μ m. The electron micrographs were kindly taken by Richard P. Elinson.

small disk of cells, that originates from the meroblastic cleavage of these large eggs. *Xenopus* embryos do not develop an embryonic disk, the processes of archenteron formation and expansion occur simultaneously, and the entire embryo is involved in the formation of the archenteron and in all of the morphogenetic movements that originate the body (Fig. 7B).

In *Gastrotheca*, the archenteron at the time of its formation is symmetrical around the closing blastopore

and there is no clear indication of the anterior region (Fig. 5E, F). As a result, the embryonic disk surrounds the closing blastopore (Fig. 7A). However, as in other frogs, the blastopore signals the posterior end of the body. Movement of the blastopore to a posterior position occurs during expansion of the disk (del Pino and Elinson, 1983), and seems to be accompanied by formation of the notochord. Although the cell movements involved in notochord formation have not been



Fig. 7. Cross-section through the gastrula of *Gastrotheca* (left) and *Xenopus* (right) at the time of blastopore formation. In *G. riobambae*, the archenteron (a) is formed first, and after two days it expands. This situation is associated with the formation of an embryonic disk (d), from which the body of the embryo will derive. The blastocoel (bl) is still large and it is invaded by yolky cells from the vegetal region. In contrast, in *X. laevis*, formation of the archenteron (a) and its expansion occur simultaneously, and an embryonic disk is not formed. Bar, 500 μ m. Redrawn from del Pino & Elinson, 1983.

studied, blastopore displacement, with the coincident formation of the notochord are reminiscent of the posterior movement of Hensen's node and notochord formation in chick embryos.

Similar to Xenopus, the mesoderm may originate from the equatorial region of the egg in Gastrotheca, since the embryonic disk can be traced back to the marginal zone of the blastula (Fig. 5A) (Elinson and del Pino, 1985). The slow pace and the changes in the timing of developmental events, however, may contribute to understand mesoderm induction. During advanced development there are new cases of altered schedules. For example, the heart is formed anterior to the head and circulation commences at this atypical location (Stages 13 to 16) in embryos of Gastrotheca. Only at stage 17, the heart is brought to the normal ventral position by the newly formed cephalic fold (del Pino and Escobar, 1981). However, formation of the heart anteriorly may not look unusual, in view of the anterior location of the heart mesoderm in Xenopus.

The speed of early development and alterations of oogenesis

Embryos of X. laevis develop faster than G. riobambae. Xenopus embryos need only 58 days from the time of fertilization to completion of metamorphosis (Nieuwkoop and Faber, 1967), while in G. riobambae maternal incubation alone lasts almost twice as long (del Pino and Escobar, 1981). Similarly, cleavage and gastrulation occur faster in Xenopus than in Gastrotheca. Eggs of X. laevis require 1.5 h to complete first cleavage and 14 h to the end of gastrulation (Nieuwkoop and Faber, 1967), while in G. riobambae, first



Fig. 8. Comparison of the early developmental rate between *X. laevis*, *F. pygmaeus* and *G. riobambae*. The data for *X. laevis* is taken from Nieuwkoop & Faber (1967).

cleavage needs 12 h and the process of gastrulation is completed in 14 days (del Pino and Escobar, 1981; Elinson and del Pino, 1985) (Fig. 8). These enormous differences in developmental rate might derive from the possible dissimilar quantities of RNA, proteins and ribosomes in the eggs of these frogs.

The *Xenopus* egg is not only supplied with about 10^{12} ribosomes that serve in protein synthesis from fertilization to the feeding tadpole stage, but with plentiful quantities of various mRNA, tRNA, snRNAs, heterologous RNA molecules of unknown function, as well as with proteins to allow 12 rounds of synchronous cleavage in absence of embryonic transcription (see Davidson, 1986 and Woodland, 1982 for reviews). At this stage, the embryo consists of about 4000 cells and from the time of fertilization, only 7h have elapsed. The progression to embryonic transcription is known as the midblastula transition. At this time, new RNA molecules are transcribed, the G₁ period of the cell cycle commences and cleavage becomes asynchronous (Gerhart, 1980; Newport and Kirschner, 1982*a,b*).

A demonstration of the importance of the mRNA and protein reserves of the egg for the process of development is shown by the immediate requirement for histones that the embryo has once the egg begins to cleave. With each cleavage cycle, the DNA is doubled and it becomes associated with an equal amount of histones to form the chromatin of blastomeres. Embryonic nuclei, therefore, need to produce large quantities of histones to support accelerated cleavage. However, the haploid genome of X. laevis contains only 50 to 100 copies of each of the nucleosomal histone genes, and in the short time of each cleavage cycle, the diploid nuclei of early blastomeres could not produce sufficient histones by de novo synthesis of histone mRNA (See Woodland, 1980, for calculations). Instead, the provision of histones depends on the translation of histone mRNAs, and on the use of histones already produced during oogenesis (see Woodland, 1980, for a review). The synthesis of large quantities of histone mRNA and of other RNA molecules might be reason why the germinal vesicle of the oocyte of X. laevis and other

animals contains a 4C amount of DNA throughout oogenesis.

With the exception of mammals, early development of most animals occurs also at fast speed, at the expense of the reserves of the egg. In mammals, in contrast, early development proceeds slowly, embryonic transcription begins in the first blastomeres and cleavage is asynchronous from the beginning. Mammalian oocytes are small, do not amplify the ribosomal genes and, in consequence, store little rRNA and, although mRNA and proteins are accumulated in oocytes, the quantities are lower than in *Xenopus* (reviewed in Davidson, 1986).

The developmental strategy of G. riobambae is comparable to that of mammals. Similar to mammalian oocytes, the oocytes of G. riobambae accumulate only a small quantity of rRNA, but rDNA becomes amplified during oogenesis. The amounts of other kinds of RNA and proteins stored in the Gastrotheca egg might be lower than in Xenopus due to dilution by the large volume of the egg. As in the case of mammals, embryonic development proceeds slowly in G. riobambae. In addition, cleavage seems to become asynchronous after the third cleavage cycle (del Pino and Escobar, 1981), suggesting that embryonic transcription may begin at this stage instead of at the midblastula stage. Embryos of G. riobambae may need 4 to 6 days, instead of 7h to reach 4000 cells, the cell number characteristic of the Xenopus midblastula transition. However, the onset of transcription in embryos of G. riobambae is unknown.

Embryonic development in F. pygmaeus is faster than in G. riobambae and slower than in X. laevis (Fig. 8). Multinucleate oogenesis can be interpreted as the method that provides the large eggs of this frog, with sufficient RNA and proteins to allow rapid development. However, the content of RNA and proteins of *Flectonotus* oocytes is unknown. Similarly, the speed of early development of Ascaphus truei is comparable to that of *Flectonotus* and faster than in G. riobambae, in spite of the low temperatures at which development occurs. Ascaphus eggs are laid in clutches of about 40 eggs in mountain streams. These eggs develop at temperatures slightly above freezing and require 4 to 5 days to the end of gastrulation, 30 days until hatching, three years of larval development and one year of development postmetamorphosis to reach the adult condition (reviewed by Duellman and Trueb, 1986). A table of developmental stages for Ascaphus is given by Brown (1975).

The modifications of oogenesis and development of the egg-brooding tree frogs is of particular interest from the point of view of evolution. Protection of embryos by the mother in these frogs is associated with an enormous increase in the size of eggs (eggs measure up to 1 cm in diameter) to provide embryos with sufficient nutrients until the formation of a new frog, eliminating in this way the dangers of the aquatic development. However, large egg size has further implications for the pattern and speed of early development, as already discussed. Analogous modifications of egg size and developmental pattern may have occurred during the evolution of the reptiles and birds, however, early development in these animals proceeds at fast speed. The evolution of maternal protection became associated with slow development in the mammals and in eggbrooding tree frogs. Rapid development, however, may be a more favorable strategy for frogs, as suggested by the evolution of multinucleated oogenesis and accelerated cleavage in some species of egg-brooding tree frogs.

Developmental adaptations of other frogs

It is difficult to guess which frog species might be particularly interesting for studies of development among the 3438 living species of frogs with their numerous reproductive adaptations (Table 1). In addition, in a given frog, many characteristics may be anuran-like but some others may be unusual. For example, the reproductive mode of *Ascaphus* follows an aquatic pattern, however, oogenesis has been modified. In this frog, the extremely low temperature of development may be associated with increase in the size of eggs to sustain the embryos for a prolonged time. As in the case of *Flectonotus*, the large size of the egg might, in turn, be coupled with the need to increase the gene dosage in oocytes to produce sufficient RNA molecules to accelerate early development.

The deposition of eggs in standing water (Table 1, reproductive mode 1) is not only the most generalized mode of anuran reproduction, but the most primitive (see Duellman and Trueb, 1986 for a discussion). Other aquatic modes of reproduction (Table 1) may be derived from this generalized mode. It might be expected, therefore, that, for a large number of anurans, early development may proceed quite rapidly and that the adaptations of oogenesis and early development may be similar to those found in X. laevis. However, some frogs develop in unusual environments that may call for changes in the size of the egg, modification of the developmental speed, or both. The marsupial frogs provide an example of the developmental changes that accompany slow development. Are there other frogs that develop slowly? What are the modifications that occur at the opposite end, that is in frogs with very fast rates of development?

At the slow end of the developmental spectrum, the African genus *Nectophrynoides* looks particularly interesting. As in the case of egg-brooding tree frogs, some species of *Nectophrynoides* have terrestrial adaptations comparable to those of mammals. The extreme situation occurs in the viviparous species *N. occidentalis* (Table 1, reproductive mode 29). Eggs of this frog, which are devoid of yolk, are retained in the oviducts of the female for 270 days, and after this period, young frogs are born (Lamotte and Xavier, 1972; Lamotte *et al.* 1964). There is a complex hormonal control of reproduction with the presence of *corpora lutea* in the ovary during the period of pregnancy (Xavier and Ozon, 1971; Xavier *et al.* 1970). However, oogenesis and the characteristics of early development are largely unknown for this species. Histological sections of the ovary, published by Lamotte *et al.* (1973), display mononucleate oocytes with few nucleoli, as may be expected on the basis of the slow developmental rate. It would be desirable to learn more about oogenesis and speed of early development in this and other species of the genus *Nectophrynoides*, particularly since the various species represent an evolutionary sequence from egg laying with free-living larvae, to direct development, to ovoviviparity and finally to viviparity in *Nectophrynoides occidentalis* (Wake, 1980).

In contrast, frogs of the genus Eleutherodactylus develop very fast, since as little as two weeks are needed for the direct development of a new frog, bypassing the aquatic larval stages (Table 1, reproductive mode 17). For example in E. coqui from Puerto Rico, fertilization is internal (Townsend et al. 1981) and 15 days are needed from fertilization to hatching of a froglet (Townsend and Steward, 1985). In E. johnstonei, the developmental process is even shorter, lasting only 12 days to hatching of a new frog (Lamotte and Lescure, 1977). To attain the adult condition, this frog needs less than one year (reviewed by Duellman and Trueb, 1986). Eleutherodactylus is abundantly represented in South America, Central America and Florida, and has direct development of terrestrial eggs, except for E. jasperi, which is ovoviviparous (Drewry and Jones, 1976; Wake, 1978). Tables of developmental stages have been made for E. coqui (Townsend and Steward, 1985) and for E. nubicola (Lynn, 1942). However, developmental studies have concentrated on the precocious development of the limbs (reviewed by Elinson et al. 1989). Oogenesis and the speed and pattern of early development are largely unknown.

Early development of *Eleutherodactylus* embryos is fast, however, acceleration of development corresponds to later developmental processes rather than to the speed of cleavage and gastrulation. In fact, the speed of early development of these species is similar to that of Xenopus, as can be inferred from the limited data available (See Lamotte and Lescure, 1977; Elinson et al. 1989). Oogenesis seems to be mononucleated in Eleutherodactylus. In E. unistrigatus, which occurs in Quito, we found mononucleated oocytes, with abundant and very large nucleoli in the germinal vesicle (Nina and del Pino, 1977). Similarly, for E. johnstonei, Gall (1968) found numerous nucleoli inside the single germinal vesicle. However, to produce large eggs with fast rates of development, oogenesis in *Eleutherodac*tylus may be modified to allow the accumulation of RNA and proteins in higher amounts than in oocytes of X. laevis. Eleutherodactylus and Nectophrynoides are just two examples of the wide array of reproductive strategies found among frogs (Table 1). Other anurans have peculiar and fascinating adaptations for reproduction (see Duellman and Trueb, 1986; and Lamotte and Lescure, 1977, for reviews), and their developmental adaptations may be equally extraordinary.

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