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## DEVELOPMENTAL DIVERSITY OF AMPHIBIANS

**Richard P. Elinson** and

Department of Biological Sciences, Duquesne University, 600 Forbes Avenue, Pittsburgh, PA 15282. elinson@duq.edu

**Eugenia M. del Pino**

Escuela de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Avenida 12 de Octubre 1076 y Roca, Quito, Ecuador

### Abstract

The current model amphibian, *Xenopus laevis*, develops rapidly in water to a tadpole which metamorphoses into a frog. Many amphibians deviate from the *X. laevis* developmental pattern. Among other adaptations, their embryos develop in foam nests on land or in pouches on their mother's back or on a leaf guarded by a parent. The diversity of developmental patterns includes multinucleated oogenesis, lack of RNA localization, huge non-pigmented eggs, and asynchronous, irregular early cleavages. Variations in patterns of gastrulation highlight the modularity of this critical developmental period. Many species have eliminated the larva or tadpole and directly develop to the adult. The wealth of developmental diversity among amphibians coupled with the wealth of mechanistic information from *X. laevis* permit comparisons that provide deeper insights into developmental processes.

### Introduction

Amphibians have long been model organisms for developmental biology. While *Xenopus laevis* is presently the amphibian model, others had previously enjoyed the spotlight.<sup>1-3</sup> Various salamanders were used in classic studies by Spemann, Vogt, Harrison, Fankhauser, Holtfreter, and others.<sup>4</sup> *Pleurodeles waltl*, the Spanish ribbed newt, was popular in French laboratories, as was *Cynops pyrrhogaster*, the fire bellied newt, in Japanese laboratories. The Mexican axolotl, *Ambystoma mexicanum*, emerged as the urodele of choice, since it could be easily bred and maintained in laboratory colonies. This permitted identification of a few mutant genes, largely through the pioneering efforts of Humphrey.<sup>5, 6</sup> Presently, the axolotl is the urodele targeted for genomic analysis (<http://www.ambystoma.org>).<sup>6</sup> Among frogs, several species of *Rana* were exploited by Pasteels, Ancel & Vintemberger, the Barths, and Briggs and King among many. *Xenopus laevis* arose through its use in pregnancy testing, and it was established as a model for development by Nieuwkoop and Fischberg.<sup>2</sup>

Even among this group of model amphibians, there are fundamental differences in development. Fertilization in most anurans, the frogs, is monospermic as in mammals, but fertilization in most urodeles, the newts and salamanders, is polyspermic.<sup>7, 8</sup> Primordial germ cells form via the germ plasm, a cytoplasmic localization in anurans, but via induction in urodeles.<sup>9-12</sup> The body form changes completely and abruptly at metamorphosis in anurans, but the body form undergoes minimal, gradual changes in urodeles.<sup>13</sup> Finally urodeles possess remarkable regeneration abilities, not found in anurans.<sup>14</sup>

We expect to find more variation in embryos of amphibians than in embryos of eutherian mammals for two reasons. First, amphibians have had a long phylogenetic history. Even representatives of model systems diverged from each other hundreds of millions of years ago. Second, all of the amphibians used as models are similar in that early development

takes place in water. There are a large number of amphibian species whose embryos develop either on land or in the body of the adult.<sup>15, 16</sup> In the evolution of these species, the embryos had to adapt to new environments, quite different from pond water. In contrast, development of eutherian mammals occurs in the conserved environment of the amnion within the uterus. We will review aspects of reproduction and embryonic development in amphibians that develop in a variety of environments.

## PHYLOGENY

There are three amphibian orders, the anurans, the urodeles, and the legless caecilians, which are part of the monophyletic Lissamphibians. While the urodeles and anurans are considered to be more closely related to each other than either is to caecilians, the last common ancestors were in the Permian, 300-250 million years ago (MYA).<sup>17</sup> By comparison, the last common ancestor of mouse and human lived about 100 MYA (<http://timetree.org>).

Within anurans, commonly used frogs of the genera *Gastrotheca*, *Eleutherodactylus*, *Bufo*, and *Rana* are part of the monophyletic clade Neobatrachia.<sup>18</sup> Species of *Gastrotheca*, *Eleutherodactylus*, and *Bufo* shared common ancestors about 55 MYA, and the last common ancestor between this group and *Rana* was present 160 MYA. *Xenopus*, which belongs to a different clade, shared a common ancestor with the Neobatrachia about 230 MYA.

The long evolutionary times between amphibian clades is coupled with the diversity of environments for development of amphibian embryos. These two features require us to examine amphibians other than the model ones in order to understand alternative developmental paths.

## DEVELOPMENTAL ADAPTATIONS IN CAECILIANS

Caecilians include 188 species (<http://amphibiaweb.org>, 2011), distributed in tropical regions of the world.<sup>15</sup> These elongate and limbless amphibians have secluded life habits, a feature that limits study of their embryos, and consequently embryonic development is little known in this group.<sup>19</sup> Their reproductive modes include oviparity with free-living larvae, direct development, and viviparity. Developmental tables for a few caecilians are available.<sup>19, 20</sup>

The independent origin of the elongated, limbless body plan in caecilians and reptiles provides opportunities to examine the developmental evolution of this morphology. Woltering et al<sup>21</sup> recently used embryos of the caecilian *Ichthyophis* to compare their elongated morphology to that of snakes. Of the 126 vertebrae, 120 are rib-bearing thoracic types. Within the somites that give rise to these thoracic vertebrae however, there are transitions in Hox gene expression which in other animals are associated with transitions in vertebral type. The contrast between the presence of Hox transitions and the absence of vertebral transitions in a caecilian and a snake implies that evolution of the elongated body plan involved alternative interpretations of the Hox code. With respect to limblessness, correlations have been made with changes in caecilian Hox gene and cluster structure<sup>22, 23</sup>, but causal connections have not emerged.

There are several feeding strategies among caecilian larvae.<sup>24</sup> Feeding by suction occurs in larvae of oviparous caecilians in aquatic habitats, and scraping of the uterine epithelium is the mode of prenatal feeding in viviparous species. Juveniles of two direct developing oviparous species feed on the skin of their mothers.<sup>25, 26</sup> Feeding switches to prey capture by biting in adult caecilians. The various feeding modes of oviparous and viviparous caecilians are in turn correlated with differences in jaw development.<sup>24</sup> Further

investigations of caecilian early development will undoubtedly reveal more unexpected characters.

## DEVELOPMENTAL ADAPTATIONS IN URODELES

Development of aquatic larvae and metamorphosis to terrestrial adults is typical of urodeles in the genera *Triturus*, *Taricha*, *Notophthalmus*, *Pleurodeles*, *Cynops*, and *Ambystoma*. This reproductive mode is not predominant however among urodeles, as 68% of all urodele species are lungless salamanders of the family *Plethodontidae* and have direct development.<sup>13, 27</sup>

### Aquatic development in urodeles and paedomorphosis

In contrast to caecilians, urodeles are classical organisms for developmental investigations. In fact, research on early amphibian embryos was concentrated on urodele species with a shift towards anurans, particularly *X. laevis*, only in the last half century.<sup>28</sup> Eggs of urodeles are larger than those of *X. laevis*, and the embryos develop slower, features which facilitate experimental manipulations such as grafting. Anyone studying neural development in *X. laevis* would look enviously at the prominent neural folds in a urodele embryo.

The most widely used urodele, *A. mexicanum*, exhibits the unusual life history of paedomorphosis, in which the aquatic larval form persists, and the axolotl becomes reproductive without metamorphosing. The failure to metamorphose is a derived condition of insufficient thyroid hormone; addition of thyroid hormone causes transformation to the terrestrial adult.<sup>29-31</sup> Natural populations of some *Ambystomid* species exhibit facultative paedomorphosis, and the frequency of paedomorphosis vs. metamorphosis is influenced by environmental factors.<sup>32, 33</sup> It is possible to cross paedomorphic and metamorphic species. These crosses indicate that metamorphosis is dominant to paedomorphosis and that there are several genetic bases for paedomorphosis.<sup>34</sup> Given the plasticity in the genus, it is of interest that metamorphosis was apparently more frequent in the original axolotls, brought to Europe in the nineteenth century.<sup>35</sup> This suggests that paedomorphosis was selected for in laboratory colonies.

Paedomorphosis arose independently several times among urodeles<sup>15</sup>, so it might be expected that the underlying molecular mechanisms differ among paedomorphic species. Indeed unlike *A. mexicanum*, the mudpuppy, *Necturus maculosus*, does not undergo metamorphosis in response to exogenous thyroid hormone. Surprisingly, thyroid hormone receptors are functional and expressed in *N. maculosus*<sup>36, 37</sup>, raising the hypothesis that key regulatory genes, downstream of receptor activity have been altered to yield paedomorphosis.

In contrast to the numerous origins in urodeles, paedomorphosis has never been found in anurans. Wassersug<sup>38</sup> argued that the unusual morphology of the anuran larva, the tadpole, precludes attaining the ability to reproduce. Nonetheless, ovaries with growing oocytes and testes with sperm occurred in giant *X. laevis* tadpoles, which lacked thyroid glands and failed to metamorphose.<sup>39</sup>

### Development in plethodontid salamanders

While reproduction as a larva in paedogenesis is one extreme, the other extreme is direct development in which the larva is eliminated, as found among the speciose plethodontid salamanders. Far less is known about development of plethodontids than of urodeles with aquatic reproduction. Plethodontids deposit large eggs with abundant yolk on land. Large egg size is associated with slow developmental rate and modifications of cleavage pattern, blastocoel roof thickness, and gastrulation.<sup>40, 41</sup> Collazo and Keller<sup>41</sup> document these

changes in *Ensatina eschscholtzii* with a 6 mm egg. These embryos appear to form an embryonic disk, which until now has only been described for the anuran *Gastrotheca riobambae*.<sup>42</sup> Plethodontids are distributed in the Americas and southern Europe.<sup>15</sup> Direct development is considered to underlie their evolutionary success.<sup>40, 43</sup>

## DEVELOPMENTAL ADAPTATIONS IN FROGS

Anurans include 5,999 species (<http://amphibiaweb.org>, 2011) with great diversity of reproductive modes.<sup>15</sup> The most familiar reproductive mode includes the aquatic larval tadpole, which eats and grows until it metamorphoses into a terrestrial adult frog. Not all tadpoles live in water, however. Some begin development on land in foam nests; others are carried by a parent or incubated in the parent's body. Some do not feed and live off the yolk in the egg. In the extreme cases, tadpoles have been deleted from the life histories, and froglets develop directly from the egg. We will first review several features of tadpoles.

### The tadpole's unusual morphology

The body plans of larval anurans, the tadpoles, look very different from adults and from any other vertebrate. Although tadpoles are aquatic, they do not look like fish. Tadpoles have a bulbous head and body, no neck, and a muscular tail lacking vertebrae. Other tadpole oddities are keratinous teeth which are not derived from neural crest, extra jaw cartilages to support this mouth designed for scraping plant material, and an elongated gut without a stomach. While there are species-specific differences between tadpoles<sup>15, 44</sup>, the shared derived characters unite tadpoles in a monophyletic grouping. In other words, there was one origin of the tadpole morphology in some ancestral anuran, and all anurans are derived from that ancestor.

The body plan of the tadpole can be compared to the body plan of the urodele larva. Larval and adult urodeles look similar. Both have elongated bodies with long tails and four legs, splayed out to the side. Vertebrae continue into the tail. The vertebral column moves horizontally in a sinusoidal motion during locomotion, when either swimming or walking. Metamorphosis in urodeles affects skin, gills, tail fins, and other structures, but the form of the body remains the same.

The evolutionary origin of the odd tadpole morphology is likely related to the existence of thyroid dependent metamorphosis. If we start from the urodele condition, any modification can be made to the larva as long as that structure is destroyed at metamorphosis. For example, tadpoles have a long intestine, useful for extracting nutrients from plants and detritus. At metamorphosis, the intestine shrinks by 75% and is remodeled to produce a gut suitable for adult carnivory. The specialized keratinous teeth and beak and the extra jaw cartilages, all designed for feeding by scraping a substrate, are destroyed at metamorphosis as are the muscles and notochord of the tail.

**Tail cartilage**—Two variations in tadpole developmental characters have recently been investigated, namely tail cartilage and carnivory. Tail vertebral cartilages are present in tadpoles of the family Megophryidae.<sup>45-47</sup> Other species can develop cartilages in the form of pelvic elements and hind limbs from amputated tails, regenerating in the presence of retinoic acid.<sup>48, 49</sup> The cartilages in the tail of megophryids or in the regeneration paradigm could arise either from sclerotome, which is normally inactive, or from a transdetermination of another cell type to cartilage. A preliminary report of expression of *pax1*, a sclerotome marker, in tails of *X. laevis* embryos, suggests that the sclerotome begins development in tadpole tails, but then arrests.<sup>50</sup>

**Mouth and digestive tract**—Although most tadpoles eat plant material and detritus, there are carnivorous forms. These require modifications of the jaw, including more massive musculature, and a fundamentally different kind of digestive tract. Carnivorous forms have shorter intestines and a true stomach. Tadpoles of *Lepidobatrachus laevis* are obligate carnivores and continue eating through metamorphosis.<sup>51, 52</sup> In addition to obligate carnivores, there are species whose tadpoles can switch from omnivores to carnivores with corresponding morphological modifications. This polyphenism has been best documented for two species of spadefoot toad which convert to carnivores when the density of shrimp for food is high.<sup>53-58</sup> The keratinous teeth are reduced but the keratinous beak thickens. Jaw muscles enlarge, and the gut shortens.

There is a parallel polyphenism in urodeles called cannibalistic morphs. Among the North American tiger salamander, *Ambystoma tigrinum*, and the Japanese salamander, *Hynobius retardus*, larvae arise with enlarged, broad heads and a greater number of larger vomerine teeth.<sup>59-61</sup> The presence of cannibalistic morphs is due to environmental factors, including the type of prey available<sup>61-63</sup>, water currents<sup>64</sup>, and egg size.<sup>65</sup>

Besides carnivorous tadpoles, there are tadpoles that do not eat, called nidicolous endotrophs.<sup>16, 66, 67</sup> The yolk in the egg is sufficient for them to metamorphose. An intermediate between feeding and non-feeding tadpoles is a facultative feeder. The most famous example is *Bufo periglenes*, the Costa Rican golden toad<sup>68</sup>, which is the poster child for disappearing amphibians. Their tadpoles ate when food was available, but they were able to metamorphose without eating. The endotrophic tadpoles, using only the maternal yolk for nutrition, are intermediates to direct developers, which have eliminated the tadpole.

We will next discuss particular terrestrial reproductive modes of frogs whose embryos have been investigated recently.

### Foam-nests in túngara frogs

The genus *Engystomops* includes 9 species (<http://amphibiaweb.org>, 2011), distributed in Central and South America.<sup>69</sup> Sexual selection, behavior, and ecology have been studied in *Engystomops* (formerly *Physalaemus*) *pustulosus*.<sup>69-71</sup> Development was analyzed in *E. pustulosus*, *E. coloradorum*, and *E. randi*.

*Engystomops pustulosus* reproduces in temporal pools of water, and during amplexus, the egg jelly is beaten into white foam by the male. The major component of the foam is ranaspumin-2, a surfactant protein compatible with developing embryos.<sup>72</sup> The foam-nest floats, has antimicrobial properties, reflects solar radiation, and camouflages eggs and embryos.<sup>70-74</sup> By means of the foam-nest, developing eggs are removed from the aquatic environment, and protected from desiccation and predators. After about two days, tadpoles fall into the water. Methods for túngara frog maintenance and embryo manipulation are given in Romero-Carvajal et al.<sup>75</sup>

*Engystomops pustulosus* has synchronous and asynchronous phases of oogenesis, a feature that has been experimentally exploited.<sup>73</sup> Oocytes contain lampbrush chromosomes, and the pattern of new RNA synthesis in embryos resembles that of other anurans.<sup>73</sup> Maternal transcripts are retained in embryos to the tadpole stage as in *X. laevis*.<sup>76</sup>

Early embryos resemble *X. laevis* albino embryos in size, developmental speed, and appearance until the neurula stage. The neural plate, neural folds, and streams of cranial neural crest cells are larger than in *X. laevis*. Somitogenesis involves small cells and cell intercalation, as found in *Bombina variegata*, *G. riobambae*, *Epipedobates* (formerly

*Colostethus machalilla*, and other dendrobatid frogs.<sup>75, 77-79</sup> In contrast, somitogenesis in *X. laevis* involves rotation of fewer, large cells that span the somite length.<sup>77, 80</sup> A table of developmental stages is given in Romero-Carvajal et al.<sup>75</sup> Pigment granule development and gastrulation are discussed in a later section.

The yolk is incorporated into the gut during early development in embryos of *X. laevis* and other small amphibian eggs.<sup>81, 82</sup> Túngara frogs are an exception, as yolky cells bulge into a yolk sac at the tail bud stage in spite of the small size of eggs. Tail bud embryos of túngara and dendrobatid frogs and of *G. riobambae* (Figure 1) resemble amphibian embryos with large telolecithal eggs.<sup>15, 75, 81, 83-85</sup> The mass of yolky cells resembles nutritional endoderm of *E. coqui*, reviewed later.

### Terrestrial nests of dendrobatid frogs

Dendrobatid frogs include 282 species, distributed in Central and South America (<http://amphibiaweb.org>, 2011).<sup>15</sup> Many dendrobatids are brightly colored and about one third of the species are poisonous.<sup>86</sup> Skin toxins are derived from the diet and are chemically known.<sup>87-89</sup> Other species are non-poisonous and darkly colored, such as *E. machalilla*.<sup>86, 90</sup> Methods for frog maintenance and handling of embryos are given in del Pino et al.<sup>85</sup>

Dendrobatid frogs exhibit parental care, and the adult releases the contents of its bladder to moisten the embryos. At hatching, tadpoles attach to the dorsum of the parent in charge and are transported to water, where development advances to metamorphosis.<sup>15</sup> Eggs have a darkly pigmented animal pole and range in size from 1.6 mm in diameter in *E. machalilla* to 3.5 mm in diameter in other species.<sup>79</sup> Nests of *E. machalilla* contain 15 eggs on average, and terrestrial development lasts about 20 days.<sup>85</sup> Early development until neurula resembles that of *X. laevis*. Thereafter, development follows the large telolecithal egg pattern, described earlier. A table of developmental stages is given in del Pino et al.<sup>85</sup> Dendrobatid gastrulation is analyzed in a later section.

### Egg-brooding in hemiphractid frogs

Hemiphractid frogs are characterized by brooding of eggs on the female's back and by the membranous external gills of the embryos, named bell gills (Figure 1f). These frogs occur in Central and South America.<sup>15</sup> Eggs are exposed on the mother's back in *Cryptobatrachus*, *Hemiphractus* and *Stefania*, but they are enclosed inside a dorsal pouch in *Flectonotus* and *Gastrotheca* (Figure 2). Because of this pouch, these latter frogs are known as marsupial frogs.<sup>15, 91</sup> *Gastrotheca* includes 60 of the 95 species of hemiphractid frogs (<http://amphibiaweb.org>, 2011).

Only *Flectonotus*, *Fritziana*, and a few species of *Gastrotheca* give birth to tadpoles. *Flectonotus* tadpoles, however, complete metamorphosis in a few days, without feeding.<sup>15, 92, 93</sup> Other hemiphractids are direct-developers. Their embryos develop rudimentary tadpole characters, such as the tail and larval mouth structures.<sup>93</sup> The tadpole was lost early in the phylogeny of hemiphractids but reappeared within *Gastrotheca*.<sup>91</sup> Accordingly, certain tadpole features would have been lost in the evolution of *Gastrotheca* tadpoles. Larval mouth parts, however, have not been modified.<sup>93</sup>

The pouch of marsupial frogs may derive in evolution from lateral foldings of the dorsal skin that would have enclosed embryos on the female's back.<sup>94</sup> The pouch anatomy in *Flectonotus* (Figure 2a, b) and the pouch ontogeny of *G. riobambae* resemble this condition.<sup>95, 96</sup>

Pouch development is triggered by gonadotropins, and thereafter, the pouch is a permanent structure of the *G. riobambae* female.<sup>95</sup> Progesterone induces the incubatory changes of the



pouch. Long-lived post-ovulatory follicles may secrete progesterone, allowing embryonic incubation and inhibiting further growth of oocytes during incubation.<sup>97, 98</sup>

The non-incubating pouch structure of *Gastrotheca* and *Flectonotus* resembles frog skin. During incubation, the pouch develops vascularized chambers that adhere tightly to each embryo (Figure 2a, c). The fertilization membrane and thin layers of egg jelly separate the pouch from the bell gills of embryos. The nature of exchanges in the pouch is not known.<sup>99</sup> After birth of tadpoles, the pouch acquires the non-incubatory morphology. Reproductive changes are similar in the pouch of *Gastrotheca* species that give birth either to tadpoles or froglets.<sup>96, 100</sup> The dorsal skin of the female in *Hemiphraactus* and *Stefania*, frogs that do not have pouches, form vascularized depressions for each embryo during incubation.<sup>96</sup> Skin incubation evolved independently in Pipidae. Changes of *Pipa* dorsal skin for incubation parallels the reproductive changes of pouch morphology in marsupial frogs.<sup>101</sup>

In *G. riobambae*, fertilization is external, and eggs are moved inside the pouch by the male during amplexus. Embryos of *G. riobambae* of 2.5 to 3 mm in diameter are the smallest among hemiphractids. Cleavage in *G. riobambae* displays modifications associated with large eggs and with slow developmental rate.<sup>102</sup> Embryos develop from an embryonic disk over the mass of cleaved yolk, and remain flat during the neurula stages, allowing observation of neural and cranial neural crest development (Figure 1).<sup>99, 103</sup> A table of developmental stages was modified to allow comparison of *G. riobambae* gastrulation with *X. laevis*.<sup>84, 104</sup> Oogenesis and gastrulation of hemiphraetid frogs are reviewed later.

Incubation in *G. riobambae* lasts about 4 months. Nitrogen waste excretion was changed to ureotelic in *G. riobambae* embryos and tadpoles.<sup>105</sup> Ureotelism is an adaptation for prolonged incubation in the maternal pouch of *G. riobambae* and favors development with limited water.<sup>105</sup> Embryos can be cultured in vitro in a physiological saline solution that contains urea.<sup>106</sup> At birth, the female aids in the emergence of tadpoles with her feet.<sup>99</sup> Methods for the maintenance of adults and handling of *G. riobambae* embryos are given in Elinson et al.<sup>107</sup>

### Direct development in frogs

Larvae have been deleted from the life history of all three orders of amphibians, producing the pattern known as direct development.<sup>15, 20, 27, 40, 41, 43, 108-110</sup> The differences in morphology between the larva and the adult are much greater in frogs than in either urodeles or caecilians, so the appearance of anuran direct developers is particularly striking.<sup>16, 111-116</sup> Although there have been multiple origins of direct developing anurans, their embryos look similar indicating convergent evolution.

The taxon Terrarana is a large group of New World direct developing frogs (900 species in four families).<sup>117-119</sup> An opportunity to examine the developmental modifications that have occurred for this reproductive mode is provided by *Eleutherodactylus coqui* (Figure 3). Mating occurs freely in captivity, and after internal fertilization, eggs are deposited on land. The clutch of embryos is guarded by the male for about three weeks, until the froglets hatch from their jelly capsules. Adults in a laboratory colony remain reproductively active for a year or two, producing clutches of 30 eggs each month. *Eleutherodactylus coqui* has invaded Hawaii, and the Hawaiians are unsuccessfully trying to get rid of them. As a result, it will be easy to collect adult frogs from Hawaii for the foreseeable future.

*Eleutherodactylus coqui*, has been used recently to examine development. A staging table was prepared by Townsend and Stewart<sup>112</sup>, and methods for the maintenance of adults and embryos are given in Elinson et al.<sup>107</sup> The embryos can be cultured in low salt solutions.<sup>120</sup> Features of *E. coqui* development are discussed in other sections of this paper.

## MULTINUCLEATED OOGENESIS

Different organisms, particularly insects, have a variety of mechanisms for making an oocyte.<sup>121</sup> In insects with panoistic ovaries, there is only one nucleus per oocyte, whereas in the meroistic ovary, oocytes accumulate transcripts and other products derived from oocyte sister cells, the nurse cells.<sup>122-125</sup> In contrast, a single nucleus provides the required gene products in oocytes of most vertebrates.<sup>121</sup> Variation of this strategy occurs in frogs with multinucleated oocytes.<sup>126</sup>

### Mononucleated oocytes

Oocytes of frogs and urodeles characteristically have a single large nucleus, known as the germinal vesicle (GV). During diplotene, chromosomes become transcriptionally active and acquire the lampbrush configuration.<sup>127</sup> Additionally, the GV contains a very large number of nucleoli, Cajal bodies, snurposomes and other structures.<sup>128, 129</sup>

*Xenopus laevis* oocytes accumulate ribosomes that support protein synthesis in the embryo until tadpole stages. Amplification of *rRNA* genes during pachytene generates thousands of copies of the major *rRNA* genes that become incorporated into extrachromosomal nucleoli. In contrast to this amplification, the *5S rRNA* genes are repeated in the *X. laevis* genome.<sup>130</sup> Ribosomal gene amplification and the high copy number of *5S rRNA* genes contribute to generate the extraordinary number of ribosomes of *X. laevis* oocytes.

### 8-nucleated oocytes of *Ascaphus truei*

The multinucleate condition may derive from a common pattern of incomplete cytokinesis of the last primary oogonia that are thus connected by cytoplasmic bridges, as observed in *X. laevis* and other organisms.<sup>131</sup> In the tailed frog of North America, *Ascaphus truei*, there is lack of cytokinesis during the last three oogonial divisions, giving rise to 8-nucleated oocytes. Each nucleus has GV features, with rDNA amplification, nucleoli, lampbrush chromosomes, and RNA synthesis. The level of rDNA amplification of each GV amounts to about 1/8 of the rDNA amplification of *X. laevis* oocytes, and the overall rDNA amplification is comparable with *X. laevis* oocytes. Oocytes remain 8-nucleated until the oocyte measures 2 to 2.5 mm in diameter, when nuclei degenerate. Only one GV remains in the full grown *A. truei* oocyte.<sup>132</sup>

### Oogenesis in hemiphractid frogs

The mode of oogenesis was screened in 36 species of hemiphractid frogs. In 14 species, oocytes were multinucleated with 4 to about 3000 GVs per oocyte. Oocytes of the 22 remaining species were mononucleated. No particular reproductive difference was detected in hemiphractid frogs with multinucleated oocytes in comparison with those with mononucleated oocytes.<sup>99, 126</sup>

Oocytes of *G. riobambae* are mononucleated throughout oogenesis, with lampbrush chromosomes, nucleoli, and amplification of *rRNA*.<sup>133</sup> The genome contains about 500 copies of one major repeat of *5S rRNA* genes, similar to the somatic *5S rRNA* gene of *X. laevis*. A limited amplification of ribosomal genes correlates with the low number of *5S rRNA* genes in the *G. riobambae* genome.<sup>133, 134</sup>

Oocytes of *F. pygmaeus* are multinucleated with up to 3000 meiotic nuclei per oocyte (Figure 4). Each GV amplifies the ribosomal genes, and the level of amplification varies among nuclei. The overall amplification of an oocyte with 2500 nuclei is 280 times higher than in *X. laevis*.<sup>135</sup> As the *F. pygmaeus* oocyte grows, peripheral nuclei enlarge and develop lampbrush chromosomes, whereas centrally located nuclei remain small (Figure 4).



All nuclei are active in RNA synthesis. Oocyte growth is accompanied by nuclear degradation until one final GV remains in full grown oocytes (Figure 4a).<sup>126, 135</sup>

The clue to the multinucleated condition most likely relates to acceleration of the process of oogenesis. The single GV of a *X. laevis* oocyte contains only 4 copies of each gene, whereas the genome is repeated 32 times in 8-nucleated oocytes of *A. truei* and 12,000 times in an oocyte of *F. pygmaeus* with 3000 GVs. The many nuclei of a multinucleate oocyte may accelerate the accumulation of gene products during oogenesis resembling the function of nurse cells in the meroistic ovary of insects. There are many unsolved questions concerning multinucleated oogenesis. It is unknown whether nuclei of a multinucleated oocyte are derived from the same oogonial cell. The mechanism of nuclear degradation and the features that protect the final GV from degradation are also unknown. The limited access to frogs with this type of oogenesis hampers further investigations.

## EGG SIZE

There is enormous variation in amphibian egg sizes, ranging in diameter from the small *Xenopus tropicalis* egg with a diameter of 0.7-0.8 mm to several species of marsupial frogs with egg diameters of 9-10 mm.<sup>84, 94</sup> These extremes represent a difference in egg volume of 1500-3000 times. Anurans that lay their eggs in water and which develop and feed as tadpoles generally have eggs with diameters of 1-2 mm.<sup>15</sup> Those that begin development out of the water but enter the water as feeding tadpoles usually have eggs that are 2-3 mm in diameter. Some species of anurans develop directly to frogs with no tadpoles, and the eggs of these direct developers are usually 3-4 mm in diameter. Urodeles with aquatic development tend to have larger eggs (2-3 mm) than anurans with aquatic development, and there are even some in the 5-6 mm range.<sup>15</sup> Particularly among anurans, the larger the egg, the smaller the number of eggs.

While these egg size parameters hold for most species, variations and exceptions abound. For example, a variation in reproductive mode is found in the Surinam toad, *Pipa pipa*, which is in the South American branch of same family as *Xenopus*, a representative of the African branch. Although little frogs emerge from capsules on the back of the female, *P. pipa* is not a direct developer. Tadpoles develop in those capsules from eggs that are 5-6 mm in diameter.<sup>109</sup> The egg of the tailed frog, *Ascaphus truei*, is 4 mm despite its aquatic, tadpole development. At the other extreme, *Sooglossus gardineri* is a direct developing frog, but its egg is only 1.8 mm in diameter.<sup>109</sup>

### Egg size and yolk

Very little is known about how egg size for a species is determined, but it can obviously change with evolution. Much of the variation in egg size is due to increased amounts of yolk, provisioned in the egg for development of the embryo. In the absence of a feeding larva, more yolk is required to generate a terrestrial, carnivorous form that is big enough to capture and eat live prey. Yolk platelets in the oocyte are derived from vitellogenin, synthesized in the liver. Vitellogenin is transported through the blood, and taken up by the growing ovarian oocyte. The oocyte is surrounded by follicle cells, and these can regulate yolk uptake as demonstrated by Wallace and co-workers. Wallace and Misulovin<sup>136</sup> succeeded in growing *X. laevis* oocytes in vitro in a defined medium, supplemented with vitellogenin. These oocytes, lacking follicle cells, continued to grow in volume linearly beyond the normal size. More remarkably, ovarian oocytes, which were already fully-grown in vivo, resumed growth when placed in vitro.<sup>137</sup>

Beside the role of the follicle cells in regulating yolk uptake and oocyte size, the ploidy of the oocyte can have an effect. Laboratory hybrids between *X. laevis* and *Xenopus gilli*<sup>138</sup>

and natural hybrids between *Lithobates* (formerly *Rana*) *lessonae* and *Lithobates* (formerly *Rana*) *ridibunda*<sup>139-142</sup> sometimes lay diploid eggs. Diploid eggs are easy to detect because they are larger than the haploid eggs.

### Lack of pigment in large eggs

Amphibian eggs that give rise to aquatic larva usually have a pigmented animal half, where the nucleus resides, and a non-pigmented, yolkier vegetal half. The animal half can be dark brown or black as with many *Rana* or *Bufo* species, or tan as in *X. laevis*. The vegetal half can be non-pigmented as in *Xenopus* or *Rana*, or contain a considerable amount of pigment granules as in some *Bufo*. Most large eggs, that give rise to non-feeding tadpoles or larvae or directly to adult forms, are unpigmented. The ecological explanation for this difference between aquatic eggs and large eggs is that a dark top and a light bottom provide camouflage in the water. Large eggs are usually brooded in a protected site on land or inside a body cavity of the adult. Like cave animals, pigmentation, that is not needed, would be lost.

Whether or not the ecological explanation is correct, it may be that the processes enriching pigment granules in the animal cortex of the oocyte are linked to yolk uptake, which also involves the cortex. When yolk uptake increases massively, pigment granule production or localization may decrease. Dependence of pigment granule localization on cortical activities is suggested by the recent comparison between *X. laevis* and *E. pustulosus*. *Engystomops pustulosus* begins development on land in foam nests. Their small (1.5 mm) eggs are white, because the pigment granules are accumulated around nuclei of blastomeres.<sup>75</sup> The localization of pigment granules in the animal cortex of *X. laevis* oocytes depends on shroom2, an actin-binding protein.<sup>143</sup> Conversely, oocytes of *E. pustulosus* have little shroom2, and both spectrin and pigment granules are concentrated near nuclei in blastulae. It would be interesting to see the distribution of shroom2 and spectrin in other unpigmented early embryos, particularly those developing from large eggs.

Egg pigmentation is used to identify the prospective dorsal side of the amphibian early embryo. In many species, the gray crescent arises before first cleavage due to the rotation of the egg cortex relative to the cytoplasm. The cortical rotation depends on a transient array of parallel microtubules in the vegetal half.<sup>144</sup> At the onset of gastrulation, the dorsal lip of the blastopore forms near the juncture of the gray crescent and the non-pigmented vegetal half. A gray crescent is not visible on the lightly pigmented *X. laevis* zygote, but pigment granules accumulate near the site of sperm entry, identifying the prospective ventral side. There is no direct evidence that a cortical rotation, which causes dorsal specification, occurs in large, unpigmented zygotes. The presence of an array of parallel microtubules in *E. coqui* zygotes, however, suggests that cortical rotation occurs even in very large eggs.<sup>145</sup>

### Egg size and cleavage

Regardless of egg size, eggs of all amphibians undergo holoblastic cleavage, in which the whole egg is divided into small cells. In some large eggs, there appears to be less cleavage of the yolk-rich vegetal region, leading some to call these vegetal divisions pseudo-meroblastic or meroblastic.<sup>41, 146</sup> True meroblastic cleavage, as in teleosts, reptiles, and birds, has not been found in amphibians.<sup>147, 148</sup> Cleavage in amphibian eggs, greater than 7 mm diameter, has not been examined, however.

In most amphibians, cleavage divisions are synchronous until the mid-blastula transition (MBT). Although most intensively investigated in *X. laevis*, the MBT was defined originally in the urodele, *A. mexicanum*.<sup>149</sup> Each plane of cleavage tends to be perpendicular to the previous plane, yielding stereotypical appearances of morulae at 2-64 cells. A major

deviation from the standard amphibian pattern occurs in *G. riobambae*, which exhibits both asynchrony and early pattern irregularity.<sup>102</sup> Nucleoli are present as early as 8 cells, suggesting a lack of an MBT. Cleavage in *G. riobambae* is very slow, taking about half a day for the first cleavage.

The cleaving *G. riobambae* embryo resembles a mammalian embryo with respect to both slow cleavage and lack of an MBT.<sup>102</sup> The correlations between slowness, irregularity, and asynchrony of cleavage extend to other amphibians, such as the tailed frog *Ascaphus truei*.<sup>150</sup> Cleavage, particularly first cleavage, is much slower in most urodeles compared to most anurans<sup>7</sup>, so asynchronous, irregular cleavage may be more likely among urodeles. Reports on cleavage in several urodeles support this possibility<sup>41, 151-153</sup>; however, the relationships between egg size, cleavage timing, and cleavage pattern require fuller analysis.

## LOCALIZED RNA

Early development of the model amphibian *X. laevis* depends on RNAs localized to the vegetal cortex of the oocyte. These RNAs are of two types: germ layer patterners and germ cell determinants. The former include *vegt*, *vg1*, and *wnt11* RNAs. The latter include *nanos1*, *dazl*, *ddx25*, and *pat* RNAs, which are localized to islands of germ plasm. Based on limited data, some of these RNA localizations are likely basal for anurans. Both *vegt* and *dazl* RNAs are localized to the vegetal cortex of the *Lithobates* (formerly *Rana*) *pipiens* oocyte.<sup>154</sup> In addition, germ plasm has been identified cytologically in various anurans.<sup>155</sup> Deviations from the *X. laevis* paradigm occur in both the direct developing frog *E. coqui* and the axolotl *A. mexicanum*.

In the large 3.5 mm oocytes of *E. coqui*, *vegt* and *vg1* RNAs are present diffusely near the animal pole of the oocyte and not at the vegetal cortex.<sup>156</sup> This distribution correlates with the more animal location of mesoderm in the fate map and the lack of mesoderm inducing activity in most of the vegetal cells of the late blastula and early gastrula (Figure 5).<sup>157, 158</sup> The animal location of *vegt* and *vg1* RNAs indicates that the great amount in yolk has altered the oocyte architecture and the subsequent embryonic patterning. There is presently no information of germ plasm, either cytologically or from RNAs of orthologues, for *E. coqui* or for any other anuran with large eggs.

Localization of RNAs to the oocyte vegetal cortex has not been found in *A. mexicanum*, the only urodele examined in this way. Urodeles lack germ plasm and form primordial germ cells by induction in the ventral marginal zone.<sup>9, 12, 159</sup> Johnson et al<sup>12</sup> proposed that germ plasm protects primordial germ cells from somatic influences in the early embryo and permits higher levels of evolvability in organisms that use germ plasm. The fact that there are almost ten times more species of anurans than urodeles may be a reflection of this greater evolvability.

Corresponding to the lack of germ plasm, RNA of *A. mexicanum dazl* is present in oocytes but not localized.<sup>160, 161</sup> RNA of the ortholog *vegt* is also present in oocytes but not localized<sup>162</sup>, indicating that lack of RNA localization extended to a transcription factor which in *X. laevis* determines formation of both endoderm and mesoderm. Based on these few shards of information, there appear to be fundamental differences in the molecular organization of urodele and anuran oocytes.

## GASTRULATION, THE ORGANIZER, AND MODULARITY

The predominant movements of gastrulation in vertebrates are epiboly, internalization, convergence, and extension.<sup>163</sup> Although these movements are highly conserved, variation

occurs in amphibians with different reproductive modes. Particularly the timing of dorsal convergence and extension (CE) varies among frogs, as analyzed in this section.

### Speed of gastrulation

The speed of early development varies among frogs. *Xenopus laevis* and túngara frogs take 14 hrs and 24 hrs respectively to advance from fertilization to the end of gastrulation.<sup>75, 81</sup> In contrast, the dendrobatid frog *E. machalilla* and the marsupial frog *G. riobambae* develop more slowly and require 4 and 14 days respectively to complete the same process.<sup>42, 85</sup> The outlined differences in developmental time may relate to modifications of gastrulation patterns.

### LIM homeobox 1 and Brachyury as gastrulation markers

*LIM homeobox 1 (lhx1)* and its expression pattern are conserved in animals.<sup>164-168</sup> *Lhx1* is implicated in the evolution of the Spemann–Mangold organizer, and its blastoporal expression is conserved from cnidarians to chordates.<sup>167</sup> In *X. laevis*, *lhx1* induces a secondary axis and acts as transcriptional activator of organizer genes, such as *gooseoid*, *chordin*, *otx2*, *cerberus* and *paraxial protocadherin*.<sup>169</sup> *Lhx1* has a conserved role in specifying neural identity in flies, nematodes and vertebrates<sup>170</sup>, and it is expressed in intermediate mesoderm, pronephros, and kidney.<sup>166, 168, 171-174</sup> Expression of *lhx1* in gastrula stage embryos of various frogs allowed identification of the dorsal blastopore lip, mesoderm induction, location of the presumptive mesoderm, involution, dorsal mesoderm, including prechordal plate, and notochord, and the separation of endomesoderm from ectodermal cells at Brachet's cleft, in comparison with *lhx1* expression in *X. laevis*.<sup>168, 171, 172, 175, 176</sup>

*Brachyury (T)* has a conserved role in Bilaterian blastopore formation and gastrulation.<sup>177, 178</sup> A regulatory N-terminal domain of *brachyury* orthologues plays a role in blastopore formation that correlates with *brachyury* circumblastoporal expression. A subset of *brachyury*-positive cells acquired mesodermal specification functions during evolution.<sup>178</sup> *Brachyury* is an early response gene to mesoderm induction in *X. laevis*<sup>179</sup>, and it is upstream of the planar cell polarity pathway (PCP) and dorsal convergence and extension (CE).<sup>180, 181</sup> Its expression in the notochord indicates the onset of CE in the *X. laevis* mid-gastrula.<sup>182</sup> Convergence and extension movements lead to vertebrate body elongation.<sup>163, 183</sup>

In gastrulae of *E. machalilla*, *Epipedobates anthonyi*, and *G. riobambae*, *brachyury* was detected in a superficial ring around the blastopore. *Brachyury* deep expression in the likely prospective mesoderm was detected after blastopore closure, followed by expression in the elongating notochord. These expression patterns may relate to the function of *brachyury* in blastopore formation, prospective mesoderm development, and body elongation by CE. *Brachyury* expression in the prospective mesoderm and notochord of *G. riobambae* and *E. machalilla* was delayed in comparison with *X. laevis*.<sup>104, 184, 185</sup> Superficial expression of *brachyury* is unknown for *X. laevis*.

### Conserved gastrulation features

**The dorsal blastopore lip and external morphology**—Frog and urodele embryos develop a dorsal blastopore lip that shares organizer properties<sup>186, 187</sup> and has conserved expression of the organizer gene *Lhx1*.<sup>79, 168, 171, 172</sup> The blastopore lip closes around a yolk plug in most frogs and urodeles. Exceptions include the giant salamander *Megalobatrachus maximus* and the frog *Rhacophorus*, where the ventral blastopore lip never forms or its formation is significantly delayed.<sup>188</sup>

Another exception is the embryonic disk of small cells that develops around the closing blastopore in the large embryos of the marsupial frog, *G. riobambae* (Figure 1a).<sup>42, 104, 189</sup> The embryonic disk is reviewed later. Patterns of gastrulation, however, do not associate strictly with egg size, as the large eggs of *E. coqui* develop an equatorial blastopore lip and do not form an embryonic disk.<sup>190</sup>

**Involution and blastopore formation**—Involution at the blastopore lip is conserved as demonstrated by vital dye staining.<sup>42, 191, 192</sup> *Lhx1* expression around the blastopore is required for involution movements in *X. laevis* embryos.<sup>171, 172, 176</sup> By comparison, expression of *Lhx1* around the blastopore is an indication of involution in embryos of túngara frogs, *E. machalilla*, and *G. riobambae*.<sup>79, 168</sup>

**Vegetal contraction**—Contraction at the vegetal pole is a morphogenetic movement of frog and urodele embryos.<sup>189, 193</sup> The vegetal surface of the *G. riobambae* gastrula undergoes 50% contraction, reducing vegetal surface. This movement is associated with bottle-like cells in the vegetal region, and with formation of a pit at the vegetal pole.<sup>104, 189</sup> Contraction pushes the vegetal mass inside the embryo, likely contributing to endoderm internalization and vegetal rotation.

**Brachet's cleft**—Separation of endomesoderm from ectoderm occurs at Brachet's cleft.<sup>182, 194</sup> Brachet's cleft was detected in *E. machalilla* and *G. riobambae* gastrulae, indicating separation between neuroectoderm from endomesoderm in embryos of these frogs.<sup>104</sup> Further analysis is required to determine whether tissue separation at Brachet's cleft is controlled by non-canonical Wnt signaling, as in *X. laevis*.<sup>195, 196</sup>

### Variable gastrulation features

**The transparent blastocoel roof**—The blastocoel roof is a pigmented epithelium that consists of several cell layers in amphibians with small and aquatic eggs. The blastocoel roof becomes thinner during gastrulation, due to the movements of epiboly.<sup>197, 198</sup> In *X. laevis*, a change in cell shape of the outer layer and radial intercalation from the inner cell layers contribute to thinning and expansion of the blastocoel roof, as it surrounds the whole embryo.<sup>197</sup> Despite this thinning, the blastocoel roof epithelium remains opaque. In contrast, the blastocoel roof thins to a transparent cell-monolayer in embryos of frogs and urodeles that are derived from large eggs. Amphibians that develop transparent blastocoel roofs include the anurans *G. riobambae*, *E. machalilla*, and *E. coqui* (Figure 6), and the urodeles *Andrias japonicus*, *Cryptobranchus allegheniensis*, and *E. eschscholtzii*.<sup>41, 85, 189, 199, 200</sup> The transparent roof provides a window that allows observation of internal cell movements. This property could be exploited to observe in vivo cell migration during gastrulation.

In *G. riobambae*, an increase in the volume of the blastocoel causes most of the thinning of the blastocoel roof, prior to the epibolic movements of gastrulation.<sup>189</sup> The blastocoel roof is derived from yolk-poor cells near the animal pole. The monolayered blastocoel epithelium will cover the embryonic disk and the entire yolk mass at later stages<sup>189</sup> (Figure 6b, c), a morphology that resembles blastoderm thinning and spreading, due to epiboly, to enclose the entire yolk cell of zebrafish embryos.<sup>183</sup>

Similarly, in *E. coqui*, most of the single-celled blastocoel roof ends up as an epithelium covering the large mass of yolky cells.<sup>201</sup> This epithelium undergoes apoptosis, and is replaced by the body wall. The blastocoel roof of these embryos is more like an extraembryonic tissue, whose function is to encase the large mass of yolk-rich cells. Accordingly, it is not surprising that the pluripotency of the blastocoel roof differs from *X. laevis*. Cells of the *X. laevis* blastocoel roof are pluripotent and can be induced to follow



many developmental pathways in animal cap experiments. The pluripotency of the animal cap is true for the urodele, *A. mexicanum*, as well.<sup>202, 203</sup> Unlike *X. laevis* and *A. mexicanum*, the *E. coqui* animal cap does not respond to inducing signals in tissue recombinants.<sup>158</sup> Pluripotency does not seem to be the case for the blastocoel roof of either *G. riobambae* or *E. coqui*.

**Presumptive mesoderm and mesoderm induction**—The Nodal gene family plays the most important role in mesendoderm induction in vertebrates, with one Nodal family member in chick, mouse and axolotl, three different Nodal genes in zebrafish and six in *Xenopus*.<sup>203, 204</sup> Diversification of the Nodal gene family during the course of evolution allowed division of labor. For example, in *X. laevis*, different nodal genes play sequential roles in mesendoderm induction and gastrulation movements in contrast with the one-gene situation of mammals and axolotl.<sup>203, 204</sup> Swiers et al.<sup>203</sup> propose that mesoderm specification by a single nodal gene is the vertebrate ancestral state, as it is conserved between urodeles and mammals.

In the axolotl, nodal activates *mix*, an endodermal transcription factor. *Mix*, in turn, is necessary for *brachyury* expression for mesoderm. This sequential regulation contrasts with the situation in *X. laevis*, where nodal signaling activates both *mix* and *brachyury*, and they are mutually inhibitory.<sup>203</sup>

The nature of mesoderm inducing signals in large frog embryos is unknown. In blastula and early gastrula of *E. coqui*, mesoderm inducing activity is present only in superficial, equatorial cells (Figure 5). The large vegetal cells lack this activity.<sup>157, 158</sup>

In the frogs *E. randi*, *E. machalilla* and *G. riobambae*, mesoderm induction may coincide in time with *X. laevis*, according to *lhx1* expression in the likely prospective mesoderm.<sup>168, 171, 172, 176</sup> In contrast, *brachyury* expression in the prospective mesoderm is delayed until blastopore closure in *E. machalilla* and *G. riobambae*, frogs that delay CE and notochord elongation until after blastopore closure.<sup>104, 184, 185</sup> We conclude that retardation of body elongation in these frogs associates with the retarded expression of *brachyury* in the prospective mesoderm.

**Surface vs. deep mesoderm**—In *X. laevis*, most of the presumptive mesoderm is located internally in the blastula.<sup>188, 192</sup> In contrast, the amount of presumptive mesoderm found on the embryonic surface varies greatly among anurans, and the presumptive mesoderm is located on the surface of the blastula in urodeles.<sup>192</sup> Internalization of the urodele surface mesoderm occurs during gastrulation and neurulation through subduction, a specialized form of ingression that involves apical constriction of cells.<sup>188, 192</sup> As cells become internalized, subduction guides closure of the blastopore, and this process occurs through a bilateral primitive streak. This mechanism differs from *X. laevis* and surprisingly resembles ingression through the single primitive streak of chick and mammalian embryos.<sup>192</sup>

**Organizer variation**—The organizer of amphibian embryos develops from the dorsal blastopore lip, and according to inductive properties, it is divided into head, trunk, and tail organizers. The head and trunk organizers are represented by the prechordal plate and notochord, respectively.<sup>186, 187, 205</sup> Head and trunk organizers are separable as indicated by transplantation of early and late blastopore lip in urodele and *X. laevis* embryos. The early lip induced only head structures, and the late lip induced only the tail.<sup>186, 187, 205</sup> *Lhx1* expression revealed the simultaneous presence of both organizers in the *X. laevis* and túngara frog mid-gastrula.<sup>168, 175</sup> In contrast, natural separation of head and trunk organizers

was observed in embryos of *E. machalilla*. The head organizer develops during gastrulation, and the trunk organizer was detected after blastopore closure (Figure 7).<sup>168</sup>

Movement of the prechordal plate away from the blastopore and towards the animal pole in *E. machalilla* may occur by the highly conserved pattern of active migration of mesendodermal cells onto the extracellular matrix of the blastocoel roof as in *X. laevis* and other vertebrates.<sup>163, 188, 206</sup> In contrast, the trunk mesoderm remains in the thickened circumblastoral collar (CBC) of *E. machalilla* embryos until the end of gastrulation, when CE allows elongation of the notochord and dorsal tissues (Figure 7).

**Elongation of the archenteron and notochord**—Archenteron elongation in amphibian embryos results from a combination of epiboly, vegetal rotation and CE.<sup>207</sup> The archenteron elongates starting in mid-gastrula in *X. laevis*, *E. coqui*, and túngara frog embryos.<sup>75, 79, 104</sup> In contrast, archenteron elongation is delayed until the end of gastrulation in *E. machalilla*, and *G. riobambae*.<sup>79, 104</sup>

Elongation of the notochord in vertebrates is guided by the non-canonical Wnt/PCP and CE.<sup>183</sup> The notochord starts to elongate in the mid-gastrula of *X. laevis* and túngara frog embryos.<sup>75, 79, 104</sup> In contrast, notochord elongation occurs after blastopore closure in *E. machalilla*, *G. riobambae*, and *E. coqui* as detected by brachyury and *lhx1* expression.<sup>75, 158, 184, 185</sup>

A possible explanation for the divergent pattern of notochord elongation derives from differences in the onset of CE. An early expression of *brachyury* is required to activate the non-canonical Wnt/PCP and CE in the *X. laevis* mid-gastrula<sup>180, 181</sup>, leading to accelerated body elongation. In contrast, brachyury expression in the presumptive mesoderm and CE are delayed until the end of gastrulation in *E. machalilla* (Figure 7), *G. riobambae* and *E. coqui* embryos<sup>79, 104, 158</sup>, allowing for delayed elongation of the trunk.

**Separation of convergence and extension from gastrulation**—In *X. laevis*, cells that involute during gastrulation move away from the blastopore lip along the elongating archenteron. This pattern is due to active cell migration of head mesoderm and to CE movements of trunk mesoderm.<sup>163, 188, 208, 209</sup> Once the blastopore closes, the CBC is small. Dorsal CE is the major force for blastopore closure on the *X. laevis* dorsal side.<sup>210, 211</sup> It may also be the leading force for closing the blastopore in túngara frog embryos, as suggested by elongation of the notochord in the mid-gastrula and similar gastrula morphology.<sup>75, 79</sup>

The *X. laevis* ventral blastopore lip undergoes convergence and thickening (CT), and this movement may guide blastopore closure in the ventral side.<sup>188</sup> Thickening of the ventral blastopore lip results from convergence in absence of extension, and cells of presumptive mesoderm are maintained in the blastopore lip for later addition to the dorsal axis.<sup>188, 212</sup>

In embryos of *E. machalilla* and *G. riobambae*, involuted cells remain for the most part in the blastopore lip, as only the prechordal plate migrates anteriorly during gastrulation (Figure 1a). Consequently, the blastopore lip thickens, and forms a large CBC (Figure 1b).<sup>79, 104</sup> This morphology apparently results from a major role of CT during gastrulation and retardation of CE until blastopore closure. The comparison suggests that in slow developing frogs, CT and other forces may close the blastopore in absence of CE.

Gastrulation and CE are naturally separated in *G. riobambae*, and *E. machalilla*, and can be separated experimentally in *X. laevis* and zebrafish embryos. Dorsal development of *X. laevis* embryos is inhibited by ultraviolet irradiation in the vegetal region of the fertilized

egg or by injection of suramin into the blastocoel.<sup>213, 214</sup> Similarly, *X. laevis* embryos deficient for *dishevelled (dvl2)*, a component of the PCP, do not undergo CE or elongate the notochord, and the blastopore lip thickens.<sup>207</sup> In zebrafish, Wnt/PCP mutants go through normal epiboly and internalization without disturbing cell fates. The resultant embryos have shortened anterior–posterior body axis and wider dorsal structures like the notochord and somites.<sup>183</sup> The thick blastopore lip of ventralized *X. laevis* embryos and shortened body axis of these zebrafish mutants resemble gastrulae of *G. riobambae*, and *E. machalilla*. Tada and Kai<sup>196</sup> propose that there is separation of CE from gastrulation in axial and non-axial tissues of zebrafish and mouse embryos, processes that occur simultaneously in *X. laevis*. Therefore, it is not surprising that in the evolution of several frogs, CE movements have been moved to post-gastrula stages, delaying elongation of the body. The comparison additionally indicates that gastrulation is modular, as previously proposed.<sup>104, 207</sup>

**The embryonic disk of *Gastrotheca riobambae***—At the onset of gastrulation, embryos of *G. riobambae* develop a blastoporal-rim at the vegetal border of the one-cell epithelium that covers the blastocoel and cleaved yolk. The blastoporal-rim is a uniform circumferential structure that consists of several tiers of surface elongated cells around the future yolk plug.<sup>104</sup> Later, bottle cells are detected in the likely dorsal side of the blastoporal-rim, and a small dorsal blastopore lip develops.<sup>104</sup> Dorsal lip formation is followed by bottle cell formation and involution all around. The blastopore lip becomes thick with involuted cells, and the archenteron is very small. Bottle cells are found at the anterior tip of the archenteron as in *X. laevis* embryos.<sup>189</sup> Embryos of *X. laevis* or *E. machalilla* do not develop a circumferentially symmetric blastoporal rim. Instead, circumferentially elongated cells appear gradually first in dorsal side and then in lateral and ventral regions of the involuting marginal zone prior to blastopore lip development.<sup>104, 215</sup> Cell involution around the blastopore lip of *G. riobambae* resembles internalization around the entire circumference of the blastoderm margin in zebrafish embryos.<sup>183</sup>

The thick blastopore lip constitutes the embryonic disk at blastopore closure (Figure 1a, b). On the surface the embryonic disk consists of small cells (Figure 1c).<sup>42</sup> Underneath, the CBC is large, and the tiny archenteron is slightly larger on the dorsal side (Figure 1b).<sup>104</sup> When the archenteron elongates along with the notochord, due to CE, the margin of the archenteron expands anisotropically. This expansion results in the displacement of the CBC in the embryonic disk from a medial to a posterior location, resembling the displacement of Hensen's node in chick and mouse embryos.<sup>42, 104</sup> In spite of the similar development of a large CBC and retardation of CE and notochord elongation, an embryonic disk was not detected in embryos of *E. machalilla*.<sup>104</sup> Formation of an embryonic disk in embryos of *G. riobambae* uses the same forces that shape the gastrula of *X. laevis*, and provide an extreme example of gastrulation modularity.

## ADVANCED DEVELOPMENT IN *E. coqui*

Omission of the tadpole stages in *E. coqui* is associated with numerous changes in embryos. For example the tail has been modified into a vascularized and membranous structure that allows gas exchanges during embryonic development (Figure 3c). Precocious development of the limbs and head and other changes associated with this reproductive mode are reviewed in this section.

### Limb development

Direct developing anuran embryos all develop on top of a large yolk mass, which is surrounded after gastrulation by epidermis and lateral plate mesoderm as in embryos of frogs with tadpoles. Large limb buds form early, and the development of the limbs is continuous through embryogenesis (Figure 3). In some species, the forelimb is covered by

the operculum as in tadpoles, but in *E. coqui*, the operculum never completely covers the forelimb.<sup>216</sup> Tadpoles all initiate limbs late, and limb development is slow until metamorphosis. Since the different direct developers were derived independently from ancestors with tadpoles, the inhibition of limb development in tadpoles must be relatively easy to modify in evolutionary time. That suggests that only a small number of genes or molecular and cellular interactions suppress limb development in tadpoles.

Limb development in *E. coqui* has been described in some detail.<sup>201, 217-221</sup> In general, limb developmental characters are conserved with chicken, mouse, zebrafish, and other animals. These characters include *shh* expression<sup>219</sup>, a retinoic acid requirement for forelimb initiation<sup>222, 223</sup>, and the migration of *Ibx1* expressing cells to form limb muscle.<sup>224</sup> Unlike chicken or mouse, *E. coqui* limb buds lack an apical ectodermal ridge (AER).<sup>218</sup> This is likely a lack of the morphological structure only, since expression of distal-less at the distal tip suggests the presence of an AER.<sup>219, 225</sup>

### Head development

A second feature of direct developing anuran embryos is a froglike head with big eyes and a gaping jaw (Figure 3). In *E. coqui*, cells in both the retina and the corresponding optic tectum proliferate rapidly and continuously from eye initiation, contributing to the relative prominence of the eye in the head.<sup>226, 227</sup> This early proliferation contrasts with eyes in tadpoles, where rapid proliferation is delayed until after feeding begins.

With respect to jaws, those of tadpoles and frogs are radically different. Tadpoles have extra cartilages, the suprarostrals and the infrarostrals, to support their mouths with the keratinous beak and teeth. The lower jaw of tadpoles contains a large palatoquadrate cartilage, which joins the skull at an acute angle, and a short Meckel's cartilage. At metamorphosis, the suprarostrals and infrarostrals are lost, and Meckel's cartilage elongates. The elongation of Meckel's cartilage shifts the palatoquadrate posteriorly, so that it now joins the skull at a slightly obtuse angle.<sup>228</sup>

Development of jaws in *E. coqui* has been investigated at multiple levels by Hanken and co-workers, including immunocytochemistry for collagen and muscle, in situ for skeletal regulatory molecules and collagen, and stains for cartilage and bone.<sup>229-231</sup> The palatoquadrate and Meckel's cartilages are in a mid-metamorphic position, when they are first detectable.<sup>229</sup> Tadpole-specific muscles do not appear, and adult muscles first form in a mid-metamorphic position.<sup>230</sup> There is no trace of the suprarostrals cartilage, although early anterior expression of *bmp4*, *sox9*, and *runx2* suggests a potential cartilaginous domain, whose differentiation is not realized.<sup>231</sup> These results show that most of the tadpole-specific jaw structures have been cleared from the derived ontogeny of *E. coqui*.

Jaw cartilages develop from cranial neural crest cells, raising the question as to whether evolution of the *E. coqui* jaws has involved neural crest changes. Tracing populations of cranial neural crest cells by both morphological and molecular markers has not revealed substantive differences between cranial neural crest in *E. coqui* and species with tadpoles.<sup>225, 232, 233</sup> A more critical test would be transplantation of cranial neural crest between embryos of *E. coqui* and those of a tadpole species, as has been done for quail and chick beaks.<sup>234</sup>

As might be expected from the number of independent origins of direct development, there is variation in the degree to which tadpole-specific structures have been eliminated. Elimination of the tadpole is very complete in *Eleutherodactylus*. In contrast, the embryo of the direct developer, *Philautus silus*, retains both suprarostrals and infrarostrals cartilages and the tadpole orientation of the lower jaw cartilages.<sup>235</sup>

## Thyroid hormone in direct development

Thyroid hormone causes metamorphosis of the tadpole to the frog, which raises the question as to whether thyroid hormone plays a role in a direct developer. Indeed it does. Inhibition of thyroid hormone synthesis by methimazole blocks many developmental changes in *E. coqui*, including transformation of the skin, growth of muscles, resorption of the tail, and differentiation of the stomach and intestinal lining.<sup>236, 237</sup>

In addition to the inhibition by methimazole, there are other indicators that *E. coqui* utilizes thyroid hormone. The thyroid gland is differentiated when the embryo in its jelly capsule is about two weeks old<sup>238</sup>, and the gene for thyroid hormone receptor, *thrb*, is expressed.<sup>236</sup> *Thrb* is upregulated by thyroid hormone in *E. coqui*<sup>237</sup>, as it is in *X. laevis* metamorphosis.<sup>239, 240</sup> Its expression is a molecular indicator of thyroid hormone activity in these embryos.

An open question is whether thyroid hormone plays a role in the early development of limbs, jaws, eyes, and other structures of the early *E. coqui* embryo, before the thyroid gland has developed and before upregulation of *thrb*. Maternal levels of both *thra* and *thrb* RNAs are high in *E. coqui*.<sup>236</sup> Thyroid hormone receptor RNA and protein are also present in *X. laevis* oocytes<sup>240-243</sup>, and thyroid hormone is detected in eggs of anurans, fish, and birds.<sup>244-250</sup> These results suggest that thyroid hormone signaling occurs prior to development of the embryo's thyroid gland. This signaling could play important roles in early development not only of *E. coqui* but also of other animals. The best way to test whether maternal thyroid hormone and its receptors are important in early development would be to use a specific inhibitor of the receptors. Unfortunately, such an inhibitor is not presently available, despite its obvious utility in treatment of hyperthyroidism.

## Nutritional endoderm

A feature of *E. coqui* direct development is the presence of a novel tissue called nutritional endoderm.<sup>251</sup> Nutritional endoderm is a mass of cells, filled with yolk platelets, attached to the developing intestine (Figure 3f). Once the yolk is used, the cells disappear and do not contribute to any frog tissues. The nutritional endoderm is derived from the vegetal region of the blastula (Figure 5). As discussed earlier, this region differs from the vegetal region of a *X. laevis* blastula in that it lacks both *vegt* RNA as well as mesoderm inducing activity. The nutritional endoderm cells are likely not exposed to signals that cause the development of definitive endoderm.

A further characteristic of the nutritional endoderm is that utilization of its yolk depends on thyroid hormone.<sup>237</sup> *Thrb* is expressed in the nutritional endoderm, and methimazole blocks yolk utilization. The effect of thyroid hormone is late, so much of the yolk in the nutritional endoderm is used only after the froglet has hatched from its jelly capsule. Whether nutritional endoderm and its thyroid hormone dependency exist in any other amphibian is not known. There are many lineages of both direct developing frogs as well as species with non-feeding, nidicolus tadpoles that have a large mass of yolk-filled cells. These species could easily be examined for thyroid dependency of late yolk utilization by treating them with methimazole. It is possible that even in *X. laevis* and other species with feeding tadpoles, a careful mapping of endodermal cell fate would detect nutritional endodermal cells.<sup>251</sup>

## Conclusion

A major difficulty in the analysis of embryonic development in less studied amphibians is obtaining embryos. In some cases, it may be easy to collect embryos from nature, whereas in other cases such as caecilians, this represents a major drawback. Frogs, for which handling



and reproduction are known such as Dendrobatids and several species of foam-nesting frogs of the genus *Engystomops*, represent promising species for further analysis. Dendrobatid frogs are particularly interesting since several species are available in pet shops worldwide. Similarly, the African frog *Hyperolius* is a favorite pet, whose early development is known only in its basic aspects.<sup>146</sup>

Besides amphibians available through the pet trade, zoos, or amateur herpetologists, any amphibian that breeds in an urban environment in tropical or sub-tropical regions such as *E. coqui*, would be a candidate for laboratory use. Urban amphibians are relatively insensitive to noise and disrupted light cycles, so they are likely to breed freely in laboratories. The reason for the tropical and sub-tropical stipulation is the greatest diversity of reproductive adaptations occurs in those regions.

There are so many adaptations waiting to be analyzed now that we have an important base line for developmental comparison in the frog *X. laevis*, and other intensively studied organisms. It is important and interesting to make use of the natural experiments of amphibian diversity to understand better the fundamental features of development.

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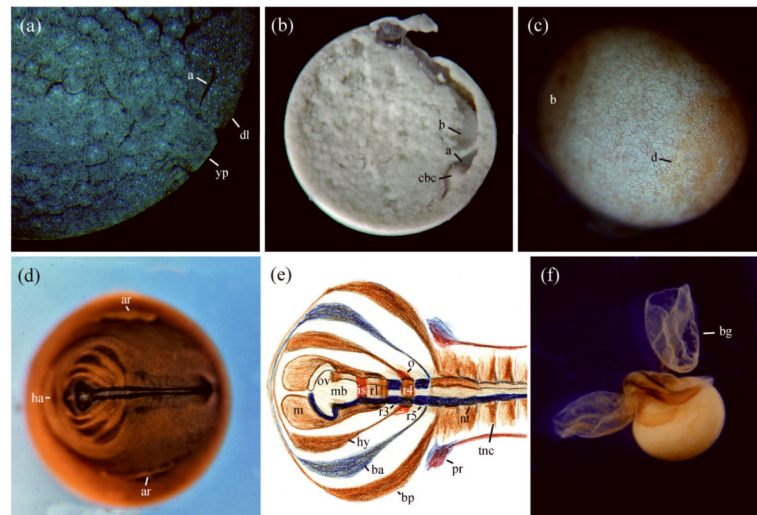
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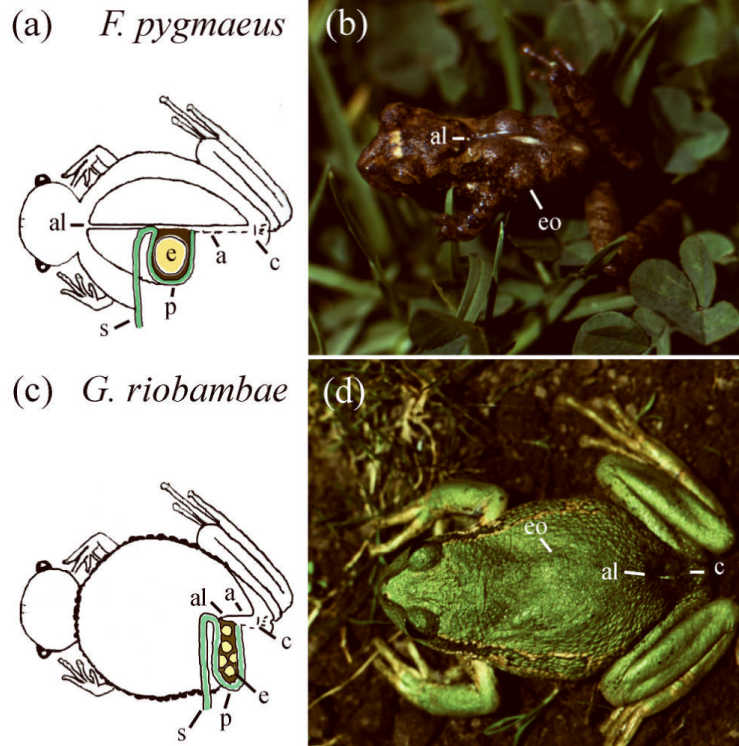
### Further Reading/Resources

252. del Pino EM. Marsupial Frogs. *Sci Am*. 1989; 260:110–118.
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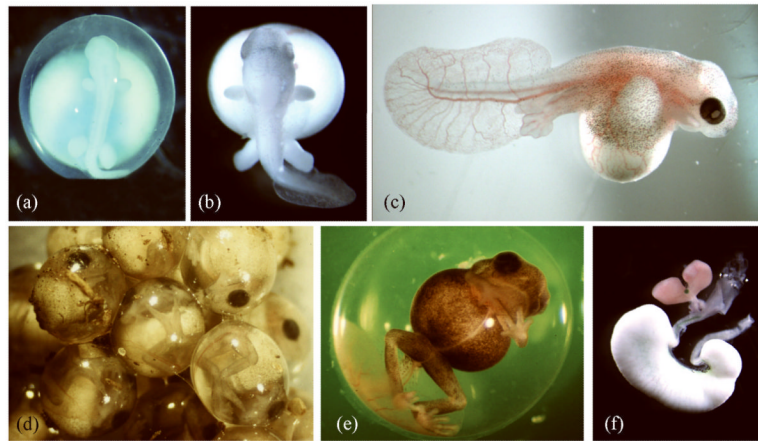
**FIGURE 1.**

Development of the marsupial frog *G. riobambae*. (A) Sagittal section of a mid gastrula embryo photographed with differential interference contrast and fluorescence to detect cell borders and Hoechst 33258 stained nuclei. Involuted cells remain in the blastopore lip. The small archenteron (a), dorsal blastopore lip (dl), and yolk plug (yp) are present in the subequatorial region. (B) Sagittal bisection of a late gastrula. The archenteron (a) remains small and cells that involuted during gastrulation form a large circumblastoporal collar (cbc) around the closed blastopore. The blastocoel (b) is still visible. This image was reproduced from BiosciEdNet Digital Library Portal for Teaching and Learning in the Biological Sciences 2010 (<http://www.apsarchive.org/resource.cfm?submissionID=3000&BEN=1>) (C) The embryonic disk (d) of a late gastrula, stained for cell borders according to del Pino and Elinson.<sup>24</sup> The body of the embryo is derived from the embryonic disk. The blastocoel (b) is still detectable. (D) Embryo immunostained for a neural antigen with antibody P3. The embryo is flat, and the heart anlage (ha) develops anterior to the head. On the sides of the embryonic disk, there are preparation artifacts (ar). (E) Composite diagram of neural expression, according to del Pino and Medina.<sup>84</sup> The mandibular (m), hyoid (hy), branchial anterior (ba) and branchial posterior (bp) streams of cranial neural crest, neural crest of the trunk (tnc), optic vesicle (ov), midbrain (mb), isthmus (is), rhombomeres (r), neural tube (nt) and pronephros (pr) were detected by expression of antigen 2G9 (brown), ncam protein (dark blue), epha7 (light blue) and pax2 protein (red). Epha7 expression on r3 and r5 is not shown. (F) Advanced embryo immunostained for myosin. In the living condition the disk-shaped bell gills (bg) enveloped the embryo in a vascularized sac.

**FIGURE 2.**

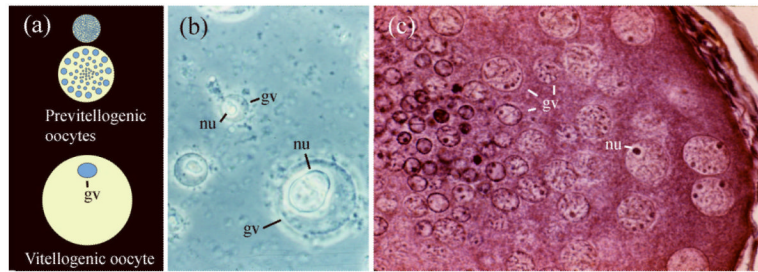
Brooding females of marsupial frogs. (A) Diagram of the pouch and embryos in *F. pygmaeus*. The anterior limit (al) of the pouch aperture (a) is located behind the head, and the posterior limit is above the cloaca (c). This morphology suggests that the pouch developed from foldings of the dorsal skin during evolution.<sup>75</sup> The pouch lining (p) is continuous with the dorsal skin (s). Embryos (e) are brooded inside the pouch. (B) A brooding female of *F. pygmaeus*. The embryo outlines (eo) are detectable. This small frog, of about 2.5 cm in snout-vent length, carries 6 embryos, each of 3 mm in diameter. (C) Diagram of the pouch and embryos in *G. riobambae*. The anterior limit (al) of the pouch aperture (a) is located near the cloaca (c). The pouch lining (p) is continuous with the dorsal skin (s) as in *F. pygmaeus*. Embryos (e) are brooded inside the pouch, which occupies the dorsal and lateral sides of the body in a brooding female. (D) A brooding female of *G. riobambae*. The embryo outlines (eo) are detectable. The pouch opens above the cloaca (c). This frog measures about 5 cm in snout-vent length and broods about 100 embryos, each of 3 mm in diameter, for about 4 months.<sup>65</sup>





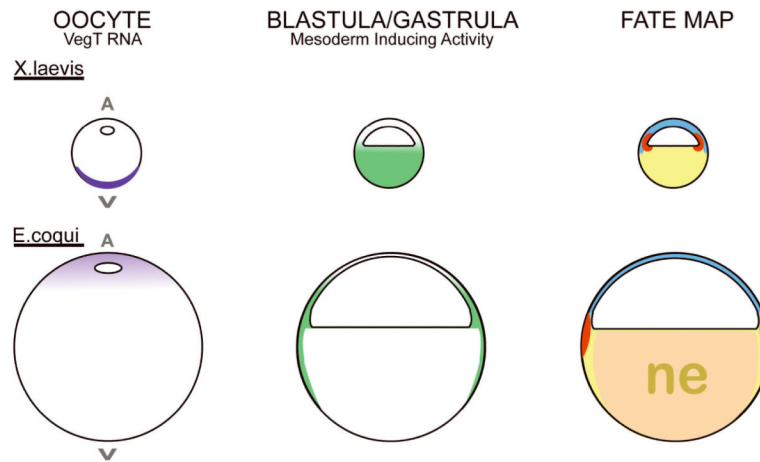
**FIGURE 3.**

Embryos of the direct developing frog *E. coqui*. (A) An early *E. coqui* embryo at Townsend-Stewart (TS) stage 4/5 has developed limb buds and a broad head. (B) By TS7, foot paddles are evident as well as large froglike eyes. (C) This TS10 embryo has been removed from its jelly capsule. The thin, highly vascularized tail serves as a respiratory surface. The pigmented body wall containing somite-derived musculature is extending over the yolk mass to form a secondary coverage. Digits are present and the eye is darkly pigmented. (D) This picture of a clutch of eggs shows TS12 embryos, as they appear naturally in their jelly capsules. (E) A TS14 froglet is about two days from hatching. (F) A digestive tract, dissected from a newly hatched froglet, shows the yolk cells (white) of the nutritional endoderm, attached to the small intestine. Two lobes of liver (pink) and the gall bladder (green) lie between the stomach and the nutritional endoderm.



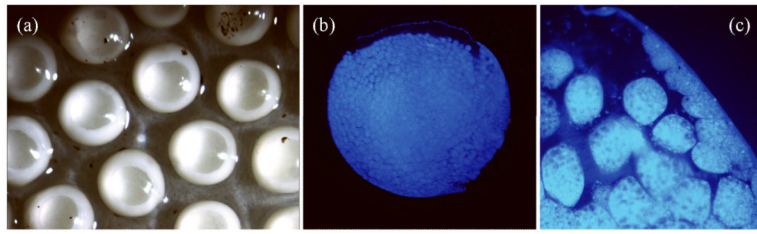
**FIGURE 4.**

Multinucleate oocytes of *F. pygmaeus*. (A) Diagrams of oocytes. Small oocytes contain about 2000 germinal vesicles of similar diameter, depicted in blue. As oocytes grow, germinal vesicles located toward the periphery enlarge, whereas the centrally located ones remain small. With vitellogenesis, the number of germinal vesicles decreases until only one remains in the full grown oocyte. (B) Germinal vesicles (gv) of different sizes, extruded from a living oocyte. Nucleoli (nu) occur in large and small gvs. (C) Section through a multinucleate oocyte with gvs of various sizes.



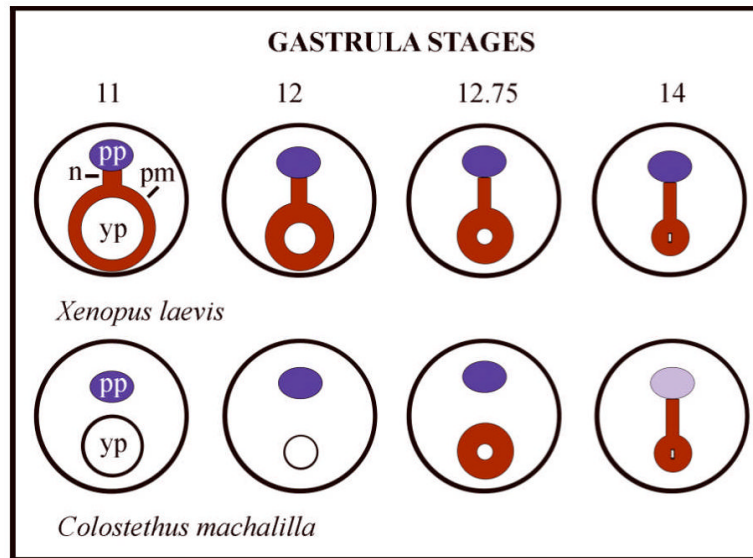
**FIGURE 5.**

Pattern of mesendodermal induction in *X laevis* and *E. coqui*. All diagrams are sagittal views, drawn to scale. In *X. laevis*, *vegt* RNA (purple), localized to the oocyte vegetal (V) cortex, leads to nodal signaling (green) in the vegetal half of the blastula/gastrula. This signaling in turn leads to endoderm (yellow) and mesoderm (red) in the fate map. In *E. coqui*, *vegt* RNA (purple) is near the oocyte animal pole (A) and mesoderm inducing activity (green) is restricted to the peripheral marginal and sub-marginal zones. The absence of *vegt* activity and nodal signaling is hypothesized to lead to development of nutritional endoderm (ne) (pale orange) in the vegetal core.



**FIGURE 6.**

Transparent blastocoel roof. (A) In this animal pole view of *E. coqui* midgastrulae, the blastocoel roofs are transparent, allowing the interior cavity of the blastocoels to be visible. (B) A section through a *G. riobambae* late blastula, treated with Hoechst 33258 to stain cell nuclei, reveals the thin blastocoel roof (top) as a single cell thick epithelium. (C) In this enlargement of (B), the thin blastocoel roof extends over large, yolky cells.



**FIGURE 7.** Brachyury and Lhx1 expression in the gastrula of *X. laevis* and *E. machalilla*. Brachyury expression in the notochord (n) and presumptive mesoderm (pm) is indicated in red. Lhx1 expression in the prechordal plate is indicated in purple. The yolk plug (yp) is indicated in white. In stage 14 embryos of *E. machalilla*, the pp expression of lhx1 is downregulated<sup>150</sup>, as indicated in light purple.