RESEARCH ARTICLE

Analysis of Cell Size in the Gastrula of Ten Frog Species Reveals a Correlation of Egg with Cell Sizes, and a Conserved Pattern of Small Cells in the Marginal Zone

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ABSTRACT

We investigated the relationship between egg and cell sizes in the early gastrula of ten species of frogs with eggs of 1,100–3,500 μm diameters. We asked whether differences in cell size of the vegetal region, blastocoel roof, and marginal zone of the early gastrula were associated with egg size. Alternatively, we proposed that cell size differences may associate with gastrulation characteristics. The analyzed species were as follows: *Xenopus laevis, Engystomops randi, Engystomops coloradorum, Espadarana callistomma, Epipedobates machalilla, Epipedobates anthonyi, Epipedobates tricolor, Dendrobates auratus, Gastrotheca riobambae,* and *Eleutherodactylus coqui.* A positive correlation between egg and cell size was detected in the three regions of the gastrula. The correlation was strong in the vegetal region and blastocoel roof, and weak in the marginal zone. Large eggs allowed the evolution of frog terrestrial reproductive modes by storing nourishment for the developing embryos. Large cells, laden with yolk, occur in the vegetal region. However, small cell size characterized the marginal zone and blastocoel roof. We proposed that small cells of the marginal zone are required for involution and blastopore formation. The evolution pressure toward small cells in the marginal zone contributed to maintain the blastopore as a universal feature of frog gastrulation in eggs of different sizes and gastrulation modes. Our comparative analysis revealed two fundamental and conserved properties of the frog early gastrula, the correlation of egg with cell sizes, and the general small size of cells in the marginal zone. *J. Exp. Zool. (Mol. Dev. Evol.) 00:1–9, 2016.* © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

On the basis of the timing of convergent extension, two different modes of gastrulation have been identified in frogs, gastrulation mode 1 and gastrulation mode 2 (del Pino et al., 2007). In frogs with gastrulation mode 1, exemplified by *Xenopus laevis*, convergent extension is an intrinsic morphogenetic process of gastrulation (Keller and Danilchik, '88; Wallingford et al., 2002; del Pino et al., 2007). In gastrulation mode 2, convergent

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extension and notochord elongation start after blastopore closure (del Pino et al., 2007). We investigated whether cell size correlates with early versus late convergent extension.

We analyzed the cell size of the early gastrula in ten frog species from seven genera and six families. These frogs exemplify the two modes of gastrulation and six different modes of reproduction (Table 1). Frogs with gastrulation mode 1 included the aquatic frog *X. laevis* (Pipidae), the túngara frogs, *Engystomops randi* and *Engystomops coloradorum* (Leptodactylidae), with embryos deposited in floating–foam nests, and the glass frog, *Espadarana callistomma* (Centrolenidae), with embryos suspended in vegetation above stream banks (Keller and Danilchik, '88; Keller and Shook, 2004; Romero-Carvajal et al., 2009; Salazar-Nicholls and del Pino, 2015). Eggs of these frogs range from 1,100 to 2,100 μm in diameter (Table 1).

The analyzed frogs with gastrulation mode 2 included *Epipedobates machalilla, Epipedobates anthonyi, Epipedobates tricolor*, and *Dendrobates auratus* (Dendrobatidae). (Benítez and del Pino, 2002; del Pino et al., 2004, 2007; Moya et al., 2007; Hervas et al., 2015). Their eggs range from 1,600 to 3,500 μm in diameter. Embryos of these frogs develop in terrestrial nests, and the tadpoles are transported to water by an adult (Duellman and Trueb, '86). Embryos of the marsupial frog, *Gastrotheca riobambae* (Hemiphractidae) (3,000 μm in diameter), were also analyzed. Embryos of this frog develop inside the pouch of the female and followed gastrulation mode 2 (del Pino, '96). Gastrulation mode 2 also characterizes the development of *Eleutherodactylus coqui* (Eleutherodactylidae: Terrarana) (Ninomiya et al., 2001). Embryos of this frog develop from large eggs (3,500 μm in diameter) directly into frogs, without the aquatic tadpole stages (Elinson and del Pino, 2012) (Table 1).

Eggs of the analyzed frogs are telolecithal with holoblastic cleavage and have marked differences in egg size (Nieuwkoop and Faber, '94; del Pino et al., 2004; Romero-Carvajal et al., 2009; Elinson and del Pino, 2012; Hervas et al., 2015; Salazar-Nicholls and del Pino, 2015) (Table 1). Egg size varies according to the amount of yolk stored during oogenesis. Yolk provides nourishment to the developing embryo until the free-living tadpoles are able to feed or until development reaches more advanced stages (Elinson and del Pino, 2012). A large variation in egg size occurs in species of the families Dendrobatidae, Hemiphractidae, and Eleutherodactylidae: Terrarana. Eggs of hemiphractid frogs range from $<$1,600 to about 10,000 μm in diameter (Schmid et al., 2012). Similarly, eggs of dendrobatid frogs range in size from 1,600 to 4,500 μm in diameter (del Pino et al., 2004; Guayara-Barragán and Bernal, 2012). Likewise, cell sizes in *Eleutherodactylus* frogs range from 2,700 to 5,000 μm in diameter (Wake, '78). The smallest eggs that we analyzed belong to *E. randi* (of 1,100 μm diameter). Eggs of this frog are about 0.8 times the volume of those of the model frog, *X. laevis*, whereas the large eggs of *E. coqui* (of 3,500 μm diameter) are almost 25 times the volume of *X. laevis* eggs. The large differences in egg size between species and their different modes of gastrulation stimulated our analysis.

We investigated the relationship between gastrulation modes, egg, and cell sizes. We hypothesized a direct relationship between the egg size and the cell sizes of the blastocoel roof, marginal zone, and vegetal region of the early gastrula. A direct relationship between egg and cell sizes would indicate that gastrulation begins when the embryos of different frogs attain comparable cell numbers. Accordingly, we expected that embryos derived from larger eggs would have larger cells in the analyzed regions of the early gastrula. In contrast, a lack of correlation between egg and cell sizes would suggest that differences in cell size are not associated with egg size and may relate to other aspects of development such as the morphogenetic movements of gastrulation or to the mode of gastrulation. We chose the early gastrula for cell size analyses because the gastrula is a period of development without cell growth, and blastomeres do not need extracellular signals to survive (Conlon and Raff, '99). Moreover at this stage, cell division slows and the morphogenetic movements of gastrulation begin, as demonstrated in *X. laevis* (Kurth, 2005).

The reproductive variation in frogs provides natural experiments, whose analysis can improve our knowledge of embryo patterning and adaptive evolution. For this reason, we characterized the cell size of the blastocoel roof, marginal zone, and vegetal region of the early gastrula in the above-mentioned species. A comparative analysis of cell size of the frog gastrula in relation to egg size has not been previously undertaken.

MATERIALS AND METHODS

Frog Species and Collection Sites

Frog species are listed according to their reproduction and gastrulation modes (Table 1). Pipidae: Adults of *X. laevis* (Daudin, 1802) were obtained from commercial dealers and maintained in the laboratory of Richard P. Elinson, Duquesne University, Pittsburgh, PA, USA. Leptodactylidae: Two species were analyzed. Adults of *E. randi* (Ron et al., 2004) were collected off the road to the Puyango Forest, W 80.083319, S 3.88184, 288 m, Province of El Oro, Ecuador. Adults of *E. coloradorum* (Cannatella and Duellman, '84) came from near a secondary road near Tinalandia, north bank of the Toachi River, W 79.05028, S 0.29546, 938 m, Province of Pichincha, Ecuador. Centrolenidae: Adults of *E. callistomma* (Guayasamin and Trueb, 2007) were collected in San Lorenzo, Durango, along the banks of the Río Durango and its tributaries, W 78.62405, N 1.04186, 243 m, Province of Esmeraldas, Ecuador. Dendrobatidae: We analyzed four species. Adults of *E. machalilla* (Coloma, '95) were collected from three localities: Rio Cuaque, 68 km NW from El Carmen, near Pedernales, S 0.018000, W 79.929000, 100 m; Pedernales, N 0.066670, W 80.050000, 75 m; and Machalilla, S 1.483330, W 80.766670, 55 m, Province of Manabí, Ecuador. Adults of *E. anthonyi*

Table 1. Frog reproduction and developmental characteristics

Family and species	Reproduction	Egg diameter (μm)	Gastrulation time (hr)[a]	Onset of notochord elongation	References
Gastrulation mode 1: Early elongation of the notochord					del Pino et al. (2007)
Pipidae					
Xenopus laevis	Aquatic	1,200	5.5	Mid gastrula[b]	Nieuwkoop and Faber ('94)
Leptodactylidae					
Engystomops randi	Floating foam-nest	1,100	12.5	Mid gastrula[b]	Romero-Carvajal et al. (2009)
Engystomops coloradorum	Floating foam-nest	1,300	12.5	Mid gastrula[b]	Romero-Carvajal et al. (2009)
Centrolenidae					
Espadarana callistomma	Leaves upperside	2,100	23	Mid gastrula[b]	Salazar-Nicholls and del Pino (2015)
Gastrulation mode 2: Late elongation of the notochord					del Pino et al. (2007)
Dendrobatidae					
Epipedobates machalilla	Terrestrial nest	1,600	65	After gastrulation[c]	del Pino et al. (2004), Hervas et al. (2015)„
Epipedobates anthonyi	Terrestrial nest	2,000	36	After gastrulation[c]	Hervas et al. (2015)
Epipedobates tricolor	Terrestrial nest	2,000	36	After gastrulation[c]	Hervas et al. (2015)
Dendrobates auratus	Terrestrial nest	3,500	72	After gastrulation[c]	Hervas et al. (2015)
Hemiphractidae					
Gastrotheca riobambae	Egg brooding	3,000	168	After gastrulation[c]	Moya et al. (2007)
Eleutherodactylidae: Terrarana					
Eleutherodactylus coqui	Terrestrial eggs	3,500	24	After gastrulation[c]	Townsend and Stewart ('85)

[a]Gastrulation time from stages 10–13. Embryo culture temperatures for *X. laevis* 23°C and 18–23°C for other frogs.
[b]Stage 11.
[c]Stage 13.

(Noble, '21) were collected from three localities: Sarayunga, 41 km from Santa Isabel, road to Pasaje, S 3.314306, W 79.580694, 557 m, Province of Azuay, Ecuador; Santa Isabel, S 3.260167, W 79.311233, 1,677 m, Province of Azuay, Ecuador; El Progreso, S 3.288330, W 79.758120, 95 m, Province of El Oro, Ecuador. *Epipedobates tricolor* (Boulenger, 1899) was collected from Moraspungo, S 1.172913, W 79.221423, 1,800 m, Province of Cotopaxi, Ecuador. Adults of *D. auratus* (Girard, 1855) were donated by The Atlanta Zoo to the Centre of Amphibian Investigation and Conservation of the Pontificia Universidad Católica del Ecuador (CICA-PUCE). Hemiphractidae: *Gastrotheca riobambae* (Fowler, '13) were purchased from Hyla, Quito, Ecuador or were collected from Quisapincha, 12 km SW from Ambato, S 1.233330, W 78.683330, 3,000 m, Province of Tungurahua, Ecuador. Eleutherodactylidae (Terrarana) (Schmid et al., 2010): *Eleutherodactylus coqui* (Thomas, '66) was obtained from Puerto Rico and Hawaii and maintained in the laboratory of Richard P. Elinson, Duquesne University.

Elinson provided fixed embryos of *X. laevis* and *E. coqui* for our work. Adults of *G. riobambae* and *E. machalilla* were maintained in the Laboratory of Developmental Biology, PUCE. Adults of the remaining species were maintained at the "Balsa de los Sapos," CICA-PUCE. The Balsa de los Sapos donated egg clutches of the various frogs for our studies. The Faculty of Exact and Natural Sciences of PUCE approved procedures for the maintenance and handling of frogs and embryos. The permit, 016-IC-FAU-DNBAP-MA, from the Ministry of the Environment, Ecuador, allowed the collection and maintenance of frogs.

Embryo Culture and Staging

Embryos of the dendrobatid frogs *E. machalilla, E. tricolor, E. anthonyi,* and *D. auratus,* glass frog *E. callistomma*, and marsupial frog *G. riobambae* were cultured in humid chambers at room temperature (18–23°C), as described for embryos of the dendrobatid frog, *E. machalilla* (del Pino et al., 2004). Embryos of the túgara frogs *E. coloradorum* and *E. randi* were cultured

within the foam nests in deep plastic dishes half filled with chlorine-free water (Romero-Carvajal et al., 2009). The egg jelly was manually removed before fixation. Fixed embryos of *X. laevis* and *E. coqui* were donated by Elinson. Gastrulae were staged according to the normal tables of development of *X. laevis, E. randi, E. machalilla, E. callistomma, G. riobambae,* and *E. coqui* (Townsend and Stewart, '85; Nieuwkoop and Faber, '94; Ninomiya et al., 2001; del Pino et al., 2004; Moya et al., 2007; Romero-Carvajal et al., 2009; Salazar-Nicholls and del Pino, 2015). We measured egg diameter in fixed and dejellied embryos at the one-cell stage with the measuring tool of the program, Axiovision (Carl Zeiss, Oberkochen, Germany).

Fixation, Embryo Sectioning, and Staining

For each species, three gastrulae (stage 10), taken from different egg clutches, were fixed and sectioned. The embryos were fixed in Smith's solution at room temperature for about 12 hr (Smith, '12). Smith's solution was prepared immediately before use by mixing solutions A and B in equal proportions (solution A: 1% $K_2Cr_2O_7$ in distilled H_2O; solution B: 200 mL 37% formaldehyde, 50 mL acetic acid, and 750 mL distilled H_2O). After fixation, the embryos were washed three times with distilled H_2O and stored in 4% formaldehyde in phosphate-buffered saline solution at 4°C (PBS; 137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4; pH 7.4).

Fixed embryos were dorsally hemibisected in a mixture of glycerol and PBS in equal parts. The bisected embryos were incubated in 7.5% gelatin in PBS at 45°C for 4 hr. Gelatin filled in the spaces between the large yolky blastomeres of the vegetal region, and facilitated the handling and cutting of sections. Moreover, the blastocoel that collapsed during fixation became inflated by ingression of gelatin into this cavity. The embryos were embedded in 6% agarose in PBS and sections of 50–100 μm thickness were cut with a Vibratome 1000 (Technical Products International, Inc., St. Louis, MO) (Moya et al., 2007).

Sagittal sections were stained for 10 min with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO), extensively rinsed in PBS, mounted in glycerol, and examined with a Stemi SV6 stereo microscope (Carl Zeiss, Oberkochen, Germany) or with fluorescent ultraviolet optics using a Z1 Axio Observer microscope (Carl Zeiss). Embryos were photographed with Axiocam cameras attached to microscopes and the image capture program, Axiovision (Carl Zeiss). The images were edited with Adobe Photoshop CS6.

Cell Size Analysis Methods

Cell size was analyzed in micrographs of the sagittal sections of early gastrulae, comparable to stage 10 gastrulae of *X. laevis* (Nieuwkoop and Faber, '94). Cells diameters of the blastocoel roof, marginal zone, and vegetal hemisphere were measured with the measuring tool of the program, Axiovision (Carl Zeiss). For each species, three sagittal sections of gastrulae (stage 10) were analyzed. Gastrulae came from different egg clutches. Embryo sections from previous work were included in the analysis. Fixation in Smith's solution followed by vibratome sectioning allowed clear identification of individual blastomeres. In each gastrula region, we measured the majority of cells characterized by round shape and the presence of the cell nucleus. For the small cells of the blastocoel roof and marginal zone, cell diameter was estimated as the distance between cell nuclei. Cell measurements of each region from the three embryos of each species were pooled. The total number of cells analyzed per species range from 101 cells in the marginal zone of *E. anthonyi* to 323 cells in the blastocoel roof of *E. randi*.

The data were tabulated in Excel. Analysis of variance was used to determine the statistical significance of cell size variation between embryos of each species, between species, and gastrulation modes. The relationship between egg size, cell diameters, and gastrulation modes were quantified using least squares regression.

RESULTS

Morphology of the Early Gastrula

The internal morphology of the early gastrula was similar in the various frogs (Fig. 1). The blastocoel cavity was surrounded by the blastocoel roof and the blastocoel floor. The blastocoel cavity separated vegetal cells from the blastocoel roof. The vegetal region was flanked by the marginal zone. Cells of the vegetal region were measured in the central core of the vegetal hemisphere. Vegetal cells were qualitatively larger in comparison with cells of the blastocoel roof and marginal zone, as shown for several species (Figs. 1B–H). We measured cells of the marginal zone from the level of the blastocoel floor to the subequatorial region of the blastopore lip. The marginal zone was equatorial in embryos of *E. coqui* in contrast with other frogs (Figs. 1H). A monolayer of small and elongated cells delimited the marginal zone in the analyzed frogs (Figs. 1 B, C, G, and H). These cells were not included in the analysis of cell size. In addition, the bottle cells, observed at the dorsal blastopore lip, were not included in the cell size analysis. The blastocoel roof, marginal zone, and vegetal region were depicted in blue, red, and yellow, respectively, in the diagram of the *X. laevis* early gastrula (Fig. 1A). The cell monolayer borders the marginal zone, and bottle cells were shown in green (Fig. 1A). Areas shown in green were not included in the analysis of cell size. Cells in mitosis were not detected in the stage 10 gastrula of the analyzed species. In contrast, cells in mitosis were observed but not analyzed in cleavage and blastula stage embryos, after Hoechst 33258 staining.

The blastocoel roof consisted of several cells in thickness in stage 10 gastrula of all species, with exception of *G. riobambae* (Fig. 1). The *G. riobambae* blastocoel roof likely underwent extensive epiboly and was already a translucent cell monolayer

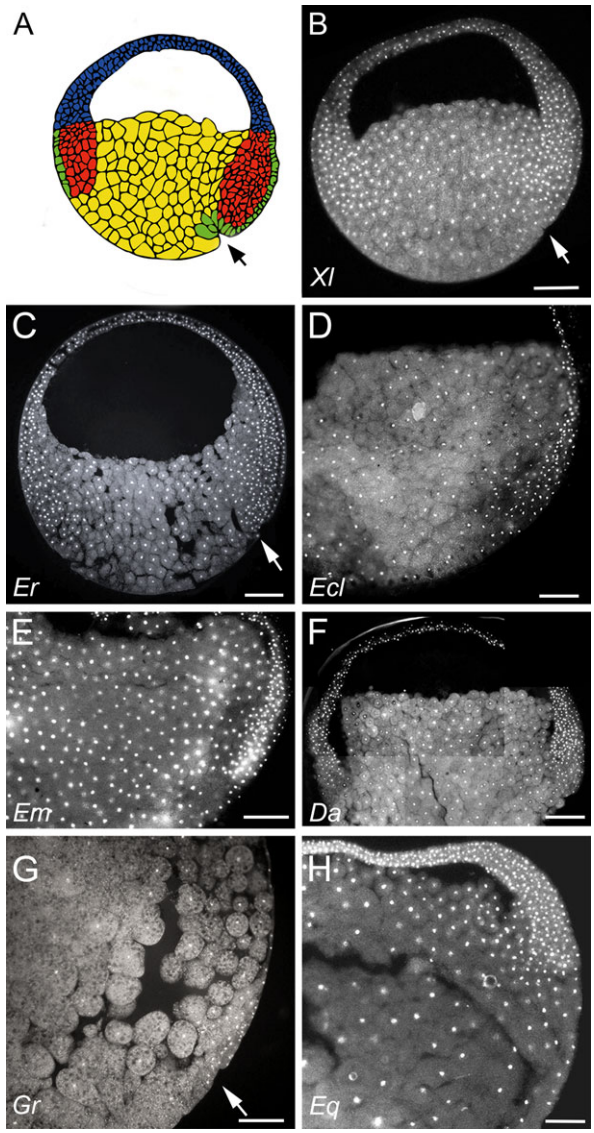

Figure 1. Internal morphology of the gastrula stage 10 of several frogs. The dorsal side is oriented to the right in all images. (A) Diagram of the *X. laevis* gastrula. The blastocoel roof was shown in blue, the marginal zone in red, and the vegetal region in yellow. A cell monolayer located at the surface of the marginal zone and bottle cells of the dorsal blastopore lip were highlighted in green and not included in the cell size analysis. (B–D) Sagittal sections of the gastrula of frogs with gastrulation mode 1. (B) *Xenopus laevis* (*Xl*), (C) *Engystomops randi* (*Er*), (D) *Espadarana callistomma* (*Ecl*), (E–H) sagittal sections of the gastrula of frogs with gastrulation mode 2, (E) *Epipedobates machalilla* (*Em*), (F) *Dendrobates auratus* (*Da*), (G) *Gastrotheca riobambae* (*Gr*), and (H) *Eleutherodactylus coqui* (*Eq*). Arrows point to the dorsal blastopore lip. Bars represent (C and E) 150 μm, (B, D, G, and H) 200 μm, and (F) 500 μm.

at this early stage. In contrast, the *E. coqui* blastocoel roof was several cells thick (Fig. 1H). In more advanced embryos, the *E. coqui* blastocoel roof becomes a translucent cell monolayer, as in *G. riobambae*. We excluded the *G. riobambae* blastocoel roof from this analysis because the cells were narrow and elongated. In contrast, cells of blastocoel roof of the remaining nine species were round in shape. Therefore, we were able to measure their diameters.

We did not analyze the possible changes in cell diameter that were due to fixation. We expect that the fixation changes in cell diameter were uniform because the fixation methodology was identical for all embryos. Differences in cell size attributable to variability in egg diameter between frogs of each species would weaken our comparative analyses of cell size. For this reason, we compared the cell diameters of the vegetal region in the embryos of each species. We choose the vegetal region because the vegetal cells account for most of the gastrula volume. We detected no significant differences in vegetal cell diameters between embryos of each species (statistical test with *P*-value) (Table S1 in the Supporting Information). Consequently, we were able to pool the measurements of three embryos per species for the statistical analyses of cell size in the vegetal region, blastocoel roof, and marginal zone.

Cell Size Analysis

The comparative analysis revealed increases in cell size in relation to egg size in the vegetal region, blastocoel roof, and marginal zone of the early gastrula (Figs. 2A–C). The numbers of cells analyzed in each gastrula region per species are given in Fig. 2A–C. Cells of the vegetal region were proportionally larger in the gastrula of frogs derived from larger eggs in comparison with smaller eggs (Fig. 2A). Similarly, cells of the blastocoel roof exhibited a clear increase in cell size in relation to egg size (Fig. 2B). In contrast, the increase in cell size in relation to egg size was moderate in the marginal zone in comparison with other regions of the gastrula (Figs. 2A–C). In fact, the marginal zone had the lowest correlation of cell size with egg diameter (regression statistics $r^2 = 0.66$ $P < 0.001$) in comparison with the blastocoel roof ($r^2 = 0.80$ $P < 0.001$) and the vegetal region ($r^2 = 0.97$ $P < 0.001$) (Fig. 2).

We analyzed the relation between egg size, cell size of the vegetal region, and mode of gastrulation. We analyzed cells diameters of the vegetal region because this region represents the main component of the gastrula volume. We found that cell diameters of the vegetal region were significantly different in species with gastrulation mode 1 in comparison with species with gastrulation mode 2 (statistical tests with *P*-value) (Table S2 in the Supporting Information). This difference may derive from the great differences in egg size between species. In fact, most frogs with gastrulation mode 1 had small eggs, and consequently small cells, in the three regions of the gastrula in comparison with frogs with gastrulation mode 2 (Figs. 2A–C).

We detected cell size divergences in frogs with eggs of about 2,000 μm diameter representing the two modes of gastrulation. For example, the gastrula of *E. callistomma* (2,100 μm egg diameter) had larger cells in the three regions of the gastrula in comparison with other species with gastrulation mode 1 (Figs. 2A–C). In contrast, cells of marginal zone of some frogs with gastrulation mode 2 (eggs of 1,600–2,000 μm diameter) were of somewhat similar size with gastrulation mode 1 species (Fig. 2C). In addition, the cells of the marginal zone of *G. riobambae* (eggs of 3,000 μm egg diameter, and gastrulation mode 2) were of similar size with the marginal zone of smaller embryos that represented both modes of gastrulation (Fig. 2C). Only *D. auratus* and *E. coqui* embryos (of 3,500 μm egg diameter) had larger cells in the marginal zone in comparison with the remaining species (Fig. 2C). In spite of this cell size difference, the marginal zone of all frogs was characterized by small cells in comparison with vegetal cells (Figs. 2A and C).

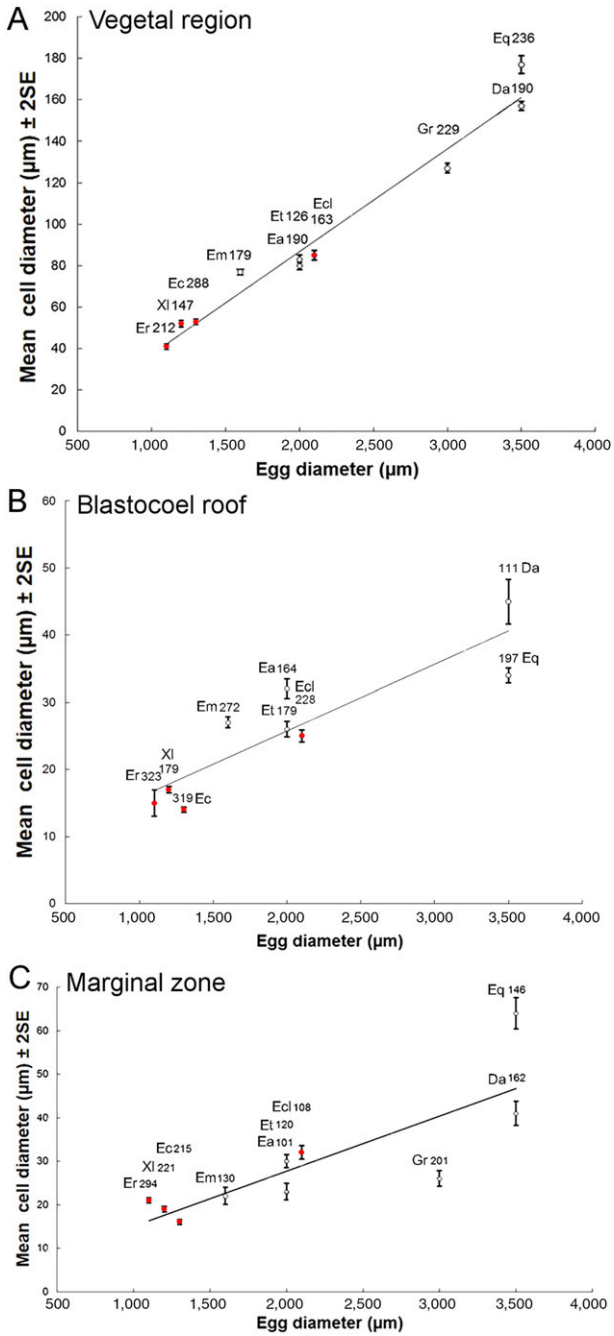

Figure 2. Cell size in the early gastrula (stage 10) of ten frog species. Data represent the mean cell diameters taken from sagittal sections of three embryos ± 2 standard errors of the mean. (A) Cell size of the vegetal region. The linear regression is plotted (regression statistics $r^2 = 0.97$, $P < 0.001$). (B) Cell size of the blastocoel roof. The linear regression is plotted (regression statistics $r^2 = 0.80$, $P < 0.001$). The blastocoel roof cells of *G. riobambae* gastrulae were not included because reliable estimates of cell diameters were not possible for this species. (C) Cell size of the marginal zone. The linear regression is plotted (regression statistics $r^2 = 0.66$, $P < 0.001$). Species identification and the number of cells analyzed and are given above each bar. Solid circles in red gastrulation mode 1; white circles gastrulation mode 2.

DISCUSSION

Cell Size of the Gastrula and Reproductive Adaptations

Reproductive adaptations were associated with gastrulation mode and cell size in the analyzed frogs (Table 1). Frogs with gastrulation mode 1 developed from comparatively small eggs and had small cells in the gastrula. An early elongation of the notochord may be required to rapidly elongate the body of frogs with gastrulation mode 1, thus allowing rapid development of tadpoles. In the aquatic media, tadpole movements are likely required for the search of food, avoidance of predators, and survival strategies. In contrast, frogs with gastrulation mode 2 develop in terrestrial environments (del Pino et al., 2007). These frogs had a tendency toward larger eggs, larger cells in the gastrula, and delayed body elongation (Table 1).

Frog adaptations to reproduce on land are associated with large egg size (Duellman and Trueb, '86; Gomez-Mestre et al., 2012) and with larger cells in the gastrula, according to our analysis. These characteristics are associated with the separation of notochord elongation from gastrulation (del Pino et al., 2007; Elinson and del Pino, 2012). It may be that the environmental pressures to precociously elongate the body are lacking in frogs that reach an advanced tadpole stage before the free-living larval period, as in the marsupial frog *G. riobambae* and in dendrobatid frogs. In the case of *E. coqui*, direct development without the tadpole stages apparently eliminates the requirement for

an early onset of body elongation, and the notochord elongates after blastopore closure (Ninomiya et al., 2001).

Egg and Cell Sizes

Differences in cell size between the small animal cells and the larger and yolk-rich cells of the vegetal region develop during cleavage in *X. laevis* embryos. Planes of cell division are displaced toward the less yolky animal region, and give rise to cell size differences between animal and vegetal blastomeres. In addition, there is an early transition to asynchronous cleavage in the vegetal hemisphere. The altered onset of asynchronous cleavage contributes to size differences between animal and vegetal blastomeres (Nieuwkoop and Faber, '94; Masui and Wang, '98). We observed differences in cell size between regions of the gastrula in the analyzed frogs (Fig. 2). However, gastrulation in none of the analyzed species has been examined at the same level of detail as in *X. laevis*.

The analysis of cell size and C-values would complement this analysis. However, the lack of information prevented this investigation. The DNA content of the haploid genomes (C-values) correlates positively with cell size, cell cycle length, developmental rate, and developmental complexity of amphibians (Cavalier-Smith, '78; Pagel and Johnstone, '92; Gregory, 2002; Zeng et al., 2014). However, the C-values of only *X. laevis* and *G. riobambae* are known, with values of 3.1×10^{-12} and 4.6×10^{-12} g DNA, respectively (del Pino, '89; Gregory, 2015).

The correlation between egg and cell sizes of the gastrula suggests a link between the relative timing of gastrulation and cell division cycles. However, we do not provide support for this possible correlation because we did not count the cells in the different embryo regions. We only measured blastomeres that were round in shape and displayed the cell nucleus. Moreover, amphibians display a diversity of cleavage patterns that may obscure this possible relationship (del Pino and Loor-Vela, '90; Collazo and Keller, 2010). For example, *X. laevis* embryos undergo 12 cycles of synchronous cleavage before the mid-blastula transition. In contrast, the synchrony of cleavage is lost after the eight-cell stage in embryos of the marsupial frog, *G. riobambae*, and this frog does not have a mid-blastula transition (Newport and Kirschner, '82; del Pino and Loor-Vela, '90).

The Marginal Zone and the Blastopore

The comparative analysis confirmed our hypothesis. We found that cell size had a high correlation with egg size in the blastocoel roof and vegetal region. In contrast, the correlation of egg with cell sizes was weak in the marginal zone (Figs. 2A–C). We detected that frogs with gastrulation mode 1 developed from small eggs and had small cells in comparison with most frogs with gastrulation mode 2. In spite of this difference, we found that cells of the marginal zone were of somewhat similar size in frogs that represented both modes of gastrulation (eggs of 1,100–3,000 μm diameter) (Fig. 2C). Thus, cell size of the marginal zone of the early gastrula was unrelated with the onset of dorsal convergent extension that defines the gastrulation modes. Instead, we propose that cells of the marginal zone may attain a small size to facilitate the morphogenetic movements of gastrulation in frogs with eggs of different sizes and gastrulation modes. In addition, the weaker correlation between egg size and marginal zone cell size in different frog species may reflect the fact that the marginal zone cells are derived from blastomeres situated at the transition between the more yolk-laden vegetal core of the embryo and the less yolky animal pole cells.

Reported experimental evidence supports the contention that there is a maximum marginal zone cell size that allows normal development in *X. laevis* (Harris and Hartenstein, '91). Cell division during embryogenesis is highly regulated in the early embryos of *X. laevis* (Saka and Smith, 2001; Murakami et al., 2004). Cell proliferation slows down at gastrulation, and there is total suppression of cell proliferation in dorsal mesodermal cells of the gastrula (Saka and Smith, 2001). Moreover, Wee1-mediated inhibition of cell proliferation is essential for *X. laevis* gastrulation (Murakami et al., 2004). Accordingly, the experimental blocking of cell division in the *X. laevis* embryo at or after gastrulation had relatively little effect on neural development (Harris and Hartenstein, '91). However, blocking of cell division before gastrulation did not allow normal development to proceed (Cooke, '73; Harris and Hartenstein, '91). The body of the embryo develops from the marginal zone. Therefore, marginal zone cells must cleave to get under a certain maximum volume in order to support the morphogenetic movements that embryos with gastrulation mode 1 undergo, that is linked extension and blastopore closure (convergence). Cells of the marginal zone undergo involution, allowing the formation of the blastopore around the conspicuous yolk plug. The blastopore is a universal feature of frog gastrulation and represents a changing population of cells at the blastopore lip. New cells from the marginal zone replace those cells that internalize by involution at the blastopore lip. Cell internalization begins at the dorsal blastopore lip, and later extends all around the blastopore lip (Keller and Shook, 2004). Small cell size in the marginal zone of the gastrula is required for normal development in *X. laevis* and likely in other frogs, according to the experimental evidence (Cooke, '73; Harris and Hartenstein, '91) and our findings.

Frog embryos develop a blastopore, including the species with highly modified reproductive modes. For example, embryos of the marsupial frog *G. riobambae* have a highly modified mode of gastrulation, with development of an embryonic disk of small cells that derive from the blastopore lip (del Pino and Elinson, '83). However, a normal looking blastopore characterizes the gastrula of this frog (del Pino et al., 2007). Similarly, dendrobatid frogs develop a blastopore. The large circumblastoporal collar of dendrobatid frog embryos consists of small cells derived from the blastopore lip (del Pino et al., 2007; Moya et al., 2007). We propose that selection favored small cells in the marginal

zone of frog embryos regardless of egg size and gastrulation mode to allow involution and other gastrulation movements. The small cells of the blastocoel roof also undergo morphogenesis, specifically epiboly, which can involve cell rearrangements and changes in cell shape. This population of cells could also impact blastopore closure via epiboly forces.

Selection acted during evolution in favor of larger cells in the vegetal region and smaller cells in the marginal zone and blastocoel roof. We interpret that large vegetal cells store nourishment for the developing embryos, a requirement for the evolution of the wide range of frog terrestrial reproductive modes. The evolution pressures favored smaller cells in the marginal zone in eggs of different sizes to allow the morphogenetic movements of gastrulation. The plasticity thus generated allowed the great diversification of egg size and frog reproductive modes while maintaining the universal pattern of blastopore formation in the frog gastrula. Our study revealed a correlation between egg and cell sizes, and small cell size in the marginal zone of the early gastrula.

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