Int. J. Dev. Biol. 34: 391-398 (1990)

Original Article

Bifurcation of the amphibian embryo's axis: analysis of variation in response to egg centrifugation

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ABSTRACT ***Xenopus*** **embryos have been reported to vary widely in their developmental response to centrifugation. Variation in response to centrifugation, as measured by embryo survival and twinning of axial structures, was monitored in different spawnings of *Xenopus laevis* eggs. A convenient method for quantifying the egg cytoplasm's potential for displacement in a centrifugal field was employed. It involved testing small batches of eggs from each spawn under carefully controlled conditions for displacement of the cytoplasm while held in an inverted orientation. The cytoplasmic immobility (CIM) values thus measured in samples from each spawn were correlated with the spawning's developmental success (survival of embryos) and the twinning frequency after centrifugation. Those spawnings with high CIM values (*i.e.* a rigid or stiff cytoplasm) had the highest survival rates and the lowest frequency and severity of twinning in centrifuged eggs. Variations in CIM account for the broad variation in response to centrifugation previously noted in several reports and further emphasize the role cytoplasmic compartments play *vis-à-vis* egg organization and early embryonic pattern formation.**

KEY WORDS: *Xenopus eggs, twinning, egg centrifugation, bifurcation of axis, egg variation*

Introduction

A remarkable number of features of early *Xenopus laevis* embryonic development routinely exhibit substantial egg-to-egg variation. Although often overlooked in everyday experimentation, numerous authors have cited the lack of a correlation between several of the so-called «textbook version» features of pattern specification and the morphogenesis regularly observed among different batches of eggs. Table 1 summarizes several of those variant features.

At least three factors, and probably even more, can account for that substantial variation in morphogenetic pattern. First, the intrinsic nature of the morphogenetic information and its cytoplasmic organization system which is built into the egg during oogenesis may contribute to egg-to-egg variation. Perhaps morphogenetic determinants are organized as gradients, as has been proposed by several authors (reviewed by Meinhardt, 1984). If the gradient components are not tightly anchored to the egg's cytoskeleton, they would be expected to be highly susceptible to reorganization by environmental influences, such as physical shock or drastic temperature changes, or by the physiological conditions of the female. That reorganization could easily account for a large amount of egg-to-egg variation in several of the properties listed in Table 1.

Second, the large size of the egg provides substantial physical forces, such as the torque exhibited during the egg's natural gravity rotation which follows fertilization. These physical forces might exceed the limit of the cytoskeleton to maintain the rigid organization system for the egg's cytoplasm. In smaller eggs, such as those of several marine invertebrates (e.g. *Styela*), the cytoskeleton is capable of guiding a wholesale relocation of major cytoplasmic components throughout the entire egg. In the larger eggs, including those of most amphibians, the large yolk platelets often provide a gravity-driven force of sufficient magnitude to disrupt morphogenesis if they cause a reshuffling of the cytoplasm when the egg is inverted (Neff *et al.*, 1983).

Third, one may speculate that females which spawn collections of eggs that include individual eggs with variations in key features are, on average, better suited for perpetuating the species in environments which occasionally pose overwhelming challenges for one or another type of egg.

The intrinsic nature of the amphibian egg's morphogenetic determinants is due largely to a passive phylogenetic history. The large, yolk-laden character of the egg is the product of a more active natural selection. Those two factors, as well as the selective advantage that spawns with broad-spectrum eggs offer can perhaps account for the tendency of the *Xenopus* egg to display variations of the type listed in Table 1. Viewed in this context, it is easy to understand why the amphibian egg is generally considered to be an example of a regulative rather than determinative type of egg.

Abbreviations used in this paper: CIM cytoplasmic immobility, TI twinning index, %T percent twinning.

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0214-6282/90/$02.00

Printed in Spain

TABLE 1

EGG CHARACTERISTICS THAT VARY AMONG *XENOPUS* SPAWNINGS

Feature	Comment	Reference
size	up to 50% difference in volume	Malacinski, 1990
cytoskeleton	cytokeratin organization varies from barely detectable to robust array	Klymkowsky *et al.*, 1987
egg (oocyte) surface	membrane resting potential and sensitivity to neurotransmitters vary enormously	Kusano *et al.*, 1977
pigmentation pattern	eggs from same spawning occasionally show variations	Palecek *et al.*, 1978
sperm entrance site/ axis orientation	good but not necessarily absolute correlation	Elinson, 1980 Vincent *et al.*, 1986
germ plasm	up to x18 difference in volume of germ plasm in eggs of single spawning	Akita and Wakahara, 1985
subcortical rotation of egg periplasm	substantial variation suggests a «rotation threshold»	Vincent and Gerhart, 1987
gray crescent/axis orientation	gray crescent often not observed	Elinson, 1989
cytoplasmic compartments	differences in size, shape and apparent viscosity among spawnings	Smith and Neff, 1986
1st cleavage furrow/ axis orientation	good but not necessarily absolute correlation	Vincent *et al.*, 1986

One way to gain insight into the manner in which morphogenetic information is stored in the *Xenopus* egg, and later deployed to specify pattern, is to employ experimental methods which exploit that egg-to-egg variation. Indeed, the very variation in egg characteristics that *Xenopus* spawnings offer, but is usually overlooked, forms the basis for our strategy for understanding how the cytoplasm of the egg is organized. Two methods that we have previously employed are gravity orientations such as egg rotation and egg inversion. From those analyses a model emerged which depicts the egg cytoplasm as being organized into a set of zones, domains or compartments (Neff *et al.*, 1984). By examining the histological features of eggs that exhibited various responses to rotation/inversion, and comparing these features with subsequent morphological patterning, a «cytoplasmic compartment» model for egg cytoplasm organization has been formulated (Malacinski and Neff, 1986). In the present report, centrifugation of the uncleaved egg was employed as a probe for understanding egg cytoplasm organization. The strategy was to analyze eggs with different cytoplasmic consistencies. Studies which employ mild centrifugal force to alter amphibian embryonic axis formation have been previously carried out (reviewed in Black and Gerhart, 1986). The extensive variability in response of different batches of eggs to hypergravity has, however, not been analyzed. The variation is often substantial, and if percentage axial twinning is employed as an index, 0 to 100% twins are produced from eggs of different spawnings (Black and Gerhart, 1986).

In this report a correlation is discovered between twinning frequency and the cytoplasmic compartmentation of the egg cytoplasm. This report represents the first formal attempt to correlate variation in specific egg characteristics (*e.g.*, CIM value) with development of morphogenetic pattern. The insights gained will be valuable for future studies on the ultrastructure and function of individual cytoplasmic compartments.

Results

Each experiment was designed to ask a specific question about the relationship between cytoplasmic immobility-CIM (Fig. 1) and twinning (Fig. 2) in centrifuged eggs. Initial questions concerned the characterization of CIM values among different spawnings. The second set of questions deals with survival of centrifuged embryos. Later questions examine axis bifurcation in spawnings which exhibit different CIM values.

CIM values

To what extent does the CIM value vary among spawnings?

CIM values were derived for the eggs of 20 spawnings. The data in Fig. 3 reveal a broad range of values. The exact meaning of the four-fold spread in CIM values (52μm-192 μm) is uncertain. However, this amount of spread provides a basis for speculation that variations in twinning frequency are related to the cytoplasmic consistency of test eggs. The CIM values for fertilized eggs are much higher than for activated eggs. This substantial difference presumably reflects the decrease in apparent viscosity of the egg cytoplasm triggered by activation (Elinson, 1985).

To what extent does the CIM value vary within a single spawning?

For the 20 spawnings represented in Fig. 3, the mean standard deviation is 27%. The range of standard deviations is 13%-51% of the mean. The extent of scatter about the mean is independent of CIM value. Furthermore, the range of variation appears to be independent of sample size. The lowest CIM ever measured was 25 μm; the highest 350 μm.

Survival rates

To what extent does centrifugation diminish survival rate?

For the two centrifugal forces employed, 15g and 30g, survival of various developmental stages was monitored. The data in Fig. 4 reveal that survival is reduced by centrifugation. Interestingly, even control (not centrifuged) eggs exhibit slightly reduced survival rates.

Is the survival rate (to the tailbud stage) related to CIM value?

Percentage survival was monitored for various CIM values, at 1g (control) and the centrifugal forces of 15g and 30g. In general, eggs with higher CIM values exhibited enhanced survival rates (Fig. 5). Curiously, even at 1g eggs with higher CIM values displayed higher survival rates.

CIM values and twinning

Do two different scoring systems for twinning provide comparable data?

The data in Fig. 6A reveal that twinning index (TI) and percent twinning (%T) do indeed generate comparable data. In addition, the range of the TI and %T data for the 20 spawnings is very broad, as illustrated in Fig. 6B. This observation of a broad range of twinning is consistent with previous reports (see Introduction).

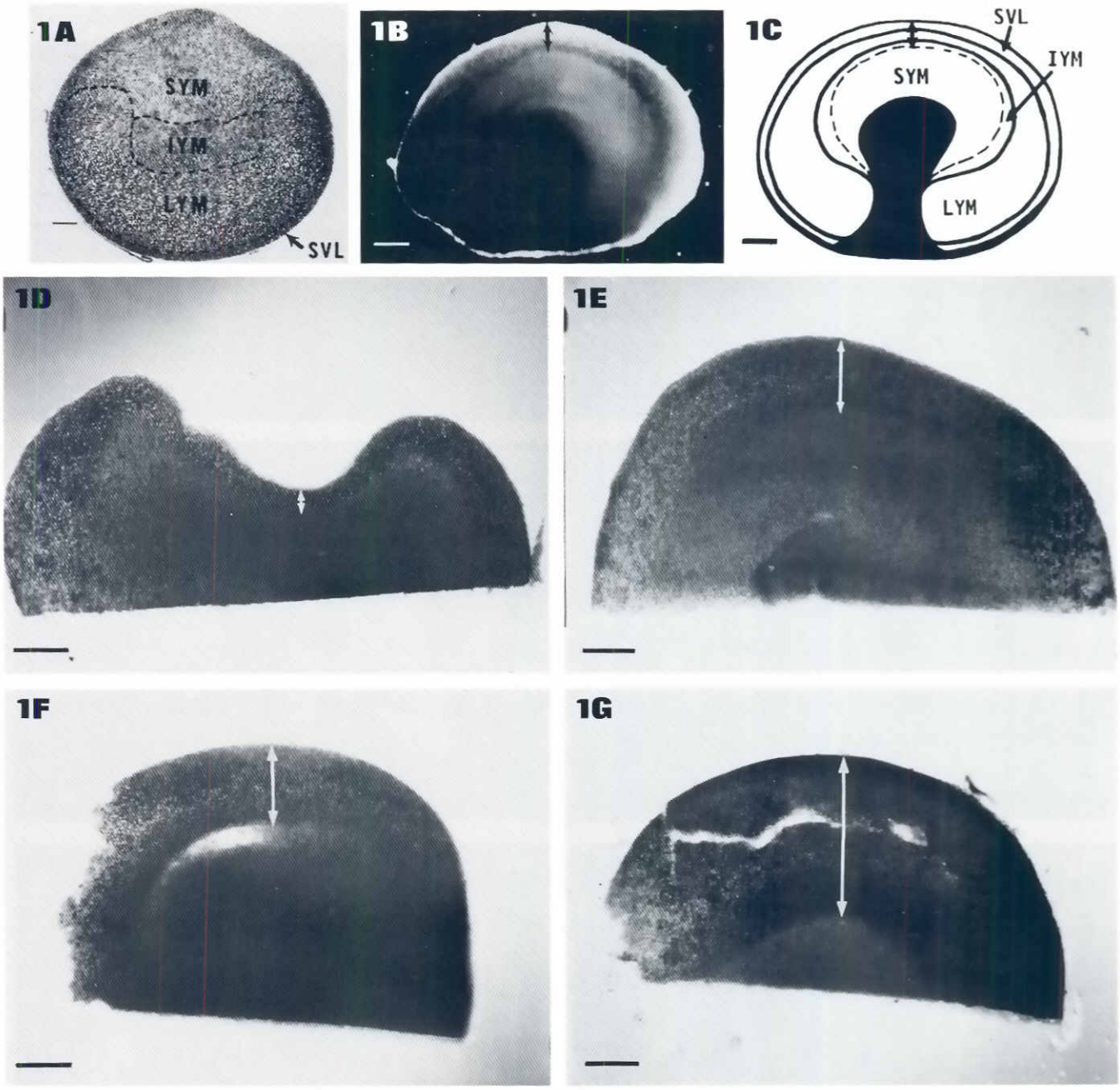

Fig. 1. Cytoplasmic compartment shifts and measurement of cytoplasmic immobility (CIM) in inverted eggs. (A) *Plastic section (1μm thick) of typical unfertilized egg illustrating cytoplasmic compartments which are based primarily on yolk platelet distribution: SYM, small yolk mass; IYM, intermediate yolk mass; LYM, large yolk mass; and SVL, subcortical vitelline layer (see Neff* et al., *1984 for description of the compartments).* **(B)** *Phase micrograph of a mid-sagittal thick section through a T= 0.5 inverted egg shows the movement of the LYM and IYM with the gravity vector and the movement of the SYM against the gravity vector (diagrammed in* **C**). *The combined thickness of the SVL, IYM and LYM left in the vegetal hemisphere of inverted eggs at T= 0.5 (double arrows) is used to measure CIM.* **(D-F)** *Mid-sagittal sections through the vegetal half (hemisphere) of T= 0.5 inverted eggs from different spawnings showing different CIMs.* **(G)** *Control unfertilized egg inverted until T= 0.5. Magnification bars= 100 μm.*

Is the range in twinning frequency related to CIM values?

Using the two scoring systems, the relationship between twinning and CIM was analyzed. Figs. 7A and C illustrate the result when «twinning index» was scored. A strong correlation is observed between CIM and TI. Eggs with higher CIM values exhibit substantially diminished TIs. Likewise, when the «percentage twinning» scoring system was employed, a similar strong direct correlation was observed between higher CIM values and lower twinning percentage (Figs. 7B and D).

From the above data it is clear that the organization of the egg

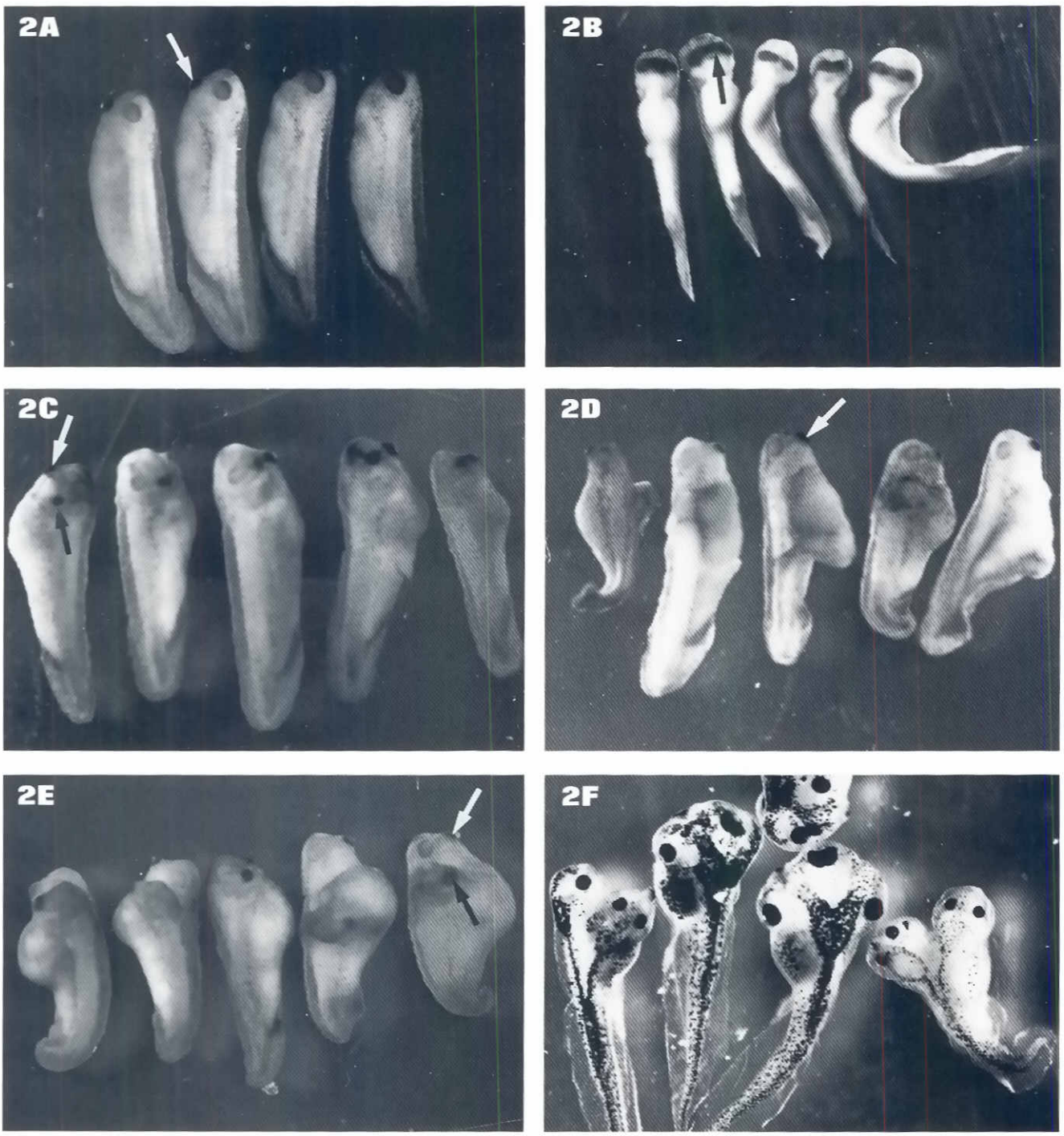

Fig. 2. Scoring system for twinning. **(A)** *Category 1 - normal tailbud embryos.* **(B)** *Category 2 - embryos with enlarged cement glands.* **(C)** *Category 3 - embryos with two eyes and two cement glands (twinning of the cement gland).* **(D)** *Category 4 - embryos with a secondary axis. The secondary axis is however incomplete.* **(E)** *Category 5 - complete twinning (embryos have complete secondary axis with two sets of eyes and two cement glands.* **(F)** *Representative twins at the tadpole stage. Arrows indicate typical cement glands.*

cytoplasm varies substantially among *Xenopus* eggs. That variation, as defined herein in terms of CIM value, can account for the variation in twinning response of different batches of eggs to centrifugation.

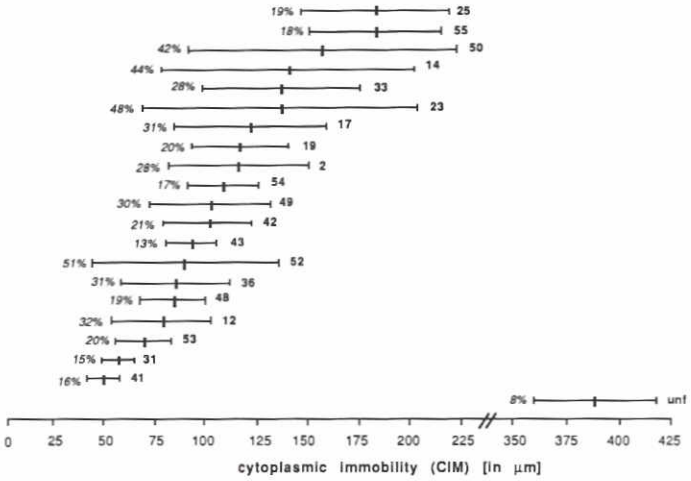

Fig. 3. Cytoplasmic immobilities (CIMs) of spawnings of fertile eggs utilized for twinning studies. *The bold numbers to the right of the lines indicate the spawning number. The center vertical bold dash lines represent the mean CIM in µm units for each spawning. The mean CIMs ranged from 52 to 192. Unfertilized eggs (unf) had a mean CIM of 388 (n= 12). The dashes on the ends of the lines represent the range of one standard deviation. The italicized percent numbers to the left of the standard deviation lines indicate one standard deviation (sd) in terms of a % of the mean CIM (%sd). The mean number of samples measured per spawning was 15 [range: 4-36]. There was no significant correlation between the %sd and sample size (r= 0.24).*

Discussion

A variety of experimental treatments have been employed to induce twinning in amphibian embryos. Table 2 provides a summary of several such studies. Most use alterations in either the force of gravity and/or the direction of the gravity vector. In all cases, substantial variation in twinning frequency was observed. Although it is not known what component of the axis induction system is finally altered by these treatments, the initial effect of the treatments is probably on the organization system of the egg's cytoplasm.

The data presented in this report provides the first direct correlation between a property of the egg cytoplasm (cytoplasmic rigidity) and twinning that can account for variation in twinning frequencies among different spawnings. The CIM data offer a plausible explanation for what several physical (*i.e.* gravity) perturbation methods have in common – they alter the distribution of the egg's cytoplasm. This explanation is consistent with two features of the centrifugal force-induced twinning method. First, the twinning index and percent twinning are related to CIM. Second, as previous reports have indicated, centrifugation is maximally effective during a limited period in the first cleavage period (approx. 30%-60% of the time line between fertilization and furrow appearance) (Black and Gerhart, 1986; Kuneida and Wakahara, 1987). This period corresponds to the polymerization part of the polymerization/depolymerization cycle for tubulin (Elinson 1985), and may explain why, during an interval when the egg's cytoskeleton is undergoing a temporary reorganization, the egg is labile to physical perturbation.

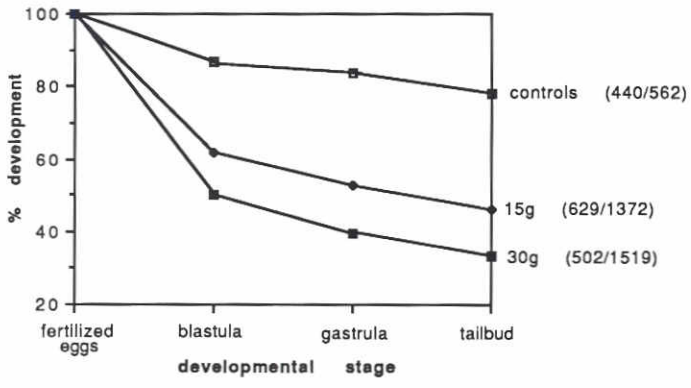

Fig. 4. Survival of centrifuged embryos. *Percent survival was calculated from pooled data from all twenty spawnings by the following formula: # surviving embryos to a given stage/total number of fertilized eggs at beginning of experiment x100. Number of embryos surviving to the tailbud stage is indicated. A total of 3,453 embryos were scored.*

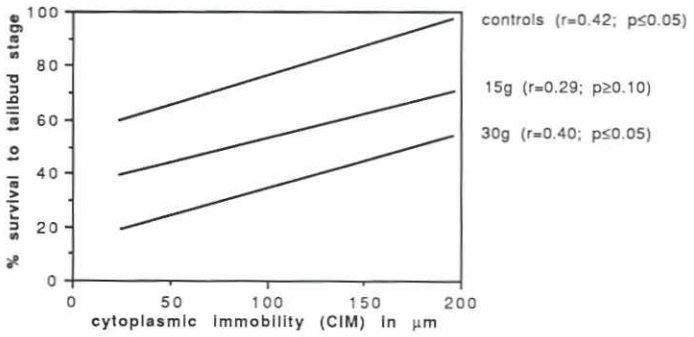

Fig. 5. Correlation between % survival and centrifugal force. *Lines represent simple linear regression line that can be drawn through the data points. The correlation coefficient (r) for each line is given for each experimental manipulation. The range in % survival was as follows: controls: 24%-100%; 15g : 6%-92%; 30g : 5%-93%. P value is a two-tailed significance test.*

The correspondence between the survival data and twinning data further substantiates the above explanation. Eggs with a high CIM value (*i.e.*, relatively «firm» or «rigid» internal cytoplasm) exhibit higher survival rates. Those eggs are less likely to twin. It should also be noted that spawnings with a large standard deviation in CIM value (see Fig. 3) will include a relatively high proportion of high CIM eggs which twin less and survive better. Those spawnings are likely to generate data which underestimates the twinning frequency in centrifuged eggs.

The primary target of centrifugation could include any of a number of components, including the cytoplasmic yolk compartments (see Fig. 1), morphogenetic determinants, the sperm aster, and/or cytoplasm/cortex interactions. Disruption of the primary target most likely cascades into alterations in the primary embryonic induction system. Although it might be tempting to speculate that

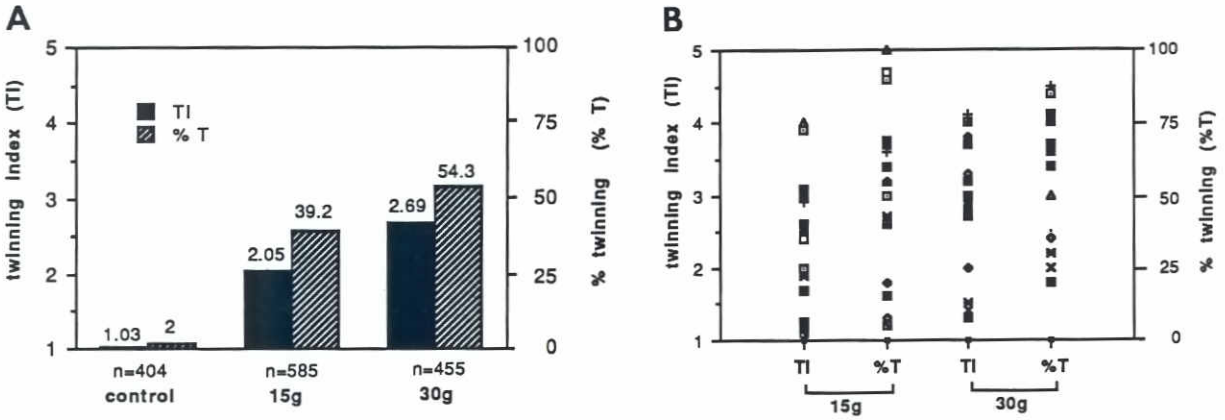

Fig. 6. Comparison of twinning index (TI) and % twinning (%T). (A) *Comparison of TI and %T scoring systems. TI and %T were calculated by summing all the twinning data derived from the 20 spawnings listed in Fig. 3. Number of embryos scored for each experimental group is given on the abscissa.* **(B)** *Range in TI and %T in individual spawnings. The range in the TI and %T for individual spawnings depicted by symbols. Each spawning has a unique symbol. The range in TI at 15g was 1.0 to 4.0; at 30g it was 1.0 to 4.1. The range in %T at 15g was 0.0 to 100% and at 30g it was 0.0 to 87%.*

modifications of a primary target in the egg eventually influence the embryonic organizer such that it induces two sets of axial structures, caution is called for. Embryonic induction consists of two components, the inducer system and the competence for response system. Since it is possible that both are altered in twinned embryos, centrifugation can be presumed to have effected a wholesale re-ordering of both the inducer and responder systems. As elucidated in this study, eggs with high CIM values resist the challenge to re-ordering provoked by centrifugation.

There are of course alternative methods for generating twinned embryos. The highly regulative character of the early *Xenopus* embryo can be witnessed when a second primary embryonic organizer is grafted to the future ventral side of a late blastula-stage embryo. A twin set of axial structures usually develops. Also, microinjection of the proto-oncogene *int-1* RNA into *Xenopus* eggs, which is presumably translated during cleavage, causes twinned axes (McMahon and Moon, 1989). These observations reinforce the notion that a regulatory cascade for axis development is set into place shortly after fertilization. Organizer grafts and proto-oncogene expression no doubt act late. Centrifugation presumably affects one of the earliest steps in that cascade.

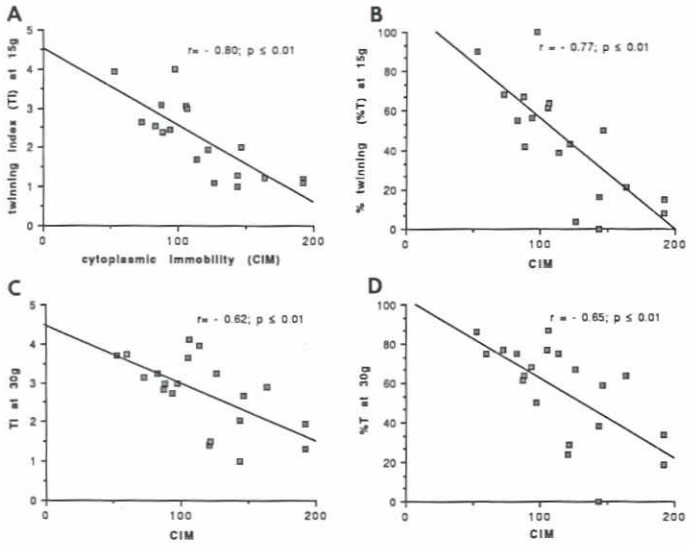

Fig. 7. Relationship between twinning and CIM (cytoplasmic immobility). (A) and (C) *Correlation between TI and CIM at 15 and 30 g respectively.* **(B) and (D)** *Correlation between %T and CIM at 15 and 30g respectively. Simple linear regression lines are illustrated. The correlation coefficient (r) for each line is given. P value is a two-tailed significance test.*

The general strategy of the present analyses, as explained in the Introduction, involves exploiting egg variation as a method for gaining insight into features of the egg cytoplasm. This theme might be further expanded in the future by comparing the cytoskeleton of eggs with low CIM values (and presumably a very weak cytoskeleton) to those with the highest CIM values (*e.g.* Neff *et al.*, 1989). By comparing eggs at each end of the CIM spectrum, additional insight into the cytoplasmic organization system might be gained.

Why such egg-to-egg variability exists in the first place, when laboratory raised (and therefore presumably uniformly treated) *Xenopus* were employed, is not easily understood. One explanation pertains to large, yolk-laden eggs, including many amphibian ones (*e.g. Xenopus*), which undergo holoblastic cleavage. Natural selection forces perhaps favor eggs that contain large stores of yolk. Considering the enormous number shed in each spawning, it is conceivable that those large eggs approach the upper size limits of the quality control mechanisms of the ovary. A balance is perhaps struck between suitability for survival and fidelity in production of high quality eggs. Previous observations on repeated spawnings from individual females revealed that the cytoplasmic features discussed in this report are not inherited traits (Malacinski and Neff, 1986). Rather, the egg type from a single female varied from one spawning to the next. In order to accommodate egg variability two types of regulatory mechanisms can be employed: redundancy – which provides multiple, overlapping processes to insure that a process is carried out (*e.g.* Malacinski and Neff, 1990); and thresholds – which require only that a minimum level of a regulatory process be achieved in order to drive a process to completion (*e.g.* Meinhardt, 1984). It is possible that combinations of those two

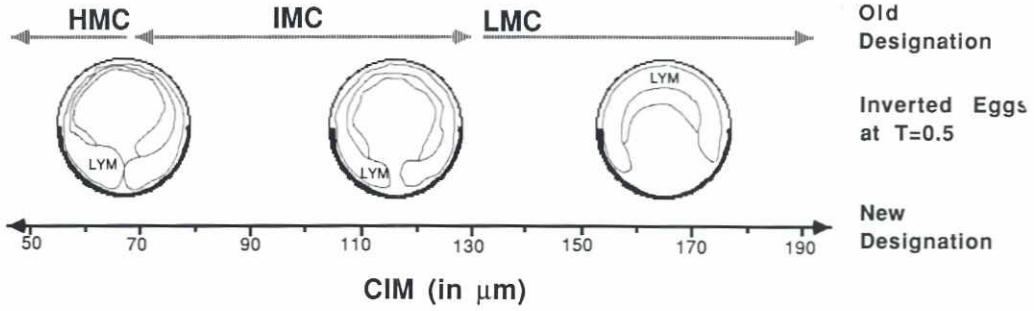

Fig. 8. Comparison of previous and present scoring systems for the rigidity of the egg cytoplasm. *The old scoring system utilized 33 μm minimal measuring units and included three classes of eggs: high mobility cytoplasm (HMC), intermediate mobility cytoplasm (IMC) and low mobility cytoplasm (LMC) eggs. That system emphasized the fluidity (mobility) of the cytoplasm. The new scoring system utilizes 5 μm minimal measuring units and therefore has increased resolution. The new designation, termed cytoplasmic immobility (CIM) emphasizes the rigidity (and resistance to displacement) of the cytoplasm, which is a more accurate representation of what is actually measured than the previous scoring system. The three diagrams show examples of typical inverted eggs at T= 0.5 with low CIM, intermediate CIM and high CIM values. The vegetal hemisphere large yolk mass is indicated in the diagrams as LYM.*

processes act to generate the remarkable degree of uniformity in developmental pattern typically displayed by different spawnings, despite the initial variability in egg features listed in Table 1.

In addition to providing an explanation for variability in twinning, and offering a focus for further studies on the cytoskeleton, the present analysis forms part of a comprehensive space biology program designed to learn more about the amphibian egg's responses to alterations in the gravity force vector (Malacinski *et al.*, 1989). Whether the egg's natural gravity-oriented rotation response to activation and the asymmetric displacement of the cortex (Vincent *et al.*, 1986) are prerequisites for normal pattern formation will be tested during spaceflight. The present studies provide background information, which will be useful in interpreting the results, especially if a varied response to microgravity is obtained.

Materials and Methods

Manipulation and centrifugation of fertile eggs

Eggs derived from hormonally stimulated *Xenopus* females were fertilized by standard methods. Eggs and embryos were maintained at 15°C. Fertile eggs before first cleavage were staged according to proportion of time between fertilization (T= 0.0) and first cleavage (T= 1.0).

TABLE 2

VARIOUS TREATMENTS TO EGGS INDUCE TWINNING IN AMPHIBIAN EMBRYOS

Treatment	Comment	Reference
delayed fertilization	overripeness of ovarian eggs	Witschi, 1952 Wakahara (unpublished)
rotation	axolotl and *Rana* eggs	Pasteels, 1964 Kubota, 1967
inversion	*Rana* eggs	Schultze, 1894; Penners and Schleip, 1928
demembranation/rotation		Kirschner *et al.*, 1980
centrifugation	long history, but no explanation for variability of response	see Table 1 in Black and Gerhart, 1986
D_2O treatment	substantial egg-egg variation	Scharf *et al.*, 1989

Dejellied (2.5% thioglycolic acid – pH 8.34) eggs were embedded in 9% gelatin (Kanto Chemicals, Japan) in 20% Steinberg's solution (pH 7.4, penicillin G potassium – 100 IU/ml, and streptomycin sulfate – 0.1 mg/ml). Approximately 60 eggs were pipetted into molten 9% gelatin (25°C) and transferred to a 35 mm plastic dish (Falcon 1008) containing 1.5 ml molten gelatin. These eggs were aligned along one diameter of the dish with their animal hemispheres facing up. Sperm entrance sites were also aligned such that they all faced in one direction. Aligned eggs were chilled on ice for 2 minutes and then placed at 15°C for approximately 10 minutes.

T= 0.4 gelatin-embedded eggs were centrifuged at right angles to their animal/vegetal axis with their sperm entrance sites facing the center of the centrifuge (centripetal side). Eggs were centrifuged at 15 g (475 rpm), and 30 g (750 rpm) for 4 minutes at 10°C. Control eggs were also dejellied, embedded in gelatin, and aligned but not centrifuged.

After centrifugation, eggs were incubated at 15°C in their normal orientation with animal hemisphere facing up. At the 8-cell stage embryos were removed from the gelatin (35°C, for 1 to 2 minutes). Embryos were observed at the blastula and tailbud stages. Dead and highly abnormal embryos (abnormal cleavages, cytoplasmic leakage, partial lysis) were counted and incorporated into the data base before being discarded.

Twinning index

Surviving tailbud embryos were classified into 5 categories based on the degree of twinning (Fig. 2). The twinning index is derived by the following formula: number of embryos within a given category times (x) the category values (1 through 5) divided by total number of embryos. Twinning index ranges from 1.0 (100% normal) to 5 (100% «category 5» twins). Alternatively, «percent twinning» (percentage of embryos in categories 2-5) was employed as a scoring system.

Cytoplasmic immobility determination

For each spawning an aliquot of eggs was used to determine the apparent cytoplasmic viscosity or, in a true operational sense, the cytoplasmic immobility (CIM). Freshly spawned eggs were mechanically inverted in plastic dishes and fertilized with standard methods. Six minutes after fertilization the eggs were incubated in 20% Ficoll until T= 0.5 (15°C). At T= 0.5 eggs were fixed in PBFG (4% formalin, 2.5% glutaraldehyde, phosphate buffer, pH 7.4) overnight, washed in phosphate buffer, and transferred to 25% ethanol in 75% phosphate buffer. Eggs were sectioned with a razor blade into 50 to 100 μm thick mid-sagittal sections along the tilt axis of the inverted egg (Fig. 1). The thickness of the SVL (subcortical vitelline layer), LYM (large yolk mass), and IYM (intermediate yolk mass) (Fig. 1) remaining in the vegetal hemisphere was measured with an inverted microscope (x10 lens) with an ocular measuring device (x10 ocular lens: 1 unit= 5 μm). Figs. 1B and C (double arrows) indicate the measurements made to quantitate CIM. Unfertilized eggs as well as eggs injured or activated during the egg manipulations were discarded. The cytoplasmic immobility of eggs from a

single spawning is expressed as the mean of all the cytoplasmic immobility measurements.

It should be noted that this designation – CIM – represents a change from that employed in previous reports from this laboratory (*e.g.* Neff *et al.*, 1984; Smith and Neff, 1986). Previously, cytoplasmic mobility (CM) was employed as the scoring system. Fig. 8 illustrates a comparison of the two scoring systems. The CIM determinations and scoring for twinning of axial structures were done in a single blind fashion. After the data was collected by different persons, it was plotted and interpreted.

Acknowledgments

This study was supported in part by a grant from NASA (NAGW-1548). The discussion generated during this study by Gary Radice, Hiroki Yokota and Susan Duhon is gratefully appreciated.

References

AKITA, Y. and WAKAHARA, M. (1985). Cytological analyses of the factors which determine the number or primordial germ cells (PGCs) in *Xenopus laevis*. *J. Embryol. Exp. Morphol. 90:* 251-265.

BLACK, S.D. and GERHART, J.C. (1986). High-frequency twinning of *Xenopus laevis* embryos from eggs centrifuged before first cleavage. *Dev. Biol. 116:* 228-240.

ELINSON, R.P. (1980). The amphibian egg cortex in fertilization and early development. *Symp. Soc. Dev. Biol. 38:* 217-234.

ELINSON, R.P. (1985). Changes in levels of polymeric tubulin associated with activation and dorso-ventral polarization of the frog egg. *Dev. Biol. 109:* 224-233.

ELINSON, R.P. (1989). Microtubules and specification of the dorsoventral axis in frog embryos. *BioEssays 11:* 124-127.

KIRSCHNER, M., GERHART, J.C., HARA, K. and UBBELS, G.A. (1980). Initiation of the cell cycle and establishment of bilateral symmetry in *Xenopus* eggs. *Symp. Soc. Dev. Biol. 38:* 187-215.

KLYMKOWSKY, M.W., MAYNELL, L.A. and POLSON, A.G. (1987). Polar asymmetry in the organization of the cortical cytoskeleton system of *Xenopus laevis* oocytes and embryos. *Development 100:* 543-557.

KUBOTA, T. (1967). A regional charge in the rigidity of the cortex of the egg of *Rana nigromaculata* following extrusion of the second polar body. *J. Embryol. Exp. Morphol. 17:* 331-340.

KUNIEDA, M. and WAKAHARA, M. (1987). Twin formation in *Xenopus laevis* eggs centrifuged before first cleavage. *Zool. Sci. 4:* 489-496.

KUSANO, K., MILEDI, R. and STINNAKRE, J. (1977). Acetylcholine receptors in the oocyte membrane. *Nature 270:* 739-741.

McMAHON, A.P. and MOON, R.T. (1989). Ectopic expression of the proto-oncogene *int-1* in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell 58:* 1075-1084.

MALACINSKI, G.M. (1990). Reproduction, development and growth of animals in space. In *Fundamentals of Space Biology* (Eds. M. Asashima and G.M. Malacinski). Japan Sci. Press, Tokyo/Springer-Verlag, Berlin, pp. 123-138.

MALACINSKI, G.M. and NEFF, A.W. (1986). Effects of gravity perturbation on developing animal systems. *Adv. Space Res. 6:* 29-36.

MALACINSKI, G.M. and NEFF, A.W. (1990). An essay on redundancy within developmental processes. In *Cytoplasmic Organization Systems* (Ed. G.M. Malacinski). McGraw Hill Pub. Co., New York, pp. 123-153.

MALACINSKI, G.M., NEFF, A.W., ALBERTS, J.R. and SOUZA, K.A. (1989). Developmental biology in outer space. *BioScience 39:* 314-320.

MEINHARDT, H. (1984). Models for pattern formation during development of higher organisms. In *Pattern Formation* (Eds. G.M. Malacinski and S.V. Bryant). MacMillan Publ. Co., New York, pp. 47-72.

NEFF, A.W., MALACINSKI, G.M., WAKAHARA, M. and JURAND, A. (1983). Pattern formation in amphibian embryos prevented from undergoing the classical «rotation response» to egg activation. *Dev. Biol. 97:* 103-112.

NEFF, A.W., RITZENTHALER, J.D. and ROSENBAUM, J.F. (1989). Subcellular components of the amphibian egg: insights provided by gravitational studies. *Adv. Space Res. 9:* 177-186.

NEFF, A.W., WAKAHARA, M., JURAND, A. and MALACINSKI, G.M. (1984). Experimental analyses of cytoplasmic rearrangements which follow fertilization and accompany symmetrization of inverted *Xenopus* eggs. *J. Embryol. Exp. Morphol. 80:* 197-224.

PALECEK, J., UBBELS, G.A. and RZEHAK, K. (1978). Changes of the external and internal pigment upon fertilization in the egg of *Xenopus laevis*. *J. Embryol. Exp. Morphol. 45:* 203-214.

PASTEELS, J. (1964). The morphogenetic role of the context of the amphibian eggs. *Adv. Morphogen 3:* 363-388.

PENNERS, A. and SCHLEIP, W. (1938). Die Entwicklung der Schultzeschen Doppelbildungen aus dem Ei von *Rana fusca*. Teil I-IV. *Zeit. Wiss. Zool. 130:* 305-454.

SCHARF, S.R., ROWNING, B., WU, M. and GERHART, J.C. (1989). Hyperdorsoanterior embryos from *Xenopus* eggs treated with D_2O. *Dev. Biol. 134:* 175-188.

SCHULTZE, O. (1894). Die künstliche Erzeugung von Doppelbildungen bei Froschlarven mit Hilfe abnormal Gravitation. *Wilhelm Roux Arch Entwicklungsmech. Org. 1:* 269-305.

SMITH, R.C. and NEFF, A.W. (1986). Organization of *Xenopus* egg cytoplasm: response to stimulated microgravity. *J. Exp. Zool. 239:* 365-378.

VINCENT, J-P. and GERHART, J.C. (1987). Subcortical rotation in *Xenopus* eggs: an early step in embryonic axis specification. *Dev. Biol. 123:* 526-539.

VINCENT, J-P., OSTER, G.F. and GERHART, J.C. (1986). Kinematics of gray crescent formation in *Xenopus* eggs: the displacement of subcortical cytoplasm relative to the egg surface. *Dev. Biol. 115:* 484-500.

WITSCHI, E. (1952). Overripeness of the egg as a cause of twinning and teratogenesis: a review. *Cancer Res. 12:* 763-786.

Accepted for publication: October 1990