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Microgravity simulation as a probe for understanding early *Xenopus* pattern specification

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SUMMARY

Pattern specification in early amphibians (*Xenopus*) was monitored in embryos subjected to gravity compensation (microgravity simulation) by constant low-speed rotation on a horizontal axis (clinostat). The useful range of clinostat speeds was determined empirically. The results were interpreted in terms of a set of models which account for the reorganization of the egg cytoplasm that follows fertilization and that correlates with the establishment of dorsal/ventral polarity. Large percentages of clinostated eggs displayed a positive result (normal axial structure morphogenesis). Consequently, normal development of amphibian eggs in the microgravity environment of space should be possible. Models which depend upon gravity-driven rearrangements for cytoplasmic organization (e.g. dorsal/ventral polarization) of the early embryo should, therefore, not be favoured. At several clinostat speeds symmetrization of the egg in accordance with the site of sperm penetration, a natural phenomenon, was altered. The results at those clinostat speeds indicate that models which employ sperm entrance as an obligatory feature of the cytoplasmic rearrangements that generate egg polarity are not applicable.

INTRODUCTION

The amphibian egg displays a dramatic response to gravity: once activated, it rotates within the perivitelline space which surrounds the egg so that the darkly pigmented animal hemisphere opposes gravity. Internal cytoplasmic rearrangements follow egg rotation (Ubbels, Hara, Koster & Kirschner, 1983). Novel gravity orientation (e.g. egg inversion) has recently been employed as a probe for understanding the general features of the organization of the egg cytoplasm (Neff, Malacinski, Wakahara & Jurand, 1983). From those studies (e.g. Neff, Wakahara, Jurand & Malacinski, 1984), as well as others which employed 90° rotation (e.g. Gerhart *et al.* 1981), it appears that gravity may play a role in determining the manner in which the radial symmetry of the egg cytoplasm

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is reorganized into a bilaterally symmetrical format following gravity-driven rotation.

One way to test whether gravity plays a role in bilateral symmetrization would be to subject fertile eggs to a gravity-free environment. Besides the freefall weightless environment of space, eggs can be subjected to simulated microgravity conditions by rotation on a *horizontal clinostat*. A horizontal clinostat is an instrument which rotates a biological specimen about a horizontal axis at a slow constant speed. Horizontal clinostat rotation simulates microgravity by compensating for the influence of the gravity vector. Amphibian eggs subjected to horizontal clinostat rotation at a speed that does not in itself create appreciable centrifugal forces should theoretically experience a time-averaged force vector of 0 and therefore should not be able to get a biological fix on the g vector. In the appropriate speed range, the egg should respond to horizontal clinostat rotation much as it would respond to the hypogravity of outer space. Comparisons of various plant responses to clinostat rotation and weightlessness (space flight) have shown that there are very striking qualitative similarities between the clinostat and space flight (as well as small, perhaps insignificant quantitative differences) (Brown, 1979; and Brown, Chapman & Lin, 1974).

Clinostat experiments have been carried out by previous workers. Most of the earlier efforts were, however, inadequate – for one reason or another. Table 1 lists several of the earlier clinostat experiments. Limitations included a narrow range of clinostat speeds, rotation beginning after fertilization, failure to monitor dorsal/ventral polarity, and failure to consider batch-to-batch variability.

The ideal clinostat experiment would have the following characteristics: rotation beginning at the time of sperm penetration; eggs oriented on horizontal axis of clinostat with respect to the sperm entrance site (SES); employment of complete range of rotation speeds; comparison of the effects of short- and long-term rotation; performance of vertical axis control experiments; and analyses of egg cytoplasm characteristics included in data interpretations.

Unfortunately, all of those features cannot be incorporated into the design of a single clinostat experiment. Four clinostat procedures (see Materials and Methods) were therefore employed. Several improvements over previous microgravity simulations were achieved: (1) the use of four separate versions of the clinostat permitted appropriate comparisons with data collected in earlier studies; (2) an expanded speed range (96 000 fold) was tested; (3) in one version, eggs were fertilized on the clinostat; (4) the effect of very slow speeds (e.g. 0·45 r.p.h.) on egg polarization was monitored; (5) *Xenopus laevis* eggs, which have been chosen for future space shuttle experimentation, were employed; (6) characterization of the egg cytoplasm was performed to control batch-to-batch variability; and (7) in contrast to most previous clinostat investigations, the present authors consider the normal development of eggs on horizontal clinostats as a 'positive result'.

The results of the present studies serve two purposes: first, they provide a data base for further refining egg polarity models that have been proposed to account for amphibian egg bilateral symmetrization; and second, they allow a prediction as

to whether *Xenopus* eggs will display normal pattern specification in the microgravity of the space shuttle experiments presently contemplated by the European and American space agencies (see Materials and Methods).

MATERIALS AND METHODS

Eggs

Xenopus laevis eggs were chosen as an experimental system. They have recently been employed extensively for studies on egg polarity (reviewed by Malacinski, 1984*b*), and are scheduled to be employed in space shuttle experiments by the European and American space agencies (ESA: Ubbels *et al.* 1983; NASA: Souza, K. A., personal communication). Collection

Table 1. *Historical review of the use of the clinostat for simulated microgravity for amphibian eggs**

Reference	Species	Clinostat Design†	Speed	Comments
Roux (1884)	*Rana esculenta*	FreRo	12–30 r.p.h.	normal development; eggs not immobilized; narrow speed range
Schultze (1900)	*Rana fusca*	PreFO	0·25 r.p.h.	all eggs lysed; only one slow speed; late addition to clinostat
Kathariner (1901)	*Rana fusca*	FreRo	18–40 r.p.m.	normal development; random axis of rotation; narrow speed range
Morgan (1904)	*Rana fusca*	FreRo	12–16 r.p.m.	normal development; narrow speed range
Tremor & Souza (1972)	*Rana pipiens; Xenopus laevis*	FreRo	4, 7·5, 15 r.p.h.; 1, 2, 10 r.p.m.	more than 50 % normal development; emphasized abnormalities; first use of *X. laevis*; eggs not immobilized at slow speeds
Popov, Palmbackh & Kuznetsov (1975)	*Rana temporaria*	FreRo	99 r.p.h.	67 % normal; one speed only
Dorfman & Cherdantsev (1977)	*Rana temporaria; Rana arvalis; Rana esculenta; Xen. laevis*	FreRo	12, 17, 24, 35 r.p.m.	more than 50 % normal; emphasized abnormalities; narrow speed range
Neubert (1979)	*Rana temporaria*	FreRo	90–130 r.p.m.	normal development; eggs not immobilized narrow speed range

* Only experiments in which microgravity was simulated and in which eggs were placed on the clinostat before first cleavage are reviewed. In each case, fertilization occurred prior to the initiation of clinostat rotation.

† See Design of Clinostat Procedures in Materials and Methods.

Abbreviations: FreRo – Free rotation within jelly coat; PreFO – Prefertilization orientation within jelly coat.

of eggs, fertilization, and egg manipulations followed standard procedures (e.g. see Neff *et al.* 1984).

Design of clinostat procedures

The key features of each of the four clinostat protocols are illustrated in Fig. 1. It was considered essential to examine the performance of eggs in each of the four different clinostat protocols. Variables among the protocols were: time of clinostat rotation initiation; whether eggs were immobilized or allowed to rotate freely; and rotation speeds. The nomenclature for each of the three general procedures is: *FreRo* (free rotation, within jelly coats) – loaded into cellophane bags and fertilized; *NaRReO* (natural rotation, re-orientation) eggs fertilized, dejellied, and then immobilized in gelatin prior to initiation of rotation; *PreFO* (prefertilization orientation, within jelly coats) – eggs oriented prior to fertilization.

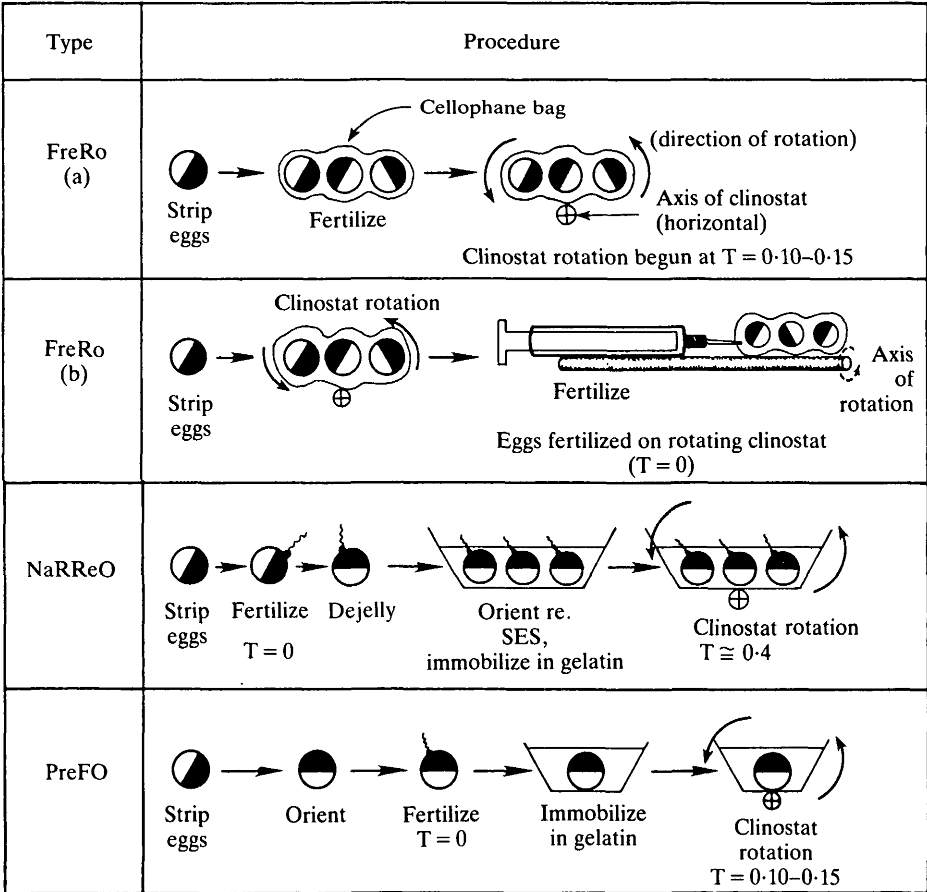

Fig. 1. Experimental designs of the four clinostat procedures. Times given are fractional values of the interval between fertilization (T = 0) and subsequent cleavages (e.g. T = 1: first cleavage; T = 3: 3rd cleavage). All rotation, unless otherwise noted, was carried out on the horizontal axis. Clinostats were constructed from constant or variable speed motors to which shaft extensions were attached that accommodated either cellophane bags (FreRo design) or small plastic dishes. In the NaRReO and PreFO clinostats eggs were oriented with the darkly pigmented animal hemisphere opposing gravity (natural postactivation orientation) at the start of the rotation.

Details of clinostat protocols

The *FreRo* protocol has several advantages over the others for the following reasons: easy to perform; can be carried out with large numbers of eggs; and rotation can be initiated very early (version (a) approx. T = 0·1 and version (b) T = 0·0). Because the eggs are not immobilized and thus are free to rotate in their perivitelline spaces, this procedure is not valid at speeds slower than the egg's intrinsic rotation rate (< 30 r.p.h.) or at the higher speeds, where eggs may orient with the centrifugal field (>24 r.p.m.). In the *FreRo* (*a*) version, newly stripped eggs were placed in small dry cellophane bags (Nalgene cat. no. 500-0100) and fertilized. Ten minutes after fertilization the bags were filled with 20 % Steinberg's solution and heat sealed. The bags were then attached with their long axis parallel to the clinostat shafts. The eggs remained attached to the inner surface of the bag (via the jelly membranes) throughout the rotation. The bags contained enough 20 % Steinberg's solution for embryonic survival. In the *FreRo* (*b*) version, newly stripped eggs in cellophane bags were placed on the shaft of the clinostat. A 1 ml plastic syringe containing 0·5 ml sperm suspension and a 5 ml plastic syringe containing 4·0 ml 20 % Steinberg's solution (both with no. 27 needles) were then attached to the clinostat shaft so as to pierce the sides of bag. After 2 min of clinostat rotation the eggs were fertilized on the rotating clinostat. At 10 min postfertilization, the 20 % Steinberg's solution was added. Air bubbles were removed with the 5 ml syringe.

The *NaRReO* protocol immobilizes eggs by shrinking their perivitelline spaces after fertilization and their natural rotation response. This procedure permits a wider range of clinostat speeds and allows the experimenter to orient eggs on the clinostat with respect to the egg's SES. The disadvantages of this procedure are that eggs cannot be rotated on the clinostat before T = 0·35, and large numbers of eggs cannot be manipulated. Eggs were dejellied in 2·5 % cysteine–HCl (pH 7·6) after they had naturally rotated within the perivitelline space (following fertilization). Eggs with SES were then placed, animal hemisphere opposing gravity, in plastic petri dishes (3·5 cm diam.) and flooded with 6 % (w/v) gelatin (2 parts 175 Bloom: 1 part 300 Bloom in 20 % Steinberg's solution). The dishes were placed on melting ice for approximately 2·5 min to solidify the gelatin. The dishes were then mounted on the horizontal axis of the clinostat. Eggs were removed from the gelatin prior to gastrulation by placing the dishes on a 32 °C warm plate for approximately 5 min to liquify the gelatin. Eggs were then permitted to develop in 20 % Steinberg's solution. For polarity studies, NaRReO eggs were oriented with respect to the SES, and were scored for the dorsal lip location in gelatin.

The *PreFO* protocol immobilizes eggs after fertilization but before the egg's natural rotation response. This procedure allows for a wide range of clinostat speeds and for an early initiation of clinostat rotation (approx. T = 0·1). Eggs in their jelly coats were manually oriented (forceps) with animal hemispheres opposing gravity in the plastic dish, then fertilized and immobilized in gelatin as in the NaRReO protocol. The gelatin was removed manually with forceps prior to gastrulation.

All experiments were performed at 15 °C in an environmentally controlled room. At that temperature, survival is enhanced (Neff *et al.* 1983), and the interval between fertilization and first cleavage is prolonged. That expanded interval permitted more precise timing and the use of larger numbers of eggs than would be possible at room temperature. In tabulating the data, uncleaved eggs were not considered.

Non-clinostated FreRo, PreFO, and NaRReO eggs as well as eggs clinostated on a vertical axis at 1, 2, 6, 12, and 300 r.p.m. (depending on experiment) were used for controls.

The 'failure speed' was defined as the speed at which 50 % of the clinostated embryos failed to complete gastrulation.

The *external* morphogenesis (gross morphology) of the clinostated embryos was observed at the tailbud stage. Clinostated embryos that were morphologically indistinguishable with respect to axial development were considered normal. Internal morphogenesis was not monitored.

Determination of cytoplasmic mobility

Eggs inverted prior to fertilization were immobilized in 20 % Ficoll 6 min after fertilization as previously described (Neff *et al.* 1984). At T = 0·50 inverted PreFO eggs fixed in PBFG (4 %

formalin, 2·5 % glutaraldehyde in 0·05 M-phosphate buffer, pH 7·4) for 4 to 18 h and were sliced (razor blade) midsagittally along the plane of egg tilt in 100 % Steinberg's solution. The thickness of the subcortical vitelline layer (SVL) and residual vitelline mass (RVM) (Neff *et al.* 1984) at the gravity opposing apex of 150° to 180° inverted eggs was measured directly at ×38 (dissecting microscope with low-angle illumination). The following classification of cytoplasmic mobility was utilized: High-mobility cytoplasm (HMC) inverted eggs had a combined RVM and SVL thickness of <67 μm. Intermediate mobility cytoplasm eggs (IMC) had a RVM and SVL thickness of between 67 and 133 μm. Low-mobility cytoplasm eggs (LMC) had a RVM and SVL thickness of >133 μm.

RESULTS

I. *Determination of the appropriate clinostat speed(s) for microgravity simulation*

In order to determine the range of useful speeds which mimic hypogravity, analyses were carried out on the rate at which internal cytoplasmic components (e.g. yolk) shift in response to abnormal gravity orientation. It was assumed that in order to mimic microgravity, eggs immobilized on the clinostat (PreFO and NaRReO protocols) should rotate at speeds faster than the rate at which internal cytoplasmic components shift in response to novel gravity orientation.

Inverted eggs that were fertilized in the inverted position and then immobilized in that position were chosen as a model system for these preliminary determinations. The rate of shift of the major yolk compartments in a batch of intermediate cytoplasmic mobility (IMC) eggs at 15 °C was analysed by histological methods. Fig. 2A indicates that the large yolk compartment shifted at an average of 0·19 r.p.h. (range: 0·15–0·24). Therefore, clinostat speeds faster than 0·2 r.p.h. were employed for the PreFO and NaRReO protocols.

In the case of the FreRO clinostat procedure, a different assumption is necessary. Eggs are free to rotate within their vitelline membranes in the FreRo protocol. It was assumed that the minimum clinostat speed needed to mimic microgravity should be faster than the speed at which eggs rotate in their perivitelline spaces. Fig. 2B shows that inverted eggs rotated within their perivitelline spaces at an average speed of approximately 0·25 r.p.m. (15 r.p.h.) (range: 1·56–31·7 r.p.h.). Accordingly, clinostat speeds faster than 0·5 r.p.m. (30 r.p.h.) were employed in the FreRo version.

The optimal clinostat speed which simulates microgravity is difficult to calculate because of the vast number of unknowns concerning the microenvironment and physical properties of cellular components. Evidence indicates that crucial properties such as cytoplasmic viscosity, for example, change during egg activation (Elinson, 1983). That observation suggests that the optimal clinostat speeds may actually change during development. A whole range of clinostat speeds was therefore employed. The upper and lower limits of the proper clinostat speed range was determined by observation. The upper effective speed was 24 r.p.m. At 24 r.p.m. the eggs in a few batches (3/50) oriented with respect to the centrifugal field, while at 100 r.p.m. all FreRo eggs oriented with respect to the centrifugal field. The lower effective speed is 1 r.p.m. At speeds slower than 1 r.p.m. the egg polarity was influenced by the direction of rotation (see Figs 5 and 6 below).

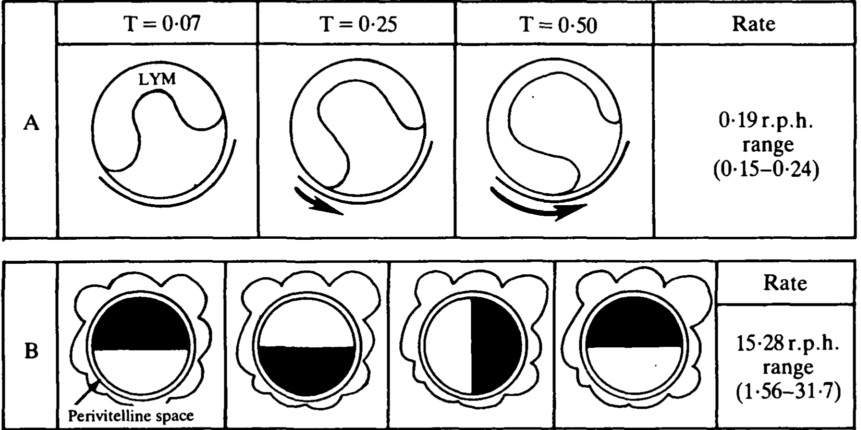

Fig. 2. (A) Rate of shift of major density compartments in fertile inverted eggs with average intermediate cytoplasmic mobility. Line around lower half of egg indicates darkly pigmented animal hemisphere. Arrow length illustrates extent of movement of leading edge of large yolk platelet density compartment (LYM). The r.p.h. was calculated from rate of increase in arrow length from T = 0·07 to T = 0·25 and from T = 0·25 to T = 0·50 at 15°C. Measurements were made on 0·5 μm plastic midsagittal sections parallel to the SES and maturation spot (Neff *et al.* 1983). (B) Rate of rotation of eggs in perivitelline space for FreRo version. Fertile eggs in their jelly coats were inverted at T = 0·3 and T = 0·6 and photographed from the side during their rotation response. The average r.p.h. was calculated from 30 to 60 s interval rotation rates at 15°C. The rate per egg was not uniform. The greatest rates were observed 1 to 2 min after inversion.

II. *Selection of eggs for clinostat protocols*

Neff *et al.* (1984) have emphasized the variability in response to novel gravity orientation (e.g. inversion) of different spawnings of eggs. The extent to which early pattern formation is reversed varied in proportion to the mobility of the major cytoplasmic density compartments. Three classes of eggs were designated according to internal cytoplasmic mobility: LMC (low-mobility cytoplasm); IMC (intermediate); and HMC (high).

Since the extent of pattern formation reversal displayed by inverted eggs varied among these three categories (Neff *et al.* 1984), a special effort was made to insure that each egg type, including both HMC and LMC versions, was tested in each of the three major clinostat protocols.

III. *Morphogenesis of clinostated embryos*

The frequency of normal *external* morphogenesis (tailbud stage) among approximately 3000 eggs which were clinostat rotated over a wide range of speeds by the PreFO protocol from approximately T = 0·1 to T = 3·0 is shown in Fig. 3. Because the PreFO rotated eggs were immobilized, clinostat rotation at low (< 1 r.p.m.) and high speeds (>24 r.p.m.) was also possible. Substantial numbers

of eggs developed normally over a wide range of clinostat speeds. From 0·25 r.p.h. to 12 r.p.m. more than 50 % of the embryos developed normally. At speeds of 24 r.p.m. and greater, however, more than 50 % displayed developmental abnormalities. At 24 r.p.m. approximately 60 % of the embryos were abnormal at the tailbud stage. At 100 r.p.m. approximately 70 % were abnormal. A wide range of abnormalities were observed. These included cleavage-furrow defects which resulted in cytolysis prior to gastrulation, incomplete gastrulation, endoderm oedema, and axis deformities such as microcephaly and tail defects. Since the major significance of the clinostat results is the observation that large proportions of the eggs develop normally, detailed descriptions of the nature of developmental abnormalities are not provided.

Of the approximately 1500 T = 0·1 fertile eggs clinostat rotated by the FreRo protocol, a substantial number showed normal *external* morphogenesis (Fig. 4). Because FreRo eggs are not immobilized, they can rotate within their perivitelline spaces below 1 r.p.m. and above 24 r.p.m. Eggs rotated by the FreRo method at low speeds, for example 15 r.p.h., kept adjusting their animal vegetal axes to gravity on the horizontal clinostats. At 100 r.p.m. virtually all eggs oriented their vegetal poles centrifugally away from the horizontal clinostat axis. Therefore, over the useful range of clinostat speeds (1 r.p.m. to 24 r.p.m.) for microgravity simulation, *Xenopus* eggs typically display normal external morphogenesis.

Since they are unable to rotate to accommodate the enhanced centrifugal forces, PreFO eggs may undergo a breakdown in the cytoplasmic component(s) which generate pattern specification. Such breakdown may account for the lower failure

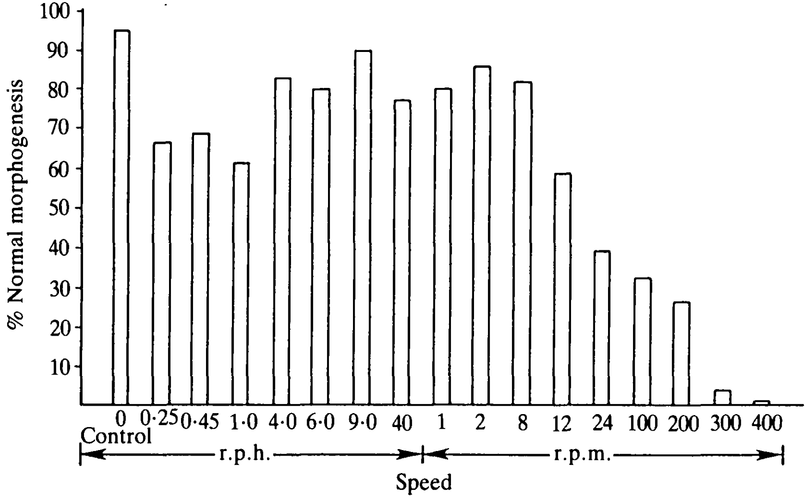

Fig. 3. Summary of data for the PreFO version of the clinostat procedure. Clinostats were stopped at T = 3·0 to 4·0. Approximately 3000 eggs (6 experiments) are included in the data. During preliminary experiments additional speeds were employed (e.g. 0·60 r.p.h., 10 r.p.m.). Since a consistent pattern was observed the 16 speeds illustrated above were chosen for detailed study.

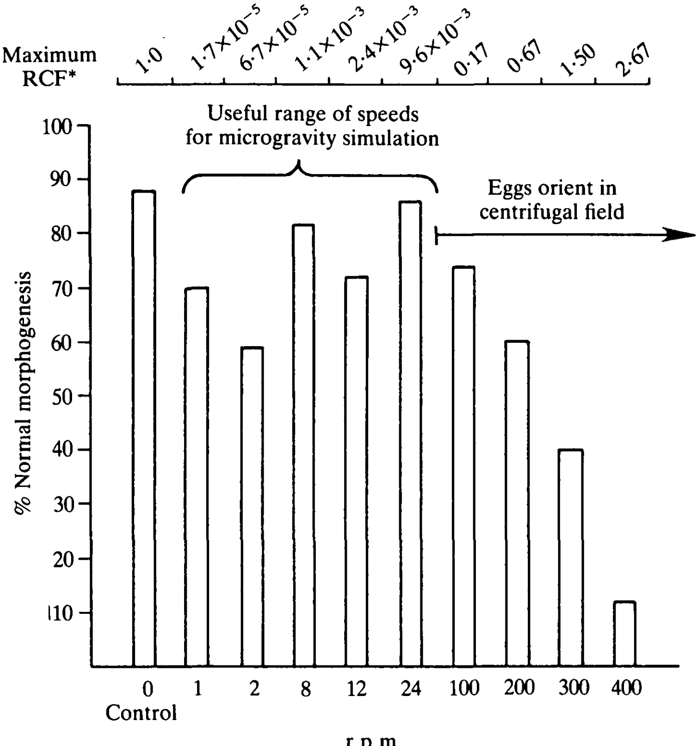

Fig. 4. Summary of data from the FreRo clinostat procedure. Clinostats were stopped at T = 3·0. Approximately 1500 eggs (5 experiments) are included in the data. *RCF (Relative Centrifugal Force = 11·17 (r) $(N/1000)^2$, where r = radius in cm and N = r.p.m.) is given in gravitational units (*g*).

speed of the PreFO protocol (i.e., PreFO failure speed = 24 r.p.m.; FreRo failure speed = 200 r.p.m.).

IV. *Analyses of responses of eggs to clinostat treatments*

A series of specific questions were answered in order to facilitate analyses and comparisons of data generated by the various clinostat protocols:

(1) Do each of the four clinostat protocols generate similar data re. normal/abnormal morphogenesis and failure speed? Generally, *yes*. The frequency of normal *external* morphogenesis was greater than 50 % for each protocol up to the speed of approx. 24 r.p.m. In the PreFO and NaRReO protocols the failure speed was usually 24 r.p.m. For the FreRo protocol eggs often displayed >50 % normal development up to 200 r.p.m.

The T = 0·1 to T = 0·15 time interval that the typical FreRo and PreFO clinostated eggs spend at 1 *g* before gravity compensation does not affect normal external development. Eggs fertilized on the rotating clinostats (2·5, 6, 8, 10, 12, and 24 r.p.m.) according to the FreRo (*b*) protocol developed normally. These

results demonstrate that the typical PreFO and FreRo experiments in which clinostat rotation begins by T = 0·1 to T = 0·15 mimic the ideal clinostat experiment.

(2) Do all three cytoplasmic mobility classes of eggs respond similarly to clinostat treatment? *Yes*. At least two or more batches of each egg type (HMC, IMC, LMC) were tested in each of the three major clinostat protocols. No appreciable difference in survival frequency was detected among the three protocols. Likewise, the nature of the defects in morphogenesis observed at higher speeds (>24 r.p.m.) was similar among the three protocols.

(3) Does the amount of time on the clinostat affect the results? *No*. Eggs were taken off the clinostats (four versions; 1 r.p.m.–400 r.p.m.) at either the first or third cleavage, or after overnight clinostating (embryos at late blastula stage). Their development was carefully monitored at the gross *external* morphological level. Neither the 'failure speed', nor the frequency of abnormalities, nor the pattern of development differed appreciably with regard to the time at which the clinostat treatment ceased.

(4) Do eggs which are rotated around a vertical rather than horizontal axis respond differently to clinostat treatment? *No*. Eggs of each type (HMC, IMC, LMC) were routinely mounted on vertical axis clinostats (FreRo, PreFO, NaRReO). Speeds of 1, 6, 10, and 300 r.p.m. were usually employed. At speeds of 1–10 r.p.m. development was typically normal. At 300 r.p.m., however, virtually 100 % of the vertical clinostated eggs cytolysed during cleavage stages. These data indicate that the failure of eggs at high clinostat speeds may, in part, be due to an effect of the high-speed clinostats other than gravity compensation.

(5) Do very slow (e.g. <1 r.p.m.) clinostat speeds mimic the polarization effect of 90° rotation? *Yes*. Previous reports (Scharf & Gerhart, 1980; Chung & Malacinski, 1980, 1981) revealed that a brief 90° rotation of uncleaved eggs establishes the dorsal side in the region of the egg which opposed gravity during the rotation period. Using the NaRReO protocol it was possible to orient eggs with respect to the sperm entrance site on the horizontal clinostat. Eggs were placed on clinostats with their animal hemispheres facing gravity and their SESs perpendicular to the horizontal clinostat axis. Rotation was carried out at 0·1, 0·5, 1·0, 2·0, 6·0 and 12·0 r.p.h. with the SES either leading or trailing in a plane perpendicular to the clinostat axis. At the dorsal lip stage, the eggs were scored for location of the involution site with respect to the SES. Fig. 5 shows that at the slow speeds (0·1 to 12·0 r.p.h.), the polarity (involution site) was determined by the direction of rotation.

(6) At clinostat speeds that simulate microgravity, is the invagination site (d/v polarity) strictly determined by the SES? *No*. Using the NaRReO protocol at speeds of 2 r.p.h.–12 r.p.m., the location of the dorsal lip with respect to the SES was monitored. Fig. 6 reveals that the very slow clinostat speed (2 r.p.h.) mimics the effect of 90° rotation (clinostat rotation determined egg polarity). At higher speeds (1 and 2 r.p.m.) the natural polarization of the eggs with the invagination site 180° opposite the SES was observed. At speeds of 8 and 12 r.p.m., however,

SES rotated "up"

SES rotated "down"

or

Score for location of dorsal lip

r.p.h.	No. of surviving embryos	Direction of rotation of SES	Location of lip			
				or		?
0 (control)	55	no rotation	37	7	1	10
0·1	32	up	6	4	5	2
		down	11	1	0	3
0·5	45	up	1	10	5	5
		down	12	7	2	3
1·0	71	up	1	9	22	10
		down	10	5	1	13
2·0	78	up	2	23	18	5
		down	19	7	0	4
6·0	60	up	6	12	17	2
		down	10	6	1	6
12·0	44	up	11	13	10	3
		down	3	1	2	1

Fig. 5. Direction of rotation of egg in NaRReO clinostat experiment determines, at low speeds, the location of the dorsal lip.

the site of the dorsal lip was dissociated (randomized) with respect to the SES. The following controls indicate that this randomization of polarity was not a side effect of clinostat rotation: (1) the shift of polarity at 2 r.p.h. and 2 r.p.m. provided the internal controls; (2) eggs rotated on *vertical* clinostats at 2 r.p.h., 2 r.p.m., and 12 r.p.m. displayed the majority of involution sites 180° opposite the SES. It appears that over the wide range of clinostat speeds that best mimic microgravity (1–12 r.p.m.), fertile eggs behave in two distinct ways with respect to the d/v polarity. At low speeds (1–2 r.p.m.), the polarity was normal with the dorsal side of the embryos 180° opposite the SES. At the higher clinostat speeds (8–12 r.p.m.), the eggs displayed abnormal (random) polarization. This randomization or reorientation of polarity was not determined by either the SES or the direction of clinostat rotation.

DISCUSSION

Because the fertilized amphibian egg displays a distinct rotation response to gravity, it has been considered feasible that gravity might drive internal cytoplasmic rearrangements. Previous attempts at using the clinostat to cancel the effects of gravity have, however, been incomplete for several reasons, including

employment of a narrow range of clinostat speeds, rotation beginning after fertilization, and dorsal/ventral polarity not being monitored. In the present study a variety of clinostat protocols designed to correct those inadequacies were

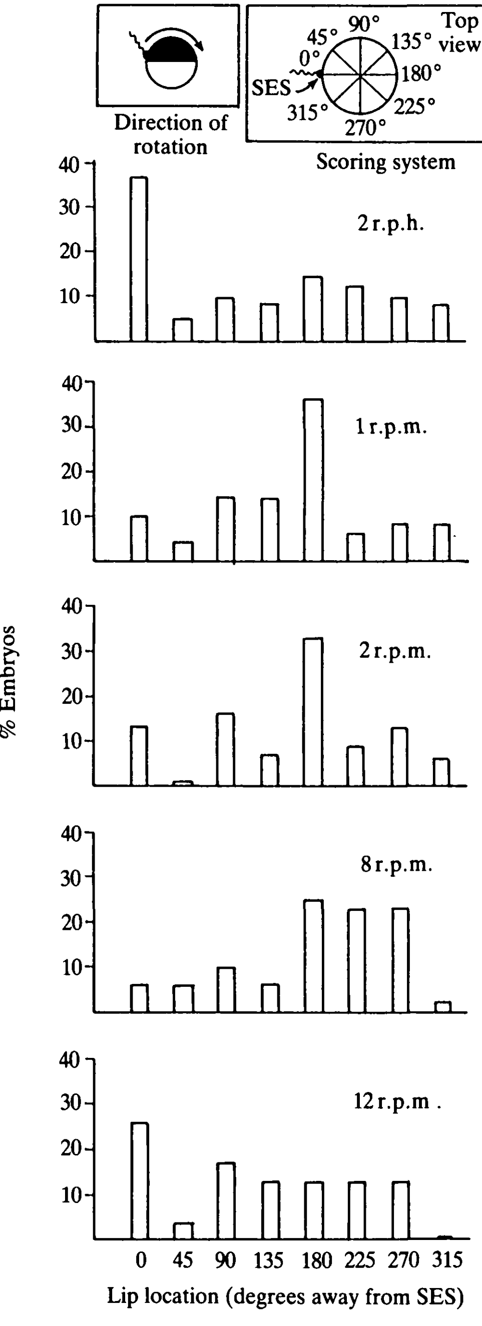

Fig. 6. Location of the site of invagination in clinostated NaRReO eggs. Approximately 250 embryos (5 experiments) were scored.

employed, including a novel one (FreRo (b)) which involved actually fertilizing the eggs during clinostat rotation. By comparing the results of the various procedures it is apparent that at the useful clinostat speeds which gravity compensate, early pattern specification is normal in a majority (>50 %) of eggs. These positive results taken together with the previous published clinostat experiments (see Table 1) and with the normal development of *Rana* eggs placed in the microgravity environment of space at around T = 1·0 (Young & Tremor, 1968) predict that *Xenopus* eggs fertilized and allowed to develop in the microgravity of space will develop normally.

Since *Xenopus* eggs display differences in the apparent viscosity of their cytoplasm (Neff *et al.* 1984), it was considered important that all types of eggs (HMC to LMC) be represented in the data. Accordingly, the cytoplasmic mobility of each batch of eggs was quantified. Even HMC eggs, which might be expected to show the greatest sensitivity to gravity effects, developed normally throughout the same broad range of speeds at which LMC eggs developed normally with respect to *external* morphogenesis.

Various models have been proposed which explain the manner in which the radial symmetry of unfertilized egg cytoplasm about the animal/vegetal axis is reformated to generate bilateral symmetry (dorsal–ventral polarity) in the zygote. The salient features of three of those models are illustrated in Fig. 7. In each instance a rearrangement of major cytoplasmic regions ('density compartments'; Neff *et al.* 1984) occurs. In each model the effects of the zygote's dorsal/ventral polarity on later pattern specification (e.g. mesoderm induction) are visualized to emerge from the novel compartment/compartment interactions which result from the breaking of the radial symmetry of the egg derived from oogenesis (reviewed by Malacinski, 1984*a*). The predicted behaviour under microgravity conditions is included. Should significant gravity-driven rearrangements of the cytoplasm be a prerequisite for egg polarization, as, for example, the 'gravity propulsion' model B in Fig. 7 predicts, early embryogenesis of clinostat rotated eggs would be abnormal. Conversely, normal pattern specification in clinostated eggs would favour the 'push-pull' and 'compartment shift' models A and C shown in Fig. 7.

In the 'gravity propulsion' model B (Fig. 7), some compartments built into the egg cytoplasm during oogenesis are not stratified according to buoyant density. Following egg activation (fertilization), the fluidity change of the cytoplasm (Elinson, 1983) permits gravity-driven compartment rearrangements to occur (Malacinski, 1984*b*; Neff *et al.* 1984). The data derived from the PreFO and FreRo clinostat experiments, which showed substantial normal development of clinostated eggs over the clinostat range that mimics microgravity, reveal that the 'gravity propulsion' model is not appropriate for describing the forces which rearrange the egg cytoplasm during the interval between activation and early cleavage.

In the 'push pull' model A (Fig. 7), the growth of the sperm aster away from the sperm entrance site (SES) physically pushes the cytoplasm compartments. Subsequent cortical contraction toward the SES pulls up yolk compartments on the

Model	Driving force	Predictions/microgravity	
		Normal development	Polarity SES = ventral
A. Push-pull T = 0 → T ≅ 0·75 Sperm entrance site (SES); Large yolk compartment; Cortical contraction; Sperm aster; Large yolk	Sperm aster; cortical contraction (Gravity accommodated)	+	+
B. Gravity propulsion Large yolk; Subcortical vitelline layer (SVL); Tilt (eg. Large yolk density > SVL/SVL provides tilt force)	Gravity (Gravity employed)	–	–
C. Compartment shift Intermediate yolk; Large yolk; SVL Fluidity increase/compartment movements under subtle control	No primary driving force; multiple subtle overlapping sequential forces	+	–

Fig. 7. General types of models that explain the manner in which the radial symmetry of the unfertilized egg cytoplasm about the animal/vegetal axis is reformated to generate bilateral symmetry (dorsal/ventral polarity) in the zygote. See Discussion for details.

future dorsal side (e.g. Gerhart *et al.* 1981; Ubbels *et al.* 1983). Although this model may be applicable at 1*g* (Ubbels *et al.* 1983) and at the lower clinostat speeds (1 and 2 r.p.m.) this mechanism does not necessarily provide indispensable driving forces for the cytoplasmic rearrangements which are necessary for providing the eggs with a dorsal/ventral axis. Clinostated eggs at 8 and 12 r.p.m. (Fig. 6) displayed an uncoupling of the SES from the future dorsal side. Clinostating does not represent the only way in which D/V polarity can be uncoupled from the SES. In inverted eggs the direction of shift of the major yolk components rather than the SES determines D/V polarity (Neff *et al.* 1984). In *Rana esculenta* the tilt of the animal hemisphere with respect to the animal/vegetal axis, rather than the SES, determined the dorsal/ventral polarity (Pasteels, 1937) of selected normally oriented eggs. Other examples of uncoupling of SES and D/V polarity include: 90° rotation and lateral centrifugation (Gerhart *et al.* 1981); and as little as 15° tilt (Malacinski & Neff, 1985).

The data collected in this series of clinostat experiments are valuable in the following ways: They have predictive value with respect to future microgravity space flight experiments. They exclude model B and place model A in doubt. They cause us to think about amphibian egg pattern specification in novel ways such as model C (Fig. 7). This model is heavily based on the data presented in this paper, on previous compartment data produced by this laboratory (Neff *et al.* 1984), and on experimental manipulations of D/V polarity such as egg rotation (Gerhart *et al.* 1981). This model requires no single indispensable driving force to bring about the compartment shifts that allow for the asymmetric compartment localizations and/or interaction (activations) (Neff *et al.* 1984). Rather, multiple, overlapping, sequential forces, some of which may be dispensable, establish the necessary compartment asymmetry. The desired end result, compartment asymmetry, is the end state that the egg requires to establish a normal dorsal/ventral axis. Therefore, several combinations of egg properties and/or forces such as intrinsic egg asymmetries (Pasteels, 1937), viscosity changes (Elinson, 1983), aster growth, cortical contraction (Ubbels *et al.* 1983), egg tilt (Malacinski & Neff, 1985), and gravity orientation (Gerhart *et al.* 1981) etc. may be sufficient. Although this model might be considered vague and perhaps less susceptible to simple direct experimentation, it emphasizes the complexity of the problem of understanding egg polarization.

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