Adv. Space Res. Vol.4, No.12, pp.9-18, 1984
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0273-1177/84 $0.00 + .50
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CYTOSKELETON AND GRAVITY AT WORK IN THE ESTABLISHMENT OF DORSO-VENTRAL POLARITY IN THE EGG OF *XENOPUS LAEVIS*

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ABSTRACT

The establishment of polarities during early embryogenesis is essential for normal development. Amphibian eggs are appropriate models for studies on embryonic pattern formation. The animal-vegetal axis of the axially symmetrical amphibian egg originates during oogenesis and foreshadows the main body axis of the embryo. The dorso-ventral polarity is epigenetically established before first cleavage. Recent experiments strongly suggest that in the monospermic eggs of the anuran *Xenopus laevis* both the cytoskeleton and gravity act in the determination of the dorso-ventral polarity. In order to test the role of gravity in this process, eggs will be fertilized under microgravity conditions during the SL-D1 flight in 1985. In a fully automatic experiment container eggs will be kept under well-defined conditions and artificially fertilized as soon as microgravity is reached; eggs and embryos at different stages will then be fixed for later examination. Back on earth the material will be analysed and we will know whether fertilization under microgravity conditions is possible. If so, the relation of the dorso-ventral axis to the former sperm entry point will be determined on the whole embryos; in addition eggs and embryos will be analysed cytologically.

INTRODUCTION

The establishment of embryonic axis is essential for normal development of plants and animals. Since all organisms on earth are subjected to gravity, for more than a century embryologists have considered gravity as one of the possible sources of embryonic polarity. Amphibian eggs are very suitable for studies on the origin of polarities for various reasons: they are easily available and because of their size are relatively easy to manipulate.

Amphibian oocytes acquire radial symmetry during oogenesis; dorso-ventral (D/V) polarity is induced by the sperm and develops epigenetically before first cleavage, as becomes evident from characteristic pigment shifts /12/. In order to analyse the genesis of D/V polarity many egg rotation, inversion and centrifugation experiments have been performed in many different species of amphibians. The results were not unambiguous /18/, often because it was not realized that upon fertilization the egg orients itself inside the vitelline membrane and thus returns to its natural position during the experimental treatment. However, more recent egg rotation and centrifugation experiments in the monospermic eggs of *Xenopus laevis* have shown that gravitationally induced rearrangements of the yolk predictably determine the orientation of the D/V axis of the embryo, regardless of the original position of the sperm entry point (SEP) or the grey crescent /12/. These results strongly suggest a role of gravity in the determination of bilateral symmetry. Logically, only an experiment under zero-g conditions can definitively prove or disprove whether indeed gravity is involved.

Experiments with frog eggs have been performed during the space flights of Gemini VIII and XII and Biosatelite II /40,41,42,43,44/. In all these cases fertilization of *Rana pipiens* eggs was performed on earth and the zygotes were kept at low temperature until they had reached microgravity. The temperature was then raised, development proceeded at the normal rate and morphogenesis was normal /42/, although recent experiments with cell cultures and *Paramecium* suggest that microgravity does influence the length of the mitotic cell cycle /7,8,27,32/.

Biorack experiment A08/52NL of the Hubrecht Laboratory has been selected as part of the payload in Spacelab during the D1 mission scheduled for 1985. The experiments focus on two questions (a) whether fertilization under microgravity conditions is possible, and if so (b) whether under such conditions the sperm is the sole agent that determines bilateral

symmetry. Since the time available for the performance of experiments on the first day of the mission is limited and the viability of the biological material restricted, eggs and testes will be sent up separately and fertilization will be performed in space in a fully automatic closed container /6,35/.

In this paper data relevant to axis formation during the early development of the egg of *Xenopus laevis* are summarized, the conditions required for storage of eggs and testes, fertilization, and raising embryos will be discussed, and information on the experiment container used will be presented.

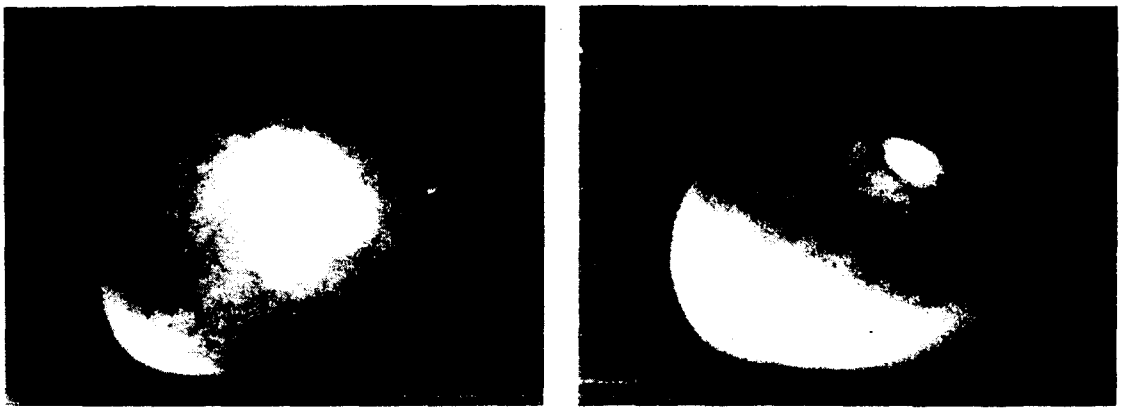
CYTOSKELETON AND POLARITIES

The axially symmetrical mature oocyte of *Xenopus laevis* exhibits a pigmented animal and an almost unpigmented vegetal hemisphere. The egg is radially symmetrical about its animal-vegetal (An/Veg) axis, which foreshadows the main body axis of the embryo /25/. This axial symmetry originates during oogenesis through the pattern of yolk formation and is independent of gravity /13/: the full-grown oocytes in the ovary as well as unfertilized eggs after oviposition are in an arbitrary position with respect to gravity. The vitelline membrane and the innermost of the surrounding jelly layers are firmly attached to the egg surface, preventing the egg from rotating freely. In this phase the differential distribution of the yolk is presumably maintained by structures of the cytoskeleton /36/. The pigmented animal hemisphere contains the nucleus and is rich in cytoplasm containing small and relatively light yolk granules, many mitochondria and other metabolically active organelles, ribosomes, glycogen, etc. The vegetal hemisphere is relatively poor in such organelles, substances and cytoplasm; it mainly comprises the larger and heavier yolk platelets. The cell membrane of full-grown oocytes, unfertilized and fertilized eggs exhibits an An/Veg polarity on the molecular level /9/. Experimentally, when the An/Veg polarity is inverted through 180° /2/ the D/V polarity is also reversed.

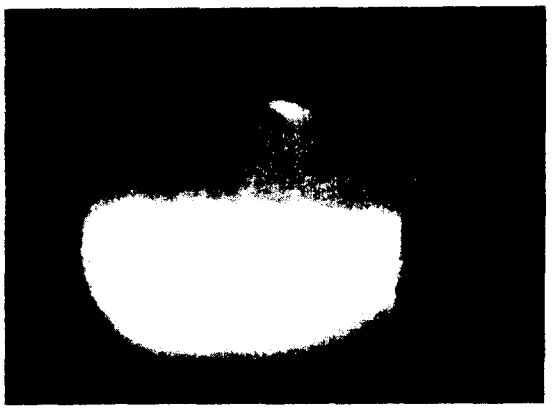
Development of dorso-ventral polarity starts with the penetration of the sperm, which can occur anywhere in the animal hemisphere. It switches the negative membrane potential to a positive one, thus creating a 'fast block' to polyspermy /14/, and subsequently initiates extrusion of the cortical granules. This causes an additional 'slow block' to polyspermy through the elevation of the vitelline membrane and the formation of the perivitelline space around the egg. Under gravity the egg then rotates, heavy vegetal half down and animal half up (Fig. 1,2): 'rotation of orientation' /1/. The extrusion of the cortical granules can be visualized by video-time-lapse and time-lapse cinematography as the 'activation wave' (AW), which starts from the sperm entry point (SEP) and spreads over the egg surface at a speed of 10 µm/sec /16/. After passage of the AW the pigment first contracts towards the animal pole region (activation contraction), but disperses again during egg rotation. Meanwhile some of the pigment concentrates on the SEP /26/, which from then on clearly marks the future ventral side of the embryo /18/, where much later structures like the intestine and the blood cells will form. A secondarily depigmented cortical region denoted as the 'grey crescent' appears on the opposite side, where about ten hours post fertilization the blastopore forms and where much later dorsal structures like neural tube, notochord, and somites appear. The AW is thus an early expression of the D/V polarity.

Soon after the AW a perceptible change in reflectivity again starts from the SEP and proceeds over the egg surface towards the opposite side, but now at a speed of 1 µm/sec /15/. The slower speed of this 'post-fertilization wave' (PFW) suggests that its nature differs from that of the AW. It is accompanied internally by a ventro-dorsally directed shift of cytoplasmic components which symmetrizes the egg. Cytological analysis of eggs fixed after video-time-lapse observation of the PFW has revealed that this wave reflects the extension of the spermaster /36/, an array of microtubules organized by the sperm, presumably through its centriole. The spermaster progressively expands into the animal hemisphere (Fig. 3), directing yolk relocations, among them a shift of a yolk-poor cytoplasmic area located centrally in the unfertilized egg towards a more dorsal and animal position ('dorsal cytoplasm') /34/. These rearrangements of yolk components foreshadow the D/V axis of the embryo. They do not occur upon prick activation, when there is no centriole present, nor in fertilized eggs when microtubule assembly is blocked by vinblastine incubation. In such eggs there is no spermaster, the rearrangements of yolk can be directed by gravity alone, the PFW is absent, and cortical pigment movements are abnormal /36/.

All this suggests that the rigid structure of the cytoplasm, that keeps the differential yolk distribution in ovarian and unfertilized eggs intact is disturbed by egg activation, and that the sperm is needed to organize an extensive aster (and possibly additional cytoskeletal structures) in order to direct determinative yolk rearrangements. Recently published experiments /28/ support this view. By applying cold shocks, ultra-violet irradiation, hydrostatic pressure, or agents breaking down constituents of the cytoskeleton, under-differentiated embryos of radial symmetry were obtained, the axis of symmetry coinciding with the original An/Veg axis. These results are also consistent with recent observations by Elinson /10,11/, who found a steep decrease in the amount of polymerized tubulin at fertilization and activation, followed by a rather steep increase some time after fertilization, and also after



a) b)



c)

Fig. 1 Rotation of orientation in the fertilized egg of *Xenopus laevis*.
(a) A few minutes after fertilization;
(b) After passage of the activation wave the pigment cap first contracts and then the egg starts to rotate in the vitelline membrane under gravity;
(c) Gravitational rotation nearly finished.
(Sequential stills reproduced from a 16-mm time-lapse film)

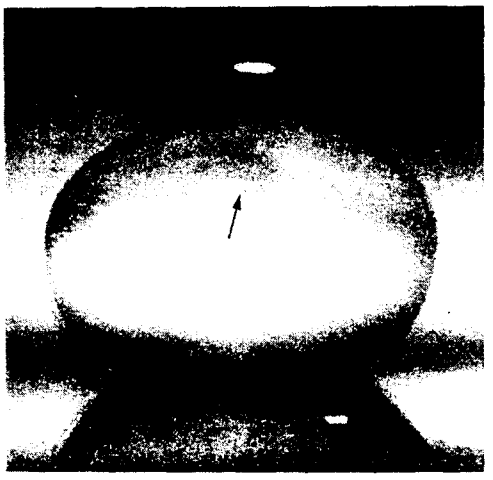
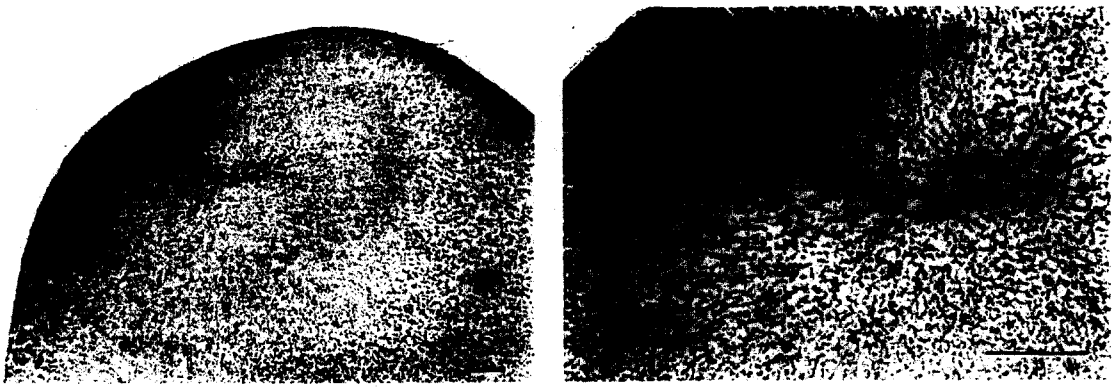


Fig. 2 Fertilized egg of *Xenopus laevis* in its perivitelline space. Arrow points to the sperm entry point (SEP).



a) b)

Fig. 3 a) Spermaster in artificially fertilized *Xenopus* egg at relative time t=0.26 ☆. In the cortical region pigment granules are arranged along the blue-stained aster fibrils. ♂: male pronucleus
b) Detail of a).
(Bouin d'Hollande fixative; 6 μm, para-median, histowax section. Bar = 50 μm in both figures).

activation only, though somewhat later. Applying conventional antisera against *Xenopus* egg tubulin and actin in an immunocytochemical assay during the period of spermaster formation, an additional array of progressively lengthening fibrillar structures could be visualized. These fibrils grew in the same direction as the spermaster rays and reached their maximal length between t=0.3 and t=0.4 ☆. From then on the package of fibrils moved as an entity towards the dorsal region of the egg, where between t=0.7 and t=0.8 only a diffuse staining was observed. The yolk shifts described that lead to dorso-ventral polarization are essential for normal development /36/. The dorso-ventral differentiation of the cytoskeleton may serve to redistribute maternal messengers and small molecules during the precleavage period (c.f. /17/).

Grey crescent formation may be caused by an asymmetric cortical contraction drawing pigment from the animal hemisphere towards the SEP. The pigment movements involved may thus reflect modifications in the cytoskeleton during grey crescent formation, which might also involve deeper cytoplasmic materials and, through this, establish regional dorso-ventral differences /12,31/, resulting in cytoplasmic localizations which finally act in selective gene activation. The asymmetric cortical contraction is assumed to occur in a particular phase of the cell cycle that is initiated by egg activation. However, in a number of species the grey crescent forms through rotation of the cortical layer around the internal egg mass /1,19/. Vincent & Gerhart /38/ recently showed that in *Xenopus laevis* the peripheral layer of the vegetal yolk mass moves away from the future dorsal side with respect to the cell membrane in the period t=0.4 to t=0.8, exactly the time during which the spermaster is maximally developed and the grey crescent is clearly visible. U.V. irradiation applied in a dose that causes radialization of the embryo stops the subcortical material from moving, which suggests that the movement of the subcortical layer is essential for dorso-ventralization. It has therefore been suggested /29/ that in *Xenopus laevis* and in *Rana pipiens* first, during spermaster enlargement, force-generating structures are oriented in the egg cortex, which might subsequently operate in the rearrangement of the egg's contents, thus creating a dorsal cytoplasmic localization.

GRAVITY AND POLARITIES

Changing D/V polarity by gravitational force.
The above data emphasize the role of the cytoskeleton in the D/V polarization of the egg prior to first cleavage. However, it is possible to overrule the dominating influence of the sperm by egg rotation after dehydration of the perivitelline space by Ficoll /12,18/. Eggs were selected for near-equatorial SEPs and rotated through 90°, so that the An/Veg axis was horizontal with the SEP either up or down for fixed periods of time. In such rotated eggs the blastopore always formed in the region which was uppermost during treatment. Thus, D/V polarity was reversed when the SEP was up, normal when the SEP was down, and twin embryos also developed. Cytological analysis revealed that the dorsal side develops where under the

☆ t=x refers to a normalized time scale (fraction of the total duration of the first cleavage cycle)

influence of gravity the heavy yolk had slid down beneath the egg cortex /36/, in some way creating or reinforcing the conditions for dorsal development /12,18/. The dorsal cytoplasm retains its original position while the outer yolk layers move about the inner mass. This experiment demonstrates that neither the egg cortex nor the dorsal cytoplasm contain long-lasting particulate dorsal determinants.
The progressively increasing stiffness of the cytoskeleton /10/ makes axis reversal less successful when approaching first cleavage /12,18/. However, when eggs are first embedded in a dish of molten gelatin, which also dehydrates the perivitelline space, and are oriented before solidification, the egg is held in a fixed position. When the dish is then placed in a centrifuge with the SEP towards the centre of the rotor, the centrifugal force will move materials away from the side of the SEP, thus imitating an egg rotation experiment. Application of stronger centrifugal forces (up to 30 g) overrules the strength of the cytoskeletal elements and reverses D/V polarity even shortly before first cleavage /12/. These experiments strongly argue for a role of gravity in the determination of D/V polarity. However, this can only be definitively proven or disproven by an experiment under 0 g conditions.

MICROGRAVITY VERSUS CLINOSTAT

Several authors suggest that 0 g conditions can be simulated by the use of a clinostat /5,22, 30,33/. Briegleb *et al.* /4/ allowed eggs of *Rana temporaria* to develop inside the rotor of a clinostat at 90 r.p.m. and concluded from the results that the establishment of bilateral symmetry is independent of gravity.

Although the clinostat may be an interesting tool to investigate behaviour and development of organisms, it remains difficult to predict what will actually happen in the relatively large *Xenopus* egg (1.2 mm), which contains yolk granules of various sizes (1-2 µm; 3-4 µm; 6-12 µm) and densities. These granules are embedded in cytoplasm of inconstant consistency (partly due to the variable composition of the cytoskeleton and associated organelles). Clinostating the eggs may easily cause torque (c.f. /23/) and the fast-rotating clinostat probably approaches conditions of centrifugation rather than microgravity. Cytological analysis following a clinostat experiment performed during the period of symmetrization (c.f. /21/) might reveal the primary effect of such treatment.

BIORACK EXPERIMENT AO8/52NL

The automatic experiment container will be mounted inside a Biorack type 1E container. Eggs, testes, various salt solutions, and the histological fixative will be contained in six separate cylindrical compartments in a perspex block (Fig. 4). Four of the compartments are equipped with spring-loaded plungers. A pre-tensioned spring will produce the driving force for the plunger, which is kept in an upward position by a nylon wire. A resistance wire twisted around the latter functions as a wire heater. The experimental cycle is controlled by a microprocessor with an electronic timer, which sequentially initiates the wire heaters for release of the plungers in the various compartments. The wall of the compartment below the plunger is covered by a rolling sleeve to make it leak-proof and to reduce the friction between wall and plunger. In the testes compartment (TC) the plunger bears a pricker that will puncture the testes. In the egg compartment (EC) monolayers of eggs are separated by vinathene sieves inside a ceramic cylinder that allows exchange of O_2 and CO_2 between the inside of the Biorack type 1E container and the medium in the egg compartment. Small channels in the bottom plate connect the various compartments, and non-return valves in the silicon rubber sheet between block and bottom plate prevent uncontrolled mixing of the fluids. The microprocessor will start the experiment as soon as the temperature in the container reaches 22°C /6,35/.

Preservation

A period of 18-22 hours must be bridged between the delivery of the experiment containers with the living material and the start of the experiment. After stripping the eggs from the females and removal of the testes from the males, eggs from two different females and two testes each from different males will be kept in 'dormant' condition until fertilization. During this period the containers will be stored at 11°C inside a passive thermal canister on the midflight deck of the Space Shuttle.
Although a delay of fertilization for such a long period is highly unnatural, a large series of experiments have shown that unfertilized eggs can be preserved at 11°C /35/ in full-strength MMR, a modified amphibian Ringer's solution (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Na-HEPES buffer, 0.1 mM EDTA; optimal osmolarity 230 mOsm., pH 7.8) /18/. After preservation at 11°C for 17-18 hours or longer, fertilized eggs sometimes show an abnormal pigmentation and more often an incomplete rotation of orientation: the pigmented hemispheres do not turn up completely. Cytological examination of such eggs revealed rearrangements of yolk and cytoplasm with respect to the pigmented cortex, which suggests a shift of their centre of gravity during the period of storage /35/. The primary deleterious effect of delayed fertilization apparently is a change in cytoplasmic consistency, probably due to a lower resistance of cytoskeletal components. Such yolk rearrangements would interfere with the goal of the experiments, but the eggs will be under microgravity conditions within 7-12 hours after stripping and this is long before spontaneous rearrangements of yolk

Fig. 4 Experiment container with electronics mounted on top.

The six compartments will contain (in order of operation):

LC: 0.6 ml labelling-fluid. Activation of the plunger in LC pushes the fluid to the testes compartment (TC). As a consequence, fluid from TC is pushed through a channel in the bottom plate into the egg compartment (EC). Fluid from EC is pushed away through a channel between the two halves of the perspex block into the now available space above the plunger of LC.

TC: 0.7 ml full-strength MMR with two testes. Upon activation of the pricker in this compartment the testes are punctured and sperm is released into the labelling-fluid. After release of the pricker the volume in TC is 0.4 ml.

AC: 1.5 ml distilled water buffered with HEPES. Activation of the plunger in AC pushes distilled water via TC into the egg compartment (EC). The sperm is mobilized through dilution of the full-strength MMR. (Note nylon thread and wire heater above the plunger and rolling sleeve below).

EC: 0.9 ml full-strength MMR with 40-50 eggs on five layers of sieves. The moment the distilled water carries the sperm into EC is considered as the actual time of fertilization.

MC: 2.0 ml 25% MMR. Activation of the plunger in MC replaces sperm and labelling-fluid by fresh culture medium, in which the embryos will develop until the time of fixation.

GC: 1.0 ml 2% glutaraldehyde. Activation of the plunger in GC moves the glutaraldehyde into EC. The biological material is fixed and will stay in the fixative until the experiment containers are opened after completion of the mission.

may be expected.
During launch the stored biological material has to withstand an acceleration of 3 g, many types of vibration, and acoustic noise. Their possible effect on freshly stripped eggs and prepared testes as well as on material preserved for 11-12 hours has been tested. In all cases the fertilization rate was similar to that in the control group /6/. However, it is evident that this only holds when the viability of the material is optimal, and this ultimately depends on the animals used.

Selection of females

Observations in the amphibian facility of the Hubrecht Laboratory made clear that some females produce egg batches of higher quality than others. Therefore all females of the 1981 class have been marked /37/ and the various features of the eggs produced can thus be followed for individual females (Fig. 5). Certain females always produce egg batches from which 80-100% normal embryos develop, but even within such a group susceptibility to storage effects can vary /6/. This emphasizes the need for an additional selection for storage resistance (e.g. cytoplasmic consistency), which is supported by recent data in the literature /24/. Males can only be used once and cannot be selected.

Fixation times

Six containers will be flown in the microgravity experiment:

1) Unfertilized eggs will be fixed as soon as the container temperature is 22°C. Cytological analysis will reveal whether eggs survived storage in a proper state and whether their inner structure is unchanged.
2) Fixation at 60 minutes after fertilization. This is about half-way between fertilization and first cleavage, for in eggs preserved at 11°C for 17-22 hours first cleavage at 22°C

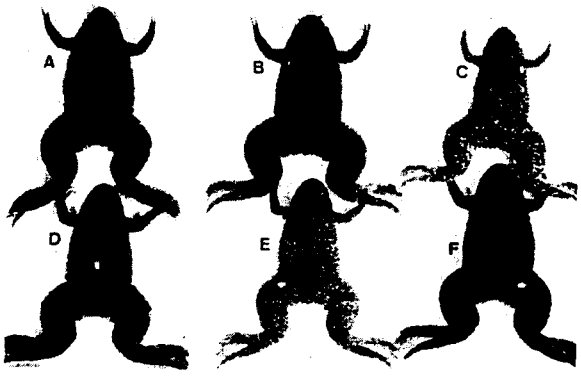


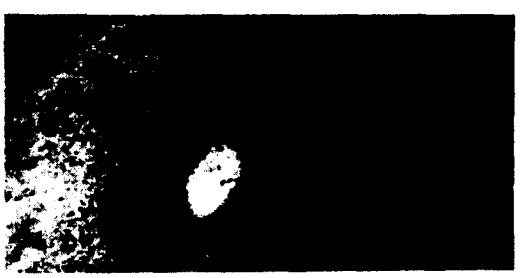
Fig. 5 Skin autografts as markers in *Xenopus laevis*: a group of six females coded by graft location. In five individuals (B-F) a piece of white belly skin was transplanted to the back of the same toad.

is delayed from 90 to 105-120 minutes post fertilization /6/. This fixation will be performed at 0 g and on the 1 g centrifuge. These samples should prove whether fertilization under microgravity conditions is successful and pronuclear movement normal.

3) The remaining fixations are made around gastrulation. The storage period only delays first cleavage and does not interfere with subsequent cleavages /6/. This means that the time elapsing between first cleavage and the beginning of gastrulation in embryos developing from stored eggs is similar to that in embryos that develop from freshly stripped eggs. The timing of gastrulation in the experiment container depends on the ambient temperature: fluctuations of 1°C cause a time shift of approximately 45 minutes /6/. In order to be sure that the fixed gastrulae do exhibit clear incipient blastopores, fixations will be made at 11 and 12 hours after fertilization. A third sample will be fixed on the 1 g centrifuge.

The position of the SEP in relation to the D/V axis

For the determination of the position of the SEP in relation to the dorso-ventral axis, its location should still be recognizable at the gastrula stage. The sperm should thus in some way mark its own entry site, because fertilization takes place inside the container. It is possible that during fertilization (part of) the sperm membrane is locally incorporated into the egg membrane. We assume that when this is the case, the location will hardly change before the beginning of gastrulation, as the membrane is still very rigid at the early gastrula stage /3/. Different approaches for labelling the SEP are being tried out: labelling of sperm prior to fertilization or visualization of the SEP in gastrulae, both by immunocytochemical methods (Fig. 6).



a)

b)

Fig. 6 SEP labelling in *Xenopus* eggs at t=0.3
a) SEP stained after fixation by incubation in antiserum against *Xenopus* sperm, visualized by FITC;
b) Location of SEP after fertilization with TNBS-labelled sperm, visualized by incubation in antiserum to DNP-BSA.
However, the obtained results are not yet consistent.

Experiments with prototypes of the experiment container /6/ have convincingly shown that (a) freshly stripped eggs can be successfully fertilized inside the container and that (b) fertilized eggs can be successfully raised inside the egg compartment, which shows that the gas exchange is sufficient. The fertilization rate of newly stripped eggs is similar to that in the controls. However, fertilization in the container after storage periods longer than six hours had a success rate of 1% only. It could be experimentally ruled out that this was caused by inappropriate mixing of fluids in the egg compartment, which might lead to inadequate osmolarity. Contamination of the medium inside the container by minimal amounts of methylmetacrylate released by the perspex block during the period of egg preservation was far below the permitted level. During the experimental sequence only minimal pH changes were noticed in the medium in the egg compartment (the full-strength MMR is buffered with 5 mM HEPES). However, it seems possible that during storage periods longer than six hours CO_2 produced by the metabolizing eggs would locally lower the pH in the closely surrounding medium, and would thus interfere with sperm-egg interaction and the succeeding cell divisions, processes that are known to be sensitive to pHs lower than 7 /20,39/. A significant increase of the fertilization rate to 20-50% was obtained by raising the buffer capacity of the preservation medium and by adding buffer to the distilled water compartment.
We expect that further adaptation of the buffer capacity of the solutions in the experiment container will improve the fertilization rate sufficiently, and through this the yield of gastrulae in our Biorack experiment.

ACKNOWLEDGEMENTS

The financial support of the Netherlands Ministry of 'Onderwijs & Wetenschappen' through the Agency for Aerospace Programs (NIVR) and the fruitful cooperation with the 'Centrum voor Constructie en Mechanisatie' (CCM), Nuenen, The Netherlands, in developing the automatic experiment container, are gratefully acknowledged. Thanks are due to Dr. J. Faber for editorial assistance, to Mr. C.H. Koster for valuable cooperation and discussion, to Ms. R. Verhoeff and staff for animal supply, and to Ms. C.L. Kroon for photography.

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