

Amphibian development in the virtual absence of gravity

(embryonic axis/morphogenesis/swimming behavior)

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Communicated by John C. Gerhart, University of California, Berkeley, CA, December 2, 1994

ABSTRACT To test whether gravity is required for normal amphibian development, *Xenopus laevis* females were induced to ovulate aboard the orbiting Space Shuttle. Eggs were fertilized *in vitro*, and although early embryonic stages showed some abnormalities, the embryos were able to regulate and produce nearly normal larvae. These results demonstrate that a vertebrate can ovulate in the virtual absence of gravity and that the eggs can develop to a free-living stage.

Is gravity required for normal embryonic development? Upon fertilization, most amphibian eggs rotate inside the fertilization membrane so that the animal-vegetal axis is aligned with gravity. This "rotation of fertilization" (1) is not a requirement for normal development, since eggs prevented from rotating can develop normally (2). Nevertheless, the direction of the rotation of fertilization normally has a role in determining the polarity of the embryonic axis (1), and eggs inclined with respect to gravity form the dorsal structures on the side of the egg uppermost in the gravitational field (3, 4). Thus, for over a century scientists have questioned whether gravity was required for amphibian embryogenesis (5, 6). Recent space-flight experiments have successfully fertilized eggs of *Xenopus laevis* and reared the embryos to the gastrula stage. The morphology of the gastrulae was somewhat abnormal (7). We report here that ovulation and subsequent development to a free-living stage can occur at microgravity ($<10^{-3} \times g$). We also examined larval swimming behavior to determine whether abnormal behaviors of *Xenopus* tadpoles previously observed during and following space flight (8) would occur with larvae that began their lives in the virtual absence of gravity.

MATERIALS AND METHODS

Preflight Procedures. *X. laevis* frogs were obtained from J. Gerhart (University of California, Berkeley) and Nasco (Fort Atkinson, WI). In the year before flight, the frogs were spawned at 2- to 3-month intervals. Frogs with a history of yielding high-quality eggs were shipped to the Kennedy Space Center. Thirty-six hours before launch, four female frogs were placed in a foam-lined container. The foam was saturated with water, and 30 hr before launch the container was loaded into a special incubator, the frog environmental unit (FEU), aboard the Space Shuttle. Air was pumped through the container at a rate of 100 ml/min, and the temperature was maintained at 18°C throughout the preflight period.

Flight Procedures. Eighteen hours into the mission, the frogs were injected subcutaneously with human chorionic gonadotropin. Eggs were collected from each frog and groups of 15-30 eggs were placed on small screens. The eggs were fertilized with a sperm suspension and inserted into Lexan chambers, and 50 ml of 0.2× modified Ringer's solution was added to each chamber (modified Ringer's solution is 100 mM

NaCl/1.5 mM KCl/0.18 mM MgCl₂/0.75 mM CaCl₂/10 μM ZnCl₂/5 mM sodium Hepes, pH 7.4). Half of the chambers were incubated on the FEU centrifuge (rotating at 60 rpm to create a force of $1 \times g$) and half were incubated at microgravity (gravity levels ranged between 10^{-3} and $10^{-5} \times g$ in the Spacelab). The temperature difference between the $1 \times g$ centrifuge and the microgravity compartment was maintained within $\pm 0.25^\circ\text{C}$. After the first 50 hr of the mission, the FEU temperature was raised to 21°C and held there for the duration of the flight, to increase the rate of development. Some embryos were fixed inflight according to the procedure of Black and Gerhart (4) and sectioned and stained postflight.

Postflight Procedures. Chambers with live embryos were returned to our laboratory within 3.5 hr of landing. Embryos were staged according to Nieuwkoop and Faber (9). Normality of tadpoles was assessed by applying criteria of the dorso-anterior index, a measure of the extent of dorso-anterior differentiation (10).

Optomotor behavior of the tadpoles was determined following the removal of the tadpoles from the flight chambers. The tadpoles were individually tested for their tendency to track a moving stimulus. The stimulus pattern, consisting of alternating vertical light and dark strips of 20-mm and 6-mm width, respectively, was imprinted on a clear plastic cylinder of 5-cm diameter. The cylinder was placed over the vial with the tadpole and was rotated at 10 rpm. The percentage of time spent following the rotating pattern was determined during a 1-min test with the cylinder rotating in a clockwise direction and 1 min in the counterclockwise direction. The initial direction of rotation was alternated to compensate for any innate handedness of the tadpoles in either the flight or the centrifuge groups.

RESULTS

Oviposition occurred in all four frogs by 16 hr after hormone injection at 18°C, just as it occurs in our ground-based laboratories. Fertilization was achieved at high frequencies in both the centrifuge and microgravity groups (Table 1). This confirms the findings of Ubbels *et al.* (7) in their previous sounding-rocket and Space Shuttle experiments that gravity is not required for fertilization in this species. Two-cell embryos from both groups showed a cleavage furrow in the normal position, originating at the animal pole and extending toward the vegetal pole. Gastrulae showed no gross abnormalities, but embryos developed in microgravity had thicker blastocoel roofs; the microgravity group averaged 3.7 cell layers versus an average of 2.6 cell layers in the $1 \times g$ group ($n = 65$, $P = 0.0001$, Mann-Whitney *U* test) (Fig. 1). In addition, the blastopore lip formed at a slightly more vegetal latitude in the microgravity group (34°) than in the $1 \times g$ sample (41°) ($n = 65$, $P = 0.02$, Mann-Whitney *U* test). Despite these differences, development to the neurula stage was unimpaired, as all fixed neurulae

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Abbreviation: FEU, frog environmental unit.

Table 1. Fertilization and development during the Spacelab-J mission

Group	Total no. of eggs	No. of fertilized eggs (%) [*]	Normality of tadpoles [†]
μg	325	285 (88)	4.91
$1 \times g$	288	210 (73)	4.99

Data from four frogs are combined. μg , Microgravity.

^{*}Contingency table analysis showed a statistically significant difference between the two groups ($\chi^2 = 20.5$, $P = 0.0001$). However, we doubt that this difference is biologically significant, as batches of eggs from different frogs commonly differ in their fertilization efficiency under standard laboratory conditions. For example, data from a ground control run during the flight but with different frogs showed a difference in percent fertilization between the sham microgravity and sham $1 \times g$ groups. Eggs from one frog were divided into two groups and fertilized at the same time with the same sperm preparation. Nevertheless, the two groups differed in fertilization efficiency (71% vs. 85%, $P = 0.03$).

[†]Normality was assessed by the criterion of the dorso-anterior index, a measure of the extent of dorso-anterior differentiation (10). Values in this index range from 0 (no dorsal structures) to 10 (radial hyperdorsal development), with a score of 5 being normal. Contingency table analysis showed no statistically significant difference between the two groups ($n = 209$, $\chi^2 = 1.26$, $P = 0.26$).

looked normal. Moreover, virtually all tadpoles in both groups appeared normal externally (Table 1).

There appears to be no difference in the rate of development between the two groups. The temperature difference between the $1 \times g$ centrifuge and the microgravity compartment was maintained within $\pm 0.25^\circ\text{C}$, and both groups were at the same stages when fixed inflight (stages 2, 10 $\frac{1}{4}$, 20, 45) or immediately postflight (stage 46) or when the 156 tadpoles that hatched in space subsequently metamorphosed.

During the flight, astronauts made video recordings of swimming tadpoles. These recordings and additional recordings made within 4.5 hr of landing show essentially normal swimming behavior. In particular, there was almost no looping. Prior studies have documented a stereotypic looping behavior

(forward somersaults) by *Xenopus* larvae that were exposed to microgravity on parabolic flights or during previous spaceflights (where the tadpoles developed at microgravity from the blastula stage onward) or that were raised on a centrifuge and observed at $1 \times g$ (11–14). The discrepancy between our results and those reported previously may reflect differences in the developmental stages of the tadpoles observed and their general physical condition in the various studies.

The postflight behavior of the tadpoles raised in microgravity differed from those raised on the $1 \times g$ centrifuge in a number of ways. Tadpoles reared at microgravity swam at a lower position in the water column than did the $1 \times g$ controls. This positioning is consistent with a difference in lung volume. Serial sections of the lungs from tadpoles fixed shortly before landing (stage 45; Fig. 2) reveal significantly smaller lungs in the microgravity tadpoles than in the $1 \times g$ tadpoles ($n = 40$, $P = 0.023$, Mann-Whitney U test). Tadpoles in both groups were observed taking breaths of air during the first day after landing, and differences in swimming posture for the two groups diminished in the following days.

The microgravity tadpoles tended to show a slightly stronger optomotor response than the centrifuge controls during the first testing period, 4.5–9.5 hr postflight ($n = 70$, $P = 0.06$, multiple ANOVA). During the first 2 hr of testing on that day, the average optomotor response improved in both groups ($r^2 = 0.38$, $P = 0.007$ for the microgravity group; $r^2 = 0.44$, $P = 0.03$ for the centrifuge group; regression analysis). The optomotor responses of both groups differed significantly on the second day of testing ($n = 71$, $P = 0.04$, multiple ANOVA), with the microgravity group again showing the stronger response. However, the response intensity for both groups did not change significantly during the testing. During the third testing period (9 days postflight), the two groups showed an even greater tendency to follow the stimulus cylinder, although they no longer differed in their mean response. No biases for clockwise or counterclockwise rotation were found in either group ($P > 0.1$, repeated measures ANOVA).

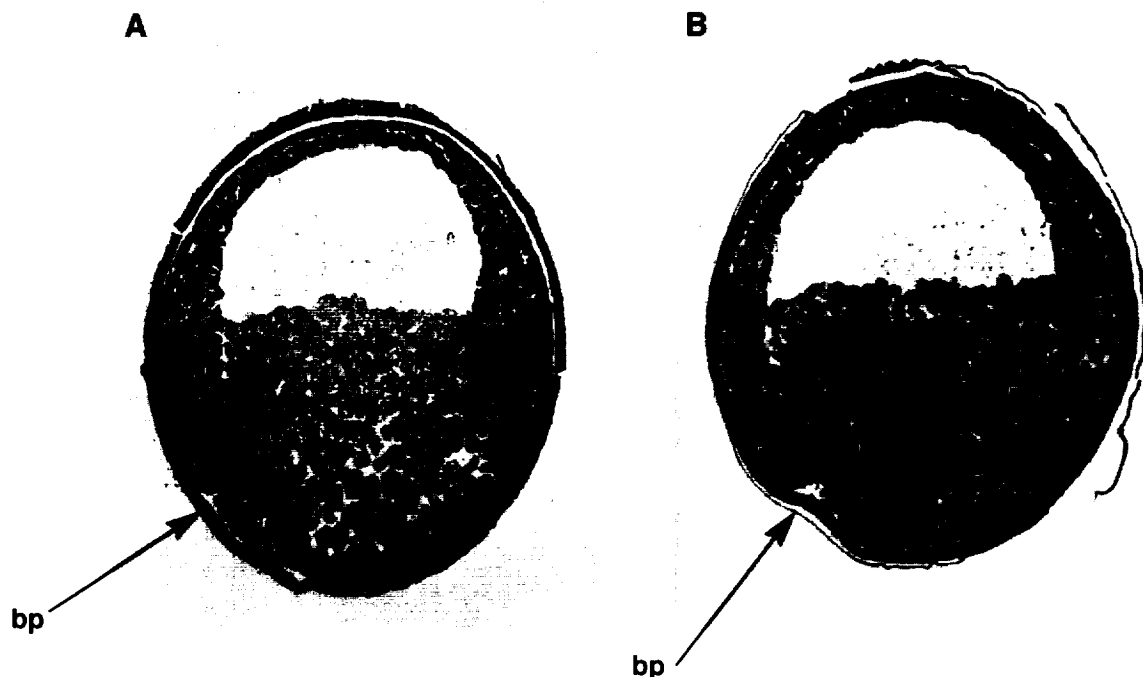


FIG. 1. Sagittal sections of gastrulae (stage 10 $\frac{1}{4}$) showing differences between embryos developed on the $1 \times g$ centrifuge (A) and those developed at microgravity (B). The microgravity sample generally showed thicker blastocoel roofs comprising more cell layers than in the $1 \times g$ controls; and the dorsal lip of the blastopore (bp) formed nearer the vegetal pole than in the $1 \times g$ controls. Embryos were fixed inflight and stained with hematoxylin, eosin-B, phloxine-B, and fast green.

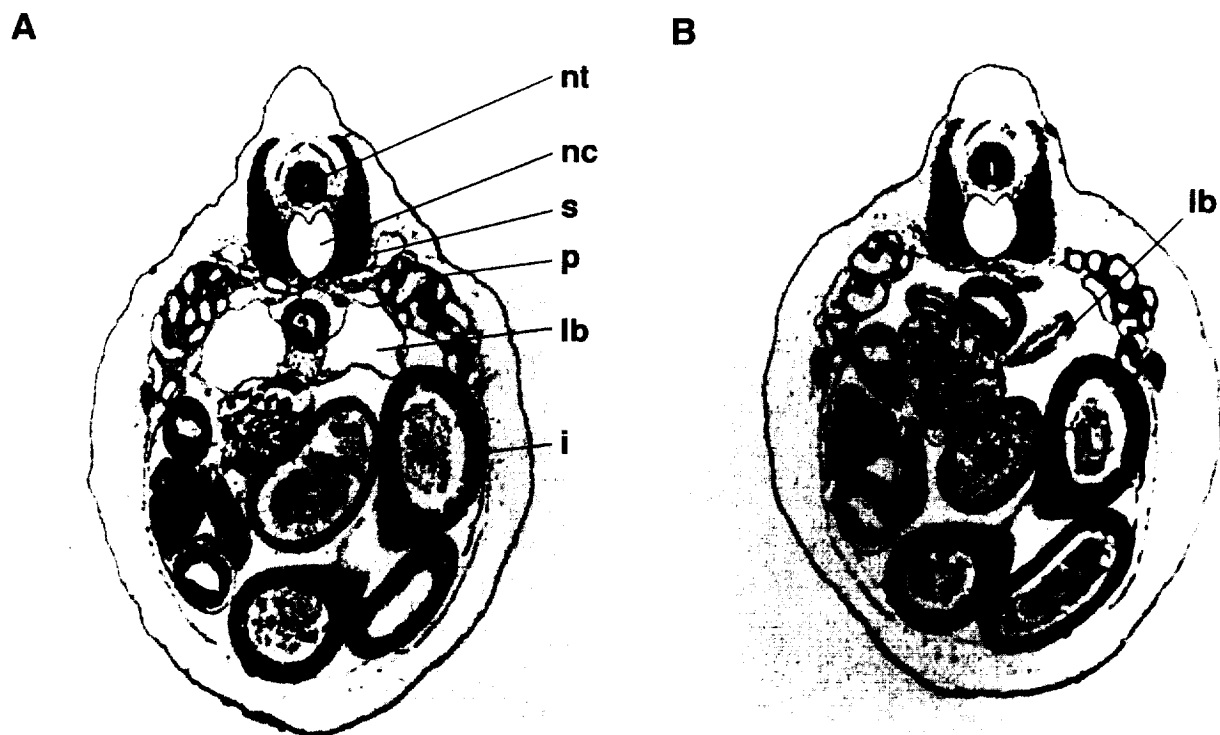


FIG. 2. The lung buds and tracheae of tadpoles developed in microgravity were generally not inflated. Transverse sections of tadpoles fixed in flight at stage 45 showed inflated lung buds in the $1 \times g$ group (A) and uninflated lung buds in the microgravity group (B). Tadpoles were fixed in flight and stained with nuclear fast red, aniline blue, and orange G. Anatomical features noted are neural tube (nt), notochord (nc), somite (s), pronephros (p), lung bud (lb), and intestine (i). Six loops of the intestine are visible in this section.

The tadpoles returned from spaceflight metamorphosed and matured normally. Reproductive function was unimpaired, and no abnormalities were found in the Earth-born F_1 tadpoles. The four adult frogs returned from flight in excellent condition. They were spawned at 2-month intervals during the 6 months after flight and continued to yield high-quality eggs.

DISCUSSION

The availability of astronauts to conduct experiments in flight allowed us to design our study and the supporting equipment so that we could test whether gravity is required for the formation of the embryonic axis in amphibian embryos. Earlier experiments either launched embryos fertilized on the ground after the most gravity-sensitive window had passed (15) or were limited by the constraints of small automated experiment containers which did not allow development to proceed beyond the gastrula stage (7). The $1 \times g$ centrifuge in the FEU allowed us to expose embryos from the same spawnings to both microgravity and $1 \times g$ conditions.

Fertilization rates were high at both microgravity (88%) and at $1 \times g$ (73%). We do not consider the differences in percent fertilization to be biologically significant (Table 1). By the two-cell stage the microgravity group showed a slight displacement of the mitotic asters toward the vegetal pole (data not shown). If this more vegetal position was maintained through the third cleavage, the third cleavage plane would occur at a lower latitude than normal (the third cleavage plane is the first horizontal cleavage plane). Since the blastocoel forms at the intersection of the first three cleavage furrows (16), a lower third cleavage plane would presumably cause the blastocoel to form at a position lower than normal. This would result in a thicker blastocoel roof, as seen in gastrulae developed at microgravity, both in our study and in that of Ubbels *et al.* (7).

A similar relationship has been documented for *Xenopus* eggs subjected to clinostat rotation (17). It is also possible that the thickened blastocoel roof results from a failure of its cells to undergo radial interdigitation at the normal time. Radial interdigitation is the mechanism driving epiboly and thinning of the blastocoel roof (18). Whatever the explanation, the embryos were able to repair this abnormality, as no significant differences in morphology were observed at the neurula stage.

The tadpoles returned from space were virtually normal in behavior and morphology, with the notable exception of position in the water column and lung size. *Xenopus* tadpoles on Earth normally come up to the surface to fill their lungs within 2–3 days of hatching. But in space, there are no gravitational clues to direct tadpoles to the air/water interface, and it is likely that the tadpoles generally did not fill their lungs. Tadpoles were observed to swim to the surface after the flight, and presumably inflated their lungs, as the difference in swimming position disappeared after a few days. Behavioral differences observed between the two groups during the immediate postflight period disappeared within 9 days. However, our tadpoles were only 2–3 days posthatching at the time of landing. Ground-based experiments have shown that *Xenopus* tadpoles deprived access to air for a period of ≈ 12 days immediately after hatching have great difficulty inflating their lungs and ultimately develop lungs half the size of controls (19). Thus, although our experimental tadpoles were functionally indistinguishable from controls 9 days after landing, we cannot conclude that they would have been normal had they spent a longer portion of their posthatching larval life in microgravity.

Tadpoles raised in microgravity showed a stronger optomotor response than their $1 \times g$ siblings or ground controls for 2 days after landing. This difference disappeared within a week, as does the accentuated optomotor response reported by

astronauts (20). Our results suggest that tadpoles raised in microgravity either receive less, or rely less on, vestibular information to assess their position in the optomotor apparatus. With visual information predominating, the microgravity tadpoles showed a stronger tendency to follow a visually moving target than did controls.

Our study demonstrates that gravity is not necessary for early amphibian embryogenesis. More-extended experiments in microgravity will be necessary to determine how microgravity affects other stages in the growth and reproduction of these and other vertebrates.

We wish to thank the many people associated with bringing this flight experiment to fruition, especially the Spacelab-J crew for their superb execution of the inflight experiment. We thank Dr. Richard Young for his pioneering work in Space Biology and for his long-standing support and encouragement. We acknowledge the assistance of Scott Pronych in the design, execution, and analysis of the optomotor portion of the experiment, as well as the support of Sally Ball, Sam Black, Kay Larkin, Mel Mack, and Michael Wu throughout this study. This work was supported by the National Aeronautics and Space Administration (K.A.S. and S.D.B.) and the Canadian Space Agency and the Natural Sciences and Engineering Research Council of Canada (R.J.W.).

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