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Development and Swimming Behavior of Medaka Fry in a Spaceflight aboard the Space Shuttle Columbia (STS-107)

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ABSTRACT—A space experiment aimed at closely observing the development and swimming activity of medaka fry under microgravity was carried out as a part of the S*T*A*R*S Program, a space shuttle mission, in STS-107 in January 2003. Four eggs laid on earth in an artificially controlled environment were put in a container with a functionally closed ecological system and launched on the Space Shuttle Columbia. Each egg was held in place by a strip of Velcro in the container to be individually monitored by close-up CCD cameras. In the control experiment, four eggs prepared using the same experimental set-up remained on the ground. There was no appreciable difference in the time course of development between space- and ground-based embryos. In the ground experiment, embryos were observed to rotate in place enclosed with the egg membrane, whereas those in the flight unit did not rotate. One of the four eggs hatched on the 8th day after being launched into space. All four eggs hatched in the ground unit. The fry hatched in space was mostly motionless, but with occasional control of its posture with respect to references in the experimental chamber. The fry hatched on ground were observed to move actively, controlling their posture with respect to the gravity vector. These findings suggest that the absence of gravity affects the initiation process of motility of embryos and hatched fry.

Key words: medaka, space experiment, microgravity, development, swimming behavior

INTRODUCTION

Animals on earth have evolved in an environment that has developed on a specific planet in the universe. Among environmental factors, gravity is unique in that it has never undergone global change. Therefore, evolution on earth would have inevitably included the process of adaptation to a 1×g environment.

Recently, space utilization has become possible for the scientific investigation of gravitational biology. In the 2nd International Microgravity Laboratory (IML-2) mission in 1994, Ijiri (1994; 1995a) confirmed that medaka laid eggs which hatched safely and the hatched fry grew to produce many offspring after the mission. The IML-2 Medaka Experiment also encompassed mating behavior followed by the laying of eggs. This was the first observation of the reproductive behavior of a vertebrate under microgravity, and showed that reproduction is possible in space (Ijiri, 1995a; 1998). Scientists are aiming to complete the life cycle of higher animals in space. In order to precisely observe the development and motile activity of medaka fry under microgravity, an experiment was carried out in a space shuttle mission, STS-107, in January 2003.

In the IML-2 Medaka Experiment, pairs of adult fish were reared in an aquarium with a continuous circulation flow for the oxygenation and filtration of water, which is required if adult fish are to be kept in a small experimental setup. Eggs were spawned and carried away by the water flow to a nesting space separated from the adults, which otherwise would eat them (Ijiri, 1995a). This situation made it difficult to observe the development of individual embryos. In addition, the water flow in the aquarium might have had some mechanical effects, which could counteract the loss of gravitational forces. Since the effects of gravity are size-dependent and subtle for smaller organisms (Albrecht-Bue-

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hler, 1991), the effects of microgravity on small organisms such as medaka eggs and fry should be investigated under conditions in which mechanical agitation is suppressed as much as possible.

In the present experiment, we used a closed ecological life support system. The system, the Autonomous Biological System (ABS; Paragon Space Development Corporation, AZ, USA), had been previously tested in US space shuttle and Russian Mir experiments. It has no circulation mechanisms so one could expect to observe the development and motile activity of medaka fry under microgravitational conditions without additional mechanical disturbances. In this mechanically 'quiet' environment, we placed four eggs, which had been fertilized before launch, and observed closely the development of the medaka embryo from each individual egg. We also compared the motile activities of embryos and hatched fry between space- and ground-based experiments.

The experiment was a part of the S*T*A*R*S (Space Technology and Research for Students, http://www.spacehab.com/stars/index.htm) Program, a program promoted by JUSTSAP (Japan-US Science, Technology & Space Application Program). In the S*T*A*R*S experiments, omnibus users shared the experimental facilities administered by a commercial agency (SPACEHAB Inc, TX, USA). In spite of highly limited resources and the loss of an opportunity to recover space hatched fry with the Shuttle Columbia tragedy, observations in our experiment may play important roles in making future space experiments, which aim at animal reproduction outside of the earth. Preliminary results were already reported in a short paper (Niihori *et al.,* 2003).

MATERIALS AND METHODS

Preparation of eggs on the ground

Medaka (*Oryzias latipes,* strain d-rR) were reared at the Paragon Space Development Corporation. They were artificially induced to mate under a 16L/8D cycle at 25°C. Eggs were collected directly from the female's abdomen, and maintained in the standard embryo rearing salt solution for freshwater teleosts (Carolina Biological Supply Company, NC, USA) with an added mold inhibitor. Eggs were transported to the launch site (Astrotech Facility, FL, USA) two days before launch.

Life support system

A closed ecological system was prepared based on the Autonomous Biological System (ABS) technology, which has been previously tested in US Space Shuttle and Russian Mir experiments by Paragon Space Development Corporation (Poynter *et al.,* 2001; Ishikawa *et al.,* 1996, 1998a, b). The ecological system was prepared in an AHAB (Aquatic animal Habitat) (Poynter *et al.,* 2001; Goulart *et al.,* 2002), a sealed transparent container of 111×76×198 mm (Fig. 1A to C), which contained artificial pond water, detritus, a water plant (hornwort, *Ceratophyllum demersum*), snails (*Physa sp.*), and daphnia (*Daphnia pulex*). After the establishment of equilibrium in the ecological system kept for five days, paramecia and rotifers were added to the system as food sources for hatched fry, together with wheat kernels and coexisting bacterial flora as nutrients for the microbes (Pendergrass, 1980), and sealed with medaka eggs being held in place two days before launch.

Throughout the experiment, the development of embryos and the swimming behavior of hatched fry were observed by watching downlinked video recordings. These embryos and fry were enclosed in a "Nest", which retained them in a place suitable for close observation (Fig. 1C to E). The Nest is made of polyethylene mesh with appropriate pores (526 μm in diameter) for observation and the diffusive exchange of materials, sandwiched in between two layered polystyrene disposable weighing dishes, which are in turn attached to the lid of the AHAB (Fig. 1E). The Nest also functions to hold eggs and hatched fry in place for close-up camera recordings as well as to keep eggs away from the snails, which might otherwise eat them. The mesh walls of the Nest enable the exchange of water and microbes freely between the Nest space and the other part of the AHAB.

The Nest space was separated into two areas: Egg and Fry areas. (Fig. 1D, E) In the Egg area (35×35 mm), two strips of Velcro tape are attached to the substratum to hold eggs between their fine plastic projections (Figs. 1F, G). The Fry area (70×70 mm) is used for observing the swimming behavior of the hatched fry. These two areas were connected by a narrow gap (ca. 2.5 mm) between the lid of the AHAB and the Nest (Fig. 1E). Once hatched, the fry were expected to swim into the Fry area through the gap. The Fry area had three meshed bags containing wheat kernels (Fig. 1D) for supplying organic matter to microbes.

The AHAB was completely sealed with 34 screws and installed in the ICM (Isothermal Control Module; BioServe Space Technologies, CO, USA) (Poynter *et al.,* 2001; Homhen *et al.,* 1999; Woodard *et al.,* 2003) (Fig. 1H). The ICM controlled the temperature of the AHAB (23.3–27°C) with a L/D cycle of 16L/8D via fluorescent lamps (10 W×2 at both sides of the AHAB).

Space experiment

Eggs from the Paragon Space Development Corporation were cleaned, and healthy eggs were selected for use under a microscope. Two days before launch, four eggs, which had been randomly selected from the healthy ones, were stuck on the Velcro strips in the Egg area of the Nest, and the whole was put in the AHAB with the ecological system being well equilibrated. Two of the eggs were at the DS3 stage (3 Days after Spawning) and the others at DS1. The developmental stage was determined according to Iwamatsu (1994).

The AHAB was equipped with two on-board video cameras for recording the eggs and fry during the experiment (Goulart *et al.,* 2002). Axes of the recording cameras were set at right angles to the lid of the AHAB (Fig. 1E). One camera was used for a close-up recording of the development of the eggs in the Egg area and the other for recording the behavior of the hatched fry in the Fry area. The Egg area was observed through an 8 mm focal length lens, which gives a field of view of 29×38 mm (3:4 aspect ratio). For the Fry area, a 3.8 mm lens was used, covering a field of view of 69×92 mm. Still images were taken once every hour, while movies were recorded about four to eight minutes every day. Movie capturing was managed following NASA's operation system. Since the timing of the capturing and downlinking of the image data depended on the schedule of the whole mission, it was not exactly fixed at the same time of the day. Recordings were made only during the light phase of the L/D cycle.

Two identical experimental units, flight and ground units, were prepared using the same procedures, one of which was flown in the SPACEHAB module (SPACEHAB Inc, TX, USA) in the Space Shuttle Columbia.

Ground control

The control experiment on the ground was conducted using the same procedure as the space experiment. The AHAB was set vertically in the ICM, so that the axes of recording cameras were set horizontally. During the mission, the ICM for the ground experiment

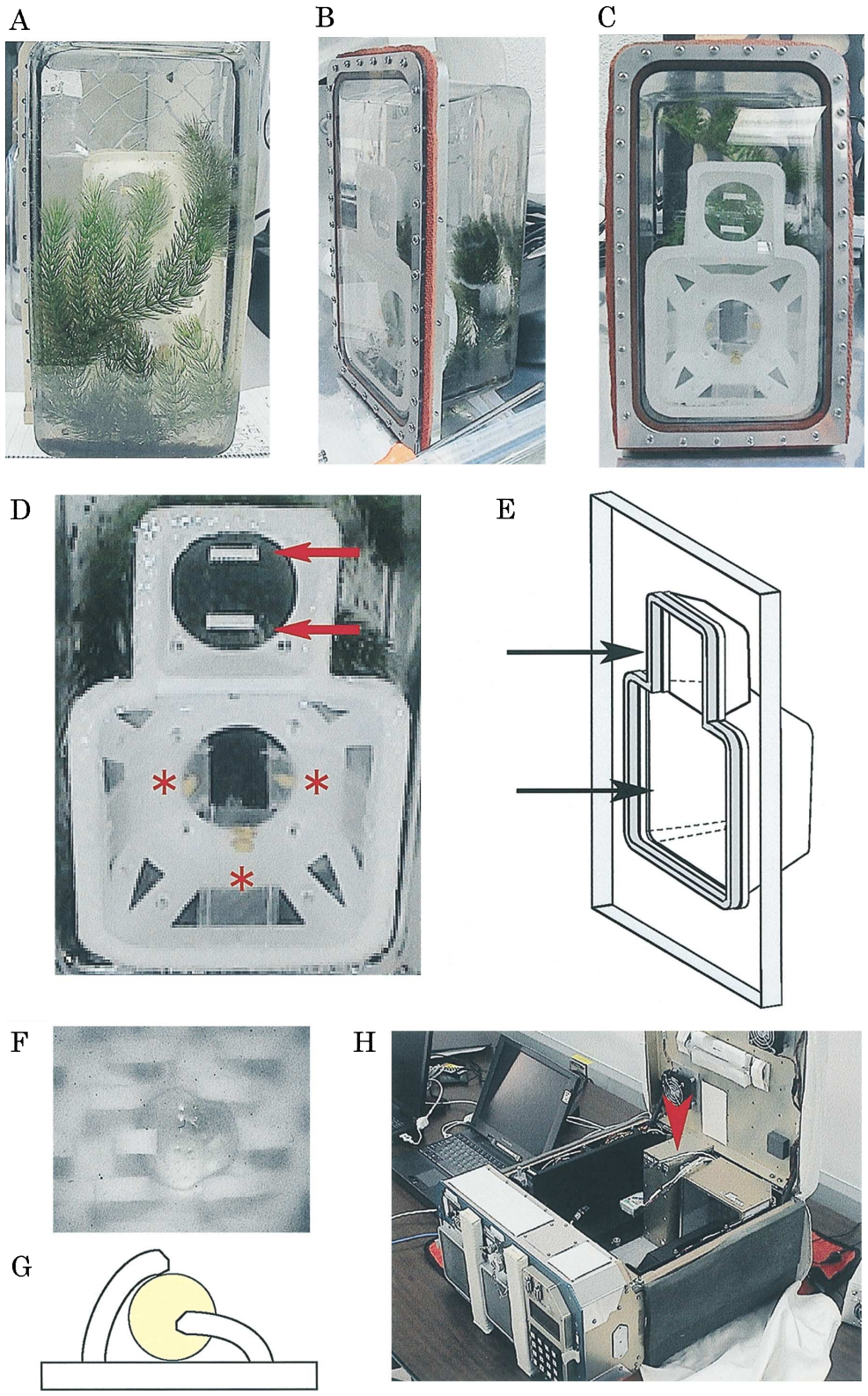

Fig. 1. Closed ecological system in the AHAB. A: back view showing a water plant (hornwort) for oxygen production. B: side view. C: front view showing the Nest for separating the nesting spaces in the AHAB. D shows the Nest having the Egg area (upper smaller area) and the Fry area (lower larger one). In the Egg area two strips of Velcro tape (arrows) were stuck on the mesh wall. Asterisks in the Fry area in D indicate wheat kernels in the mesh bags. E shows a schematic drawing of the Nest attached to the lid of the AHAB. A 2.5 mm gap is located between the Egg and Fry areas. Arrows in E represent the directions of viewing by the recording cameras. F shows an egg fixed between plastic projections on the Velcro tape (as shown in a schematic drawing in G). H shows the ICM in which the AHAB was installed in a housing box indicated by an arrowhead.

was maintained in the SPACEHAB Payload Facility (FL, USA).

Analysis of video images

In order to estimate the swimming activity of the fry, downloaded movies were analyzed by means of a laboratory made autotracking software, Bohboh (Shiba *et al.,* 2002). The details of the software will be published elsewhere. Briefly, the recorded videos were analyzed frame by frame, and the center of mass of the fry image was determined every frame and both the mean velocity and direction of swimming were calculated at adequate time intervals.

RESULTS

The Space Shuttle Columbia was launched at 10:39 on January 16, 2003 (EST). The research mission period was sixteen days. The days of the flight mission are shown, in this paper, with reference to launch (L±0): L-n and L+n mean n days before and after launch, respectively.

For the flight unit, still images were taken from L-1 to L+15, and downlinked at the rate of 7 to 32 frames per day. We obtained 444 images but missed receiving a small number of images sent to ground during the mission. For the ground unit, still images were taken according to the same protocol and a total of 480 images were obtained safely as well as two post-mission images. The total length of time of video recordings from L±0 to L+14 was 157 and 154 min-

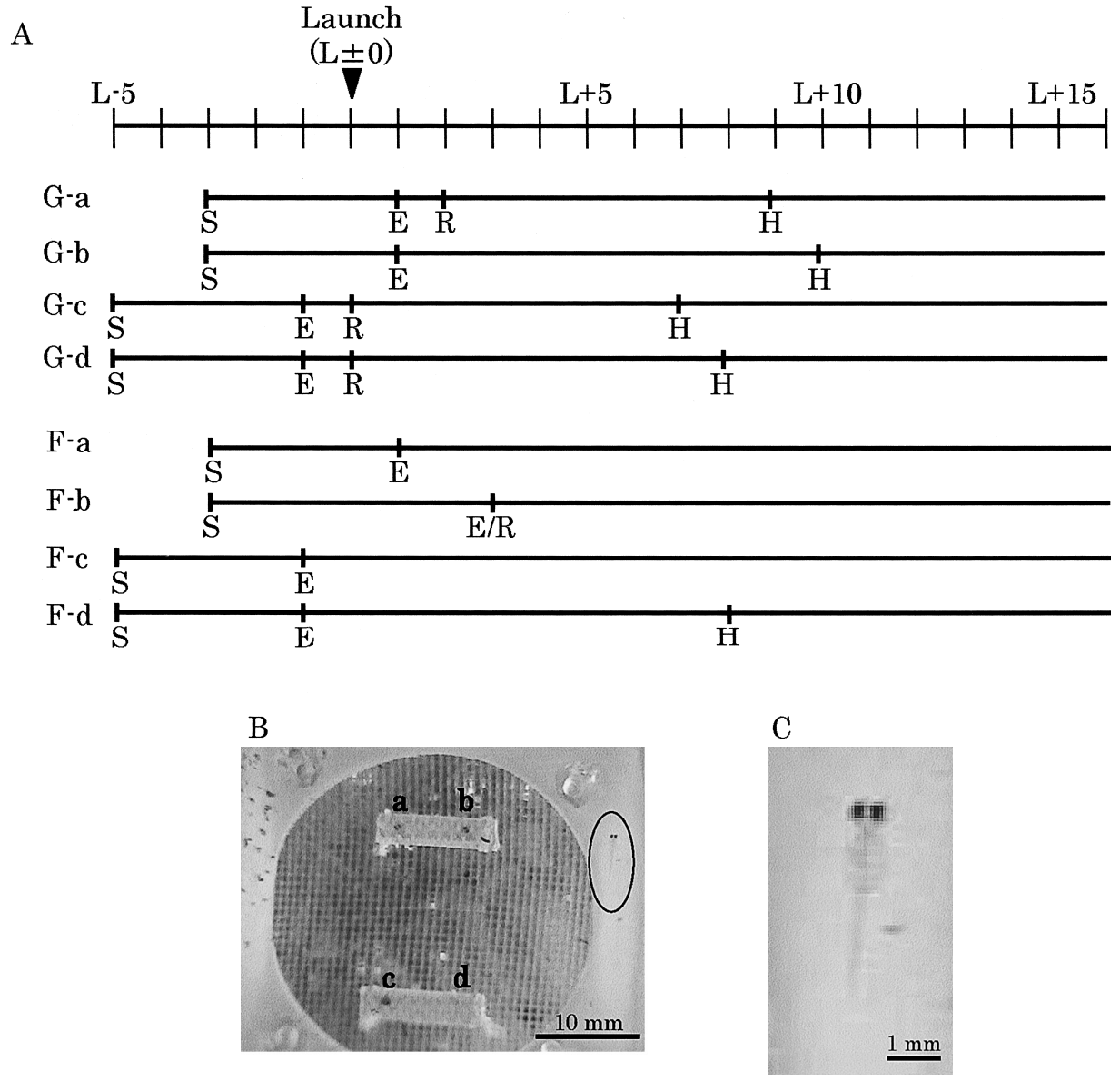

Fig. 2. Summary of the observed events of development. In A, remarkable events of development (S, spawning; E, full development of eyes; R, initiation of rotation beneath the egg membrane; H, hatching) are shown in days with reference to launch (L±0). The AHAB was closed on L-2. Each egg was discriminated by the position of setting in the Egg area and designated by the character a to d (B). Flight and ground eggs are labeled with F- and G- in front of each distinguishing character. B shows a space-hatched fry (corresponding to egg F-d). The appearance of eyes as parallel bars in the enlarged image (C) shows that the fry had its back (or abdomen) to the camera.

utes in the flight and ground unit, respectively.

Development of medaka embryos under microgravity in space

In this experiment, eggs were fixed in the field of view of CCD cameras so that the development of each individual embryo could be observed. Fig. 2 summarizes the time course of the development of the flight and ground specimens. Individual specimens were designated as F-a to F-d for flight and G-a to G-d for ground. In spite of the rather limited resolution of the video recordings, we could clarify three distinct events of development: development of the eyes, rotational movement beneath the egg membrane, and hatching.

The development of the eyes is a good marker of embryogenesis, and developmental stages can be defined based on the black granule speckle on the optic cup. In the ground control, full development of the eyes was confirmed not later than DS4 (Fig. 2A). In the flight experiment, the completion of eye development was also observed at the same stage for three of four specimens, although in the remaining embryo it seemed to take up to 2 days longer (F-b in Fig. 2A). The experimental procedure mentioned above, in which recordings were made only in the light phase of the L/D cycle, may explain a part of the delay, one day at maximum. There is also a possibility that the eyes were positioned in a blind spot and could not be observed until the embryo began to rotate. In fact, the eyes were confirmed to

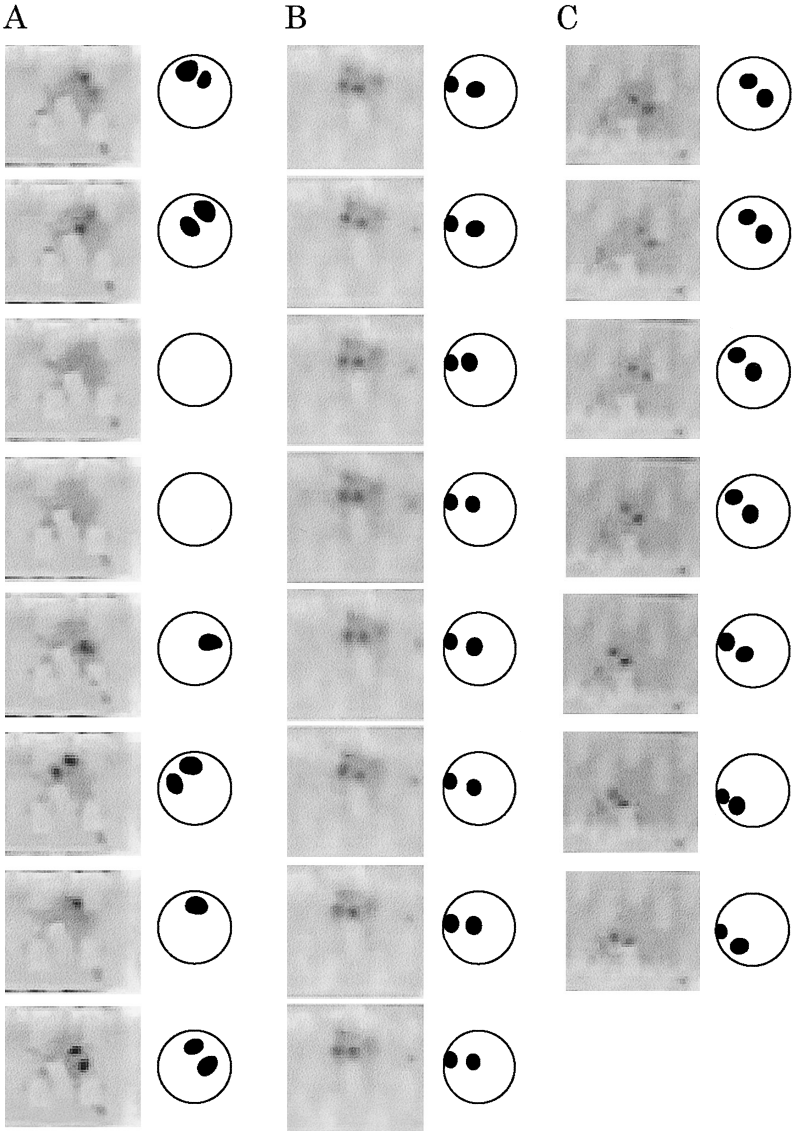

Fig. 3. Movement of embryos beneath the egg membrane. Sequential photographs of embryos in the ground (A) and flight (B) experiments taken once every hour on L+2. These embryos were photographed on DS7 and from the eggs G-d in A and F-d in B, respectively. C shows sequential photographs of another embryo in the flight experiment taken once every hour on L+4. This embryo was photographed on DS7 and from the egg F-b. Schematic drawings of the photographs are shown in which circles indicate the egg membrane and black dots the eyes of the embryo.

have developed at the beginning of the rotation in F-b. It is therefore likely that the time course of embryogenesis was almost the same in both the flight and ground-based embryos.

There was a remarkable difference in the amount of activity beneath the egg membrane. In the ground control, three embryos began to rotate within a day after the full development of the eyes (Fig. 2A). The rotation was obvious from a change in the position of the eyes beneath the egg membrane as shown in Fig. 3A for one of the three, but was hardly discernible in the others (not shown). On the other hand, in the flight experiment, embryos did not show any remarkable rotational movement (Figs. 2A and 3B). Only one embryo showed slower and less frequent movement than the ground controls (Fig. 3C). This specimen turned about one quarter of a rotation in several hours whereas the ground controls turned a full rotation within the same period.

In the ground unit, all embryos hatched between DS12 and 13 and two of them were recovered alive after the experiment (L+18). In the flight unit, however, only one egg hatched on DS13. Hatching was not confirmed with the other three eggs at least until L+15.

Swimming behavior of space-hatched fry

The fry that hatched in space (Fig. 2B) was found in the video image recorded on L+8. The fry stayed in the Egg area for a while but swam away in the next video recording. However, it could not be found in the Fry area. This was partly because of the reduced contrast of the image due to the turbid water in the AHAB with small particles free from sedimentation, which were derived from the organisms in the ecological system. Analysis of the swimming behavior was therefore done inevitably using the recordings taken on L+8 of this fry swimming before leaving the Egg area. The fry could be seen in all three shots of a movie (84 seconds in total) taken in a twenty-minute observation time window open according to the recording schedule. The movie must have been taken more than 10 hours after the time of hatching since 10 hours had elapsed after the first still image of that fry was taken. The fry in the AHAB on the ground swam actively (Fig. 4A). They moved to the Fry area immediately after hatching. The fry were observed to swim freely in all directions. They were found frequently in front or side view, when swimming with the dorsoventral axis parallel to the gravity vector, and sometimes in dorsal or ventral view when

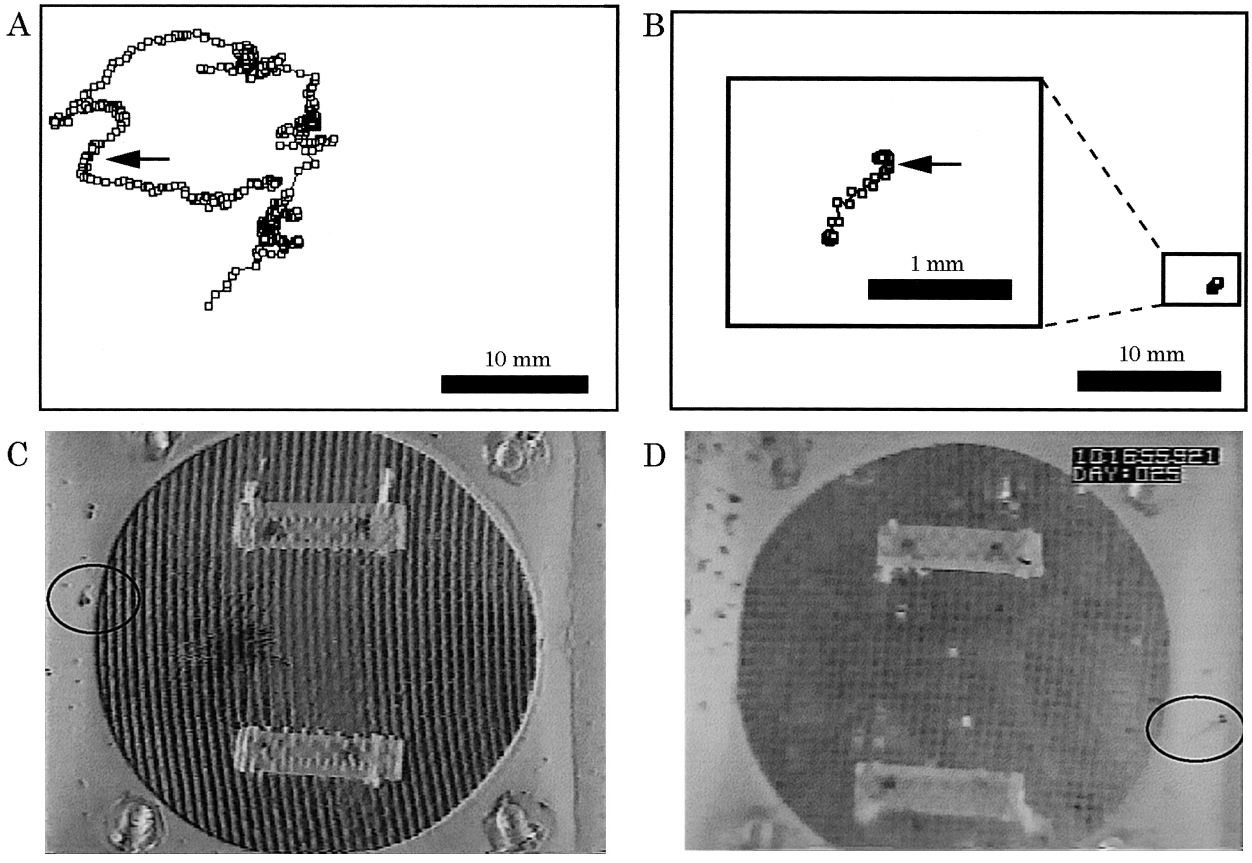

Fig. 4. Swimming behavior of the fry that hatched on the ground (A and C) and in space (B and D). A and B: swimming trajectories for 27 seconds of the fry that hatched on ground (G-c on L+8, <24 hrs after hatching) and in space (F-d on L+9, >10 hrs after hatching), respectively. The inset in B shows an enlarged trajectory in a rectangle. C and D: frames of the video images of swimming fry on ground and in space, respectively. The positions of the fry in C and D correspond to those indicated by arrows in the trajectories in A and B. The mean velocities calculated on the basis of the trajectories shown here were 3.16 and 0.06 mm/sec for the ground and space-hatched fry, respectively. The maximum velocities were 16.7 and 1.6 mm/sec, respectively.

swimming up and down the chamber. On the other hand, the space-hatched fry was mostly motionless, although it slowly changed its position in the Nest (Fig. 4B). The ground-hatched fry oscillated their tails actively, while the space-hatched fry moved its tail in a twitch-like manner only when changing its orientation. The total time in which the space-hatched fry was observed to swim with its tail actively undulating was 1.7 seconds in video recordings totaling 84 seconds, while the ground-hatched fry showed active undulation of the tail throughout the full length of the recordings. The mean velocity of the space-hatched fry calculated from all the recordings on L+8 was 0.05 mm/sec. The mean velocities of the ground control were 4.06, 3.94 and 4.30 mm/sec for fry on L+7, L+10 and L+13, respectively. The velocity was calculated based on the recording of 62 seconds with one fry on L+7, and of 250 seconds and 159 seconds each with four fry on L+10 and L+13, respectively.

These great differences in velocity may indicate that the swimming activity was much reduced in the space-hatched fry. Interestingly, the space-hatched fry was seen mostly in dorsal or ventral view in the recording frame fixed to the AHAB (cf. Fig. 2C). This may suggest active control of the body axis so as to fix it with respect to a reference in the Nest, even though the fry exhibited very little swimming locomotion.

Footage of the movement of the fry shown in Fig. 4 is available from supplementary materials of this electrical version.

DISCUSSION

In this experiment, an ecological system was used to maintain embryos in a closed environment and hatched fry obtained in the flight unit as well as on the ground. This indicates that the closed ecological system is effective as a life support system for small aquatic vertebrates. As mentioned earlier, two of the four fry that hatched in the ground unit were found alive even after a 78-hour delay in the opening of the unit due to the loss of the Space Shuttle Columbia. The death of the fry was not due to a shortage of food because, paramecia and daphnia was found inside the Nest when the AHAB was opened. The water in the flight unit appeared more turbid throughout the experiment than that in the ground unit, probably because it contained a lot of small particles floating under microgravity, which otherwise would have sedimented to the bottom. It would be a great help in future microgravity experiments to remove floating particles by adding filter feeders, such as fresh water sponges, to the ecological system.

We focused our attention on the development of the eyes for the assessment of early embryogenesis. Embryos on the ground as well as in space showed a similar time course in the development of the eyes. Two embryos (F-a and F-b) experienced microgravity before full development of the eyes. They formed eyes of almost the same DSs as the embryos in the ground unit (G-a to d), and also as the embryos that formed eyes before the launch in the flight unit (F-c and F-d). This may suggest that the experience of microgravity in the course of embryogenesis did not affect the time course of development if already initiated on earth.

The development of late stages was assessed by studying the movement of embryos beneath the egg membrane and the hatching ability. The embryo begins to rotate beneath the egg membrane as the neuromuscular system develops before hatching. In the ground unit, three out of four embryos began to rotate on DS5 (Fig. 3A). There were no detectable changes in the position of the eyes of the remaining embryo until hatching. However, no distinct rotational movement was observed in three embryos in the flight unit (Fig. 3B), although slower and less frequent rotation was observed in the remaining one (Fig. 3C). This rotational movement was observed until the end of the recording schedule. The motionless embryos appeared yellowish and nearly transparent so that they were thought to be alive because dead embryos can be distinguished from live ones by their milky opaque appearance. In fact, one of the motionless embryos (F-d) hatched afterwards. These results suggest that the motile activity of the embryos was reduced in the environment of the flight unit, probably by microgravity.

Differences between ground and flight specimens at the late developmental stages were also found in the hatching ability. In the ground unit, all four fry hatched on DS12 to 13, whereas in the flight unit, only one hatched on DS13.

It is supposed that the hatching of medaka is controlled by the activity of a hatching enzyme as well as by the mechanical stress powered by the embryo itself. The release of the hatching enzyme is influenced by the autonomic nervous system stimulated by lighting and also by dissolved oxygen, which is determined in close relation to the water temperature. In this experiment, eggs were put under constant L/D cycles and at a controlled temperature (25.0±1.6°C in the flight unit and 25.3±2°C in the ground unit). It is therefore fair to relate the lower hatching ability in space not to the chemical factor but to a decreased mechanical stress added to the egg membrane, because such stress may help embryos to tear the egg membrane, which can be partly dissolved by the hatching enzyme (Iwamatsu, 1994, 1998). The lower motile activity may reduce the possibility of hatching in the flight unit. No similar reduction in hatching ability was found in the IML-2 MEDAKA Experiment, in which 30 eggs fertilized on the ground were flown with two pairs of adult fish. Ijiri (1995a) has reported that most of the ground-born eggs became hatched fry around five days after launch. In the IML-2 MEDAKA Experiment, an aquarium was used which was equipped with water circulation to maintain adult fish. It is likely that forces due to the water current around the egg help embryos to hatch more easily by breaking the egg membrane mechanically rather than chemically only in such an experiment, while such efforts of water current cannot be expected from an environment of low mechanical disturbance characteristic of

the closed ecological system used here.

We reported in the present paper that the motility of hatched fry as well as embryos beneath the egg membrane was much reduced in space. This reduction in motility of space-hatched fry was not reported in the IML-2 MEDAKA Experiment, where most of the fry hatched in space from ground-born eggs were observed to swim normally (Ijiri, 1995a, c [with movie data on the Web at http://cosmo.ric.u-tokyo.ac.jp/SPACEMEDAKA/IML2/e/table/P11_E.html]). The reduced motility might indicate defects in a motor function of the fry. However, the fry was observed occasionally to move with its tail bending sharply. In addition, the fry showed some active controls to fix its body axis with respect to a reference in the Nest. The fixed posture resulting in the persistent dorsal or ventral view of the fry in the recordings (cf. Fig. 2C and Movies) may be due to the fixed direction of illumination. The AHAB was illuminated laterally from both sides in the ICM, which might cause the fry to orient its dorsoventral axis halfway in between the two light sources due to its dorsal light response capability. These findings and explanations suggest that the motile activity of the space-hatched fry might not be suppressed in rolling but in locomotion although the latter could be initiated occasionally by some unknown environmental cues. Interestingly, it is found in the video recordings of the IML-2 MEDAKA experiment that a space-born and space-hatched fry was motionless when alone, but swam with its tail moving normally when disturbed mechanically by the water current created by nearby adult fish (Ijiri, 1995b). As shown in the movie at the above URL, the fry hatched from ground-born eggs in separate chambers of limited volume, so that the swimming motion of the fry was observed under much crowded conditions. It might be, therefore, inferred that the swimming motion observed among the ground-born and space-hatched fry happened to be induced through mechanical interactions between nearby fry. In the closed ecological system used in our experiment, in which the AHAB had no active water circulation, embryos and resultant hatched fry were maintained almost free from mechanical disturbances. In space, specimens were basically free from the effects of gravity. It is therefore inferred that the motile activities before as well as after hatching, that is, the rotation of embryos beneath the egg membrane and the locomotion of fry, were little initiated in such an extremely 'quiet' environment.

Gravity has been assumed to be a crucial stimulus to initiate development of the behavior of organisms by providing an absolute framework for their space recognition (Manadillo *et al.*, 2003). The reduced motility of space-hatched fry in this experiment might imply a lack of sufficient stimuli required for the development of behavior and hence may support this assumption. However, further evidence is needed before accepting this hypothesis on the role of gravity, which will require more investigations inevitably including microgravity experiments. The results obtained in this experiment suggest that the investigation of the effects of virtual weightlessness will need substantial efforts to reduce mechanical disturbances. A closed life support system as used here would provide a good mechanically-isolated environment for the research of gravitational biology.

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