Biological Sciences in Space, Vol.15 Suppl. (2001)

Japanese Red-Bellied Newts in Space - AstroNewt Experiment on Space Shuttle IML-2 and Space Flyer Unit

Masamichi Yamashita[1], Akemi Izumi-Kurotani[1], Mari Imamizo[2], Hajime Koike[3], Makoto Okuno[3], Carl J. Pfeiffer[4], Shinji Komazaki[5], Fumie Sasaki[6], Yoshinobu Ohira[7], Isamu Kashima[8], Sakae Kikuyama[9], Takeo Ohnishi[10], Yoshihiro Mogami[11], and Makoto Asashima[3]

1 Institute of Space and Astronautical Science; 2 Toho University; 3 University of Tokyo, 4 Virginia Polytechical Institute, State University; 5 Saitama Medical College; 6 Tsurumi University; 7 National Institute of Fitness and Sports; 8 Kanagawa Dental College; 9 Waseda University; 10 Nara Medical College; 11 Ochanomizu University

***Abstract*:** Biological effects of gravity was examined in embryonic development of Japanese red bellied newt. Two space newt missions were conducted in 1994 and 1995. The Second International Microgravity Laboratory was flown in 1994 as one of the SpaceLab missions. Space Flyer Unit, a Japanese space platform, was delivered to the earth orbit by the third launch of the H-II rocket and retrieved by Space Shuttle in 1996. Female newts were induced to lay eggs in orbit at these two space missions. Eggs were successfully obtained on both missions, and exposed to space environment from its early developmental stages. Morphology of the embryos was found not deviated from those developed on ground, as long as in the images taken in orbit or the examined specimen retrieved to ground. On the other hand, pathological changes were discovered in several organs of the adult newts that returned alive from their space flight.

Key words; newt, gravity, development

Introduction

Gravity has long been believed to have an inevitable role on the developmental process of amphibian. Early stages of development, especially before the first cleavage of a single egg cell to two cells, are sensitive to gravity. Since amphibian eggs have quite large mass and density difference along their vegetal and animal hemisphere, they have been considered as a good model to assess direct actions of gravity on the cellular processes. Amphibian embryos exposed to hypergravity for a short time during the early stage have the higher rate of mortality and anomaly such as formation of bicephalous embryos. In order to explore possible effects of microgravity on early embryogenesis, newt eggs were exposed to space environment from the step of their fertilization. Female newts keep spermatophore in their body cavity for a long period. When they wake up from hibernation in spring, they lay eggs which are fertilized by the stored sperm. Taking advantage of this characteristic in Japanese newt, natural spawning of fertilized eggs was induced without having male newts on orbit. Morphology of newt embryos and time course of their development were analyzed by close up video images taken in orbit. The experiment with a code name of AstroNewt was originally proposed for Space Flyer Unit (SFU). It turned out to be the first launch of animal from the Japanese territory. AstroNewt on the Second International Microgravity Laboratory, IML-2, expanded the opportunity to survey biological effects of space on adult newts.

Address for correspondence: Masamichi Yamashita
Institute of Space and Astronautical Science
Yoshinodai, Sagamihara, Kanagawa, 229-8510, Japan
Tel.: +81-42-759-8230
Fax: +81-42-759-8449
E-mail: yamashita@surc.isas.ac.jp

Materials and Method

Newt: Female of the Japanese red-bellied newt, *Cynops pyrrhogaster*, takes spermatophore inside cloaca when she mates with male. She keeps it during hibernation. Eggs are laid when temperature and light/dark ratio tells start of spring. Female newt does not require further mating with male to fertilize eggs at the time of spawning. Life support of hibernating newt is feasible without feeding for a long period. Female newts were collected from water creek near rice paddies in late autumn or winter after their copulation. They were bred at 5℃ under dark in laboratory until the space experiments.

Fig. 1 Abdominal view of Japanese red bellied newt, *Cynops pyrrhogaster*

Fig. 2 Experimental operation on IML-2

IML-2: Four female newts were induced to lay eggs in the cassettes of Aquatic Animal Experiment Unit, AAEU. One of three newt cassettes in Aquarium Package, A-3, housed two newts, which were treated before launch with a suprathreshold dosage of hormone for egg laying. Newts in the other two cassettes, A-1 and A-2, were given about a half of the dosage. Additional injection of hormone was planned to be conducted in orbit, in case no egg was laid. These four newts shared a single water circulation loop. Aquarium Package was equipped with biological filtration, gas exchange, and temperature control function for its circulated water.

Egg spawning and survival ratio of newt has a strong dependence on temperature. Also, oxygen consumption increases at the initial phase of ovulation. Several measures were taken to reduce the risk for newts that were submerged under water at temperature relatively high for keeping them. Slow release of hCG (human chorionic gonadtrophins) from the hormone-containing capsule (Minipellet) was verified effective to suppress acute increase of hormone in their body and to prolong period of egg spawning. Use of Minipellet for controlled release of hormone was essential for this experiment to meet the requirements for possible launch slip. Newts flown to space were selected through several tests, and warmed up for a week with adopting acclimation process before the space flight.

In orbit, close-up video images of egg and embryo were taken by crew. Ground control experiment was conducted at Kennedy Space Center using Bread Board Model, BBM, of AAEU. Configuration of this BBM was almost same as Flight Model, except several points such as use of an external water circulation pump and a water sampling port open to room air, All of the procedures executed in orbit was applied to the ground control with a delay of four hours.

Soon after the recovery of sample to ground, the adult newts both of flight and ground control were pictured by a X-ray imaging system, and dissected to examine biological effects of space flight. Specimens fixed or frozen for the post flight analysis were; brain, eye, lower jaw, vertebral column, heart, lung, liver, stomach, intestine, ovary, abdominal muscle, blood, skin, limb, and tail. The two newts recovered in frozen state from orbit were not subjected to this histological survey.

SFU: The hibernation state of two female newts was maintained until the start of experiment in orbit. Female newts were treated with the sustained release pellet of hormone just before the loading onto SFU at three weeks prior to the launch. The period from this loading to the induction of egg laying was a constraint of the launch site operation of SFU. Spawning and fertilization of egg in orbit were induced by raising temperature in a quite natural way. Close up image of the newt vessel was taken by a CCD camera and down-linked to ground. Observation was terminated after 12 days from its initiation in orbit. Specimens recovered to ground were examined to survey the effects of space environment on morphology of embryo.

SFU Experimental System: Two female newts were kept in two rectangular vessels (30 x 27 x 140 mm). Circulated water was filtered by active charcoal, zeolite and porous glass beads to hold filtrate bacteria. Air was exchanged at an artificial lung made of porous polypropylene hollow-fiber. Temperature of circulating water was regulated by a Peltier device. Temperature and dissolved oxygen were monitored by a sensor unit in order to control the Peltier device and an air pump that fed air into the artificial lung. Mixed pellets of potassium peroxide and calcium peroxide contained in a stony matrix were placed in the air feeding line. These chemicals generated oxygen and absorbed carbon dioxide. Fixative solution was isolated in its reservoir at the water circulation loop by electric valves. Those valves were opened at the termination of experiment in orbit.

In order to keep temperature low for hibernation at launch site, heat dissipation was enhanced by forced feeding of ambient air to the external side of the Peltier device. Newt Experiment Unit, NEU was mounted on the main panel of Special Payload Unit of SFU as shown in Fig. 2. Experiment computer and power unit were installed on the side panel. Continuous supply of electric power was essential to keep biological specimen all through the phase. Electrical contacts to the newt system on ground was made through an umbilical connector at the fairing of H-II rocket (Fig. 3).

SFU Flight Operation: When SFU was projected into its orbit by the H-II rocket, electric power was turned on to activate the life support. Total length of power outage was 22 minutes during this launch phase. Temperature of circulating water for newts was 4 ℃, when data collection of BIO system was initiated at 3 hours 55 minutes. The BIO experiment was activated without delay from the completion of checkout of the SFU system in orbit. Water temperature was raised to 20 ℃ . First scan of the newt vessels showed no eggs laid in the captured images.

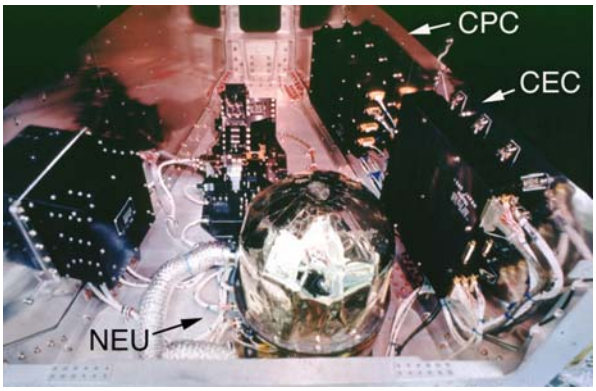


Fig. 2 BIO experiment system installed on Special Payload Unit-2. NEU: Pressurized container of Newt Experiment Unit, CEC: Experiment dedicated computer to interface data and command, and CPC: Power controller.

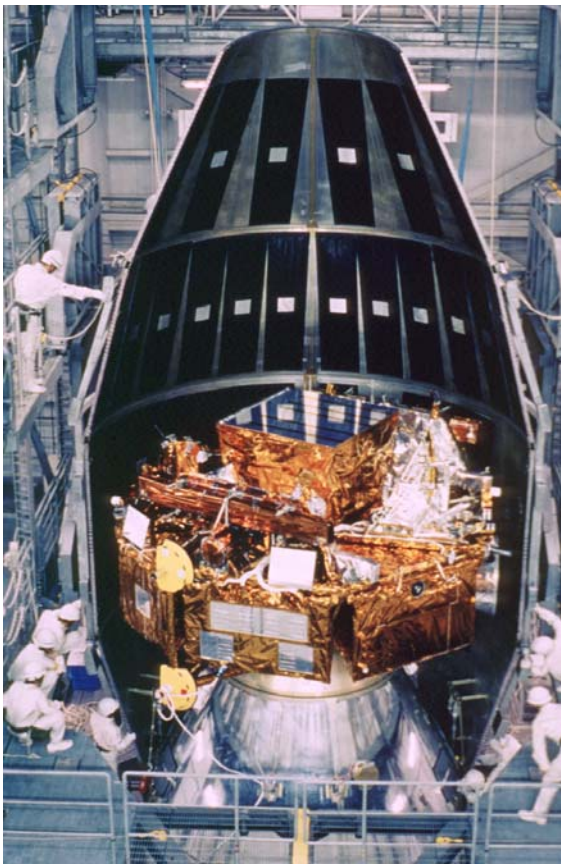


Fig. 3 Space Flyer Unit configured in the fairing of H-II rocket

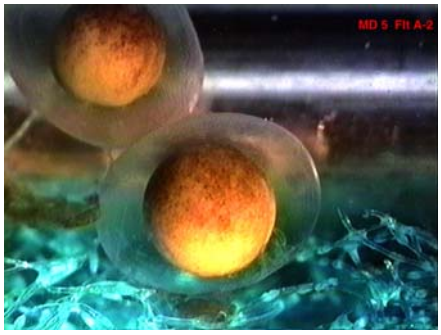
Result

IML-2 Embryos:

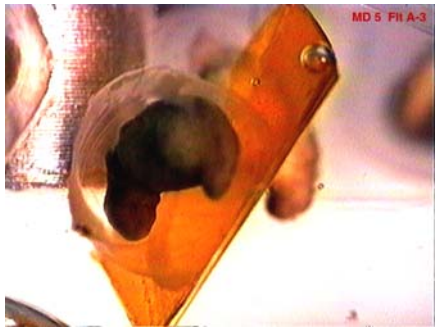
Three cassettes, A-1 throgh A-3, carried four female newts both in the set-up for flight and ground control. Development of newt eggs in Flight A-3 cassette was video recorded on MD (Mission Day) 2, 5 and 12. Eggs was estimated to be laid between MD0 and MD2. Close up image showed three embryos at tail bud stage 26 on MD5, and at stage 36 on MD12. However, two developed embryos could not be found when the cassette was opened after its recovery. Time course of development was 3 to 5 days from spawning to stage 26, and 7 days from stage 26 to 36.

In Flight A-2 cassette, most of the eggs were spawned between MD2 and 3. They were video documented on MD5 after the cassette was detached from AAEU AP water loop at the death of the adult newt held in it. Among 37 eggs in the cassette, two or three were at stage 8 or 9; late morula or early blastula. Those developed eggs were probably laid after MD3. In that case, the eggs developed to stage 8 or 9

a



b



c

Fig. 4 Embryos in Flight AAEU Cassettes
a; F A-2 on MD5, **b**; F A-3 on MD5, **c**; F A-3 on MD12
(MD: Mission Day; A-2 and A-3 are the name of the newt vessels)

in no more than two days.

Based on recorded video image of the embryos in those two flight cassette, their morphology was judged to be normal. Time course of development did not deviate from those of eggs and embryos grown under earth gravity at a same temperature range. It was shown that the fundamental process of early development occurs normally without gravity. Neither morphology of embryo nor time course of development differed from those on ground.

IML-2 Adult Newts: Harsh effects of space flight were found in the adult newts. Two newts were lost from the flight group in the middle of the mission. In the ground control experiment, four newts survived through the mission. In addition to this, eggs were laid in all three newt cassettes. By visual inspection at dissection, no abnormality was evidenced in body surface and abdominal organs of the two flight newts recovered in alive, and neither of the ground controls. However, those flight newts suffered severe damage on their liver, lung and stomach at cytological level even they returned from space in alive. No such damage was found in the ground controls.

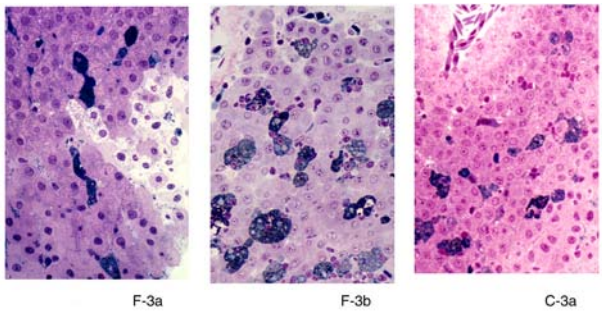


Fig. 5 Stained section of newt liver. F-A3a and F-A3b are two newts of flight group that were recovered in alive. C-A3a is one of ground control group on which same procedures were applied during the space mission. Darker cells are osmiophilic granular cells (OG cells.) Note vacuolated OG cells found in the flight group.

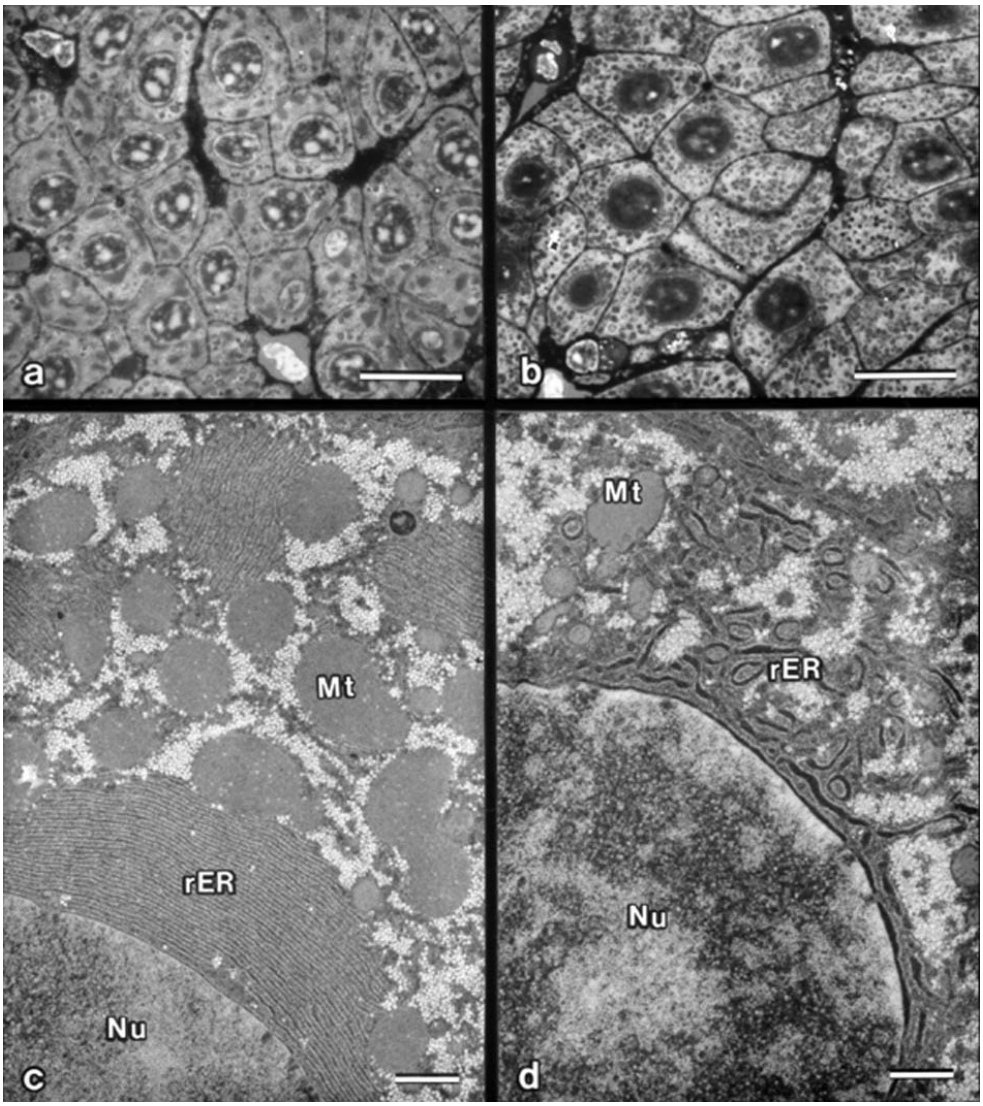


Fig. 6 Morphological changes in hepatic cells **a**, **c**; ground control, **b**, **d**; flight group retrieved in alive to ground. Compared to hepatic cells of ground control, the cells of flight group had smaller nucleolus. Rough ER of flight group was less in amount and showed distinct deformation. Morphological changes of mitochondria were also shown. Bar in **a** and **b**; 50 μm, **c** and **d**; 1 μm. Mt; Mitochondrion, Nu; Nucleus, rER; Rough Endoplasmic Reticurum.

In the liver of the two flight newts, a certain portion of osmiophilic granular cells (OG cells) were vacuolated. OG cells might function to scavenge free radicals similar to Kupffer cells in mammals. The features of these hepatic cells might be relevant to the reaction against toxin. In ordinary cells, fine layered membrane of rER is developed on nucleus. This fine structure of rER was largely deformed in hepatic cells of the flight group. The amount of rER was decreased in the flight group, but not in the controls. Morphological changes observed in nucleolus and mitochondrion, in addition to rER, suggest that the activity for protein synthesis in liver was supressed during the space flight. Similar findings were reported in the liver of treefrog flown on Mir space station in 1990.

Stomach of Flt A-3a had ulcer in several parts and edema of lamina propria (Fig. 7). Intestine was entirely normal in all the specimen. Ulcer formation in stomach reminds that such pathogenic changes are commonly recognized as one of stress reaction widely seen in animals.

Lung was another organ which had pathological changes in ultrastructure of cells (Fig. 8). Disruption of pulmonary epithelial lineage and degeneration of epithelial cells were observed exclusively in the flight group. At submersion in the cassettes, hypoxic condition was same burden for flight and ground control newts. Furthermore, in the ground set-up, no air layer or bubble was formed in the cassettesl where the newts were held. In the flight vessel, air bubbles were formed in the cassettes regardless of accessibility by the specimen to it.

There were organs which did not show notable difference between the flight and the ground control groups. Muscles had no significant change in the flight group. Bone analyzed by X ray imaging showed no sign of space flight found in its fine structure. Expression of heat shock protein (HSP) was not detected in liver, limb, and vertebra of the space flown newts.

Post flight control experiment was conducted with FM and BBM. The newts were kept healthy, laid eggs, and showed no significant pathology in both specimen held in FM and BBM on ground. Pathological changes in the two flight newts that were recovered in alive from orbit were confirmed to be an influence of space flight on newts.

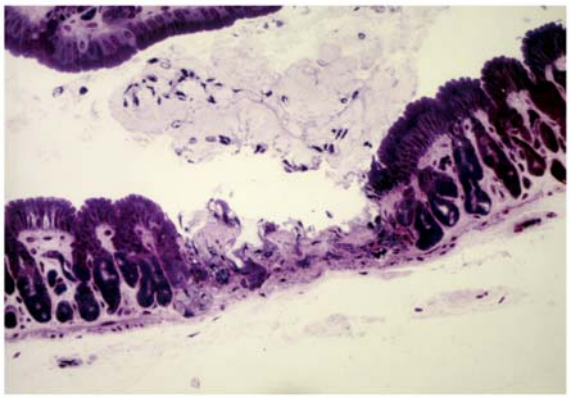


Fig. 7 Stomach of the newt, Flight A-3a
Deep ulcer in the center. Mucosa on right shows normal histology.

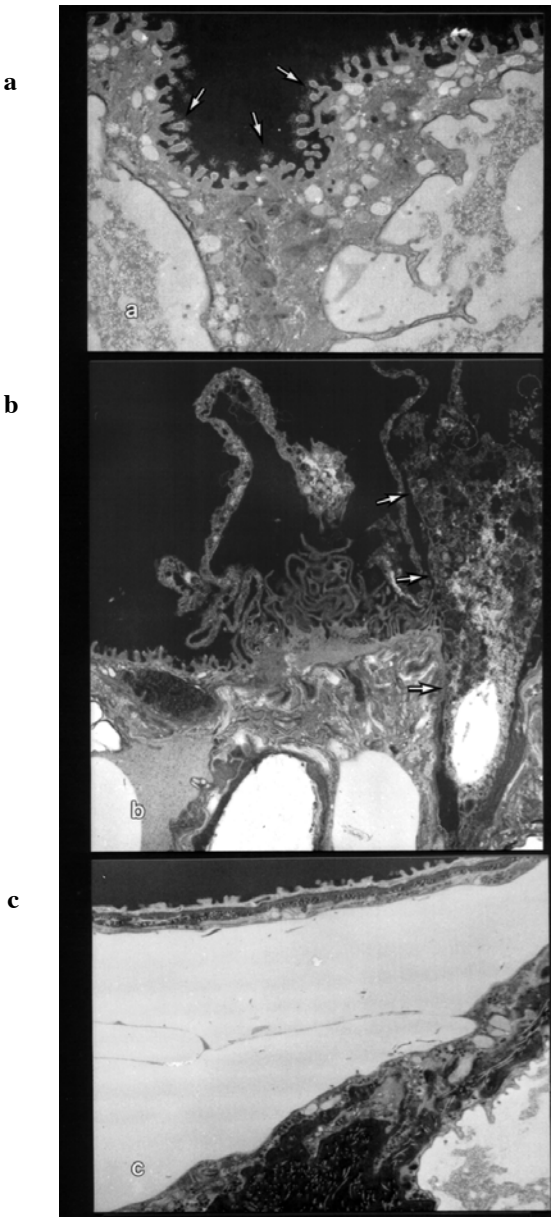


Fig. 8 Ultrastructure of pulmonary epithelia
a; Normal newt. Microvilli and glycocalyx (arrows). **b**; Flight group, F A-3b, showing the degeneration of a cell (arrow). **c**; ground control, C A-2.

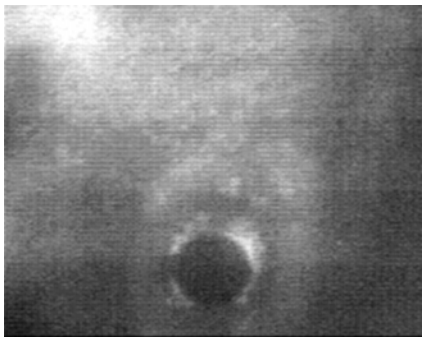


Fig. 9 Image of newt egg downlinked from SFU.

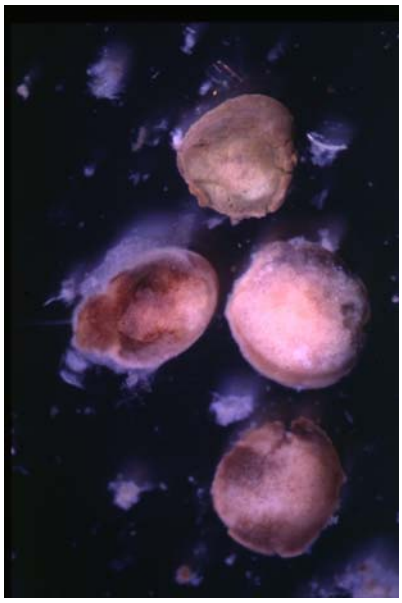


Fig. 10 Newt embryos recovered from orbit. Developmental stages were identified to neurula to tail-bud.

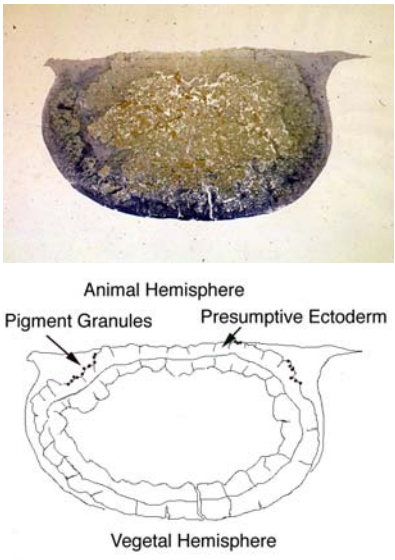


Fig. 11 Microscopic image of sectioned embryo. Identified to late morula to early blastula.

SFU: An image of spawned egg was obtained on the fifth day from the start of experiment. Spherical egg and jelly layer observed are shown in Fig. 9. Developmental stage of this egg was estimated to be at quite early stage, probably before the first cleavage.

In order to fix specimen at rather early stage of development, observation was terminated on the 12th day. Electric power supply dropped by safety management task at sending an improper parameter in commanding. It brought the experiment system to a frozen state. Because of the excessive opening of the heat radiation panel of the payload box unit, the system could not be warmed up again. The last step of experimental procedure was introduction of fixative solution into the circulating water, and it was not made by this happening.

Monitoring of environmental parameters for biological specimen was continued until the retrieval of SFU by Space Shuttle. Fixation of the specimen was not completed in orbit. However, it was kept frozen until the retrieval of SFU by Space Shuttle. After SFU was placed in the cargo bay of Space Shuttle, status of the experiment system was not monitored, except temperature at the hydrazine feed line of SFU. Temperature swung largely during the maneuver of Space Shuttle for another mission conducted to simulate thermal environment for crew working on International Space Station.

As one of post-landing operations of SFU, early access to the BIO system was made after one month from its landing. Inner pressure of the container was slightly less than atmospheric pressure at the opening. Crack was developed at the newt vessel, and water was flooded out to the internal volume of the container. It was suspected that temperature swing experienced on Space Shuttle might cause and develop the crack in plastic parts.

Inner volume of the vessel and specimen were partially dried out. Newt embryos found in the vessel were transferred into fixative solution of para-folmaldehyde and glutar-aldehyde. Figure 10 shows several embryos retrieved at neurula and tail bud stage. Specimens were embedded in Epon resin after additional fixation made by osmium tetraoxide. It was sectioned at thickness of 1 μm, and stained for microscopic examination. One of sectioned specimens is shown in Fig. 11.

Twenty one specimens found in the vessel were judged to be either egg or embryo. Developmental stage of each specimen was determined from morphology of embryo and size of cells. The stage distributed over morula, blastula, gastrula, neurula, and tail bud.

Conclusion

Newt eggs were spawned in orbit and exposed to space environment from their early stage of development at both IML-2 and SFU mission. Spawning of egg was confirmed by image downlinked after the activation of experiment. Microgravity and space environment do not affect early developmental process of amphibians in terms of

morphology and the time course of development. This conclusion is rather restricted, because of the small sample size and lack of histological survey on the embryos in detail. However, these results enforced similar findings obtained by other experiments conducted in space using different amphibian species. Fundamental step of development such as determination of embryonic axis does not require the presence of gravity. The working hypothesis on the role of gravity in the determination of embryonic axis was clealy denied by those studies. Since developmental biology has been advanced in a great degree during the recent years, new methodology could be implemented in space biology experiments. The mechanism for the gravitational effects on amphibian development could be revealed by those new advanced experiments, and the robustness would be explained in the embryonic development.

In contrast to the results of embryos, distinct effects of space flight were found in adult newts. Mortality rate of newts was high (two losses out of four) for the flight group in IML-2. Furthermore, severe pathological damages were evidenced in liver, stomach and lung of the two newts that were recovered in alive from orbit. Ground control group had neither loss nor pathological damages in organs, even at a complete submerged condition without any air bubble in the cassettes. It should be reminded that a single water circulation loop was shared by four AAEU AP cassettes. Dead newts were left in the loop for a while. We can not deny possible propagation of damages in Flight AP initiated by the death of the two newts in it. Since newt embryos and Medaka bred in the same water loop were in quite good condition, effects should be highly specific to adult newt. Independent observation on the liver of the frogs recovered from a space flight showed certain similarities with the results obtained in this study. One speculation is the stress reaction of the adult newts in flight group relevant to the thigmotactic behavior of the animal. Density of newt body is slightly higher than that of water. It produces physical sense for thigmotaxis. In microgravity, this sensation was lost, and might cause stress for the animal. Physiological effects of gravity at organismal level should be recognized important. AstroNewt experiment contributed to provide such a scope for future strategy of space biology.

Acknowledgment

AstroNewt experiment on SFU was the first time for Japanese space community to experience launching animals on the own space life support system and carrier from its territory. The authors express sincere thanks to people and organizations who lead SFU mission in success. Among them, SFU system management and operation group of ISAS and Mitsubishi Electronics Company, and launch and retrieval team at NASDA and NASA are deeply appreciated for their warm support given to our newt experiment. The authors share gratification with Fujitsu, who developed the BIO flight hardware. For IML-2 mission, hardware development and operation were managed by NASDA. We sincerely thank to Mitsubishi Heavy Industries, Toray Research Center and other companies for their support.

Publications on AstroNewt

Masamichi Yamashita, Akemi Izumi-Kurotani, Michael Wiederhold, Hajime Koike, Makoto Asashima, Yoshihiro Mogami, Makoto Okuno, Yukishige Kawasaki and Satoshi Komada; Preparatory Studies for Newt Experiment in Space, *Proc. Space Utiliz. Symp.*, **9**, 132-134 (1992).

Mari Imamizo, Hajime Koike, Yoshihiro Mogami, Akemi Izumi-Kurotani, Masamichi Yamashita, Shunji Nagaoka, Mitsutaka Yoshida and Makoto Asashima; Egg Laying of Newt in Space, *Proc. Space Utiliz. Symp.*, **10**, 49-52, (1993).

Mari Imamizo, Hajime Koike, Yoshihiro Mogami, Akemi Izumi-Kurotani, Michael L. Wiederhold, Shunji Nagaoka, Masamichi Yamashita and Makoto Asashima; How to Induce Egg Laying in Space, *Biol. Sci. Space*, **7**, 256-257, (1993).

Masamichi Yamashita, Akemi Izumi-Kurotani, Mari Imamizo, Yoshihiro Mogami, Makoto Okuno, Hajime Koike and Makoto Asashima; Experimental Operation of "Fertilization and Embryonic Development of Japanese Newt in Space" , *Biol. Sci. Space*, **8**, 210-211, (1994).

Masamichi Yamashita, Akemi Izumi-Kurotani, Mari Imamizo, Yoshihiro Mogami, Makoto Okuno, Hajime Koike and Makoto Asashima; Operation of AstroNewt experiment on IML-2, *Proc. Space Utiliz. Symp.*, **11**, 13-16, (1994).

Mari Imamizo, Mitsutaka Yoshida, Keiji Fujioka, Yoshihiro Takada, Akihiko Hisada, Akemi Izumi-Kurotani and Masamichi Yamashita; Sustained Release of hCG Minipellet for Newt Experiment in Space, *Biol. Sci. Space,* **8**, 226-230, (1994).

Fumie Sasaki, Sumio Nishikawa, Junko Hirata and Yuji Takahashi; Cytological analysis of IML-2 AstroNewt lung, *Proc. Space Utiliz. Symp.*, **12**, 12-15, (1995).

Masamichi Yamashita, Akemi Izumi-Kurotani and Shunji Nagaoka; Air bubble under microgravity: AstroNewt experiment onboard IML-2, *J. Jpn Soc. Microgravity Appl.,* **12**, 150-156, (1995).

Carl J. Pfeiffer, M. Yamashita, A. Izumi-Kurotani, H. Koike and M. Asashima; Cytopathologic observations of adult newts (Cynops pyrrhogaster) on-board the Space Shuttle, Columbia, during the Second International Microgravity Laboratory experiment, *J. Submicrosc. Cytol. Pathol.*, **27**, pp. 501-509, (1995).

Carl J. Pfeiffer, M. Yamashita, M. Asashima, A. Izumi-Kurotani and H. Koike; Cytopathology of lungs of newts on-board Columbia Space ShuttleSTS-65, *ASGSB Bull.*, **9**(1) 88, (1995).

Yoshihiro Mogami, Mari Imamizo, Masamichi Yamashita, Akemi Izumi-Kurotani, Michael L.Wiederhold, Hajime Koike and Makoto Asashima; AstroNewt: Early Development of Newt in Space, *Adv. Space Res.*, **17**, No 6/7, pp. (6/7)257-(6/7)263, (1996).

Yoshihiro Mogami, H. Koike, M. Yamashita, A. Izumi-Kurotani, and M. Asashima; Early Embryogenesis of Amphibians in Space: AstroNewt for the Space Embryology in IML-2 and SFU, *Adv. Astron. Sci.*, **91**, 1089-1097, (1996) .

Masamichi Yamashita, A.Izumi-Kurotani, M. Imamizo, Y. Mogami, H. Koike, M. Asashima and S. Komazaki; AstroNewt Experiment on SFU, *Space Utiliz. Res.*, **13**, 1-4, (1996).

Makoto Asashima, S. Komazaki, Y. Mogami, M. Yamashita, A. Izumi-Kurotani, M. Imamizo and H. Koike; Scientific Results from AstroNewt Experiment onboard SFU, *Biol. Sci. Space,* **10**, 206-207, (1996).

Masamichi Yamashita, Akemi Izumi-Kurotani, Mari Imamizo, Yoshihiro Mogami, Hajime Koike, Makoto Asashima and Shinji Komazaki; BIO (Biological experiment on Space Flyer Unit :Effects of gravity on early development of newt,) *ISAS Report*, **36**, 30-39, (1997).

Masamichi Yamashita, Akemi Izumi-Kurotani, Mari Imamizo, Yoshihiro Mogami, Hajime Koike, Shinji Komazaki and Makoto Asashima; BIO experiment on Space Flyer Unit :Effects of gravity on early development of Japanese red bellied newt, *J. Space Tech. Sci.*, **13**-2, 12-17, (1997).

Fumie Sasaki, Sumio Nishikawa, Junko Hirata, Hachiro Iseki and Yuji Takahashi, Electron microscopic observation of the lung and the tail skin of adult newts (*Cynops pyrrhogaster*) after spaceflight, *Tsurumi Univ. Dent. J.*, **23**(3), 377-388, (1997).