Early Development of a Gravity-Receptor Organ in Microgravity (OTOCONIA)

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The object of this experiment was to determine what role the gravitational field might have on the initial development of the gravity-sensing portions of the inner ear. Particular emphasis was placed on the formation of the otoliths, test masses on which gravitational and linear-acceleration forces act. If the growth of the otoliths is somehow regulated by their weight, their mass should be increased in reduced gravity. Pre-fertilized eggs of the Japanese red-bellied newt, some at developmental stages before any portion of the inner ear had formed and others just before the otoliths are formed were flown in the Aquatic Animal Experiment Unit. During the fifteen day flight, these eggs reached larval stages in which the inner ear neared its adult form. The otoliths of the saccule and utricle, the two gravity-sensing portions of the inner ear, were not of significantly different volumes when prepared for histologic study within the week after return of the shuttle. The endolymphatic duct and sac extend dorsally from the saccule and, in the adult newt, cover the brain stem and portions of the spinal cord. In the adult, these structures are filled with a different form of otoconia. This system develops earlier and is significantly larger in the flight-reared larvae, compared to ground-controls. At the stages the larvae reached at the end of the flight, endolymphatic otoconia begin to appear in the saccule and contribute to the saccular otolith. In X-ray microfocus studies performed by our Japanese collaborators, the saccular otolith and endolymphatic otoconial mass are seen to be significantly larger in one flight-reared larvae maintained in Japan for several months post-flight. Thus, the system of otoconia formed in the endolymphatic sac is greatly accelerated in the flight-reared animals and leads to a long-lasting increase in the saccular otolith in the one specimen maintained for months afterlanding.

OBJECTIVES

1) To maintain developing larvae in microgravity through a period starting before any otoconia are formed and ending after the inner ear is substantially developed.

2) To compare rates of development in microgravity to those on earth.

3) To compare the cumulative volumes and locations of otoconia in flight and ground-reared larvae.

4) To compare the sensory structures of the inner ears of flight and ground-reared larvae.

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5) To compare the otolith-ocular reflex in flight- and ground-reared larvae.

6) To follow the further development of the otolith systems after return to 1-*g* conditions of larvae reared in microgravity.

BACKGROUND

All animals sense gravity and linear acceleration by the interaction of a specialized mass with sensory receptor cells. In vertebrates the masses are the otoliths (collections of small $CaCO_3$ stones called otoconia or statoconia) in the utricle, saccule and, in non-mammalian species, the lagena. The size (mass) of the otoliths appears to be "appropriate" for each species and stage of development; in species which grow throughout their life, such as fish, the otoliths also continue to grow. This implies that there is some mechanism which regulates otolith growth, enabling growth where needed and preventing excessive growth in a full-sized adult. If this regulation is, in some way, based on the otolith's weight, larger-than-normal otoliths would be expected in animals which develop their inner ears in microgravity (μ-*g*). Previous studies of this hypothesis have been either inconclusive or contradictory.

A major function of the otoliths is to send signals to the brain stem cell bodies of the motor neurons to the extra-ocular muscles. These muscles serve to stabilize the eyes when an animal moves, keeping an image stable on the retina. One set of reflexes which accomplish this are the otolith-ocular reflexes. It is known in higher animals, including man, that the gain (degree of "effectiveness") of these reflexes can be modified in conditions such as a subject wearing prismatic lenses. If the reflexes rely upon comparison of gravity and linear acceleration with visual inputs during their development, it is of interest to determine how they will develop in the absence of gravity.

The Japanese red-bellied newt, *Cynops pyrrhogaster*, is a favorable model system in which to study inner-ear development, in that fertilized eggs of any desired developmental stage can be readily obtained and the major development of the inner ear occurs from days 4 to 20 after eggs are laid, within the range of space shuttle missions. Thus, viable fertilized eggs which either have not yet begun to form the inner ear, or in which no otoconia have yet been formed, can be launched and will develop nearly adult inner ears during the duration of a shuttle mission. Upon return, the anatomy of the inner ear as well as its function can be studied and compared to ground-reared control specimens.

METHODS

Female newts were collected from rice paddies in Japan in the spring of 1994. After the fall mating, these newts go into hibernation and the female keeps sperm and eggs separate through the period of hibernation. The animals were kept in hibernation under refrigeration until eggs were desired, when they were moved to room temperature for one or two days and injected with human chorionic gonadotropin (HCG), either 100 IU/6g on two successive days or with 200 IU/6g on one day (the average weight of the adult newts is 6 g).

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Eggs were then laid over the next 3 to 5 days. Their developmental stage was determined by external morphological characteristics, as described by Okada (1947, 1989). Eggs were reared in the laboratory until loading for flight.

At approximately 36 hours before launch, eggs at stages 10 to 25 were loaded into individual egg holes (6 mm diameter, approximately 12 mm deep) in an egg container in each of three cassettes of the Aquatic Animal Experiment Unit (AAEU). This unit circulated fresh, aerated water at 24 °C continuously. A similar unit was maintained at KSC Hangar L as a ground control facility. Forty-eight eggs were loaded into each cassette. Approximately 100 eggs from the same group of females were maintained in the laboratory as additional controls.

During the flight, the developmental stages of eggs in both flight and ground control AAEU's were determined from high-magnification video taping. Using a Canon L1 video camera (which will accept any Canon EOS lens) video could be obtained in which the 6 mm eggs hole took up most of the field. This allowed identification of the gill and limb development characteristic of the relevant stages. With optimal lighting, which was difficult to obtain in the Spacelab, blood flow could even be assessed in the developing gills.

The flight cassettes were retrieved about 6 hours after shuttle landing. Some larvae were fixed with 0.5 % paraformaldehyde and 1.0 % glutaraldehyde, dehydrated and embedded in Medcast plastic for sectioning. Some larvae were tested to estimate the gain of the otolith-ocular reflex. These larvae were restrained in a device that allowed rotation about the longitudinal body axis, and counter-rotation of the eyes was measured from high-magnification video records. Six flight and six ground-control larvae were studied on post-flight days 1, 3 and 5 by X-ray microfocus imaging of the otoliths.

Otolith volumes and areas of associated sensory epithelia were calculated from three-dimensional reconstructions of serial sections through the inner ears at the stages available. Each section was stained with methylene blue, traced with a camera lucida attachment to the microscope and then traced into a computer, using a digitizing pad. The reconstructions were computed using Jandel PC3D software.

FLIGHT RESULTS

A total of 148 fertilized eggs were loaded into the 3 AAEU cassettes 30 hours before launch. Each cassette also contained one or two adult newts. The adult in cassette A2 died on flight day 5 and malfunction procedures called for the removal of a cassette in which an adult had died, so this cassette was removed and placed in the freezer. Thus, all 48 eggs from A2 were lost to further study. On mission day 9, the adult in cassette A1 also died. A modified malfunction procedure was developed to remove the cassette, open one side panel, remove the dead adult, replace the panel, and replace the cassette in the AAEU. This was accomplished successfully and approximately half of the eggs in this cassette survived. Sixty-two of the original eggs survived to the end of the flight.

The progression through the developmental stages was assessed from high-magnification down-linked video and video tapes reviewed after the flight. Similar observations were made on the ground-control equipment at KSC Hangar L at the same Mission Elapsed Times

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(MET's). Figure 1 demonstrates, for cassette A3, that the flight and ground-control specimens developed at rates that were indistinguishable from one another by external morphological criteria. Thus, if temperature is well controlled and identical between ground and space-reared newts, they appear to develop at the same rate. By extrapolation between the stages a loading and the first in-flight observations, the flight larvae were divided into two groups, one from stages 17 to 27 and the other from stages 29 to 30 at launch, whereas the ground larvae were in groups from stages 19 to 23 and from 28 to 31. Since the otic vesicle is first seen at stage 25, all but one larva in the two younger groups were in μ-g before any of the otic vesicle formed and in the older groups, the larvae were all in μ-g before any otoconia were formed (otoconia are first seen at stage 33).

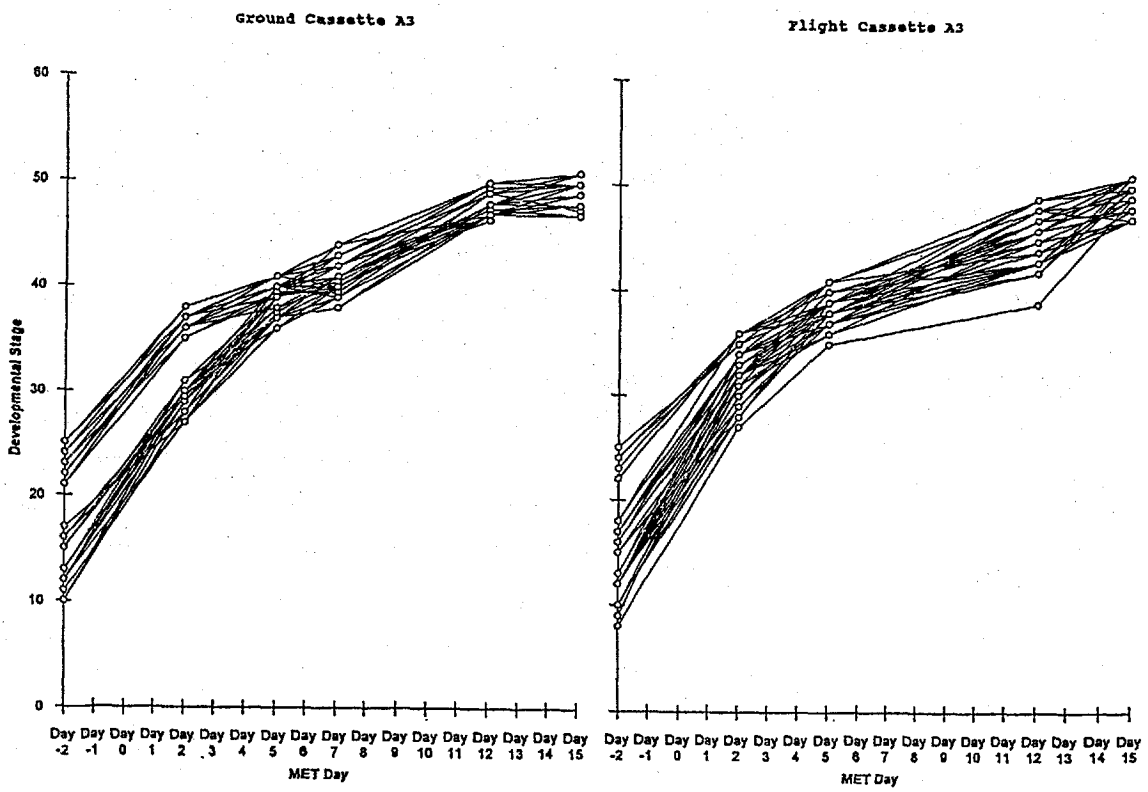

Fig. 1

Cassettes A2 and A3 were retrieved approximately 6 hours after shuttle landing. The surviving larvae had all hatched and swam vigorously, so it was not possible to clearly identify each larva with the egg hole in which it had been reared. Thus, post-flight samples were staged as they were studied or fixed for anatomical study. Sixteen flight and 12 ground-control specimens were fixed and embedded for sectioning on landing day (R + 0), 8 flight and ground on R + 3 and 18 flight and 25 ground larvae on R + 5.

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Figure 2 illustrates three-dimensional reconstructions of the inner ears of a ground- and a space-reared larva at stage 52, both fixed on R + 3. Note that there is no apparent difference in the volume of the saccular or utricular otoliths between these two specimens. It is clear that the endolymphatic sac and the volume of otoconia within the sac are larger in the flight-reared specimen and there are many otoconia found in the semicircular canals, identified as "ectopic otoconia", in the flight specimen.

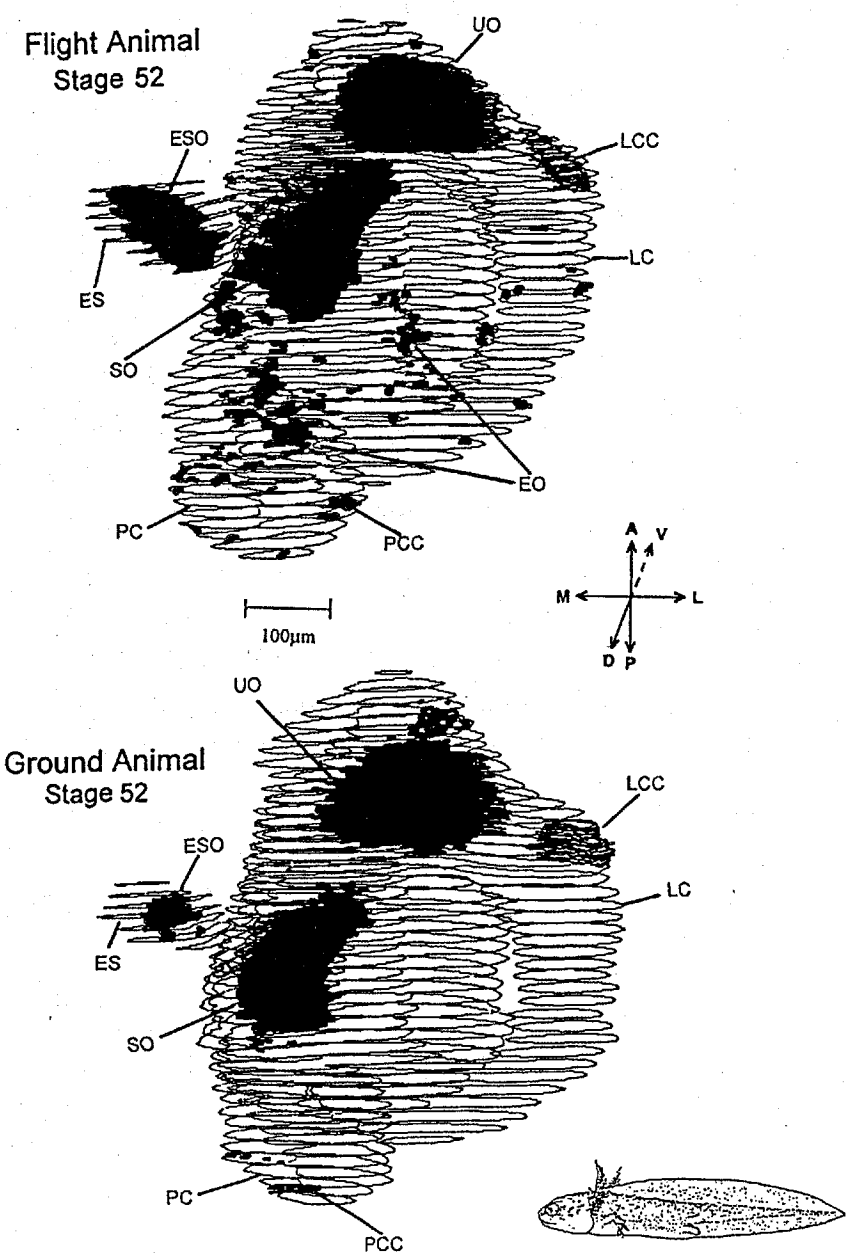

Fig. 2 Three dimensional reconstruction of serial sections through the developing otic vesicle of flight and ground-reared stage 52 larvae. Abbreviations: ES: endolymphatic sac; ESO: endolymphatic sac otoconia; EO: ectopic otoconia; UO: utricular otolith; SO: saccular otolith; LC: lateral semicircular canal; LCC: lateral canal crista; PC: posterior semicircular canal; PCC: posterior canal crista; D: dorsal; V: ventral; M: medial; L: lateral; A: anterior; P: posterior. Drawing at lower right is a stage 52 *Cynops* larva.

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There is considerable variation in the volumes of otoliths within specimens at a given developmental stage. Figure 3 is a scatter plot of the volumes of the saccular and utricular otoliths for flight and ground specimens for the 21 specimens reconstructed to date (2 flight and 2 ground at stages 49 - 53 and one ground larva at stage 54). This plot illustrates the variability as well as the clear growth of otolith volume with developmental stage but does not indicate a systematic difference between the flight and ground specimens. Possibly due to the variability between specimens, it was not possible to demonstrate a significant difference in the volumes of the utricular or saccular otoliths between flight- and ground-reared specimens between stages 49 and 53 fixed within one week after return to earth. Figure 4 shows plots of the mean volumes, +/- the standard error of the mean (SEM), at each stage, along with plots (dashed lines for ground and dotted for flight-reared) of the 95 %

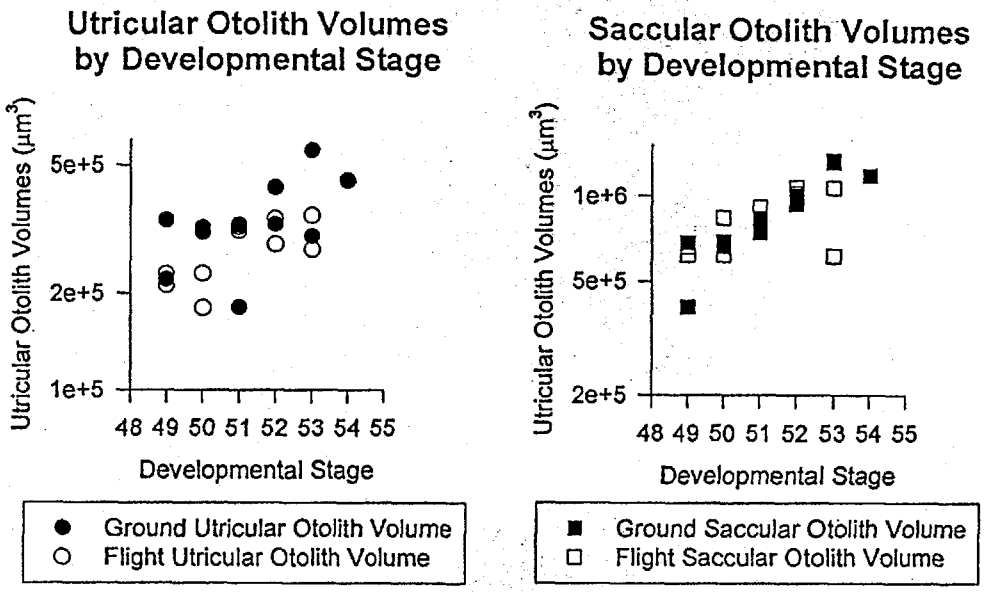

Fig. 3

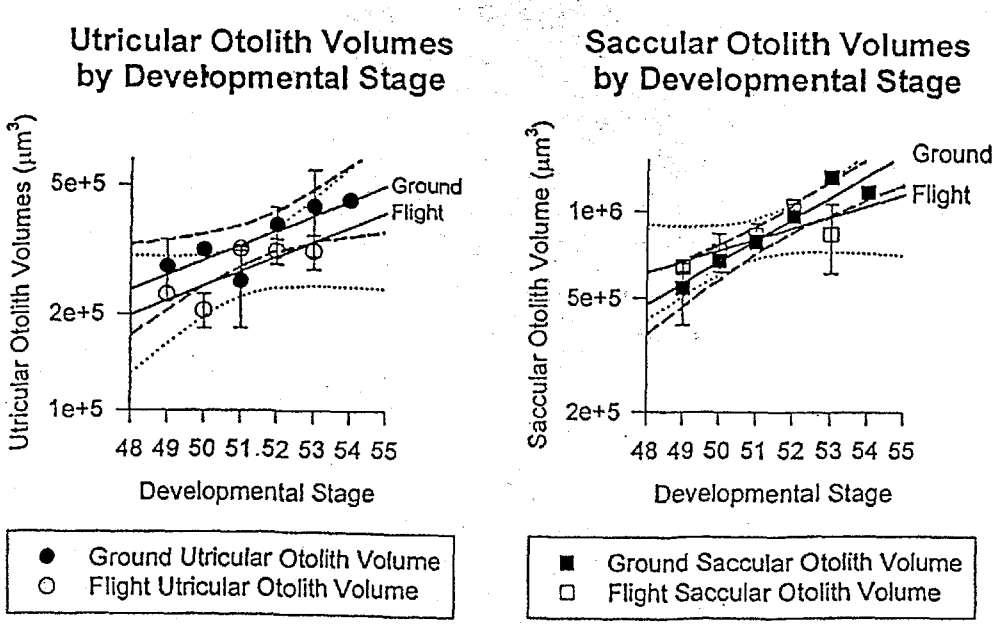

Fig. 4

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confidence interval for the relationship of volume to developmental stage. It is clear that the confidence intervals for flight and ground animals in both the utricle and saccule overlap significantly.

From Figure 2, it is apparent that the flight-reared larva has a larger endolymphatic sac (ES) and duct and a larger volume of otoconia in the sac (ESO). Figure 5 shows scatter plots of ES and duct volumes for the same specimens as in Figures 3 and 4. Means and SEM's are also plotted, with 95 % confidence intervals. One flight specimen at stage 50 had an exceptionally large sac and duct, which causes a large dispersion in the 95 % confidence interval for the flight larvae. However, the average flight sac and duct volumes are above the upper 95% interval for ground larvae at stages 49 through 53. Figure 6 shows the scatter plots and regression plots, with 95 % confidence intervals, for the volumes of otoconia within

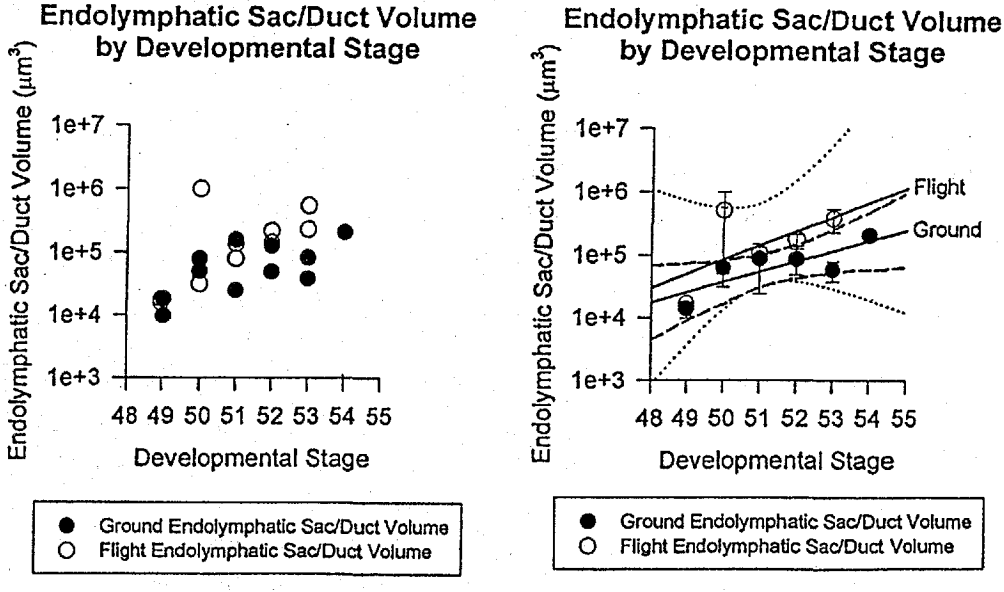

Fig. 5

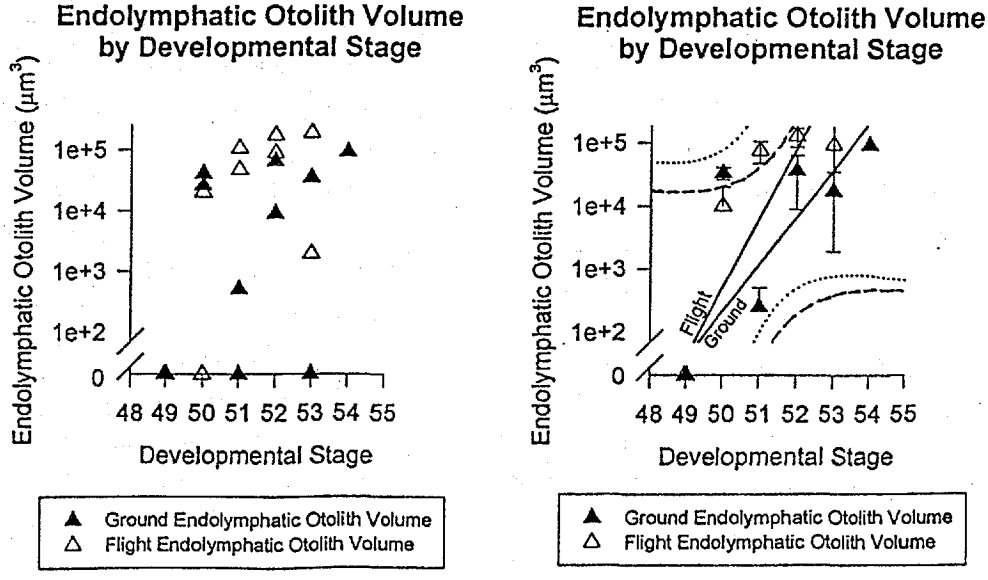

Fig. 6

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the ES in the flight- and ground-reared larvae. Again, there is a strong trend for the total volume of otoconia in the sac to be larger in the flight-reared larvae. As with the saccular and utricular otoliths, there is considerable variation between specimens. Three out of 11 ground animals had no otoconia in the sac, whereas only one of 10 flight animals had no ES otoconia. As a further example of the variability, the stage 50 larva with the very large ES had no otoconia within the sac and the two ground stage-50 larvae had a relatively large volume of ES otoconia. Thus both ground-reared larval otoconial volumes are larger than the two flight-reared specimens. Neither the two ground nor the two flight larvae at stage 49 had any ES otoconia. At stage 51, the mean ESO volume of flight-reared larvae is nearly 300 times as large as in the ground larvae, at stage 52 the mean flight volume is 3.6 times larger and at stage 53 the mean flight volume is 5.5 times larger.

The above analyses combine specimens fixed on days R + 0, R + 3 and R + 5. However, there was a systematic progression across the five post-flight days in the probability of there being externally visible ESO. Before the flight, we had never seen otoconia within the endolymphatic system before stage 57. When flight-reared larvae were examined, either live or after fixation and embedding, it was noted that otoconia in the endolymphatic sac could often be seen using a dissection microscope with bright direct illumination, as reflected light similar to that from the utricular and saccular otoliths. Figure 7 shows plots of the number of specimens in which ES otoconia could be seen in ground and flight larvae on the three fixation days. On day R + 0, no ES stones were seen in either ground-control or flight animals. On day R + 3, 56 % of ground and 86 % of flight larvae had visible stones and on day R + 5, 21 % of ground and 70 % of flight larvae had visible stones. A group of larvae from the same group of females which laid the flight and ground-control eggs were maintained in the laboratory in plastic dishes on the counter top. Significantly, none of the laboratory-reared larvae, from stages 48 to 54, had visible ES otoconia. None of the lab-reared specimens has been reconstructed yet. Thus, the percentage of specimens with visible ES otoconia increases with time after return of the specimens to 1-g conditions on earth, and at days R + 3 and R + 5, the percentage of specimens with visible ES stones is substantially higher in flight, compared to ground-control specimens. On days R + 3 and R + 5, 37 % of the specimens raised in the ground AAEU had visible ES otoconia while the lab-reared larvae had none. Endolymphatic sac otoconia were visible as early as stage 49 (one case) and 50 (14 cases) in flight and ground-control larvae, whereas none were visible in the laboratory-reared larvae, up to stage 54, consistent with our previous observations from sectioned material indicating that otoconia do not appear in the endolymphatic sac before stage 57.

Using the Japanese X-ray microfocus system, Drs. Kashima, Nakamura, Nishimura and Mr. Koike have studied the areas and total X-ray absorption of the otoliths. Their observations will be reported separately, but they do agree with our observations in the first week, in that the utricular and saccular otoliths are not of significantly different sizes between flight and ground-control specimens. However, the endolymphatic system is clearly larger in the flight-reared larva, and the saccular and utricular otoliths are also larger at 2 and 3 months after return, compared to lab-reared controls from the same original stock of eggs.

Otolith-ocular reflexes were measured in the week after return, but those data are still being analyzed.

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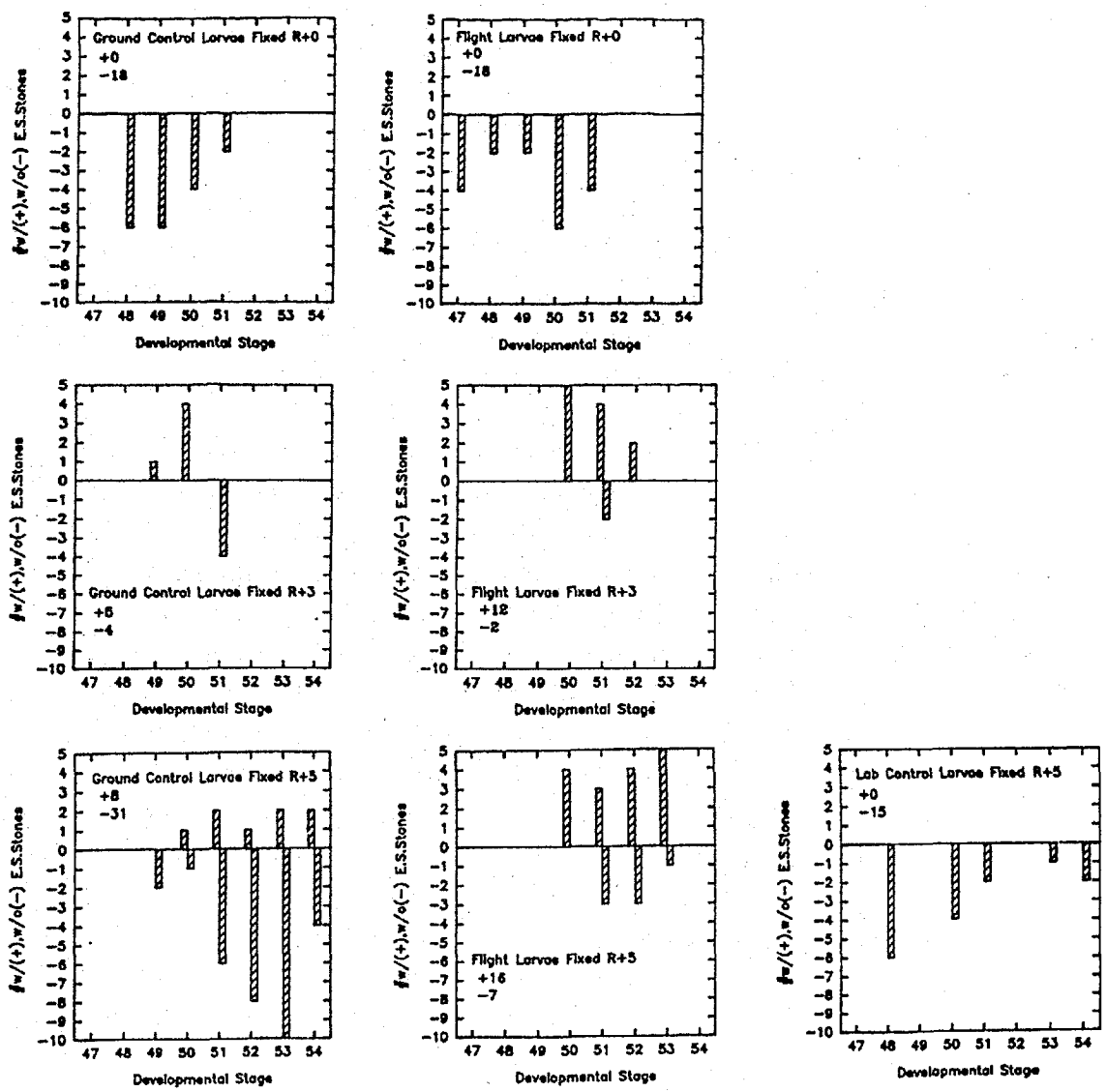

Fig. 7

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CONCLUSIONS

The newt eggs developed normally and survived well during the flight. In cassette A1, which was not opened during the flight, approximately 80 % of the eggs survived and had hatched by the end of the flight. Thus, fertilized newt eggs appear to be excellent specimens in which to study development in mgravity. Over the fifteen day flight, the animals went from early embryonicstages to hatched, swimming late-stage larvae.

One hypothesis upon which these experiments were designed was that an animal which developed its otoliths in reduced gravity would, by some mechanism, increase the mass of the otolith developed, to compensate for its reduced weight. This did not appear to happen within the first week after flight. However, the production of otoconia in the endolymphatic system was accelerated in the flight-reared larvae. In ground-based studies, we have shown that the otoconia produced in the ES are made of $CaCO_3$ in the aragonite crystal form, which is different from the calcite form found in the utricle and early-larval stage saccule (see refs. 8) and 11), below). In normal laboratory-reared larvae, aragonitic otoconia are first seen in the saccule at stage 51 and the first noticeable collection of otoconia within the ES was seen at about stage 57 [8)] . In the adult newt, all of the otoconia found in the saccule are made of aragonite. We have interpreted these findings to indicate that the aragonitic otoconia are produced in the ES and transported to the saccule through the endolymphatic duct.

Apparently the system which produces the aragonitic otoconia in the ES is enhanced inspace-reared larvae. Amphibians store calcium in the ES otoconia since they lack trabecular bone, where calcium is exchanged in mammals. Perhaps there is some change in calcium metabolism in these larvae growing in μ-g conditions which causes them to store more calcium than normal in the ES. Since the ES (aragonitic) otoconia contribute to the saccular otolith in later stages, the changes induced during two weeks of development in space appear to lead indirectly to a larger saccular otolith several months after return to earth, as shown by the X-ray micro-focus studies.

To increase the statistical power of the results presented here, more specimens must be analyzed. Over the next six months, we will double the number of flight and ground specimens reconstructed at each stage.

For future flight experiments, we plan to fly additional newt larvae on the ARF-02 flight, scheduled for early 1997. We hope to have the X-ray micro-focus system available for that flight also and will plan to keep more of the larvae alive for extended periods after the flight, to verify the long-lasting effects after return to 1-g conditions. The data of Fig. 2 indicate that the increase in ES otoconia began after return to earth. The determine whether this is a response to entering 1-g conditions after development in μ-g, or might be even greater with continued development in μ-g, will require much longer flight experiments. These could be accomplished in the Aquatic Habitat on Space Station Alpha.

Endolymphatic sac otoconia were more prevalent in the ground AAEU, compared to laboratory-reared larvae. This suggests that the AAEU egg chambers might act somehow to mimic the effects of μ-g. In a post-flight control experiment run in Japan, the ground AAEU cassette was attached to an extender and selected larvae were video taped for two hours every other day during the 15-day flight simulation. The dorsal axis of the larvae was identified and its vector noted, relative to "up". It was found that the larvae were within 45°

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of up 20 % of the time, were between 45° up and 45° down 70 % of the time and within 45° of down 10 % of the time. Thus, the orientation of the larvae was nearly random in the ground AAEU. When raised in dishes in the laboratory, newt larvae always remain dorsal-side up [2]. Thus, the constraint of the egg hole appears to act as a clinostat, averaging out the direction of gravity with respect to body axes of the developing larva. Somehow this randomization may lead to enhancing the storage of calcium in the ES. Perhaps, since the larvae do not need to support themselves in the egg holes, calcium is lost from, or diverted from the developing skeleton to the endolymphatic storage system.

PUBLICATIONS

The following publications describe ground-based studies performed as a baseline against which to compare the flight results. References 11, 13 and 14 include flight results (paper 11 is now being modified to satisfy reviewer suggestions).

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