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Michael L. Wiederhold, Ph.D.
Dept. O-HNS
University of Texas Health Science Center at San Antonio
7703 Floyd Curl Dr.
San Antonio, TX 78284-7777

Telephone: (210) 567-5655
Fax: (210) 567-3617

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DEVELOPMENT OF GRAVITY-SENSING ORGANS IN ALTERED GRAVITY

M.L. Wiederhold, W.y. Gao, J.L. Harrison and R. Hejl. Dept. of Otolaryngology-Head & Neck Surgery, Univ. Texas Health Science Center, San Antonio, TX 78284-7777

ABSTRACT

Experiments are described in which the development of the gravity-sensing organs was studied in newt larvae reared in μg on the IML-2 mission and in Aplysia embryos and larvae reared on a centrifuge at 1 to 5 g. In Aplysia embryos, the statolith (single dense mass on which gravity and linear acceleration act) was reduced in size in a graded fashion at increasing g. In early post-metamorphic Aplysia or even in isolated statocysts from such animals, the number of statoconia produced is reduced at high g. Newt larvae launched before any of the otoconia were formed and reared for 15 days in μg had nearly adult labyrinths at the end of the IML-2 mission. The otoliths of the saccule and utricle were the same size in flight and ground-reared larvae. However, the system of aragonitic otoconia produced in the endolymphatic sac in amphibians was much larger and developed earlier in the flight-reared larvae. At later developmental stages, the aragonitic otoconia enter and fill the saccule. One flight-reared larva was maintained for nine months post-flight and the size of the saccular otolith, as well as the volume of otoconia within the endolymphatic sac, were considerably larger than in age-matched, ground-reared newts. This suggests that rearing in μg initiates a process that continues for several months after introduction to 1-g, which greatly increases the volume of otoconia. The flight-reared animal had abnormal posture, pointing its head upward, whereas normal ground-reared newts always keep their head horizontal. This suggests that rearing for even a short period in μg can have lasting functional consequences in an animal subsequently reared in 1-g conditions on Earth.

INTRODUCTION

Several aspects of the systems animals use to orient to gravity might development differently in μg. If the growth of the "test masses" on which gravity acts (otoliths, in vertebrates, statoliths or statoconia in most invertebrates) is controlled on the basis of their weight, larger otoliths (or their analogs) would be expected to develop in μg. The otolith-mediated reflexes, which stabilize images on the vertebrate retina and control gravitactic responses in many invertebrates, are mediated by synaptic reflex circuits established early in development. These reflexes could develop abnormally without the steady gravitational forces exerted on the test mass during development on Earth.

The literature on the effects of rearing in altered gravity on the gravity-sensing organs is rife with inconsistency. Early Russian reports of Xenopus larvae reared in space indicated no qualitative differences in the vestibular organs, compared to ground-reared controls (Vinnikov et al., 1976, 1983). Neubert (1986) reported a similar lack of differences in Xenopus. Lychakov and Lavrova (1985) did report that the utricular otolith was 30% larger in space reared Xenopus. Lim et al. (1974) found no differences in saccular otolith volume between centrifuged and control adult rats. Howland and Ballarino (1981) reported a delay in otoconial development in chick embryos reared at 2g on a centrifuge, but in a later report (Ballarino and Howland, 1984) found no difference in otolith weight between 2g and control chicks. Souza, et al. (1995) report increased optokinetic responses in flight-reared Xenopus tadpoles, suggesting that the animals reared in the absence of gravity made greater relative use of their visual system, rather than the vestibular system, in orienting to a moving stimulus.

To test early development in microgravity, fertilized eggs of the Japanese newt, Cynops pyrrhogaster, were maintained in orbit for 15 days on the IML-2 mission in 1994. All specimens reached orbit before the inner ears formed and all major components of the inner ear were formed by the end of the flight. The rates of development of the larvae reared in space was compared to that of ground-control specimens. The volume of the otoliths in the utricle and saccule, computed from

3-dimensional reconstruction of serial sections, were compared between space- and ground-reared specimens. In amphibians, a separate set of otoconia is produced in the endolymphatic sac (ES), which, in adults, fills the saccule. The volume of the ES and of the otoconia contained in the ES were also compared in the flight- and ground-reared larvae. An X-ray microfocus system was used to monitor both otoconial systems of one larva maintained for several months after the flight and compared to age-matched ground controls (Koike et al., 1995b).

In ground-based studies of the Aplysia statocyst, the volume of the statolith in embryos reared at high g and the number statoconia in post-metamorphic animals is smaller in post-metamorphic animals reared at high g. These studies have been published or are in press (Pedrozo and Wiederhold, 1994, Pedrozo et al., 1995, 1996), so will not be presented here. The effects of μg on statoconia development and the ability to develop gravitactic crawling behavior in pond snails will be studied on the Neurolab mission.

Space Station extensions of these experiments, involving longer periods of development and multi-generational studies are discussed. As we report here, the effects on the system of ES otoconia in the newt reared for 15 days in μg appear to be greater several months after return to Earth. With long-term development on the ISS, we could separate effects of extended periods in μg from reactions to the first exposure to 1-g conditions after return to Earth.

METHODS

For the IML-2 Shuttle mission, 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 (AAEU). Female newts were collected from rice paddies in Japan in the spring of 1994. 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. 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. One hundred and forty-four eggs were launched, each in a 6mm diameter, 12 mm deep chamber, through which aerated fresh water, at 22-24 °C, was circulated. The same number of eggs were loaded into a ground-control AAEU, maintained at the same temperatures.

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 egg 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. 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 as described by Koike, et al. (1995a and b).

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 1 μm 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, NIH Image and ROSS (Ross et al., 1990) software.

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 Kennedy Space Center Hangar L (KSC) at the same Mission Elapsed Times (MET's). 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 at loading and the first in-flight observations, the flight larvae were divided into two groups, one from stages 17 to 27 (Okada, 1989) 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 μg before any of the otic vesicle formed and in the older groups, the larvae were all in μg before any otoconia were formed (otoconia are first seen at stage 33, Wiederhold et al., 1995).

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 8 ground specimens on R + 3 and 18 flight and 25 ground larvae on R + 5.

Figure 1 illustrates a three-dimensional reconstructions of the inner ear of a ground control larva at stage 52, both fixed on R + 3, prepared using the ROSS reconstruction system. Figure 2 is a similar reconstruction from a flight-reared stage 52 larva also fixed at 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 ES 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 in the flight-reared specimen.

Figure 1

Figure 1. Three dimensional reconstruction of serial sections through the developing otic vesicle of a ground-reared stage 52 larva. Abbreviations: AC: Anterior semicircular canal; ES: endolymphatic sac (ES); LC: lateral canal; PC: posterior canal; SAC: saccule; UTR: utricle; D: dorsal; V: ventral; M: medial; L: lateral. Scale bar = 50 μm. The pixelated areas are otoconia in the utricular and saccular otoliths whereas the lighter gray areas are otoconia within the ES and areas of the vestibule not normally containing otoconia. The latter are thought to be aragonitic otoconia, as opposed to the calcitic otoconia in the early-stage utricle and saccule (Wiederhold et al., 1996).

Figure 2

Figure 2. Reconstruction, similar to Figure 1, of a flight-reared stage 52 larva. Same abbreviations as in Figure 1. Scale bar = 50 μm.

There is considerable variation in the volumes of otoliths within specimens at a given developmental stage. In Figure 3 the mean +/- 1 standard error of the utricular (A) and saccular (B) otolith volumes are plotted v developmental stage for stages 48 - 53. The log of volume is well fit by a first-order regression with developmental stage but the differences between ground- and flight-reared specimens are not significant at the $P<0.05$ level at any stage using the unpaired Students t-test. The amount of variation of these volumes is apparent in the scatter plots in Figure 3 C and D.

Figure 3

Figure 3. Plot of mean volume of utricular otolith (A) for flight-reared (open symbols) and ground-control (closed symbols) and of the saccular otolith (B) for the same groups. Error bars indicate +/- one standard error of the mean. Lower plots show individual specimen volumes for the utricle (C) and saccule (D). The differences between flight and ground-reared means were not significant at the $P<0.05$ level at any stage. Lines indicate linear regression plots for the logarithm of otolith volume for flight and ground specimens. All measurements from specimens fixed within 5 days of Shuttle landing.

Figure 4 presents the mean +/- 1 standard error of the volume of otoconia within the ES (A) and of the lumen of the ES and its duct (B) in flight-and ground-reared larvae. It is apparent that the flight-reared larvae have larger mean ES and duct volumes and a larger average volume of otoconia within the sac. The differences between flight and ground ES otoconia (labeled "otolith") are significant at the $P<0.05$ level at stage 52 and at the $P<0.001$ level at stages 50 and 51. The lumen of the ES and duct is also larger in the flight-reared larvae, the difference being significant at the $P<0.05$ level at stage 52 and the $P<0.01$ level at stage 53. The ratio of flight-to-ground mean volumes of the ES otoconia are 0.2, 7.7, 5.6 and 2.1 at stages 50 through 53, respectively. For the volume of the ES and duct lumen, the ratios are 2.7, 1.5, 2.6 and 6.2 for the same stages. If stages 50 through 53 are grouped together, the mean volume of otoconia within the ES is 3.9 times larger in the μg reared larvae and the mean volume of the ES and duct is 3.3 times larger in the space-reared larvae.

Figure 4

Figure 4. Plots similar to those of Figure 3 for volume of otoconia within the ES (Endolymphatic Otolith) (A and C) and of the lumen of the ES and duct (B and D). The

means are fit with a second-order regression. Differences between flight and ground mean volumes which are significant at the $P<0.001$ are indicated by "d", $P<0.01$ by "b" and $P<0.05$ by "a".

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 otoconia in the ES. 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. 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. 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, through stage 54, consistent with our previous observations from sectioned material indicating that otoconia generally do not appear in the endolymphatic sac before stage 57.

Using the X-ray microfocus system (Koike et al., 1995a,b), the area of the utricular and saccular otoliths were not significantly different between flight and ground-reared specimens within

the first week after Shuttle landing. One specimen was maintained for nine months after the flight. The volume of otoconia within 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 (Nakamura et al., 1994, Koike et al., 1995b)

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 microgravity. Over the fifteen day flight, the animals went from early embryonic stages 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 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 (Wiederhold et al., 1996). 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 (Steyger, Wiederhold and Batten, 1995). 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 (Wiederhold et al., 1996).

Apparently the system which produces the aragonitic otoconia in the ES is enhanced in space-reared larvae. Amphibians store calcium in the ES otoconia since they lack trabecular bone, where calcium is exchanged in mammals (Guardabassi, 1960). Perhaps there is some change in calcium

metabolism in these larvae growing in μg 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 (Nakamura et. al., 1994, Koike et al., 1995b).

Our finding that the likelihood of finding externally visible otoconia in the ES increases during the week after return and that the size of particularly the saccular otolith increase in the months after return, indicate that the greatest increase in ES otoconia began after return to Earth. The results presented here indicate that this process begins during development in flight, since the ES system is larger in larvae fixed soon after landing. To determine whether this is a response to entering 1-g conditions after development in μg, or might be even greater with continued development in μg, will require much longer flight experiments. These could be accomplished in the Aquatic Habitat on the International Space Station.

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 μg. 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° 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 (Wiederhold et al., 1992). Thus, the constraint of the egg hole appears to act as a clinostat, allowing the larvae to orient in any direction and averaging out the direction of gravity with respect to body axes of the developing larva. Somehow this randomization appears to enhance 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.

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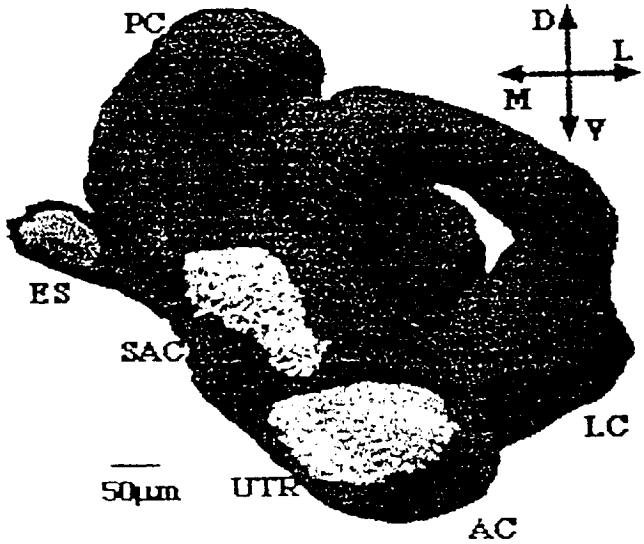
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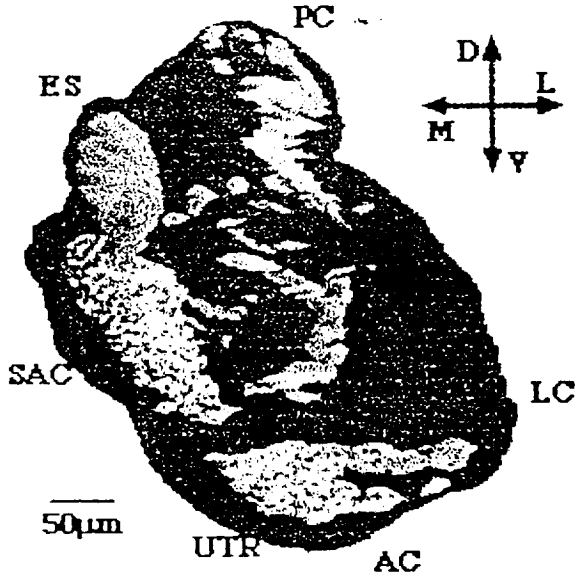
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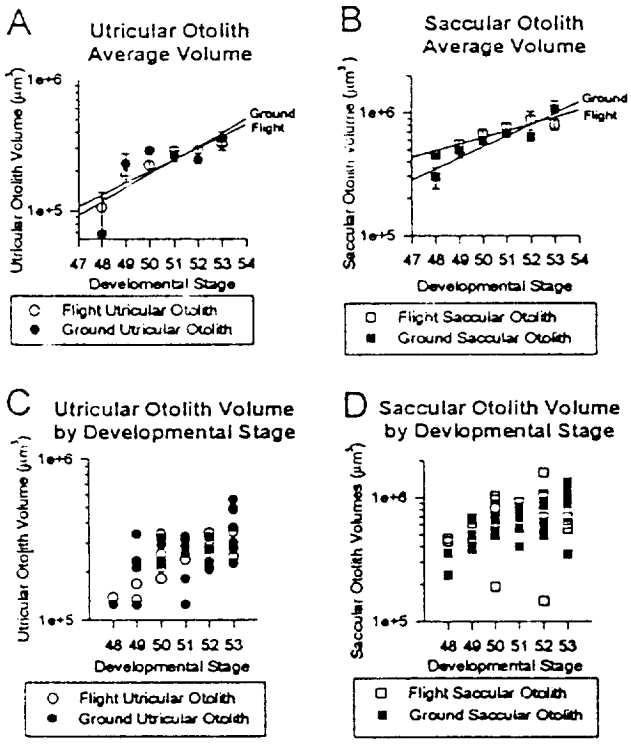

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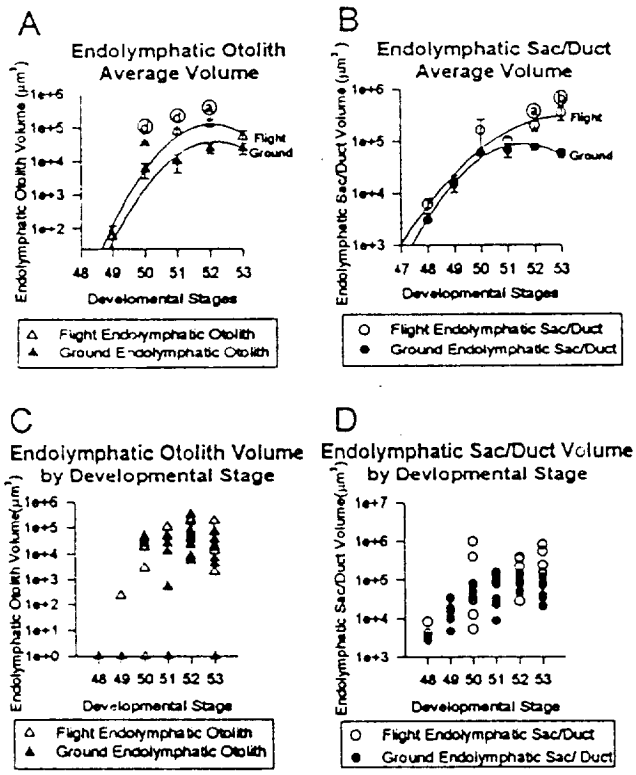

DEVELOPMENT OF GRAVITY-SENSING ORGANS IN ALTERED GRAVITY CONDITIONS: OPPOSITE CONCLUSIONS FROM AN AMPHIBIAN AND A MOLLUSCAN PREPARATION

NAG 2-730

Michael L. Wiederhold, Hugo A. Pedrozo, Jeffrey L. Harrison, Robert Hejl and Wenyuan Gao

University of Texas Health Science Center
San Antonio, Texas 78284-7777, USA

INTRODUCTION

Several components of the systems animals use to orient to gravity might develop differently in µg. If the growth of the "test masses" on which gravity acts (otoliths, in vertebrates, statoliths or statoconia in most invertebrates) is controlled on the basis of their weight, larger otoliths (or their analogs) would be expected to develop in µg.

The vestibular systems in animals reared in altered gravity have been studied in several species, with varied results being reported. Early Russian reports of *Xenopus* larvae reared in space indicated no qualitative differences in the vestibular organs, compared to ground-reared controls (1,2). Neubert (3) reported a similar lack of differences in *Xenopus*. Lychakov and Lavrova (4) did report that the utricular otolith was 30% larger in space-reared Xenopus. Lim et al. (5) found no differences in saccular otolith volume between centrifuged and control adult rats. Howland and Ballarino (6) reported a delay in otoconial development in chick embryos reared at 2g on a centrifuge, but in a later report (7) found no difference in otolith weight between 2g and control chicks. Souza et al. (8) report increased optokinetic responses in flight-reared *Xenopus* tadpoles, suggesting that the animals reared in the absence of gravity made greater relative use of their visual system, rather than the vestibular system, in orienting to a moving stimulus.

To test early development in microgravity, fertilized eggs of the Japanese newt, *Cynops pyrrhogaster*, were maintained in orbit for 15 days on the IML-2 mission in 1994. All specimens reached orbit before any otoconia were formed and all major components of the inner ear were formed by the end of the flight.

In ground-based studies of the *Aplysia* statocyst, the volume of the statolith in embryos and the number statoconia in post-metamorphic animals were compared between 1-g controls and specimens reared at 2 to 5.7 g.

METHODS

For the IML-2 Shuttle mission, 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 (AAEU). Spawning was induced by injection of human chorionic gonadotropin, after warming from hibernation. The developmental stage was determined by external morphological characteristics, as described by Okada (9, 10). One hundred and forty-four eggs were launched, each in a 6mm diameter, 12 mm deep chamber, through which aerated fresh water, at 22-24 °C, was circulated. The same number of eggs were loaded into a ground-control AAEU, maintained at the same temperatures.

The flight cassettes were retrieved about 6 hours after shuttle landing. Some larvae were fixed with 2.5% paraformaldehyde and 1.5% glutaraldehyde, dehydrated and embedded in Medcast plastic for sectioning. Some larvae were tested to estimate the gain of the otolith-ocular reflex. 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 as described by Koike (11, 12).

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 3 µm 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, NIH Image and ROSS (13) software. The same analysis techniques were used to measure statolith volume in *Aplysia* embryos reared in artificial seawater either statically or on a centrifuge at 2, 3 or 5.7 g.

RESULTS

In the marine mollusk *Aplysia californica*, the statocyst is a spherical organ whose wall is comprised of 13 mechanoreceptor cells and numerous smaller supporting cells. The lumen is filled with fluid and a single statolith from about 4 days after the eggs are laid through metamorphosis (60 - 100 days) after which multiple statoconia are formed in the supporting cells and exocytosed into the cyst lumen (14). Embryos reared for 10 days on a centrifuge had consistently smaller statoliths, compared to 1-g controls, as illustrated in Figure 1 (see also 15, 16). The difference between control and centrifuge-reared statolith diameters was significant at the $P = 0.0001$ level for all three speeds. The differences between the effect of centrifugation at 2 and 3 g, 2 and 5.7 g and 3 and 5.7 g were all significant at $P \leq 0.0005$. Thus, the statolith is smaller, in a graded fashion the higher the g in which the embryos are reared. Similarly, when the post metamorphic *Aplysia*, just beginning to form multiple statoconia, were reared at high g, the number and size of statoconia were reduced. This was even found in isolated statocysts reared in culture at high g (17).

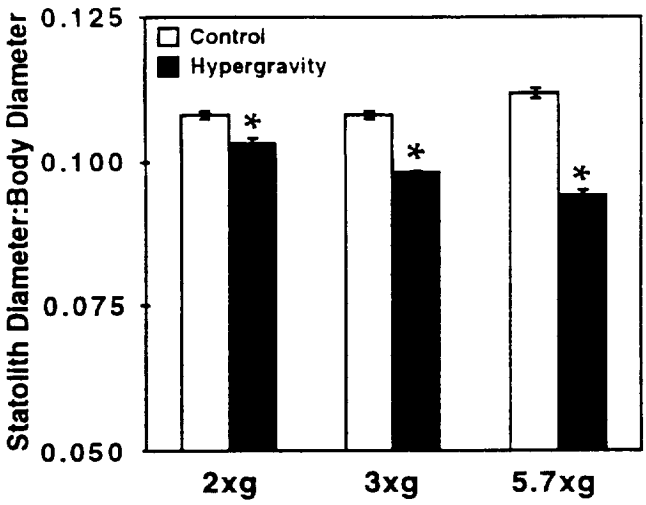

Figure 1. Ratio of statolith-to-body diameter of *Aplysia* embryos reared on a 15.5" diameter centrifuge for 10 days before fixation. * indicates significance at $P \leq 0.0001$ level between control and high-g animals at each g level.

Of the 144 newt eggs loaded into the AAEU on IML-2, 62 survived to the end of the flight. Progression through the developmental stages, assessed from high-magnification video recordings, was equivalent between flight and ground-reared specimens. Flight specimens were retrieved approximately 6 hours after shuttle landing. The surviving larvae had all hatched and swam vigorously. Post-flight samples were staged as they were studied physiologically and/or when they were fixed. Sixteen flight and 12 ground-control specimens were fixed and embedded for sectioning on landing day (R + 0), 8 flight and 8 ground specimens on R + 3 and 18 flight and 25 ground specimens on R + 5.

To illustrate changes in the inner ear between flight- and ground-reared larvae, Figure 2 shows 3-d reconstructions of serial sections through two stage-52 larvae. A: ground-control specimen, B: flight-reared larva, both fixed on R + 3.

There is considerable variation in the volumes of otoliths within specimens at a given developmental stage. In Figure 3 the mean +/- 1 standard error of the mean of the utricular (A) and saccular (B) otolith volumes as well as the volume of otoconia within the ES ("ES Otolith") (C) and of the ES lumen (D) are plotted v developmental stage for stages 48 - 53. In (A) and (B) the log of volume is well fit by a first-order regression with developmental stage but the differences between ground- and flight-reared specimens are not significant at the $P < 0.05$ level at any stage using the unpaired Students t-test. In (C) and (D), log of volume is fit with a 2nd order polynomial. The volume of the ES and its duct is significantly larger in flight-reared, compared to ground-control specimens ($P < 0.05$) at stage 53 (D). Although there is a clear tendency for the volume of otoconia within the ES to be larger in flight-reared larvae at the later stages (C), the difference is significant ($P \leq 0.05$) at stage 51, but not at stages 52

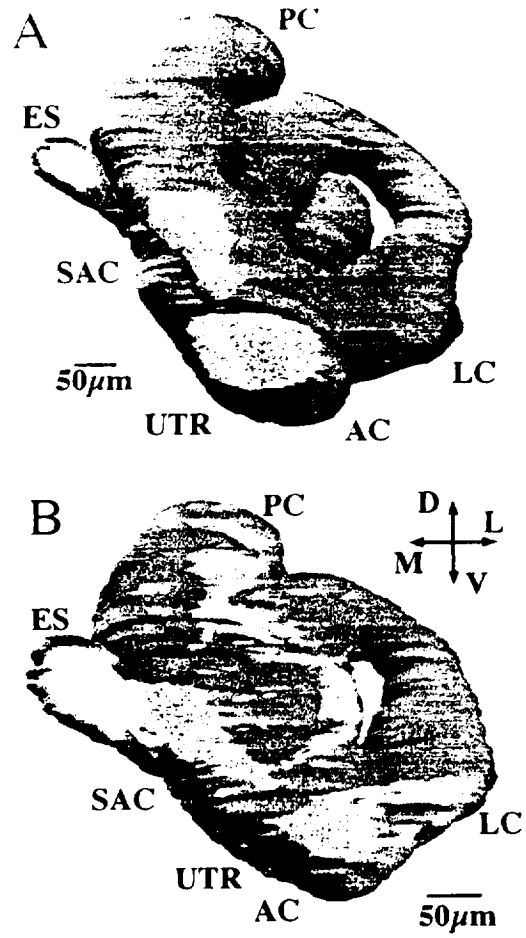

Figure 2. Reconstruction of serial sections through the developing otic vesicle of a ground-reared (A) and a flight-reared (B) stage 52 larva. Abbreviations: AC: Anterior semicircular canal; ES: endolymphatic sac; LC: lateral canal; PC: posterior canal; SAC: saccule; UTR: utricle; D: dorsal; V: ventral; M: medial; L: lateral. The pixelated areas are otoconia in the utricular and saccular otoliths whereas the lighter gray areas are otoconia within the ES and areas of the vestibule not normally containing otoconia.

and 53. Including all of the specimens at stages 51, 52 and 53, the average volume of ES and duct is 4.3 times greater and the average volume of endolymphatic otoconia is 3.0 times greater in flight-reared larvae than in ground controls. These differences are significant at the $P < 0.05$ level for the ES and duct and $P < 0.01$ for the ES otoconia.

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 otoconia in the ES. 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. The ES otoconia reflect light similar to that from the utricular and saccular otoliths. On day R + 0, no ES stones were seen in either ground-control or flight animals. On day R + 3,

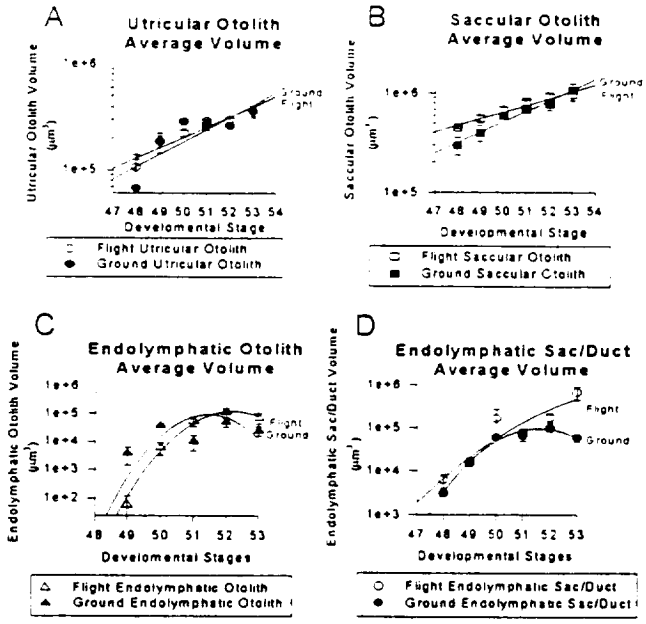

Figure 3. Plot of mean volume of utricular otolith (A) for flight-reared (open symbols) and ground-control (closed symbols), of the saccular otolith (B), volume of otoconia within the ES (C) and of the lumen of the ES and its duct (D) for the same groups. Error bars indicate +- one standard error of the mean. Lines in A and B indicate linear regression plots for the logarithm of otolith volume for flight and ground specimens. Lines in C and D are 2nd order polynomial fits to data. All measurements from specimens fixed within 5 days of Shuttle landing.

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. 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.

Using an X-ray microfocus system (11, 12), the area of the utricular and saccular otoliths were not significantly different between flight and ground-reared specimens within the first week after Shuttle landing. One specimen was maintained for nine months after the flight. The volume of otoconia within 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 (12, 18).

CONCLUSIONS

The hypothesis that an animal which develops in hyper-g would, by some mechanism, decrease the mass of the "otolith" developed, to compensate for its increased weight, was confirmed in the *Aplysia* statocyst. However, within the first week after rearing in µg, the utricular and saccular otoliths in the newt were clearly not of increased size. However, the production of otoconia in the endolymphatic system was enhanced in the flight-reared larvae. The ES otoconia 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 (19). 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 (20). 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 (19).

Apparently the system which produces the aragonitic otoconia in the ES is enhanced in space-reared larvae. Amphibians store calcium in the ES otoconia since they lack trabecular bone, where calcium is exchanged in mammals (21). Perhaps there is some change in calcium metabolism in these larvae growing in µg 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 (12, 18).

Endolymphatic sac otoconia were more prevalent in the ground AAEU, compared to laboratory-reared larvae. This suggests that the AAEU egg chambers might somehow have an effect similar to that of µg. 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° 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 (22). Thus, the constraint of the egg hole appears to act as a clinostat, allowing the larvae to orient in any direction and averaging out the direction of gravity with respect to body axes of the developing larva. Somehow this randomization appears to enhance 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.

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