Early Development of Gravity-Sensing Organs in Microgravity

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ABSTRACT

Most animals have organs that sense gravity. These organs use dense stones (called otoliths or statoconia), which rest on the sensitive hairs of specialized gravity- and motion-sensing cells. The weight of the stones bends the hairs in the direction of gravitational pull. The cells in turn send a coded representation of the gravity or motion stimulus to the central nervous system. Previous experiments, in which the eggs or larvae of a marine mollusk (*Aplysia californica*, the sea hare) were raised on a centrifuge, demonstrated that the size of the stones (or "test mass") was reduced in a graded manner as the gravity field was increased. This suggests that some control mechanism was acting to "normalize" the weight of the stones. The experiments described here were designed to test the hypothesis that, during their initial development, the mass of the stones is regulated to achieve a desired *weight*. If this is the case, we would expect a larger-than-normal otolith would develop in animals reared in the weightlessness of space. To test this, freshwater snails and swordtail fish were studied after spaceflight. The snails mated in space, and the stones (statoconia) in their statocysts developed in microgravity. Pre-mated adult female swordtail fish were flown on the Space Shuttle, and the developing larvae were collected after landing. Juvenile fish, where the larval development had taken place on the ground, were also flown. In snails that developed in space, the total volume of statoconia forming the test mass was 50% greater than in size-matched snails reared in functionally identical equipment on the ground. In the swordtail fish, the size of otoliths was compared between ground- and flight-reared larvae of the same size. For later-stage larvae, the growth of the otolith was significantly greater in the flight-reared fish. However, juvenile fish showed no significant difference in otolith size between flight- and ground-reared fish. Thus, it appears that fish and snails reared in space do produce larger-than-normal otoliths (or their analogs), apparently in an attempt to compensate for the reduced weight of the stones in space. The fish data suggest that there is a critical period during which altered gravity can affect the size of the test mass, since the larval, but not the juvenile, fish showed the changes.

INTRODUCTION

Most animals sense gravity using dense stones (called otoliths, statoliths, or statoconia) that rest on the sensitive hairs of specialized gravity- and motion-sensing cells. The weight of the stones bends the hairs in the direction of gravitational pull. This excites the hair cells and their associated nerve fibers. The brain uses this information to determine position in the gravity field (i.e., upright vs. lying down) and the direction of movements. In many species, from snails to humans, the stones are either one or a collection of dense calcium carbonate ($CaCO_3$) crystals. These crystals can be separate (in many mollusks), held together by a gelatinous structure (in amphibians and mammals), or fused into three large otoliths in each ear (in fish). Little is known about the mechanisms that control the production and growth of the otoliths and their analogs. In species that grow continually, such as fish, the otoliths continue to grow throughout life; whereas in species that reach a final size, such as humans, the otoliths also stop growing. Thus, there must be mechanisms for initiating and terminating the mineralization of the otoliths.

The bulk of existing data suggests that maintaining *adult* animals in altered gravity, either in hypergravity on a centrifuge or in the microgravity in space, does not substantially affect the otoliths or their analogs (Sondag, 1996). In *developing* animals, however, there may be profound effects. To test the hypothesis that gravity could influence the early development of the stones, we first studied the marine mollusk *Aplysia californica*. This mollusk has a gravity-sensing organ (statocyst) very similar to the pond snail's. In *Aplysia*, there is a single stone (statolith) when the mollusk hatches; multiple stones (statoconia) are not developed until the mollusk undergoes metamorphosis 60 to 100 days later. Developing eggs or isolated statocysts were reared on a centrifuge with G-values ranging from one to five (Pedrozo, 1994). The diameter of the statolith and the volume and number of statoconia were all reduced, in a graded manner, as the centrifuge speed (G-level) was increased.

On a previous Space Shuttle mission (IML-2, 1994), we flew developing eggs of the Japanese red-bellied newt, *Cynops pyrrhogaster*. The eggs were staged so they would reach orbit before any stones were formed. Particularly in the stones made of aragonite (another crystal form of $CaCO_3$), the otoliths were as much as five times the volume of those in ground control newts of the same developmental stage (Wiederhold, 1997 a,b, Koike, 1995 a,b). Lychakov et al. found the utricular otolith to be 30% larger in space-reared frog (*Xenopus*) larvae (Lychakov, 1985); and Anken et al. have reported that in cichlid fish reared on a centrifuge, the saccular otolith was smaller than in one-G controls (Anken, 1998).

These previous experiments suggested that gravity may affect the stones in the developing gravity sensor. Our Neurolab experiments were designed to test the hypothesis that during the initial development of the otolith, the mass of the stones is regulated to achieve a desired *weight*. If this is the case, we would expect a larger-than-normal otolith to be developed in animals reared in the microgravity of space. This modification would produce an average stimulus to the otolith organs in space closer to what they would receive at one-G on Earth. On the Neurolab (STS-90) and the preceding STS-89 Shuttle flights, we had the opportunity to study pond snails reared or conceived in space and juvenile and embryonic fish reared in space.

We used animals that develop rapidly so that they would undergo a significant portion of their development during the 10–8 days usually available on Space Shuttle missions. Like many gastropod mollusks, the pond snail, *Biomphalaria glabrata*, has a very simple gravity-sensing organ, the statocyst. Figure 1 is a schematic drawing of the statocyst. The organ is spherical; the wall is made of 13 sensory receptor cells with smaller supporting cells between them. The lumen of the cyst is filled with fluid (statolymph) and dense stones (statoconia), which fall to the bottom of the cyst in the one-G environment of Earth. The statoconia are produced in the supporting cells and are ejected (exocytosed) into the cyst lumen. The cilia on the receptor cells are motile, beating continuously. On Earth, the statoconia sink to the bottom of the cyst. They interact with the beating cilia of the receptor cells at the bottom. It is this interaction between the beating cilia and the dense statoconia that leads to stimulation of the receptor cells and initiation of impulses in their axons (Wiederhold, 1974, 1978). These impulses give the central nervous system information on the direction of gravity and the direction and magnitude of accelerations due to movement. Larval snails can have as few as two or three statoconia, but adults have 300–400 statoconia in each statocyst (Gao, 1997b). The snails are hermaphrodites, so any pair can mate. They produce

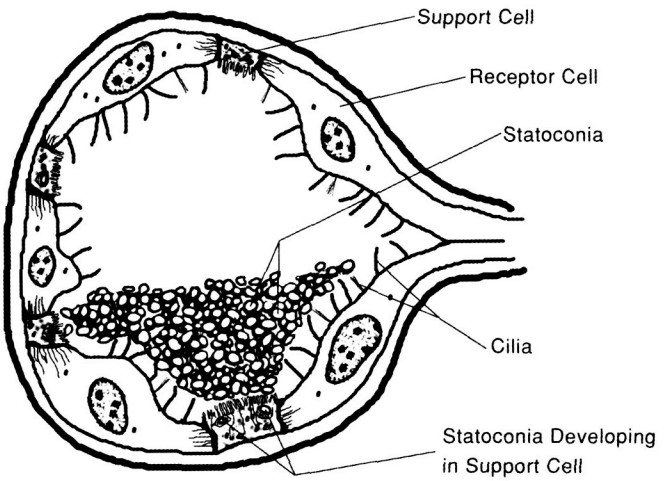

Figure 1. Schematic drawing of the gastropod molluscan statocyst. The cyst is approximately 150 μm in diameter in an adult *Biomphalaria* snail. The cyst wall contains 13 ciliated mechanoreceptor cells, separated by numerous supporting cells, which bear microvilli. The statoconia are produced in the supporting cells and ejected (exocytosed) into the cyst lumen. On Earth, gravity holds the statoconia in the bottom third of the cyst lumen, although the beating cilia keep them in constant motion.

approximately 20 to 30 eggs almost every day. About five days after the eggs are laid, the larvae hatch. The hatched larvae have a fully formed statocyst with three to 10 statoconia.

On Earth, snails tend to crawl downward on an aquarium wall. This is an example of behavior controlled by the statocysts. Instead of crawling in any direction, the snails show a clear preference for crawling downward. After crawling to the bottom, the snails detach from the wall, inflate an air bubble under the shell, and float to the surface. When they contact the wall again, the cycle recurs. This preferential downward crawling is exhibited immediately after they hatch from the egg.

The swordtail fish (*Xiphophorus helleri*) has two gravity-sensing organs similar to those in mammals (the saccule and utricle), as well as a third otolith organ, the lagena, which is often seen in fish, birds, and reptiles. These three organs are sometimes referred to as the sagitta (saccule), lapillus (utricle), and astericus (lagena). The three otoliths are each a solid stone, made of $CaCO_3$, that continues to grow throughout the life of the fish. As in mammals, the otolith lies above a sensory epithelium containing hair cells. Due to the stones' inertia, when the head is accelerated or pulled upon by gravity, the hair bundles of the hair cells are bent, leading to excitation of the cell. Synapses on the hair cells depolarize the endings of the vestibular nerve fibers and increase or decrease the firing of impulses in its fibers, which carry the coded acceleration information to the brain. The location and orientation of the otolith organs and the three otoliths are illustrated in Figure 2.

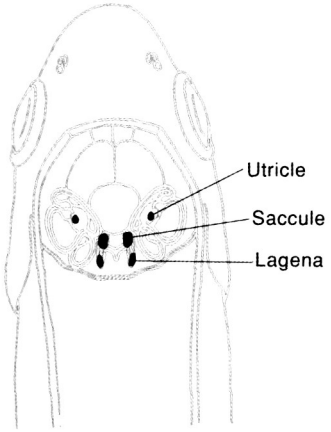

Figure 2. Schematic drawing of fish inner ear, showing the three semicircular canals and the three otolith organs in each ear, with the otoliths represented in solid black.

By studying the pond snails and swordtail fish, our studies addressed the effect of the relative absence of gravity on the early development of the stones in gravity-sensing organs. On Earth, problems such as motion sickness and balance disorders can often be caused by gravity sensors in the inner ear. Basic research into how and when gravity sensors develop will help us better understand inner ear disorders. In space, it will be important to know whether animals born and raised in zero-G will have permanently altered gravity sensors. This knowledge will be especially vital in the future, when planners try to determine whether humans could be born and raised in low gravity.

METHODS

Thirty-five adult pond snails (*Biomphalaria glabrata*), four previously mated adult female swordtail fish (*Xiphophorus helleri*), and 50 to 200 juvenile swordtail fish, approximately one-centimeter long, were loaded into the closed equilibrated biological aquatic system (CEBAS) (Slenzka, 1999) two days before the Shuttle launch. The CEBAS contains seven liters of spring water and 50 grams of the hornweed plant, *Ceratophylum*. The CEBAS has four chambers. The adult fish and snails were in the front chamber; juvenile fish and adult snails were in the second animal chamber. The third chamber was a plant chamber, containing the hornweed plants and also some adult snails. The fourth chamber was filled with filter material. A system of lights illuminated the plant chamber, and dissolved oxygen (DO) was monitored in the circulating water. When DO dropped below four mg/L, extra lights were turned on until DO reached six mg/L. The system was equipped with a video camera that monitored fish and snail behavior throughout the two Shuttle missions. STS-89 was a 10-day flight, and Neurolab, STS-90, was 16 days. On both flights, many snails mated and produced a large number of spawn packs. Our results came from young snails retrieved on landing day with shell diameters ranging from 0.5 to 2.0 mm. Ground control animals were maintained in a functionally identical CEBAS unit maintained at the Kennedy Space Center (KSC).

The swordtail fish, *Xiphophorus*, reproduce by internal fertilization, and the embryos in the ovary develop to a stage where the otoliths are well formed before the fry are hatched. After a single mating, the female generally produces three broods at one-month intervals. The four mated, adult female fish were selected from previous matings such that the fry would not be hatched until after Shuttle landing. Our results are from fish embryos with spinal cords from three to 10 mm long.

The CEBAS unit was removed from the Shuttle and received in the laboratory at KSC approximately 3.5 hours after Shuttle landing. Half of the small snails were preserved for anatomical study on landing day, and the other half were preserved five days later. Details of fixation of snail statocysts for light and electron microscopy are given in Gao et al. (Gao, 1997a, b). Adult fish ovaries were removed and preserved in alcohol on landing day, as were the juvenile fish. Fish embryos were removed from the ovaries after returning to our home laboratory. Snails were removed from their shell during fixation and sectioned at 1–2 μm. The total volume of statoconia was estimated by outlining the area occupied by statoconia in serial sections through the statocyst, adding the areas, and multiplying by the section thickness.

After removing the otoliths from the fish specimens, the otoliths were rinsed in distilled water, dried, and mounted either on glass slides, in well slides, or on stubs for scanning electron microscopic (SEM) analysis. All otoliths were placed with the flatter side down, and the area of each was measured using either Bioquant or UTHSCSA ImageTool software. Images were obtained with a Spot 2 digital camera. The area of each otolith in these micrographs was measured and plotted against total body length for juveniles or against spine length for embryos. A linear regression analysis was performed for the flight- and ground-reared specimens for each group, relating area to animal or spine length. The flight and ground regression lines were compared by an analysis of covariance for the significance of differences in their slopes. When measurements of

both otoliths (of any type) from one animal were available (the usual case), the two were averaged. If the slopes were not significantly different ($p>0.05$), both groups were fit with a line of the same slope and the difference in mean otolith area at the mean length was tested for significance.

RESULTS

Videotaping of the animal chambers in the CEBAS revealed that, during orbital flight, the snails were easily dislodged from the aquarium walls. On Earth they spend most of their time attached to the walls. Once separated from the wall, the water circulation carried them in a rotating pattern around each chamber. In the front two chambers, it was evident that snails kept their feet extended, apparently searching for a substrate to which to attach. In this situation, they were likely to come into contact with another snail, and mating pairs were often seen floating attached to one another. Thus, it was not surprising that a large number of small snails were retrieved on Shuttle landing. After the STS-89 (10-day) mission, 251 snails from 0.5 to 4.0 mm diameter were retrieved; and on the STS-90 (16-day) mission, 206 snails in the same size range were retrieved. In the ground control run, 308 small snails were retrieved for STS-89 and 155 ground control snails were retrieved during STS-90.

Figure 3 is a photomicrograph of a 1.0-µm section through the statocyst of a two-mm-diameter snail, taken using Nomarsky optics. The statoconia appear brightly colored in the polarized light due to their ability to refract light. This

Figure 3. 1.0-µm section through the statocyst of a two-mm shell-diameter pond snail, *Biomphalaria glabrata*. The micrograph was taken with Nomarsky optics. The polarized light makes the statoconia appear bright and multicolored, due to their highly birefringent optical properties. This makes the statoconia easy to identify and outline, from which the cross-sectional area is calculated and multiplied by the section thickness to obtain total statoconia volume.

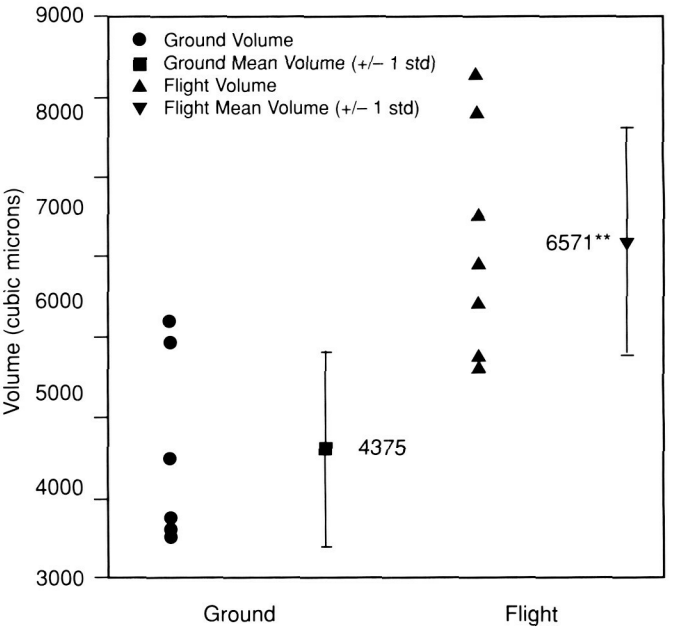

Figure 4. Plot of total statoconia volume for six ground-reared and seven flight-reared snails of two-mm-shell diameter, fixed on landing day after STS-89. The mean and one standard deviation for each group are indicated to the right of the individual data points. The mean total volume is 50% greater for the flight-reared snails in this case. The difference between flight- and ground-reared mean volume is significant at $p<0.01$.

makes it easy to identify the statoconia and outline the area occupied by statoconia in each section. Figure 4 plots total statoconia volume for flight- and ground-reared two-mm snails fixed on landing day of STS-89. Each measurement for six ground- and seven flight-reared specimens is displayed. It is apparent that considerable variation in total volume exists within both groups, but the difference in mean values for the two groups is statistically significant. In this case, the average volume of statoconia is 50% greater in the flight-reared snails. Sufficient snails were available to make meaningful comparison between flight- and ground-reared animals of one and two or 1.5-mm-diameter snails, both on landing day (R+0) and five days after return to Earth (R+5). The ratios of total statoconia volume for flight and ground-control snails fixed on R+0 and R+5 are shown in Table 1. Note that the mean volume of statoconia is significantly greater in the flight-reared snails in all groups, except for those from STS-90 fixed at R+0, where no significant difference between the groups was seen. Note also that for three of the four groups, excepting only the one- and 1.5-mm snails from STS-90, the ratio of flight- to ground-reared statoconia volume was larger at R+5 than at R+0. That is, the increase in statoconia volume, which occurred in space, was even more pronounced in snails allowed to develop for another five days on Earth before they were fixed.

Table 1. Total Statoconia Volume Ratio: Flight/Ground

STS-89 R+0		R+5	
1 mm:	1.36*	1 mm:	2.16*
2 mm:	1.50*	2 mm:	1.87*
STS-90 R+0		**R+5**	
1 mm:	1.67	1.5 mm:	1.20*
2 mm:	0.95	2 mm:	1.38*
Average:	1.37	Average:	1.73

Overall Average = 1.55
*$p \leq 0.05$ to 10^{-6}

Figure 5 shows a plot of the distribution of sizes of statoconia. This is the size distribution for statoconia from a two-mm snail fixed on landing day from STS-90. The general shape of the distribution of areas is similar between flight- and ground-reared specimens. The distribution is slightly shifted to smaller sizes in the flight-reared snails, but by less than two bin widths; i.e., by less than 10 μm^2, with a modal value of 20 to 30 μm^2. In this case, the mean statoconia area is 33 μm^2 for ground-control snails and 29 μm^2 for flight-reared snails. Figure 6 shows the distribution of total number of statoconia in flight- and ground-reared two-mm snails. It is apparent that the flight-reared snails had approximately 50% more statoconia than size-matched, ground-reared snails. These results indicate that rearing in microgravity causes the supporting cells to produce more statoconia, which are of approximately the same size as those in ground-control snails. This implies that the effects are mediated within the supporting cells and probably do not affect the growth of statoconia once they have left the supporting cells and are in the lumen of the statocyst.

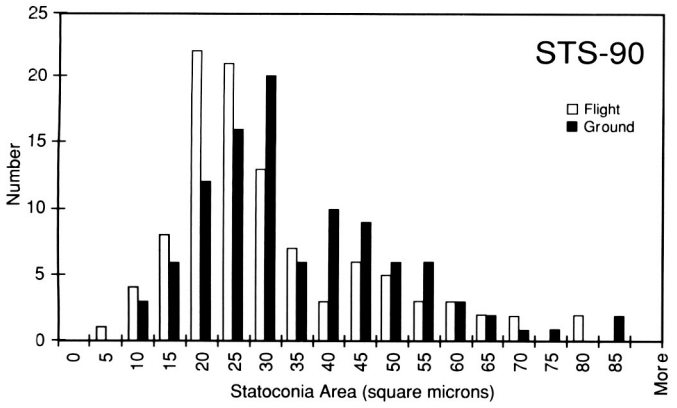

Figure 5. Histograms showing the distribution of areas, as seen in light micrographs, of statoconia lying on their flattest surface, isolated from two-mm-shell-diameter snails, removed on landing day after STS-90. The overall distribution is little different between flight and ground control groups, with the flight-reared statoconia being slightly smaller. The mean area for flight-reared statoconia is 29 μm^2; and for the ground-reared animals, the average is 33 μm^2.

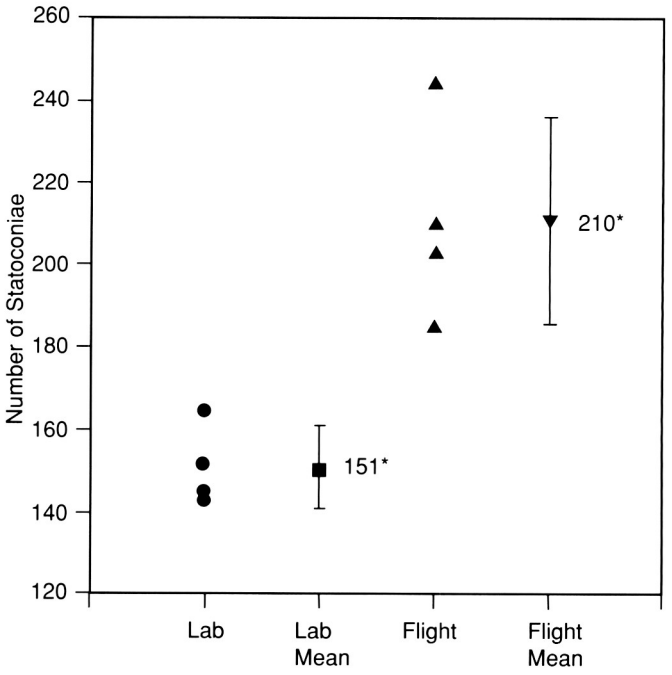

Figure 6. Plot of number of statoconia retrieved from two-mm-shell-diameter snails retrieved from the flight and ground-reared groups from STS-90. In this case, the average number of statoconia was 39% greater in the flight-reared snails.

The data shown above suggest that the process of statoconia development in the supporting cells is modified in snails reared in microgravity. We had only two flight-reared specimens available for transmission electron microscopy (TEM); but the four statocysts from these animals, one fixed on (R+0) and the other on (R+5), demonstrate significant modifications in the flight-reared statocysts. Figure 7a is an electron micrograph from a two-mm flight-reared snail fixed on landing day. It shows three large vacuoles in a thin section through a supporting cell at the ventral side of the statocyst. These vacuoles are from 0.8 to 1.3 µm in diameter. In ground-reared snails, very few vacuoles are seen in the supporting cells, and their maximum size is 0.1 µm in diameter. Thus, the vacuoles in this flight-reared snail have approximately 1000-fold greater volume than those of ground control animals. The flocculent material seen in the upper vacuole suggests that the formation of statoconia is initiated within the vacuoles and that the increased number of statoconia is likely a direct result of the larger volume of vacuoles.

Consistent with the increased number of statoconia in flight-reared snails, more statoconia were seen in the supporting cells of these animals. Figure 7B is an electron micrograph of a thin section through another supporting cell from the same snail as in Figure 7A. This illustration shows three intracellular statoconia. These statoconia appear as "ghosts," probably demineralized during fixation. In an adjacent one-µm section, these statoconia displayed brightly colored birefringence (refraction) as shown in Figure 3, indicating that the statoconia were fully mineralized. In ground control snails, it is generally difficult to find statoconia in the supporting cells; and we have never seen more than one statoconium in a supporting cell.

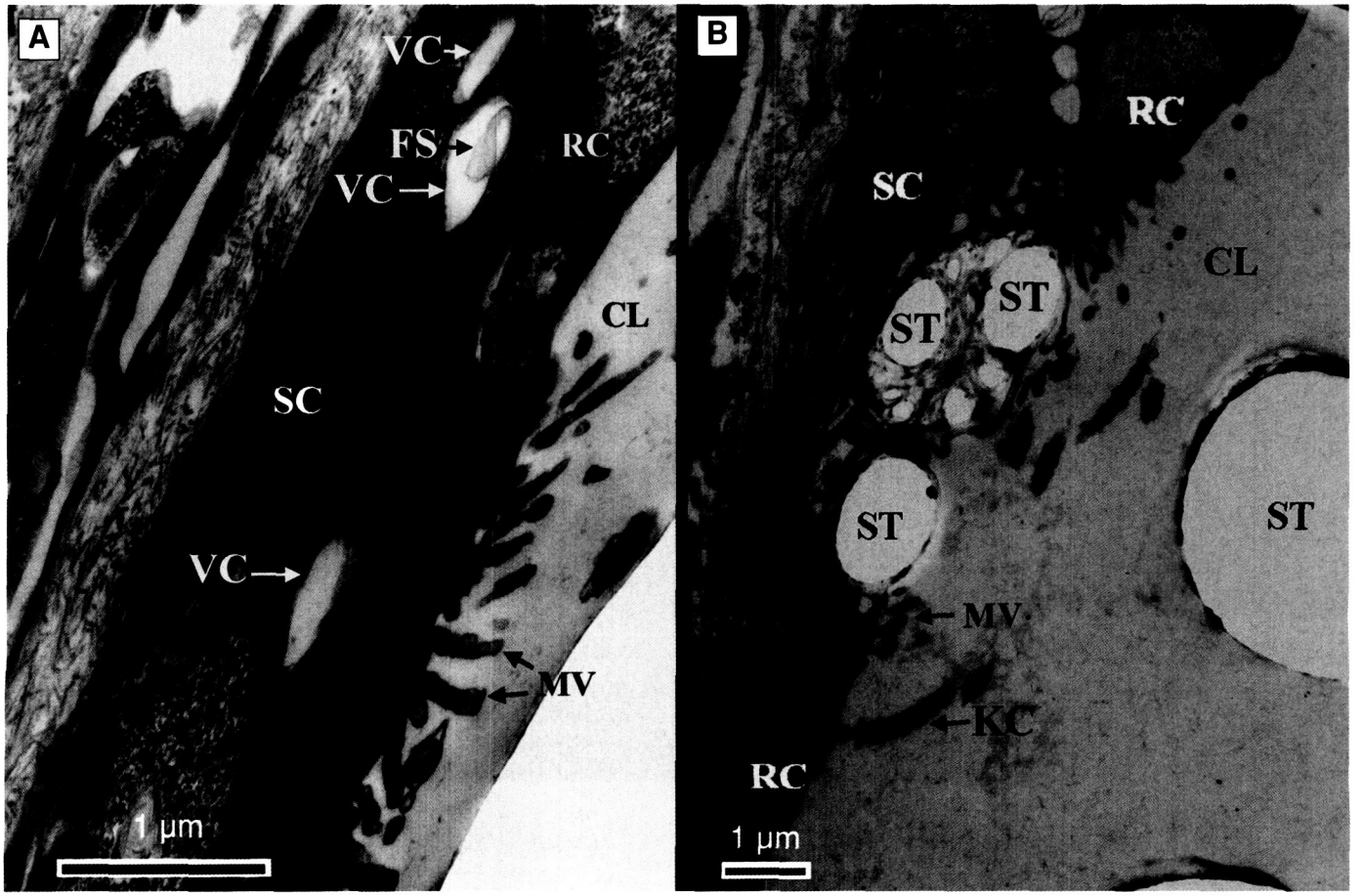

Figure 7. TEMs of supporting and receptor cells from a statocyst of a two-mm flight-reared snail. A. Note three large vacuoles (VCs) in the supporting cell (SC). Flocculent substance (FS) seen in one vacuole. MV: microvilli seen on supporting cells. Receptor cell (RC) is seen adjacent to the middle supporting cell. CL: statocyst lumen. B. Ghosts of three forming statoconia (ST) seen in the supporting cell. Two receptor cells, bearing cilia, are seen on either side of the supporting cell. Ghost of another statoconium is seen is the cyst lumen. Ghosts represent demineralized statoconia.

Thus, increased vacuoles and intracellular statoconia are seen in the supporting cells of flight-reared snails.

Figure 8 shows light micrographs of the three pairs of otoliths from a one-centimeter-long juvenile *Xiphophorus* and from an embryo with a spine length of five mm.

The juvenile fish retrieved from the STS-89 flight ranged from 8.2 to 10.7 mm in length. Figure 9 plots the area of 42 utricular otoliths from 23 ground-reared juvenile fish and 39 otoliths from 20 flight-reared juvenile fish from the STS-89 flight. The area of the otolith (measured from the micrographs of the otoliths resting on their flattest surface) is plotted against the total fish length (measured when the fish were retrieved from the CEBAS.) Neither the slopes nor the mean values for the two regression lines were significantly different between flight and ground control groups (p=0.7904 for slopes, p=0.3068 for means). The same conclusions were drawn for the saccular otolith size for STS-89 juvenile fish (p=0.3974 for slopes, p=0.3553 for means), although the lagenar otoliths were marginally larger in the flight-reared juveniles compared to ground controls, with p=0.0806 for slopes and p=0.0222 for means. The slope for the flight lagenas is 4342±1017 µm²/mm (fish length, mean±SEM). For the ground controls, the slope is 890±1633 µm²/mm.

In contrast to the juvenile fish, there was a significant difference in otolith size between ground- and flight-reared fish embryos from the STS-90 flight. The measurements of utricular otolith area for different size embryos from STS-90 are illustrated in Figure 10. Here the slope of the fit to the flight-reared embryos is significantly larger (p=0.008) than the fit to the ground-reared embryos. Thus, for embryos with spine length >4.5 mm, the flight-reared embryos did have significantly larger utricular otoliths. Similarly, the slope of the best fit to the flight-reared saccular otoliths was significantly larger than that for the ground controls (p=0.035). For the lagenar otoliths, the slope of the ground-reared otoliths was significantly larger than that for the flight specimens (p=0.0028), although there was a very weak relationship between spine length and otolith size in the group of flight animals available. The ground-control group contained three "outliers," with otoliths only a fraction of the area of the smallest otoliths in the rest of the ground and flight specimens. If these three points were deleted from the analysis, there was no significant

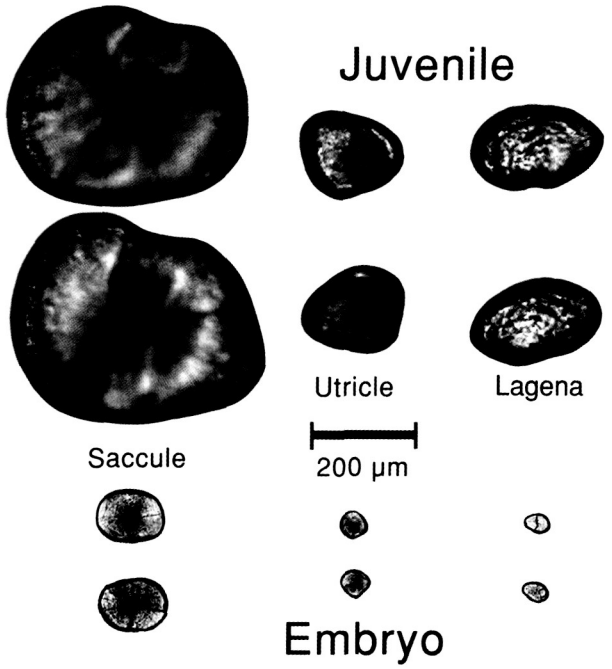

Figure 8. Light micrographs of otoliths of the saccule (sagitta), utricle (lapillus), and lagena (astericus) from a one-cm-long juvenile fish and from an embryo with a spine length of five mm. Both are shown at the same magnification. Scale bar is 200 µm.

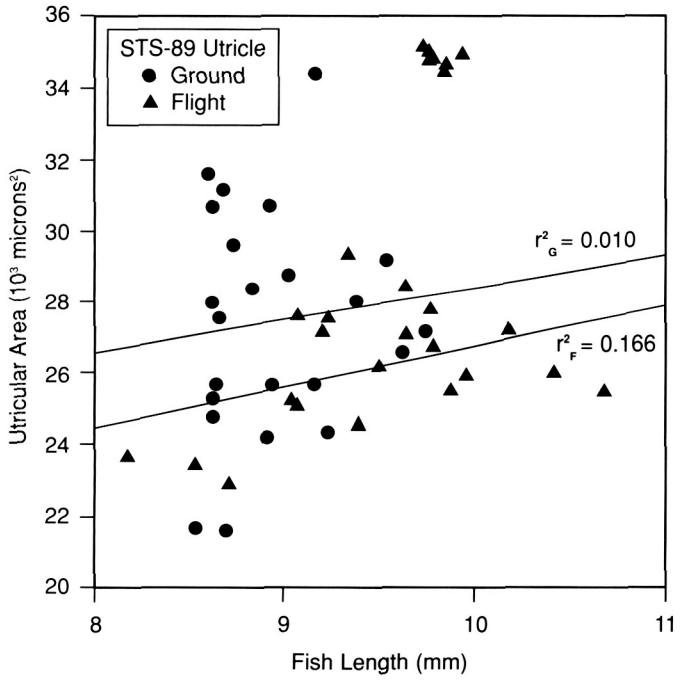

Figure 9. Plot of utricular otolith vs. total fish length for ground- (filled circles) and flight- (filled triangles) reared juvenile fish retrieved from STS-89. Each group was fit with a linear regression line. The square of the correlation coefficient for each regression line is given. An analysis of covariance indicated no significant difference in either slope or mean values between ground and flight groups.

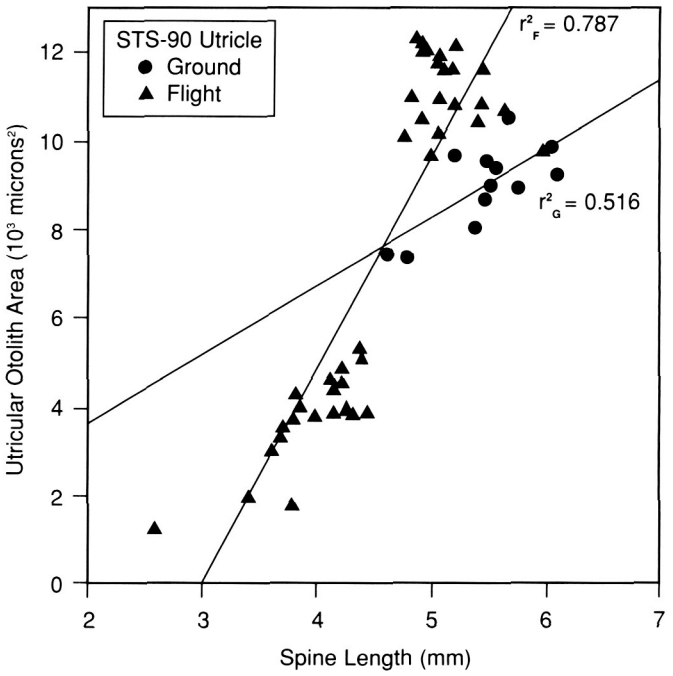

Figure 10. Plots of utricular otolith area vs. spine length from flight- and ground-reared fish embryos from STS-90. As in Figure 9, each group is fit with a linear regression line. Here the slope of the fit to the flight-reared group is significantly larger than that for the ground-reared controls (p=0.008). For flight-reared specimens, 82 otoliths from 42 embryos, and for ground-reared specimens, 24 otoliths from 12 embryos, were analyzed. For fish in which two utricular otoliths were retrieved and measured, the area is the mean of the two.

difference in slope between the flight and ground-reared groups.

Embryos were also retrieved from adult female fish on STS-89. For this nine-day flight, the embryos were considerably smaller than those obtained from STS-90 (16 days). Plots of utricular otolith area vs. spine length for the two groups of embryos from STS-89 are presented in Figure 11. In this case, the slope of the regression line fit to the flight-reared embryos is smaller than that for the ground control group. This difference is significant (p=0.0160). For the saccular otoliths in the STS-90 embryos, the growth of otolith area with spine length is also significantly greater for the ground-reared specimens (p=0.0054).

Some insight to the mechanisms that might underlie the production of larger otoliths in the larger embryos reared in space can be gained from an examination of the surface of the otoliths. This was done using an SEM. Figure 12 shows SEM images of saccular otoliths from a flight-reared embryo with a spine length of 5.5 mm and a ground-reared embryo with a spine length of 5.2 mm. The surface of the flight-reared otolith is covered with octahedral crystals with a square base of approximately one µm on a side. Such crystals were found

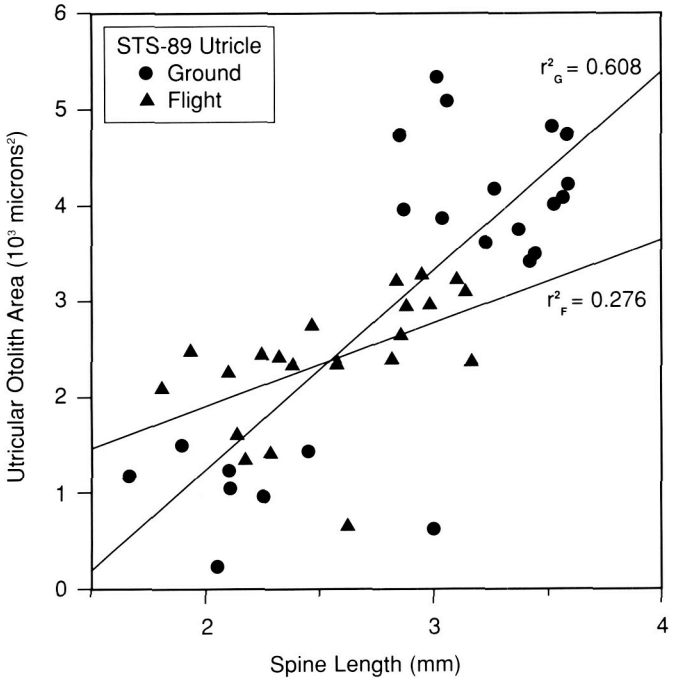

Figure 11. Plots of utricular otolith area vs. spine length from flight- and ground-reared fish embryos from STS-89. Regression lines as in Figures 9 and 10. Here the slope of the fit to the flight-reared group is significantly smaller than that for the ground-reared controls (p=0.0160). Note that embryos in this experiment were smaller than those retrieved from STS-90. For flight-reared specimens, 40 otoliths from 21 embryos, and for ground-reared specimens, 41 otoliths from 23 embryos were analyzed. Where both otoliths were measured, the mean was plotted.

on 110 of 207 flight otoliths (53%) but not on any of 75 ground otoliths examined. Among the flight otoliths, 39/88 (44%) of saccular otoliths, 39/72 (54%) of utricular otoliths, and 32/47 (68%) of lagenar otoliths exhibited these crystals. Thus, there is little difference among the three otoliths in the likelihood of forming surface crystals.

DISCUSSION

The finding of larger otoliths in later-stage embryos from STS-90 is consistent with our own experience with pond snails (Wiederhold, 1997 a,b), *Aplysia* (Pedrozo, 1994), and Anken's report (Anken, 1998) on centrifuged cichlid fish. Here the mass of the otoliths was increased in animals reared in the microgravity of space. The fact that significant differences were not seen in the juvenile fish (Figure 9) is compatible with Anken's conclusion that centrifugation did not affect older (juvenile) fish otoliths. The finding of smaller otoliths in flight-reared, early-stage embryos (Figure 11) is surprising. It is difficult to imagine a mechanism by which gravity (or the relative lack of gravity) could affect mineralization of the smallest otoliths, whose weight would be minimal. It is even more surprising to find an effect opposite to the effect on otoliths in embryos approximately one week older. These findings clearly illustrate that the effects of rearing in microgravity depend critically on the developmental stage at which the animal is exposed to microgravity.

The appearance of crystals on the surface of flight-reared otoliths suggests that the mechanism by which mineral is added to the otolith differs in the low gravity of space.

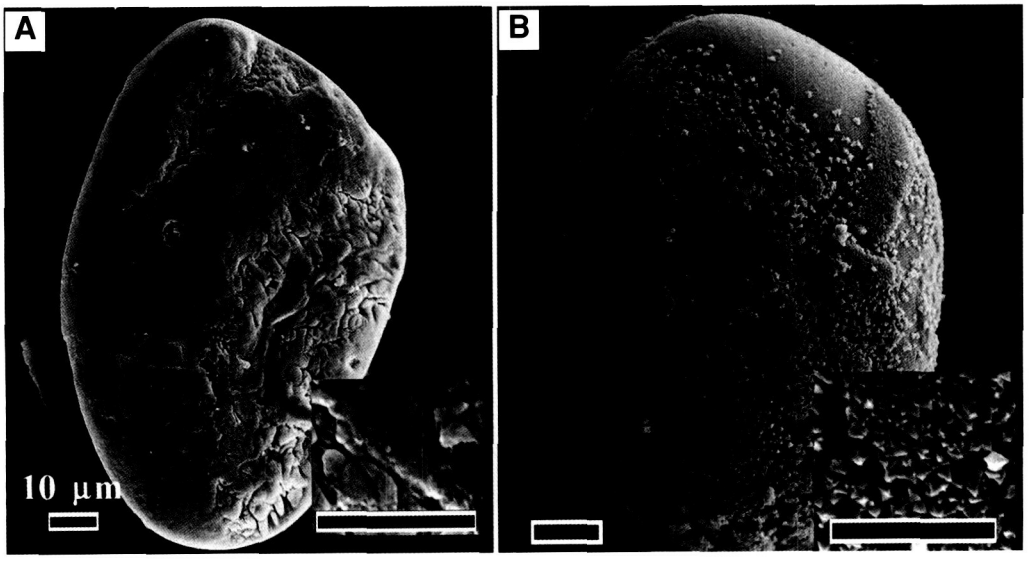

Figure 12. Scanning electron micrographs of saccular otoliths from a ground- (A) and a flight-reared (B) fish embryo with spine length of 5.5 mm. Insets in each panel show surface at 4× higher magnification. Note octahedral crystals seen on the flight-developed otolith. All scale bars are 10 µm.

It has been suggested that the supporting cells in the macula secrete the organic material in the otoliths (Harada, 1998). This secretion could be enhanced in low gravity, due to a reduced otolith input to the macula. The actual crystallization of the mineral component could also be altered in microgravity.

The results presented here and those previously published on newt larvae reared in space all indicate that early growth of the stones (test mass) in the gravity-sensing organs is regulated to produce an otolith, or its analog, of the appropriate *weight*. Thus, in microgravity, where a given mass has almost no weight, a larger mass is produced in an attempt to compensate for the reduced weight. Similarly, animals reared on a centrifuge produce smaller otoliths, since the increased G-force produces a greater weight with the same mass. The intriguing results with the fish embryos (Figures 9–11) suggest that there is a critical period, relatively early in development, in which the regulatory mechanism, which optimizes otolith weight, can act. The very early-stage fish data (Figure 11) suggest that the control mechanisms can even be reversed at the earliest stages. At the earliest stages, when the otoliths do not have sufficient mass to activate the postulated control mechanisms, we had expected to see no effect of altered gravity, so the reverse effect is all the more surprising. The statistical significance of the effects at the early stages suggests that as soon as the otoliths appear, they have sufficient mass to activate the control mechanism(s), presumably through the hair cells, but that a positive feedback is working to produce smaller otoliths at reduced gravity. Centrifuge experiments at the earliest stages have not been reported, so we do not know what the effects would be of rearing at hypergravity during the earliest stages of development.

It is important to note that in all of the systems in which we have demonstrated gravitational effects on otolith growth, the test mass is composed of $CaCO_3$ in the aragonite crystal form (Pedrozo, 1997; Wiederhold, 1994). In mammals, both the utricular and saccular otoliths are constructed of $CaCO_3$ in the calcite form (Carlstrom, 1963). In frogs and newts, the adult saccular otolith is aragonite whereas the utricular otolith is calcite (Wiederhold, 1994). In the newt larvae flown in 1994, we found an increase in the volume of the saccular otolith but not in the utricular otoliths of newts fixed at R+5. Repeated x-ray microfocus imaging (Koike, 1995b) showed a slight increase in the utricular otoliths of one newt at nine months postflight, but a much more pronounced increase in the saccular otoliths. If our findings do apply to calcite otoliths, this will need to be considered before attempts are made to have humans conceived and brought up in space. The size of the saccular otoliths was followed in groups of newts for nine months after a 15-day Shuttle mission. The size of the saccular otoliths was maximal, relative to ground controls, at two to three months postflight and only returned to "normal" size six months postflight. If mammals, including humans, were to be conceived and brought up in microgravity, we do not know whether their otoliths would be abnormally large while in space or whether they would revert to normal size at some time after introduction to one-G. We also do not know if the otolith-related reflexes developed in microgravity would function normally in one-G.

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