J. GRAVITATIONAL PHYSIOL. IN PRESS

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

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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).

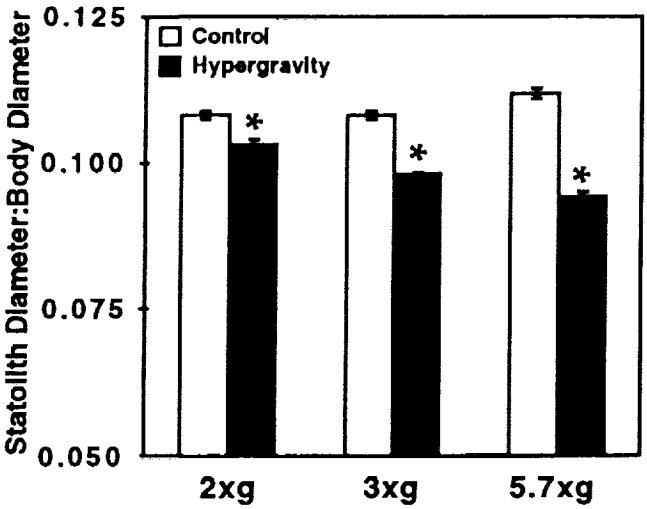

Figure 1. Ratio of statolith-to-body diameter of *Aplysia* embryos reared on a 15.5" diameter centrifuge for 10 days before fixation. * indicates significance at $P \leq 0.0001$ level between control and high-g animals at each g level.

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

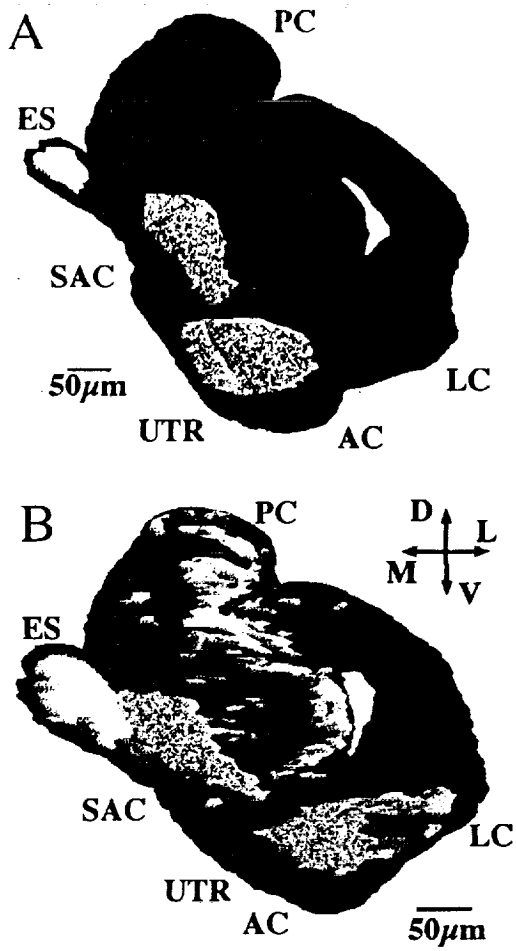

Figure 2. Reconstruction of serial sections through the developing otic vesicle of a ground-reared (A) and a flight-reared (B) stage 52 larva. Abbreviations: AC: Anterior semicircular canal; ES: endolymphatic sac; LC: lateral canal; PC: posterior canal; SAC: saccule; UTR: utricle; D: dorsal; V: ventral; 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,

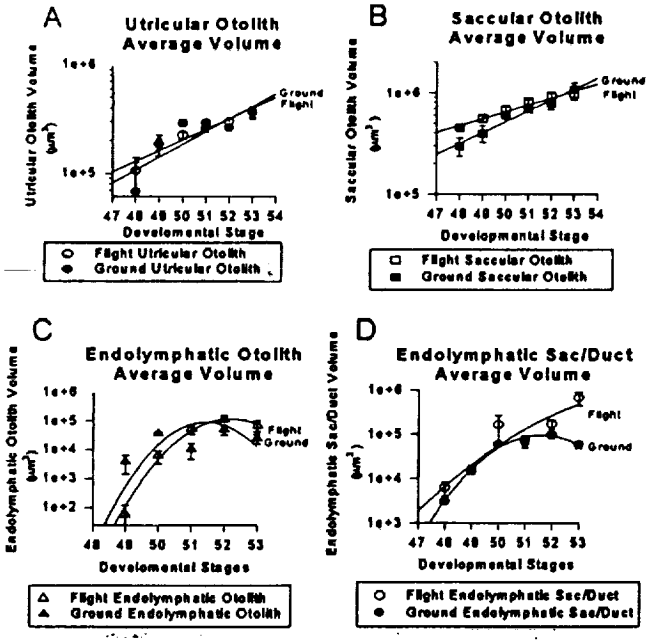

Figure 3. Plot of mean volume of utricular otolith (A) for flight-reared (open symbols) and ground-control (closed symbols), of the saccular otolith (B), volume of otoconia within the ES (C) and of the lumen of the ES and its duct (D) for the same groups. Error bars indicate +/- one standard error of 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.

ACKNOWLEDGEMENTS

We wish to thank Dr. Muriel Ross and her staff for sharing the ROSS software and helping us implement it. Supported by NASA: NAG 2-730 and 2-952 and NSF.

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