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Effects of hypergravity on statocyst development in embryonic *Aplysia californica*

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Abstract

Aplysia californica is a marine gastropod mollusc with bilaterally paired statocysts as gravity-receptor organs. Data from three experiments in which embryonic *Aplysia californica* were exposed to $2 \times g$ are discussed. The experimental groups were exposed to excess gravity until hatching (9–12 day), whereas control groups were maintained at normal gravity. Body diameter was measured before exposure to $2 \times g$. Statocyst, statolith and body diameter were each determined for samples of 20 embryos from each group on successive days. Exposure to excess gravity led to an increase in body size. Statocyst size was not affected by exposure to $2 \times g$. Statolith size decreased with treatment as indicated by smaller statolith-to-body ratios observed in the $2 \times g$ group in all three experiments. Mean statolith diameter was significantly smaller for the $2 \times g$ group in Experiment 1 but not in Experiments 2 and 3. Defective statocysts, characterized by very small or no statoliths, were found in the $2 \times g$ group in Experiments 1 and 2.

Key words: Statocyst; Statolith; Mineralization; Gravity; *Aplysia californica*

1. Introduction

The gravity-sensing organ in *Aplysia californica* consists of bilaterally paired statocysts. These organs are fluid-filled sacs containing dense stones in the form of either a single statolith or multiple statoconia. Under the effects of gravitational pull, these calcium-rich inclusions fall and interact with beating cilia on the underlying sensory cells. This interaction causes an increase in conductance, leading to depolarization of the receptor cells (Gallin and Wiederhold, 1977; Wiederhold, 1974).

Fertilized *Aplysia* eggs begin to develop a mesoderm after the fourth cell division and by the sixth cell division the three germ layers have been originated, i.e., mesoderm, endoderm and ectoderm. Soon after, the developing embryo reaches gastrulation and by day two or three it enters the trochophore stage. At this

stage (300 cell stage) it begins to rotate inside the egg case by ciliary movement of the velar cells, the prevelum. It soon begins to develop an internal cavity followed by internal organs and a nervous system. The statocyst develops at this stage (day 3) (Kandel, 1979). By day four or five the statolith becomes visible under the microscope. By day 5–9 the embryos enter the veliger stage and by days 9–14 they begin to hatch. The developmental time varies from one egg mass to another according to environmental conditions. The free-swimming veligers undergo 5 stages of development and by stage 6 ($5\frac{1}{2}$ weeks after hatching) they begin to metamorphose, becoming pelagic, i.e., crawlers.

The statocyst in embryonic *Aplysia californica* contains a single stone called the statolith. This statolith is the only stone within the statocyst until the animal reaches stage 10. At this point (60–100 days), production of multiple statoconia begins (Wiederhold et al., 1990). Using FTIR (Fourier Transform Infra-red Spectroscopy), it has been determined that the statoconia are composed of calcium carbonate in the aragonite form (unpublished data). The single stone (statolith) found in embryonic and newly metamorphosed animals has been reported to be aragonite (Bidwell et al.,

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1990). Otoconia, like other biominerals, contain an organic and an inorganic component. The inorganic phase in most vertebrates is a mineral salt of calcium carbonate; however the type and shape of the calcium carbonate mineral varies with the species (Carlström, 1963; Marmo, 1983; Pote and Ross, 1991). The organic phase of amphibian aragonitic otoconia is composed of two major proteins, 22 and 10 kDal (Pote and Ross, 1991). It is expected, but not yet shown, that the statolith and statoconia have an organic phase similar to other aragonitic otoconia.

Exposure to weightlessness during space flight has been reported to induce changes in vestibular function and decrease calcium mineralization in bones when compared to $1 \times g$. Russian investigators have reported vestibular asymmetry and disorders of mechanisms of opto-vestibular interaction (Gorgiladze and Matveev, 1991), as well as changes in optokinetic, opto-oculomotor and vestibulo-oculomotor reactions in humans (Kornilova et al., 1990). Exposure to weightlessness has also been shown to affect the bones in supporting limbs by decreasing their formation, volume (Morey-Holton et al., 1992; Wronski et al., 1980; Patterson-Buckendahl et al., 1987), and mineralization as well as by altering biomechanical properties (Morey-Holton et al., 1992; Patterson-Buckendahl et al., 1987). However, rat mandibles did not show any of the deficits of bone formation associated with weight-bearing bones (Simmons et al., 1980), whose function is to oppose the influence of gravity. This indicates that decalcification during exposure to weightlessness occurs in some systems whose function is related to gravity. Because of the effects of microgravity on calcium (Ca^{2+}) deposition and vestibular function, it is of interest to investigate whether changes in Ca^{2+} deposition contribute to changes in vestibular function. In order to approach this problem, we have investigated the effects of gravity on the development of the gravity-sensing organ, the statocyst, in *Aplysia*. The objective of this study was to determine whether an increase in the magnitude of the gravitational force affects statocyst and statolith formation and dimensions. This was achieved by studying the effects of exposure to $2 \times g$ on the gravity-sensing organ in embryonic *Aplysia californica*.

If the mechanisms which control mineralization are based on statolith weight, it is expected that in $2 \times g$ smaller statoliths would be produced. Previous research in this area is controversial. Hara and collaborators (1994) report a delay in otoconial development in centrifuged ($2 \times g$) chick embryos followed by the formation of 'giant' otoconia (40–100 μm in length). Howland and Ballarino (1981) found that chick embryos of more than 20 g, when exposed to $2 \times g$, showed a smaller utricular otolithic weight than their control counterparts. However, in a later study, these investigators found no significant change in otoconial

weight in chick embryos exposed to $2 \times g$, compared to controls (Ballarino and Howlland, 1984). Lim et al. (1974) found no significant difference in saccular otolith volume between centrifuged and control rats; the utricle was not examined. In space-reared animals, the evidence is equally contradictory. Lychacov and Lavrova (1985) found that space-reared *Xenopus* had 30% larger utricular otoliths and greater asymmetry than their ground-reared counterparts. On the other hand, Vinnikov et al. (1983 and 1976) found no qualitative differences in the vestibular organ of space-reared *Xenopus laevis*, and *Rana temporaria* during very early stages of development. Similarly, Neubert and co-investigators (1986) reported no significant qualitative difference between the otolithic membranes in *Xenopus laevis* larvae developed in micro-gravity (μg). However, they reported the presence of an unknown structure next to the vestibular capsule in near-zero gravity reared animals. This structure was found to contain irregular crystals with otoconia-like electron density and was surrounded by sensory-looking cells. When found in control animals, this structure was underdeveloped in comparison to μg animals.

2. Methods

Embryonic *Aplysia californica* were exposed to excess gravity ($2 \times g$) by placing them in sealed petri dishes with artificial sea water (Instant Ocean) on a 16-inch-diameter centrifuge with a vertical axis of rotation. The water within the dish had a maximum and minimum distance from the axis of rotation of 8 and 7.25 inches, respectively. Because during rotation the specimen is not always flush against the outer margin of the petri dish, the rate of rotation was calculated based on a diameter of $15\frac{1}{2}$ inches. The experimental gravitational force was calculated by vectorially summing the centrifugal and normal gravitational accelerations:

a: centrifugal acceleration, f: frequency,
r: radius.
$$a^2 + (1g)^2 = (2g)^2$$
$$a = 1.732(32.174) = 55.7184 \text{ ft/sec}^2$$
$$r = 7.75 \text{ in} = 0.6458 \text{ ft}$$
$$a = v^2/r; \quad v = 2\pi rf$$
$$a = (2\pi rf)^2/r$$
$$f = \sqrt{ar}/2\pi r$$
$$f = 5.9986/4.0577 = 1.48 \text{ sec}^{-1}$$

Therefore, the centrifuge rotated on the vertical axis at a rate of 1.48 revolutions per second, monitored by a digital counter.

Embryonic *A. californica* were obtained from the *Aplysia* Rearing Facility at the University of Miami Marine Division. Many embryos are contained within

individual egg cases (approximately 20), which in turn are grouped together inside a gelatinous sac, forming egg-strands many centimeters in length. A single 16-cm-long egg-strand was divided into four segments of approximately equal length. The first and third segments were exposed to $2 \times g$, and the second and fourth segments constituted the control group. The same selection procedure was followed in all the experiments reported here. There were approximately 20 animals per egg case and 40 egg cases per cm; therefore, the total number of embryos in each group was approximately 6,400. Of these approximately 260 embryos (3 mm of egg-strand) were sampled each day from each group. From these (260), 20 were randomly selected from those laying in the right orientation (see below).

The experiment was performed three times, each with eggs from different progenitors and following the same protocol. Embryos from different progenitors vary significantly in size. This explains why there are differences in the same parameter among experiments and why the data from all three is not grouped but rather presented as three separate experiments. The experiments will be referred to as Experiments 1, 2 and 3. Embryos were exposed to high gravity until the time they began to hatch. Body diameters were determined before exposure to hypergravity in Experiments 1 and 2 but not in Experiment 3. Statocyst and statolith were measured from the day they were first visible until the day the embryos hatched. These days vary with the egg-strand; therefore, the measurements were taken on different days for each experiment. This was done in the following fashion: Body diameter in a sample of 20 randomly chosen embryos was measured upon arrival of the egg-strand and prior to exposure to $2 \times g$. This corresponds to day 3 of life for Experiment 1, and day 2 for Experiment 2. For Experiment 3 the body diameter was not determined prior to exposure to $2 \times g$. The statocyst, statolith and body diameters were measured on days 6 through 12 for Experiments 1 and 2, days 6 through 9 for Experiment 3. The measurements were taken from a random sample of 20 embryos from each experimental group and 20 from their control (uncentrifuged) groups.

All measurements were made using Java™, Jandel's image analysis software. In order to prepare each specimen for light microscopy, a 3 mm long egg-strand sample was cut off from each group. Each strand was cut longitudinally in two halves, and a coverslip was gently pressed over the sample. Only specimens that were oriented with their shell opening facing up and whose statocysts and statoliths were clearly seen through this opening were used in the experiments. Fig. 1 is a schematic representation of how these measurements were taken in embryos.

Body diameter (D_b) was measured along the longest
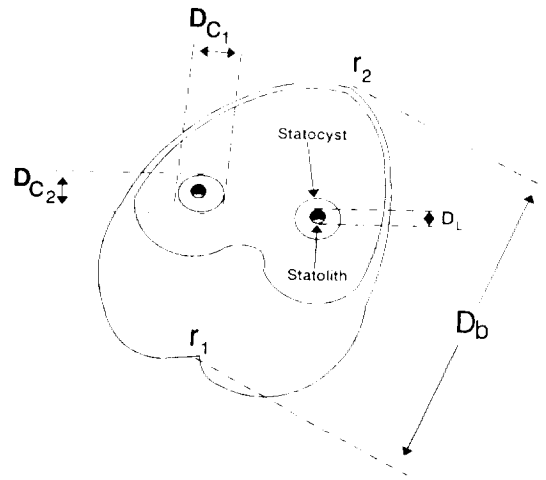
Fig. 1. Schematic representation of an embryonic *Aplysia californica*. Shell opening faces up making statocyst and statolith visible. D_b, body diameter; r_1 and r_2, reference points from which body diameter was determined; D_L, statolith diameter, and D_{c1} and D_{c2}, statocyst diameters.

axis between two reference points on the embryo's shell (r_1 and r_2). The statocyst width was measured along both the longest (D_{c1}) and the shortest (D_{c2}) axis. The mean of the two values was taken as statocyst diameter (D_c). Under light microscopy and after 1 μm sectioning, the statolith appears as a perfect circle. Therefore, only one statolith diameter was measured (D_L). Both left and right statocyst and statoliths were measured.

The data were analyzed with the general linear module procedure of the statistics analysis software, SAS system. Statistical differences in body diameter were analyzed using a two-factor analysis of variance (ANOVA), assuming a normal distribution. For the statocyst and the statolith diameters, a three-factor ANOVA was used. Differences were established as a function of day, group and side (left vs. right). The statocyst- and statolith-to-body ratios were treated in the same fashion.

There is asymmetry between the left and right organs in all parameters measured. The means of the left organ, i.e., statocyst and statolith, as well as the ratios, are always significantly larger than the right organ (Data not shown). This asymmetry is consistent in all three experiments. The shape of the plot for the left organ, i.e. statocyst or statolith diameter vs. time in days, is similar to the right organ (data not shown) in all three experiments. In comparing the control to the hypergravity groups, the statistical results (ANOVA) are almost identical, when the left and right organs are grouped together, to that when they are considered separately, i.e., comparing left to left and right to right. Therefore, to facilitate the presentation of the data, the left and right values for the statocyst were grouped
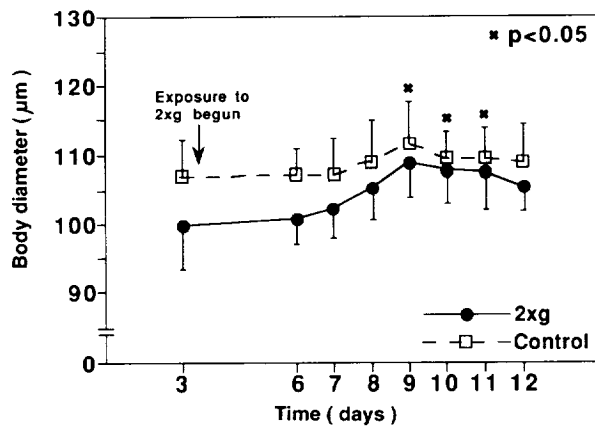
Fig. 2 (Experiment 1). This figure depicts the mean body diameter in μm before exposure to $2\times g$ (day 3) and on days 6 through 12. The values represent the mean ± 1 standard deviation of 20 embryos chosen randomly. These means were statistically analyzed in relationship to their baseline values (day 3). An analysis of variance (ANOVA) indicates the $2\times g$ group is statistically greater than their baseline value on days 9, 10, and 11. ($P < 0.05$).

together (n = 40) for each day and group, i.e., control and hypergravity. The statolith, statocyst- and statolith-to-body ratio were treated in the same fashion.

Differences between the control and hypergravity groups for each day were established using Sidak's multiple comparison tests in all cases, i.e., statocyst and statolith diameters, and statocyst- and statolith-to-body ratios.

Only the results from Experiment 1 are presented graphically while the other two are presented in tables.

3. Results

Fig. 2 shows the mean body diameters (D_b measured as indicated in Fig. 1) ± 1 standard deviation, in μm, at day 3 and from days 6 through 12. Day 3, before the larvae were exposed to $2\times g$, is considered baseline. On day 3, the control group had significantly greater ($P = 0.0001$) mean body diameter than the $2\times g$ group. Due to this, the statistical analysis (ANOVA) was done using the baseline value for each group as its reference. The $2\times g$ group is statistically greater ($P < 0.05$) on days 9, 10 and 11. The whole egg strands were selected in alternate order as explained under Methods; it is not possible to select eggs to equalize body diameter between groups before initiating the experiment, since they are encapsulated.

Tables 1 and 2 represent the same type of information for Experiments 2 and 3, respectively. For Experiment 2 (Table 1) the body diameter for the $2\times g$ group is greater than $1\times g$ control on days 7 through 12, however, this difference is not statistically significant

Table 1
Body diameter (μm), Experiment 2

Day	$2\times g$	Control
1	115.56±2.71	115.74±4.24
6	113.66±4.13	114.50±4.09
7	114.12±5.16	112.75±4.24
8	114.96±5.82	113.69±4.92
9	118.46±5.44	115.59±4.72
10	117.58±5.24	117.91±5.43
11	116.51±5.16	114.11±4.26
12	117.23±5.18	115.83±4.78

Mean body diameter (μm) ± 1 standard deviation of 20 measurements per day on days 6–12 for Experiment 2. Although the data are not presented in graphic form, the plots follow a pattern similar to that in Fig. 2 (Experiment 1).

on any day (ANOVA). For Experiment 3, the body diameters were not determined before exposure to $2\times g$ as in Experiments 1 and 2. In this experiment, the mean body diameter is greater for the $2\times g$ group every day (Table 2). In all three Experiments, the body diameter of embryonic *Aplysia californica* is greater for the $2\times g$ group than the $1\times g$ control.

Fig. 3 shows the mean statocyst and statolith diameters (D_L, n = 40) for Experiment 1. Fig. 3A shows the mean ± 1 standard deviation of the control and $2\times g$ statocyst vs time. Time is the days on which the measurements were taken, which corresponds to the age of the animal. There is no statistically significant difference between the groups on any day except day 6 (Sidak's multiple comparison, $P < 0.01$). Fig. 3B depicts the same for the statolith. The mean of the $2\times g$ statolith was significantly smaller every day (Sidak's multiple comparison, $P = 0.0001$). In the case of the statocyst (Fig. 3A), an analysis of variance indicated no interaction between the day and group affecting the dependent variable, mean statocyst diameter. This is indicated by similar curves for the control and the $2\times g$ groups. The same statistical test indicated that the statolith diameter is affected by an interaction between the day and the group, i.e. the statolith diame-

Table 2
Body diameter (μm), Experiment 3

Day	$2\times g$	Control
6	119.5±4.2**	113.4±4.3
7	118.7±5.7*	115.0±6.2
8	128.4±4.7***	110.3±4.4
9	122.8±4.4***	109.2±3.8

Mean body diameter (μm) ± 1 standard deviation of 20 measurements per day on days 6–9 for Experiment 3. Although the data on Tables 1 and 2 are not presented in graphic form, the plots 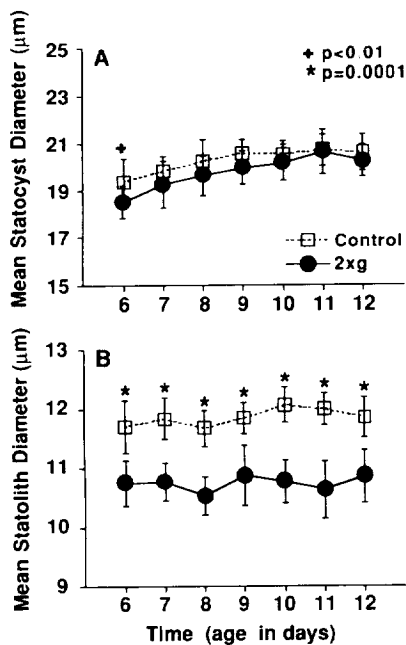follow a pattern similar to that in Fig. 2 (Experiment 1). * indicates $P < 0.05$; **, $P < 0.001$; and ***, $P = 0.0001$ (Sidak's multiple comparisons test).

Figs. 3A and 3B (Experiment 1). These figures depict the mean ± 1 standard deviation of the statocyst and statolith diameters on days 6–12. Each value represents the mean ± SD of 40 measurements. Figs. 3A represent the mean statocyst diameters. Fig. 3B represents the mean statolith diameters. The means for the control statoliths are different to $2 \times g$ ($P = 0.0001$).

Table 3
Statocyst diameter (μm), Experiment 2

Day	$2 \times g$	Control
6	20.258 ± 0.90	19.027 ± 0.94
7	20.461 ± 1.04	20.301 ± 0.98
8	20.593 ± 0.916	20.483 ± 0.99
9	21.081 ± 1.12	20.649 ± 1.04
10	20.629 ± 1.19	20.665 ± 0.98
11	20.508 ± 1.13	20.776 ± 1.00
12	21.017 ± 1.34	20.961 ± 0.91

Mean statocyst diameter ± 1 standard deviation for the $2 \times g$ and the control groups on days 6–12 in Experiment 2. All the values represent the mean of 40 measurements. * indicates a significant statistical difference of $P < 0.05$ (Sidak's multiple comparisons test).

Table 4
Statocyst diameter (μm), Experiment 3

Day	$2 \times g$	Control
6	21.615 ± 0.82 *	21.021 ± 0.99
7	21.307 ± 0.75	21.210 ± 0.77
8	21.623 ± 0.97 *	20.867 ± 0.75
9	21.210 ± 0.58	20.876 ± 0.8

Mean statocyst diameter ± 1 standard deviation for the $2 \times g$ and the control groups on days 6–9 in Experiment 3 (Table 4). All the values represent the mean of 40 measurements. * indicates a significant statistical difference of $P < 0.05$ (Sidak's multiple comparisons test).

Table 5
Statolith diameter (μm), Experiment 2

Day	$2 \times g$	Control
6	11.648 ± 0.57	11.778 ± 0.51
7	11.722 ± 0.47 *	12.092 ± 0.58
8	11.738 ± 0.51	11.948 ± 0.58
9	11.713 ± 0.52 *	11.997 ± 0.55
10	11.121 ± 0.64 ***	12.189 ± 0.47
11	12.069 ± 0.59	12.068 ± 0.59
12	11.406 ± 0.61 ***	12.122 ± 0.59

Mean ± 1 standard deviation of the statolith diameter for the $2 \times g$ and the control groups on days 6–12 in Experiment 2. All the values represent the mean of 40 measurements. * and *** indicate $P < 0.05$ and $P = 0.0001$, respectively (Sidak's multiple comparisons test).

Table 6
Statolith diameter (μm), Experiment 3

Day	$2 \times g$	Control
6	11.825 ± 0.56	11.733 ± 0.55
7	11.824 ± 0.49 *	11.554 ± 0.44
8	11.751 ± 0.46	11.569 ± 0.47
9	11.840 ± 0.48	11.624 ± 0.51

Mean ± 1 standard deviation of the statolith diameter for the $2 \times g$ and the control groups on days 6–9 in Experiment 3 (Table 6). All the values represent the mean of 40 measurements. * and *** indicate $P < 0.05$ and $P = 0.0001$, respectively (Sidak's multiple comparisons test).

ter varies differently with time in the control and the $2 \times g$ groups. In short, these data indicate that the statolith diameter is smaller with exposure to $2 \times g$, and that the rate of growth is also lower. On the other hand, statocyst diameter is not significantly affected by exposure to $2 \times g$.

The results are similar for Experiment 2. Tables 3 and 5 show the mean statocyst and statolith diameters (μm) for Experiment 2, respectively. There is no statistical difference in statocyst diameter between the two groups on any day (Table 3). However, mean statolith diameter is always smaller in the $2 \times g$ group than control. This difference is significant on days 7, 9, 10 and 12 (Sidak's multiple comparison, $P < 0.01$). Table 4 shows the mean statocyst diameter (μm) and Table 6 shows the mean statolith diameters (μm) for Experiment 3. In this experiment, the statocyst was significantly bigger for the $2 \times g$ group on days 6 and 8 (Table 4), and the statolith (Table 6) was the same except on day 7 (Sidak's multiple comparison).

Fig. 4 summarizes the statolith diameter distribution for the control and the $2 \times g$ groups for Experiment 1. The values indicate the number of statoliths within a given size range as a percent of the total number of embryos selected at random throughout the experiment, i.e., 280. The statolith size distribution for the $2 \times g$ group is shifted to the left, thus indicating smaller statoliths than control ($1 \times g$). The distribution pattern

Fig. 4. This figure depicts the overall statolith diameter distribution in both 2× g and control group. The results are indicated as a percentage of the total number of statoliths measured in samples randomly selected, i.e. 280 per group. The left and right statoliths were grouped together.

is similar for both groups except for the zero values, which are indicative of statocysts without statolith (4% of total), in the 2 × g group.

Because of differences in body diameter between the control and the 2 × g group, and based on previous research demonstrating that the number of statoconia
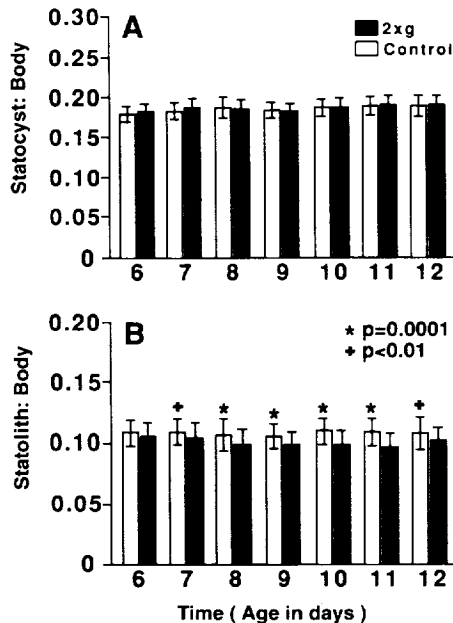
Figs. 5A and 5B (Experiment 1). These figures depict the statocyst- and statolith-to-body ratios on days 6 through 12 of exposure to 2× g. Each value represents the mean ± SD of 40 measurements. The hollow bars represent the control groups, and the filled bars represent the 2× g groups. Fig. 5A depicts the statocyst-to-body ratio and Fig. 5B depicts the statolith-to-body ratio. * and + indicate statistical differences in ratio between the control and the 2× g groups (P < 0.001 and P < 0.01, respectively).

Table 7
Statolith-to-body ratio, Experiment 2

Day	2 × g	Control
6	0.103 ± 0.006	0.103 ± 0.005
7	0.102 ± 0.005 **	0.107 ± 0.005
8	0.102 ± 0.007 *	0.105 ± 0.007
9	0.099 ± 0.006 **	0.104 ± 0.005
10	0.095 ± 0.005 ***	0.104 ± 0.006
11	0.104 ± 0.008	0.106 ± 0.005
12	0.097 ± 0.006 ***	0.105 ± 0.006

Mean ± 1 standard deviation of the statolith-to-body ratio for the 2× g and the control groups on days 6–12 in Experiment 2 (Table 7). All the values represent the mean of 40 measurements. * indicates $P < 0.05$; **, $P < 0.001$; and ***, $P = 0.0001$ (Sidak's multiple comparisons test).

vary according to body size throughout the animals' lifespan (Wiederhold et al., 1990), both statocyst and statolith diameters were normalized to body diameter in order to compare groups. These results (Experiment 1) are presented graphically in Fig. 5. Figs. 5A and 5B shows the mean ± 1 standard deviation of the statocyst- and statolith-to-body ratio for days 6 through 12, respectively. There are no significant differences in statocyst-to-body ratio between control and 2 × g on any day (Fig. 5A). The statolith-to-body ratio was significantly smaller (Sidak's multiple comparison, $P <$ 0.01) for the 2 × g group, compared to the controls every day except day 6.

In Experiment 2, the results for the statocyst- and statolith-to-body ratios are very similar to Experiment 1. There is no significant difference in statocyst-to-body ratio on any day (data not shown); however, the statolith-to-body ratio is smaller for the 2 × g group on days 7 through 12 (Table 7). This difference is statistically significant (Sidak's multiple comparison) on days 7 and 9 ($P < 0.01$), and 10 and 12 ($P = 0.0001$). Thus the results are similar to the mean statocyst and statolith, i.e. without normalization to body diameter (see Tables 3 and 5). In Experiment 3, the statolith-to-body ratio is smaller every day for the 2 × g group than control (Table 8). However, this difference is statistically significant on days 6 ($P = 0.0025$), 8, and 9 ($P =$

Table 8
Statolith-to-body ratio, Experiment 3

Day	2 × g	Control
6	0.099 ± 0.005 *	0.104 ± 0.0.006
7	0.099 ± 0.007	0.101 ± 0.006
8	0.092 ± 0.004 ***	0.105 ± 0.006
9	0.096 ± 0.005 ***	0.107 ± 0.006

Mean ± 1 standard deviation of the statolith-to-body ratio for the 2× g and the control groups on days 6–9 in Experiment 3 (Table 8). All the values represent the mean of 40 measurements. * and *** indicate $P < 0.05$ and $P = 0.0001$, respectively (Sidak's multiple comparisons test).
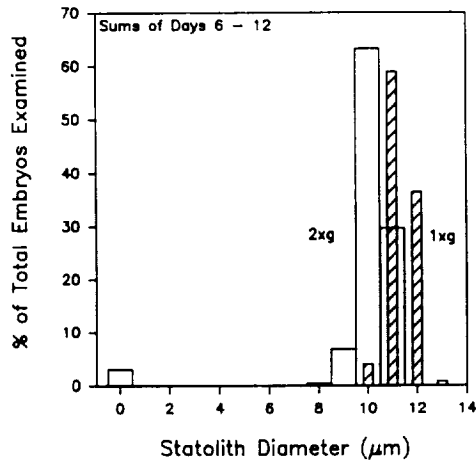
0.0001), but not on day 7 (Sidak's multiple comparison). In this experiment the $2 \times g$ statocyst-to-body ratio was statistically smaller than control on days 8 and 9 only (Sidak's multiple comparison, $P = 0.0001$) (data not shown).

In Experiments 1 and 2, defective statocysts were found in the $2 \times g$ groups only. Statocysts containing statoliths with oval appearance and with small dimensions (5–9 μm in diameter), were considered to be defective. Furthermore, statocysts lacking statoliths altogether were also considered defective. For both experiments, the results are expressed as a percentage of the total number of embryos examined per day, i.e.,

approximately 260. In Experiment 1, embryos with defective statoliths were found on days 10, 11, and 12. On day 10, approximately 1.0% of all embryos examined had defective statocysts. On day 11, 1.40% had defective statocysts. Each one of these two days, the embryos with defective statocysts were predominantly found in two egg cases. Finally, on day 12, 6.44% of all embryos had defective statocysts, and they were predominantly found in four egg cases. In Experiment 2, defective statocysts were found on days 11 and 12 only. These values are 6.7% for day 11 and 7.6% for day 12. In both cases most of the embryos with defective statocysts were found in two egg cases.

Figs. 6A and 6B. Photomicrographs of embryonic *Aplysia californica* with a normal statocyst (right organ) and a defective one in which the statolith is missing (left organ). Both photomicrographs correspond to the same specimen at different magnifications, which is a sample of those found in the $2 \times g$ groups in Experiments 1 and 2. In Fig. 6A the magnification factor is $125 \times$ whereas in Fig. 6B it is $62.5 \times$.

Figs. 7A and 7B. Photomicrographs of embryonic *Aplysia californica* lacking statoliths in both statocysts. These are samples of those found in the 2 × g group on days 10, 11 and 12 in Experiment 1 and days 11 and 12 in Experiment 2. Both are photomicrographs of the same embryo at different magnifications. Fig. 7A is magnified 125 × . Fig. 7B is magnified 62.5 × .

Defective statoliths were not included in the calculations of mean statolith or mean statolith-to-body ratio presented above. If included, differences between control and hypergravity groups would have been much greater in both experiments. Figs. 6 and 7 are photomicrographs of some embryos with defective statoliths. In Fig. 6, the right statolith is normal, whereas the left one is missing. Fig. 7 shows abnormal statoliths.

4. Discussion

The mean body diameter (Fig. 2) remained relatively constant throughout the experimental period.

After treating the initial mean body diameter, i.e. prior to exposure to excess gravity, as baseline for each group, the 2 × g group had bigger differences from its own baseline than control every day. However, there was no significant difference between control and 2 × g (ANOVA) until days 9, 10 and 11. Hence, the rate of growth was different between the two groups. This suggests that exposure to excess gravity caused an increase in the rate of body growth in the 2 × g group. This increase in growth rate is not so apparent in Experiment 2 (Table 1) and the differences are not statistically significant. In Experiment 3, the mean body diameter for the 2 × g group was also significantly

greater than the control group (Table 2). However, for this particular experiment, no conclusion can be drawn because of the lack of a baseline measurement prior to exposure to $2 \times g$. These results could be related to other research in which filamentous fungus grew at a faster rate when chronically exposed to hypergravity (Pence et al., 1992). It is, however, in contrast with results obtained by Howland and Ballarino (1981) who found no differences in body weight of chick embryos after exposure to $2 \times g$ in a centrifuge.

Fig. 3A depicts the mean statocyst diameters as a function of time for Experiment 1. There are no significant differences between the hypergravity groups and their controls except on day 6. The results are similar in Experiments 2 and 3 (Tables 3 and 4).

In Experiment 1, mean statolith diameters are smaller for the $2 \times g$ group than for the control group every day (Figs. 3B). The statolith diameter distribution (Fig. 5) is shifted to the left in the $2 \times g$ group in relationship to the control group, which shows the decrease in statolith diameter caused by exposure to $2 \times g$. In Experiment 2, the mean statolith diameter in the $2 \times g$ group was always smaller than control; however, the differences in statolith diameter were not statistically significant (Table 5). In Experiment 3, there are no statistically significant differences in mean statolith diameter between the control and $2 \times g$ groups, except on day 7 (Table 6). However, when normalized to body diameter, the statolith is smaller in the $2 \times g$ than the control groups on each day. Therefore, we conclude that hypergravity led to an inhibition of statolith growth so that the statoliths grew at a lower rate than in the control group. These results are similar to previous research in which the utricular otolithic weight of chicks exposed to $2 \times g$ was found to be less than control animals exposed to normal gravitational force (Howland and Ballarino, 1981). The results are also in accordance with research reported by Lychakov et al. (1988a), in which mean utricular otolith size in rats tended to diminish with chronic exposure to $2 \times g$.

Fig. 5A depicts the statocyst diameters normalized to body diameter, i.e., the statocyst-to-body ratios on days 6 through 12. There is no significant difference in these ratios between the control and the $2 \times g$ group. However, the statolith-to-body ratios (Fig. 5B) are smaller for the $2 \times g$ group than for the control group. This difference is not statistically significant on day 6, but it is significant on days 7 through 12. The results of Experiment 2 and 3 are very similar to these (see Tables 7 and 8). In short, this difference in ratios indicates that exposure to $2 \times g$ affects statoliths by inhibiting their growth relative to total growth of the animal. In approximately 3% of the embryos reared at $2 \times g$, the statolith failed to develop at all. Similar results were observed in a current study with exposure to $5.7 \times g$. In the latter, 9% of embryos had no sta-

tolith. This indicates a more dramatic effect of hypergravity on the development of the gravity-sensing organ in *Aplysia*. However, the current data cannot distinguish whether lack of statolith is a continuous extension of the more moderate effects of hypergravity on most specimens, or a more pronounced stress response.

Taken together, these data show that exposure to $2 \times g$ affects statolith but not statocyst size. This indicates that the statolith is an important site for regulation during development in hypergravity, and therefore, it raises the question of whether statolith formation and stability are dynamic processes. In higher animals, there is substantial evidence that otoliths are in dynamic equilibrium with their environment. Rat calcitic otoconia have been shown to incorporate $^{45}Ca^{2+}$ in a time-dependent manner at rates similar to those in bone (Ross, 1979; Ross and Williams, 1979). Ross et al. have also shown that saccular and utricular otoconia *in vitro* show an increase in Ca^{2+} incorporation with an increase in K^+/Na^+ ratio (1980). There is also evidence suggesting that carbonic anhydrase, which is responsible for the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$ $(H_2CO_3 \rightleftharpoons H^+ + HCO_3^-)$, may play a role in otolithic homeostasis. The use of specific carbonic anhydrase inhibitors prevented normal otolith morphogenesis from occurring in chicks (Toshishige et al., 1991; Vincentiis and Marmo, 1968). In spite of this, there is no evidence that otoliths from lower animals are in equilibrium with their environment. In fact, it is widely accepted that fish otoliths are metabolically inert, meaning that once deposited, the crystal structure remains fixed (Gauldie and Nelson, 1990). From the data presented here no conclusion can be drawn about the nature or mechanisms by which statolith dimensions are affected by $2 \times g$. Therefore, the question raised by our results remains to be answered.

From the present study, it can be concluded that, first, exposure to excess gravity leads to an increase in body diameter. Second, statocyst size is not affected by excess gravity independently of body size. Third, excess gravity causes a decrease in statolith size. And fourth, exposure to $2 \times g$ leads to the development of some embryos with defective statocysts; however in most instances a normal statocyst and statolith develop under $2 \times g$.

The implication is that these embryos have the ability to adapt to an increase in the force of gravity by regulating statolith dimensions. Therefore, animals reared in reduced gravity might be expected to show the opposite effect, i.e., larger otoliths. Space flight might affect not only the formation, but also the homeostasis of these organs. Reports of results of space flight are controversial. However, the results reported here support the work of Lychakov, et al. (1985, 1988a, 1988b). They reported that space-reared *Xenopus* lar-

vae had larger utricular otoliths, compared to ground-reared controls.

Acknowledgement

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