Hypergravity induces vertebrae and otolith deformation in medaka fish
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Abstract
Teleost fish live under a constant force derived from gravity, with hard tissues playing important roles to help maintain body balance. However, the mechanism of hard tissue formation induced by gravity remains unclear. To examine the effects of gravity in aquatic animals, we performed experiments with medaka fish reared in a hypergravity environment, in which the force of gravity exceeded that present on the surface of the Earth, and analyzed hard tissue formation. Medaka fish were reared for 6 months under a normal gravitational force (1G) or that 5.29 times greater than normal (5.29G) using a centrifuge designated for small fish rearing. Micro-CT analysis results showed that hypergravity induced a vertebral curvature towards the dorsal side and asymmetric formation of otoliths in which the cross-sectional area was increased. Our findings indicate that the process of adaptation to a hypergravity environment results in spinal and otolith deformation in medaka fish. ©2019 Jpn. Soc. Biol. Sci. Space; doi:10.2187/bss.33.12

Introduction
For survival of animals on Earth, it is necessary to maintain body posture and balance (Thompson 1917), as body formation as well as physical behavior are completely controlled by the strength of gravity (Crookes 1987). Terrestrial animals support their body by normal force on the ground against gravity, while aquatic animals require a balance of gravity and buoyancy for body support, with hard tissues playing important roles in all vertebrate animals. One of those hard tissue structures is the vertebrae, which is composed of several different bones that support the body against mechanical stress in all vertebrates including fish (Carter 1987; Fiaz, et al 2010). Another type of hard tissue structure is otoliths, composed of crystalline calcium carbonate, which bend hair cells in the direction of gravity to maintain a sense of body balance (Anken 2006). It is considered that the combination of skeleton and otoliths is indispensable for adaptation to gravity, though the effects of gravity on hard tissue formation remain largely unknown. To elucidate the mechanism related to regulation of hard tissue formation by gravity in aquatic animals, we examined structural changes in medaka fish under the influence of hypergravity.

The teleost fish medaka is well suited for study of formation of hard tissues, such as the spine and otoliths, as it is easy to perform examinations of the vertebrae phenotype as part of the structure of the skeleton (Chatani et al. 2011) and also observe otolith formation in the inner ear (Nemoto et al. 2008). A study of bone metabolism under a microgravity environment in space for a period of approximately 2 months showed results indicating osteoclast activation in these fish (Chatani et al. 2015). Furthermore, an investigation of medaka reared for a relatively short term in a space environment showed changes in gene expression levels (Chatani et al. 2016). These results indicate that medaka is a suitable animal model for gravity experiments.

To clarify the effects of gravity in aquatic animals, we reared medaka in an enhanced gravity environment and performed experiments. The effects of chronic hypergravity on skeleton development (Canciani et al. 2015; Tominari et al. 2019) as well as the inner ear system in mice, rats, and fish (Anken et al. 2001; Wubbels et al. 2002) have been examined, though no dynamic changes in skeletal structural characteristics were observed in those, except for differences in regard to mineral density. For the present study, we investigated vertebrae and otolith shape following exposure to a hypergravity environment using micro-CT analysis. For this purpose, we established a fish rearing system capable of supporting medaka for approximately 6 months.

Materials and Methods

Hypergravity environment
Twenty-four days after hatching, 4 medaka fish (10 mm total length) were placed into 220 ml of rearing water in a 225-cm² flask for the suspension culture (IWAKI, Japan, 1160-225). The fish were then kept for 6 months under a hypergravity environment provided by use of a centrifuge customized for medaka fish (Tomy Seiko Co., Ltd, Japan, LIX-140SP), which produced an approximately 5.29 times gravity (5.29G) environment by rotating at 160 rpm. In results of preliminary tests, we have found that 5.29G is an ideal condition for research of the effects of gravity on hard tissue formation, because medaka are able to maintain body posture and a curvature toward the dorsal side in those fish appears over time. The rearing water was kept at 28°C and

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shaded for the entire experimental period. Feeding for 30 minutes was done twice a day, during which time the centrifuge was stopped. Except for the feeding time, centrifuge rotations were not interrupted. The experiments were performed in accordance with policies and protocols approved by the Institutional Animal Care and Use Committee of Showa University.

**Video imaging under hypergravity**

The behavior of the medaka while being reared in the centrifuge machine was captured using a video camera (GoPro, USA, GoPro HERO Session 5) placed on the flask (ThermoFisher, USA, 159934).

**Soft-X analysis**

Radiography images were obtained with a soft X-ray apparatus (Bruker BioSpin Co., Ltd., Japan, in vivo Xtreme). The shooting conditions for 4% PFA fixed medaka samples included an exposure time of 1.2 seconds, filter size 0.4 mm, and 45 kVp.

**Micro-CT analysis**

Three-dimensional digital images of the 4% PFA fixed medaka samples after rearing under hypergravity were reconstructed from micro-CT analysis findings obtained using a X-ray computed tomograph apparatus (Comscantecno Co., Ltd., Japan, ScanXmate-L090H), with the voltage set at 80 kV and current at 71 μA. Histomorphological analysis of the micro-CT images was performed with a three-dimensional reconstruction imaging for bone system (RATOC System Engineering Co., Ltd., Japan, TRI/3D-Bon-FSC(R)).

**Statistical analysis**

To estimate the curvature of the spine shown in images obtained with micro-CT analysis, the farthest vertebra in a straight line connecting the 1st to 12th vertebra was defined as the Xth vertebra, then the angle formed by the line connecting the Xth with the 1st vertebra and the line connecting the Xth with the 12th vertebra was measured. Otolith area was displayed in red color in the micro-CT images, because otoliths have a high-density level. The cross-sectional area was determined as pixel number using the ImageJ software package (National Institutes of Health, USA, imagej v1.51[8]), with otolith size divided by that of the fish head for normalization. All values are shown as the mean±standard error. Statistical comparisons were performed using an unpaired t-test or the Tukey-Kramer method (Statistical Discovery, JMP Pro14), with differences considered to be significant at p<0.05.

**Results**

To investigate the effects of gravity on hard tissues, medaka fish were reared in a hypergravity environment created by use of a centrifuge (Fig. 1A, B). Medaka were placed in flasks containing breeding water (Fig. 1C), which were then placed in the centrifuge machine and
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rotated at 160 rpm, which according to our theoretical calculation resulted in a load of 5.29G to the flask (Fig. 2A-C). To observe the behavior of medaka under the 5.29G environment, video imaging was performed, which showed that the fish were able to maintain body posture and position. However, a curvature toward the dorsal side appeared over time (Fig. 3).

To examine changes in skeletal structure, fish reared for 6 months under 1G as well as those under 5.29G conditions were fixed using 4% PFA, then examined by soft-X and micro-CT. The vertebrae in the 5.29G group showed a large amount of curvature toward the dorsal side as compared with the 1G group (Fig. 4A-F). Based on the measured vertebrae angle, the 5.29G group showed a curvature of about 8.5% to the rear (Fig. 4I), while there was no curvature in a left or right direction seen in the dorsal view (Fig. 4G, H).

In micro-CT findings, the lateral view showed otoliths colored red, because of the high density of calcium carbonate crystals (Fig. 5A, B). Furthermore, the cross-sectional area of the sagitta was significantly increased in the 5.29G group (Fig. 5C). In the horizontal view, the 5.29G group showed asymmetrical positioning of otoliths on the right and left (Fig. 5D, E). The cross-sectional left and right areas of the lapillus, sagitta, and asteriscus were significantly increased by approximately 35% and 36%, respectively, in the 5.29G group (Fig. 5F). There was no significant difference between the left and right areas in medaka raised under the hypergravity environment.

Discussion

In the present hypergravity experiments with medaka fish, we noted changes in regard to formation of hard tissue in the spine and otoliths. Fish generally respond to pitching force, i.e., longitudinal lift moments, as a stimulus due to both weight and buoyancy (Brix et al. 2009; Goolish 1992). Considering that buoyancy is mainly produced by the swimbladder, an internal gas-filled organ, the spine curvature around the swimbladder noted in the present 5.29G group likely came from attempts by the fish to maintain posture against longitudinal lift moments in the hypergravity environment. This phenotype of vertebra curvature caused by hypergravity suggests that medaka prefer to resist hypergravity and swim, even when the skeleton becomes deformed, which demonstrates the gravity tolerance of these fish. Since vertebral bones in medaka contain no cartilage (Ekanayake et al. 1987), it is thought that there are some differences in regard to vertebrae bending between

![Fig 2.](image)

**Fig 2.** Theoretical calculation of load caused by acceleration at center of flask bottom. (A) Pattern diagram of acceleration loaded at center of flask bottom (red circle) caused by centrifuge. (B) Acceleration loaded at center of flask bottom (red circle) shown as three vectors. The synthetic vector indicates total acceleration (Gt) summed as centrifugal acceleration (Gc) and gravitational acceleration (Gg) with centrifuge rotation at 160 rpm. (C) Equations used to determine acceleration with centrifuge rotation at 160 rpm.

![Fig 3.](image)

**Fig 3.** State of swimming by medaka under 1G and 5.29G conditions. (A) Medaka under normal gravitational force (1G). (B) Medaka under gravitational force 5.29 times greater than normal (5.29G). Arrows show vertebrae curvature.
mammals and aquatic animals.

As for otolith formation, the increased cross-sectional area in both the left and right regions noted after rearing under hypergravity suggests a dependence on the magnitude of gravity. A previous study found that Coriolis force produced by a centrifuge promoted deformation of otoliths (Helling et al. 2003), thus we considered that the asymmetric otolith deformation seen in the present experiment was due to that force. When the fish were returned to a 1G environment for feeding, they occasionally swam in tight circles, known as looping behavior (data not shown), which was similar to behavior seen when under microgravity (Ijiri 1995) and considered to indicate that formed otoliths had adapted to the 5.29G environment. In the future, we intend to investigate the mechanisms of response to hypergravity by rearing several bone-related transgenic medaka lines under various levels of gravity.

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Declaration of Interests
The authors have no competing financial interests to declare in regard to this study.

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Fig 5. Asymmetric formation of otoliths in hypergravity environment.
Microcomputed tomography (micro-CT) images of medaka head. Colored scale represents bone mineral density. (A) Lateral view of head region of medaka reared under normal gravitational force (1G). (B) Lateral view of head region of medaka reared under gravitational force 5.29 times greater than normal (5.29G). (C) Quantitative analysis to compare cross-sectional area of otolith (sagitta) in lateral view between 1G and 5.29G conditions. (D) Horizontal view of head region of medaka reared under 1G. (E) Horizontal view of head region of medaka reared under 5.29G. (F) Quantitative analysis to compare cross-sectional areas of otoliths in left and right otocyst regions in horizontal views between 1G and 5.29G. Asterisk indicates significant difference (p<0.05) as compared with 1G [pairwise comparison using Student’s t-test of results shown in (C) and Tukey-Kramer method of results shown in (F)]. 1G, n=4; 5.29G, n=4. Error bars, s.e.m. NS: not significant difference, L: lapillus, S: sagitta, A: asteriscus. Scale bars=1 mm.
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