Acta Astronautica Vol. 45, No. 3, pp. 167–170, 1999

Printed in Great Britain
PII: S0094-5765(99)00119-8
0094-5765/99 $ - see front matter

THE ASYMMETRICAL GROWTH OF OTOLITHS IN FISH IS AFFECTED BY HYPERGRAVITY

R. H. ANKEN† T. KAPPEL and H. RAHMANN

Zoological Institute, University of Stuttgart-Hohenheim, Garbenstr. 30, D-70593 Stuttgart, Germany

(Received 9 January 1998)

Abstract—Size and asymmetry (size difference between the left and the right side) of inner ear otoliths of larval cichlid fish were determined after a long-term stay at moderate hypergravity conditions (3***g***; centrifuge), in the course of which the animals completed their ontogenetic development from hatch to freely swimming. Both the normal morphogenetic development as well as the timely onset and gain of performance of the swimming behaviour was not impaired by the experimental conditions.

However, both utricular and saccular otoliths (lapilli and sagittae, respectively) were significantly smaller after hyper-***g*** exposure as compared to parallely raised 1***g*** control specimens. The asymmetry of sagittae was significantly increased in the experimental animals, whereas the respective asymmetry concerning lapilli was pronouncedly decreased in comparison to the 1***g*** controls.

These findings suggest, that the growth and the development of bilateral asymmetry of otoliths is guided by the environmental gravity vector.

1. INTRODUCTION

Since the vertebrate inner ear statolithic organs are gravity sensors, numerous studies have been adressed to the question, if altered gravity may have any impact on the formation of the otolithic (or statolithic) mass itself [e.g. 1–4]. By far most of these investigations were focused on the qualitative evaluation of the morphology of amphibian, avian or mammalian otoconia, yielding contradictory results [2,3].

Quantitative analyses on inner ear otoconial masses after altered gravity exposure have never been satisfactorily performed. The area covered with otoconia can methodologically not be correlated with their total mass or "physical capacity", since the otoconia (or statoconia) can be redistributed in the direction of the gravitational force by hypergravity experiments [4]. The actual mass of a statolith — comprising a large number of virtually minute otoconia (some 200,000 with a size ranging from 4 to 30 μm in length in the utricle of a hatched chick [3]) — can hardly be determined, since some otoconia can be overlooked in the course of a dissection, and the dissected material may contain additional tissue, which cannot readily be separated (M. Ross, personal communication, 1997).

Surprisingly, fish otoliths have never been investigated regarding the topic, although they possess compact otoliths, the physical capacity of which (i.e. weight, size) can be quantitatively easily assessed. Otoliths have played a major role in fisheries science for decades [5] and reliable techniques to determine the size of a given otolith as a parameter of its physical capacity are readily available [5].

Therefore, the present study was undertaken in order to clarify, whether or not developing fish otoliths might be quantitatively affected by a long-term hypergravity exposure. Particular focus was attributed to a possible effect of altered gravity on the development of otolith asymmetry (bilateral differences in the otolith sizes from the left to the right side of the body), since vestibular afferents ought not to be too asymmetric due to asymmetrical otoliths in order to stay in the range of the central nervous vestibular compensation of bilaterally different inputs [6] for postural control.

2. MATERIAL AND METHODS

2.1. Experiment and dissection of specimens

Left and right utricular and saccular otoliths (i.e. lapilli — gravity perception [7], and sagittae — hearing process [7], resp.; the sensory inputs, however, do overlap to some extent) to be analysed morphometrically were dissected from larval cichlid fish siblings (*Oreochromis mossambicus* PETERS, Perciformes), which had developed at 22° and at a 12 h:12 h day:night cycle from developmental stage 14 [8] (2 days after hatch and 6 days post fertilization, prominent yolk-sac) until stage 23 [8] (about 24 days after hatch, yolk-sac completely absorbed, all fins except pelvic fin revealing rays) either at 1***g*** normal earth gravity (1***g*** controls) or at 3***g*** hypergravity (centrifuge; hyper-***g*** animals). The centrifuge was equipped with an on-board video camera,

†Corresponding author. Tel.: +49-711-459-2255; fax: +49-711-459-3450.

which allowed a direct observation of the animals during hypergravity exposure (details on this Video Observation Centrifuge for Aquatic Vertebrates, VOCAV, which was constructed in the Zoological Institute, where published elsewhere [9]).

The 1*g* control siblings were maintained within the centrifuge setup, but without rotation.

Details on rearing the specimens during the experiment were published earlier [10]. In brief, they were housed in Petriperm cell culture vessels (content: 35 ml; Bachofer, Reutlingen, Germany), some 5 specimens each. The vessels were equipped with a semipermeable membrane for gas exchange and have so far successfully been used in our earlier hypergravity experiments as well as in the course of the Second German Spacelab Mission D-2 [11].

2.2. Morphometry of otoliths

After dissection, the otoliths were mounted (flat side up) in glycerine on microscopical slides.

Following widely used standard procedures [12,13], the maximum radius (r_{max}) of the otoliths examined in the present study was evaluated, as it is routinely performed in fishery science when the otoliths are too small to be weighted. For the determination of the maximum radius of a given otolith, the circumference of an otolith was drawn at a ×400 magnification at a Standard 14 compound microscope (Zeiss, Oberkochen, Germany) equipped with a camera lucida (Zeiss), and the nucleus was marked. By means of a pair of compasses and a metric ruler, the maximum distance from the nucleus to the circumference was determined. The true metric units (µm) as given in Fig. 1 were back-calculated via a micrometer-slide (Zeiss). "Asymmetry" was calculated by determining the absolute difference in metric units between the maximum radii of the left and right saccular and utricular, respectively, otolith of each animal. All preparations were scored blind to avoid observer bias.

Statistics were based on Student's *t*-test with a 95% confidence interval. The nomenclature as used for preparation of the figure is: * = significant ($p < 0.05$–0.01), ** = very significant ($p < 0.01$–0.0001), *** = extremely significant ($p < 0.0001$). See caption of figure for *n*-numbers.

3. RESULTS

3.1. Behaviour and general development of cichlid fish at hypergravity (3g)

Both in the experimental animals and in the 1*g* controls, the morphogenetic development was identical, as has been quantitatively demonstrated in the course of our earlier centrifuge experiments [14]. The development of the swimming behaviour (timely onset and gain of performance) was qualitatively identical as well. Both animal groups began increasingly to explore their miniaquaria in the course of the resorption of the yolk-sacs, which force younger stages to lie on the ground due to their weights. Like the later-staged larvae at 1*g*, the siblings at hyper-*g* explored the entire space of their habitat without being centrifuged down.

3.2. Otolith size and asymmetry in hyper-g animals vs. 1g controls

Qualitatively observed lightmicroscopically, morphological differences between hyper-*g* and 1*g* control otoliths concerning parameters such as the general shape of the otoliths and the overall pattern of daily incremented ring-layers were not obtained.

Regarding the size (maximum radius, r_{max}), however, the sagittae of experimental animals were by 14.1% smaller than those of the 1*g* control speci-

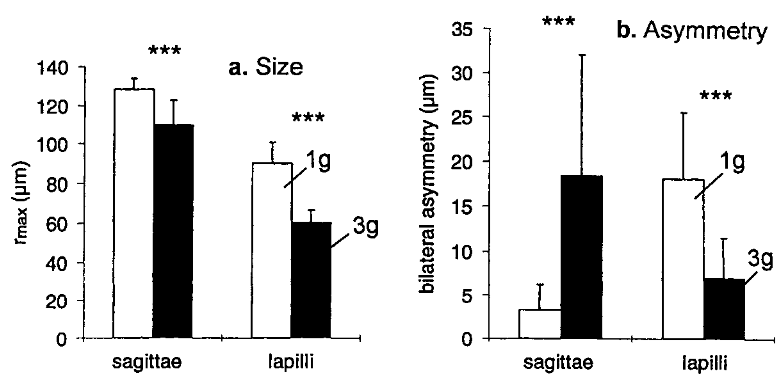

Fig. 1. Effects of increased gravity on the growth of inner ear otoliths of cichlid fish, which had been raised under 3*g* within a centrifuge from developmental stage 14 to stage 23, in comparison to 1*g* controls, reared at normal Earth gravity conditions. (a) Size (maximum radius, r_{max}). Data are expressed as standard deviations; $n = 54$ otoliths (each sagittae and lapilli) in case of 1*g* (white bars) and $n = 36$ (each sagittae and lapilli) in case of hyper-*g* (black bars) otoliths. ***: $p < 0.0001$. (b) Asymmetry (absolute bilateral difference in r_{max} between the left and the right side otoliths). Same animals as in (a); $n = 27$ control and $n = 18$ hyper-*g* animals. ***: $p < 0.0001$.

mens (Fig. 1a; $p < 0.0001$). Their asymmetry was dramatically increased after hyper-$\boldsymbol{g}$ (Fig. 1b; $p < 0.0001$).

The lapilli were by 33.2% smaller after centrifugation (Fig. 1a; $p < 0.0001$). Their asymmetry was pronouncedly decreased after long-term hypergravity exposure (Fig. 1b; $p < 0.0001$).

4. DISCUSSION

It is well known that environmental parameters, e.g. feeding and abiotic factors such as light supply and temperature are reflected in the maximum radius (r_{max}) of otoliths [5]. However, all previous works clearly outline the general finding that the environment affects the development of a given fish in general, thus revealing parallel developmental effects on otoliths [5]. Therefore, otoliths can be used for back-tracing the individual life history of a given fish [5].

In the course of the present study, the morphological and behavioural development of the hyper-$\boldsymbol{g}$ fish was indistinguishable from that of the 1$\boldsymbol{g}$ controls. The r_{max}-values (absolute r_{max} and asymmetries) as reported concerning hyper-$\boldsymbol{g}$ sagittae (involved in the hearing process [7]) and lapilli (gravity perception [7]) are moreover not concommittantly resembled in any developmental stage. Exemplarily, the asymmetry of the sagittae ranges around some moderate 5 μm in the complete course of the normal development at 1$\boldsymbol{g}$ and thus never reaches the values found after hypergravity exposure (own, unpublished observations). Thus, the present findings do not indicate any general effect of altered gravity (i.e. general environmental stress). In fact, the results indicate a differential adjustment of lapilli and sagittae in their growth towards the altered gravitational environment.

Under increased gravity, given otoliths will exert an increased pressure on the underlying sensory epithelia. A recent study, which was focused on the neuronal activity of the sensory hair cells of cichlid fish utricles after long-term hypergravity exposure, revealed that hypergravity had no effect on the energy metabolism [15]. This previous finding may be explained by the present results, according to which the utricular otoliths were considerably decreased in their size after centrifugation. Both results taken together suggest, that an otolith formed at hypergravity exerts about the same impact on the epithelium as a respective normally sized otolith at 1$\boldsymbol{g}$.

This in its turn strongly indicates the existence of a feedback mechanism affecting the growth of an otolith depending on the surrounding gravity vector. The interpretation is in complete agreement with a study on the inner ear otoconia of higher vertebrates, in the course of which it was argued that the mass and the degree of mineralisation of otoconia might be regulated to maintain a consistent output from the sensory maculae [16].

The results regarding lapillar asymmetry after long-term hypergravity strongly support this view. At hypergravity, a given bilateral asymmetry between two utricular otoliths will be increased. For a correct interpretation of the afferent inputs to the brain for postural control and spatial orientation, any asymmetry ought to be not too pronounced in order to stay in the range of the neuronal vestibular compensation, which has thoroughly been analysed using hemilabyrinthectomized animals [6]. The feedback mechanism proposed above might reduce a hypergravity-based increased asymmetry in order to fulfill the requirements for a sufficient central vestibular compensation. The efficiency of the system influencing the growth of otoliths by reducing the asymmetry, however, seems to be individually different, since the interindividual differences were remarkable (consult the prominent standard deviations in the figure). The efficiency of the feedback mechanism proposed might be correlated with the efficiency of the individual neuronal compensation and the efficacy of the latter in its turn might be correlated with the visual performance. It is known from various studies [see [17] and further references therein] that a given individual (man or fish) may be more visually or more vestibularily related.

From a functional point of view, it remains ambiguous why the asymmetry of the sagittae was tremendously increased after hypergravity. Since the biological basis of more or less asymmetric structures in bilateral elder larval or adult animals is largely unknown [18], it might be argued that asymmetry may simply develop whenever there is no system counterreacting it. Further studies are sorely needed to clarify this topic.

The structural basis of the proposed feedback mechanism adjusting size and asymmetry of lapillar otoliths towards altered gravity remains unclear as well, since the basic mechanisms of otolith (or statolith) formation in vertebrates so far remains largely unknown. In developing chick it was found [19], that precursors of the otoconia are secreted by the supporting cells of the sensory epithelium and that particular glycoconjugates within these precursors may play their role in calcium carbonate deposition. Acidic amino acids, present on glycoproteins, are known to attract both calcium and carbonate ions, facilitating crystal nucleation and potentially providing a template for crystallization [20, 21]. Calcium carbonate deposition itself might be regulated by the concentrations of Ca^{2+} and HCO_3^- in the endolymph [22].

As it is probably the case in higher vertebrates [19], the organic matrix of otoliths in fish is most likely provided by the membrane of vesicles which are released from the sensory epithelium [23]. Own, unpublished investigations revealed that the membranes of these vesicles contain Ca^{2+}, which details a recent finding [24] according to which small granules of calbindin-like immunoreactivity were observed in the area of the otolithic membrane of squirrel monkey. Thus, the onset of calcium carbonate deposition might be based on a nucleating process, initiated by Ca^{2+}, which is transported to the later deposition area together with the organic otolith matrix by the vesicles mentioned.

An adjustment of otolith size (and asymmetry) towards the endogenous requirements to cope effectively with the environmental gravity might thus be regulated by the secretion of the vesicles.

Further studies have to be adressed to the question, if the secretion of these vesicles correlate with the formation of otoliths at altered gravity, and if the secretion is regulated by the central nervous vestibular efferent system or within synaptic microcircuits at the level of the sensory epithelia. A most recent study, performed on spaceflown rats, speaks in favour of the latter possibility, because microgravity yielded a particular increase of synapses in type II hair cells, which are known to be inserted in the local circuits [25] and thus might influence the otolith's growth directly on the level of the sensory epithelium. Type I cells, however, which are not involved in the microcircuits of the epithelia, also responded to spaceflight (yet to a lesser extent [25]). Therefore, the involvement of the central nervous system in the feedback control of otolith growth must not be precluded at present.

Acknowledgements—This work was financially supported by the Deutsches Zentrum für Luft- und Raumfahrt (DLR) e.V. (FKZ: 50 WB 9533). We thank Katja Werner for her excellent technical assistance.

REFERENCES

1. Ross, M., Implications of otoconial changes in microgravity. *The Physiologist*, 1987, **30**(Suppl.), 90–93.
2. Hara, H., Sekitani, T., Kido, T., Endo, S., Ikeda, T. and Takahashi, M., Fine structures of utricle of developing chick embryo exposed to 2G gravity. *Acta Otolaryngol. (Stockh.)*, 1995, **519**(Suppl.), 257–261.
3. Ballarino, J. and Howland, H., Centrifugation of 2G does not affect otoconial mineralization in chick embryos. *Am. J. Physiol.*, 1984, **246**, 305–310.
4. Lim, D., Stith, J. and Stockwell, C., Observations on sacculus of rats exposed to longterm hypergravity. *Aerospace Med.*, 1974, **45**, 705–710.
5. Kingsmill, S., Ear stones speak volumes to fish researchers. *Science*, 1992, **260**, 1233–1234.
6. Darlington, C., Flohr, H., Smith, H. and Smith, P., Molecular mechanisms of brainstem plasticity. *Molecular Neurobiol.*, 1992, **5**, 355–368.
7. Platt, C., The peripheral vestibular sytem of fishes. In *Fish Neurobiology*, ed. R. Northcutt and R. Davis. University of Michigan Press, Ann Arbor, 1983, pp. 89–123.
8. Anken, R., Kappel, T., Slenzka, K. and Rahmann, H., The early morphogenetic development of the cichlid fish, *Oreochromis mossambicus* (Perciformes, Teleostei). *Zool. Anz.*, 1993, **231**, 1–10.
9. Hilbig, R., Ibsch, M., Flemming, J., Anken, R., Bremen, D. and Rahmann, H., Hypergravity research in aquatic animals with a video observation centrifuge for aquatic animals (VOCAV). In *Proc. C.E.B.A.S. Workshop Conf.*, 11, 1995, pp. 91–99.
10. Paulus, U., Körtje, K. and Rahmann, H., Effects of development and altered gravity conditions on cytochrome oxidase activity in a vestibular nucleus of the larval teleost brain: A quantitative electronmicroscopical study. *J. Neurobiol.*, 1993, **24**, 1131–1141.
11. Rahmann, H., Slenzka, K., Anken, R., Appel, R., Flemming, J., Hilbig, R., Kappel, T., Körtje, K., Nindl, G. and Paulus, U., Structure- and function-related neuronal plasticity of the CNS of aquatic vertebrates during early ontogenetic development under microgravity conditions. In *Scientific Results of the German Spacelab Mission D-2*, ed. P. Sahm. Wissenschaftliche Projektführung D-2, Aachen, 1995, pp. 621–637.
12. Alemany, F. and Alvarez, F., Formation of initial daily increments in sagittal otoliths of reared and wild *Sardina pilchardus* yolk-sac larvae. *Marine Biol.*, 1994, **121**, 35–39.
13. Hare, J. and Cowen, R., Ontogeny and otolith microstructure of bluefish *Pomatomus saltatrix* (Pisces: Pomatidae). *Marine Biol.*, 1994, **118**, 541–550.
14. Bäuerle, A., Anken, R. and Rahmann, H., Morphological and histochemical investigations on the influence of hyper-gravity on the central nervous system of cichlid fish larvae (*Oreochromis mossambicus*). In *Proc. 1995 Microgravity Summerschool Conf. IB 333-95/04*, 1995, pp. 127–130.
15. Nindl, G., Körtje, K., Slenzka, K. and Rahmann, H., Comparative electronmicroscopical investigations on the influence of altered gravity on cytochrome oxidase in the inner ear of fish: a spaceflight study. *J. Brain Res.*, 1996, **37**, 291–300.
16. Pote, K., Weber, C. and Kretsinger, R., Inferred protein content and distribution from density measurements of calcitic and aragonite otoconia. *Hearing Res.*, 1993, **66**, 225–232.
17. Hilbig, R., Schüle, T., Ibsch, M., Anken, R. and Rahmann, H., New approaches to the gravity relevant behaviour of the swordtail fish (*Xiphophorus helleri*). In *Proc. C.E.B.A.S. Workshop Conf.*, 12, 1996, pp. 51–57.
18. Tsuda, T., Philp, N., Zile, M. and Linask, K., Left-right asymmetric localization of flectin in the extracellular matrix during heart looping. *Develop. Biol.*, 1996, **173**, 39–50.
19. Endo, S., Sekitani, T. and Yamashita, H., Glycoconjugates in the otolithic organ of the developing chick embryo. *Acta Otolaryngol. (Stockh.)*, 1991, **481**(Suppl.), 116–120.
20. Crenshaw, M., Mechanisms of normal biological mineralization of calcium carbonates. In *Biological Mineralization and Demineralization*, ed. G. Nancollas. Springer, New York, 1982, pp. 243–257.
21. Steyger, P. and Wiederhold, M., Visualization of newt aragonitic otoconial matrices using transmission electron microscopy. *Hearing Res.*, 1995, **92**, 184–191.
22. Gauldie, R. and Nelson, D., Aragonite twinning and neuroprotein secretion are the cause of daily growth rings in fish otoliths. *Comp. Biochem. Physiol. A*, 1988, **90**, 501–509.
23. Dunkelberger, D., Dean, J. and Watabe, N., The ultrastructure of the otolithic membrane and otolith in the juvenile mummichog, *Fundulus heteroclitus*. *J. Morphol.*, 1980, **163**, 367–377.
24. Usami, S., Shinkawa, H., Inoue, Y., Kanzaki, J. and Anniko, M., Calbindin-D28 K localization in the primate inner ear. *ORL*, 1995, **57**, 94–99.
25. Ross, M., Studies of neuroplasticity in space. In *Workshop Neurosci. Paris 22–24 April*, 1997, p. 7.