The effects of sound pollution as a stress factor for the Baikal coregonid fish

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ABSTRACT. We have studied morphological features of the hearing epithelium affected and unaffected by increased long-term sound (at 160 dB re 1 μPa) on the example of the Baikal omul (Coregonidae, *Coregonus migratorius*). The sensory epithelium was analyzed using the 3D confocal laser scanning techniques. We observed local epithelium damages in the rostral, central and caudal regions of the saccule, e.g. sticking stereocilia, vacuolization and round shape gaps. This article discusses the reasons for the local effects of sound on different regions of the sensory epithelium. We assume that using of morphological screening of sensory acoustic system in the artificial rearing of the Baikal coregonid fishes under the conditions of intense noise could contribute to indicating the most stress-resistant forms, which are promising for high-tech industrial aquaculture, and developing more gentle approaches to its creation.

1. Introduction

The study of fish hearing and their acoustic behavior is important due to increasing effects of sound pollution on fish in their natural habitat (Hastings et al., 1996; Popper and Hastings, 2009; Ladich, 2013; Sapozhnikova, 2018). Intensive production of fish in aquaculture also involves the use of equipment, such as aerators, air and water pumps, combines, blowers, and filtration systems, which increase the noise level in fish storage tanks (Wysocki et al., 2007). Constant exposure to intense noise levels can adversely affect the cultivated species. Possible consequences include impairment of hearing sensitivity, increased stress, and reduced growth rates (Popper and Hastings, 2009). As a result, the acoustic conditions of larvae maintaining during artificial reproduction of fish populations determine their further survival and population replenishment. After their release into the wildlife, they use their sensory organs in order to locate direction, select suitable habitat, settle at locations with sufficient shelter, and avoid the immediate attention of many voracious predators (Montgomery et al., 2006; Caiger et al., 2012).

Fish estimate the locomotion of their body in the acoustic field relative to the otoliths in the inner ears, the utricle, saccule, and lagena (Popper, 2011; Ladich and Schulz-Mirbach, 2016). Nevertheless, the saccule has the main auditory load in many fishes (Zhongmin and Xu, 2002). Sagitta is usually larger than other parts of the labyrinths in these fishes; the removal of both sagittae sharply reduces the auditory sensitivity (Lu and Xu, 2002). These results demonstrate that the saccule plays significant roles in hearing and frequency distinction. In addition, the largest diversity in ultrastructural features for teleosts is characteristic of the saccule (Platt and Popper, 1981; Popper and Coombs, 1982; Popper and Fay, 1999; Sapozhnikova et al., 2017).

The saccular otoliths lag in their locomotion relative to the fish body in the acoustic field and thus stimulate the sensory saccular cells by deflecting their ciliary (hair) bundles (Fig. 1). This mediated process limits the detectable frequencies to a few hundred hertz and restricts the sound intensities to higher levels (Schuijf and Hawkins, 1976; Bradbury and Vehrencamp, 2011).

Lake Baikal was previously suggested as an ideal location for investigation of the application of ocean sound propagation models and assessment of

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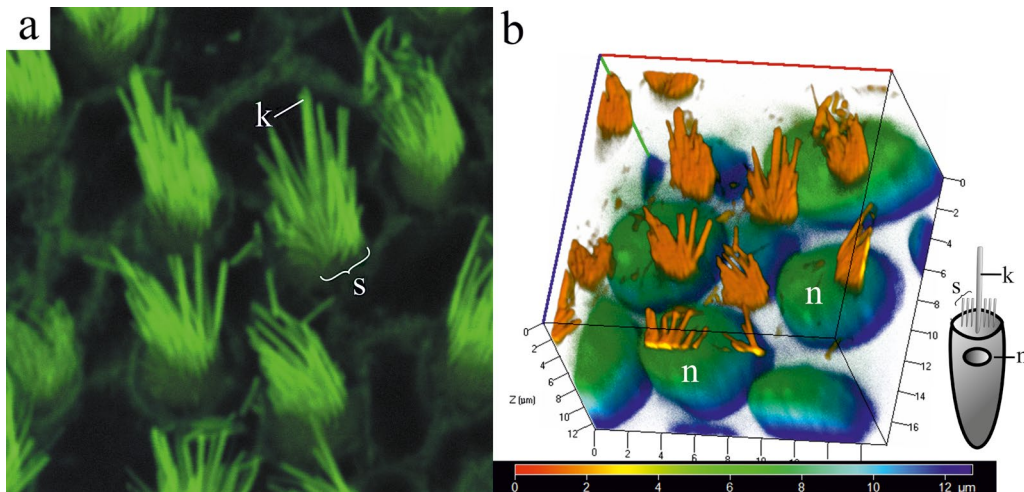E-mail address: jsap@mail.ru (Yu.P. Sapozhnikova)

Fig. 1. Sensory epithelium of the Baikal omul (LSM 710, Carl Zeiss): a − sensory elements on the surface of the epithelium; b − 3D model of sensory epithelium. k – kinocilium, s – stereocilia, n – nucleus.

hearing adaptations in hydrobionts (Glotin et al., 2017). Although some sounds are natural for Lake Baikal (ice crackle, natural gas seepages, surf noise in the splash zone, voices of seals and birds), now, there is often a cacophony of transient and continuous man-made sound from boating, shipping, construction, nearby roadways, tunnels, etc. Thus, another question is whether these sounds potentially interfere with the behavior of fish populations. The greatest effect may result from acoustic masking which would shorten the distances over which animals can detect sounds of biological importance, for example, from potential predators (Engas et al., 1996; Hawkins et al., 2014; Hughes et al., 2014; Voellmy et al., 2014; Glotin et al., 2017).

The fish with high hearing characterstics may be affected by this noise up to a distance of several hundred meters (Amoser et al., 2004; Ladich, 2013), but fish lacking hearing characterstics, such as lacus-trine and riverine coregonid fishes, would be affected predominantly at close distance, which is also typical for aquaculture cultivation.

Among Baikal coregonid fishes, omul is more popular for growing in aquaculture, particularly due to the reduction of its populations in the natural condi-tions and the introduction of a ban on its catch in 2017 for Lake Baikal. Omul is one of the most important species to the subsistence fisheries throughout the world, including Lake Baikal (Tallman and Reist, 1997; Smirnov et al., 2009; Sukhanova et al., 2017).

Therefore, this study was aimed at the evalua-tion of long-term effects of increased sound on the audi-tory epithelium of the Baikal omul. In previous studies, control hearing thresholds showed that coregonid fishes (on the example of broad whitefish Coregonus nasus Pallas, 1776) had far less sensitive hearing and broader bandwidth of hearing than other investigated fish (Popper et al., 2005). This was expected, since the core-gonid fish is not a member of the superorder Otophysi, a group of hearing specialists that have a set of bones, the Weberian ossicles, which acoustically couple the swim bladder to the saccule of the inner ear. The initial analysis of hearing sensitivity in coregonid fish showed that they could detect sounds up to 1600 Hz (Popper

et al., 2005). However, they have a very poor hearing at 1600 Hz. Therefore, in our work the specimens were exposed to the 500 and 1 kHz tones at 160 dB re 1 μPa, which are within the range of hearing of this species and are usually observed in artificial cultivation.

2. Materials and methods

The study objects were adult specimens of the artificially obtained Baikal omul (Coregonidae, *Coregonus migratorius*). Coregonid fishes was artificially inseminated under controlled conditions of the Unique Facility «Experimental Freshwater Aquarium Complex of Baikal Hydrobionts» at the Limnological Institute Siberian Branch of the Russian Academy of Sciences.

To conduct the evaluation experiments of the effects of increased sound on the auditory organs of the Baikal fish under the conditions of the Aquarium Complex, the installation consisting of two round pools with a diameter of 2 m and a height of 0.5 m was constructed. These two pools were located in sepa-rate rooms. One pool had the control fish (without the sound stimulation) and another one had sound-exposed fish (an experimental pool). In both pools, fish were maintained with daily 50% water changes. The walls of the pools were made of plastic.

Tone signals with the frequencies of 500 Hz and 1 kHz (corresponding to determined ranges of acoustic sensitivity) at 160 dB re 1 μPa were generated through a portable computer using the Sound Forge program (Fig. 2). The sound was radiated continuously for 18 days in the experimental pool. After the amplifier, the signal was fed to the UW30 Electro-Voice hydroacoustic emitter with operating frequencies of 100 Hz − 100 kHz and nameplate capacity of 120 W. The emitter was set under the water at half the depth of the experimental pool for noise-exposed fish. The form and intensity of the emitted signal were controlled using the RESON TC 4013 piezoceramic transducers. After the linear ampli-fier, the signal from the transducer was fed to the LCard E-440D analog-digital converter. The amplitude and frequency analysis were carried out using the specia-lized LCard PowerGraph software. The sensitivity of fish to the sound signal was determined visually and
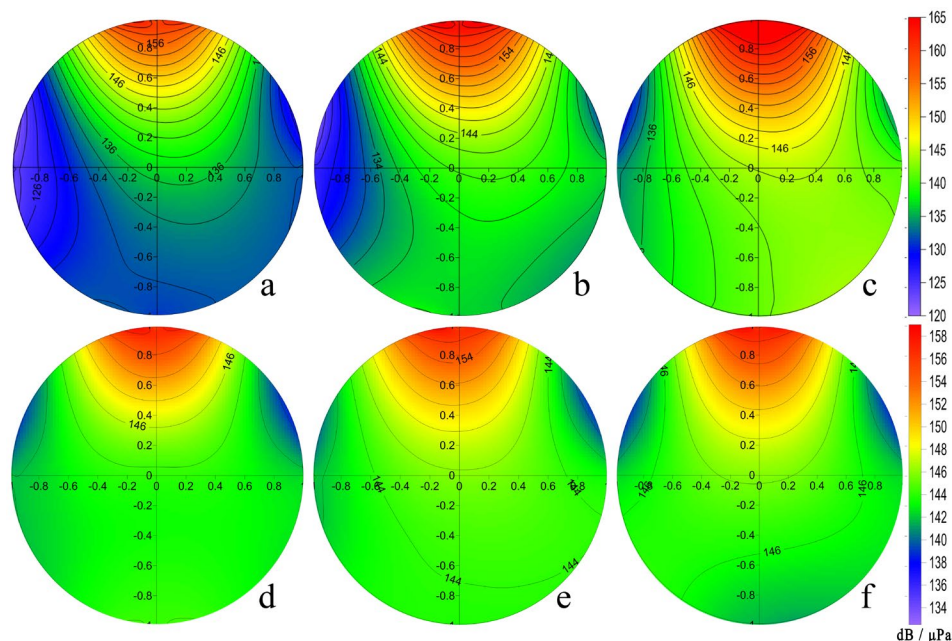
Fig. 2. Uneven sound field of 500 Hz (a, b, c) and 1 kHz (d, e, f) in the horizontal plane, scale in dB / µPa: a, d − the surface, b, e − half the depth, and c, f - the bottom.

by the EthoStudio density maps, as well as by activity of the specimens and a positive reaction to a certain acoustic signal (active movements and jerks) (Kulikov et al., 2014).

Fish from both experimental and control pools were collected with a dip-net, euthanized by using clove oil (0.02-0.05 ml / l, a sedative, contains eugenol) and killed by cutting the spine in accordance with Guidance on the use of clove oil as an anesthetic in aquaculture (Mikodina et al., 2011) and the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (2013).

Immediately prior to the preparation of the auditory organ samples, the cranium was opened from the ventral side, the brain was removed, the ear capsules were uncovered, the right and left labyrinths were extracted, and the saccule was removed together with the sagitta (otolith). Hair cell bundle loss was determined using laser confocal scanning microscopy LSM 710 (Carl Zeiss).

Histological processing of the samples with sensory epithelium was performed according to the standard technique (Klimenkov et al., 2018). The sensory epithelium was fixed for 30 min in a 2% paraformaldehyde (Sigma-Aldrich Co. LLC, USA, Cat. No. 158127) solution in 0.1 M phosphate buffer (pH 7.4) and permeabilized for 20 min in 0.25% Triton™ X-100 (Sigma-Aldrich Co. LLC, USA, Cat. No. T8787). Actin microfilaments were stained for 40 min with FITC-Phalloidin (Sigma-Aldrich Co. LLC, USA, Cat. No. P5282). The nuclei were stained for 15 min with DAPI (Sigma-Aldrich Co. LLC, USA, Cat. No. D9542). Functionally active mitochondria were stained with MitoTracker® Orange CMTMRos (Termo Fisher Scientifc Inc., USA, Cat. No. M7510) by 25-min incubation in medium 199 with Hank's salts (Kompaniya PanEko, Russia, Cat. No. S230p); the medium contained 100–500 nM dye at 37 °C. After each step, the samples were washed three times in Hank's solution without phenol red (PanEko, Russia,

Cat. No P020p). The stained samples were mounted on glass slides in ProLong® Gold Antifade Mountant (Termo Fisher Scientifc Inc., USA, Cat. No. P36930) and covered with a coverslip. The slides were analyzed using a Carl Zeiss LSM 710 laser confocal microscope; Plan-Apochromat 20 × /0.8 and 63× /1.40 Oil DIC M27; lasers: track 1, 405 nm: 3.0%; track 2, 488 nm: 3.0%; track 3, 561 nm: 3.0%.

The morphometric analysis yielded 10-50 images of each macula along the length and depth (depending on the length of the epithelium) at an interval of 30 µm. The density of hair cells on the macula, the length of sensory elements (kinocilium and stereocilia) and hair cell bundle loss were measured using program Image-Pro Plus.

To classify hair cells, cluster analysis was used, in particular, k-means and hierarchical clustering, using the Statistica 8.0 program. Quantitative similarity indicator of hair cells belonging to the same cluster was calculated from the lengths of the kinocilia (k) and the maximum length of the stereocilia (s). Statistical processing of the obtained data was carried out using the single-factor analysis of variance in the R Project. The Kruskal-Wallis test was used for the verification of the null hypothesis.

3. Results and discussion

Confocal scanning microscopy revealed regional distinctions in native hair cells of fish based on the different density of hair cells in central and caudal regions of the epithelium, as well as the presence of different types of cells in these regions. The mean density of the intact hair cells in the rostral region was 571500 ± 8975 cells/mm2, $M \pm m$ in the Baikal omul. In the central region of the macula, hair cells were considerably distant from each other. The hair cells of the caudal region were located more densely.
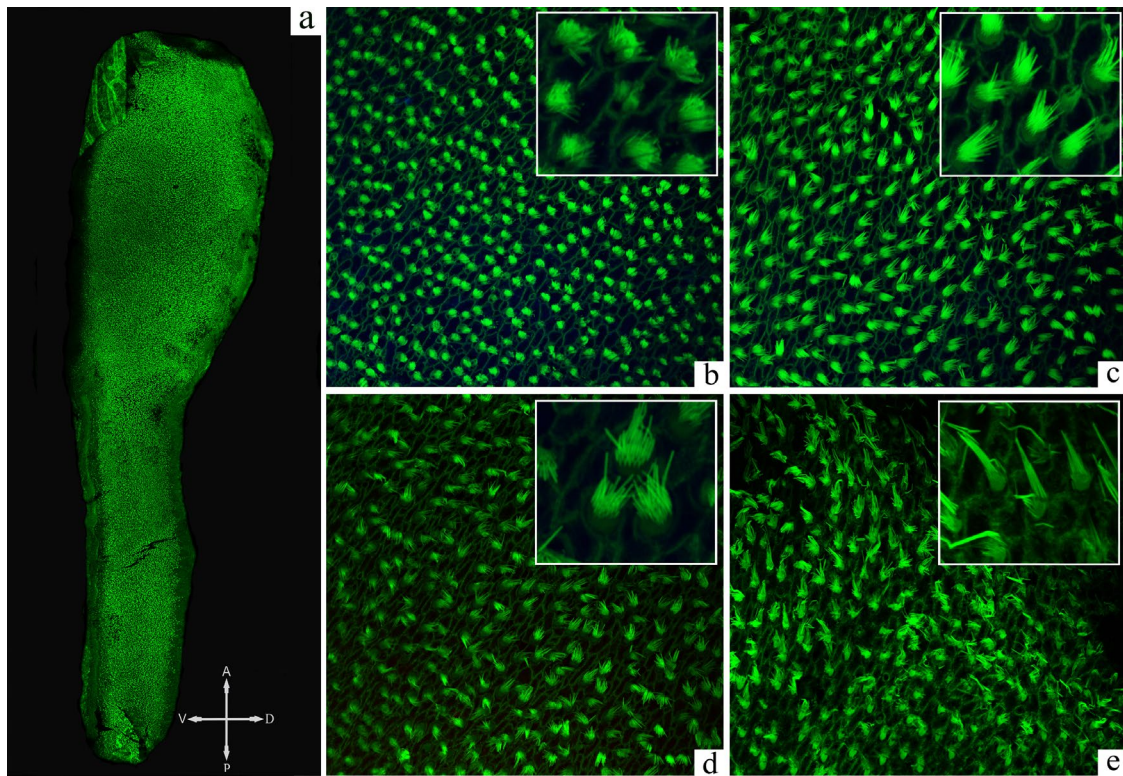
Fig. 3. Different types of the sensory saccular cells in Baikal omul: a – general view of the saccule; b – k3s2 type; c – k6s5 type; d – k7s2 type; e – k11s4 type. k – kinocilium, s – stereocilia. Orientation of the saccule: V – ventral, D – dorsal, A – anterior (rostral), P – posterior (caudal). LSM 710, Carl Zeiss.

Cluster analysis combined sensory cells into classes (types) in such a way that similar cells were included in one class. The length of kinocilium (k) and maximum length for stereocilium in one sensory bundle (s) were the most significant characteristics for the integration of sensory cells into different clusters (designation for cilia was used by Platt and Popper (1981)). These most variable characteristics were regarded as criteria for identification of types of sensory saccular cells. Four cell morphotypes were identified in the Baikal omul, k3s2, k6s5, k7s2, and k11s4 (Fig. 3).

These hair cell types vary across the peripheral, rostral, central and caudal areas of the saccule. The cell types k3s2, k6s5, k7s2, and k11s4 were the most typical for the peripheral and caudal areas of the saccule. The type k7s2 was found in the rostral region of the saccule. Finally, in the central region of the saccule, we identified the cell types k3s2 and k7s2. Thus, in the Baikal omul cells, stereociliar and kinociliar length varies by location: shorter stereocilia are more characteristic of the central and rostral regions of the saccule, longer stereocilia were found in the peripheral and caudal regions of the saccule.

In the course of the experiment on the increased acoustic stimulation of the Baikal omul, there were no exposure effects on mortality. The swim bladders were intact in all experimental and control specimens. But after confocal scanning of sensory hearing epithelium, we found varieties of local epithelial damages: rounded ruptures emerging on the surface of the epithelium, the coalescence of stereocilia described previously for other animals damaged by highly intense sound or ototoxic antibiotics (Wysocki et al., 2007). Damage of various parts of the sensory epithelium of the inner ear occurs unevenly. The hair cell bundle loss in the Baikal omul was individual and varied in different parts of the rostral, central, and caudal regions, at the saccule of each fish. In some specimens, the damages are represented by small foci, in other ones significant areas were injured. However, there were some patterns: the damaged areas were localized and usually varied as a function of exposure sound frequency. The cell damages were minimal at 1 kHz. The small areas of damaged hair cells were observed throughout the epithelium, and, primarily, in the central and rostral areas.

Visible changes in the epithelium occurred only on the 10th day of the experiment at 500 Hz, and then gradually depending on the day of stimulation: evident vacuolization was registered on the 10th day; obvious ruptures of the sensory epithelium were observed on the 18th day after the onset of sound stimulation (Fig. 4).

The zones of saccular hair cell loss were larger for fish exposed to 500 Hz (up to 44% in the different parts of the peripheral area on the 10th day after the onset of sound stimulation). This tone destroyed hair cells predominantly in the peripheral part with the largest cell injuries occurring in the caudal region (Fig. 4g-i). The destructive changes occurred in the hair cells on the 18th day after the onset of sound stimulation (up to 60% in the different parts of the peripheral area) (Fig. 4j-l). Thereby, there was a hypothesis about the tone selectivity in different areas of the auditory epithelium with the different cell types (Hawkins and Sand, 1977; Smith et al., 2011; Sapozhnikova et al., 2016). The changes in cells of the different saccular
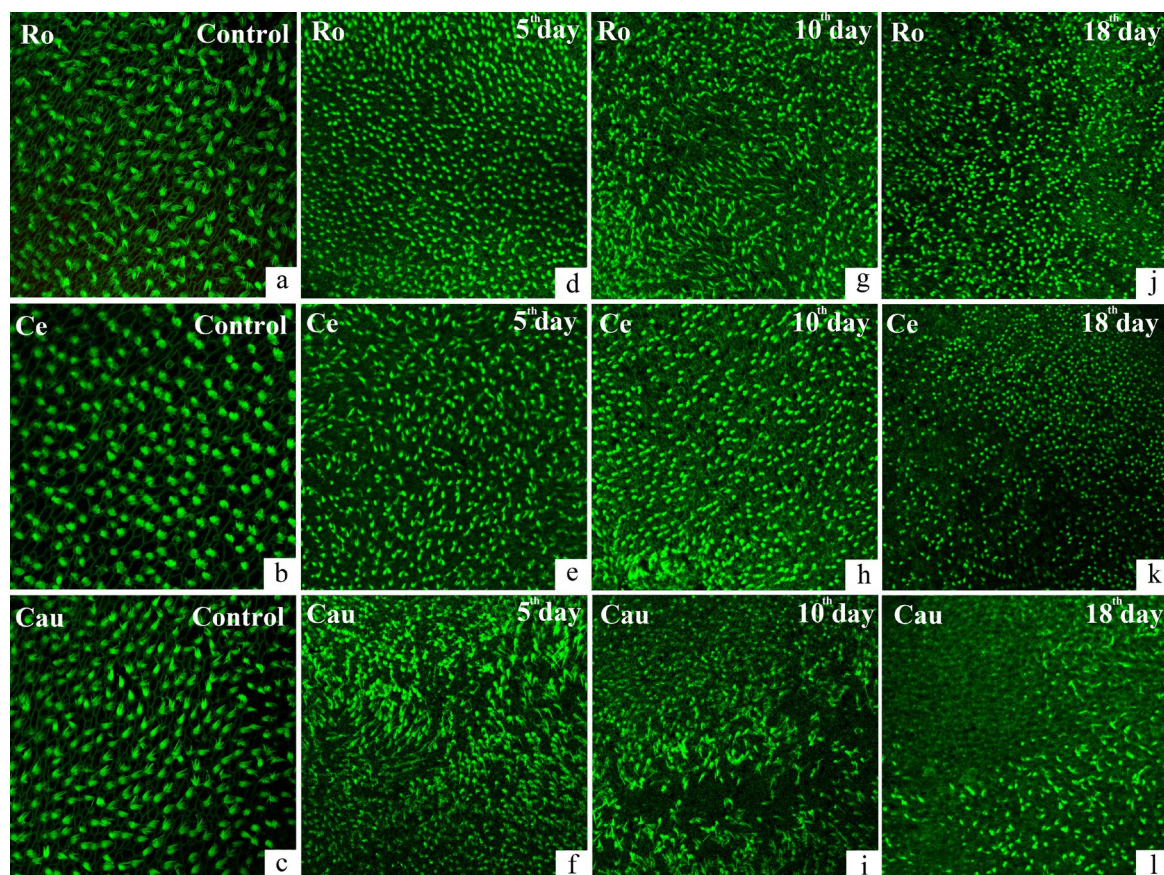
Fig. 4. Images obtained by LSM 710 (Carl Zeiss) showing hair cells destructive changes in the Baikal omul due to stimulation by increased sound (500 Hz, 160 dB) in rostral (Ro), central (Ce) and caudal (Cau) regions of the saccule: a-b – control; d-f – epithelium after 5 days of sound exposure; g-i – epithelium after 10 days of sound exposure; j-l – epithelium after 18 days of sound exposure.

regions are likely to be a result of their damages due to the increased stimulation by various sounds, which confirms the involvement of these regions in the perception of relevant frequency sound oscillations.

4. Conclusions

The obtained results can be used for further study of the acoustic communication of the Baikal fishes, including conditions of acoustic pollution. Unfortunately, anthropogenic increased sound under natural conditions may dramatically reduce acoustic communication of fish reducing their ability to acoustically detect incoming hazards (Hastings et al., 1996; Popper and Hastings, 2009; Ladich, 2013). As it was previously shown, 50% of aquatic noise is generated by 15% of ships, and in some coastal and other high-traffic areas, ship noise has reached levels that degrade habitat for endangered species (Glotin et al., 2017). In particular, prolonged exposure to noise leads to stress, which may affect the nervous and immune systems of hydrobionts (Popper and Hastings, 2009).

The conducted experiment allows us to successfully record the response of specimens to the presence of sensitivity to a particular sound signal, including increased sound evoking anxiety. Moreover, the experiments, in which the increased acoustic stimulation caused a different degree of hair cell damages in different regions of the macula, presumably showed the existence of the regions with different tonal specia-

lization in the auditory maculae of the Baikal omul. So far, we can make only preliminary conclusions about the presence of tonotopic specialization in different regions of the epithelium in coregonid fishes. However, a better understanding of the physiological processes causing cell damage and regeneration, which affect the behavior and lead to stress under the influence of different acoustic effects, requires additional studies. This work may be ultimately used to mitigate the effects of increased sounds on fish in aquaculture or in the natural environment, and contribute to the development of new approaches for the management of lake environment.

Irkutsk Region «Obtaining a High-Tech Aquaculture of Whitefish ...» (Youth Forum Baikal-2020).

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