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Characterization and variations of organic parameters in teleost fish endolymph during day–night cycle, starvation and stress conditions

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Abstract

The aim of the present work was to examine the modifications of the organic composition of fish endolymph under environmental conditions (day–night cycle, starvation and Cl_2-stress) known to modify otolith growth. Endolymph electrophoretic patterns were compared. An antibody raised against the trout otolith organic matrix allowed examining the variations of organic matrix precursors in the endolymph under the above conditions. Western blot analysis showed bands around 60–80 kDa. A 50% decrease of immunolabelling was observed during the night whereas increases were seen after starvation (factor 3) or stress (factor 2) suggesting that these variations could be related to the organic matrix deposit. A factor retarding *in vitro* $CaCO_3$ crystallization (FRC) was shown to co-precipitate with endolymph proteins and its apparent molecular mass (determined by measuring the activity after electro elution of gel electrophoresis) was estimated around 20 kDa. The FRC activity was stable during day–night cycle whereas it decreased by 70% and nearly 100% under starvation and stress respectively. These results suggest that the FRC, although retarding *in vitro* crystallization, plays a major role in the process of otolith calcification and that the decreases measured after starvation and stress are responsible for the decreases of the otolith growth. The variations of these two parameters (precursors and FRC) could contribute for the changes in the microstructure of the otolith.

Keywords: Endolymph; *In vitro* crystallization; Otolith calcification; Organic matrix; Day–night cycle; Starvation; Stress; Trout; Turbot

1. Introduction

The inner ear is composed of 3 semi-circular canals and 3 compartments (utricule, saccule and lagena), each one containing an otolith bathing in endolymph. Otoliths in teleost fish are calcified structures involved in hearing and maintenance of equilibrium (Fay, 1984; Popper and Fay, 1993). Otolith consists of a predominant (>90%) mineral phase of calcium carbonate in aragonite form (Carlstom, 1963) incorporated into an organic matrix (OM), which accounts for 0.01% to 10% of the total weight (Degens et al., 1969; Borelli et al., 2001). The saccular epithelium, containing the largest otolith, is characterized by an asymmetric distribution of ionocytes (Mayer-Gostan et al., 1997; Takagi, 1997) and abundant secreting-cells (Pisam et al., 1998; Takagi and Takahashi, 1999). As otoliths are spatially separated from the saccular epithelium, the mechanism of their calcification is an acellular process taking place in the endolymph. Consequently, authors suggested that the specific composition of the endolymph was involved in the otolith calcification process (Romanek and Gauldie, 1996; Payan et al., 1997).

The endolymph is a peculiar medium, with concentrations of Na^+ and Cl^- comparable to those of classical extracellular compartments, but characterized by a high K^+ concentration, an alkaline pH, and a high HCO_3^- concentration (Enger, 1964; Kalish, 1991; Payan et al., 1997). Payan et al. (1999) showed that endolymph components (proteins and ionic parameters)

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displayed a lack of uniformity in their spatial distribution within the saccule. Recently, Borelli et al. (2001) showed that trout endolymph not only contained various proteins but also proteoglycans and collagens that were not uniformly distributed either.

In order to better understand the process of the otolith growth and to clarify which components of endolymph were involved in the calcification process, the approach chosen in the present work was to examine the variations of the endolymph organic composition in conditions known to induce modifications in the otolith growth. Three conditions have been selected: the circadian cycle, the effect of starvation and the effect of stress. During the day–night cycle, Mugiya and Takahashi (1985) showed simultaneous diurnal variations of pH and of total CO_2 concentration in trout plasma and pooled endolymphs. Edeyer et al. (2000) confirmed the variations of total CO_2 in turbot proximal and distal endolymphs, and also showed protein variations during the day–night cycle, with a maximum reached during the day. Recently, Borelli et al. (2003b) showed that cyclic variations of Ca^{2+} and CO_2 concentrations induced an increase in aragonite saturation in the proximal endolymph during the night period whereas proteins and collagen were in favor of the matrix formation during the day period. However, the $CaCO_3$ deposit in otolith only took place at the beginning of the day period (Wright et al., 1992). Starvation was shown to induce modifications in otolith microstructure in relation to the event intensity and duration (Pannella, 1980). Payan et al. (1998) showed a relationship between the decrease of pH and total CO_2 concentration in trout pooled endolymph (proximal plus distal), suggesting that ionic parameters were involved in otolith calcification. They also showed that protein concentration remained unchanged in pooled endolymph. A stress induced by exposure to Cl_2 gas was recently shown to induce increases in protein and total CO_2 concentrations in the proximal endolymph, and a decrease in otolith growth with a discontinuity in the microstructure (check) (Payan et al., 2004).

Although these three conditions have been already partially studied, they were reexamined in the present study to evaluate the variations in the endolymph of two other parameters that could play a major role in the otolith growth: a factor controlling the calcium carbonate crystallization, and proteins that can be considered as precursors of the otolith organic matrix.

The presence of a factor inhibiting the *in vitro* $CaCO_3$ crystallization was shown in the organic matrix of various calcareous structures : oyster shell (Wheeler et al., 1981), fish otolith (Wright, 1991; Borelli et al., 2001), chicken egg shell (Gautron et al., 1996, 1997), coccolith (Okazaki et al., 1998), molluscs and corals (Marin et al., 2000). Among these biominerals, only egg shell and the fish otolith are suitable for analytical study since there is access to the fluids surrounding them (uterine fluid and endolymph respectively). The activity of a factor, that we prefer to call « factor retarding crystallization » (FRC), was found in the endolymph and in the soluble matrix of the otolith of trout and turbot (Borelli et al., 2001). FRC activity per μg protein was found 65 and 45 times greater in the otolith than in the endolymph of trout and turbot respectively. Although this factor is not yet known, it has been shown to co-precipitate with proteins in TCA (Borelli et al., 2003a). In order to obtain more information about this factor, we carried out the three following experiments: 1) proximal and distal endolymphs were treated with ethanol or acetone and the FRC recoveries were determined in the different phases of each treatment, 2) endolymph proteins, run on an electrophoresis gel, were electro-eluted and an estimation of the FRC apparent molecular weight was undertaken by measuring the calcification activity contained in each sample and 3) FRC activity was determined in proximal and distal endolymphs under the 3 different environmental conditions.

Takagi and Takahashi (1999) showed the presence of a specific precursor (>94 kDa) of the otolith EDTA-soluble OM detectable in the trout endolymph. Borelli et al. (2003b) showed by western blots, using an antibody raised against the otolith acetic acid-soluble fraction of OM, that some proteins (75 and 65 kDa) present in the endolymph were precursors of the OM and that they would be incorporated in the otolith. Borelli et al. (2003b) also showed that there was a difference in the amount of OM precursors between night and day endolymph samples. In the present work, protein concentrations in the proximal endolymphs collected from animals adapted to the three different conditions were measured and the respective protein electrophoresis patterns analyzed. Western blots, using the same antibody, were carried out and differences in the labeling intensity observed in the different conditions were discussed with reference to the day-control.

The results taken together will be discussed in relation to otolith growth and microstructure.

2. Materials and methods

2.1. Fish handling

Turbot (*Psetta maxima*) of 140 to 320 g body mass and 1 to 2-year old were reared at IFREMER Brest, and kept in running seawater at 14 °C for at least 3 weeks before experimentation. Fish were maintained under a constant photoperiod (12 h light: 12 h dark) and fed once a day.

Trout (*Oncorhynchus mykiss*) of 220 to 360 g body mass and 12-month old were obtained from a local fish farm (Auribeau-sur-Siagne). Trout were maintained in running tap water at 17 °C for at least 3 weeks before experimentation and under a constant photoperiod (12 h light: 12 h dark), the day period starting at 08.00 h. Trout were fed once a day every morning. The withdrawals were performed in February and March between 11.00 and 15.00 h, except for night study (04.00 and 06.00 h).

About 80 trouts were divided at random in four groups. The first group, used for control (day period), was maintained in the conditions described above. The second group was collected during the night period. The third group had been starved for 6–7 weeks prior to experimentation. The fourth group was submitted to stress. For achieving the latter experiments, we deliberately made use of the fact that the Municipal Water Board of Nice told us in advance any change in the water treatment (from ozonization to chlorination). The dissolution of Cl_2 gas in water gives hypochlorous acid (HOCl, actually free chlorine), the sterilizing form of chlorine (Bass and Heath, 1977). Hypochlorous acid can also be obtained from chloramine-T, widely used as a disinfectant in aquacultural therapeutics (Booth and Mc Donald, 1988). Chloramine-T breaks