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Restricted fish feeding reduces cod otolith opacity

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Summary

The purpose of this work was to examine the effect of reduced feeding and constant temperature on cod otolith opacity. Three groups of juvenile cod were given restricted food rations at different times for 4 months, resulting in depressed somatic growth. Otolith opacity was measured on pictures of the otolith sections. The otolith carbonate deposited during the experimental period was generally opaque compared to the more translucent otolith material deposited prior to and after the experimental period, when the fish were kept in a pond and in sea-cages at higher temperatures. Large variations in otolith opacity were found between individual fish both within groups and between groups. In two of the three groups significantly more translucent otolith material was deposited in response to reduced feeding. Our results show that variations in feeding and hence fish growth resulted in variation in otolith opacity, but the effect was minor compared to that of variations in ambient temperature. The combined influence of these effects, which both act on fish metabolism, are most likely controlling the seasonal opacity changes observed in wild fish. Our results help explain the variations seen in fish at constant temperatures.

Introduction

Estimation of fish age is often done by counting growth structures in the otoliths. Primary increments are formed by deposition of light, mineral-rich (L) and dark, matrix-rich (D) zones that can be used to age fish at the daily level (Wright et al., 2002). Age estimation on an annual level is based on the number of seasonal growth structures defined as one opaque and one translucent zone as observed in the otolith macrostructure. Translucent zones absorb less light than opaque zones, therefore the translucent zones appear bright when viewed with transmitted light and dark when viewed with reflected light. Opaque zones appear dark when viewed with transmitted light and bright when viewed with reflected light (Panfili et al., 2002). The optical properties of seasonal zones should be a function of the sum of the underlying primary increments that constitute the seasonal zones. Varying amounts of water-soluble and water-insoluble amino acids as well as the total otolith structural matrix proteins might affect the optical properties of the primary increments. The seasonal translucent and opaque zones can differ with respect to primary increment widths, thickness and size of aragonite crystals (Morales-Nin, 1987), amount of protein matrix (Dannevig, 1956a) and elemental ratios (Kalish, 1991), which in sum leads to the difference in opacity between the zones (Wright et al., 2002). Hüssy et al. (2004) defined the term 'otolith opacity' and developed a standardized method to measure it. At the primary increment level they found that altered temperature did not influence otolith opacity for young juvenile cod, although a change in the amino acid composition was observed with increasing temperatures. However, they found indications of fish growth rate influencing otolith opacity. A conceptual model for otolith growth predicts more translucent otolith growth at periods of reduced fish feeding (Hüssy and Mosegaard, 2004).

The physiological mechanism behind altered otolith opacity in response to feeding is not clear, but protein syntheses most likely play a major role (Hüssy and Mosegaard, 2004; Hüssy et al., 2004). Early laboratory experiments showed that less protein was found in the translucent cod otolith zones compared to the opaque zones (Dannevig, 1956a), and Dannevig (1956b) also reported that the more opaque centre of the otoliths contained more organic compounds than the opaque otolith material deposited later in life. A general decrease in cod otolith opacity with fish age has been confirmed by others (Mina, 1968; Høie and Folkvord, 2006). Analyses of otoliths from wild-caught cod have also demonstrated that the seasonal translucent zones contain less protein than opaque zones (Johnsen, 1981). Laboratory experiments with herring (*Clupea harengus*) otoliths showed that only the insoluble proteins in the otoliths affect the otolith opacity, either by different amounts of proteins or by differences in the physical properties of proteins (Tomás et al., 2004). A physiological basis for otolith translucency coupled to fish metabolism is concurrent with the general change in opacity and density in otoliths at increasing fish age and size (Hoff and Fuiman, 1993; Høie and Folkvord, 2006) as the fish metabolism changes with age (Wootton, 1990).

Despite knowledge of different properties of the seasonal otolith zones the underlying cause of the varying opacity is poorly understood. Factors associated with seasonality like temperature, light conditions, feeding and sexual maturation have often been linked to changes in otolith opacity (Beckman and Wilson, 1995). Temperature is a controlling factor on fish physiology that most likely correlates with the deposited otolith opacity (Høie and Folkvord, 2006), but its influence is most likely modulated by other factors. To our knowledge only two laboratory experiments have examined effects of feeding on otolith opacity at the seasonal scale, but with conflicting results. Starvation of Chinook salmon (*Oncorhyncus tshawytscha*) led to more translucent otoliths (Neilson and Geen, 1985), but periods of slow growth for bluegill (*Lepomis macrochirus*) were associated with opaque zone formations (Schramm, 1989). Variable feeding

influencing otolith opacity has also been supported by field observations (Reis, 1986; Admassu and Casselman, 2000; Colloca et al., 2003; Johnson and Belk, 2004). Reduced food availability is ecologically more relevant for cod than are long periods with no food, as cod is an opportunistic feeder that switches to other available food sources in periods of low food abundance (Mehl, 1991; Pálsson, 1994). In this study we examined the effect of altered feeding for juvenile cod (*Gadus morhua* L.) on otolith opacity by offering three groups of fish reduced food quantity for 4 months at different times while keeping temperature constant. Based on predictions of the model of Hüssy and Mosegaard (2004), we expected to find more translucent otoliths during periods of reduced feeding.

Material and methods

Experimental setup

Juvenile cod reared in a production pond (Parisvatnet, 60°38'N, 4°49'E) northwest of Bergen, Norway were transferred to laboratory tanks at the Austevoll research station, Institute of Marine Research, where the experiment was performed. Two hundred fish with mean weight and length of 32.8 g and 145 mm, respectively, were distributed into round 1500-L tanks in early October 2002. The fish were allowed to acclimate to the laboratory conditions for 1 week before the experiment started. They were co-reared in the same tanks with other cod used for another study on sexual maturation.

The experimental design produced fish with different feeding and growth histories (Fig. 1). In October 2002 all fish were placed in a bath of 50 mg l^{-1} Alizarine red S (ArS) to induce a mark in the otoliths corresponding to the initiation of the experiment. All fish were injected with pit tags (Trovan) for later identification of individual fish. Three groups of fish were reared under reduced feeding conditions for 4 months, during which the fish were given a food ratio corresponding to 40% of maximum food consumption based on the growth model of Jobling (1988) using the food conversion factor for cod of Rosenlund et al. (2004).

At the start of the experiment, 50 of the fish were placed in a separate tank (Group: Oct40) and fed at the restricted ration for 4 months. The remaining 150 fish were fed *ad libitum*. In December 2002 another 50 fish were transferred to a tank (Group: Dec40) and fed at reduced ration for 4 months, and in February 2003 the final group was transferred to reduced ration (Group: Feb40) for the last 4 months of the experiment. One control group was fed *ad libitum* during the whole period. The experiment presented in this paper was part of a larger laboratory study where fish food quantity was altered at different times and durations for studying the effects on sexual maturation (B. Norberg, unpublished data). The effective fish densities were 200 fish per tank at start of the experiment. Every second month, at the initiation and termination of the different feeding periods, all fish were placed in an ArS bath for 24 h to induce otolith marks which would later delineate the carbonate deposited during the four different periods. Five ArS marks were thus induced in the otoliths during the 8-month laboratory experiment, representing the start and termination of the four feeding periods for each group (Fig. 1). Every month all fish were anesthetized, also at the same time as the ArS marking, and measured for length and weight. In June 2003 all fish were transferred to a sea cage where they remained until harvest in February 2004.

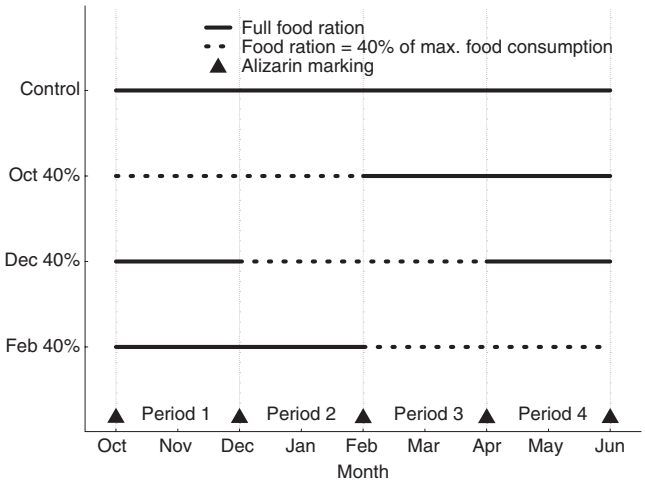

Fig. 1. Experimental design showing time and duration of different feeding periods. Triangles on X-axis show time when all fish were kept 24-h in Alizarine red S bath to induce marks in otoliths. Fish were kept an additional 8 months in sea-cages after the last marking

The water supplied to the tanks during the feeding manipulations was pumped from 165 m depth where temperature was stable at 8.0 ± 0.4°C (±SD). The temperature during the time thereafter in the sea cages varied due to normal seasonal fluctuations in the upper water layer, reaching a maximum of nearly 20°C in early August before gradually decreasing below 5°C by February the following year.

A total of 33 tagged fish were recovered at termination of the experiment, when the fish were 22 months old. The control group suffered from a disease outbreak in the experimental period and only one fish of that group was recovered at the end of the experiment. Sampling of fish during the experimental period for other purposes as well as some mortality reduced the number of fish in all groups. Six of the otoliths were excluded from further analyses due to one or more undetectable ArS bands. The remaining numbers of otoliths for analyses were: 11, Oct40%; 10, Dec40%; and 7, Feb40%.

Otolith preparation

The sagittal otoliths were removed at harvest and cleaned with distilled water. All left sagittae were imbedded in resin (Epofix) and sectioned through the core using a low speed diamond wafering saw blade. The slides were then mounted on glass-slides, and reduced to 200 μm thickness by grinding and polishing.

The otolith sections were photographed with reflected white light and UV epi-illumination (Fig. 2). For photographing the otoliths using reflected white light, the otoliths were placed under a dissection microscope connected to a monitor, a digital camera (Leica DFC 320), and a frame-grabber (Leica IM50). The setup was standardized using a fixed exposure time (31.1 ms, 8 bit channel) with a frame of 2088 x 1550 pixels. The light intensity and aperture were kept constant as was the angle of the incoming reflected light (from the right at an angle of 0 degrees). When photographing the otoliths with transmitted light the reflector mirror was kept at a constant angle of 45 degrees. The position of the otolith under the dissection microscope was fixed, thus all otoliths were photographed under the same light conditions.

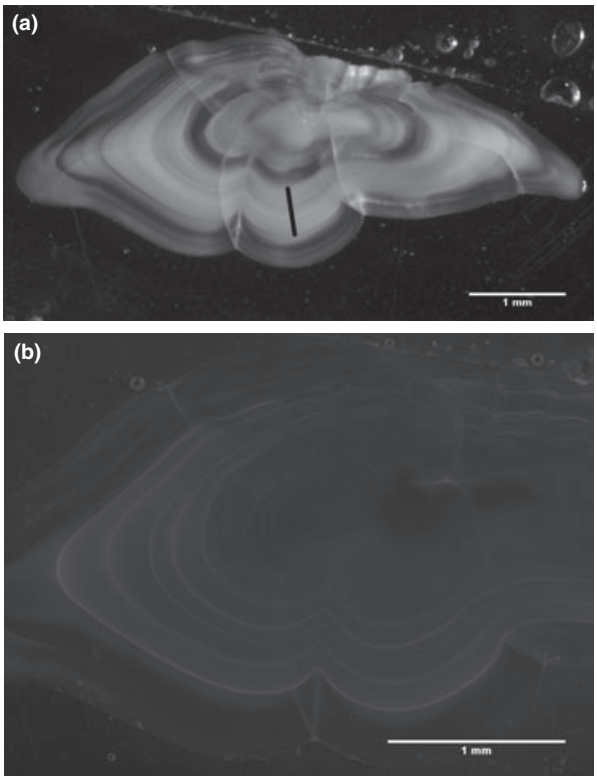

Fig. 2. Otolith section from Oct40 group photographed with (a) reflected light, and (b) UV epi-illumination light. Area between the two distinct translucent bands marked with a black line in (a) corresponds to experimental period with manipulations of feeding levels. Scale bars = 1 mm

The otolith opacity was measured from the digital images taken with reflected light, along a transect from the core to the distal edge. First, a line was drawn between two easily recognizable landmarks at the distal and proximal side on the pictures taken at UV epi-illumination light, and the distances from the otolith edge and the alizarin bands were measured. Then a line between the same landmarks was drawn on the pictures taken with reflected light, and the positions that corresponded to the alizarin bands were marked with a small (1 μm) black line perpendicular to the line between the landmarks. The pictures were then converted to grayscale, and a line crossing all the marked positions was drawn using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2006.). The gray values on the 0–255 scale were then recorded along the transect line; the transitions between the feeding periods (i.e. the alizarin bands) were easily recognized and deleted due to a strong signature of the inserted small black line.

In order to better compare otoliths within each group, the otolith opacity data were normalized for each individual otolith by dividing the otolith opacity for each feeding period by the mean otolith opacity for all four feeding periods. Differences in otolith opacity between groups within periods and between periods within groups were analysed by one-way analysis of variance (ANOVA), after testing for homogeneity of variances (Levene's *F*-test, Brown and Forsythe, 1974). One value of normalized otolith opacity in the Oct40 group in period 4 was more than eight standard errors lower than the group mean value and was regarded as an outlier and excluded from the analyses.

Results

Fish growth

Fish growth responded well to the feeding regimes (Fig. 3). The Oct40 group increased on average 25.8 g and reached a mean weight of 58.6 g after 4 weeks at restricted feeding in February. In comparison, the Feb40 group, which were fed *ad libitum* in the same period, reached a mean weight of 137.5 g in February, but the mean weight then decreased 2 g by the end of the restricted feeding period in June. Fish in the Dec40 group had a mean weight of 77.0 g at onset of the restricted feeding, and reached 98 g mean weight at the end of 4 months restricted feeding in April.

Otolith opacity

Based on the first ArS band the fish had deposited a translucent zone in the otoliths prior to their transfer to the

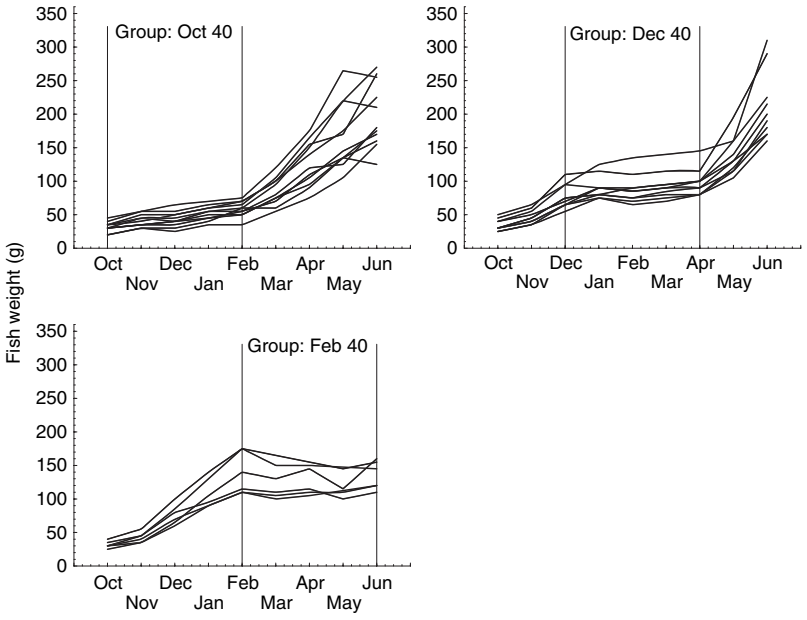

Fig. 3. Fish weight (g) during experimental period based on measurements at monthly intervals. Period of reduced feeding indicated for each group within vertical lines

laboratory (Fig. 2). Opacity of the otolith material deposited during the experimental period was generally high (opaque), with areas of local maxima. After the experimental period, when the fish were transferred to a sea cage, the otolith material deposited was again translucent. The mean otolith opacity of material deposited during the experimental period for all otoliths was 137, varying from 86 to 188 overall. In comparison, the mean opacity of the translucent otolith carbonate deposited prior to onset of the experiment, measured in ten randomly-chosen otoliths, was 72, with minimum and maximum values of 48 and 103.

Otolith opacity varied within the otoliths of a single fish and within each group, as well as between groups (Fig. 4). For the Oct40 group, with restricted food for the two first feeding periods, opacity was less in the two first periods compared to the two last periods in 9 of 11 otoliths. For the Feb40 group, with reduced feeding in the two last periods, the opposite pattern was evident. In four out of six fish from this group the otolith opacity was lower in the two last periods compared to the first two. Fish in the Dec40 group were kept at restricted feeding in periods 2 and 3; 4 months of reduced feeding did not produce a consistent pattern of lower otolith opacity.

Normalized opacity in the Oct40 group in feeding periods 1 and 2 was significantly lower statistically than in feeding period 3, and normalized opacity was significantly lower statistically in feeding period 1 compared to feeding period 4 (one-way ANOVA, $P < 0.05$, Fig. 5). In the Feb40 group normalized otolith opacity was lower in feeding period 4 compared to the other periods (one-way ANOVA, $P < 0.05$). No difference in normalized otolith opacity was found among the feeding periods in the Dec40 group (one-way ANOVA, $P > 0.05$, Fig. 5).

Normalized opacity was also compared between the feeding groups during each feeding period. There was no significant difference in normalized opacity among the three groups during the first feeding period (one-way ANOVA, $P > 0.05$).

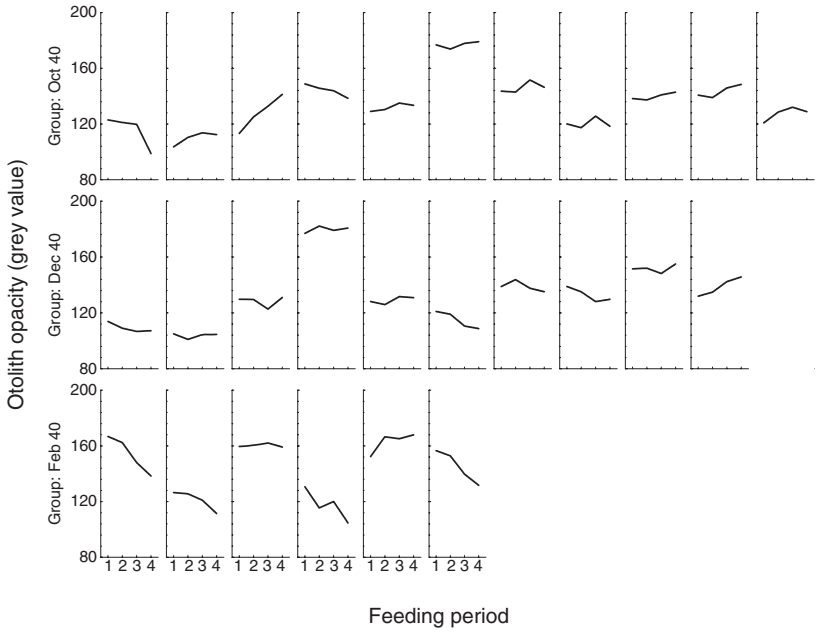

Fig. 4. Mean otolith opacity (grey values) during the four feeding periods for individual otoliths in the three feeding groups. Measurements were done from pictures taken with reflected light, thus low opacity indicates more translucent carbonate

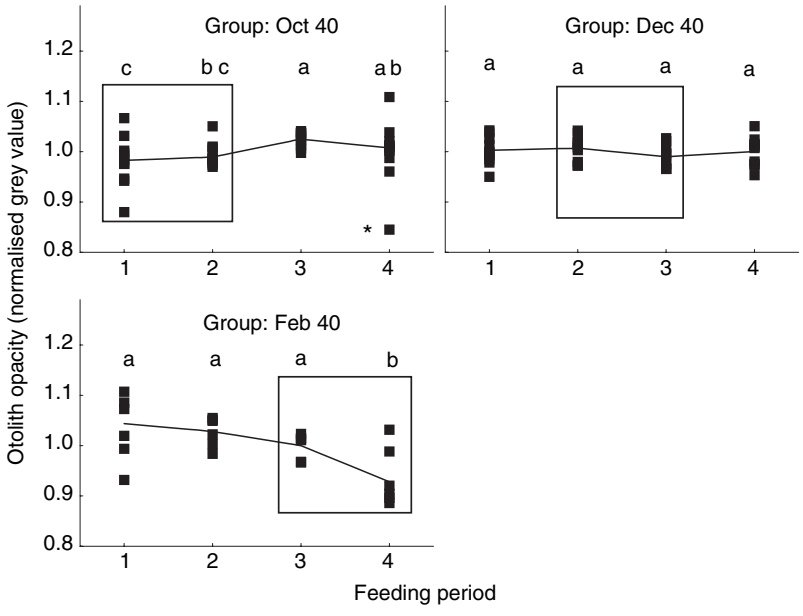

Fig. 5. Mean normalized otolith opacity (grey values) during four feeding periods for three feeding groups. Different numbers within each group reflect significant differences between feeding periods. Point marked with * in the Oct40 group represents an outlier more than eight standard errors lower than the group mean value not included in the statistical analyses. Large squares indicate period of restricted feeding for each group

The normalized opacity of otolith material deposited in the second feeding period was lower in the Oct40 group compared to the Feb40 group (one-way ANOVA, $P < 0.05$). In the third feeding period, the normalized opacity was significantly higher in the Oct40 group than in the Dec40 and Feb40 groups (one-way ANOVA, $P < 0.05$). The normalized opacity of the material deposited in the fourth feeding period was also significantly lower in the Feb40 group than in the Oct40 and Dec40 groups (one-way ANOVA, $P < 0.05$).

Discussion

In this study juvenile cod deposited more translucent otolith material in periods of restricted feeding, but otolith opacity varied considerably among individuals and feeding effects were indicated in only two of three experiments. The otolith growth model by Hüssy and Mosegaard (2004) predicts that starvation will result in more translucent otolith growth, but in a shorter experiment lasting for 40 days, young juvenile cod fed 65% of food required for maximum growth failed to show an effect of starvation on otolith opacity (Hüssy et al., 2004). However, individual fish growth rates were related to otolith opacity. The effect of feeding on the otolith opacity might therefore be weak and only triggered by severe food restriction. This experiment lasted longer and used lower rations during the periods of restricted feeding; the results indicate that reduced feeding can lead to more translucent otoliths. However, reduced somatic growth associated with opaque zone formation has also been reported (Schramm, 1989).

The effect of feeding on otolith opacity is probably relatively weak. Otolith material deposited prior to and after the experimental period was much more translucent compared to otolith material deposited during the experimental period. Those periods when the fish were kept in pens in the sea were characterized by higher water temperatures, which suggests that the temperature effect is stronger that the effect of reduced feeding. The combined effect of high temperature and reduced feeding was not addressed in this study, however such a combination could be important since temperature above a given level is associated with reduced growth and loss of appetite in fish (Brett, 1979). In the absence of fluctuating water temperature, Utah chub (*Gila atraria*, Girard) still formed annual translucent and opaque growth otolith rings (Johnson and Belk, 2004). However, feeding might not be the main reason for the seasonal otolith zones in the absence of temperature fluctuations. Circannual processes under endogenous physiological control might also be a controlling factor (Johnson and Belk, 2004).

Translucent otolith features that do not conform to the seasonal opaque and translucent bands of annuli, such as false and split or double zones, are called secondary structures (Panfili et al., 2002). Identification of such secondary structures is a major challenge for age estimation, and failure to separate the true seasonal structures from the secondary structures leads to inaccurate age estimates. Results from the present study demonstrate that periods of severe food reductions influence otolith opacity, which means that feeding can be one factor responsible for formation of secondary growth structures.

Standardized procedures are important when analysing otolith opacity in a quantitative manner. Much confusion of the terms 'opaque' and 'translucent otolith zones' in the literature is due to poorly defined light settings and the use of alternative terminology (Beckman and Wilson, 1995). In this study, we ensured that the otolith slides had the same thickness, and applied a standardized method when taking the pictures. This minimized opacity variation between otoliths caused by methodological artefacts. However, some individual variation in otolith opacity could be caused by different light scatter from the adjacent translucent carbonate that was deposited prior to and after the laboratory period. Variation in the size of these translucent zones as a result of individual variation in fish growth rates is likely. Wider translucent zones allow more light to penetrate into the otolith and possibly allow more light scatter into the neighbouring carbonate deposited during the experimental period. This could have influenced the recorded opacity for carbonate deposited during the experimental period.

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