PHASE DIFFERENCE BETWEEN CALCIFICATION AND ORGANIC MATRIX FORMATION IN THE DIURNAL GROWTH OF OTOLITHS IN THE RAINBOW TROUT, *SALMO GAIRDNERI*

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ABSTRACT

The relative role of calcium and organic matrix deposition in the formation of daily increments in otoliths was studied in in vitro preparations of otolith-containing sacculi of rainbow trout, *Salmo gairdneri*. Sacculi were incubated in a Ringer solution containing both ^{45}Ca and ^{3}H-glutamic acid for 2 hours at 6-h intervals throughout a 24-h period and then the uptake of these isotopes was determined for both otolith and saccular tissue fractions. Serum calcium and sodium concentrations were also analyzed for diurnal variations.

Serum calcium concentrations varied diurnally by 8% in a single phasic pattern, reaching a peak at dusk (1600 h) and a nadir at night (2200 h), while sodium concentrations remained almost constant throughout a 24-h period. Diurnal variation in the otolith's uptake of calcium and glutamic acid showed discrete, antiphasic cycles. The rate of calcium uptake varied in a pattern closely resembling that of serum calcium (the peak at 1600 h and the nadir at 2200 h); glutamic acid uptake remained almost constant during the daytime and peaked at night (2200 h). The results indicate that in rainbow trout daily increments of otoliths are formed by the antiphasic deposition of calcium and organic matrix.

Teleost otoliths consists of calcium carbonate in aragonite form and an organic matrix in which acidic amino acids dominate (Degens et al. 1969). Concentric rings within the microstructure of otoliths are commonly laid down on a daily basis (Campana and Neilson 1985; Jones 1986). A unit increment comprises one light and one dark ring when observed under transmitted light. These bipartite structures are also observable by scanning electron microscopy. After etching with weak acids or decalcification with calcium-chelating agents, they usually appear as an alternating pattern of well-calcified zones with elongated crystals perpendicular to the otolith periphery (accretion zone) and narrow grooves which intersects the crystal development at right angles (discontinuous zone). However, some recent studies (Mugiya and Muramatsu 1982; Watabe et al. 1982; Takahashi 1982; Morales-Nin 1987) showed that if the etching and subsequent treatments were carried out carefully, the organic matrix could be preserved in the discontinuous zone, appearing as a raised ridge. After complete decalcification of the otolith, Dean et al. (1983) and Radtke and Targett (1984) observed incremental features in the remaining matrix. Thus, stated in relative terms, the accretion and discontinuous zones appear to be alternatively calcium-dominant and matrix-dominant structures. However, Watabe et al. (1982) observed that morphologically similar matrix material extended continuously between accretion and discontinuous zones, and proposed a possible mechanism for otolith increment formation. For their recently proposed model, Campana and Neilson (1985) also assumed continuous matrix formation in diurnal otolith growth.

Based on these morphological studies, three hypotheses might account for the formation of the bipartite structure of otolith increments: 1) both organic matrix and calcium deposition show diurnal variations occurring in antiphase, 2) calcium deposition varies diurnally, while matrix deposition does not, and 3) calcium deposits at a constant rate throughout a 24-h period, while matrix deposition varies diurnally. All these would result in the formation of alternate zones where calcium or matrix deposition predominated. Of these, the last possibility can be excluded. Physiological studies indicate that the rate of calcium uptake by otoliths varies diurnally in goldfish and rainbow trout (Mugiya et al. 1981; Mugiya 1984).

The present study was undertaken to investigate diurnal variation in matrix formation and to

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relate its phase, if apparent, to otolith calcification. Because diurnal variations in otolith calcification show seasonality (Mugiya 1984), an ideal way to examine such a phase relationship is to determine the rates of calcium and matrix deposition on a single otolith simultaneously, using a double-tracer method and in vitro, isolated sacculi from rainbow trout. Diurnal profiles of glutamic acid and calcium uptake were examined in the otoliths and the remaining saccular tissue. Serum calcium and sodium concentrations were also measured for diurnal variations.

MATERIALS AND METHODS

Rainbow trout, *Salmo gairdneri*, 29-31 cm in standard length, were obtained from a commercial dealer and reared in a pair of outside ponds supplied with 14°C running water. They were fed trout food pellets once a day at around 0845 h. Two females showed maturing ovaries, so their data were omitted. No males were excluded because maturing testes have little, if any, effect on the level of serum calcium. The experiment was carried out in December 1984. Dusk occurred at 1600 h and dawn at 0700 h.

At each sampling time, five or six fish were gently netted one at a time. Blood was immediately collected from the caudal vessels by cutting the tail of the fish and draining it into test tubes. After centrifugation, the separated sera were stored at −30°C for 6-24 hours and analyzed for calcium and sodium concentrations by flame photometry using an atomic absorption spectrophotometer (Hitachi[2] 518).

After blood collection, the head was severed, trimmed, and placed in an oxygenated Ringer solution kept at 14°C. The sacculi were dissected under a binocular microscope according to a previously described technique (Mugiya 1984). The pair of sacculi were placed in the incubation medium, and the next fish was netted. Time was recorded to ensure that sacculi from each fish were incubated for the same length of time.

Isolated sacculi were placed in a glass vessel and incubated in 50 mL of a Ringer solution (Mugiya 1986) containing ^{45}Ca and ^{3}H-glutamic acid (New England Nuclear) at concentrations of approximately 0.17 μCi/mL and 0.33 μCi/mL respectively. The incubation was carried out with oxygenation at 14°C for 2 hours. To determine proper incubation times, the uptake of ^{3}H-glutamic acid by otoliths was plotted against time; although in this preliminary experiment, sacculi were incubated for periods of up to 3 hours, steady-state levels were obtained in less than 2 hours.

After incubation, sacculi were rinsed several times in the radioisotope-free Ringer solution and separated into otolith and saccular tissue fractions under a binocular microscope. The separated otoliths were lightly rinsed in water, placed in individual counting vials, dried at 90°C overnight and then weighed. The saccular tissue was directly placed in the vial without a further rinse and air-dried. These samples were solubilized in a mixture of 0.2 mL perchloric acid and 0.2 mL hydrogen peroxide at about 80°C for 2 hours, and added to Scintisol EX-H (Wako) for counting (liquid scintillation spectrometer, Aloka LSC-673).

Tritium and ^{45}Ca activities were measured simultaneously using two channels with narrowed windows, 50-300 for ^{3}H and 70-900 for ^{45}Ca. Although the amount of ^{3}H activity entering the Ca channel was found to be practically negligible, ^{45}Ca would certainly affect counts on the H channel, despite the window conditions. Therefore, counts on the H channel were corrected by the equation:

$$^{3}H \text{ activity} = H - 1/\alpha \, Ca \qquad (1)$$

where H and Ca represent counts on the H and Ca channels respectively, and α (contamination ratio) is experimentally defined as log $\alpha = 0.1386R - 0.0488$ where R is a ratio determined for the quenching level of each sample. The validity of this correction was further checked by another equation based on differences in the physical half-life of the isotopes:

$$^{3}H \text{ activity} = \frac{Ca_0H_2 - Ca_2H_0}{Ca_0 - Ca_2} \qquad (2)$$

where H_0 and Ca_0 are counts on the H and Ca channels at time 0 respectively, and H_2 and Ca_2 are those recounted a few months later. Because these two methods of discrimination gave essentially the same results, the data from Equation (1) were presented in this study.

Some rainbow trout have an aberrant otolith in either or both of their sacculi (Mugiya 1972). If

[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

the highly aberrant form was found by inspection after incubation, it was excluded from the data.

RESULTS

Serum calcium concentrations varied diurnally by approximately 8% in a single phasic pattern (Fig. 1). The maximum level (5.47 meq/L) occurred at dusk (1600 h), followed by a rapid decrease ($P < 0.05$) to a nadir (5.04 meq/L) at night (2200 h). The level then gradually increased toward the next peak. In contrast, serum sodium concentrations showed a statistically insignificant variation of only 0.6% ($P > 0.05$; 148.1-149.0 meq/L) throughout a 24-h period.

When otolith-containing sacculi were incubated with ^{3}H-glutamic acid, the saccular tissue (without otoliths) was almost saturated with the isotope within the first 30 minutes or hour of incubation (Fig. 2). Otoliths also showed a considerable uptake (about 60% of the total) of the isotope in the first 30 minutes, followed by a gradual increase in radioactivity until 3 hours, when the incubation was terminated. Tritium activities were always 6-8 times higher in the saccular tissue than in the respective otolith (Fig. 2). The time-related uptake of ^{45}Ca by these tissue fractions has been reported (Mugiya 1984). In that study, the saccular tissue was saturated with ^{45}Ca within the first hour of incubation, while otoliths showed an almost linear increase in ^{45}Ca uptake during the first 5 hours at which point the incubation was terminated.

The uptake of calcium by otoliths varied diurnally (Fig. 3), and the pattern was quite similar to that of diurnal variations in serum calcium concentrations (Fig. 1). The rate of calcium uptake was intermediate at 1000 h, peaked at dusk (1600 h), and then decreased significantly ($P < 0.02$) by 37% to a nadir at night (2200 h). The low rate persisted through the night, increasing slightly at 0400 h. Clearly otolith calcification proceeded more actively during the daytime. The uptake of glutamic acid by the same otoliths also showed a diurnal variation, and its profile was almost antiphasic to that of calcium uptake (Fig. 3). The rate of the uptake remained rather low during the daytime with a small nadir at dusk (1600 h). Then the rate increased significantly ($P < 0.05$) to a peak at night (2200 h), followed by a return to the daytime level. Thus the most active deposition of otolith matrix (at least proteins) occurred during the first half of the nighttime period, when calcium deposition was at its lowest level.

The uptake of glumatic acid by the saccular tissue showed significant ($P < 0.02$), diurnal

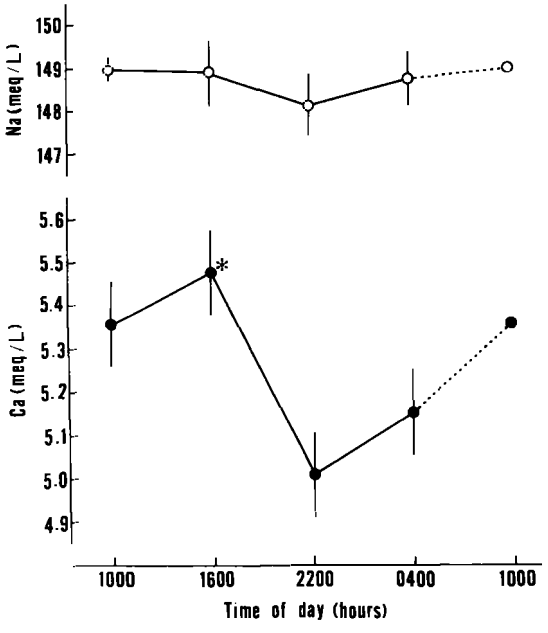

FIGURE 1.—Diurnal variations in serum calcium (●) and sodium (○) concentrations in rainbow trout. Each plotted value represents mean ± SE of 5 or 6 fish. *$P < 0.05$ for 2200 h.

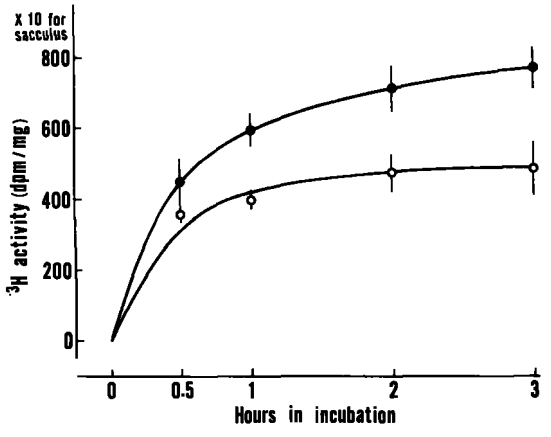

FIGURE 2.—Time course for the in vitro uptake of ^{3}H-glutamic acid by otoliths (●) and the saccular tissue (○) of isolated sacculi in rainbow trout. The radioactivity of the saccular tissue is expressed as dpm per otolith weight (mg) because the dry weight of the individual saccular tissue was too light to be determined accurately. Each plotted value represents mean ± SE of 8-10 samples.

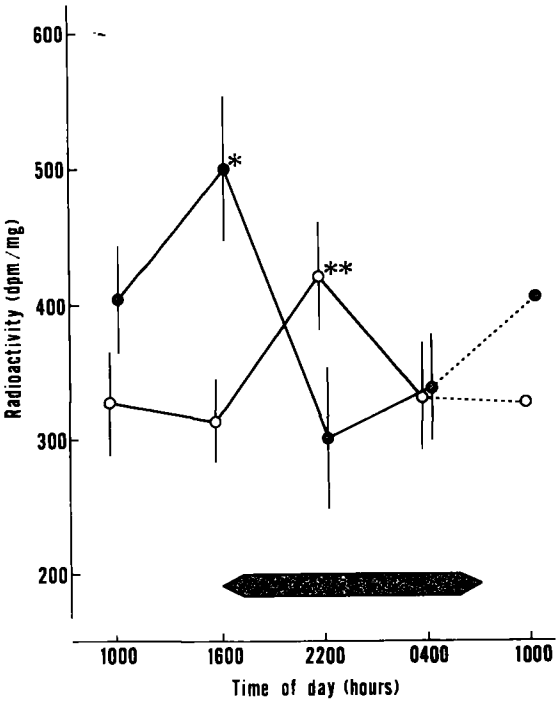

FIGURE 3.—Diurnal variations in the in vitro uptake of ^{45}Ca (●) and ^{3}H-glutamic acid (○) by otoliths in rainbow trout. Each plotted value represents mean ± SE of 8-10 samples. Dark horizontal bar indicates nocturnal and twilight periods. $*P < 0.02$ for 2200 h; $**P < 0.05$ for 1600 h.

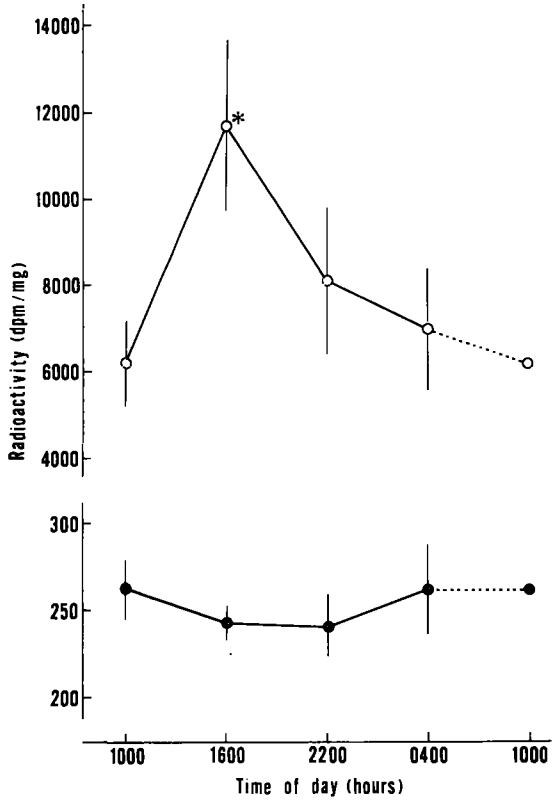

FIGURE 4.—Diurnal variations in the in vitro uptake of ^{45}Ca (●) and ^{3}H-glutamic acid (○) of saccular tissue in rainbow trout. Each plotted value represents mean ± SE of 8-10 samples. $*P < 0.02$ for 1000 h.

variation with a single peak at 1600 h when matrix deposition on the otoliths was lowest (Fig. 4). Note that the active biosynthesis of matrix proteins in the saccular tissue is not necessarily followed by their instantaneous deposition on the otoliths, suggesting the presence of cyclic secretion activity in the cells of the sacculus. The rate of calcium uptake by the saccular tissue did not vary much throughout a 24-h period (Fig. 4).

Ratios of counts of ^{45}Ca and ^{3}H-glutamic acid in the respective otoliths magnified the antiphasic relationship between ^{45}Ca and ^{3}H uptake (Fig. 5). Significant variation ($P < 0.01$) between the peak (1600 h) and the nadir (2200 h) demonstrates the much greater deposition of calcium relative to glutamic acid during the daytime, which suggests that in December the accretion zone forms during the daytime with its peak at dusk.

DISCUSSION

Although previous studies (Mugiya et al. 1981; Mugiya 1984) showed that otoliths grew by the diurnal deposition of calcium, it remained to be determined whether matrix deposition on the otoliths was diurnal or not. Histochemically, otolith matrix consists of various kinds of substances such as proteins, acid mucopolysaccharides, PAS-positive materials, and lipids (Mugiya 1968). Of these, proteins are the most dominant component and are characterized by a high content of acidic amino acids (Degens et al. 1969). In the present study the diurnal deposition of otolith matrix was evident when examined in terms of the incorporation of glutamic acid into otoliths, showing a single peak at night. Interestingly, calcium deposition on the same otoliths proceeded most actively at dusk, followed by minimum deposition at night. Thus, it is concluded, in rainbow trout kept under natural photoperiod, the pace of otolith calcification is almost antiphasal to the pace of matrix deposition on the otoliths.

The present results, where both calcium and

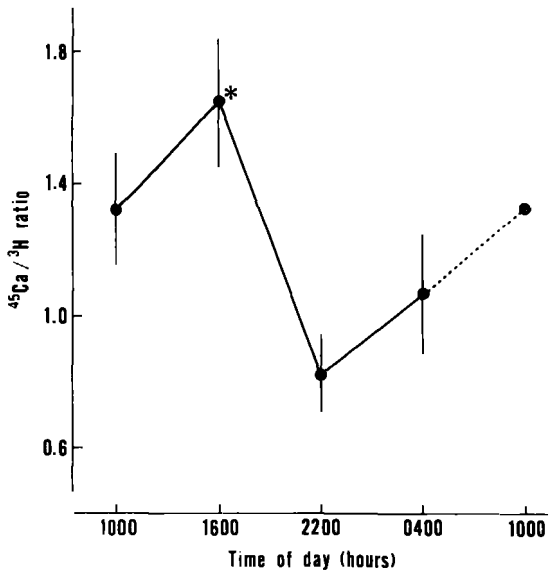

FIGURE 5.—Diurnal change in the ratio of ^{45}Ca to ^{3}H-glutamic acid activity incorporated into the same otoliths in rainbow trout. Each plotted value represents mean ± SE of 8-10 samples. *$P < 0.01$ for 2200 h.

matrix deposition on otoliths varied diurnally in antiphase, indicate the relative importance of these substances for daily increment formation in otoliths. The accretion zone is formed predominantly by calcium deposition, while the discontinuous zone results from reduced calcium and substantially increased matrix deposition on the otoliths. These findings coincide with the morphological observation that the accretion zone is a crystalline layer with organic materials and the discontinuous zone is a layer containing more organic materials and less calcium (Mugiya and Muramatsu 1982; Watabe et al. 1982). Watabe et al. (1982) observed that the matrix fibers and their aggregates were morphologically similar in the two zones and continuous throughout in *Tilapia* and *Fundulus* otoliths. Based on these observations, they have suggested that the matrix materials are identical in the zones and their deposition might be an uninterrupted event during diurnal otolith growth. They also stated that this did not necessarily imply that the rate of organic matrix secretion was diurnally constant. In fact, the present study reveals that diurnal variations in the rate of the matrix deposition, coupled with variations in calcium deposition, play an important role in otolith increment formation.

Although the sacculus contains the otolith, otolithic membrane, and endolymph, a high content of acidic amino acids is characteristic of the calcified otolith (Degens et al. 1969). Therefore, variations in the uptake of glutamic acid by the saccular tissue should be closely related to the activity of the matrix formation of the otolith, even though glutamate is also used as a neurotransmitter in the saccular macula (Potter et al. 1986). Otolith forming cells, yet to be positively identified (Mugiya 1974; Dale 1976; Dunkelberger et al. 1980; Saito 1984), synthesize the precursor of the matrix and secrete it into the lumen. The precursor may then deposit on the otolith after further biochemical modification.

The present study showed the presence of a time-lag between the matrix biosynthesis in the saccular tissue and its deposition on the otoliths. However, this does not necessarily mean that these two processes are separated in phase. In this study, calcification and matrix formation were measured in terms of "instantaneous" growth rates (Ottaway 1978). Therefore the maximum deposition of organic matrix on otoliths at night must be accompanied by the active biosynthesis of the matrix in the cellular level and its consecutive secretion into the lumen, which may rather reduce the radioactivity in the saccular tissue. The high radioactivity in the tissue at dusk might result from the accumulation of the newly synthesized matrix owing to the reduction of its transport to the otoliths. These results suggest the presence of at least three different phases in otolith matrix formation: the active synthesis of matrix proteins on the cellular level with its reduced deposition on the otoliths, active synthesis with active deposition, and inactivity in both synthesis and deposition.

Mugiya (1984) reported that the profile of diurnal otolith calcification was antiphasal between the summer and winter solstices in rainbow trout. In the winter experiment, the peak and the nadir of calcium deposition on otoliths came at 1600 h and 0400 h, respectively; while in the present winter experiment the peak at 1600 h decreased to the nadir earlier, at 2200 h, followed by a slight increase at 0400 h. Although there is a difference in the time-related profiles, the results of both experiments mainly showed that otolith calcification slowed down after the onset of darkness and remained relatively inactive until the next sunrise.

Molluscan nacre shows a laminar structure resulting from alternate accumulation of organic

matrix and calcium carbonate crystals. In the development of these bipartite structures, matrix deposition on growing crystals is known to interrupt further crystal growth (Wilbur 1980). This results in the alternate formation of calcium-rich and matrix-rich layers. In such cases, the matrix may have opposite functions in controlling crystal growth: as an inhibitor for the crystal growth along the C-axis and as a nucleator for the formation of the next crystal layer (Crenshaw 1982). The matrix appears to play a key role in controlling the formation of these different layers. Although this sequence is likely to be the case for otolith increment formation (Wilbur 1980), the rate of calcium deposition on otoliths appears to be closely related to the level of serum calcium, which is regulated by the action of hypercalcemic and hypocalcemic hormones (Oguro and Pang 1982). Serum calcium has been suggested as a trigger for otolith calcification through the calcium-calmodulin system (Mugiya 1986). Although Pickford (1953) found that hypophysectomy resulted in no otolith growth in killifish, what exactly controls the rate of matrix deposition on otoliths remains unknown.

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