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Diel changes in endolymph aragonite saturation rate and mRNA expression of otolith matrix proteins in the trout otolith organ

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ABSTRACT: In the teleost fish otolith, the alternate deposition of $CaCO_3$-rich and protein-rich layers results in the formation of daily increments. In order to clarify the mechanism of daily increment formation, a precise understanding of the relationship between ionic and organic controls of otolith growth is essential. In the present study, we studied diel variations in the aragonite saturation rate (S_a) of the endolymph and the mRNA expression of 2 major otolith matrix proteins, OMP-1 and otolin-1, in the saccular tissue. A new technique for simultaneously quantifying endolymph S_a and mRNA expression in the saccular tissue from an individual rainbow trout was developed. Endolymph Ca^{2+} levels, CO_2 partial pressure (P_{CO_2}) and pH were simultaneously measured using an automatic pH/gas/electrolyte analyzer, and the S_a was calculated. Total RNA was isolated from sacculi of individual fish after the endolymph was obtained. cDNAs were synthesized and used to quantify OMP-1 and otolin-1 mRNA expression levels by real-time PCR. Significant diel variations were observed in endolymph pH and P_{CO_2} levels in an antiphasic manner. Endolymph S_a did not exhibit significant diel variations and was maintained at a value of more than 2.0, indicating that the endolymph was kept supersaturated with respect to aragonite during the day-night cycle. Expression of otolin-1 mRNA had apparent diel variations with high levels at night, whereas that of OMP-1 mRNA was almost constant. These data strongly suggest an organic control of daily increment formation in the otolith. The most probable candidate protein for daily increment formation is otolin-1.

KEY WORDS: Calcification · mRNA expression · Endolymph · Daily increment · Aragonite · Otolith · Rainbow trout

INTRODUCTION

Teleost fish otoliths are calcified concretions composed of organic matrices and calcium carbonate aragonite polycrystals (cf. Campana & Neilson 1985, Campana 1999). Since Pannela (1971) described the occurrence of daily increments in otolith microstructure, otoliths have been used as determinants of daily-age in both larval and juvenile teleost fishes. Consequently, a large number of publications are dedicated to the use of otolith microstructures as a key for clarifying the population dynamics and life histories of teleost fishes. However, fundamental research on the mechanisms of otolith formation is insufficient and a great deal remains unknown.

Otolith microincrements consist of alternately deposited light and dark bands observed under transmitted light. The light band is generally called the L-zone,

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and the dark one is referred to as the D-zone (Kalish et al. 1995). Morphological examinations have revealed that the D-zones contain more organic matrices and less calcium carbonate relative to the L-zones (Mugiya & Muramatsu 1982, Watabe et al. 1982, Morales-Nin 1987); therefore, the alternate manner in which the deposition of mineral-rich and organic-matrix-rich layers occurs is manifested as daily increments. Indeed, Mugiya (1987) reported the discrete antiphasic diel cycles of ^{45}Ca and ^{3}H-glutamic-acid uptake into the otoliths in the rainbow trout *in vitro* and suggested that the daily increments of otoliths were formed by the antiphasic deposition of calcium and organic matrices. Daily variations in otolith calcification have also been demonstrated in the goldfish (Mugiya et al. 1981, Tohse & Mugiya 2002) and in the Atlantic salmon (Wright et al. 1992).

The mechanism of these diel variations in otolith calcification and the deposition of the organic matrix is unknown. The otolith can be defined as acellular tissue that grows in an inner ear sacculus filled with endolymph. Consequently, endolymph must contain all the precursors required for otolith development, and the physicochemical characteristics of this fluid may affect otolith growth. The aragonite saturation rate (S_a) of endolymph is considered to be one of the candidates that regulates otolith calcification, since aragonite precipitation rate from a dilute solution has been demonstrated to correlate strongly with the S_a of the solution in a non-biological system (Romanek et al. 1991). Conversely, variations in the synthesis, secretion and endolymph concentration of the matrix proteins probably lead to fluctuations in the matrix deposition onto the otolith. The otolith matrix proteins may also affect otolith calcification, because organic matrices in biominerals have been reported to affect mineral deposition in general (Wheeler & Sikes 1984, Addadi & Weiner 1985, Belcher et al. 1996, Falini et al. 1996), and because otolith matrix proteins are known to interact with Ca or $CaCO_3$ (Baba et al. 1991, Wright 1991, Asano & Mugiya 1992, Sasagawa & Mugiya 1996, Borelli et al. 2001, Murayama 2002).

The S_a is a function of the chemical composition of the solution. The first determination of the endolymph S_a in a single fish species based on the direct quantification of endolymph chemical composition was performed by Takagi (2002) in the rainbow trout. He reported that trout endolymph was supersaturated with respect to aragonite. Previous studies have also partially determined endolymph chemical compositions (Enger 1964, Mugiya 1966, Fänge et al. 1972, Watanabe & Miyamoto 1973, Mugiya & Takahashi 1985, Kalish 1991, Payan et al. 1997, 1998, 1999, Takagi 1997, Gauldie & Romanek 1998, Edeyer et al. 2000, Borelli et al. 2003), with some authors reporting diel changes in endolymph composition. Although these data suggest that the diel variations in the endolymph composition generate daily rhythm of otolith calcification through the fluctuations of S_a, no direct determination of diel variations in endolymph S_a has been conducted.

Otolith organic matrix proteins are synthesized in the epithelial cells of the sacculus and secreted into the endolymph (Takagi & Takahashi 1999, Takagi 2000, Takagi et al. 2000, Murayama et al. 2002, 2004). However, otolith matrix proteins seem to constitute rather minor components of the endolymph proteins (Takagi & Takahashi 1999, Borelli et al. 2001); thus, reported diel variations in the concentrations of total endolymph proteins (Edeyer et al. 2000, Borelli et al. 2003) may not be reasonably correlated to the diurnal rhythm in otolith matrix deposition. The only study that has dealt with the endolymph concentrations of otolith matrix proteins is the report of Borelli et al. (2003), in which antibodies were raised against the acetic-acid-soluble fraction of the otolith matrix proteins in the rainbow trout. Using densitometric analysis of Western blot samples of the endolymph, these authors demonstrated that the 2 bands that reacted with the antibody were more concentrated during the day than at night. However, no direct quantification of the synthesis and secretion of otolith proteins has been undertaken to date.

Recently, we cloned 2 cDNAs of the otolith matrix protein, OMP-1 and otolin-1, from salmonid fishes and demonstrated their specific expressions in the otolith organ (Murayama et al. 2000, 2002). Immunohistochemical studies using specific antibodies against OMP-1 and otolin-1 proteins, respectively, revealed that the differences in the densities of these proteins are responsible for the development of the concentric ring-like structures in the otolith matrix (Murayama 2002, Murayama et al. 2002, 2004).

In the present study, we studied diel variations in the endolymph S_a and the mRNA expression of OMP-1 and otolin-1 in the saccular tissue. A new technique for simultaneously quantifying endolymph S_a and mRNA expression in the saccular tissue from an individual rainbow trout was developed.

MATERIALS AND METHODS

Fish and maintenance. Rainbow trout *Oncorhynchus mykiss*, weighing about 800 to 1000 g, were obtained from a local farm in Hokkaido, Japan. They were maintained in outdoor tanks with continuous supplies of fresh water and fed to satiation with commercial trout pellets once a day at 13:00 h. Water temperature on the days of sampling fluctuated daily between 12 and 16.5°C. Fish were acclimatized to experimental

conditions for about 3 wk prior to initiation of sampling.

Sampling of blood, endolymph and sacculi. At 09:00, 15:00 and 21:00 h on August 27 and at 03:00 and 09:00 h on August 28, 2003, blood, endolymph and saccular tissue from 9 to 12 fish were sampled as described below. Our preliminary results suggested that frequent repetition of sampling at short time intervals (<15 min) was stressful to the remaining fish in the tank. Fish were, therefore, kept at rest for at least 5 h before the next sampling. Fish were deeply anaesthetized in a 0.1% solution of 2-phenoxyethanol, and blood was taken from the caudal vessels without touching the air using a heparinized syringe. The fish were then decapitated, and the blood samples and heads kept on ice until further processing. Both left and right sacculi were sampled and endolymph was collected as previously described (Takagi 2002). Briefly, the sacculi were immersed in liquid paraffin to avoid any contact of endolymph with the air. The endolymph was then sucked into capillary tubes with the liquid paraffin, sealed at one end with a capillary sealer, and centrifuged for 3 s at 10 000 × *g*. Ca^{2+}, CO_2 partial pressure (P_{CO_2}) and pH levels were measured immediately after centrifugation as described below. Pooled samples from the left and right sacculi of each individual were sufficient to assay endolymph chemicals. Once endolymph had been sampled, both sacculi were immediately frozen in liquid nitrogen and kept at under −80°C until the extraction of RNA.

Determination of blood and endolymph chemicals. Blood Ca^{2+} (mmol l^{-1}), Na^+ (mmol l^{-1}), P_{CO_2} (torr) and pH levels, and endolymph Ca^{2+}, P_{CO_2} and pH levels were measured using an automatic pH/gas/electrolyte analyzer (Model 348, Chiron Diagnostics Limited). Plasma and endolymph HCO_3^- and CO_3^{2-} levels (mmol l^{-1}) were obtained using the Henderson-Hasselbalch equation. The aragonite saturation rate (S_a) of the endolymph was calculated as follows:

$$S_a = \sqrt{\frac{(Ca^{2+})(CO_3^{2-})}{K^0_{S,a}}}$$

where (C_i) is the activity of ionic species *i*, and $K^0_{S,a}$ is the thermodynamic solubility product of aragonite. When $S_a < 1$, $S_a = 1$ and $S_a > 1$, the solution is respectively undersaturated, at equilibrium and supersaturated with respect to aragonite. A more detailed method of calculation is described in Takagi (2002).

Quantification of mRNA expression for OMP-1 and otolin-1 in saccular tissue. One sacculus per fish was used to quantify mRNA expression of OMP-1 and otolin-1. Total RNA was prepared from individual sacculi using Isogen (Nippon gene) according to the manufacturer's instructions. After DNase I (TaKaRa) treatment, 30 ng of each total RNA was reverse-transcribed using a First-Strand cDNA Synthesis kit (Amersham Biosciences) with oligo-$d(T)_{18}$ primer. Using 1/100 aliquots of each cDNA as a template, quantitative PCR was performed using gene-specific primers and TaqMan™ probes depicted in Table 1. The reaction mixture (50 µl) contains 1 × QPCR mix (Absolute™ QPCR ROX Mix, Advanced Biotechnologies), 900 nM of each primer, 450 nM of each probe, and first-strand cDNA. The conditions for PCR included an initial step at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 58°C for 1 min. Amplification plots were collected using a sequence detection system (ABI PRISM 7700 Sequence Detection System, Applied Biosystems).

Table 1. Oligonucleotide primers and probes used in quantitative RT-PCR

mRNA	Primer/Probe	Sequence (5′–3′)
OMP-1	Forward primer	GAATGGTACAGCGGATGCA
	Probe	CAGCCATGTTTGCTGATGACATCTACACAG
	Reverse primer	CTAGTCCAAAGCACCAGCCA
Otolin-1	Forward primer	TCTACGTGTTCTCCTACCACATCAC
	Probe	TGCGCAACCGCCCCCTG
	Reverse primer	GCTTCTTCACGCCGTTAATCA
β-actin	Forward primer	ATCACCATCGGCAACGAGAG
	Probe	CTCCTTCCTCGGTATGGAGTCTTGCGGTAT
	Reverse primer	TGGAGTTGTAGGTGGTCTCGTG

Detected copy numbers of OMP-1 and otolin-1 mRNAs were standardized against β-actin mRNA. Expression levels of β-actin mRNA were almost constant for the duration of the experiment.

Statistics. Data are presented as means and standard errors. To assess the significance of the variations, data were analysed using the Kruskal-Wallis test. When a significant difference was detected by the Kruskal-Wallis test, significance between 2 selected groups was tested using the Mann-Whitney *U*-test. The relationships of pH, P_{CO_2} and ion levels between endolymph and blood were analysed using a Spearman rank correlation test. StatView software (SAS Institute) was used for analyses and statistical significance was taken as $p < 0.05$.

RESULTS

Endolymph pH, P_{CO_2}, S_a and ion levels

Endolymph Ca^{2+} levels were observed to vary significantly depending on the sampling time (Fig. 1A). Endolymph pH levels exhibited significant diel fluctuations with high values observed at night (Fig. 1B). Conversely,

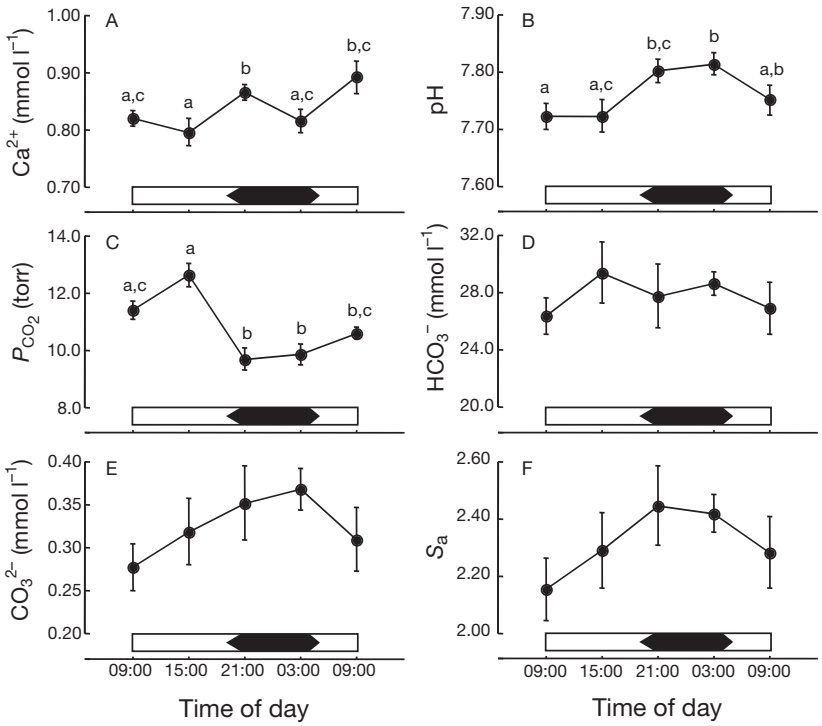

Fig. 1. *Oncorhynchus mykiss*. Diel variations in the endolymph parameters. (A) Ca^{2+}, (B) pH, (C) P_{CO_2}, (D) HCO_3^-, (E) CO_3^{2-}, (F) aragonite saturation rate (S_a). Each point and bar represent mean and SE. Number of samples measured: 9 to 12. Significant variations were observed in the levels of Ca^{2+}, pH and P_{CO_2} (Kruskal-Wallis test, $p < 0.05$), and data not sharing a common letter are significantly different (Mann-Whitney *U*-test, $p < 0.05$)

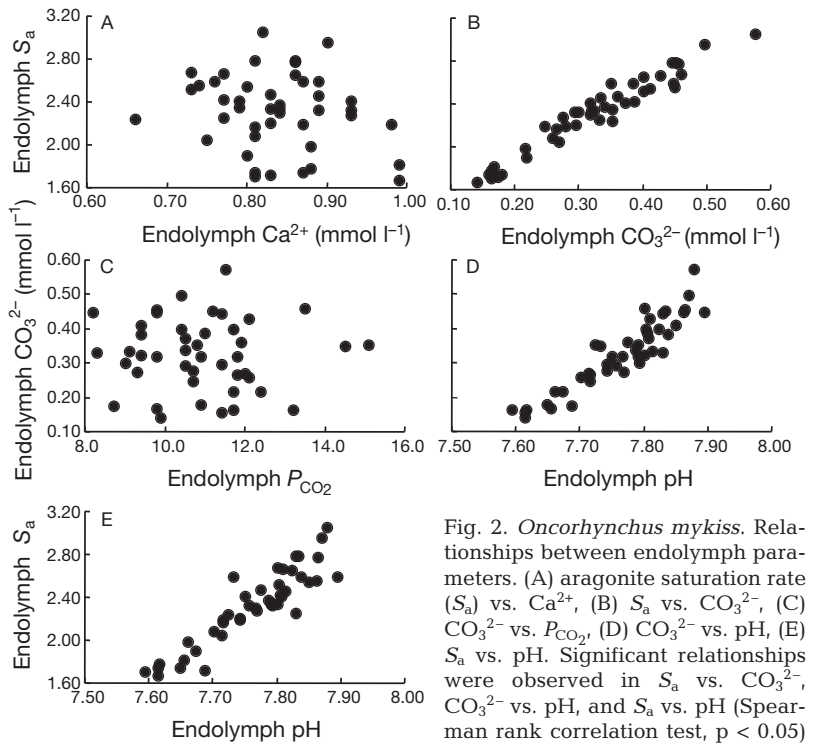

Fig. 2. *Oncorhynchus mykiss*. Relationships between endolymph parameters. (A) aragonite saturation rate (S_a) vs. Ca^{2+}, (B) S_a vs. CO_3^{2-}, (C) CO_3^{2-} vs. P_{CO_2}, (D) CO_3^{2-} vs. pH, (E) S_a vs. pH. Significant relationships were observed in S_a vs. CO_3^{2-}, CO_3^{2-} vs. pH, and S_a vs. pH (Spearman rank correlation test, $p < 0.05$)

P_{CO_2} levels were significantly higher during the day (Fig. 1C). No significant variations were observed in HCO_3^- concentrations (Fig. 1D). Levels of CO_3^{2-} exhibited diel fluctuations, with relatively higher values observed at night, but the differences were not statistically significant (Fig. 1E). Endolymph S_a did not exhibit significant diel variations and was maintained at a value of more than 2.0, indicating that the endolymph was kept supersaturated with respect to aragonite during the day-night cycle (Fig. 1F).

Since pH, Ca^{2+} concentrations, P_{CO_2} levels, CO_3^{2-} concentrations, and the S_a of the endolymph were obtained from individual fish, the relationships between these factors could be analyzed (Fig. 2). S_a correlated significantly with CO_3^{2-} levels, but not with Ca^{2+} concentrations (Fig. 2A,B). CO_3^{2-} levels were closely related to pH, but not to P_{CO_2} levels (Fig. 2C,D). Consequently, a linear correlation was observed between S_a and pH (Fig. 2E).

Plasma pH, P_{CO_2} and ion levels

Significant diel fluctuations were observed in all of the plasma parameters assayed (Fig. 3). Plasma Ca^{2+} and P_{CO_2} levels were highest during the day, decreased significantly during the night and returned to initial levels at 09:00 h of Day 2 (Fig. 3A,D). In contrast, pH, HCO_3^- and CO_3^{2-} levels had minimum values at 15:00 h, which then gradually increased to the initial levels (Fig. 3C,E,F). Plasma Na^+ concentrations were significantly higher at 09:00 h of Day 2 (Fig. 3B).

The relationships of pH levels, P_{CO_2} and ion concentrations between endolymph and plasma are shown in Fig. 4. A significant correlation was only observed between the P_{CO_2} levels in the endolymph and those in the plasma (Fig. 4C).

Expression of OMP-1 and otolin-1 mRNAs

Fig. 5 shows the diel variations in the expression of OMP-1 and otolin-1 mRNAs in the saccular tissue. The relative

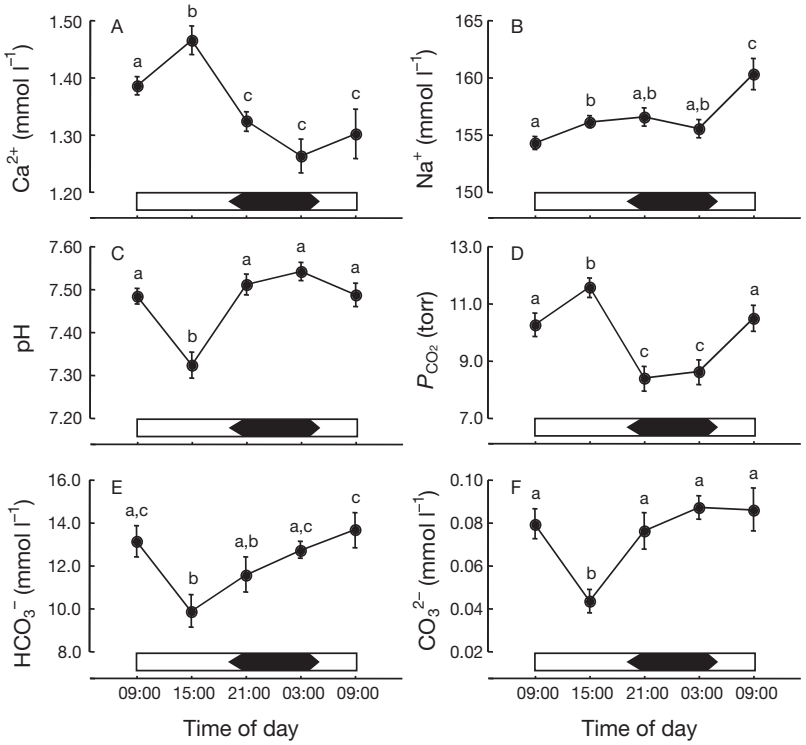

Fig. 3. *Oncorhynchus mykiss*. Diel variations in plasma parameters. (A) Ca^{2+}, (B) Na^{+}, (C) pH, (D) P_{CO_2}, (E) HCO_3^-, (F) CO_3^{2-}. Each point and bar represent mean and SE. Number of samples measured: 9 to 12. Significant variations were observed in all the parameters (Kruskal-Wallis test, $p < 0.05$), and data not sharing a common letter are significantly different (Mann-Whitney *U*-test, $p < 0.05$)

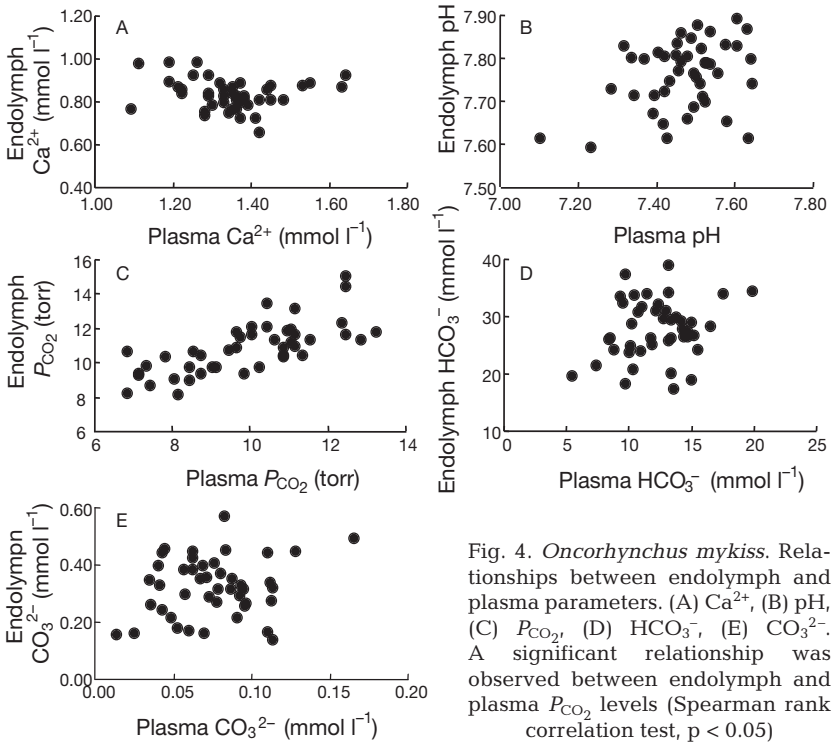

Fig. 4. *Oncorhynchus mykiss*. Relationships between endolymph and plasma parameters. (A) Ca^{2+}, (B) pH, (C) P_{CO_2}, (D) HCO_3^-, (E) CO_3^{2-}. A significant relationship was observed between endolymph and plasma P_{CO_2} levels (Spearman rank correlation test, $p < 0.05$)

expression of OMP-1 mRNA to β-actin expression was around 0.2; it was almost constant during the day-night cycle and was maintained at levels that exceeded those observed for otolin-1 mRNA expression. The expression of otolin-1 mRNA exhibited significant diel changes with higher values recorded at night. During the day (between 09:00 and 15:00 h), the relative expression of otolin-1 mRNA was approximately 0.1, half that observed for OMP-1 mRNA expression. At 21:00 h, however, expression was observed to increase markedly reaching a peak value of about 0.2 that was maintained until 03:00 h, before decreasing to approximately 0.1 at 09:00 h the next day.

DISCUSSION

The alternate deposition of mineral-rich and organic-matrix-rich layers constructs daily increments in the teleost fish otolith (Mugiya & Muramatsu 1982, Watabe et al. 1982, Morales-Nin 1987). In order to clarify the mechanism underlying the formation of daily increments, a thorough understanding of the relationship between ionic and organic controls of otolith growth is necessary. In the present study, we developed a technique to simultaneously quantify endolymph ionic composition and mRNA expression levels of otolith matrix proteins, OMP-1 and otolin-1, in the saccular tissue from an individual rainbow trout. From the quantified ionic composition, the aragonite saturation rate (S_a) of the endolymph was calculated. The results clearly showed (1) that the mRNA expression of otolin-1 exhibits a diel rhythm with higher expression occurring at night, and (2) that the saccular endolymph is supersaturated with respect to aragonite and that the saturation ratio is independent of the day-night cycle. This is the first study to simultaneously investigate the relationship between ionic and organic controls of otolith growth using data from individual fish.

The marked diel fluctuation in mRNA expression of otolin-1 indicates that the diel fluctuation reported for matrix

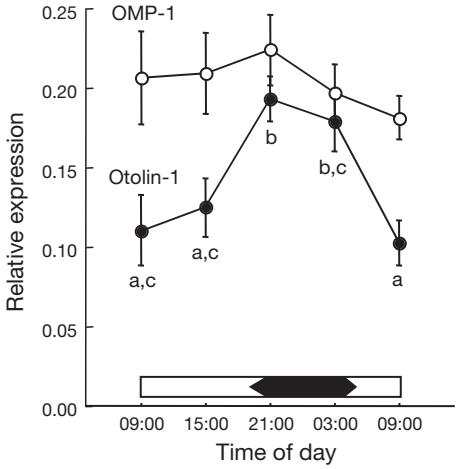

Fig. 5. *Oncorhynchus mykiss*. Diel variations in the expression of OMP-1 and otolin-1 mRNAs in saccular tissue. Detected copy numbers of OMP-1 and otolin-1 mRNAs were standardized against β-actin mRNA. Each point and bar represent mean and SE. Number of samples measured: 9 to 12. A significant variation was observed in the expression of otolin-1 mRNA (Kruskal-Wallis test, $p < 0.05$), and data not sharing a common letter are significantly different (Mann-Whitney *U*-test, $p < 0.05$)

deposition in the otolith (Mugiya 1987) is regulated at the level of mRNA-expression of matrix protein. Otolin-1 is the most abundant protein contained in the EDTA-insoluble fraction of the otolith matrices and exhibits structural similarity with collagen types VIII and X (Murayama et al. 2002). Collagen types VIII and X are short-chain collagens known to form a 3-dimensional structure (Sawada et al. 1990, Kwan et al. 1991). Consequently, it has been suggested that otolin-1 is a prime candidate for development of the otolith framework (Murayama et al. 2002). It is, therefore, reasonable to hypothesize that the diel fluctuations in otolin-1 synthesis are manifested as the formation of daily increments. Immunohistochemical studies have also implicated otolin-1 in the formation of concentric ring-like depositions of the organic matrix in the trout otolith (Murayama et al. 2004).

Although immunohistochemical data suggest that cyclical deposition of OMP-1 occurs in the fish otolith (Murayama et al. 2004), expression of OMP-1 mRNA in this study did not exhibit marked diel changes. Using northern blot analysis, Murayama et al. (2000) also demonstrated that expression of OMP-1 mRNA did not exhibit any significant diurnal changes in the saccular tissues of the rainbow trout and chum salmon. Present experimental evidence does not, therefore, support the active involvement of OMP-1 in the formation of daily increments. Rather, the role of OMP-1 may be one involved in otolith calcification, since recombinant proteins of OMP-1 have the ability to interact with $CaCO_3$ crystals (Murayama 2002). At present, the mechanism of the cyclical deposition observed in OMP-1 is unclear, and thus should be carefully examined in future.

The present study applied the method of Takagi (2002) to calculate the diel changes in the endolymph S_a. It was found that endolymph was kept supersaturated with respect to aragonite during the day-night cycle. The diel fluctuations in S_a were small and insignificant, although endolymph levels of Ca^{2+}, pH and P_{CO_2} showed significant diel variations. In such a supersaturated solution, calcium carbonate crystals can grow continuously in a non-biological system. These findings conflict with previously published reports in which clear diel fluctuations in Ca deposition into the otolith were observed (Mugiya et al. 1981, Mugiya 1984, 1987, Wright et al. 1992). The present data, therefore, strongly imply the involvement of an organic control mechanism in otolith calcification. Otolith matrix proteins probably have a crucial function in mediating or inhibiting aragonite deposition, and diel fluctuations in deposition of the matrix proteins lead to fluctuations in calcification. Although several reports have tried to explain the interaction between the otolith organic matrices and Ca^{2+} or $CaCO_3$ (Baba et al. 1991, Wright 1991, Asano & Mugiya 1992, Sasagawa & Mugiya 1996, Borelli et al. 2001, Murayama 2002), the precise nature of the relationship between them remains unknown. In order to understand the mechanism of increment formation more accurately, we would have to clarify the function of each component of the organic matrices more precisely.

In assays of the proximal and distal zones of the sacculus in rainbow trout, Payan et al. (1999) demonstrated that the chemical composition of endolymph was not spatially uniform. While the endolymph used in the present study was a mixture of proximal and distal endolymph, its electrolyte composition was close to the values reported for distal endolymph by Payan et al. (1999). This may simply be a consequence of the much larger size of the distal space relative to the proximal space in the trout sacculus used in the present study, and the diel changes in the endolymph chemicals and saturation state reported here may thus be representative of the trout distal endolymph.

Borelli et al. (2003) sampled proximal and distal endolymph separately, estimated saturation states using total Ca, CO_2, protein levels and pH, and compared the saturation states between day and night. Similar to the results of our study, the data obtained in their experiment suggested that the distal endolymph was supersaturated with respect to aragonite. In the proximal endolymph, however, the S_a was in equilibrium (around 1.0), clearly lower than that observed in the distal zone. From this result, they suggested that otolith calcification in the proximal side only occurred when the

S_a exceeded 1.0. They proposed that the pH remained unchanged, and instead, that changes in Ca^{2+} and CO_3^{2-} levels might lead to the changes observed in endolymph S_a. These suggestions contrast markedly with the findings of this study that are representative of the distal endolymph. In the present study, endolymph was always supersaturated, and diel S_a variations were small and insignificant. Moreover, S_a was largely determined by the CO_3^{2-} levels that were dependent on pH levels, and variations in Ca^{2+} levels had insignificant effects on S_a. At present, technical constraints mean that direct quantification of S_a in proximal endolymph is impossible. However, these constraints need to be overcome in the future so that the chemical composition and saturation state of the proximal endolymph can be quantified directly, and for the assumptions of Borelli et al. (2003) to be verified.

The present study demonstrated that changes in the concentrations of endolymph chemicals, except for P_{CO_2} levels, were independent of those in the plasma. This would suggest that the regulatory mechanisms for electrolyte transport are at the level of the saccular epithelium. Several previous studies have suggested the existence of energy-dependent electrolyte transport systems in the saccular epithelium. Mugiya & Yoshida (1995) proposed a transcellular route for Ca^{2+} entry from plasma into the endolymph, involving a receptor-operated Ca^{2+} channel, a Na^+/Ca^{2+} exchanger and a Ca^{2+}-ATPase in the trout sacculus. Tohse & Mugiya (2001) suggested that a HCO_3^--ATPase and a Cl^-/HCO_3^- exchanger are involved in the transepithelial transport of HCO_3^- to the endolymph in the sacculus of the masu salmon. Recently, Tohse & Mugiya (2004) indicated that the metabolic inhibitor, cyanide, reduced the incorporation of Ca^{2+} and carbonate ions into the endolymph and otolith, suggesting that active transport mechanisms of both ions are found in the salmon sacculus. Conversely, Payan et al. (2002) studied the kinetic and pharmacological characteristics of Ca^{2+} fluxes across the saccular epithelium of the rainbow trout and suggested that endolymph, especially the proximal one, was supplied with Ca^{2+} in a concentration-gradient-dependent manner via a paracellular pathway. In the present study, the Ca^{2+} levels in the endolymph were not correlated with those of the plasma. In fact, variations in endolymph Ca^{2+} levels were smaller than those observed in the plasma, suggesting the strict control of endolymph Ca^{2+} levels. These data suggest the existence of regulatory mechanisms of Ca^{2+} transport into the endolymph, and thus support the idea of a transcellular pathway for Ca^{2+} entry, at least in the distal zone of the sacculus.

In conclusion, from the present results, we propose revision of a previous model to describe otolith growth and increment formation. The endolymph, at least in the distal zone, is kept supersaturated with respect to aragonite during the day–night cycle. On the other hand, some of the otolith matrix proteins are incorporated into the otolith in a diel, cyclical manner and regulate otolith growth, calcification and increment formation. Otolin-1, the synthesis of which is characterised by diel fluctuations at the level of mRNA expression, is the most likely candidate protein for increment formation.

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