

Otolith formation and endolymph chemistry: a strong correlation between the aragonite saturation state and pH in the endolymph of the trout otolith organ

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ABSTRACT: This is the first report on the aragonite saturation state of the endolymph in a single fish species, the rainbow trout *Oncorhynchus mykiss*, based on the direct quantification of electrolyte concentrations in the saccular endolymph. The Ca^{2+} level, CO_2 partial pressure and pH of the saccular endolymph in 1+ and 2+ yr old trout were simultaneously determined using an automatic pH/blood gas/electrolyte analyzer. From the values of CO_2 partial pressure and pH, HCO_3^- and CO_3^{2-} levels were obtained using the Henderson-Hasselbalch equation. In addition, Na, K, Cl, Mg and inorganic P levels were measured in order to determine ionic strength of the endolymph. The aragonite supersaturation rate (S_a) was calculated from the Ca^{2+} and CO_3^{2-} concentrations and the ionic strength. In both age groups, Ca^{2+} and CO_3^{2-} concentrations were around 0.75 and 0.68 mmol l^{-1} , respectively. Small differences in Na, P, and HCO_3^- concentrations were observed between the 2 age groups, but endolymph ionic strength was similar. The S_a ratio was 2.885 to 3.507 in 1+ yr old fish and 2.027 to 4.303 in 2+ yr old fish. Therefore, the endolymph is supersaturated with respect to aragonite. S_a was significantly correlated with CO_3^{2-} levels, which were largely determined by pH. As a consequence, S_a was strongly dependent on pH, indicating that endolymph pH-regulation is important in the aragonite crystallization of the otolith.

KEY WORDS: Otolith · Endolymph · Chemical composition · Aragonite saturation state · Teleost

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INTRODUCTION

The inner ear in teleosts consists of 3 otolith organs, the utriculus, sacculus and lagena, each containing a single dense calcareous otolith (Lowenstein 1971). Teleost otoliths are considered as time-keeping, biological records. They are commonly used for age and growth estimations and population studies (Secor et al. 1995). Since Pannella (1971) found the presence of daily increments in the microstructure, otoliths have

also been used as daily-age determinants of larvae and juveniles. Moreover, the microanalysis of otolith trace elements enables us to discriminate fish population stocks (Campana et al. 1995, Severin et al. 1995) and to characterize environmental events that an individual fish has experienced (Kalish 1989, Radtke et al. 1990, 1998, Kalish 1992, Secor & Piccoli 1996). In order to increase the accuracy of age determination and other information gained from the otolith, an understanding of the physiological mechanism of otolith formation is indispensable.

The major component of the teleost otolith is calcium carbonate polycrystals in the form of aragonite (Carlström 1963). The otolith also contains a small amount of

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organic matrix including proteins, carbohydrates and lipids (Mugiya 1968, Degens et al. 1969, Baba et al. 1991, Asano & Mugiya 1992, Sasagawa & Mugiya 1996, Takagi & Takahashi 1999, Murayama et al. 2000, Takagi et al. 2000). Otoliths are bathed in endolymph within the inner ear sacs. The otolith grows in the endolymph without touching any cells. It is generally believed that the organic matrix is first constructed and then aragonite crystallization occurs. The cells of the membranous wall of the otolith organ synthesize components of the otolith matrix (Takagi & Takahashi 1999, Takagi 2000, Takagi et al. 2000). The components are secreted into the endolymph (Takagi 2000, Takagi et al. 2000) and a framework is constructed. Then, aragonite crystallization occurs on the matrix framework. Therefore, otolith growth may be greatly dependent on the endolymph chemical composition.

Aragonite precipitation rate from a dilute solution strongly correlates with the aragonite saturation state of the solution in a non-biological system (Romanek et al. 1991). Therefore, growth of the otolith aragonite may also be strongly related to the aragonite saturation state of the endolymph. Calculation of the saturation state requires determination of endolymph chemical composition. Previous studies have partially characterized the electrolyte composition of the endolymph in several teleost species (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). The endolymph electrolyte composition is characterized by strikingly high potassium and low sodium concentrations compared with other body fluids such as plasma. Calcium concentration is lower in the endolymph than in plasma, whereas carbon dioxide concentration is higher in the endolymph. Using values reported for endolymph chemicals, Shichiri (1985) and Romanek & Gauldie (1996) tried to quantify the saturation state of the endolymph. Shichiri roughly estimated the aragonite saturation state of goldfish endolymph from values reported for the blood electrolyte composition of carp and the endolymph/blood ratio of electrolyte concentrations in rainbow trout. He concluded that goldfish endolymph was highly supersaturated. Since data for all ion species were not available for any single fish species, Romanek & Gauldie established the minimum, average and maximum concentrations of endolymph electrolytes from values reported for several fish species, including teleosts and elasmobranchs. Then they estimated the range of aragonite saturation state of fish endolymph and proposed a predictive model of otolith growth. Their calculated value was significantly lower than that reported by Shichiri.

However, research into the endolymph aragonite saturation state remains incomplete. Accurate quantification of the ionized forms of calcium and carbonate in the endolymph is necessary to calculate the saturation state, since only these ions can participate in the crystallization of calcium carbonate. Nevertheless, the ionized calcium level of endolymph had not been determined when Shichiri (1985) and Romanek & Gauldie (1996) calculated the aragonite saturation state. It has been measured only in the orange roughy *Hoplostethus atlanticus*, a deep-sea species (Gauldie & Romanek 1998). This species seems to have a rather special composition of endolymph electrolytes; the ionized calcium and ionized sodium levels ranged from 1.8 to 7.9 mM and 94 to 276 mM, respectively, which are significantly higher than the total calcium and total sodium concentrations of endolymph in freshwater and seawater species reported in recent studies (Mugiya & Takahashi 1985, Kalish 1991, Payan et al. 1997, 1998, 1999, Takagi 1997, Edeyer et al. 2000). Ionized carbonate could be calculated from total dissolved carbon dioxide and pH. However, endolymph pH and dissolved carbon dioxide have only been determined once in rainbow trout (Mugiya & Takahashi 1985), when Romanek & Gauldie (1996) calculated the aragonite saturation state. Shichiri (1985) estimated ionized carbonate concentration using the values for human plasma. Therefore, the aragonite saturation state for any single fish species, based on the direct quantification of electrolyte concentrations, has not yet been determined.

The present study quantified calcium and carbonate ion concentrations, as well as other major electrolyte (sodium, potassium, magnesium, chloride and inorganic phosphate) concentrations and pH in the saccular endolymph of the rainbow trout. Then, the aragonite saturation state of the trout endolymph was calculated. Relationships among the aragonite saturation state, pH, calcium ion concentration, and carbonate ion concentration were analyzed.

MATERIALS AND METHODS

Fish. Rainbow trout *Oncorhynchus mykiss* were purchased from a local breeder in Iwate, Japan, and reared in outdoor tanks with continuous supplies of fresh water at about 15°C. They were fed to satiation with commercial trout pellets once a day. Fish in 2 year-classes, 1+ and 2+ yr old, were used. The 1+ yr old fish weighed 418 ± 11 g (mean \pm SE) and the 2+ yr old fish weighed 1313 ± 28 g.

Endolymph sampling. Fish were deeply anesthetized in a 0.1% solution of 2-phenoxyethanol and decapitated. The head was opened dorsally and the

brain removed by forceps. Both the right and left sacculi, each containing an otolith, were removed using forceps, blotted with tissue paper to remove body fluids around the sacculi, and immersed in liquid paraffin. After opening the distal end of the sacculus using fine scissors in liquid paraffin, a capillary tube, which was filled with a small amount of liquid paraffin, was inserted into the sacculus. Then endolymph was sucked into the capillary. Extra liquid paraffin was sucked at the end of the endolymph collection so that the endolymph was sandwiched by paraffin. After collection, one end of the capillary was sealed with a capillary sealer and immediately centrifuged at 12 000 rpm for 3 s. Liquid paraffin is lighter than the endolymph. Thus, after centrifugation, endolymph was collected at the bottom of the capillary, covered by a layer of liquid paraffin. In this way, the endolymph could be collected without coming in contact with air, which would have changed the endolymph pH and carbonate ion concentrations. In rare cases, trout sacculus contains several small otoconia. These otoconia, along with cellular debris, were removed by this short centrifugation. The amount of endolymph collected from each sacculus was 5 to 10 μl in 1+ yr old trout and 20 to 30 μl in 2+ yr old trout.

Determination of endolymph Na, K, Mg, Ca, Cl, and Pi concentrations. Total sodium (Na_T) and potassium (K_T) concentrations (mmol l^{-1}) were determined by flame emission spectrophotometry, and total magnesium (Mg_T) and calcium (Ca_T) concentrations (mmol l^{-1}) by atomic absorption spectrophotometry (Hitachi 170-50, Tokyo, Japan). Total chloride (Cl_T) concentration (mmol l^{-1}) was determined by coulometric titration using a Buchler (Fort Lee, New Jersey, USA) model 4-2500 digital Chloridometer. For the measurements of Na_T , K_T , Mg_T , Ca_T and Cl_T concentrations, the left and right endolymph of three 1+ yr old fish was pooled. In 2+ yr old fish, the left and right endolymph of each individual was pooled. Similarly, the pooled samples were also prepared and used to measure inorganic phosphate (Pi) concentration (mmol l^{-1}), which was determined by spectrophotometry following Goldenberg & Fernandez (1966).

Determination of endolymph pH, Ca^{2+} , P_{CO_2} , HCO_3^- and CO_3^{2-} levels. Endolymph pH, ionized calcium (Ca^{2+}) concentration (mmol l^{-1}) and CO_2 partial pressure (P_{CO_2}) (torr) were determined using an automatic pH/blood gas/electrolyte analyzer (Model 348, Chiron Diagnostics Limited, England), which was equipped with pH and selective ion electrodes. In 1+ yr old fish, the left and right endolymph of 3 fish was pooled and used for the measurement. In 2+ yr old fish, the left and right endolymph of each individual was pooled. Immediately after the collection of the endolymph, samples were pooled and loaded on the analyzer.

The analyzer was equipped with a heater to maintain the sample temperature at 37°C. Therefore, pH and P_{CO_2} levels at the temperature at which trout were maintained were calculated using the following equations listed in the operator's manual:

$$\text{pH} = \text{pH}_{\text{at}37} + [-0.0147 + 0.0065 \times (7.4 - \text{pH}_{\text{at}37})](t - 37) \quad (1)$$

$$P_{\text{CO}_2} = 10^{\log P_{\text{CO}_2\text{at}37} + 0.019 \times (37-t)} \quad (2)$$

where pH and P_{CO_2} are values at temperature $t^\circ\text{C}$, and $\text{pH}_{\text{at}37}$ and $P_{\text{CO}_2\text{at}37}$ are values measured by the analyzer at 37°C.

From the corrected pH and P_{CO_2} , the bicarbonate ion (HCO_3^-) concentration can be calculated from the Henderson-Hasselbalch equation:

$$\text{pH} = \text{p}K_1' + \log \frac{[\text{HCO}_3^-]}{\alpha_{\text{CO}_2} P_{\text{CO}_2}} \quad (3)$$

$$[\text{HCO}_3^-] = 10^{\text{pH} - \text{p}K_1'} \alpha_{\text{CO}_2} P_{\text{CO}_2} \quad (4)$$

where $\text{p}K_1'$ is the apparent first dissociation constant of carbonic acid, $[\text{HCO}_3^-]$ is the concentration of HCO_3^- in mmol l^{-1} , and α_{CO_2} is the solubility coefficient of CO_2 in $\text{mmol l}^{-1} \text{ torr}^{-1}$. The α_{CO_2} is affected by the temperature and ionic strength of the solution. Since our preliminary study showed that the ionic strength of the endolymph is similar to that of plasma in the rainbow trout (about 0.18 in the endolymph and 0.15 in the plasma), the following formula for the rainbow trout plasma (Boutilier et al. 1984) was applied to obtain α_{CO_2} in the endolymph:

$$\alpha_{\text{CO}_2} = 1.0064 \times 10^{-1} - 5.4431 \times 10^{-3}t + 2.1776 \times 10^{-4}t^2 - 4.9731 \times 10^{-6}t^3 + 4.5288 \times 10^{-8}t^4 \quad (5)$$

where t is the temperature ($^\circ\text{C}$) at which the trout were maintained. $\text{p}K_1'$ is influenced by temperature, ionic strength and pH, and the following formula for the rainbow trout plasma (Boutilier et al. 1984) was applied to obtain $\text{p}K_1'$ in the endolymph:

$$\text{p}K_1' = 6.4755t^{-0.0187} + \log t(1.1704 - 0.1672\text{pH}) + 0.1073\text{pH} - 0.7511 \quad (6)$$

From the calculated value of $[\text{HCO}_3^-]$, the carbonate ion (CO_3^{2-}) concentration was calculated from the following equations:

$$\text{p}K_2' = \text{pH} - \log \frac{[\text{CO}_3^{2-}]}{[\text{HCO}_3^-]} \quad (7)$$

$$[\text{CO}_3^{2-}] = 10^{\text{pH} - \text{p}K_2'} [\text{HCO}_3^-] \quad (8)$$

where $\text{p}K_2'$ is the apparent second dissociation constant of carbonic acid and $[\text{CO}_3^{2-}]$ is the CO_3^{2-} concentration in mmol l^{-1} . $\text{p}K_2'$ is also influenced by temperature and ionic strength. The following formula

(Truchot 1976) was used to obtain pK_2' in the endolymph:

$$pK_2' = 10.183 - 1.1\sqrt{I} \quad (9)$$

where I is the ionic strength of the endolymph. Eq. (9) is valid only at 15°C (Truchot 1976), and rainbow trout were maintained at a similar temperature in the present experiment. I is expressed as follows:

$$I = \frac{\sum_i (Z_i^2 [C_i])}{2} \quad (10)$$

where Z_i and $[C_i]$ are the electric charge and concentration (mol l^{-1}) of ionic species i , respectively. In the present study, endolymph was assumed to contain Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , PO_4^{3-} , HCO_3^- , and CO_3^{2-} . I was calculated in each sample using $[\text{Ca}^{2+}]$, $[\text{HCO}_3^-]$ and $[\text{CO}_3^{2-}]$ and the mean value of $[\text{Na}_T]$, $[\text{K}_T]$, $[\text{Mg}_T]$, $[\text{Cl}_T]$, and $[\text{Pi}]$, assuming Na, K, Mg, Cl and Pi all existed in ionized forms in the endolymph.

Calculation of the aragonite saturation state. In order to calculate the aragonite saturation state, concentrations of electrolytes should be given in mol kg^{-1} H_2O . Using gravity (1.029 kg l^{-1}) and water content (98.1% by weight) of the endolymph of chum salmon *Oncorhynchus keta* (author's unpubl. data), electrolyte concentrations of trout endolymph given in mol l^{-1} solution were converted into mol kg^{-1} H_2O .

The formation of aragonite is expressed as follows:



The aragonite saturation state of the endolymph can be expressed by the supersaturation ratio, S_a , which is expressed as follows:

$$S_a = \sqrt{\frac{(\text{Ca}^{2+})(\text{CO}_3^{2-})}{K_{S_a}^0}} \quad (12)$$

where (C_i) is the activity of ionic species i , and $K_{S_a}^0$ is the thermodynamic solubility product of aragonite. When $S_a < 1$, $= 1$ and > 1 , the solution is respectively undersaturated, in equilibrium and supersaturated with respect to aragonite. (C_i) is expressed as follows:

$$(C_i) = \gamma_i [C_i] \quad (13)$$

where γ_i and $[C_i]$ are the activity coefficient and concentration (mol kg^{-1} H_2O) of ionic species i respectively. γ_i was estimated according to Truesdell & Jones (1974) using modified Debye-Hückel equations:

$$\log \gamma_i = -\frac{AZ_i^2 \sqrt{\mu}}{1 + Ba_i \sqrt{\mu}} + b_i \mu \quad (14)$$

where A and B are constants defined by Hamer (1968), Z_i is the electronic charge of ionic species i , and a_i and b_i are parameters specific to individual ions. μ is the ionic strength of the endolymph calculated in Eq. (10), but in mol kg^{-1} H_2O . According to Truesdell & Jones (1974),

values of a_i and b_i for Ca^{2+} are 5.0 and 0.165, respectively, and those for CO_3^{2-} are 5.4 and 0.0, respectively.

$K_{S_a}^0$ is influenced by temperature, and the following formula (Plummer & Busenberg 1982) was used to obtain $K_{S_a}^0$ at the temperature, T (in K), at which the rainbow trout were maintained:

$$K_{S_a}^0 = -171.9773 - 0.077993T + 2903.293/T + 71.595 \log T \quad (15)$$

In the present study, both $[\text{Ca}^{2+}]$ and $[\text{CO}_3^{2-}]$ were obtained from the same sample simultaneously. Therefore, S_a was calculated for each sample.

Statistics. Data are presented as means \pm SE (N = number of determinations). Differences between the values of 1+ and 2+ yr old fish were compared by the Mann-Whitney U -test. The relationships between endolymph aragonite saturation state and endolymph Ca^{2+} or CO_3^{2-} concentrations, and between endolymph CO_3^{2-} concentrations and endolymph P_{CO_2} levels or pH, were analyzed by the Spearman rank-correlation test. Statistical significance was set at $p < 0.05$.

RESULTS

Electrolyte composition of trout endolymph

Electrolyte concentrations, pH and ionic strength (I) of saccular endolymph in 1+ and 2+ yr old rainbow trout are listed in Table 1. The major electrolytes in the

Table 1. *Oncorhynchus mykiss*. Electrolyte concentrations, P_{CO_2} , pH and ionic strength (I) of the saccular endolymph in the rainbow trout. Means \pm SE (n). Na_T : total sodium concentration; K_T : total potassium concentration; Mg_T : total magnesium concentration; Ca_T : total calcium concentration; Ca^{2+} : ionized calcium concentration; Cl_T : total chloride concentration; Pi : inorganic phosphate concentration; P_{CO_2} : partial pressure of carbon dioxide; HCO_3^- : bicarbonate ion concentration; CO_3^{2-} : carbonate ion concentration

	1+ yr old fish	2+ yr old fish
Na_T (mmol l^{-1})	82.5 \pm 1.1 (6)	92.0 \pm 1.6 (12)*
K_T (mmol l^{-1})	85.8 \pm 0.8 (6)	88.8 \pm 2.1 (12)
Mg_T (mmol l^{-1})	0.319 \pm 0.006 (6)	0.337 \pm 0.014 (12)
Ca_T (mmol l^{-1})	1.60 \pm 0.09 (6)	1.61 \pm 0.05 (12)
Ca^{2+} (mmol l^{-1})	0.753 \pm 0.005 (6)	0.741 \pm 0.016 (19)
Cl_T (mmol l^{-1})	140 \pm 1 (6)	142 \pm 2 (11)
Pi (mmol l^{-1})	0.421 \pm 0.015 (6)	0.242 \pm 0.024 (12)*
P_{CO_2} (torr)	12.48 \pm 0.77 (6)	8.42 \pm 0.24 (19)*
HCO_3^- (mmol l^{-1})	44.6 \pm 1.8 (6)	35.4 \pm 2.3 (19)*
CO_3^{2-} (mmol l^{-1})	0.691 \pm 0.039 (6)	0.673 \pm 0.077 (19)
pH	7.90 \pm 0.02 (6)	7.95 \pm 0.03 (19)
I	0.182 \pm 0.001 (6)	0.184 \pm 0.001 (19)

* $p < 0.05$ compared with the values for 1+ yr old fish: Mann-Whitney U -test

endolymph were Na, K, Cl, and HCO_3^- , whereas Ca, Mg, CO_3^{2-} , and Pi were minor electrolytes. $[\text{Ca}_T]$ and $[\text{Ca}^{2+}]$ were around 1.6 and 0.75 mmol l^{-1} , respectively, in both 1+ and 2+ yr old fish. Therefore, about 47% of Ca in the endolymph was ionized. The CO_3^{2-} concentrations were about 0.68 mmol l^{-1} , and pH was about 7.9 in both fish age groups. Although significant differences in $[\text{Na}_T]$, $[\text{Pi}]$, P_{CO_2} levels, and $[\text{HCO}_3^-]$ were observed between 1+ and 2+ yr old fish, the differences were small. Thus, I was similar in both fish age groups.

Aragonite saturation state of the endolymph

The aragonite supersaturation ratio, S_a , is shown in Table 2. The endolymph of both 1+ and 2+ yr old fish were similarly supersaturated with respect to aragonite; the S_a ranged from 2.885 to 3.507 in 1+ yr old fish and from 2.027 to 4.303 in 2+ yr old fish.

Relationships among pH, $[\text{Ca}^{2+}]$, $[\text{CO}_3^{2-}]$ and S_a

As described in 'Materials and methods', pH, $[\text{Ca}^{2+}]$, $[\text{CO}_3^{2-}]$, and S_a were obtained for individual 2+ yr old fish. Therefore, relationships among these factors were analyzed. S_a correlated significantly with $[\text{CO}_3^{2-}]$ ($p <$

Table 2. *Oncorhynchus mykiss*. Aragonite supersaturation ratio (S_a) of the saccular endolymph in the rainbow trout. Means \pm SE (n) and range given

	1+ yr old fish	2+ yr old fish
S_a	3.300 \pm 0.090 (6)	3.088 \pm 0.173 (19)
range	2.885–3.507	2.027–4.303

0.0001, Spearman rank correlation test), but not with $[\text{Ca}^{2+}]$ (Fig. 1). It is obvious that $[\text{CO}_3^{2-}]$ is closely related to pH ($p < 0.0001$, Spearman rank correlation, but not to P_{CO_2} (Fig. 2). As a consequence, S_a correlated with endolymph pH almost linearly ($p < 0.0001$, Spearman rank correlation test; Fig. 3).

DISCUSSION

The present study clearly showed that the saccular endolymph of the rainbow trout is supersaturated with respect to aragonite. In 2+ yr old fish, S_a , which is an indicator of the degree of saturation, was obtained for 19 individuals and ranged from 2.027 to 4.303. In 1+ yr old fish, S_a was obtained for 6 pooled samples, each consisting of the left and right endolymph of 3 individuals, and ranged from 2.885 to 3.507. This is the first

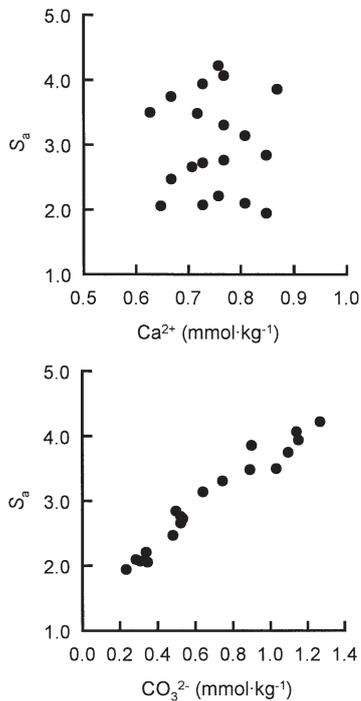


Fig. 1. *Oncorhynchus mykiss*. Relationships between aragonite supersaturation ratio (S_a) and Ca^{2+} or CO_3^{2-} concentrations in the endolymph of rainbow trout inner ear

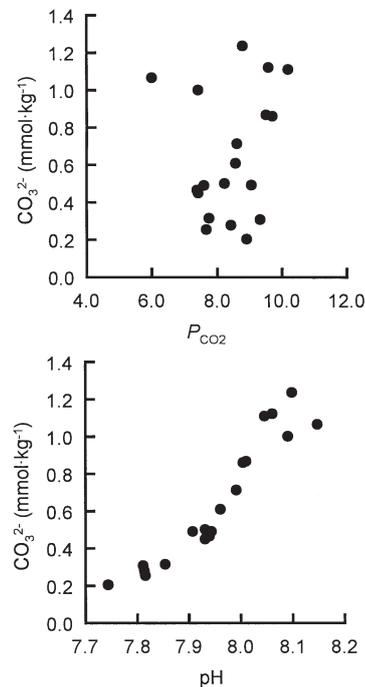


Fig. 2. *Oncorhynchus mykiss*. Relationships between CO_3^{2-} concentration and CO_2 partial pressure (P_{CO_2}) or pH in the endolymph of rainbow trout inner ear

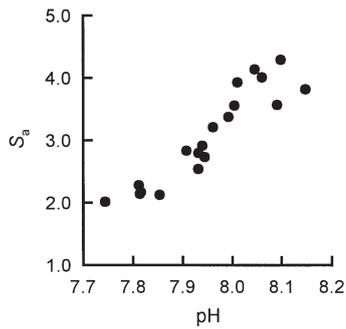


Fig. 3. *Oncorhynchus mykiss*. Relationship between aragonite supersaturation ratio (S_a) and pH in the endolymph of rainbow trout inner ear

calculation of the aragonite saturation state of the endolymph in a single fish species based on the direct quantification of endolymph electrolyte composition.

Payan et al. (1999) revealed that endolymph chemical composition of the rainbow trout was not spatially uniform. They compared the composition of endolymph chemicals between the proximal and distal zones of the sacculus. The proximal zone is the endolymphatic space between the otolith and sensory macula. Proteins, phosphate and magnesium levels were more concentrated in the proximal zone. In contrast, potassium and total CO_2 levels were significantly higher in the distal zone. The endolymph used in the present study was a mixture of proximal and distal endolymph. The electrolyte composition was close to the value for distal endolymph reported by Payan et al. This may be simply because the distal space is much larger than the proximal space in the sacculus. The separate quantification of S_a in the proximal and distal endolymph was impossible due to the limited amount of endolymph for pH, P_{CO_2} and $[\text{Ca}^{2+}]$ measurements, which were the key factors for the determination of S_a . Further technical improvement is needed to study spatial differences of S_a in the sacculus.

During the calculation of S_a , all the HCO_3^- and CO_3^{2-} ions were assumed to be free. However, some of the HCO_3^- and CO_3^{2-} ions should exist as ion pairs such as CaHCO_3^+ , NaHCO_3^0 , CaCO_3^0 and other chemical forms (Truesdell & Jones 1974). Therefore, $[\text{HCO}_3^-]$ and $[\text{CO}_3^{2-}]$, and hence S_a , may be slightly overestimated in this study. At present, accurate concentrations of such minor chemical forms cannot be obtained for the endolymph, since no reliable method for quantifying or calculating these concentrations in biological fluids exists. Biological fluids contain a significant amount of organic acids and macromolecules, which are potential chelators of cations. The interactions among cations and their chelators should be examined carefully. However, rough estimation is possible if we neglect the macromolecules and organic acids. This

gives us the highest amount of cations that bind HCO_3^- and CO_3^{2-} to form ion pairs, and thus produces the lowest estimation of free HCO_3^- and CO_3^{2-} concentrations. For example, Garrels & Thompson (1962) calculated the distribution of dissolved species in seawater at 25°C. In their study, dissociation constants involving Na^+ , K^+ , Mg^{2+} , Ca^{2+} , HCO_3^- , CO_3^{2-} , and SO_4^{2-} ions, and individual ion activity coefficients were used to calculate the proportion of free ions and ion pairs. Applying their methods to the mean electrolyte composition of the endolymph, the proportion of free HCO_3^- and CO_3^{2-} ions can be estimated as 97 and 59% respectively, in 1+ yr old trout, and 97 and 57%, respectively, in 2+ yr old trout. Using these values, S_a for 1+ yr old trout is calculated as 2.162 to 2.631, and that for 2+ yr old trout as 1.509 to 3.203. Therefore, I conclude that endolymph is clearly supersaturated with respect to aragonite, even when the formation of ion pairs is taken into account.

There are several computer programs that calculate the saturation states and activities of individual ion species (including ion pairs) of a solution from the total concentrations of major electrolytes. However, these programs deal with non-biological fluids that contain no organic molecules. Interactions among organic molecules and ion species in the endolymph are not clear at present. Therefore, the present study did not use such programs and directly measured ionized calcium concentration, and I calculated $[\text{HCO}_3^-]$ and $[\text{CO}_3^{2-}]$ using apparent first and second dissociation constants determined for biological fluids.

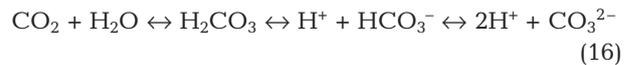
Before the present study, Shichiri (1985) estimated the saturation state of goldfish endolymph using values reported for the blood electrolyte composition of carp and the endolymph/blood ratio of electrolyte concentrations in the rainbow trout. He calculated the S_a ratio of goldfish endolymph to be 12.9. Such a high S_a value resulted from high values of $[\text{Ca}^{2+}]$ and $[\text{CO}_3^{2-}]$, which were estimated to be 1.8 and 28.6 mmol l^{-1} , respectively. These seem to be an overestimation compared with the values obtained in the present study. Romanek & Gaudie (1996) established the minimum, average and maximum concentrations of endolymph electrolytes from values reported for several fish species and calculated the range of the saturation state of fish endolymph. Since only $[\text{Ca}_T]$ had been reported, they estimated $[\text{Ca}^{2+}]$, taking into account the formation of ion pairs such as CaHCO_3^+ . However, they neglected the presence of organic compounds in the endolymph, which may also bind calcium. They calculated Ω , which is expressed as $(\text{Ca}^{2+}) \cdot (\text{CO}_3^{2-}) / K_{S_a}^0$ to be 7.8 to 40.5. Since the relationship between Ω and S_a is expressed as $\Omega = S_a^2$, the S_a of trout endolymph obtained in the present study is close to the lower limit of Ω in their study. However, the sodium and chloride concentrations used

in their study were extremely high compared with those measured in the present study and with those reported for teleost endolymph in recent studies (Payan et al. 1997, 1998, 1999, Edeyer et al. 2000). Such high levels of sodium and chloride probably originated from the values for elasmobranch endolymph (Fänge et al. 1972, Peterson et al. 1978). Higher levels of sodium and chloride make the ionic strength of the solution high and result in low Ω . Therefore, direct comparison of Ω in Romanek & Gauldie's 1996 study and S_a in the present study is difficult.

What is the key that determines the endolymph saturation state? The present study showed that endolymph pH determines the saturation state. The results revealed that the endolymph $[Ca^{2+}]$ was well regulated, and hence $[CO_3^{2-}]$ determines S_a . Further, endolymph $[CO_3^{2-}]$ correlated well with pH, but not with P_{CO_2} . CO_3^{2-} is dissociated from H_2CO_3 , which is a dissolved form of CO_2 . The dissociation rate of CO_3^{2-} is largely dependent on pH, as indicated in Eqs. (4), (6) & (8). Thus, the present results indicate that individual variation in P_{CO_2} levels is rather small, and variation in the pH largely determines $[CO_3^{2-}]$. As a consequence, endolymph S_a is strongly related to pH. For continuous aragonite precipitation, S_a levels must be maintained above 1.0. Therefore, fine regulation of endolymph pH is important for continued aragonite crystallization. The importance of endolymph pH to otolith growth has been repeatedly assumed. Gauldie & Nelson (1990) proposed an otolith-growth model whereby the pH gradient in the endolymph leads to deposition of aragonite. Romanek & Gauldie (1996) proposed a predictive model of otolith growth in fish using Ω and the precipitation rate-saturation state relationship of aragonite in a non-biological system (Romanek et al. 1991). In their model, endolymph pH greater than 7.8 rapidly increases the theoretical otolith-growth rate. Gauldie & Romanek (1998) measured endolymph pH, calcium and sodium levels (both in ionized form) of individual orange roughy, and calculated the theoretical otolith-growth rate of each fish using the Romanek-Gauldie model. They pointed out that the theoretical otolith-growth rate was significantly related to the measured endolymph pH, but not to ionized calcium levels. Although they did not compare the relationships between endolymph pH and observed otolith-growth rates directly, they showed that the theoretical and observed growth rates correlated well. Thus, endolymph pH and observed otolith-growth rate may have some correlation. However, all these reports did not directly quantify either the pH, $[Ca^{2+}]$, or $[CO_3^{2-}]$ of the endolymph. Instead, values for different fish species reported in the literature were used. Therefore, the present result is virtually the first evidence to show the pH dependency of the endolymph saturation state

from direct measurements of endolymph chemicals for a single fish species. In future, the relationship between S_a and otolith growth rate should be examined in the rainbow trout in order to further validate the Romanek-Gauldie model of otolith-growth, because the electrolyte composition of endolymph obtained in the present study does not fit the hypothetical composition used in the model. In the present study, the S_a of 2 year-classes of the fish was determined. The otolith-growth rate is assumed to be different between 2 age groups. However, the large individual variation in S_a makes it impossible to compare the difference between the 2 age groups. Precise determination of individual otolith growth rate is needed to clarify the relationship between S_a and otolith-growth rate. Edeyer et al. (2000) proposed that circadian variations of endolymph electrolyte composition, especially those in total CO_2 and protein levels, result in the daily increment formation. It is also important to re-examine their proposal from the point of endolymph S_a , in order to clarify mechanism of daily-increment formation in the otolith.

It is worth mentioning that aragonite precipitation itself potentially decreases endolymph pH. Aragonite precipitation is expressed in Eq. (11), whereas the chemical reaction of CO_3^{2-} generation is expressed as follows:



When aragonite precipitation continues in the endolymph, $[CO_3^{2-}]$ in the endolymph decreases and the dissociation of CO_3^{2-} from HCO_3^- proceeds. As a result, H^+ is produced and the pH goes down. Therefore, in order to keep the endolymph pH constant, the released H^+ should be removed from the endolymph. Payan et al. (1999) found a concentration gradient of HCO_3^- in the trout endolymph, with higher HCO_3^- levels in the distal zone, and proposed a model of H^+ removal from the endolymph. Based on the gradient of HCO_3^- , they presupposed that a concentration gradient of H^+ would exist in the endolymph, with lower levels in the distal zone. In their model, H^+ diffuses away from the otolith surface to the distal zone depending on the concentration gradient. Then, H^+ is buffered by HCO_3^- to form CO_2 gas and H_2O as in Eq. (16). Tohse & Mugiya (2001) proposed a hypothetical model of HCO_3^- transport in the teleost otolith organ. In their model, HCO_3^- ions are transported into the endolymph by energy-dependent mechanisms involving HCO_3^- -ATPase and Cl^-/HCO_3^- exchangers. However, localization of HCO_3^- transporters has not been clarified. Thus, the connection between these 2 models is still incomplete. Further detailed studies on the mechanism of pH regulation in the teleost fish otolith organ are needed.

Calcium carbonate has several crystal morphs, such as aragonite, calcite and vaterite. In teleost fishes, aragonite is the most common crystal morph of the otolith (Carlström 1963). Vaterite is also found commonly in the asteriscus, an otolith formed in the lagena (Lowenstam & Weiner 1989). In contrast, calcite has been found only in aberrant otoliths of several fishes (Gauldie 1993). Using the endolymph electrolyte composition obtained in the present study and the thermodynamic solubility products of calcite ($K_{S,c}^0$) and vaterite ($K_{S,v}^0$) reported in Plummer & Busenberg (1982), the supersaturation ratio of the endolymph with respect to calcite (S_c) and vaterite (S_v) can be calculated. For example, in 2+ yr old trout, endolymph S_c , S_a and S_v are 2.413–5.126, 2.027–4.303, and 1.217–2.578, respectively. When formation of ion pairs is taken into account, S_c , S_a and S_v are 1.796–3.817, 1.509–3.203, and 0.906–1.919, respectively. Compared with $K_{S,a}^0$, $K_{S,c}^0$ is smaller and $K_{S,v}^0$ is larger. Therefore, S_c becomes the largest, S_v is the smallest, and S_a is in between. These data indicate that calcite is physicochemically the most easily precipitated crystal morph. The fact that aragonite or vaterite is the specific crystalline morph in the teleost otolith suggests the existence of strict control over polymorph formation. Ions in the endolymph, other than Ca^{2+} and CO_3^{2-} , are possible regulators of the polymorph. For example, magnesium ions in the solution inhibit calcite precipitation and favor the formation of aragonite (Kitano et al. 1976). However, the magnesium levels examined by Kitano et al. were significantly higher than those in fish endolymph. The effects of magnesium and other ions, within the levels of teleost endolymph, on the crystalline morphs should be carefully studied in the future. On the other hand, molluscan-shell organic matrices, which are mainly glycoproteins, are crucial in determining aragonite or calcite polymorphism in the shell (Belcher et al. 1996, Falini et al. 1996). Otolith organic matrices of teleosts are also possible regulators of specific polymorph precipitation. Moreover, organic matrices in biominerals are believed to regulate nucleation and growth of crystals (Weiner 1986). It is important to clarify the functional significance of organic matrices to otolith mineralization. Although amino acid composition of several otolith organic matrices has been reported (Degens et al. 1969, Baba et al. 1991, Asano & Mugiya 1992, Sasagawa & Mugiya 1996), only 1 matrix protein of the teleost otolith has been identified and characterized (Murayama et al. 2000). Further identification of otolith organic matrices is necessary to allow the study of the functions of otolith organic matrices.

In conclusion, the saccular endolymph of the rainbow trout is supersaturated with respect to aragonite. The endolymph S_a is largely determined by pH, suggesting that pH regulation is important in maintain-

ing aragonite crystallization in the otoliths of rainbow trout.

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