

# 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  $\text{Ca}^{2+}$  level,  $\text{CO}_2$  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  $\text{CO}_2$  partial pressure and pH,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  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  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  concentrations and the ionic strength. In both age groups,  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  concentrations were around 0.75 and 0.68 mmol  $\text{l}^{-1}$ , respectively. Small differences in Na, P, and  $\text{HCO}_3^-$  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  $\text{CO}_3^{2-}$  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 utricle, saccule 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  $418 \pm 11$  g (mean  $\pm$  SE) and the 2+ yr old fish weighed  $1313 \pm 28$  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  $\mu\text{l}$  in 1+ yr old trout and 20 to 30  $\mu\text{l}$  in 2+ yr old trout.

**Determination of endolymph Na, K, Mg, Ca, Cl, and Pi concentrations.** Total sodium ( $\text{Na}_T$ ) and potassium ( $\text{K}_T$ ) concentrations ( $\text{mmol l}^{-1}$ ) were determined by flame emission spectrophotometry, and total magnesium ( $\text{Mg}_T$ ) and calcium ( $\text{Ca}_T$ ) concentrations ( $\text{mmol l}^{-1}$ ) by atomic absorption spectrophotometry (Hitachi 170-50, Tokyo, Japan). Total chloride ( $\text{Cl}_T$ ) concentration ( $\text{mmol l}^{-1}$ ) was determined by coulometric titration using a Buchler (Fort Lee, New Jersey, USA) model 4-2500 digital Chloridometer. For the measurements of  $\text{Na}_T$ ,  $\text{K}_T$ ,  $\text{Mg}_T$ ,  $\text{Ca}_T$  and  $\text{Cl}_T$  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 ( $\text{mmol l}^{-1}$ ), which was determined by spectrophotometry following Goldenberg & Fernandez (1966).

**Determination of endolymph pH,  $\text{Ca}^{2+}$ ,  $P_{\text{CO}_2}$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  levels.** Endolymph pH, ionized calcium ( $\text{Ca}^{2+}$ ) concentration ( $\text{mmol l}^{-1}$ ) and  $\text{CO}_2$  partial pressure ( $P_{\text{CO}_2}$ ) (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  $37^\circ\text{C}$ . Therefore, pH and  $P_{\text{CO}_2}$  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  $P_{\text{CO}_2}$  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  $37^\circ\text{C}$ .

From the corrected pH and  $P_{\text{CO}_2}$ , the bicarbonate ion ( $\text{HCO}_3^-$ ) 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  $\text{HCO}_3^-$  in  $\text{mmol l}^{-1}$ , and  $\alpha_{\text{CO}_2}$  is the solubility coefficient of  $\text{CO}_2$  in  $\text{mmol l}^{-1} \text{ torr}^{-1}$ . The  $\alpha_{\text{CO}_2}$  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  $\alpha_{\text{CO}_2}$  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 ( $\text{CO}_3^{2-}$ ) 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  $\text{CO}_3^{2-}$  concentration in  $\text{mmol l}^{-1}$ .  $\text{p}K_2'$  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 ( $\text{mol l}^{-1}$ ) of ionic species  $i$ , respectively. In the present study, endolymph was assumed to contain  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{HCO}_3^-$ , and  $\text{CO}_3^{2-}$ .  $I$  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  $\text{mol kg}^{-1}$   $\text{H}_2\text{O}$ . Using gravity ( $1.029 \text{ kg l}^{-1}$ ) 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  $\text{mol l}^{-1}$  solution were converted into  $\text{mol kg}^{-1}$   $\text{H}_2\text{O}$ .

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  $\gamma_i$  and  $[C_i]$  are the activity coefficient and concentration ( $\text{mol kg}^{-1}$   $\text{H}_2\text{O}$ ) of ionic species  $i$  respectively.  $\gamma_i$  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.  $\mu$  is the ionic strength of the endolymph calculated in Eq. (10), but in  $\text{mol kg}^{-1}$   $\text{H}_2\text{O}$ . According to Truesdell & Jones (1974),

values of  $a_i$  and  $b_i$  for  $\text{Ca}^{2+}$  are 5.0 and 0.165, respectively, and those for  $\text{CO}_3^{2-}$  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  $\text{Ca}^{2+}$  or  $\text{CO}_3^{2-}$  concentrations, and between endolymph  $\text{CO}_3^{2-}$  concentrations and endolymph  $P_{\text{CO}_2}$  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,  $P_{\text{CO}_2}$ , pH and ionic strength ( $I$ ) of the saccular endolymph in the rainbow trout. Means  $\pm$  SE (n).  $\text{Na}_T$ : total sodium concentration;  $\text{K}_T$ : total potassium concentration;  $\text{Mg}_T$ : total magnesium concentration;  $\text{Ca}_T$ : total calcium concentration;  $\text{Ca}^{2+}$ : ionized calcium concentration;  $\text{Cl}_T$ : total chloride concentration;  $\text{Pi}$ : inorganic phosphate concentration;  $P_{\text{CO}_2}$ : partial pressure of carbon dioxide;  $\text{HCO}_3^-$ : bicarbonate ion concentration;  $\text{CO}_3^{2-}$ : carbonate ion concentration

	1+ yr old fish	2+ yr old fish
$\text{Na}_T$ ( $\text{mmol l}^{-1}$ )	82.5 $\pm$ 1.1 (6)	92.0 $\pm$ 1.6 (12)*
$\text{K}_T$ ( $\text{mmol l}^{-1}$ )	85.8 $\pm$ 0.8 (6)	88.8 $\pm$ 2.1 (12)
$\text{Mg}_T$ ( $\text{mmol l}^{-1}$ )	0.319 $\pm$ 0.006 (6)	0.337 $\pm$ 0.014 (12)
$\text{Ca}_T$ ( $\text{mmol l}^{-1}$ )	1.60 $\pm$ 0.09 (6)	1.61 $\pm$ 0.05 (12)
$\text{Ca}^{2+}$ ( $\text{mmol l}^{-1}$ )	0.753 $\pm$ 0.005 (6)	0.741 $\pm$ 0.016 (19)
$\text{Cl}_T$ ( $\text{mmol l}^{-1}$ )	140 $\pm$ 1 (6)	142 $\pm$ 2 (11)
$\text{Pi}$ ( $\text{mmol l}^{-1}$ )	0.421 $\pm$ 0.015 (6)	0.242 $\pm$ 0.024 (12)*
$P_{\text{CO}_2}$ (torr)	12.48 $\pm$ 0.77 (6)	8.42 $\pm$ 0.24 (19)*
$\text{HCO}_3^-$ ( $\text{mmol l}^{-1}$ )	44.6 $\pm$ 1.8 (6)	35.4 $\pm$ 2.3 (19)*
$\text{CO}_3^{2-}$ ( $\text{mmol l}^{-1}$ )	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  $\text{HCO}_3^-$ , whereas Ca, Mg,  $\text{CO}_3^{2-}$ , and Pi were minor electrolytes.  $[\text{Ca}_T]$  and  $[\text{Ca}^{2+}]$  were around 1.6 and 0.75  $\text{mmol l}^{-1}$ , respectively, in both 1+ and 2+ yr old fish. Therefore, about 47% of Ca in the endolymph was ionized. The  $\text{CO}_3^{2-}$  concentrations were about 0.68  $\text{mmol l}^{-1}$ , and pH was about 7.9 in both fish age groups. Although significant differences in  $[\text{Na}_T]$ ,  $[\text{Pi}]$ ,  $P_{\text{CO}_2}$  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  $P_{\text{CO}_2}$  (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

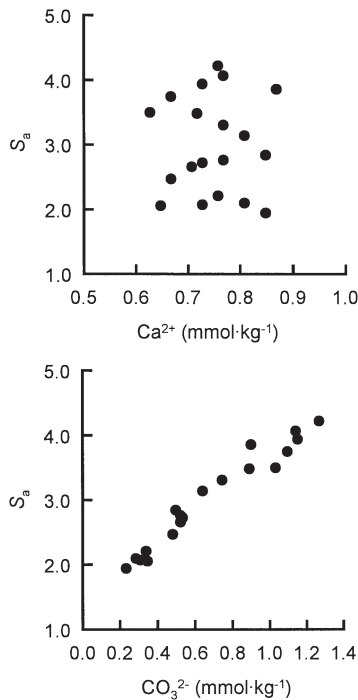


Fig. 1. *Oncorhynchus mykiss*. Relationships between aragonite supersaturation ratio ( $S_a$ ) and  $\text{Ca}^{2+}$  or  $\text{CO}_3^{2-}$  concentrations in the endolymph of rainbow trout inner ear

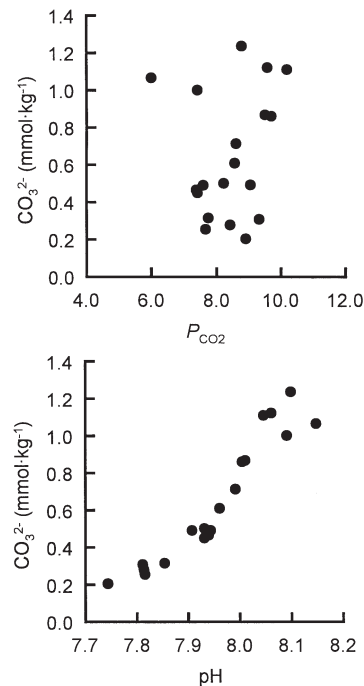


Fig. 2. *Oncorhynchus mykiss*. Relationships between  $\text{CO}_3^{2-}$  concentration and  $\text{CO}_2$  partial pressure ( $P_{\text{CO}_2}$ ) or pH in the endolymph of rainbow trout inner ear



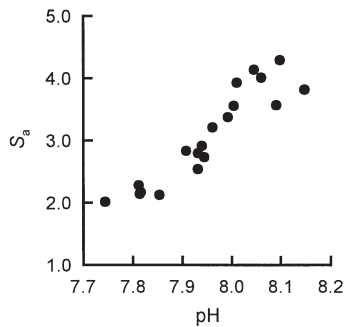


Fig. 3. *Oncorhynchus mykiss*. Relationship between aragonite supersaturation ratio ( $S_a$ ) and pH in the endolymph of rainbow trout inner ear

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  $\text{CO}_2$  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,  $P_{\text{CO}_2}$  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  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  ions were assumed to be free. However, some of the  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  ions should exist as ion pairs such as  $\text{CaHCO}_3^+$ ,  $\text{NaHCO}_3^0$ ,  $\text{CaCO}_3^0$  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  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  to form ion pairs, and thus produces the lowest estimation of free  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  concentrations. For example, Garrels & Thompson (1962) calculated the distribution of dissolved species in seawater at 25°C. In their study, dissociation constants involving  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ , and  $\text{SO}_4^{2-}$  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  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  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.

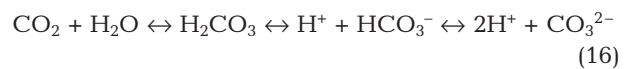
Before the present study, Shichiri (1985) estimated the saturation state of goldfish endolymph using values reported for the blood electrolyte composition of carp and the endolymph/blood ratio of electrolyte concentrations in the rainbow trout. He calculated the  $S_a$  ratio of goldfish endolymph to 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  $\text{mmol l}^{-1}$ , respectively. These seem to be an overestimation compared with the values obtained in the present study. Romanek & Gauldie (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  $\text{CaHCO}_3^+$ . However, they neglected the presence of organic compounds in the endolymph, which may also bind calcium. They calculated  $\Omega$ , 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  $\Omega$  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  $\Omega$  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  $\Omega$ . Therefore, direct comparison of  $\Omega$  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  $\Omega$  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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