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Otolith $\delta^{18}O$ of Pacific bluefin tuna *Thunnus orientalis* as an indicator of ambient water temperature

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ABSTRACT: To detect the relationship between ambient temperature and otolith stable oxygen isotope ($\delta^{18}O$), Pacific bluefin tuna *Thunnus orientalis* larvae were reared at 6 different temperatures (between 23 and 28°C at 35 psu; mean $\delta^{18}O$ value, +0.31‰) for 2 to 8 d after hatching. For the first time, otolith $\delta^{18}O$ was measured using a continuous-flow isotope ratio mass spectrometry system. The linear relationship between otolith $\delta^{18}O$ and temperature was determined as follows: $\delta_{otolith} - \delta_{water} = -2.70\ (T\ °C) + 5.193$ ($r^2 = 0.806$, $p < 0.01$). The temperature-dependent fractionation of otolith $\delta^{18}O$ was close to that reported for inorganic aragonite, indicating that the vital effects of isotopes are small. The otolith $\delta^{18}O$ values of juveniles reared at a mean of 26.5°C were not significantly different from those of larvae reared at 26°C. With regard to the 2 pairs of otoliths within an individual fish, the differences in $\delta^{18}O$ values between the left and right otoliths were subtle. The otolith $\delta^{18}O$ values of larvae reared in $\delta^{18}O$-depleted seawater (24 to 26°C at 32 psu; $\delta^{18}O$, –0.17‰) were lower, but were not significantly different from those of larvae reared in +0.31‰ water, which is the $\delta^{18}O$ value of the water in which the spawners were raised. These results suggest that the sample size for the 32 psu experiment was too small and that the rearing durations were too short to affect the otoliths completely. Our results demonstrate that otolith $\delta^{18}O$ values of Pacific bluefin tuna larvae can provide precise and accurate estimates of their ambient temperature experience.

KEY WORDS: Pacific bluefin tuna · *Thunnus orientalis* · Otolith · $\delta^{18}O$ · Temperature indicator

INTRODUCTION

Pacific bluefin tuna *Thunnus orientalis* are one of the most important international fishery resources; however, the catch has declined in recent years due to overfishing. Therefore, adequate management is necessary to maintain present stock abundance. In 2010, it was agreed that the fishing should be decreased to below 2002 to 2004 levels, particularly for juvenile age classes (Anon 2010). The primary spawning ground of this species is in the northwest region of the Philippine Sea, between eastern Tai-

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wan and Ryukyu Islands in the southern part of Japan (roughly the western North Pacific, Fig. 1, Bayliff 1994, Chen et al. 2006, Tanaka et al. 2006, Kitagawa et al. 2010, Shiao et al. 2010). Spawning occurs in late April to July at a sea surface temperature of 26 ± 2°C, which has been confirmed in rearing experiments (Kimura et al. 2010). In addition, considerably large-scale spawning occurs in July to August in the Sea of Japan (Bayliff 1994). The relative contributions of fish hatched at the 2 spawning grounds to the total Age-0 catch have been tentatively estimated by Itoh (2009) from fertilization dates by counting increases in otolith daily values. The subcohort fertilized up to early July (76%) was considered to originate from the Pacific spawning ground and those fertilized subsequently were considered to originate from the Sea of Japan (24%) (Itoh 2009), indicating that the contribution of fish hatched in the Sea of Japan was higher than that expected initially. Therefore, the stock structure in the Pacific must be clarified for proper management of this species. In particular, it is important to determine Pacific bluefin tuna ecological spawning characteristics, such as spawning migration, spawning period, duration, and geographic scale, in detail.

Although electronic devices, such as archival tags, can accurately log daily geo-location, swimming depth, ambient water temperature, and other data (Kitagawa et al. 2000, 2004, 2006, 2009, Block et al. 2011), data for a fish's entire life are not available from these devices because very small fish cannot carry these devices, as noted by Shiao et al. (2010). Moreover, only a single fish can be tracked with one electric device at a time, which is generally expensive, and released tags are not always recovered. Hence, this method is not suitable for fish population, stock, or cohort analyses.

Fish otoliths, which are composed of aragonite, a crystalline form of calcium carbonate, record environmental information as well as age (Struhsaker & Uchiyama 1976, Radtke & Dean 1982, Volk et al. 1984, Tanaka et al. 2006) throughout a fish's life. The chemical and isotopic compositions that accumulate on the growing surface of the otolith are permanently retained after being deposited because otoliths are acellular and metabolically inert (Campana & Neilson 1985, Mugiya 1994, Campana 1999). Rooker et al. (2001) analyzed the otolith chemistry of juvenile Pacific bluefin tuna and clarified that otolith elemental composition differs between samples collected from the Pacific Ocean and from marginal seas, suggesting that otolith elemental composition could be an indicator for distinguishing nursery grounds.

Oxygen stable isotope ratios ($\delta^{18}O$) in otoliths are used as an indicator of the ambient environment (Campana 1999). Oxygen isotope ratios in otoliths are affected by the water oxygen isotope ratio and ambient water temperature (Patterson et al. 1993, Radtke et al. 1996, 1998, Thorrold et al. 1997, Høie et al. 2004). These otolith oxygen isotopes have been used to discriminate populations of Atlantic bluefin tuna *Thunnus thynnus* that hatched and fed in the Mediterranean Sea or western Atlantic Ocean (Rooker et al. 2008, Schloesser et al. 2010) and to discriminate the natal origins of Pacific bluefin tuna feeding in the western North Pacific or Sea of Japan (Shiao et al. 2010). Furthermore, it should be experimentally proven whether different isotope compositions between the 2 grounds could be reflected in otolith $\delta^{18}O$, in which hydrological characteristics, such as temperature and salinity ($\delta^{18}O$), are different.

Data collected from various fish species provide convincing evidence of a relationship between $\delta^{18}O$ and ambient water temperature (Høie et al. 2004). $\delta^{18}O$ in the aragonite of fish otoliths appears to be in equilibrium with seawater $\delta^{18}O$ values. Similar conclusions were made by Kalish (1991a); however, more precise determinations from laboratory experiments performed under controlled temperature conditions are needed to conclusively determine the equilibrium deposition of oxygen isotopes in otoliths. Studies evaluating the validity of fish otolith $\delta^{18}O$ as a temperature recorder are still sparse, and the methods have not been applied to Pacific bluefin tuna. Furthermore, bluefin tuna maintain their brain and body temperature above water temperature through their ability to conserve heat (Carey et al. 1984, Kitagawa et al. 2001, 2006). The otolith $\delta^{18}O$ values of immature (>20.0 cm fork length, Kubo et al. 2008) and adult Pacific bluefin tuna may reflect brain or body temperature rather than the ambient water temperature (Radtke et al. 1987).

Therefore, to examine the validity of otolith $\delta^{18}O$ as a thermometer, it is necessary to use hatched larvae (<20.0 cm in length) because their brain and body temperatures are likely to track ambient temperatures. However, larvae otoliths are too small to measure $\delta^{18}O$ using conventional methods. Ishimura et al. (2004) developed an analytical system to determine stable isotope compositions of submicrogram quantities of calcium carbonate. Using this method for the first time, we assessed the relationship between the stable oxygen isotope composition of otoliths in Pacific bluefin tuna larvae reared at constant temperatures of 23 to 28°C and the ambient temperature. Our main objective was to examine the validity of

otolith $\delta^{18}O$ as a thermometer for estimating the ambient temperature experienced by fish. The results were compared with those for other species and inorganic aragonite. The effect of differences in seawater (assuming 2 spawning areas) $\delta^{18}O$ values on otolith $\delta^{18}O$ values was also considered.

MATERIALS AND METHODS

Pacific bluefin tuna eggs and hatched larvae were reared at the Amami Station, National Center for Stock Enhancement (NCSE), Fisheries Research Agency (current name: Seikai National Fisheries Institute, Amami branch, Fisheries Research Agency, Fig. 1). The rearing experiments were performed from June 26 to July 2, 2010. Fertilized eggs were obtained from spontaneous broodstock spawning in a net pen at the Amami Station. The eggs were transferred to 1000 ml bottles at stocking densities of 100 eggs per bottle. The eggs were at the one- or 2-cell stage of development. Seawater for rearing was pumped up from the inlet where the net pen is located and sterilized by activated carbon filtration. The salinity of the pumped water was 35 psu.

Two rearing experiments (main and subtopic) were performed in the present study. The main experiment was performed at a salinity of 35 psu (that is, using the seawater pumped up from the inlet), and the second experiment was performed at 32 psu to test the effects of an $\delta^{18}O$-depleted environment on the early developmental stage. In the main experiment, to assess the relationship between constant and ambient temperatures and the stable oxygen isotope composition of otoliths, the eggs were reared in incubators (CN25C Mitsubishi Electric Engineering) at 6 different mean temperatures (23, 24, 25, 26, 27, 28, and 29°C). Three or 4 uncovered bottles were placed in each incubator. The temperatures in the incubators reached the preset temperatures in 5 h after the bottles were placed in the incubators. The temperatures were then controlled within 0.2°C during the experiments.

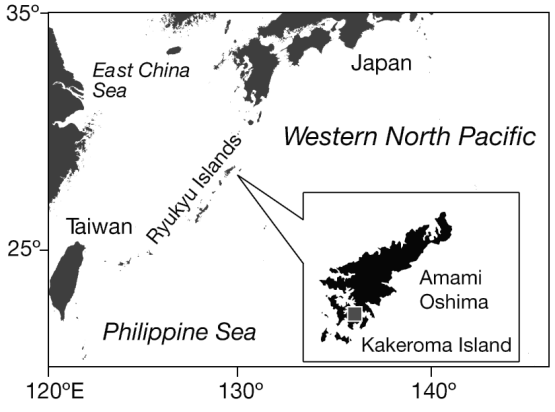

Fig. 1. Location of the Amami Station, NCSE (current name: Seikai National Fisheries Institute, Amami branch), Fisheries Research Agency, Kakeroma Island, Kagoshima, Japan

A second experiment was performed to detect the effect of differences in water (assuming 2 spawning areas) $\delta^{18}O$ values on otolith $\delta^{18}O$ values. Eggs were reared at 3 temperatures of 24, 25, and 26°C in ^{18}O-depleted $\delta^{18}O$ water ($\delta^{18}O = -0.17 \pm 0.05$‰) prepared by adding distilled water to the pumped up 35 psu water so that salinity was 32 psu. The 32 psu water was assumed to be coastal water from the Sea of Japan, which is another Pacific bluefin tuna spawning area and where salinity is often <32.5 psu during the summer (Geospatial Information Authority of Japan, Ministry of Land, Infrastructure, Transport, and Tourism, www.gsi.go.jp/atlas/ in Japanese). $\delta^{18}O$ of the 32.5 psu surface water from the East China Sea to the Kuroshio Current region, which is in contact with the Sea of Japan, is –0.16‰ (Oba 1990, Fig. 1). The eggs were reared in the same way as in the main experiment. In both experiments, the water in the bottles was not replaced for the entire rearing period. We considered the change in the water $\delta^{18}O$ due to the evaporation or absorption by larvae to be subtle compared with the amount of water in each bottle.

Experimental larvae were fed the rotifer *Brachionus rotundiformis* from 3 to 5 d after hatching (DAH). The larvae were reared as long as possible, and dead larvae were preserved in 99% ethanol. The sagitta and lapillus of each otolith were picked from the larvae using a needle and tweezers under a stereoscopic microscope (Fig. 2a). The asterisci were not found, and thus it was not used for analysis. In total, 4218 otoliths from 1109 larvae were obtained at all preset temperatures. Of these, 3000 otoliths were used for analysis (Table 1).

Otolith $\delta^{18}O$ was measured using the continuous-flow isotope ratio mass spectrometry system at Hokkaido University; the system can determine $\delta^{18}O$ for ultra-microvolume carbonate samples (as low as 0.2 µg) with high precision and accuracy (Ishimura et al. 2004, 2008). Since the average otolith weight was much less than 0.2 µg, we pooled 100 otoliths (approximately 1.3 µg in total) in a reaction tube as a subsample for each analysis (Fig. 2b,c). Four subsamples at each temperature from 23 to 27°C, 1 subsample at 28°C, and 3 subsamples at 24 to 26°C ($\delta^{18}O$-depleted water) were used for analysis.

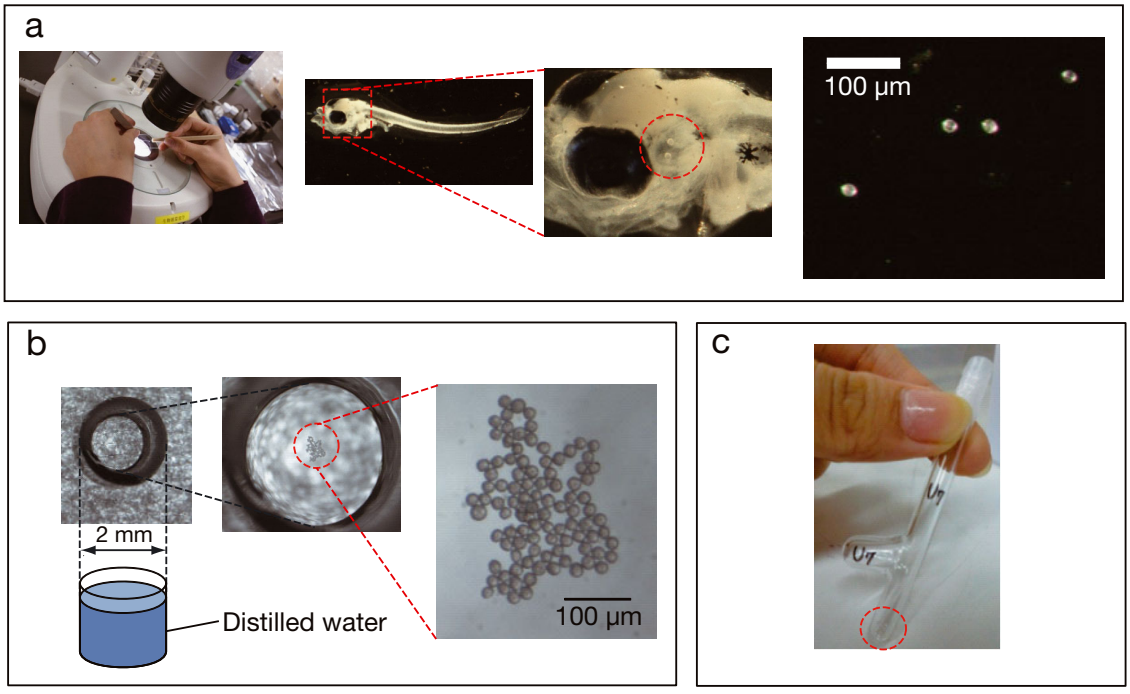

Fig. 2. Procedures for otolith removal from larvae aged 2 to 8 DAH: (a) the sagitta and lapillus were removed from the larvae using a needle and tweezers under a stereoscopic microscope, (b) 100 otoliths (approximately 1.3 µg in total) were collected in a distilled-water-filled micro-Petri dish (2 mm diameter), and (c) after drying up the water in the dish, the dish of the otoliths was placed in the bottom of a reaction tube with a branched tubule filled with 0.1 to 0.5 ml of 100% phosphate acid

Table 1. Rearing conditions, sampling data, and $\delta^{18}O_{otolith}$ of Pacific bluefin tuna larvae

Salinity (psu)	Temperature (°C)	$\delta^{18}O_{water}$ (‰)	Total no. of larvae	Total no. of otoliths	Sample size (10^2 otoliths)	Mean ± SD	
						Weight of 100 otoliths (µg)	$\delta^{18}O_{otolith}$ (‰)
35.0	23.0	+0.32	141	533	4	1.3 ± 0.13	−0.98 ± 0.27
	24.0	+0.31	128	486	4	1.2 ± 0.19	−1.43 ± 0.47
	25.0	+0.33	134	513	4	1.4 ± 0.09	−1.16 ± 0.09
	26.0	+0.30	137	523	4	1.3 ± 0.10	−1.76 ± 0.32
	27.0	+0.30	138	526	4	1.4 ± 0.07	−2.17 ± 0.41
	28.0	+0.32	32	116	1	0.7	−2.9
Mean		+0.31 ± 0.01					
32.0	24.0	−0.21	129	500	3	1.4 ± 0.16	−1.57 ± 0.16
	25.0	−0.12	130	503	3	1.4 ± 0.04	−1.65 ± 0.30
	26.0	−0.17	140	518	3	1.5 ± 0.06	−1.71 ± 0.22
Mean		−0.17 ± 0.05					

Otoliths of the bluefin tuna juveniles at 34 DAH provided by the Amami Station were also analyzed for comparison. The juveniles hatched on July 27, 2010, and were reared as mass-produced fertilized eggs at the station. The hatched larvae (<10 000 m^{-3}) were reared in an octagon-shaped tank with 2.3 m sides and a depth of 1.6 m to which 40 m^{-3} of seawater was added. The rearing conditions were the same as that described previously by Masuma (2008) and Masuma et al. (2008). The average water temperature for the 34 DAH juveniles was 26.5 ± 0.58°C (Table 2). Ten otoliths from 8 juveniles, including 2 pairs of otoliths (left and right), were used for the $\delta^{18}O$ analysis. Each otolith (sagitta) of the 34 DAH juveniles was >500 µm in length (Fig. 3), and the average weight (±SD) of 5 otoliths was 166.2 ± 16.6 µg (Table 2), which was sufficient to permit individual analysis of the samples using the continuous-flow isotope ratio mass spectrometry system.

Table 2. Rearing conditions and $\delta^{18}O_{otolith}$ of 34 DAH Pacific bluefin tuna

Salinity (psu)	Mean ± SD Temperature (°C)	$\delta^{18}O_{water}$ (‰)	Sample size	Mean ± SD Otolith weight (µg, n = 5)	Mean ± SD $\delta^{18}O_{otolith}$ (‰)
35.4	26.5 ± 0.58	+0.21	10	166.2 ± 16.6	−1.86 ± 0.10

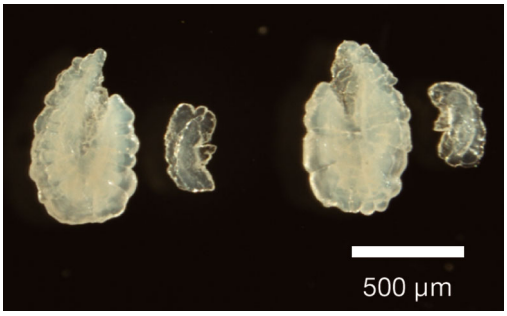

Fig. 3. Otoliths removed from a juvenile bluefin tuna aged 34 DAH. The larger otoliths are the sagittae and the others are the lapilli

CO_2 $\delta^{18}O$ values can be analyzed with SD of <0.09‰ using this mass spectrometry system (Ishimura et al. 2008); the SD of NBS 19 (international standard reference calcite) analyzed on the same day as the samples was ±0.08‰ for $\delta^{18}O$. All data are reported in standard δ notations ($\delta^{18}O$; ‰) relative to the Vienna–Pee Dee Belemnite (VPDB) standard scale. The weight of the analyzed otoliths was calculated using the volume of CO_2 gas produced during the reaction between calcite and phosphoric acid (Ishimura et al. 2004). Details of the $\delta^{18}O$ analysis for ultra-microvolume carbonate samples are described by Ishimura et al. (2008).

The stable oxygen isotope composition ($\delta^{18}O$) of water used for rearing was also measured using DELTA plusXL (Thermo Fisher Scientific, precision 0.05‰). The $\delta^{18}O$ water sample values are reported in standard δ notations ($\delta^{18}O$; ‰) relative to the Vienna–Standard Mean Ocean Water (VSMOW) standard. The values were converted from the VSMOW scale to the VPDB scale for comparison, according to Friedman & O'Neil (1977). To permit comparisons between our results and those of other studies that reported linear relationships between temperature and oxygen isotope fractionation, fractionation was expressed as 1000 ln α where α was calculated as follows:

$$\alpha = \frac{\delta^{18}O_{otolith} + 1000}{\delta^{18}O_{water} + 1000} \quad (1)$$

When expressing the relationship in terms of $\delta_{otolith} - \delta_{water}$, $\delta_{otolith}$ represented the oxygen isotope composition of otoliths on the VPDB scale, and δ_{water} represented the oxygen isotope composition of seawater on the VSMOW scale.

RESULTS

In total, 3000 otoliths from the larvae reared in both experiments were used for analysis (Table 1). The mean $\delta^{18}O$ values (±SD) of the 35 psu salinity water between 23 and 28°C used for rearing was +0.31 ± 0.01‰ and remained almost constant irrespective of temperature. The mean value of the 32 psu salinity water at 24 to 26°C was −0.17 ± 0.05‰, and the values were significantly lower than those of the 35 psu water (*t*-test, $p < 0.01$, Table 1).

The mean weights of 100 otoliths collected from larvae reared in the 35 psu water were 1.2 to 1.4 µg at 22 to 27°C (Table 1). The mean $\delta^{18}O$ values (±SD) were −0.98 ± 0.27, −1.43 ± 0.47, −1.16 ± 0.09, −1.76 ± 0.32, and −2.17 ± 0.41‰ at 23, 24, 25, 26, and 27°C, respectively (Fig. 4, Table 1), indicating an inverse relationship with temperature. The coefficients of

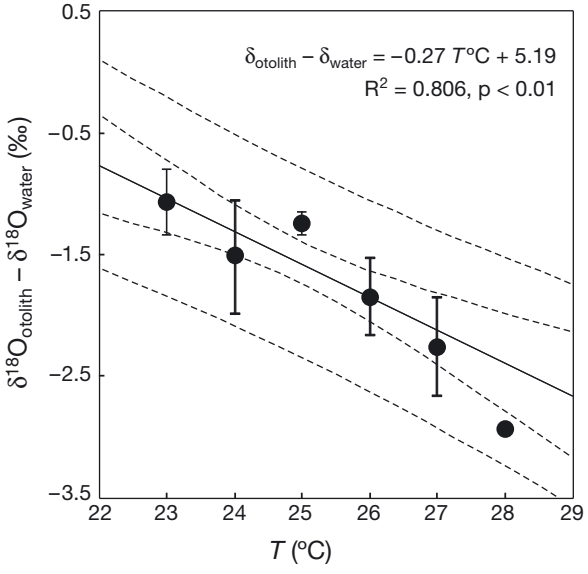

Fig. 4. Relationship between water temperature (T) and the fractionation of oxygen isotopes in otoliths of Pacific bluefin tuna larvae. Plots are shown as the mean and SD (bars). Note that only one datum was available at 28°C. Regression lines (solid line) and 95% confidence intervals (upper/lower confidence limits: inside dotted lines; prediction limits: outside dotted lines) are also shown

variation (the ratio of SD to the mean) were –0.28, –0.32, –0.08, –0.18, and –0.19 at 23, 24, 25, 26, and 27°C, respectively. Although the otolith sample weight was <1.0 µg at 28°C, only one datum was available. The $\delta^{18}O$ values at 28°C were –2.9‰ on average, which was more negative than the other mean values for the other temperatures (23 to 27°C).

Linear regression of the temperature and oxygen isotope fractionation data yielded the relationship of $\delta_{otolith} - \delta_{water}$ for a given temperature as follows:

$$\delta_{otolith} - \delta_{water} = -0.270\,(T\,°C) + 5.193 \quad (r^2 = 0.806,\ p < 0.01) \quad (2)$$

where T is the temperature in Celsius. Note that the datum at 28°C was plotted in Fig. 4 but was excluded from the regression analysis. The relationship between 1000 ln α and temperature is presented as follows to facilitate comparison of the results of this study with those of other studies:

$$1000\ln\alpha = 24.28\,(1000T^{-1}\mathrm{K}) - 52.83 \quad (r^2 = 0.805,\ p < 0.01) \quad (3)$$

where T is the temperature in Kelvin. In addition, published temperature dependencies for the oxygen isotope fractionation are provided in Table 3. The data in the present and previous studies were compared with the inorganic aragonite relationship represented as a regression equation as shown below (Kim et al. 2007) (Fig. 5):

$$1000\ln\alpha = 17.88 \pm 0.13(1000T^{-1}\mathrm{K}) - 31.14 \pm 0.46 \quad (4)$$

Compared with those presented by Kim et al. (2007), all $\delta^{18}O$ values, excluding that at 25°C, were lower but not significantly different from regression Eq. (4) (χ^2-test, $p > 0.05$). To facilitate comparison with fractionation factors derived from analyses of biogenic carbonates in Fig. 5, the aragonite–water fractionation factor determined by Kim et al. (2007) was recalculated using an acid fractionation factor of approximately 1.01025 (Sharma & Clayton 1965), a value commonly used for calcite of any origin or physical state. The biogenic aragonite–water curves of Thorrold et al. (1997), Radtke et al. (1996, 1998), and Høie et al. (2004) are slightly different from each other but are uniformly close to those of Kim et al.

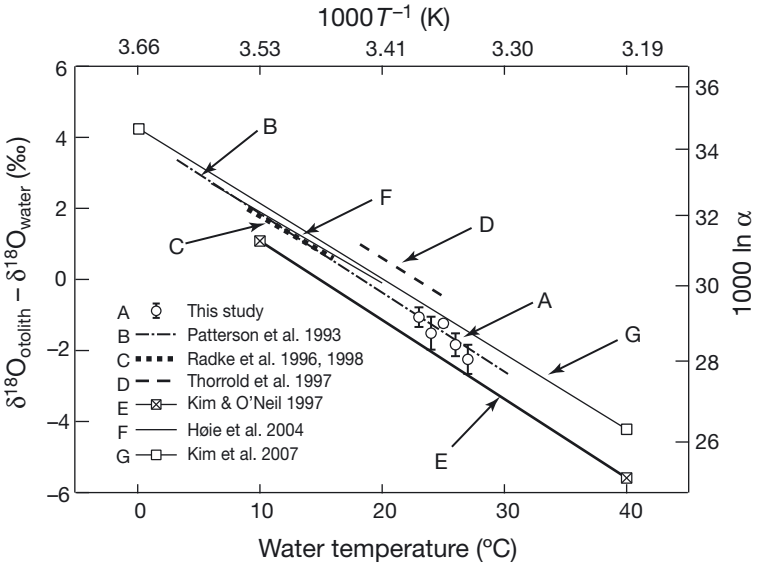

Fig. 5. Comparison of the result of this study with previously reported fractionation relationships for other fish species and inorganic aragonite–water at low temperatures. The regression line for the data in the present study (A) is not shown to avoid clutter. In addition, line G reported by Kim et al. (2007) corresponds to Eq. (4) in the text, but it was recalculated using an acid fractionation actor of 1.01025 to facilitate comparison (Sharma & Clayton 1965). The calcite–water isotope fractionation curve of Kim & O'Neil (1997) was also added

Table 3. Comparison to previous studies. α: see Eq. (1); K: Kelvin

Species	Equation	Temperature range (°C)	Reference
Thunnus orientalis	$\delta_{otolith} - \delta_{water} = -0.27\,T\,°C + 5.19$ $1000\ln\alpha = 24.28(1000\,T^{-1}\mathrm{K}) - 52.83$	23.0–27.0	Present study
Inorganic aragonite	$1000\ln\alpha = 17.88(1000\,T^{-1}\mathrm{K}) - 31.14$	0–40.0	Kim et al. (2007)
Gadus morhua	$\delta_{otolith} - \delta_{water} = -0.20\,T\,°C + 3.90$ $1000\ln\alpha = 16.75(1000\,T^{-1}\mathrm{K}) - 27.09$	6.0–20.0	Høie et al. (2004)
Gadus morhua	$\delta_{otolith} - \delta_{water} = -0.20\,T\,°C + 3.79$	9.0–16.0	Radtke et al. (1996, 1998)
Inorganic calcite	$1000\ln\alpha = 18.03(1000\,T^{-1}\mathrm{K}) - 32.42$	10–40	Kim & O'Neil (1997)
Micropofonias undulatus	$1000\ln\alpha = 18.56(1000\,T^{-1}\mathrm{K}) - 32.54$	18.2–25.0	Thorrold et al. (1997)
Several freshwater fish species	$1000\ln\alpha = 18.56(1000\,T^{-1}\mathrm{K}) - 33.49$	3.2–30.3	Patterson et al. (1993)

(2007) (line G in Fig. 5). In contrast, line G lies higher than the curve constructed by Patterson et al. (1993) from analyses of freshwater fish otoliths. Considering that the standard error (SE) of Eq. (4) was ±0.46, all the biogenic aragonite–water fractionation curves, including those of this study, were almost indistinguishable from line G. Data from Kalish (1991a) were excluded because the precision of their temperature estimates has been questioned, as indicated by Høie et al. (2004).

The mean $\delta^{18}O$ values (±SD) for otoliths collected from larvae reared in the 32 psu water at 24, 25, and 26°C were −1.57 ± 0.16, −1.65 ± 0.30, and −1.71 ± 0.22‰, respectively (Table 1). Mean values at 24 and 25°C were lower than the values of the 35 psu water, suggesting that the environmental $\delta^{18}O$ concentration was imprinted in the otoliths; however, all values were within the 95% confidence interval of the regression line Eq. (2) (Fig. 4, χ^2-test, $p > 0.05$). Due to the small sample size, regression analysis was not performed to estimate the slope for the results of the 32 psu experiment.

The otolith $\delta^{18}O$ values of the 34 DAH juveniles reared at approximately 26.5°C were compared with those of larvae reared at 26°C. The mean value (±SD) was −1.9 ± 0.13‰, which was not significantly different from the value of the larvae reared at 26°C (−1.8 ± 0.32‰, Table 1, *t*-test, $p > 0.05$). Furthermore, the differences in $\delta^{18}O$ values between the left and right otoliths were subtle (0.04 and 0.13‰, respectively).

DISCUSSION

The temperature dependence of otolith $\delta^{18}O$ values in some freshwater or demersal fish species has been investigated (Patterson et al. 1993, Radtke et al. 1996, 1998, Thorrold et al. 1997, Høie et al. 2004). However, this study is the first report on bluefin tuna larvae. In this study, 5 temperatures between 23 and 27°C were used, and a linear relationship represented by regression Eq. (2) was detected. The temperature dependence was −0.27‰°C^{-1} from the slope of Eq. (2). This value was close to that (−0.33‰°C^{-1}) for Australian salmon *Arripis trutta*, which was estimated at 13 to 22°C by Kalish (1991a), and that (−0.20‰°C^{-1}) for Atlantic cod *Gadus morhua*, which was estimated at 9 to 16°C by Radtke et al. (1996; 1998) and at 6 to 20°C by Høie et al. (2004). As with the previous studies, the otolith $\delta^{18}O$ values of Pacific bluefin tuna larvae provided precise and accurate estimates of the ambient temperature experienced by the fish.

In contrast, the slope (24.28) and intercept (52.83) of Eq. (3) were larger than those reported in other studies for aragonite (slope range, 16.75 to 18.56; intercept range, 27.09 to 33.49, Table 3). This probably resulted from statistical processing and a narrower temperature range (23 to 27°C) than that used in previous studies. Therefore, there is probably little difference to those relationships reported by other studies. In this study, the ratio of abnormal to normal larvae increased and the survival rate decreased at seawater temperatures >27°C. It has been reported that the otolith elemental composition changes when fish are under physiological stress (Kakuta 2000); therefore, physiological stress may have affected otolith $\delta^{18}O$ at temperatures >27°C. Thus, without the 27°C value, the regression equation was estimated as follows:

$$1000 \ln\alpha = 18.46\,(1000\,T^{-1}\mathrm{K}) - 33.22 \quad (r^2 = 0.611,\ p < 0.01) \qquad (5)$$

The slope of regression Eq. (5) (18.46) was much closer to that of Eq. (4) (17.88) than that of Eq. (3). The difference in $1000 \ln\alpha$ from Eq. (4) was <0.2‰, which was within the SE of Eq. (4) (0.46) and statistically indistinguishable. As indicated by Kim et al. (2007), the implications of these observations are as follows. Many biogenic aragonites are precipitated at or very near oxygen isotope equilibrium with ambient water. When deviations from the oxygen isotope equilibrium are observed, the discrepancy can be explained by species-specific vital (physiological and/or kinetic) effects operating during the precipitation of the biogenic aragonite. Regardless of vital effects, the temperature coefficients of all aragonite–water curves are the same. Of note, the coefficient of determination for Eq. (5) was high ($r^2 = 0.611$) but was slightly lower than that of Eq. (3) ($r^2 = 0.805$). This was probably because of a single influential point (outlier at 27°C), which created a spurious correlation in Eq. (3) (Cook & Weisberg 1982). As the sample size was very small in this case, the effect of the outlier was probably greater in Eq. (3) (Stockburger 1998).

The $\delta^{18}O$ values of the Pacific bluefin tuna larvae were slightly lower than the theoretical composition of aragonite formed in equilibrium (line G in Fig. 5). McCrea (1950) reported that as the total carbonate ion in seawater increases with increasing pH, total carbonate $\delta^{18}O$ decreases. It has also been reported that precipitated calcium carbonate $\delta^{18}O$ depends on the pH of the solution (Usdowski & Hoefs 1993, Takagi 2002). Therefore, the value might be lower in seawater because of the increase in carbonate ions in seawater as a result of larval respiration, although

the pH of the rearing water was not measured in this study. Another factor is precision and error of the $\delta^{18}O$ water analysis. As a whole, the analytical error was within 0.1‰, which could have affected the results in the present study. The other factor is the water temperature itself, which also could have affected the detected errors to some extent.

Rooker et al. (2008) analyzed the otolith core of adult Atlantic bluefin tuna and reported an approximately 1‰ difference in otolith $\delta^{18}O$ values between eastern and western stocks, which actually reflects the difference in surface seawater $\delta^{18}O$ values between the east and west Atlantic (LeGrande & Schmidt 2006). This difference was also observed in Pacific bluefin tuna spawning in the northwest region of the Philippine Sea, between eastern Taiwan and Ryukyu Islands located in the southern part of Japan (roughly the western North Pacific) from late April to June (Bayliff 1994, Chen et al. 2006, Tanaka et al. 2006, Kitagawa et al. 2010, Shiao et al. 2010) and in the southern Sea of Japan in late July to August (Tanaka et al. 2007, Itoh 2009). These 2 areas have different temperatures and salinities. The seawater in the western Pacific is influenced by the Kuroshio Current, which is characterized by high salinity and enriched water $\delta^{18}O_{SMOW}$ values of approximately +0.4‰ (Shen et al. 2005), which was consistent with $\delta^{18}O$ of the 35 psu water (+0.31‰ on average) used for the rearing experiment in this study.

In contrast, breeders probably spawn in the coastal waters of the southern Sea of Japan based on larval appearance frequency (Nishikawa et al. 1985, Tanaka et al. 2007). As explained by Shiao et al. (2010), water $\delta^{18}O_{SMOW}$ in the Sea of Japan is depleted in the deep waters ($\delta^{18}O_{SMOW}$ = −0.8 to −0.3‰), which can upwell to the sea surface and reduce surface water $\delta^{18}O_{SMOW}$ to values of −0.4 to 0‰ (Ki 1999). Oba (1990) also reported that the $\delta^{18}O$ value of the 32.5 psu surface water in the East China Sea, which connects with the Sea of Japan, is −0.16‰. The Sea of Japan has very weak vertical stability (Kim et al. 2002), and vertical mixing of surface and deep waters driven by the winter monsoon (Nakanishi & Minagawa, 2003, Mooers et al. 2005) can extensively reduce water $\delta^{18}O_{SMOW}$ in the euphotic zone. In addition, when the dry, relatively cold continental air mass passes over the Sea of Japan, it evaporates the surface water of the Sea of Japan, and the evaporated water (with lower $\delta^{18}O$ values) precipitates along the coastal areas of Japan (Waseda & Nakai 1983). In this regard, $\delta^{18}O$ of the 32 psu water ($\delta^{18}O$ = −0.17‰ on average) analyzed in this study was within the range of −0.4 to 0‰.

The mean $\delta^{18}O$ values in the 32 psu water at 24, 25, and 26°C were lower than those of the 35 psu water, suggesting that the environmental $\delta^{18}O$ concentration was already imprinted in the otoliths. Oxygen isotopes in otolith aragonite are deposited in or very near to equilibrium in ambient water (Kalish 1991a,b, Patterson et al. 1993, Radtke et al. 1996, Thorrold et al. 1997, Campana 1999). In general, the basic pathway of the bulk of inorganic elements into the otolith is from the water into the blood plasma via the gills or intestine, then into the endolymph, and finally into the crystallizing otolith (Campana 1999). Although the pathway of any given element or ion from the environment into otoliths is a multistage process, Tohse & Mugiya (2002) clarified that when goldfish weighing 8 to 10 g were exposed to ambient water containing $NaH^{14}CO_3$, carbon incorporation into otoliths occurred after 6 to 12 h. Therefore, in this study, $\delta^{18}O$ values could reflect otolith $\delta^{18}O$ for the 2 to 8 DAH larvae reared in the 32 psu water, since equilibrium is reached more quickly in smaller fish.

In contrast, all values of the 32 psu water were not significantly different from the regression line Eq. (2) (χ^2-test, $p > 0.05$), which may have been due to a statistical problem. The sample size for the experiment with 32 psu water was only 3, which was too small to estimate a precise slope value for the regression line and to compare with the results of the main experiment. Therefore, as a minimum requirement, sample sizes should be similar for statistical comparisons. However, 2 to 8 d might be insufficient for complete exchange of isotopic elements in the endolymph fluid surrounding otoliths and their crystallization into otoliths. In our case, the body fluid of larvae such as the endolymph fluid consisted of 35 psu seawater shortly after the hatch, which was obtained from the parent body and/or egg water. Therefore, mature adults should be reared in seawater with different $\delta^{18}O$ values and their larvae should be used for otolith $\delta^{18}O$ analysis to precisely detect differences in otolith $\delta^{18}O$ values via differences in seawater $\delta^{18}O$ values. In particular, eggs obtained from adult fish spawned in the Sea of Japan should be reared in seawater from the Sea of Japan to detect the difference in otolith $\delta^{18}O$ values between the 2 spawning areas. It would be useful to clarify the difference in otolith $\delta^{18}O$ values between the spawning areas (which actually reflects the difference in surface seawater $\delta^{18}O$ values between the western Pacific and the Sea of Japan) when the otolith core of adult Pacific bluefin is analyzed. Detecting the difference in otoliths between spawning areas will clarify Pacific bluefin tuna stock structure.

The otolith $\delta^{18}O$ values of the 34 DAH juveniles reared were not significantly different from those of the larvae reared at 26°C, and the differences in $\delta^{18}O$ values between the left and right otoliths were subtle. These results suggest that otolith $\delta^{18}O$ fractionation does not change after the juvenile stage, and indicates the validity of Pacific bluefin tuna otolith $\delta^{18}O$ as a precise and accurate estimate of ambient temperature experienced by the fish. The continuous-flow isotope ratio mass spectrometry system used in the study can determine $\delta^{18}O$ in submicrogram quantities of carbonate samples with high precision and accuracy (Ishimura et al. 2004, 2008). However, it was still impossible to rear larvae at various temperatures other than 26°C before their otoliths reached a size of approximately 1.0 µg and, thus, we had to pool 100 otoliths (approximately 1.3 µg in total) for one analysis. However, it will be possible to analyze larger-sized otoliths in wild fish using a single individual. As the density of aragonite is 2.93 g cm^{-3}, samples can be obtained by drilling the otolith core to a radius of 50 µm, i.e. fish with otolith radii >50 µm can be analyzed. Natural larvae with otoliths of sufficient size are approximately 14 DAH (Tanaka et al. 2008), and many larvae of such a size remain in the local waters because of eddy circulations that occur around their hatching area. A simulation study revealed that virtual eggs and larvae of Pacific bluefin tuna are entrained in an anticyclonic (clockwise) circulation east of Taiwan, and some particles are retained in circulation for approximately 20 d before being transported northward (Kitagawa et al. 2010). These larvae can drift in a homogeneous environment for some time after hatching. Therefore, otoliths with a radius of ≥50 µm will provide precise information — with little noise — on the waters where the larvae hatched.

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