Using segmental isotope analysis of teleost fish vertebrae to estimate trophic discrimination factors of bone collagen

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Abstract

We conducted feeding experiments with sardine (Sardinops melanostictus) and Japanese flounder (Paralichthys olivaceus) to investigate trophic discrimination factors (TDFs) in bone collagen using retrospective isotope analysis. Sardines and Japanese flounder were fed a single diet for 9 months and 4 months, respectively. At the end of the experiments, we extracted vertebral centra from six sardines and seven Japanese flounder and subdivided them into multiple sections. We extracted bone collagen from each section, and measured the $\delta^{13}C$ and $\delta^{15}N$ of each vertebral section and food source. In all fish samples, the $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen increased from the centers of vertebrae to the marginal sections. This trend was consistent with the predicted isotopic shift in the diets of these fishes. The TDFs for sardines were estimated at 5.58 ± 0.25‰ (mean ± SD) for $\delta^{13}C$ and −0.70 ± 0.25‰ for $\delta^{15}N$; those for Japanese flounder were 3.89 ± 0.14‰ for $\delta^{13}C$ and 1.18 ± 0.35‰ for $\delta^{15}N$. Our results show that segmental analysis of vertebrae can reconstruct $\delta^{13}C$ and $\delta^{15}N$ values for multiple life-stages of both fish species and is useful for estimating TDFs and turnover times of fish bone collagen.

Chemical analysis of the calcified structures in fish is useful for reconstructing individual life histories (e.g., Campana et al. 2019; Tzadik et al. 2017) and is one of the most important techniques in the fields of aquatic biology, fish paleoecology, and fisheries science. The most common and accepted method of analyzing calcified structures is the chemical analysis of otoliths; this can be used to reconstruct retrospective chemical information (e.g., element composition and isotope ratios), with aging information read from the otolith annuli (e.g., Campana 2005; Kerr and Campana 2014). The advantage of otolith analysis is that this tissue is not subject to metabolism and can provide precise chemical information for a specific age. Conversely, some of the ecologically important isotopes, such as those of radiocarbon, nitrogen, and sulfur, are extremely difficult to measure with high-temporal resolution from otoliths, because otoliths are composed primarily of calcium carbonate minerals (Campana 1999).

Recently, Matsubayashi et al. (2017) proposed segmental isotope analysis of vertebrae (SIAV) of teleost fishes as a method for reconstructing retrospective information on light isotopes. SIAV has high potential for investigating the shifts in diet and habitat of teleost fishes; however, the lack of validation studies for general applicability of this method potentially limits its utility. Specifically, understanding the trophic discrimination factors (TDFs) of the most common stable carbon and nitrogen isotopes for fish bone collagen should be the important first step in converting isotope ratios obtained from SIAV to the proportional contribution of each diet to a fish’s nutrition (e.g., Caut et al. 2009; Phillips et al. 2014).

Here, we performed controlled feeding experiments to estimate the TDFs for the stable carbon and nitrogen isotope ratios of bone collagen of sardine (Sardinops melanostictus) and Japanese flounder (Paralichthys olivaceus). We know of no...
studies to date that have empirically shown TDFs and turnover times for bone collagen of fishes. Although the duration of feeding experiments to estimate TDFs must exceed the turnover time of the target tissues, bone collagen is considered to have the longest turnover time among tissues (Schoeninger and DeNiro 1984; Sholto-Douglas et al. 1991; Gaston and Suthers 2004), and this makes it difficult to estimate TDFs of bone collagen. On the other hand, segmental isotope analysis of radially growing bone allows for reconstruction of isotopic shifts resulting from changes in diet, and TDFs can be calculated using an exponential model (e.g., Tieszen et al. 1983; Hobson and Clark 1992; Tominaga et al. 2003) even when the duration of the feeding experiment is slightly shorter than the turnover time of bone collagen.

Based on this assumption, we applied SIAV to the carbon and nitrogen stable isotope ratios that were used in feeding experiments with sardine and Japanese flounder, and estimated the TDFs for these isotopes by constructing a logistic model to describe the isotopic shifts in bone sections. The logistic model is better suited to our data than the general exponential model, which is generally used to estimate tissue-specific turnover rates of isotope ratios (e.g., Ogden et al. 2004; Browning et al. 2014), because the logistic equation includes the stationary phase prior to the shift in diet.

**Materials and methods**

**Feeding experiments**

Feeding experiments were conducted at the Fisheries Research Laboratory at Kyushu University and the Institute for East China Sea Research at Nagasaki University. Wild juvenile sardines (around 200 individuals) sampled from the coast of Kochi Prefecture, Japan on 30 October 2015 were transported by a fish tank truck (Aquaspot, Saga, Japan) and released into a 3000-liter outside tank configured as a flow-through aquaculture system (water residence time about 4 h) at Kyushu University. Water temperature and quality were routinely monitored. The water tank was shaded by black netting to prevent growth of phytoplankton and attached algae. Until 04 August 2016, the fish were fed daily with a commercially available fish food, designated “Food A” (Ezuke-ru 0, Chubu Shiryō, Aichi, Japan) and based on krill, squid, and fish meal. These sardines were fed the same type of food (Food A), but from two different food batches; we therefore measured the stable carbon and nitrogen isotope ratios of Food A from each batch (Foods A-1 and A-2) to check for differences in isotopic values between the two batches (Table 1). Some fish were sampled periodically during the experiment and measured for body length and weight (Fig. 1; Supporting Information Table S1). At the end of the feeding experiment, all remaining sardines were killed in the same manner and immediately frozen at −20°C until they could be processed.

Eggs of Japanese flounder from aquaculture stock were hatched at the end of September 2015 and were reared at Nagasaki Prefectural Institute of Fisheries from November 2015 to April 2016 (referred to as the “first period”). During this period, they were sequentially fed four commercially available foods (Foods B, C, D, and E-1) with grain sizes and ingredients differing to meet changing growth requirements, although information on the timing of switching between foods is not available. On 01 May 2016, the Japanese flounder (about 100 individuals) were moved to the Institute for East China Sea Research at Nagasaki University for the main feeding experiment. These fish were reared in a 500-liter indoor tank configured as a flow-through aquaculture system (residence time around 1 h), and their food was changed to another commercially available fish food, “Food E-2” (Kuroshio-float P-3, Higashimaru, Kagoshima, Japan), based on krill, prawn, and fish meal. Food E-2 was the same type of food as food E-1 but from a different food batch (Table 1).

The flounder were fed this food consistently for about 4 months (referred to as the “second period”). Some fish were sampled periodically during this period and measured for body length and weight (Fig. 1; Supporting Information Table S1). They were then killed on 12 September 2016 by breaking a vertebra and immediately frozen until they could

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**Table 1.** δ¹³C and δ¹⁵N values (mean ± SD) for fish foods used in feeding experiments. Foods A-1 and A-2 and E-1 and E-2 are respectively the same types of food but from different food batches. Lipids were removed from all food samples before isotopic analysis.

| Sample | Onset of feeding | Food name          | δ¹³C (‰) | δ¹⁵N (‰) | n |
|--------|------------------|--------------------|----------|----------|---|
| Food A-1 | 30 Oct 2015     | Ezuke-ru          | −20.5 ± 0.3 | 13.5 ± 0.5 | 3 |
| Food A-1 | 18 Jan 2016     | Ezuke-ru          | −20.5 ± 0.2 | 13.6 ± 0.0 | 3 |
| Food A-2 | 04 Apr 2016     | Ezuke-ru          | −20.2 ± 0.1 | 13.5 ± 0.4 | 3 |
| Food A-2 | 06 Jul 2016     | Ezuke-ru          | −20.2 ± 0.1 | 13.3 ± 0.1 | 3 |
| Food B  | Unknown         | Ambrose 600       | −21.3 ± 0.3 | 11.0 ± 0.8 | 4 |
| Food C  | Unknown         | Hiram EP F-3      | −19.8 ± 0.1 | 9.2 ± 0.2 | 5 |
| Food D  | Unknown         | Kuroshio float P-1 | N/A      | N/A      | — |
| Food E-1 | Unknown        | Kuroshio float P-3 | −17.4 ± 0.2 | 9.7 ± 0.2 | 5 |
| Food E-2 | 01 May 2016     | Kuroshio float P-3 | −17.7 ± 0.1 | 12.7 ± 0.1 | 11 |

NA, not available.
be processed. Unneeded food and fish feces automatically flowed out through the overflow system, and the tank was also cleaned daily by siphon pump to prevent disease. No other food sources were available to these fish, as the water entering the tank was filtered through sand filters.

The sardines and Japanese flounder used in this study were treated with care and killed in accordance with the guidelines for animal experiments of the Faculty of Agriculture and Graduate School of Kyushu University and the Faculty of Fisheries and Graduate School of Nagasaki University.

Sample preparation and stable isotope analysis

We used six sardines (SM-01 to SM-06) and seven Japanese flounder (PO-01 to PO-07) for stable carbon and nitrogen isotope measurements in vertebrae. The ranges of fork lengths and body weights of SM-01 to SM-06 were 178–191 mm and 73–91 g, respectively, and those of PO-01 to PO-07 were 254–306 mm and 265–358 g, respectively (Table 2). We extracted vertebral centra from fish bodies, subdivided each centrum, and extracted collagen from each bone section as described by Matsubayashi et al. (2017). Briefly, we first removed any remaining muscle, spines, and cartilage from all vertebral centra by using a microgrinder. Next, the centra were immersed in a methanol : chloroform mixture (1 : 1, vol : vol) for approximately 6 h, rinsing with methanol, and drying.

Stable isotope ratios are expressed in δ notation in accordance with the international standard scale on the basis of the following equation:

\[
\delta X = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1,
\]

where \(X\) is \(^{13}\text{C}\) or \(^{15}\text{N}\), \(R_{\text{sample}} \) is the \(^{13}\text{C} : ^{12}\text{C}\) or \(^{15}\text{N} : ^{14}\text{N}\) ratio of the sample, and \(R_{\text{standard}} \) is that of Vienna Pee Dee Belemnite for \(^{13}\text{C} : ^{12}\text{C}\) or atmospheric nitrogen for \(^{15}\text{N} : ^{14}\text{N}\). For stable isotope analysis of bone collagen, approximately 0.1–0.3 mg was put into a tin capsule; the \(\delta^{13}\text{C}\) and \(\delta^{15}\text{N}\) values were measured at the Research Institute for Humanity and Nature (Kyoto, Japan) with a Delta plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) connected to a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific) via a ConFlo III interface (Thermo Fisher Scientific). The elemental concentrations and isotope ratios of carbon and nitrogen were calibrated against those of alanine A (\(\delta^{13}\text{C} = -19.04\%\), \(\delta^{15}\text{N} = 22.71\%\); CERKU-02) and threonine (\(\delta^{13}\text{C} = -9.45\%\), \(\delta^{15}\text{N} = -2.88\%\); CERKU-05) laboratory standards (Tayasu et al. 2011), which are traceable back to international standards. The analytical standard deviations (SDs) of these standards were 0.14‰ (\(\delta^{13}\text{C}\)) and 0.14‰ (\(\delta^{15}\text{N}\)) for alanine (\(n = 22\)) and 0.10‰ (\(\delta^{13}\text{C}\)) and 0.10‰ (\(\delta^{15}\text{N}\)) for threonine (\(n = 15\)). We assessed the purity of each bone collagen sample on the basis of the C : N ratio (DeNiro 1985).

For stable isotope analysis of fish foods, approximately 0.5 mg was put into a tin capsule; the \(\delta^{13}\text{C}\) and \(\delta^{15}\text{N}\) values were measured at Nagasaki University (Nagasaki, Japan) with a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific) connected to a Flash EA 2000 elemental analyzer (Thermo Fisher Scientific) via a ConFlo IV interface (Thermo
The laboratory standards used for this analysis were alanine B ($\delta^{13}C = -19.6\%_o$, $\delta^{15}N = 8.7\%_o$) and alanine C ($\delta^{13}C = -19.6\%_o$, $\delta^{15}N = 13.7\%_o$). The SDs of these standards were less than 0.1\%_o and 0.15\%_o for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Fig. 2. Photographs of vertebrae and vertebral centra after removal of the spongy bone of (A) sardine (S. melanostictus) and (B) Japanese flounder (P. olivaceus). Vertebral centra were subdivided into 10 sections (sardines) or 15 sections (Japanese flounder), by cutting along the broken lines.

Table 2. Parameters of logistic curves (Eq. 2 in the text) fitted to $\delta^{13}C$ and $\delta^{15}N$ values of vertebral sections of sardines (S. melanostictus) and Japanese flounder (P. olivaceus).

| Isotope | Fish ID | Fork length (mm) | Body weight (g) | $K$ | $N_0$ | $r$ | $b$ | TDF |
|---------|---------|-----------------|----------------|-----|-------|-----|-----|-----|
| $\delta^{13}C$ | SM-01 | 178 | 74 | — | — | — | — | 3.03 |
|          | SM-02 | 180 | 80 | 0.073 | 0.940 | 0.207 | 0.575 | 0.091 | 0.971 | — | — | — | 14.615 | 3.18 |
|          | SM-03 | 184 | 76 | 0.228 | 0.902 | 0.775 | 0.500 | 0.100 | 0.950 | — | — | — | 15.463 | 2.58 |
|          | SM-04 | 188 | 73 | — | — | — | — | — | — | — | — | — | 3.18 |
|          | SM-05 | 191 | 90 | 0.582 | 0.009 | 0.000 | 0.966 | 5.542 | 0.804 | — | — | — | 15.349 | 2.92 |
|          | SM-06 | 191 | 91 | — | — | — | — | — | — | — | — | — | 2.59 |
|          | PO-01 | 254 | 265 | 3.466 | 0.000 | 0.315 | 0.036 | 0.645 | 0.000 | — | — | — | 17.217 | 3.94 |
|          | PO-02 | 256 | 305 | 2.653 | 0.000 | 0.300 | 0.086 | 0.545 | 0.006 | — | — | — | 16.292 | 4.05 |
|          | PO-03 | 259 | 317 | 2.412 | 0.000 | 0.023 | 0.601 | 1.960 | 0.030 | — | — | — | 16.336 | 3.76 |
|          | PO-04 | 266 | 358 | 3.476 | 0.000 | 0.332 | 0.020 | 0.507 | 0.000 | — | — | — | 17.047 | 4.12 |
|          | PO-05 | 274 | 265 | 1.469 | 0.000 | 0.001 | 0.752 | 3.449 | 0.047 | — | — | — | 15.421 | 3.74 |
|          | PO-06 | 296 | 324 | 1.712 | 0.000 | 0.030 | 0.429 | 1.865 | 0.008 | — | — | — | 15.549 | 3.85 |
|          | PO-07 | 306 | 298 | 2.667 | 0.000 | 0.006 | 0.547 | 2.836 | 0.004 | — | — | — | 16.579 | 3.78 |
| $\delta^{15}N$ | SM-01 | 178 | 74 | 3.651 | 0.000 | 0.013 | 0.076 | 1.317 | 0.000 | 9.773 | 0.67 |
|          | SM-02 | 180 | 80 | 3.430 | 0.000 | 0.001 | 0.465 | 1.593 | 0.003 | 9.838 | 0.52 |
|          | SM-03 | 184 | 76 | 2.568 | 0.000 | 0.000 | 0.191 | 1.757 | 0.000 | 9.781 | 0.39 |
|          | SM-04 | 188 | 73 | 2.688 | 0.000 | 0.000 | 0.414 | 2.057 | 0.000 | 9.681 | 0.38 |
|          | SM-05 | 191 | 90 | 2.886 | 0.000 | 0.025 | 0.296 | 1.045 | 0.008 | 9.524 | 0.34 |
|          | SM-06 | 191 | 91 | 3.804 | 0.000 | 0.005 | 0.552 | 1.557 | 0.015 | 9.036 | 0.09 |
|          | PO-01 | 254 | 265 | 4.875 | 0.000 | 0.259 | 0.011 | 0.664 | 0.000 | 9.196 | 1.32 |
|          | PO-02 | 256 | 305 | 2.842 | 0.000 | 0.016 | 0.388 | 1.076 | 0.001 | 10.983 | 1.08 |
|          | PO-03 | 259 | 317 | 2.984 | 0.000 | 0.091 | 0.027 | 0.596 | 0.000 | 10.714 | 0.95 |
|          | PO-04 | 266 | 358 | 3.924 | 0.000 | 0.215 | 0.052 | 0.524 | 0.000 | 10.576 | 1.75 |
|          | PO-05 | 274 | 265 | 2.110 | 0.000 | 0.048 | 0.010 | 0.747 | 0.000 | 11.256 | 0.62 |
|          | PO-06 | 296 | 324 | 2.012 | 0.000 | 0.014 | 0.015 | 0.999 | 0.000 | 11.746 | 1.01 |
|          | PO-07 | 306 | 298 | 2.653 | 0.000 | 0.004 | 0.240 | 0.933 | 0.000 | 11.621 | 1.52 |

The differences in $\delta^{13}C$ and $\delta^{15}N$ values among vertebral sections were tested for significance by examining the overlap between 95% credible intervals; this method is more conservative than standard methods such as the $t$-test (Schenker and Gentleman 2001). We then fitted the $\delta^{13}C$ and $\delta^{15}N$ values of the vertebral sections of each fish to the following standard form of the logistic equation:

$$
\delta X_t = K / \{ 1 + [(K-N_0)/N_0] \cdot e^{-rt} \} + b, 
$$

where $\delta X_t$ is the $\delta^{13}C$ or $\delta^{15}N$ value of vertebral section $t$, $K$ is the asymptotic value of $\delta^{13}C$ or $\delta^{15}N$, $N_0$ is the $\delta^{13}C$ or $\delta^{15}N$ Fisher Scientific).
value at the beginning of the growth curve, and \( r \) is the rate of increase of \( \delta^{13}C \) or \( \delta^{15}N \). \( b \) is the minimum \( \delta^{13}C \) or \( \delta^{15}N \) value of the vertebral sections. We fitted the isotopic data from vertebral sections to the logistic model by using the R package “growthcurver” (Sprouffske and Wagner 2016). We then estimated the asymptotic \( \delta^{13}C \) or \( \delta^{15}N \) values for each fish and calculated TDFs by subtracting the \( \delta^{13}C \) and \( \delta^{15}N \) values of their foods from the asymptotic \( \delta^{13}C \) and \( \delta^{15}N \) values (+b).

Turnover time is the time required for the isotope ratio in a specific tissue to shift to match that of the diet through both growth of the tissue (growth-based turnover: Fry and Arnold 1982; Maruyama et al. 2001) and metabolic tissue replacement (replacement-based turnover: MacAvoy et al. 2001). When stable isotope ratios show a “flat” phase before a change in diet, the contribution of replacement-based turnover to the total turnover can be calculated by using the following equation:

\[
C = 1 - (K - N_0)/(\delta X_{Food2} - \delta X_{Food1}),
\]

where \( C \) is the contribution of replacement-based turnover to the total turnover (%), and \( \delta X_{Food1} \) and \( \delta X_{Food2} \) are \( \delta^{13}C \) or \( \delta^{15}N \) values of the fish diets before and after the change in diet, respectively.

All statistical analyses were conducted with R version 3.4.2 (R Core Team 2013). The reliabilities of the asymptotic values were evaluated on the basis of the \( p \) values for the coefficient \( (\alpha = 0.05, \text{Table 2}) \). When the data could not be fitted to the logistic model, we assumed that the isotopic values of vertebral sections had already reached equilibrium and the mean isotopic value was used as an asymptotic value.

**Results**

During the feeding experiment with sardines, the average fork length increased from 154 mm to 188 mm (Fig. 1). Body sizes of Japanese flounder were measured only after the dietary shift to Food F; fork length increased from 172 mm to 274 mm (Fig. 1). The \( \delta^{13}C \) and \( \delta^{15}N \) values of the different foods used in this study are presented in Table 1.

The \( C : N \) ratios of bone collagen from internal sections (numbers 1–3 for sardines and 1 and 2 for Japanese flounder) were sometimes outside the established range for pure bone collagen (2.9–3.6; DeNiro 1985). Therefore, data for these sections were excluded from the statistical analyses shown below (Supporting Information Table S2). Stable isotope ratios of the vertebral sections of sardines showed similar trends among individuals. The \( \delta^{15}N \) values were initially low in sections 4–6 and then increased up to > 11‰ in sections 8–10, whereas the \( \delta^{13}C \) values were almost the same among all vertebral sections (Fig. 3). Comparison of 95% credible intervals showed a significant difference in \( \delta^{15}N \) values between sections 8–10 and 4–6 (Fig. 3). Vertebral sections of Japanese flounder showed almost the same isotopic trends among individuals. In all fish, internal vertebral sections (numbers 3–4) had low \( \delta^{13}C \) values; the values rapidly increased from section 3 to section 6, and then slightly increased from sections 6 to 15 (Fig. 3). The \( \delta^{15}N \) values of vertebral sections were also low in section 3 but were almost constant until section 6. The \( \delta^{15}N \) values then increased from section 6 to section 10, and were almost constant outward of section 11 (Fig. 4; Table 2). Marginal sections (numbers 11–15) had \( \delta^{13}C \) and \( \delta^{15}N \) values significantly higher than those of sections 3–5 on the basis of the overlap of 95% credible intervals.

Fitted curves could not be successfully generated for the \( \delta^{13}C \) values of some sardines (SM-01, SM-04, and SM-06) and were not used for the calculation of TDFs; mean isotopic values of these vertebral sections were used instead (Table 2). The estimated TDFs for sardines were 5.58 ± 0.25‰ for \( \delta^{13}C \) and −0.70 ± 0.44‰ for \( \delta^{15}N \), and those for Japanese flounder were 3.89 ± 0.14‰ for \( \delta^{13}C \) and 1.18 ± 0.35‰ for \( \delta^{15}N \). We found a distinct flat phase before the feeding experiment only in the fitted curve of \( \delta^{15}N \) for Japanese flounder, and the mean (± SD) difference between \( K \) and \( N_0 \) was 3.0‰ (± 0.9‰). The maximum difference between \( \delta^{15}N \) values in pre- and post-experiment diets was 3.6‰ (Food C vs. Food E-2), and the contribution of replacement-based turnover to the total turnover was thereby estimated to be lower than 16.5% per 4 months.

**Discussion**

We found that the \( C : N \) ratios of bone collagen from vertebral sections near the centrum center (section numbers 1–3 for sardines and 1 and 2 for Japanese flounder) were sometimes outside the accepted range for \( C : N \) ratios (2.9–3.6). The atypical \( C : N \) ratios in some bone sections from the centrum centers might have been caused by contamination with non-collagenous organic matter, but the nature of the contaminant was unclear. Although some bone collagen from these sections had normal \( C : N \) ratios, we uniformly excluded these sections from the comparisons, because the \( C : N \) ratios of the problematic collagen showed both positive and negative deviations from the normal range (Supporting Information Table S2) and the influence of contaminants may not appear in the \( C : N \) ratios depending on the proportions of these contaminants.

Segmental isotope analysis of sardine vertebrae showed an overall trend of \( ^{15}N \) enrichment with increasing distance from the centrum center (Fig. 3). All samples of sardine foods (Foods A-1 and A-2) had similar \( \delta^{15}N \) values (Table 1); this suggests that there were no significant isotopic differences between the two batches of food. Wild sardines feed on phytoplankton and zooplankton (Kawasaki and Kumagai 1984), which generally have lower \( \delta^{15}N \) values than consumer species (e.g., Takai and Mishima 2002; Aita et al. 2011). Therefore, the observed isotopic increase in sardine vertebral sections should correctly reflect the dietary shift from plankton to artificial diet, and this result suggests that sardine vertebrae record temporal isotopic chronologies.
Both $\delta^{13}C$ and $\delta^{15}N$ of vertebral sections of Japanese flounder increased from the vertebral center to the margin. The foods for juvenile Japanese flounder (Foods B, C, and E-1) had $\delta^{13}C$ and $\delta^{15}N$ values lower than those of Food E-2. The lower $\delta^{13}C$ values of internal vertebral sections (numbers 1–4) of Japanese flounder might reflect the isotopic values of artificial foods with low $\delta^{13}C$ values, such as Food B, which was used as food during the juvenile stage. The isotopic shift in vertebral sections 5–10 was consistent with that of their diet (from Foods B, C, and E-1 to Food E-2), suggesting that the chronology of diet was recorded in the vertebrae of Japanese flounder.

We successfully constructed logistic models for the temporal shifts in isotopic values of vertebral sections, except for the $\delta^{13}C$ values of some sardines (Fig. 4A), which were almost constant across the vertebral sections of each individual, suggesting that the $\delta^{13}C$ values of these sardines were already at equilibrium by section No. 4, even though $\delta^{15}N$ was not yet in equilibrium (Fig. 4B). There are two potential explanations for

![Graph](image-url)
the difference in turnover times between the two elements: (1) the $\delta^{13}$C values of the pre- and post-experiment diets were almost the same, or (2) the turnover time of $\delta^{13}$C is far shorter than that of $\delta^{15}$N, as has been shown in other tissues (e.g., MacAvoy et al. 2001; Suzuki et al. 2005; Guelinckx et al. 2007). We cannot determine which explanation is correct, because we lack isotopic values for the pre-experiment diet of sardines. On the other hand, $\delta^{13}$C turnover times in Japanese flounder differed among individuals; this is a complex result and is difficult to interpret. Presumably, individual differences in growth, body size at the start of the feeding experiment, and feeding rate might have influenced the individual turnover times. Interpretation of the observed isotopic turnover in vertebral sections will require tracking of the growth patterns of individuals during a feeding experiment.

Our experiments successfully estimated TDFs for both $\delta^{13}$C and $\delta^{15}$N, although there is some uncertainty in $\delta^{13}$C turnover times, as mentioned above. Determining TDFs between tissue and diet is one of the most important steps toward interpreting organismal isotope data (e.g., Bond and Diamond 2011; Phillips et al. 2014). Nevertheless, few studies have reported the TDF of bone collagen in teleost fishes, probably because stable isotope analysis of bone is used exclusively in archaeological studies (e.g., Barrett et al. 2011; Nehlich et al. 2013), and also because of the long turnover time of bone collagen. We showed that SIAV can be used to estimate TDFs of bone collagen in relatively short feeding experiments (9 months and 4 months for sardines and Japanese flounder, respectively) because the method provides retrospective isotope values that can be used to predict asymptotic values by using the logistic model. The TDFs of bone collagen estimated in this study ($\delta^{13}$C: $5.58 \pm 0.25\%o$ for sardines, $3.89 \pm 0.14\%o$ for Japanese flounder; $\delta^{15}$N: $-0.70 \pm 0.44\%o$ for sardines, $1.18 \pm 0.35\%o$ for Japanese flounder) are higher for $\delta^{13}$C and lower for $\delta^{15}$N.
than those of fish muscle (1.81 ± 0.80‰ and 3.23 ± 1.34‰, respectively; Caut et al. 2009, Guo et al. 2016). This trend is consistent with previous studies, in which the bone collagen of vertebrates generally shows lower trophic enrichment of 15N than muscle, but higher 13C enrichment than in other tissues (e.g., Sholto-Douglas et al. 1991; O’Connell et al. 2001).

Such differences in TDFs among proteinaceous tissues likely result from differences in amino acid composition (Itahashi et al. 2014).

The greater differences between minimum and maximum δ15N along the vertebral axis (average, 3.0‰) than between Foods E-2 and B (1.7‰) suggests that the contribution of Food B to the δ15N values of Japanese flounder bones during the first period of the feeding experiment was low. On the other hand, the differences in δ15N values between Foods E-2 and E-1 (3.0‰) and between Foods E-2 and C (3.6‰) were similar to the increase in δ15N values along the vertebral axis, suggesting that these were the primary nutrient source of Japanese flounder during the first period. When the primary diet during the first period was Food E-1, the contribution of replacement-based turnover to the total turnover in bone collagen was estimated to be almost 0%, whereas it was estimated to be low (16.5% per 4 months) even when the primary diet was Food C. Although these estimates were made under the assumption that TDF was constant regardless of the isotopic value of the diet, some previous studies have shown that TDFs tend to decrease with increases in dietary δ15N values (e.g., Caut et al. 2009; Mohan et al. 2016). Such differences in TDF depending on diet could have resulted in overestimation of the contribution of replacement-based turnover in our experiments, because we changed from a diet with lower δ15N to one with higher δ15N, but it would not have influenced the estimated maximum contribution of replacement-based turnover. These results are consistent with previous studies in elasmobranch species that suggested a very small effect of metabolic replacement on the isotopic values of bone collagen (Campana et al. 2002; Ardizzone et al. 2006).

Generally, the fork length of fishes is highly correlated with the height of the vertebral. On the basis of the somatic growth of Japanese flounder during the second period (Supporting Information Table S1), we estimated the position in vertebral sections corresponding to the change in diet as 9.4 out of 15 sections. However, the onset of the isotopic shift within vertebral sections was clearly in sections deposited earlier than this (Fig. 4; the isotopic shift starts at about section numbers 4 or 5). This mismatch in timing with the dietary switch suggests that a bone section constructed during a certain period can move toward the center of a centrum over time or through the growth of the fish. Although our data suggest a minimal effect from replacement-based turnover in bone collagen, it is possible that the oldest bone is subject to resorption and younger sections move toward the center to fill in empty spaces. Such resorption of the oldest vertebral bone will critically influence the results of SIAV, especially when we attempt to reconstruct the isotope information in juvenile stages from the vertebrae of adult fish.

For this reason, care is recommended in the interpretation of isotope ratios in internal vertebral sections, and more empirical studies are needed, such as those using biological markers to investigate the stability of vertebral bone.

In this study, we have shown a way to estimate TDFs of bone collagen by using a combination of SIAV and feeding experiments shorter than 1 yr. Our results also account for the mismatch in turnover times of bone collagen among previous studies (i.e., faster turnover time in juvenile fish than adult fish; Schoeninger and DeNiro 1984; Sholto-Douglas et al. 1991; Gaston and Suthers 2004, Ankjæro et al. 2012), and they emphasize the importance of considering replacement-based and growth-based turnover separately when interpreting turnover times of bone collagen. This concept is also important when investigating turnover and TDFs of other soft tissues, because fish samples from different growth stages will show different contributions from growth-based turnover. Feeding experiments in juvenile fishes can be shorter than with adults; however, it should be noted that the TDFs might also change with the growth stages of fishes (Trueman et al. 2005).

We showed that the use of SIAV has high potential to increase the quality and quantity of information obtained from isotope analysis, and it can help to reconstruct retrospective changes in diet and migration of fishes; this is not possible with traditional isotope analysis using soft tissues. We also suggest that more empirical studies to support the applicability of the method—especially studies of the stability over time of vertebral sections in juvenile stages—will greatly improve the utility and reliability of SIAV.

**Ethics statement**

This study was approved by the Animal Care and Use Committee of the Faculty of Fisheries, Nagasaki University (permit numbers NF-0003 and NF-0004), in accordance with the Guidelines for Animal Experimentation of the Faculty of Fisheries (fish, amphibians, and invertebrates) and the Regulations of the Animal Care and Use Committee, Nagasaki University.

**Data availability statement**

All data used in this study are listed in the tables and Supporting Information.

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Conflict of Interest
None declared.

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