Incremental analysis of vertebral centra can reconstruct the stable isotope chronology of teleost fishes

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Abstract
1. Isotope analysis has high potential for understanding fish ecology and food-web structure in aquatic ecosystems. The utility of isotope analysis will be greatly improved if we can reconstruct the chronology of several isotopes at multiple growth stages of individual fish. However, no practical methods exist for reconstructing the chronology of light-element isotopes (e.g. δ13C, δ15N, δ34S, and Δ14C) in teleost fishes. Here, we present and test a new analytical approach for reconstructing the isotopic ratios of light isotopes at multiple life-stages in teleost fishes.

2. We sampled an anadromous salmon species, masu salmon Oncorhynchus masou (n = 3), along with water from its natal stream and from the ocean. We subdivided the vertebral centra of the salmon equally into 10 sections and extracted bone collagen from each sample. We then measured the stable sulphur isotope ratios of each vertebral section and compared them with δ34S values of the river water and sea water. We also measured the 87Sr/86Sr ratios of otoliths as a reference indicator of salmon migration between fresh water and the ocean.

3. In all samples, the bone section closest to the centre of the centrum had the lowest δ34S values, which were similar to those of fresh water. The δ34S values gradually increased from the centre to marginal sections, finally reaching constant values similar to those of seawater. The 87Sr/86Sr ratios of sagittal otolith sections had significant inter-individual differences and were consistent with the patterns of variation of the δ34S values of the vertebral sections.

4. Our results show that the vertebral centra of teleost fishes record isotopic information from juvenile to adult life stages. We suggest that our method can provide reproducible isotopic chronology, even in teleost fishes smaller than 50 cm. This method can be used in isoscape studies and in studies of the ecology of marine teleost fishes.

Keywords
87Sr/86Sr, anadromous migration record, fish migration history, masu salmon, multi-isotope chronology, otolith, sulphur stable isotopes

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Isotope analysis is a powerful tool in ecological studies of fishes for reconstructing their dietary sources, trophic positions, and movements. For example, isotope analysis has been used to quantify the dietary contributions of resources from different habitats ($\delta^{13}C$: McCutchan & Lewis, 2002; Shin & Mitamura, 2011; $\Delta^{14}C$: Ishikawa, Hyodo, & Tayasu, 2013; Ishikawa, Uchida, Shibata, & Tayasu, 2010), to measure the trophic positions of organisms ($\delta^{15}N$ in bulk tissues: Minagawa & Wada, 1984; $\delta^{15}N$ in amino acids: Chikaraishi et al., 2009) and to estimate migration history ($\delta^{18}O$: Devereux, 1967; Eldson & Gillanders, 2002; $^{87}Sr/^{86}Sr$: Kennedy, Klaue, Blum, Bolt, & Nislow, 2002). Isotope analysis thus has high potential to increase our understanding of the ecology of fishes and food web structures in aquatic ecosystems. Furthermore, the utility of isotope analysis would be greatly improved if we could reconstruct the history of these isotopes at multiple growth stages of individual fish.

One common method for reconstructing the isotopic chronology of teleost fishes is otolith analysis (Kerr & Campana, 2014). The otoliths of teleost fishes are composed mostly of calcium carbonate (Campana, 1999) and can provide accurate chronological isotopic records because they are not subject to metabolic activity and have high temporal stability (Campana & Thorrold, 2001). However, chronological isotopic analysis of otoliths is generally applicable to the isotopes of only a few elements ({$\delta^{13}C$, $\delta^{18}O$, and $^{87}Sr/^{86}Sr$}), and there are no practical methods for providing chronological information on the isotope ratios of other elements, such as $\Delta^{14}C$, $\delta^{15}N$, and $\delta^{34}S$, in teleost fishes. For elasmobranch species, on the other hand, isotopic analysis of collagen in vertebrae is commonly used to reconstruct annual isotopic records (e.g., Estrada, Rice, Natanson, & Skomal, 2006; Kerr, Andrews, Cailliet, Brown, & Coale, 2006). Although, unlike otoliths, the vertebrae may not have perfect chronological isotope information, $\Delta^{14}C$ analysis of elasmobranch vertebrae suggests that the vertebral centra of elasmobranch fishes record a certain degree of isotopic chronology (Ardizzone et al., 2006; Campana, Natanson, & Myklevell, 2002). However, this method has been applied exclusively to elasmobranch species because separating these bones into multiple sections and measuring stable isotope ratios in the sections are technically difficult because of the small size of most teleost fishes. Nevertheless, we expect that incremental isotope analysis of the vertebral centrum can be applied to teleost fishes if enough bone collagen can be collected from their vertebral sections to measure stable isotope values.

Here, we present a new experimental protocol for preparing and extracting collagen from the vertebra sections of teleost fishes, and we test whether bone collagen in the vertebral sections provides a chronological isotopic record of teleost fish. We use the stable sulphur isotope ($\delta^{34}S$) values from an anadromous salmon species, masu salmon, Oncorhynchus masou Brevoort, 1856. The $\delta^{34}S$ values are good for identifying terrestrial and marine signals because sea water has a markedly higher $\delta^{34}S$ value than freshwater (Thode, 1991). The $\delta^{34}S$ values of fresh water are determined by the type of bedrock in the region and show no seasonal variation (Rubenstein & Hobson, 2004). Marine sulphates have uniform $\delta^{34}S$ values (21.0‰; Rees, 1978), which are spatially and temporally homogeneous (Rubenstein & Hobson, 2004). Furthermore, $\delta^{34}S$ values in bone collagen are minimally affected by trophic enrichment because methionine, which is the dominant sulphur-bearing amino acid in fish bone collagen (Eastoe, 1957), is one of the essential amino acids for which there is little prey-predator isotopic discrimination (Chikaraishi et al., 2009). Therefore, there should be negligible trophic enrichment of stable sulphur isotopes in bone collagen (Barnes & Jennings, 2007; Peterson, Howarth, & Garritt, 1985), further increasing their usefulness as an accurate reflection of marine versus freshwater habitat use by fishes.

We also used the strontium isotope ratios ($^{87}Sr/^{86}Sr$) in otoliths of the salmon as a reference record of these fishes’ migrations between fresh water and the ocean. This ratio in otoliths has been used as a record of the migration of anadromous fishes between fresh water and the ocean (e.g., Kennedy et al., 2002; Padilla, Brown, & Wooller, 2015, 2016). This is because different water bodies have distinct $^{87}Sr/^{86}Sr$ values that are reflected in the $^{87}Sr/^{86}Sr$ in the otoliths: there is little to no biological fractionation from uptake to incorporation into the otoliths (Pouilly, Point, Sondag, Henry, & Santos, 2014; but see de Souza, Reynolds, Kickza, & Bourdon, 2010; Halicz, Segal, Fruchter, Stein, & Lazar, 2008).

Masu salmon are distributed in the Western Pacific Ocean off East Asia. They hatch in fresh water and generally stay there for about 1.5 years, after which some individuals migrate to the ocean where they spend another approximately 1.5 years before returning to their natal streams to spawn. Other individuals may stay in the river mouths for several months without completing their migration to the ocean, and then return to freshwater (Machidori & Kato, 1984). Masu salmon are about 11–14 cm fork length when they migrate to the ocean, and they reach about 35–70 cm at the spawning stage (Machidori & Kato, 1984). Therefore, if chronological records of the $\delta^{34}S$ values reflecting their freshwater and seawater habitat use remain in the vertebral centra, it should be possible to detect both freshwater and marine signals from the central and marginal sections, respectively, of the vertebrae, as well as from the $^{87}Sr/^{86}Sr$ values in otoliths.

2 | MATERIALS AND METHODS

2.1 | Study site and field sampling

Field sampling was conducted on the Churui River in the town of Shibetsu, in eastern Hokkaido, Japan (Figure 1). The mean annual temperature in Shibetsu is 6.2°C and the mean annual precipitation is 1204.0 mm (Japan Meteorological Agency, 2016). Anadromous salmonids in the Churui River include masu salmon, Oncorhynchus gorbuscha Walbaum, 1792, and chum salmon Oncorhynchus keta Walbaum, 1792. Masu salmon return to their natal streams from June to July, pink salmon from August to October, and chum salmon from September to November. Pink and chum salmon are artificially hatched and stocked, but masu salmon are not. Masu salmon in Hokkaido are known for their strong homing ability, even to tributary level (Miyakoshi et al., 2012). Masu salmon are, therefore, a suitable species for the investigation of natural isotopic signatures from fresh water to the ocean.
We sampled masu salmon, river water, and sea water on 12 and 13 July 2016. We collected three masu salmon (identified as OM-01, OM-02 and OM-03) from upstream in the Churui River (Figure 1); river-water samples were collected at the same point (upstream) and from the middle section of the Churui River (Figure 1). Sea water was sampled about 10 km south of the mouth of the Churui River (Figure 1) so that the sampling would not be influenced by river-water discharge. River-water samples were initially filtered through a 0.7-μm glass-fibre filter (GF/F, Whatman, Buckinghamshire, UK); we then added 1.0 M (mol L−1) HCL at 3 ml/L of filtered sample. We next added 10% (wt:vol) BaCl₂ solution at 15 ml/L and allowed the mixture to react for about 24 hr at ambient temperature. The precipitated BaSO₄ was then collected on a 0.7-μm membrane filter (C300A025A, Advantec, Tokyo, Japan) and dried for approximately 24 hr at 60°C in a drying oven. Because of the homogeneity of sulphur isotopes in sea water, we did not use the δ³⁴S value from our seawater samples; instead we used a representative δ³⁴S value for sea water of 21.0‰ (Rees, 1978).

2.2 | Otolith preparation and stable strontium isotope analysis

Sagittal otoliths extracted from skulls were sonicated in Milli-Q water (Millipore, Bedford, Massachusetts, USA) for 30 s and then freeze-dried. The otoliths were embedded in epoxy resin (SpeciFix Resin, Struers, Ballerup, Denmark) and polished to the core on polyester sheets coated with aluminium oxide powder in decreasing particle sizes (e.g. 500, 800 and 1,000 grit). Each sample was fixed to a frosted-glass slide with epoxy-based adhesive (Quick 30, Konishi, Osaka, Japan) and ground parallel to the slide surface with a grinding machine (Discoplan-TS, Struers, Ballerup, Denmark). The slides were sonicated in pure water for 30 s and freeze-dried. We then took photomicrographs of each thin section and counted the annuli on the surface of the otoliths (Figure S1). Finally, multiple samples were taken from the otoliths using a micro-drill (Micro Mill, Electro Scientific Industries, Portland, Oregon, USA), staring at the core and ending at the rim.

Sample scrapings were digested in 1 ml 3 M HNO₃ on a hotplate at 80°C for 6 hr. After sample digestion, 0.5 ml of the solution was diluted with 5 ml 1% HNO₃ for analysis of elemental concentrations of calcium and strontium by quadrupole inductively coupled plasma mass spectrometry (7500cx, Agilent Technologies, Waldbronn, Germany). Indium was added inline as an internal standard for drift correction. External standard curves were generated by using a properly diluted multi-elemental standard solution (XSTC-622, SPEX SertiPrep, Metuchen, New Jersey, USA). Concentrations of calcium and strontium were measured with a typical precision of ± 3% to 5%. All reagents used were of high purity, and the blank contribution was <1% of the analyte.

Strontium was separated from sample solutions by using columns filled with strontium resin (Eichrom Technologies Inc., Lisle, Illinois, USA), as follows. Samples re-dissolved in 0.3 ml 3.5 M HNO₃ were loaded onto the columns. The strontium fractions were then collected with 1.8 ml 0.05 M HNO₃ after elution of other elements using 0.5 ml 3.5 M HNO₃ and 0.5 ml 7 M HNO₃ in that order. Finally, the
$^{87}\text{Sr}/^{86}\text{Sr}$ ratios of the water samples (river water and sea water) and otolith sections were measured with thermal ionization mass spectrometry (Finnigan Triton, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

The measured strontium isotope ratios were normalized to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$. Repeated analyses of the National Institute of Standards and Technology (NIST) standard NIST SRM987 yielded an average $^{87}\text{Sr}/^{86}\text{Sr}$ of 0.710250 ± 0.0000013 (2σ) during the measurement of otolith samples and 0.710261 ± 0.000013 (2σ) (n = 10) during measurement of water samples. The strontium isotope ratios of samples were corrected to $^{87}\text{Sr}/^{86}\text{Sr} = 0.710250$ of NIST SRM987.

### 2.3 Bone segregation and collagen extraction

For stable sulphur isotope analysis, all vertebrae were first extracted from the fish body; any remaining muscle, spines and cartilage were removed from the vertebral centra by using scissors and a micro-grinder (Figure 2). Relatively small vertebrae from around the tail and head were excluded from further analysis. The thickness of each vertebral centrum was measured to the nearest 1 μm with a micrometer, and then each centrum was frozen at −20°C in Milli-Q water (MC-802A Electro Freeze, Yamato Kohki Industrial Co., Ltd., Saitama, Japan). Finally, we subdivided each centrum equally into 10 sections from the centre to the margin using a sliding microtome (REM-710 Retratome, Yamato Kohki.). We combined corresponding vertebral sections from all vertebral centra to obtain enough bone collagen to determine the δ34S values.

Bone collagen was extracted from each set of combined vertebral sections by using the general gelatinization method (Longin, 1971; Yoneda et al., 2004), with slight modifications. We first immersed the samples in a methanol:chloroform mixture (1:1, vol:vol) for approximately 6 hr. The samples were rinsed twice with 99.5% methanol and then the remaining solvent was allowed to completely evaporate at ambient temperature. Next, the samples were immersed for about 12 hr in 0.1 M NaOH and then washed twice with Milli-Q water. The samples were subsequently treated with 1.0 M HCl for about 12 hr, and then rinsed twice with Milli-Q water. To obtain a high collagen yield, we did not crush the vertebral sections into powder (Sealy, Johnson, Richards, & Nehlich, 2014). Finally, samples were heated in Milli-Q water at 90°C for about 12 hr and then freeze-dried.

### 2.4 Stable sulphur isotope analysis

Approximately 3 mg of bone collagen or 0.5 mg of BaSO$_4$ were put into tin capsules. We then measured the stable sulphur isotope ratios with a Delta V Plus mass spectrometer (Thermo Fisher Scientific) connected to a Flash EA 2000 elemental analyser (Thermo Fisher Scientific) at the Research Institute for Humanity and Nature (Kyoto, Japan). Stable isotope ratios are expressed in δ notation in accordance with the international standard scale, on the basis of the following equation:

$$\delta^{34}\text{S} = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1,$$

where $R_{\text{sample}}$ is the ratio of number of atoms ($^{34}\text{S}/^{32}\text{S}$) of the sample and $R_{\text{standard}}$ is that of Vienna Canyon Diablo Troilite. Values in the samples were corrected to the international scale with a two-point calibration by using International Atomic Energy Agency (IAEA) standards IAEA-S-1 and IAEA-S-2. The overall measurement error was estimated to be <0.5‰ for collagen and <0.1‰ for BaSO$_4$ samples.

### 3 RESULTS

Masu salmon OM-01, OM-02 and OM-03 had fork lengths of 400, 410 and 466 mm and body weights of 836.4, 1171.8 and 1259.2 g respectively. The mean (±SD) height of each vertebra was 2.313 ± 0.279 mm, 2.431 ± 0.232 mm and 2.527 ± 0.220 mm for OM-01, OM-02 and OM-03 respectively. Salmon ages were estimated to be 2, 3 and 3 years for OM-01, OM-02 and OM-03 respectively (Figure S1). The stable sulphur isotope ratios of river water were 13.1‰ upstream and 12.6‰ in the middle section (mean, 12.8‰). The difference between δ34S values in river water and ocean water (21.0‰) was 8.2‰. In all salmon samples, the bone section nearest to the centre of the vertebra (section No. 1) had the lowest δ34S values (13.1‰ ± 0.5‰). The δ34S values gradually increased from the centre to marginal sections, finally reaching almost constant values after vertebral section No. 6 (18.8‰ ± 0.4‰). These increases were constant for OM-02 and OM-03, but the values in OM-01 decreased once from section No. 3 to No. 4 and then resumed increasing (Figure 3). The mean difference between the maximum and minimum δ34S values of the bone sections was 5.9‰ ± 0.6‰, which corresponds to 73% of the difference between δ34S values in river water and sea water.

The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in the Churui River water was 0.7044 upstream and 0.7050 in the middle section. The $^{87}\text{Sr}/^{86}\text{Sr}$ of seawater was 0.7092. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of all otolith sections (0.7046–0.7092) fell between the values for river water and sea water. We observed systematic changes in the value with distance from the core. All individuals had $^{87}\text{Sr}/^{86}\text{Sr}$ values of about 0.707 at the 200-μm distance from
the core. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of otoliths of OM-02 and OM-03 then decreased outward to a value at 400–500 μm from the core of about 0.7050, which coincides with that of Churui River water. From the middle section (400–500 μm) toward the marginal sections (>900 μm) of otoliths from OM-02 and OM-03, $^{87}\text{Sr}/^{86}\text{Sr}$ ratios increased dramatically with values reaching as high as 0.7091, which is almost equivalent to the value for sea water (Figure 4). The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of the otolith from OM-01 increased once to about 0.7083 at 354 μm from the core, and decreased outward again to 0.7074 at 558 μm from the core. Otolith sections of OM-01 more than 800 μm from the core had high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, as in OM-02 and OM-03 (Figure 4). Sr/Ca ratios (μg/L:mg/L$^{-1}$) of otolith sections showed a pattern of variation similar to that of the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (Figure S2).

4 | DISCUSSION

Sulphur in bone collagen and strontium in otoliths follow different physiological pathways, and therefore these elements have different origins and biological turnovers. Stable sulphur isotope ratios of fish tissues, including bone collagen, are influenced by diet rather than ambient water (Hesslein, Hallard, & Ramial, 1993; Nehlich, Barrett, & Richards, 2013) because methionine—the major sulphur-containing amino acid in bone collagen—is one of the essential amino acids, and most vertebrates cannot synthesize methionine. On the other hand, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in otoliths of marine fishes is directly determined by that of ambient water and is not influenced by diet (Berg, 1968; Farrell & Campana, 1996; Walther & Thorrold, 2006). The importance of diet and ambient water to $^{87}\text{Sr}/^{86}\text{Sr}$ ratios in otoliths of freshwater fishes has not yet been determined.

The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in otoliths, which is known to record the migration history of fishes (e.g. Kennedy et al., 2002; Muhlfeld, Thorrold, McMahon, & Marotz, 2012; Padilla et al., 2015), shows a trend concordant with the $\delta^{34}\text{S}$ of vertebrae. The lowest $^{87}\text{Sr}/^{86}\text{Sr}$ values, observed 400–500 μm from the core of otoliths from salmon OM-02 and OM-03, were comparable to the value for the ambient river water, strongly suggesting that the natal river of these fish was the Churui River. The otoliths of all three fish sampled shared $^{87}\text{Sr}/^{86}\text{Sr}$ values of about 0.707 at their cores. This is possibly due to the considerable contribution of maternal strontium to the development of otoliths in juvenile fish (Volk, Blakley, Schroder, & Kuehner, 2000). The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of the otolith sections from OM-01 did not decrease to the value of the river water; we interpret this to indicate that fish OM-01 migrated to the sea before all maternal strontium had been eliminated from its body. The unexpected decrease of $^{87}\text{Sr}/^{86}\text{Sr}$ at 558 μm from the core of the OM-01 otolith suggests that this fish returned to the river once and then went back out to sea. Such multiple returns to fresh water by masu salmon have not been reported previously but are possible given the flexible migratory pattern of this species (Machidori & Kato, 1984).

Another possible cause of the temporal increase of $^{87}\text{Sr}/^{86}\text{Sr}$ in the otolith of OM-01 is the dietary marine subsidy in freshwater. The marine subsidy potentially consumed by juvenile masu salmon in fresh

**FIGURE 3** Stable sulfur isotope ratios ($\delta^{34}\text{S}; \%$) of vertebral sections from masu salmon ($n = 3$; identified as OM-01, OM-02 and OM-03) and water collected from the Churui River. A representative $\delta^{34}\text{S}$ value for marine sulphates (21.0‰) is used as an index of seawater. Vertebral bone section numbers (abscissa) start at the centre of the vertebral centrum and increase toward the margin.

**FIGURE 4** $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of fish otolith sections from masu salmon ($n = 3$; identified as OM-01, OM-02 and OM-03) collected from the Churui River, water from the Churui River, and sea water collected approximately 10 km south of the mouth of the Churui River. Error bars represent the 95% confidence intervals for each sample.
water is the eggs of anadromous salmon such as chum, pink and masu salmon, given that juvenile salmon sometimes consume the eggs of spawning salmon (Reed, 1967). In either case, it is not clear why only OM-01 showed a different isotopic pattern, and more field surveys are necessary to reveal the specific reason.

The observed variations in $\delta^{34}S$ values of vertebral centra are explained in large part by the differences between $\delta^{34}S$ values in fresh water and sea water. Stable sulphur isotope ratios in vertebral centra showed a freshwater signal in the central sections (Nos. 1 and 2) and $\delta^{34}S$ values similar to those of sea water in marginal sections. Because the centre is the oldest part of the vertebra, this change in $\delta^{34}S$ is interpreted to reflect the migration of salmon from the river to the sea. However, there were still differences in $\delta^{34}S$ values between the marginal sections of vertebrae and sea water (about 3.1‰); these differences are likely attributable to sulphur isotopic discrimination between water and bone collagen.

Assimilatory reduction of sulphate by plants and animals in aquatic environments often results in isotopic discrimination in $\delta^{34}S$ values from +0.5‰ to ~4.4‰ in organisms, compared with the values in water (e.g. Kaplan, Emery, & Rittenberg, 1963; Mekhtiyeva & Pankina, 1968). On the other hand, given that the $\delta^{34}S$ values of bone collagen were 3.1‰ below those of ambient water, the $\delta^{34}S$ values of vertebral section No. 1 were not consistent with those of river water (Figure 3). There are several possible reasons for this discrepancy: for example, the turnover of bone collagen could have increased the $\delta^{34}S$ of older vertebral sections, as could the incorporation of proteins from egg yolk. Although we cannot confirm these explanations, an isotopic signal from marine-derived nutrients in the $^{87}Sr/^{86}Sr$ ratios of core sections of otoliths could partially substantiate the latter explanation (Figure 4). In contrast, the turnover of bone collagen presumably had little effect on our results because the $\delta^{34}S$ values for the oldest vertebral section (No. 1) were almost the same for all three salmon sampled, despite the difference in age between OM-01 and the other two.

Although, we found a positive relationship between the $^{87}Sr/^{86}Sr$ ratios of the otoliths and $\delta^{34}S$ values of the vertebral sections, there were substantial differences in the timing of increases and decreases in these values between bone and otolith. In the profiles of the $^{87}Sr/^{86}Sr$ ratios in otoliths, only OM-01 had a peak in $^{87}Sr/^{86}Sr$ ratios at an early age (Figure 4). However, the plot of $\delta^{34}S$ values of vertebral sections shows the $\delta^{34}S$ values of all fish starting to increase at the same time (from sections No. 2 to No. 3; Figure 3). It is likely that this mismatch results from differences between growth mechanisms in vertebrae and otoliths. The growth of vertebral bones is generally considered to parallel growth in total body size (Campana & Thorrold, 2001). In contrast, otoliths often grow even when somatic growth is very slow or completely stopped (e.g. Campana & Thorrold, 2001; Maillet & Checkley, 1990; Reznick, Lindbeck, & Bryga, 1989).

Presumably, all salmon in our study rapidly increased in body size during their early life stage; specimen OM-01 then immediately migrated to the ocean, whereas the other two specimens stayed in the river for a substantial amount of time. At higher latitudes, growth rates of anadromous salmon are typically higher in the ocean than in freshwater (Gross, 1987; Vallestad, Peterson, & Quinn, 2004). Therefore, despite the comparatively short residence time of OM-01 in freshwater, the body and vertebral growth patterns of all three fish specimens might have been almost the same, regardless of the time they spent in the river. However, their otoliths would have kept growing even during the period of freshwater residence with slower somatic growth, thereby resulting in different patterns of variation between the $^{87}Sr/^{86}Sr$ ratios of the otoliths and the $\delta^{34}S$ values of the vertebral sections. We therefore suggest that both the vertebral centroid and the otolith contain chronological isotopic information, but that the time resolutions obtained from these tissues differ: during the juvenile stage, analysis of the vertebrae yields a lower time resolution than otolith analysis.

The three fish sampled in this study had vertebrae of similar size but different ages and growth rates, and these differences likely influenced the isotopic patterns in the vertebral sections and otoliths. For example, OM-01 was aged 2 years, whereas the other fish were 3 years old, and therefore sub-samples from their vertebrae would reflect different time scales. Although such inter-individual differences are generally important in comparing isotopic patterns among individuals, they do not affect our conclusion that the freshwater signal was detected from the vertebral sections of all adult masu salmon sampled. However, the age and growth rate of fish are critically important when using segmental analysis of vertebrae to compare multiple fish. The utility of segmental isotopic analysis of vertebrae would be maximized if it were used together with age determination by using vertebral annuli, although the age estimate would be less accurate than that estimated from otolith annuli (Gunn et al., 2008).

To date, there has been only one study that has applied stable carbon and nitrogen isotope analysis of dorsal spines to a large teleost fish, marlin *Kajikia audax* Philippi, 1887 (Acosta-Pachón, Ortega-García, & Graham, 2015). However, no studies have tested the validity of reconstructing chronological isotopic information from the bones of teleost fishes. Although there have been several attempts to reconstruct isotopic chronology by incremental analysis of fish scales, this is likely to be inapplicable to some fish species because scales have a complex structure in which new collagen layers overlie older ones (Hutchinson & Truean, 2006), making it difficult to separate them into multiple sections that are chronologically distinct. Therefore, to the best of our knowledge, ours is the first study to illustrate that the incremental analysis of vertebral centra can be applied to teleost fishes as it has been to elasmobranches. The minimum necessary quantities of bone collagen depend on the purpose of the experiment (e.g. target isotopes, size and number of vertebrae, and life stage of interest in each study). For example, it is particularly difficult in stable sulphur isotope analysis to reconstruct isotopic chronology with high resolution by incremental analysis of vertebrae because this analysis requires large amounts of bone collagen. However, stable carbon and nitrogen isotope analyses of bone collagen are commonly used for tracking the foraging ecology and habitat use of marine vertebrates (e.g. Hesselein et al., 1993; Schoeninger & DeNiro, 1984; Tomasewicz et al., 2017) and require less collagen than stable sulphur isotope analysis (Matsubayashi et al., 2015; Tomasewicz, Calandra, Seminoff, Avens,
& Kurle, 2016). Therefore, it may be more practical to chronologically reconstruct teleost fish habitat use with incremental δ^{13}C and δ^{15}N analysis of sequential vertebral sections using the method we outline here. We believe that our method is applicable to a broad range of fish species and isotopes. However, to ensure the utility of the method it is important to conduct more validation studies, ideally based on feeding experiments, which involve other species and isotopes.

In conclusion, our method provides a useful tool not only for investigating the ecology of teleost fishes, but also for the study of isoscapes (Bowen, 2010) in the ocean. Isoscape studies generally focus on the natural variation of isotopic values in animal habitats and aim to reconstruct the migration history of target species. In particular, the stable isotope ratios of strontium in otoliths have been important indicators of the migration history of teleost fishes in freshwater ecosystems (Hegg, Kennedy, & Fremier, 2013; Hobbs, Lewis, Ikemiyagi, Sommer, & Baxter, 2010). However, stable strontium isotope ratios are homogeneous in the ocean and are therefore not effective tracers for migrations of marine teleost fishes. Significant spatial variation has been reported for the isotopes of several light elements in the ocean (δ^{13}C and δ^{15}N: McMahon, Hamady, & Thorrold, 2013; Δ^{14}C: Bayliss, Marshall, & Sidell, 2004; Kumamoto, Murata, Saito, Honda, & Kusakabe, 2002). Therefore, our method, which demonstrates the reconstruction of habitat use by teleost fishes by analysing chronologically incorporated δ^{34}S values in fish vertebrae, could be applied to the use of other stable isotopes in marine isoscape and ecosystem studies.

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AUTHORS’ CONTRIBUTIONS

J.M. and I.T. conceived the study, and J.M., Y.S. and Y.O. collected samples of water and salmon. J.H. and T.S. helped to analyse salmon samples, and J.M., Y.U. and Y.S. prepared and performed the chemical analyses of fish vertebrae and otoliths. All authors contributed to the writing of this manuscript.

DATA ACCESSIBILITY

The δ^{34}S values of water samples and fish bone collagen and δ^{87}Sr/δ^{86}Sr values of water samples and fish otoliths are deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.r6p27 (Matsubayashi et al., 2017).

CONFLICT OF INTERESTS

The authors have no conflict of interests.

ETHICS STATEMENT

Capture of masu salmon was officially approved by the Fishing Management Division, Bureau of Fisheries, Department of Fisheries and Forestry, Hokkaido Government (approval No. 120, 28 June 2016).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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