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Calcium and strontium stable isotopes reveal similar behaviors of essential Ca and nonessential Sr in stream food webs

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Abstract

Recent studies showed the potential of stable isotopes of the macronutrient calcium ($\delta^{44/40}$ Ca) and nonessential strontium ($\delta^{88/86}$ Sr) as new trophic level indicators in terrestrial vertebrates and marine teleost fishes. In this study, we tested whether similar Ca and Sr isotopic fractionation trends existed in macroinvertebrate-dominated stream food webs compared to vertebrates despite their physiological differences. We have determined the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values as well as the 87 Sr/ 86 Sr ratios of stream macroinvertebrates and small gobies and their potential metal sources (stream water, periphyton, and terrestrial plant litter) in upper and lower reaches of two streams in the Lake Biwa catchment, central Japan. The ⁸⁷Sr/⁸⁶Sr ratios revealed that stonefly nymphs, crustacea, and gobies mostly relied on aquatic Sr sources. Higher ⁸⁷Sr/⁸⁶Sr ratios of some crane fly and caddisfly larvae, mayfly, dobsonfly, and dragonfly nymphs indicated greater terrestrial contributions via plant litter. Positive correlations between the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values implied that similar Ca and Sr sources existed, and that Ca and Sr stable isotopes underwent similar fractionation trends although Sr was not essential. The $\delta^{44/40}$ Ca and partly the $\delta^{88/86}$ Sr values were positively correlated with Sr/Ca ratios and negatively with δ^{15} N values indicating trophic effects on Ca and Sr stable isotopes. The enrichment of ⁴⁴Ca and ⁸⁸Sr in large filter-feeding caddisfly larvae was a notable exception from these trophic trends. Our data confirm that the trophic ⁴⁴Ca and ⁸⁸Sr depletion observed for marine teleost fishes and terrestrial vertebrates also applied to macroinvertebrate-dominated stream food webs despite their different physiologies indicating that shared mechanisms of Ca and Sr isotopic fractionation may exist at the cellular or molecular level between these taxa.

K E Y W O R D S

calcium, feeding habit, goby, macroinvertebrate, nontraditional isotope, stream, strontium

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INTRODUCTION

Calcium (Ca) is regarded as a macronutrient in most forms of life, while strontium (Sr), another alkaline earth metal, is not (yet) considered essential in biological systems (Maret, 2016). In mineralized tissues of vertebrates, Sr can substitute Ca in small extents, but Sr is biologically purified along food chains (Balter, 2004; Burton et al., 1999; Elias et al., 1982). Less information exists on the behavior of Ca and Sr in stream food webs, which are typically dominated by aquatic macroinvertebrates (Wallace & Webster, 1996).

Aquatic macroinvertebrates can be classified based on their feeding habits. Typical specialists are algae-grazers such as mayfly nymphs (Heptageniidae), leaf-shredders (e.g., some crane fly larvae-Tipulidae), and strict predators (e.g., dragonfly nymphs-Odonata), while predatory generalist may shift their diet during maturing (e.g., stonefly nymphs—Plecoptera) (Cummins & Klug, 1979). Aquatic macroinvertebrates take up and accumulate metals whether essential or not (Goodvear & McNeill, 1999; Rainbow, 2007). Metal uptake directly from the water is possible via specific cells (referred to as chloride cells) located on the skin and the tracheal gills (e.g., Ephemeroptera, Plecoptera), on the hindgut (e.g., Odonata), via chloride epithelia, or via anal papillae (e.g., Trichoptera, Diptera) (Komnick, 1977; Nowghani et al., 2017). Furthermore, metal uptake occurs from the diet with absorption possible in the midgut epithelium (Dow, 1986; Huang et al., 2015). Earlier research found Ca uptake from the water varied 70-fold across 12 species of the mayfly Ephemerellidae and of the caddisfly Hydropsychidae (Poteat & Buchwalter, 2014) demonstrating relative Ca contributions from the water and the diet differ strongly across taxa. Calcium is crucial in living animals, and in particular in regulating aquatic insect homeostasis as Ca is a vital messenger in all cells and an important structural element (Dow, 2017). Aquatic insects lack calcified structures, and Ca levels in their chitinous exoskeleton are low compared to the Ca levels in hardened body parts of crustacea (Griffith, 2017). In the hemolymph, most Ca^{2+} is bound to organic molecules (Griffith, 2017). Molting of the exoskeleton represents a major Ca loss, and minor Ca can also be excreted with the Malpighian tubules (Taylor, 1987).

Nontraditional stable isotopes of Ca (here referred to as $\delta^{44/40}$ Ca) have previously been analyzed in both terrestrial and marine vertebrates (e.g., Clementz et al., 2003; Martin et al., 2015, 2018; Skulan et al., 1997; Skulan & DePaolo, 1999). These studies found a significant decrease in $\delta^{44/40}$ Ca values in mineralized tissues, such as bone and tooth enamel, compared to dietary sources with increasing trophic level. For vertebrates, it is assumed that this ⁴⁴Ca depletion along the trophic chain is mainly driven by isotopic fractionation during bone mineralization from the blood and by kidney regulation of the Ca cycle (Channon et al., 2015; Dodat et al., 2021; Hassler et al., 2021; Heuser et al., 2016; Reynard et al., 2010; Skulan et al., 2007; Skulan & DePaolo, 1999; Tacail et al., 2014, 2020). Furthermore, direct uptake of Ca from the water through gills could buffer the diet-bone Ca isotopic fractionation as suggested for marine teleost and elasmobranch fish species (Clementz et al., 2003; Martin et al., 2015; Tacail et al., 2020). Similarly, a significant decrease in Sr stable isotope ratios ($\delta^{88/86}$ Sr) in mineralized tissues compared to dietary sources with increasing trophic level was found (Knudson et al., 2010; Lewis et al., 2017) indicating similar isotope fractionation trends between Ca and Sr exist in vertebrates. Whether these Ca and Sr isotopic fractionation trends also apply to aquatic macroinvertebrates is not known. Aquatic macroinvertebrates are fundamentally different from vertebrates as they lack bone bioapatite, typical renal function, and gill osmoregulation. Instead, Ca and Sr isotopic fractionation could occur during the osmoregulation and excretion functions in the gut. Malpighian tubules, and rectum (Komnick, 1977). Furthermore, isotopic fractionation could be suspected during the formation and the molting of the exoskeleton and during absorption of Ca from the diet in the midgut epithelium.

With respect to sources, plants preferentially take up the light Ca and Sr isotopes, and significantly lower $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values were observed for plant leaves relative to those of stream water (Cenki-Tok et al., 2009; Holmden & Bélanger, 2010; Souza et al., 2010). Traditionally, ⁸⁷Sr/⁸⁶Sr ratios are a powerful tool in catchment studies that allow for tracking the biogeochemical cycling of Ca and Sr (Capo et al., 1998). The ⁸⁷Sr/⁸⁶Sr ratios of the bioavailable Sr to the plants and of the dissolved Sr in stream water in a given region reflect the ⁸⁷Sr/⁸⁶Sr ratios of the weighted contributions of bedrock chemical leachates in that region (Knudson et al., 2010; Ohta et al., 2018). Thus, while Ca and Sr stable isotopes could allow for assessing trophic relationships in stream food webs, ⁸⁷Sr/⁸⁶Sr ratios can be used to discern between terrestrial (plant-derived material) and aquatic (stream water, algae) Ca and Sr sources and to monitor temporal variations in those.

In this study, we determined the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values as well as the 87 Sr/ 86 Sr ratios of stream macroinvertebrates and small gobies and their potential metal sources (stream water, periphyton, and plant litter) in two streams in the Lake Biwa catchment, Japan. Upper and lower reaches were sampled to account for potential human impacts as upper reaches were pristine and dominated by forests, while residential areas and farmland were present in lower reaches. Trophic positions of aquatic macroinvertebrates and small gobies in these streams have been previously determined based on the stable nitrogen

isotopic compositions of individual amino acids (Ishikawa et al., 2014). The main goal of this study was to test whether similar Ca and Sr isotopic fractionation trends existed in macroinvertebrate-dominated stream food webs compared to vertebrates despite their physiological differences. Furthermore, we aimed for understanding trophic effects by linking Ca and Sr stable isotopes to nitrogen stable isotopes (δ^{15} N) and to Sr/Ca ratios, while we use 87 Sr/ 86 Sr ratios to assess seasonal variabilities of metal sources.

MATERIALS AND METHODS

Study site

The upper and lower reaches of Yasu River and Ado River in the Lake Biwa catchment, central Japan, were sampled in May 2018 and November 2018 (Table 1). Cypress (Cupressaceae), beeches, and oaks (Fagaceae) typically dominate the riparian areas (Ishikawa et al., 2014). Jurassic mudstone and sandstone (59%), as well as Late Cretaceous granite (41%), dominate the upper reaches of the Yasu River, while chert, sandstone, and shale of the Jurassic Tanba Group are the typical lithology in upper reaches of the Ado River (Seamless Digital Geological Map, Geological Survey of Japan, 2015). Pliocene and Pleistocene unconsolidated siliciclastic sediments of the Kobiwako Group and Quaternary sediments are present in catchments of lower reaches. Sedimentary rock cobbles from the Tanba Group remain present in stream beds of lower reaches.

Sampling

One hundred milliliters of stream water were filtered $(0.20 \ \mu m \ cellulose \ acetate \ filter; DISMIC 25CS020AS, Advantec, Tokyo, Japan) and cooled before further analyses. Aquatic macroinvertebrates and small gobies were collected using hand nets by washing cobbles. Terrestrial leaf litter from the most abundant species was collected from litter packs within the streams. Periphytic algae$

attached to rock cobbles (hereafter: periphyton) were removed from several randomly chosen submerged cobbles using a nylon brush and subsequently rinsing cobble surfaces with distilled water (DW). The slurry from the individual cobbles was mixed and transferred into 100 ml polypropylene bottles. Rock cobbles with no or minimal alteration were sampled from stream beds. The collected rock cobbles represented mudrocks, sandstones, and granites.

Sample preparation

The samples used in this study are the same as those in our previous study (Nitzsche et al., 2020). Terrestrial leaf litter was oven-dried at 65°C for 48 h after rinsing with DW to remove mineral particles. Three leaves of each species were ground with a multibeads shocker (Yasui Kikai Corporation, Japan). Plant litter was absent at lower reaches in May except for Quercus myrsinaefolia (oak). In November, Sorbus alnifolia (Korean mountain ash) was the main plant litter in Upper Ado, while Euptelea polyandra (a Japanese endemic deciduous tree species) largely dominated in Upper Yasu. The periphyton slurry was freeze-dried after decanting with DW to remove mineral particles. The dried periphyton samples were examined under the binocular and visible mineral particles, small larvae of aquatic insects and plant residues were removed. Aquatic macroinvertebrates were classified based on the species, genus, or family levels and categorized according to feeding habits based on the observations of Takemon (2005): grazer (GR), shredder (SH), filter-feeder (FF), collector-gatherer (CG), omnivore (O), predator (PR). The samples included larvae of caddisfly (Hydropsychidae spp. [FF], Stenopsyche marmorata [FF], Rhyacophilidae spp. [PR]) and crane fly (Tipulidae, not further classified [SH]), and nymphs of mayfly (Baetis spp. [GR], Heptageniidae spp. [GR]), stonefly (Kamimuria spp. [PR], Oyamia spp. [PR]), dragonfly (Gomphidae spp. [PR]), and dobsonfly (Protohermes grandis [PR]), as well as small freshwater crab (Geothelphusa dehaani [CG]) and shrimps (Atyidae spp. [O]). Hereafter, small freshwater crab and shrimp are referred to as crustacea. Collected consumers were previously

TABLE 1 Sampling locations, watershed area, and elevation of the studied streams

Stream	Location	Latitude	Longitude	Watershed area ^a (km ²)	Elevation (m)
Ado	Upper	35°12′12.0″N	135°51′30.7″E	25.4	417
	Lower	35°21′00.0″N	136°00′02.0″E	298.5	109
Yasu	Upper	35°00′04.8″N	136°23′32.0″E	4.2	503
	Lower	34° 59' 04.0" N	136°07′15.0″E	294.7	147

^aFrom Ishikawa et al. (2014).

kept in stream water in the laboratory for 24 h to purge their gut contents as far as possible prior to analysis. While Solà and Prat (2006) showed Hydropsychidae cleared its gut within 24 h, this time might be insufficient for other taxa. Larvae of crane fly, large caddisfly (S. marmorata), dobsonfly, and dragonfly were additionally dissected to remove gut contents that could still be present. Mayfly nymphs and small caddisfly nymphs sometimes died within the 24-h purging phase. Shells from crustacea, bones and muscles of freshwater gobies (Cottus pollux [PR], Rhinogobius kurodai [PR/CG]), and one chub (Rhynchocypris oxycephalus jouyi [PR]) were obtained by dissection. All specimens were freeze-dried for 48 h and subsequently weighed to obtain dry weight (dw). Several individuals of mayflies, stoneflies, and caddisflies, respectively, were pooled to obtain 2-100 mg dw for further analysis. The analysis of larvae of crane fly, dobsonfly, and dragonfly was conducted on single individuals. Specimens were ground into a powder and the ground samples were digested with concentrated HNO₃ and H_2O_2 . Several drops of concentrated HF were added to digest silicacontaining phytates in the plant litter samples. The periphyton samples were digested in 10% H₂O₂ for 48 h to avoid the leaching of metals from clay minerals by HNO₃. The periphyton samples were centrifuged at 2610g for 10 min, and the supernatant was extracted. The remaining periphyton pellet was washed with DW, centrifuged, and the supernatant mixed with the supernatant from the first centrifugation step. This washing step was repeated one more time. The combined supernatant was evaporated and redissolved in 1 M HNO₃. Rock samples were crushed with a jaw crusher (HERZOG, Germany) and about 1 cm³ of fresh pieces were ground into a fine powder with the multibeads shocker. The rock powder samples were digested with HF and HClO₄ following the protocol of Yokoyama et al. (1999).

Chemical and isotopic analyses

The N stable isotopes of solid subsamples of biological samples have been measured using an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, Bremen, Germany) connected to an elemental analyzer (Flash 1112, Thermo Fisher Scientific) at the Research Institute for Humanity and Nature (RIHN) in Kyoto, Japan. Nitrogen isotopic values are expressed in delta notation (‰) relative to N₂ in air. Isotopic calibration was to CERKU-01 (DL-alanine), CERKU-02 (L-alanine), and CERKU-03 (glycine) (Tayasu et al., 2011). Analysis of the internal laboratory standards ensured that the estimates of the isotopic values were precise to within 0.07‰ for δ^{15} N.

Aliquots of digested samples and stream water were analyzed for elemental concentrations using a quadrupole inductively coupled plasma mass spectrometry (7500cx; Agilent Technologies, Tokyo, Japan).

The Sr isotope measurement corresponds to the method described in Wakaki et al. (2017). This method involves the separate Sr isotope measurement of the natural sample for determining the ⁸⁷Sr/⁸⁶Sr and of both natural and spiked samples for determining $\delta^{88/86}$ Sr. Prior to the Sr isotope measurement, Sr was separated from sample solutions using columns filled with Sr spec resin (Eichrom Technologies, Darien, IL). For the Sr isotope measurement of natural samples, aliquots containing up to 350 ng Sr were evaporated, redissolved in 0.3 ml of 3 M HNO₃, and loaded onto the columns. For the Sr isotope measurement of spiked samples, aliquots were first mixed with aliquots of a ⁸⁴Sr-⁸⁶Sr of double-spike to achieve a Sr spike/sample ratio of 47:53. The spiked sample solution was dried, redissolved in 0.3 ml of 3 M HNO₃, and loaded onto the columns. Major cations (e.g., K, Na, Ca, etc.) were washed out from the column with 2 ml of 6 M HNO₃ followed by 0.5 ml of 3 M HNO₃. Subsequently, Sr was collected with 2 ml of 0.05 M HNO₃. The pure Sr aliquots were dried, redissolved in one drop of concentrated HNO₃, and dried again to oxidize organics from the column. A sample containing 20-350 ng Sr was loaded on a single W filament with a Ta oxide activator. The Sr isotope measurement was performed on a Thermal Ionization Mass Spectrometer (TIMS; Finnigan Triton, Thermo Fisher Scientific) at Kochi Core Center. The Sr isotopes were measured with the static multicollection mode using five faraday cups connected to amplifiers with 10¹¹ Ω: ⁸⁴Sr(L1), ⁸⁵Rb(C), ⁸⁶Sr (H1), 87 Sr + 87 Rb(H2), and 88 Sr(H3). For samples containing >270 ng Sr, the ⁸⁸Sr ion beam intensity was kept at 12 and 4 V during the natural and spiked runs, respectively. A single measurement consisted of 400 cycles (20 cycles \times 20 blocks) of 16 s integration of the ion signal. Samples containing <270 ng Sr were measured manually with sometimes lower ion beam intensities and cycle numbers. The measured ⁸⁷Sr/⁸⁶Sr ratios of natural samples were normalized to ${}^{86}\text{Sr}/{}^{88}\text{Sr} = 0.1194$ (Steiger & Jäger, 1977). The small systematic bias of ⁸⁷Sr/⁸⁶Sr ratios between analytical sessions was corrected by using ${}^{87}\text{Sr}/{}^{86}\text{Sr} = 0.710248$ for NIST SRM 987 (McArthur et al., 2001). Data processing of the double-spike runs was carried out by a data reduction scheme that consists of a matrix approach and iterative calculations (for details see Wakaki et al., 2017). The ⁸⁸Sr/⁸⁶Sr ratios were expressed in delta notation (in ‰ units) relative to the NIST SRM 987 standard following Equation (1):

$$\delta^{88/86} Sr = \left(\frac{\left({}^{88} Sr / {}^{86} Sr \right)_{sample}}{\left({}^{88} Sr / {}^{86} Sr \right)_{SRM \, 987}} - 1 \right) \times 1000. \tag{1}$$

The repeated measurement of SRM 987 yielded a $\delta^{88/86}$ Sr value of 0.00 \pm 0.02‰ (2 σ , n = 45) and a 87 Sr/ 86 Sr value of 0.710248 \pm 0.000009. Furthermore, the accuracy of the measurements was assessed by analyzing the interlaboratory North Atlantic Seawater Standard (NASS-6), which underwent the same purification protocol as our samples. The repeated measurement of NASS-6 yielded a $\delta^{88/86}$ Sr value of 0.40 \pm 0.03‰ (2 σ , n = 8) and a 87 Sr/ 86 Sr value of 0.709173 \pm 0.000006 (2 σ , n = 8) respectively. These values agree well with previously reported values (Neymark et al., 2014; Wakaki et al., 2017). The two

times standard error (SE) of 87 Sr/ 86 Sr ratio of samples was always <0.000015.

The Ca isotope ratios were measured using a 43 Ca- 42 Ca of double-spike similar to the method described in Lehn et al. (2013). Aliquots of samples containing up to 2.25 µg Ca were mixed with aliquots of a 43 Ca- 42 Ca of double-spike to achieve a spike/sample Ca ratio of 0.3. The solution was evaporated and redissolved in 1 ml of 2 M HCl. One milliliter of AG50W-X12 resin (200–400 mesh) was packed in an S size ($d = \sim 5.5$ mm, h = 42 mm) polypropylene column (Muromachi Chemicals Inc., Japan). The resin was cleaned



FIGURE 1 ⁸⁷Sr/⁸⁶Sr ratios in metal sources and in consumers of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data

by passing two volumes of 6 M HCl. After conditioning the resin with 5 ml of 2 M HCl, the sample aliquot was loaded onto the column. The resin was washed with 10 ml of 2 M HCl for matrix elution and the fraction containing Ca was collected by passing an additional 10 ml of 2 M HCl. The pure Ca aliquot was dried, redissolved in one drop of concentrated HNO₃, and dried again to oxidize organics from the column. A sample containing 1 μ g of Ca was loaded on an out-gassed single Re filament (0.03 mm h \times 0.8 mm w)

with a Ta-H₃PO₄ activator. The Ca isotope measurement was performed on a TIMS at the RIHN. The Ca isotopes were measured with the static multicollection mode using five faraday cups connected to amplifiers with 10¹¹ Ω : ⁴⁰Ca (L3), ⁴¹K(L1), ⁴²Ca(C), ⁴³Ca(H1), and ⁴⁴Ca(H3). In the mass spectrometer, the filament was heated over approximately 30 min to achieve a ⁴⁴Ca beam of 160 mV, which corresponded to approximately 5.5–6 V of ⁴⁰Ca. A gain calibration was performed before each measurement. ⁴⁰Ca was



FIGURE 2 $\delta^{88/86}$ Sr $\pm 2\sigma$ values in metal sources and in consumers of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data

corrected for ⁴⁰K interference using ⁴⁰K/⁴¹K = 0.00174 (Meija et al., 2016), but ⁴⁰K interference was found to be negligible. A single measurement consisted of 200 cycles (20 cycles × 10 blocks) of 16.8 s integration of the ion signal. Data analysis was performed equivalent to $\delta^{88/86}$ Sr based on the combination of internal normalization isotope ratio measurement and the iterative calculation protocol. The ⁴⁴Ca/⁴⁰Ca ratios were expressed relative to the NIST SRM 915b standard following Equation (2):

$$\delta^{44/40} Ca = \left(\frac{\left({}^{44}Ca/{}^{40}Ca\right)_{sample}}{\left({}^{(44}Ca/{}^{40}Ca)_{sRM\,915b}} - 1\right) \times 1000.$$
(2)

The repeated measurement of SRM 915b yielded a $\delta^{44/40}$ Ca value of $0.00 \pm 0.08\%$ (2σ , n = 16). Furthermore, the accuracy of the measurements was assessed by analyzing the OSIL Atlantic seawater standard, which underwent the same purification protocol as our samples.



FIGURE 3 $\delta^{44/40}$ Ca $\pm 2\sigma$ values in metal sources and in consumers of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data

The repeated measurement of OSIL Atlantic seawater yielded a $\delta^{44/40}$ Ca value of $1.13 \pm 0.07\%$ (2σ , n = 8), which was well in agreement with previously reported values (Lehn et al., 2013; Wang et al., 2019).

Statistical analyses

We used the Shapiro-Wilk test to test for normally distributed data and the Levene's test to test for homoscedasticity. We performed the Student's t test in order to compare the $\delta^{44/40}$ Ca. $\delta^{88/86}$ Sr values and Sr/Ca ratios in stream water and periphyton between the sampled streams (Yasu vs. Ado), stream location (upper vs. lower), sampling month (November vs. May) and in order to compare stream water with those values in periphyton. In order to test for significant differences in the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values in consumers according to the sampled stream (Yasu vs. Ado), stream location (upper vs. lower), sampling month (November vs. May), feeding habits, and their interaction effects, we performed multiway analysis of variance. We performed Tukey's honestly significant difference post hoc test to identify these differences. Owing to the large variations in the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values across different orders of predators, we explored statistical differences with order rather than with feeding habits as the explanatory variable. Crane fly larvae and Japanese freshwater crabs (only present at upper reaches), and shrimps and large caddisfly larvae S. marmorata (only present at lower reaches) were not included in this statistical analysis. We used Pearson product-moment correlation analyses to explore relationships between $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values and with 87 Sr/ 86 Sr ratios. δ^{15} N values and Sr/Ca ratios. All statistical analyses were performed using R (version 3.6.3, R Foundation for Statistical Computing, Vienna, Austria, http://www. R-project.org/).

RESULTS

Sr isotope ratios in rock cobbles

The ${}^{87}\text{Sr}/{}^{86}\text{Sr}$ ratios of mudrocks, sandstone, and granite cobbles (n = 9) ranged from 0.729949 to 0.738499, and the $\delta^{88/86}\text{Sr}$ values ranged from -0.04% to 0.51% (Appendix S1: Table S1). The $\delta^{44/40}$ Ca values of rock cobbles were not determined.

Ca and Sr isotope ratios and Sr/Ca ratios in stream water

The ⁸⁷Sr/⁸⁶Sr ratios of stream water showed slight seasonal variations (Figure 1; Appendix S1: Table S2). The ⁸⁷Sr/⁸⁶Sr ratios increased from upper to lower reaches in the Ado River but decreased from upper to lower reaches in the Yasu River, well in agreement with previously reported ⁸⁷Sr/⁸⁶Sr ratios (Nakano et al., 2008). The $\delta^{88/86}$ Sr values of stream water ranged from 0.23% to 0.37% with no seasonal variation (Figure 2), but the $\delta^{88/86}$ Sr values were 0.08% higher in the Yasu River (p = 0.004). These $\delta^{88/86}$ Sr values were in the range of those in global rivers (Krabbenhöft et al., 2010; Yoshimura et al., 2021). The $\delta^{44/40}$ Ca values ranging from 0.12% to 0.23% showed no seasonal variation and differences across sites (Figure 3).

TABLE 2 Results of the analysis of variance (ANOVA) for factors controlling $\delta^{44/40}$ Ca values and $\delta^{88/86}$ Sr values in consumers of different order

	$\delta^{44/40}$ Ca			$\delta^{88/86}$ Sr		
Explanatory variable	df	F	р	df	F	р
Stream	1	1.7	0.196	1	35.4	< 0.001***
Location	1	1.5	0.228	1	1.4	0.241
Month	1	0.5	0.470	1	1.1	0.29
Order	5	68.6	< 0.001***	5	17.9	0.007**
Stream : location	1	2.1	0.156	1	0.3	0.617
Stream : month	1	0.0	0.899	1	0.0	0.990
Stream : order	5	1.1	0.377	5	2.6	0.053
Location : month	1	0.3	0.573	1	0.8	0.372
Location : order	5	0.0	0.736	5	2.7	0.045*
Month : order	5	1.0	0.421	5	0.3	0.252
Stream : location : month	5	0.9	0.348	5	0.5	0.472

 $^{*}p < 0.05; \, ^{**}p < 0.01; \, ^{***}p < 0.001.$



TABLE 3 Results of the analysis of variance (ANOVA) for factors controlling Sr/Ca ratios in consumers of different order

	log(Sr/Ca)			
Explanatory variable	df	F	р	
Stream	1	46.6	< 0.001***	
Location	1	1.5	0.223	
Month	1	0.9	0.357	
Order	5	52.7	< 0.001***	
Stream : location	1	11.0	0.002**	
Stream : month	1	0.0	0.872	
Stream : order	5	4.4	0.004**	
Location : month	1	2.2	0.152	
Location : order	5	5.0	0.002**	
Month : order	5	0.2	0.959	
Stream : location : month	5	0.0	0.858	

p < 0.05; p < 0.01; p < 0.01; p < 0.001.

These values were comparable to those of streams and rivers that drain silicate rocks worldwide (Fantle & Tipper, 2014). The Sr/Ca ratios were significantly higher in stream water from Ado River compared to Yasu River (p = 0.001).

Ca and Sr isotope ratios and Sr/Ca ratios in primary producers

The ⁸⁷Sr/⁸⁶Sr ratios of plant litter were usually in the range of those values of stream water at lower reaches, but sometimes higher at upper reaches (Figure 1; Appendix S1: Table S3). The $\delta^{88/86}$ Sr values varied across plant species and ranged from 0.11% to 0.30% (Figure 2), while $\delta^{44/40}$ Ca values ranged from -0.39% to 0.35% (Figure 3). *Quercus salicina* and *Q. myrsinaefolia* usually had the highest $\delta^{44/40}$ Ca values and lowest Sr/Ca ratios. However, litter of *Q. salicina* and *Q. myrsinaefolia* are generally unfavorable to decomposers owing to their hardness and poor nutrient content (Motomori et al., 2001).

The ⁸⁷Sr/⁸⁶Sr ratios of periphyton were in the range of those values of stream water except for slightly higher ⁸⁷Sr/⁸⁶Sr ratios of periphyton at Upper Yasu (Figure 1; Appendix S1: Table S4). The $\delta^{88/86}$ Sr values (0.15‰–0.28‰) were significantly lower than those values in stream water (p < 0.001) (Figure 2). Similar to stream water, the $\delta^{88/86}$ Sr values in periphyton were significantly higher (average of 0.09‰) in the Yasu River compared to

FIGURE4 Boxplots of $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values and log(Sr/Ca) ratios in metal sources and consumers in all sampling locations combined. *Quercus* (*Q*) and plant litter at lower reaches were not included

Ado River (p < 0.001). The $\delta^{44/40}$ Ca values in periphyton ranged from -0.27% to 0.11% and were significantly lower than the $\delta^{44/40}$ Ca values in stream water (p = 0.004) (Figure 3). Sr/Ca ratios in periphyton were significantly higher compared to those in stream water (p < 0.001).

Ca and Sr isotope ratios and Sr/Ca ratios in consumers

The ⁸⁷Sr/⁸⁶Sr ratios of bones of goby, crustacea, and stonefly nymphs tended to reflect those ⁸⁷Sr/⁸⁶Sr

ratios in stream water and periphyton (Figure 1; Appendix S1: Tables S5 and S6). Nymphs of predatory dragonfly and dobsonfly, grazing mayfly, and larvae of filterer-feeding caddisfly, leaf-shredding crane fly often tended to have higher ⁸⁷Sr/⁸⁶Sr ratios than stream water and periphyton.

The $\delta^{88/86}$ Sr and 87 Sr/ 86 Sr ratios were not determined for all specimens because of sometimes low samples masses available. The $\delta^{88/86}$ Sr values were significantly higher in consumers from the Yasu River (0.18‰) than from the Ado River (0.11‰) (p < 0.001) (Table 2). The $\delta^{88/86}$ Sr values greatly differed across feeding habits and order (Figure 2, Table 2). We observed the lowest $\delta^{88/86}$ Sr



FIGURE 5 Scatter plots of $\delta^{44/40}$ Ca $\pm 2\sigma$ against $\delta^{88/86}$ Sr $\pm 2\sigma$ values in metal sources and consumers at the four sampling locations. Black solid lines present linear regressions through metal sources and consumers. Plant litter at lower reaches was not included in the linear regression. Filled symbols represent May 2018 data; open symbols represent November 2018 data



FIGURE 6 Scatter plots of $\delta^{88/86}$ Sr $\pm 2\sigma$ values against log(Sr/Ca) ratios in sources and consumers at the four sampling locations. Black solid lines present linear regressions through metal sources and consumers. *Stenopsyche marmorata (S. m.), Quercus (Q)*, and plant litter at lower reaches were not included in the linear regression. Filled symbols represent May 2018 data; open symbols represent November 2018 data

in bones of demersal goby $(0.08 \pm 0.03\%)$, mean \pm SE, n = 7) and in predatory stonefly nymphs $(0.06 \pm 0.01\%)$, n = 11) (Figure 4). Large filterer-feeding caddisflies (*S. marmorata*; 0.44 \pm 0.07%, n = 3) had the highest $\delta^{88/86}$ Sr values.

There were no significant differences in the $\delta^{44/40}$ Ca values of consumers between the Ado River versus the Yasu River, upper versus lower, and between May versus November (Figure 3, Table 2). Similar to the $\delta^{88/86}$ Sr values, the $\delta^{44/40}$ Ca values significantly varied with order (Figure 4). The $\delta^{44/40}$ Ca values ranged from $-1.39 \pm 0.06\%$ (n = 7) in bones of demersal goby to $1.33 \pm 0.17\%$ (n = 3) in large filterer-feeding caddisflies. Furthermore, the bones of goby

were significantly more depleted in 44 Ca by an average of $0.32 \pm 0.06\%$ compared to muscles (p = 0.003; Appendix S2: Figure S1).

Similar to the $\delta^{88/86}$ Sr and the $\delta^{44/40}$ Ca values, the Sr/Ca ratios varied greatly across order (Table 3, Figure 4).

Relationships between $\delta^{44/40}Ca$ and $\delta^{88/86}Sr$ values with $\delta^{15}N$ values and Sr/Ca ratios

We found that the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values of primary producers and consumers were positively correlated at all sampling locations (Figure 5). To test for trophic effects on



FIGURE 7 Scatter plots of $\delta^{44/40}$ Ca $\pm 2\sigma$ values against log(Sr/Ca) ratios in sources and consumers at the four sampling locations. Black solid lines present linear regressions through metal sources and consumers. *Stenopsyche marmorata* (*S. m.*), *Quercus* (*Q*), and plant litter at lower reaches were not included in the linear regression. Filled symbols represent May 2018 data; open symbols represent November 2018 data

the Ca and Sr stable isotope ratios, we correlated the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values against the δ^{15} N values and the Sr/Ca ratios. The $\delta^{88/86}$ Sr values were positively correlated with Sr/Ca ratios at Upper Ado and Lower Yasu (Figure 6), while the $\delta^{44/40}$ Ca values were positively correlated with Sr/Ca ratios at each sampling site (Figure 7). Similarly, the $\delta^{88/86}$ Sr values were negatively correlated with the δ^{15} N values at Upper Ado and Lower Yasu (Figure 8), while the $\delta^{44/40}$ Ca values were negatively correlated with the δ^{15} N values at Upper Ado and Lower Yasu (Figure 8), while the $\delta^{44/40}$ Ca values were negatively correlated with the δ^{15} N values at each sampling site (Figure 9). The $\delta^{88/86}$ Sr values were correlated with the 87 Sr/ 86 Sr ratios at each sampling site (Sigure 9). The $\delta^{88/86}$ Sr values were negatively S2: Figure S3). While there were no relationships between the $\delta^{44/40}$ Ca

with the 87 Sr/ 86 Sr ratios in consumers at upper reaches, we found the $\delta^{44/40}$ Ca values were correlated with the 87 Sr/ 86 Sr ratios at lower reaches (Appendix S2: Figure S4).

DISCUSSION

Effects of land use and season on calcium and strontium stable isotope ratios

The alkaline earth metal input into rivers and streams is largely determined by the chemical weathering of



FIGURE 8 Scatter plots of $\delta^{88/86}$ Sr $\pm 2\sigma$ against δ^{15} N values in primary producers and consumers at the four sampling locations. Black solid lines present linear regressions through metal sources and consumers. Plant litter at lower reaches was not included in the linear regression. Filled symbols represent May 2018 data; open symbols represent November 2018 data

bedrocks in upper reaches in the Lake Biwa catchment (Nitzsche et al., 2019) as well as by leaching from soils and rocks via acids generated from agricultural practices in the plain in lower reaches (Nakano et al., 2008). The absence of significant differences in the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values of stream water and periphyton, and consumers (macroinvertebrates and goby) between upper versus lower reaches (Table 1; Appendix S2: Figure S2) indicates that neither land use, nor differences in silicate rock sources significantly affected the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values in consumers although differences in the 87 Sr/ 86 Sr ratios between upper and lower reaches

indicated weathering of different rock sources (Figure 1). Plant litter was likely only of minor importance in lower reaches as indicated by the large offset in the δ^{15} N values between plant litter and consumers (Figure 8) and by the absence of shredders. In fact, downstream areas are typically dominated by autochthonous sources (Vannote et al., 1980). Furthermore, the Sr/Ca ratio in apatite of teleost fish was found to increase with water temperature (Balter & Lécuyer, 2010). Although we observed 3–8°C higher temperatures in lower reaches (Appendix S1: Table S2), a potential effect on the Sr/Ca ratios and on the Ca and Sr isotopic composition in consumers is



FIGURE 9 Scatter plots of $\delta^{44/40}$ Ca $\pm 2\sigma$ against δ^{15} N values in primary producers and consumers at the four sampling locations. Black solid lines present linear regressions through metal sources and consumers. Plant litter at lower reaches was not included in the linear regression. Filled symbols represent May 2018 data; open symbols represent November 2018 data

difficult to assess with our study design, and other factors (e.g., physiological, dietary sources) could overprint temperature effects.

Furthermore, the season did not significantly affect the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values and the 87 Sr/ 86 Sr ratios in stream water and periphyton between May and November (Figures 1–3; Tables 2 and 3) indicating that dissolved external Ca and Sr contributions and their isotopic signatures (e.g., groundwater, surface runoff, agricultural drainage, precipitation) were rather constant over the year. More frequent, for example, monthly variations in the $\delta^{44/40}$ Ca, $\delta^{88/86}$ Sr values and in the 87 Sr/ 86 Sr ratios may

be restricted to streams and rivers with carbonates dominated catchments (Cenki-Tok et al., 2009; Wei et al., 2013).

In contrast to stream water and periphyton, the ⁸⁷Sr/⁸⁶Sr ratios of plant litter showed a wider variation in upper reaches. The ⁸⁷Sr/⁸⁶Sr ratios of plant litter reflect that of the exchangeable Sr fraction in the soil, which depends on the ⁸⁷Sr/⁸⁶Sr ratio of the leachable Sr of the substrate (underlying rocks) (Capo et al., 1998; Maurer et al., 2012). Owing to the wide range in ⁸⁷Sr/⁸⁶Sr ratios of rock cobbles (Appendix S1: Table S1; Appendix S2: Figure S3), larger variations in the ⁸⁷Sr/⁸⁶Sr ratios of

plant litter could be expected. In fact, our litter samples only encompassed a mixture of three leaves. The higher 87Sr/86Sr ratios of two crane fly larvae than those of plant litter support this assumption (Figure 1). Another important dietary source in stream food webs is fine particulate organic material (fPOM), a mixture between several aquatic (periphyton, faces, fungi, and bacteria) and terrestrial (plant litter, soil organic matter) sources. Depending on its composition, ⁸⁷Sr/⁸⁶Sr ratios of fPOM could show a large variation and explain the higher ⁸⁷Sr/⁸⁶Sr ratios in primary consumers that rely on fPOM such as filter-feeding caddisfly larvae. Furthermore, Baetis spp. also collect detrital material of possible terrestrial origin (Takemon, 2005). Alternatively, it is possible that grazing mayfly nymphs ingested colloidal mineral particles with high ⁸⁷Sr/⁸⁶Sr ratios trapped in periphyton mats that could have still been present in their guts prior to the digestion by nitric acid.

However, the effect of plant litter and fPOM on primary consumers is hard to assess as Ca and Sr stable isotopes fractionate in the plant-soil system (Cenki-Tok et al., 2009; Souza et al., 2010), and the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values of plant litter in this study varied across sampled plant litter species with a tendency of lower $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values in November compared to May (Figures 2 and 3; Appendix S1: Table S3). Furthermore, dietary metal contributions (periphyton vs. plant litter) can change over the year (Ishikawa et al., 2014, 2016). Some taxa also change their diets during maturing, for example, stonefly nymphs change from CG to predatory feeding habits (Allan, 1982). The different larval stages of taxa also vary from several weeks to a few years (Merrit et al., 2008). Collectively, seasonal variations in the isotopic composition of plant litter and fPOM together with shifts in dietary contributions could have led to differences in the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values in some primary consumers between May and November.

Higher ⁸⁷Sr/⁸⁶Sr ratios were also observed for the strict predatory dobsonfly nymphs (P. grandis) which could be explained by contributions from their prey (mostly caddisfly and crane fly larvae) (Hayashi, 1988) that partly relied on fPOM and/or plant litter with elevated ⁸⁷Sr/⁸⁶Sr ratios. Similarly, the often higher ⁸⁷Sr/⁸⁶Sr ratios in predatory dragonfly nymphs (Gomphidae spp.) indicated that their prey relied on Sr sources with higher ⁸⁷Sr/⁸⁶Sr ratios. Dragonfly nymphs at upper reaches were collected from riffles, thus caddisfly and crane fly larvae and mayfly nymphs that partly rely on terrestrial sources are a possible prey. In lower reaches, dragonfly nymphs were collected from river edges, where mayfly nymphs and caddisfly larvae were absent. Thus, other prey that relies on partly terrestrial sources with elevated ⁸⁷Sr/⁸⁶Sr ratios must exist (e.g., amphibians: Kishida et al., 2009).

Calcium and strontium stable isotope ratio variability across feeding habits and trophic effects

We found positive correlations between the $\delta^{44/40}$ Ca and $\delta^{88/86}$ Sr values across all sampling sites (Figure 5) indicating that Ca and Sr originated from similar sources (stream water, periphyton, plant litter) and that Ca and Sr isotopes underwent similar fractionation trends in stream food webs although Sr is not essential. Leaf-shredding crane fly larvae do not follow this fractionation trend as they were enriched in ⁸⁸Sr, but depleted in ⁴⁴Ca relative to plant litter indicating a decoupling of Ca and Sr cycling. Furthermore, bones of gobies showed a slight offset from this fractionation trend as bones showed a larger depletion in ⁴⁴Ca than in ⁸⁸Sr (Figure 4). Bone data are likely to reflect isotope fractionations of both the trophic effect and the boneforming process as observed for Ca (Appendix S2: Figure S1). The lower $\delta^{44/40}$ Ca values in bones relative to muscles ($0.32 \pm 0.06\%$) agree with the reported offset in Ca stable isotopic composition between soft tissue and mineralized tissue (Dodat et al., 2021; Skulan & DePaolo, 1999; Tacail et al., 2014, 2020). Consequently, it is reasonable to assume that a slight ⁸⁸Sr fractionation also exists between bone apatite and blood. In marine teleost fishes, the diet-bone is on average -0.30% for $\delta^{44/40}$ Ca (Martin et al., 2015). When applying the dietbone offset of -0.30% to our gobies (-1.4%, n = 7), then the expected $\delta^{44/40}$ Ca of its diet will be -1.1%which is lower than the $\delta^{44/40}$ Ca values measured for most macroinvertebrates expect for a few stonefly nymphs (Figure 3). With respect to our gobies, C. pollux is a pure predator that consumes all kinds of available megalopteran larvae and freshwater crab (Nakagawa et al., 2012), while R. kurodai consumes aquatic macroinvertebrates (mainly Ephemeroptera and Diptera) and minor proportions of periphyton (<10%) (Maruyama et al., 2001). When using the average $\delta^{44/40}$ Ca values of bones of gobies (-1.4‰) and of aquatic insects (-0.2%, n = 61), then the average bonediet offset is -1.2%, which is comparable to terrestrial vertebrates (Tacail et al., 2020). Thus, and although this is only a rough calculation, the bone-diet offset appears to be much larger in freshwater compared to marine teleost fishes. A possible explanation for this observation could be a higher Ca intake from seawater through gills for marine teleost fishes due to two magnitudes higher Ca concentrations in seawater compared to stream water. This could lead to the postulated buffering of the diet-bone Ca isotopic fractionation for marine teleost fishes (Clementz et al., 2003; Martin et al., 2015; Tacail et al., 2020).

The positive correlations between the $\delta^{44/40}\mbox{Ca}$ and partly $\delta^{88/86}$ Sr values with Sr/Ca ratios (Figures 6 and 7) and negative correlations with δ^{15} N values (Figures 8 and 9) indicate trophic level effects on both Ca and Sr stable isotopes. $\delta^{44/40}$ Ca is a better trophic marker than $\delta^{88/86}$ Sr in stream food webs owing to the better correlations with the Sr/Ca ratios and δ^{15} N values. These correlations are partly controlled by demersal goby and omnivorous shrimps indicating that trophic depletion in ⁴⁴Ca and ⁸⁸Sr is restricted to stream food webs with taxa of wide ranges of trophic positions. In fact, trophic positions of stream food webs in the Ado and Yasu River cover only a narrow range (2.3-3.5) presumably as stream insects are generalists relying on multiple food sources (Ishikawa et al., 2014, 2016). Furthermore, the larger trophic depletion in ⁴⁴Ca and ⁸⁸Sr in stonefly and dragonfly nymphs compared to dobsonfly nymphs (Figure 4) indicates that physiological differences exist in predators that drive the Ca and Sr isotopic fractionation. A notable exception from the trophic trends is the exceptional large ⁴⁴Ca and ⁸⁸Sr enrichment in large filter-feeding S. marmorata which is opposite to the ⁴⁴Ca and ⁸⁸Sr trophic depletion observed for other orders of stream consumers and for vertebrates and marine teleosts (Knudson et al., 2010; Tacail et al., 2020). Filter-feeding S. marmorata spin silk nets underwater that comprise Ca^{2+} in silk proteins (Ashton et al., 2013; Stewart & Wang, 2010). We hypothesize that net-spinning leads to a ⁴⁴Ca (and ⁸⁸Sr) depletion in silk nets causing the large enrichment in ⁴⁴Ca (and ⁸⁸Sr) in *S. marmorata*.

Nevertheless, our data generally confirm that the observed ⁴⁴Ca and ⁸⁸Sr trophic depletion known from marine teleosts and terrestrial vertebrates (Clementz et al., 2003; Knudson et al., 2010; Lewis et al., 2017; Martin et al., 2015, 2018; Skulan et al., 1997) also existed in macroinvertebrate-dominated stream food webs despite physiological differences across these taxa. On the one hand, Ca and Sr isotopic fractionation mechanisms could for instance be related to the cellular or molecular shared features between these taxa (e.g., related to cellular Ca homeostasis mechanisms, transmembrane Ca transport proteins). On the other hand, Ca and Sr isotopic fractionation in stream insects could occur during the osmoregulation and excretion functions in the gut, Malpighian tubules, and rectum (Komnick, 1977). Furthermore, Ca and Sr isotopic fractionation could be possible during the intestinal absorption in the midgut epithelium, during the formation of the chitinous exoskeleton from the hemolymph, and during molting of the exoskeleton. For crustacea, the biomineralization of hard parts (e.g., shells, mouthparts, legs) strongly enriched in Ca and Sr (Appendix S1: Table S5) could induce an isotopic fractionation. For better understanding the Ca and Sr isotopic fractionation mechanisms

in stream macroinvertebrates, we suggest future studies to analyze different organs and the shed exoskeletons.

CONCLUSION

By determining the $\delta^{44/40}$ Ca, $\delta^{88/86}$ Sr values and 87 Sr/ 86 Sr ratios alongside with δ^{15} N values and Sr/Ca ratios as trophic indicators in stream consumers (aquatic macroinvertebrates and small gobies) and their potential metal sources (stream water, periphyton, plant litter), we found a similar uptake of Ca and Sr sources and isotopic fractionation trends of the macronutrient Ca and nonessential Sr existed in stream food webs. Our data confirmed that the trophic Ca and Sr isotope fractionation observed for marine teleost fishes and terrestrial vertebrates also applied to macroinvertebratedominated stream food webs despite physiological differences across these taxa. Although some shared mechanisms of Ca and Sr isotopic fractionation may exist at the cellular or molecular level, future studies may target exploring the fractionation mechanisms in stream macroinvertebrates. We conclude that the approach of linking Ca and Sr stable isotopes in the same specimens with $\delta^{15}N$ values and Sr/Ca ratios represent a powerful tool to understand trophic relationships, not restricted to stream consumers. The use of ⁸⁷Sr/⁸⁶Sr ratios may further help in distinguishing between aquatic (stream water, periphyton) and terrestrial (plant litter, fPOM) Ca and Sr sources.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Nitzsche et al., 2021) are available from Dryad: https://doi.org/10.5061/dryad.flvhhmgwn.

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Additional supporting information may be found in the online version of the article at the publisher's website.

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