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Possible ideas on carbon and nitrogen trophic fractionation of food chains: a new aspect of food-chain stable isotope analysis in Lake Biwa, Lake Baikal, and the Mongolian grasslands

Abstract Trophic fractionation of carbon and nitrogen isotopes ($\Delta\delta^{13}C$, $\Delta\delta^{15}N$) was examined using previously complied databases for food chains in Lake Biwa, Lake Baikal, and Mongolian grassland. The following two features were clarified: (1) For each ecosystem, the ratios of trophic fractionation of carbon and nitrogen isotopes ($\Delta\delta^{15}N/\Delta\delta^{13}C$) throughout food chain could be obtained as the slope of linear regression line on the $\delta^{15}N$–$\delta^{13}C$ plot. (2) Further, analysis of covariance (ANCOVA) revealed the slopes on $\delta^{15}N$–$\delta^{13}C$ were not significantly different among these various ecosystems and allowed us to have the regression by setting $\delta^{15}N$ as the response variable: $\delta^{15}N = 1.61\ \delta^{13}C +$ [ecosystem specific constant] with standard errors of [± 0.41] and [± 9.7] for the slope and the intercept, respectively. It was suggested that the slope of the regression (or the ratio $\Delta\delta^{15}N/\Delta\delta^{13}C$) could be applicable to more complicated food webs in case nitrogen and carbon isotope ratios of primary producers can be assumed constant in space and time within the ecosystems. The results from simple linear regression analyses coincided well with the ANCOVA results for these ecosystems, although there was some discrepancy between the results of the two statistical analyses. Possible factors that govern the linear relationship between $\delta^{15}N$ and $\delta^{13}C$ along a food chain are discussed together with a new scope for the stable isotope food chain analyses.

Keywords $\Delta^{15}N$–$\delta^{13}C$ relationship · Food chain · Lake Biwa · Lake Baikal · Mongolian grassland

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Introduction

The stable isotopes (SIs) of biogenic substances vary depending on the isotopic compositions of the reactants, the pathways and kinetic modes of the reaction dynamics, and the physical and chemical conditions. Thus, every biota has its own unique isotopic composition, known as the dynamic stable isotope fingerprint, which is determined by its function and position in the material flow through food webs in ecosystems as well as by the dynamics of metabolic processes within the organism's body (Wada et al. 1995; Wada 2009a, b).

Analysis of food-web structure using a nitrogen stable isotope ratio has provided qualitative depictions about trophic structure of natural aquatic ecosystems (Wada and Hattori 1991; Cabana and Rasmussen 1996; Fry 2006). In their pioneering works, DeNiro and Epstein (1981), and Minagawa and Wada (1984) reported significant ^{15}N enrichment during feeding processes. The latter authors reported that the $\delta^{15}N$ value of a consumer is enriched by $3.4 \pm 1.1\ ‰$ over that of its diet whether it is invertebrate or vertebrate.

Conversely, the magnitude of $\delta^{13}C$ enrichment during feeding process is known to be somewhat smaller than that of $\delta^{15}N$. Fry and Sherr (1984) reported that animals usually have $\delta^{13}C$ values within $\pm 2\,\permil$ of their diets, while Rau et al. (1983) reported a small enrichment of ^{13}C of $<1\,\permil$ in marine pelagic food chains. Including offshore food webs, it is documented that the $\delta^{13}C$ enrichment $(\Delta\delta^{13}C)/TL$ (TL = trophic level; plant TL = 1.0) lacked its consistency ranging from 0 to 1.5 $\permil$ (Fry and Sherr 1984; Rau et al. 1983; McConnaughey and McRoy 1979). Vander Zanden and Rasmussen (2001) synthesized field and laboratory $\delta^{15}N$ enrichment $(\Delta\delta^{15}N)$ and $\Delta\delta^{13}C$ estimates from aquatic systems estimated both in the field and in laboratory and examined patterns of variation in isotopic fractionation. They obtained the average $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ as 3.4 and 0.8 $\permil$, respectively. Using data from 25 north temperate lakes, Post (2002) examined how the $\delta^{15}N$ and $\delta^{13}C$ of the base of aquatic food webs varies among lakes and between littoral and pelagic food webs within lakes. The mean trophic fractionation of $\delta^{15}N$ for consumers was 3.4 ± 1 $\permil$, and the corresponding $\delta^{13}C$ was 0.4 ± 1.3 $\permil$. Summarizing the data in the literature together with original data, McCutchan et al. (2003) reported $\Delta\delta^{13}C$ as 0.5 ± 0.3 $\permil$ and range of $\Delta\delta^{15}N$ from 1.4 to 3.3 $\permil$.

Meanwhile, comparison among more various aquatic systems suggests that $\Delta\delta^{13}C/TL$ has relatively small values in lakes and coastal systems (Post 2002; Wada et al. 1993), while it would become larger in pelagic systems in the ocean (Wada et al. 1987). In fact, Wada et al. (1987) reported significantly high trophic fractionation of $\Delta\delta^{13}C/TL$ along a food chain in the Antarctic Ocean. Significant ^{13}C enrichment up to 1.5 $\permil$ along food chain was also recognized in off-shore food webs in marine ecosystems (Fry and Sherr 1984). At present, although the mechanisms and conditions to generate the variation in magnitude of the trophic fractionation of carbon isotopes remain unclear, it would be clarified by further understanding of kinetic isotope fractionation with emphasis on biochemical metabolic reactions at such levels as amino acid synthesis (Martinez del Rio et al. 2009; Wada 2009a, b). In other words, if the effect of biochemical metabolic reaction is not blurred by other external factors, we could expect a common value for $\Delta\delta^{15}N/\Delta\delta^{13}C$ as long as the animals share similar metabolic maps.

It is known that nitrogen and carbon isotope fractionations occur during biosynthesis (Minagawa et al. 1992; Caut 2009; Vanderklift and Ponsard 2003).

Mizutani et al. (1991) studied the nitrogen and carbon isotope compositions of tissues of a cormorant (*Phalacrocorax capillatus*) fed only with mackerel throughout its 23-year lifespan at the Ueno Zoo in Tokyo, from which a very clear linear relationship was obtained on the $\delta^{15}N$–$\delta^{13}C$ map for the organs of the cormorant. Wada (2009a, b) reported high reproducibility of C/N isotopic compositions of various organs of reared 16 rats. Both of these results show that distribution of C/N isotopes in animal body is isotopically ordered and this, in turn, indicates a certain positive linear relationship between carbon and nitrogen isotope effects at the protein-synthesis level, in other words, at the level of amino acid synthesis.

Chikaraishi et al. (2009) reported on "the amino acid trophic level" using variations in $\delta^{15}N$ in amino acids during a feeding process and suggested the common occurrence of apparent trophic fractionation of nitrogen in amino acid metabolism. All of the facts described above suggest the trophic fractionations of $\delta^{15}N$ and $\delta^{13}C$ of an organism are reflecting its physiological constraints and the ratio might be more stable than the respective values. This in turn suggests the existence of a linear relationship with a common slope between $\delta^{15}N$ and $\delta^{13}C$ in food chains irrespective of ecosystem types (Vander Zanden and Rasmussen 2001; Post 2002; Wada et al. 1987). Aita et al. (2011), for example, reported a similar slope value of 1.52 for oceanic food chains among different oceanic regions, including the OYASHIO area, the warm core ring, Antarctic Ocean, and Alaska Bay.

The facts shown above strongly suggest that stable isotope analyses of carbon and nitrogen are powerful not only to capture the complex interactions within ecological communities (e.g., such as trophic relationships or energy/mass flows in food webs; Peterson and Fry 1987; Cabana and Rasmussen 1996) but also they have a potential to give insight into these ecosystem features relating with the metabolic constraints on the environment conditions for the organisms consisting the ecosystems. Among various tangible measures, we here focus on C/N trophic fractionations (i.e., $\Delta\delta^{15}N/TL$, $\Delta\delta^{13}C/TL$) because they would provide us with valuable information irrespective of ecosystem types.

However, since precise examinations of $\Delta\delta^{15}N/TL$ and $\Delta\delta^{13}C/TL$, as described above, have been mostly limited to aquatic ecosystems, especially to lakes to date, we could not even discuss whether these values would serve as general indices to understand the features of wide range of ecosystems. Therefore, as the first step, it is strongly required to collect information about the trophic fractionations of carbon and nitrogen in both terrestrial and other aquatic ecosystems and compare them to elucidate the commonness and the variation of $\Delta\delta^{15}N/\Delta\delta^{13}C$ among various food webs.

Here, we conducted detailed analyses of the $\delta^{15}N$–$\delta^{13}C$ diagrams of various ecosystems including the Lake Biwa, the pelagic regions of Lake Baikal, and Mongolian grasslands together with other related information in the literature to clarify the trophic fractionation of carbon and nitrogen isotopes. Among numerous candidates, these three ecosystems were selected because they satisfied the following essential conditions for this study: (1) they are rich in biological information and the energy flows within the ecosystems were simple, so that the food chain structure could be clearly detected; (2) they were rich in data of C/N stable isotope for more than three trophic levels analyzed in the same laboratory

conditions (see "Materials and methods" for details); (3) their major herbivorous animals (such as zooplankton or livestock mammals) are known to move around for foraging. By doing so, they are averaging the spacio-temporal variations in $\delta^{15}N$ and $\delta^{13}C$ of the primary producers (see "Materials and methods").

In the light of these considerations, it could be expected to observe a similar slope for different ecosystems on $\delta^{15}N/\delta^{13}C$ maps as long as they consist of animals that share quite similar dynamics of amino acid syntheses, which are expected for animals at matured stage since C/N trophic fractionations change depending upon growth rate (Ishii, PS). Along this line, we have examined to find a common slope among several ecosystems to elucidate only kinetic isotope effect during bulk amino acid synthesis.

In this context, we need assumption to apply a simple linear regression analysis; the linear regression can be available in case we expect a common $\Delta\delta^{15}N/\Delta\delta^{13}C$ value through a food chain under such constrains to have matured animal samples and a homogenizer type of herbivorous animals at TL = 2 in time and space.

The questions arising in this paper are:

Can we regard the relationship of $\delta^{15}N/\delta^{13}C$ throughout a food chain to be linear (i.e., common $\Delta\delta^{15}N/\Delta\delta^{13}C$)?

and if so,

Does each ecosystem have a distinct specific ratio of $\Delta\delta^{15}N/\Delta\delta^{13}C$ throughout its food chain?

We here hypothesize that each ecosystem should have a linear relationship on the $\delta^{15}N$–$\delta^{13}C$ map if all animal components in the system share the same ratio of trophic fractionations of carbon and nitrogen, $\Delta\delta^{15}N/\Delta\delta^{13}C$ reflecting by the constraints of their metabolic processes provided that isotopic composition of primary producers is kept constant in time and space.

Materials and methods

All the samples analyzed in this study were recollected from those that have already been published, and therefore the detailed information about sampling protocols, sample preparation, and isotope analyses can be found in their original reports (Takai and Sakamoto 1999; Yamada et al. 1998; Yoshii et al. 1999; Ogawa et al. 2000; Kohzu et al. 2009). The greatest advantage of using the database of these three ecosystems is that the running standards of the stable isotopes are identical, and therefore we could ignore the errors from their variation. Furthermore, the precise ecological surveys have been carried out for these food chains involving stomach content examinations or age estimation of animal samples. These three ecosystems are rich in ecological knowledge, which tell that their major herbivorous animals (zooplankton in lakes Biwa and Baikal or livestock animals in Mongolian grassland) move around for foraging so that they average the spatio/temporal fluctuations of C/N isotopic compositions of primary producers. We selected data for matured animal samples in which amino acid metabolisms are at the stationary state to avoid isotopic signals specifically biased by body growth.

Study sites and sampling

In this study, we analyzed the samples from two freshwater ecosystems, Lakes Biwa and Baikal, and a terrestrial grassland system in Mongolia. Lake Biwa is an ancient Japanese lake with an age of 4 million years whose surface area and the maximum depth are 670 km^2 and 103 m, respectively. Its watershed is inhabited by two million people and it is supporting over 14 million people in its downstream region and so it must be emphasized that the lake has been highly polluted by increasing human activities during the past 50 years. The samples for the stable isotope analysis were collected over 3 years by Yamada et al. (1998) and Takai and Sakamoto (1999) whose main objectives were to understand the food-web structures and the food habit of the endemic catfish (Silurus biwaensis), respectively. According to Takai and Sakamoto (1999), gammarus (Gammarus spp.), bluegills (Lepomis macrochirus), and the catfish (S. biwaensis) consist of the main food chain in the lake. Takai and Sakamoto (1999) examined the stomach contents of 180 adults of the cat catfish.

Lake Baikal, one of the most ancient lakes, is about 30 million years old and rich in biodiversity. In the pelagic region of the lake, the food chain is well developed from diatoms in euphotic layers to floating sculpins in the deep layer, with diurnal vertical migrations of animal community. The distribution and variability of $\delta^{13}C$ and $\delta^{15}N$ in the pelagic were investigated to elucidate the food-web structures (Yoshii et al. 1999; Ogawa et al. 2000; Miyasaka et al. 2006). The pelagic food-web structure (deeper than 100 m, after Yoshii et al. 1999) is rather simple and consists of five major ecological groups: phytoplankton (Aulacoseira baicalensis), meso-zooplankton (Epischura baicalensis), macrozooplankton amphipod (Macrohectopus branickii), carnivorous fish (omul: Coregonus autumnalis migratorius and four sculpin: cottoid species), and Baikal seal (Phoca sibirica). Due to the low diversity of major animal species and hence the limited dietary options for each species, we were able to estimate the dietary composition of each animal quantitatively. First, an SI database was built from data obtained by Yoshii et al. (1999) and Ogawa et al. (2000). With respect to sculpins, only the samples collected from deeper than 100 m were selected as components of the pelagic food chain to eliminate the effects of the coastal community, where various kinds of algae with different $\delta^{13}C$ values grow (Yoshii et al. 1999).

Mongolian grassland has been always under grazing pressure of nomadic pastoralization for more than a 1,000 years in a sustainable manner. However, it is considered that recent rapid growth of human and livestock populations under rapid urbanization began to threaten the sustainability of the pasturelands and hence the method of livestock production (Fujita et al. 2009). Field sampling of the plants and mammals was conducted during summers of 1999 and 2000 at two field sites in central and northern Mongolia: the Khangai site (47°31′N, 100°56′E; 430 km southwest from Ulaanbaatar) and the Gachuurt site (48°01′N, 107°11′E; 30 km northeast from Ulaanbaatar). In our study sites, major herbivores are livestock, which mostly consist of sheep (*Ovis aries*) and goats (*Capra aegagrus hircus*) while the major carnivores are foxes (*Vulpes vulpes*) and wolves (*Canis lupus*) from which we collected hair samples for analysis. Detailed information of these study sites and samples could be found in the report by Kohzu et al. (2009). Of particular concern about the northern part of Mongolia is its importance for the conservation of Lake Baikal. Since it hosts the watershed of the Selenge River, the main inflow to the Baikal, the careful management of effluent arising from traditional nomadic pasturelands in Mongolia is essential for the lake.

Stable isotope (SI) analyses

After the extraction of lipids, the $\delta^{13}C$ and $\delta^{15}N$ values of the samples were measured using a continuous-flow isotope-ratio mass spectrometer (CF/IRMS: EA1108, Fisons; Delta S, Finnigan). Stable isotope ratios are expressed in δ notation as the difference in parts per thousand (‰) from the standard: $\delta X = (R_{sample}/R_{standard} - 1) \times 1000$, where δX is $\delta^{13}C$ or $\delta^{15}N$, R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, and the standards were Vienna PeeDee Belemnite (VPDB) and atmospheric N_2 for carbon and nitrogen, respectively. Analytical precision was better than ± 0.2 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

Statistical analyses

Statistical analyses were performed for the isotopic signatures of the samples in terms of testing our hypothesis. All statistical analyses were conducted using JMP software (version 8.0.2 for Windows, SAS Institute, Inc., Carey, NC, USA). For each ecosystem, simple linear regression analysis was applied to examine the relationships of $\delta^{15}N$ with $\delta^{13}C$. In the calculations, the average values of each species were used, as they have the same weight as TL for the regression analysis.

To examine the overall effect of sampling sites on the relationship between the $\delta^{15}N$ and $\delta^{13}C$ of the animals and plants, we also used analysis of covariance (ANCOVA), in which $\delta^{13}C$ was set as a covariate and the sampling site as the independent variable. Then, in case the interaction term between $\delta^{13}C$ and the sampling

site had no significant effects on the $\delta^{15}N$ of the samples, we discarded the interaction from the analyses, which allow us to assume no significant difference in their slopes and to newly redraw parallel regression lines (see manual of JMP software ver. 8.0.2, SAS).

Results

Figure 1 presents the regression lines for $\delta^{15}N$–$\delta^{13}C$ in Lake Biwa, Lake Baikal, and Mongolian grassland. For the three ecosystems, there were significant relationships between $\delta^{15}N$ and $\delta^{13}C$ as below:

Lake Biwa: $\delta^{15}N = 1.53\ \delta^{13}C + 47.5$ ($r^2 = 0.67$);
Lake Baikal: $\delta^{15}N = 2.05\ \delta^{13}C + 63.9$ ($r^2 = 0.36$);
Mongolian grassland: $\delta^{15}N = 1.33\ \delta^{13}C + 36.3$ ($r^2 = 0.64$).

As for the Mongolian grassland, the $\delta^{15}N$ and $\delta^{13}C$ values of mammals showed large variation within species and also varied considerably for the whole grassland ecosystem, which includes several kinds of livestock and their wild predators. In our study sites, livestock are mostly sheep and goats that are foraged by wolves. For mammal species, more detailed analysis was performed using data of individuals to investigate the intra-specific variation. As indicated in Fig. 2, the mammalian isotopic map exhibited a rather straight line, although individual trophic effects were variable and data of cattle individuals showed this trend clearly, although the isotopic signatures contain variation due to several conditions, which will be discussed below.

Inter-ecosystem comparison among Lake Biwa, Lake Baikal, and Mongolian grassland

The ANCOVA revealed no significant interactions between ecosystems and either $\delta^{15}N$ or $\delta^{13}C$ (Table 1a, b;
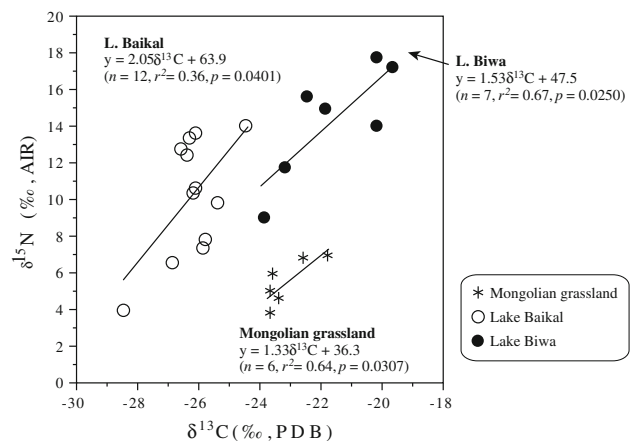

Fig. 1 Relationship between $\delta^{13}C$ and $\delta^{15}N$ values for various kinds of organism for three ecosystems (see text for detail): Lake Biwa (*filled circles*), Lake Baikal (*open circles*), and Mongolian grassland (*asterisks*). *Symbols* represent means, and *solid lines* are derived from linear regressions of each ecosystem
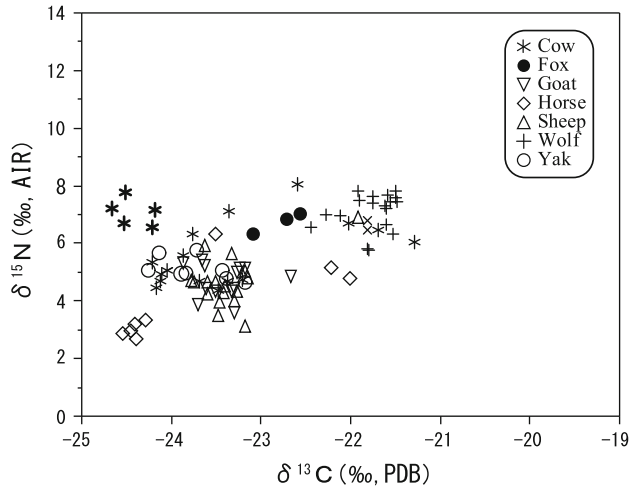
Fig. 2 The δ^{13}C and δ^{15}N map for various kinds of mammals in the Mongolian grasslands: cattle (*Bos taurus*, *asterisks*), infant cattle (*bold asterisks*), fox (*Vulpes vulpes*, *filled circles*), goat (*Capra aegagrus hircus*, *down-pointing triangles*), horse (*diamonds*), sheep (*Ovis aries*, *triangles*), wolf (*Canis lupus*, *plus signs*) and yak (*Bos grunniens*, *open circles*)

Fig. 3 Relationship between δ^{13}C and δ^{15}N values for various kinds of organism for three ecosystems: Lake Biwa (*filled circles*), Lake Baikal (*open circles*), and Mongolian grassland (*asterisks*). *Symbols* represent means, and *solid lines* are fits for each ecosystem from ANCOVAs (analysis of covariance)

Table 1 Results of ANCOVA on δ^{15}N and δ^{13}C of zooplankton among three ecosystems: L. Baikal, L. Biwa, and Mongolian grassland

Parameter	df	F	p value
(a) Response variable: δ^{15}N (‰)			
δ^{13}C (‰)	1	**10.2464**	**0.0045**
Sites	2	**11.6447**	**0.0004**
δ^{13}C(‰) × sites	2	0.2148	0.8085
(b) Response variable: δ^{13}C (‰)			
δ^{15}N (‰)	1	**12.8841**	**0.0018**
Sites	2	**17.3278**	**< 0.0001**
δ^{15}N(‰) × sites	2	2.3366	0.1225
(c) Response variable: δ^{15}N (‰)			
δ^{13}C (‰)	1	**15.5432**	**0.0006**
Sites	2	**33.0404**	**< 0.0001**
(d) Response variable: δ^{13}C (‰)			
δ^{15}N (‰)	1	**18.5309**	**0.0003**
Sites	2	**68.5644**	**< 0.0001**

Significant effects at $p = 0.05$ are indicated in *bold*

$p > 0.05$), implying we could assume parallel regression line for these three ecosystems. For δ^{15}N as response variable, the regression for the three ecosystems could be redrawn as: δ^{15}N = 1.61 δ^{13}C + [ecosystem specific constant] with standard errors of [±0.41] and [±9.7] for the slope and the intercept, respectively (Fig. 3). The constant values for each site are Lake Biwa: 1.42 ($p > 0.05$), Lake Baikal: 4.16 ($p < 0.01$) and Mongolian grassland: −5.58 ($p < 0.001$). This means that these three ecosystems can be regarded to have similar $\Delta\delta^{15}$N/$\Delta\delta^{13}$C, but have different intercepts in the δ^{15}N–δ^{13}C map (Table 1c, d and linear regression line in Fig. 1, $p < 0.001$). This slope 1.61 (±0.41) is not largely different from those obtained from simple regression for respective ecosystem, 1.53 for L. Biwa, 2.05 for L. Baikal, and 1.33 for Mongolian grassland. Thus, we could obtain
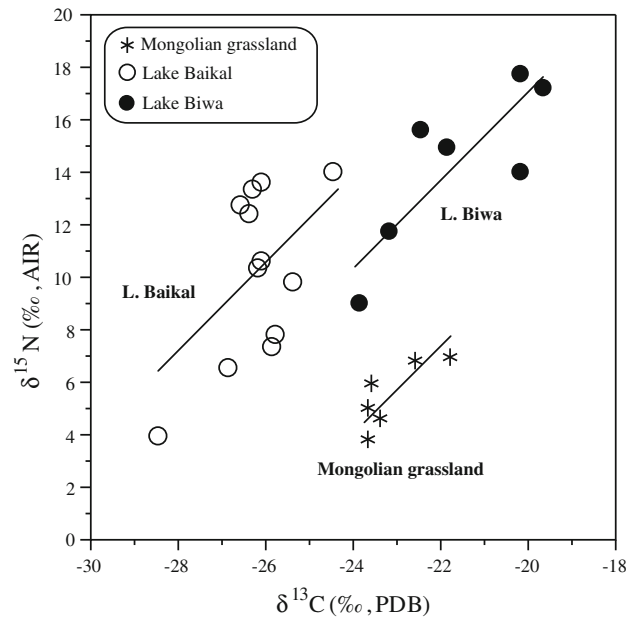
similar slopes for above different types of ecosystems by different ways of statistical analyses.

Discussion

Recall the two questions proposed in "Introduction". Here, we suggest the answers to each of the questions based on our results.

Can we regard the relationship between δ^{15}N and δ^{13}C throughout a food chain to be linear (i.e., constant $\Delta\delta^{15}$N/$\Delta\delta^{13}$C)?

Our results clearly show that all the food webs in the three ecosystems showed linear relationships between δ^{15}N and δ^{13}C values. Then, why do we observe such relationships? The reasons for the linear relationships can be discussed from three aspects of biological facts. The first involves general understandings of the dynamics of kinetic isotope effects in certain metabolic processes with different limiting steps during a feeding process (Wada et al. 1995; Pecquerie et al. 2010). It is emphasized that the observed isotope fractionation may take place during synthesis of amino acids and their carbon skeletons, the rates of which are governed by energy-producing systems such as glycolysis, the TCA cycle, and oxidative phosphorylation, that is, the ratio of the rate of amino-acid syntheses to that of energy-yielding processes. At this point, all biota share almost the same processes among these ecosystems to generate

the same magnitude of C and N isotope fractionation except some animals with different anabolic/catabolic ratios. Second explanation for the linearity involves intermolecular differences in both isotopes among amino acids (Minagawa et al. 1992; Macko et al. 1986, 1987). Furthermore, Chikaraishi et al. (2009) also reported on "the amino acid trophic level" using variations in $\delta^{15}N$ in amino acids during a feeding process and suggested the common occurrence of apparent trophic fractionation of nitrogen in amino acid metabolism. Third explanation involves carbon and nitrogen isotope ratios in animal organs. Mizutani et al. (1991) studied the nitrogen and carbon isotope compositions of tissues of a cormorant (Phalacrocorax capillatus) that fed on only mackerel ($\delta^{15}N$ of 9.4 ± 0.8 ‰; $\delta^{13}C$ of -20.1 ± 1.2 ‰) throughout its 23-year lifespan at the Ueno Zoo in Tokyo. A very clear linear relationship was obtained on the $\delta^{15}N$–$\delta^{13}C$ map for the organs of the cormorant as follows: $\delta^{15}N = 1.14\delta^{13}C + 33.0$ ($r = 0.92$, $p < 0.05$). This strongly indicated a certain positive linear relationship between carbon and nitrogen isotope effects at the protein-synthesis level, i.e., the amino acid synthetic level in the avian species. This relationship might be true for other animal organisms, although the pathways of excretion, hence nitrogen metabolism of avian group is somewhat different from other animal classes in their pathway. Therefore, it would be natural to observe the common linear relationship as indicated in Fig. 3, if we could assume that the limitations on carbon-skeleton syntheses and trans-amination processes in amino acid metabolisms with respect to the isotopic fractionations should be similar among the components of food chains from herbivores to top predators. Under these considerations described here, the linearity observed in the food chains in Lake Biwa, Lake Baikal, and the Mongolian grasslands support our hypothesis to provide an explanation for the linearity of a food chain on the isotopic map. This view is also supported by previous study, which showed similar values of $\Delta\delta^{15}N/\Delta\delta^{13}C$ (1.52) for oceanic food chains (Aita et al. 2011).

Does each ecosystem have a specific ratio of $\Delta\delta^{15}N/\Delta\delta^{13}C$? (Some difference in $\Delta\delta^{15}N/\Delta\delta^{13}C$ among ecosystems)?

As we saw, we did not observe significant difference in the ratio of trophic fractionation of carbon and nitrogen isotopes ($\Delta\delta^{13}C/\Delta\delta^{15}N$) by ANCOVA analysis for the aforementioned three ecosystems. To know whether this similarity could be general, we reviewed similar analysis results obtained from different types of ecosystems, a tidal flat system (Wada et al. 1993) and a decomposing system of wood and decomposing fungi (Kohzu et al. 2005) to compare with the three systems. Although linear relationships were observed, each food chain in Gamoh tidal flat (Miyagi prefecture, Japan) and wood decomposing fungi exhibited quite different ratio of trophic fractionation of carbon and nitrogen isotopes ($\Delta\delta^{13}C/\Delta\delta^{15}N$). From the comparison, we found two clear contrasting features for these two systems. In Gamoh tidal flat, little carbon trophic fractionation was observed along the food chain (Wada et al. 1993). The reason why we observed such small carbon trophic fractionation in the tidal flat has not been clarified, but it might be related with its eutrophic condition and rather complicated food webs. Since it induces carbon limitation, eutrophic condition generally lowers C/N ratio of autotrophs which in turn might result in smaller carbon fractionation in higher trophic levels in the food-chain. On the other hand, the slope, $\Delta\delta^{15}N/\Delta\delta^{13}C$, was very small for wood decomposing fungi due to small nitrogen isotope fractionation under the extremely nitrogen limiting conditions (Kohzu et al. 1999, 2005). Although the reasons why animals or fungi in these ecosystems exhibit different values of apparent nitrogen and carbon isotope fractionation, these facts insist the possibility that the $\Delta\delta^{13}C/\Delta\delta^{15}N$ ratio in the feeding processes might be altered dynamically with changing metabolic process. As a matter of fact, we observe distinct slope values for some food chains, typical examples of which are as follows: $\Delta\delta^{13}C/TL$ seems to be low in lakes (Post 2002) and coastal areas (tidal flat, this study), increasing to high values in pelagic areas of the ocean (Fry and Sherr 1984; Wada et al. 1987) as described above.

Our results support our hypothesis stated in "Introduction", that is, $\Delta\delta^{15}N/\Delta\delta^{13}C$ (the slopes on $\delta^{15}N/\delta^{13}C$ maps) of any food chain would be made similar if they share intrinsic metabolic processes that must have adapted to their external environmental conditions. On the other hand, the slopes, $\Delta\delta^{15}N/\Delta\delta^{13}C$, would differ if the proportions of available nitrogen and carbon for biochemical metabolic reactions varied, which in turn make the kinetic isotope fractionation different from one another. Also, ecological complexity in natural food webs, such as spatio-temporal variation in the isotopic compositions of primary producers as well as activities of omnivorous animals should make a strong effect on the variation of the slopes. Thus, in this paper we selected such food chains to have herbivorous animals to migrate and average the isotopic compositions of primary producers. To clarify these arguments, further studies in the related field are required. Potential factors that might govern the C/N trophic fractionation factors at the moment are listed in Table 2. The observed linear regression lines for the Lake Biwa, Lake Baikal, and Mongolian grassland ecosystems (Fig. 1) strongly suggested that perturbation by the abovementioned ecological factors play significant roles in governing the apparent trophic fractionation of animals throughout a food chain in each ecosystem although the principal factor is governed by the occurrence of a similar isotope effect of 1.61 for $\Delta\delta^{15}N/\Delta\delta^{13}C$. In what follows, we point out several points to be discussed which were found in our results.

Table 2 Potential factors that might govern the C/N trophic fractionation factors in food chains in ecosystems

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(1) Kinetic isotope effects at the level of metabolic map:
  Kinetic isotope effects in the processes of amino acid syntheses
  Reuse of amino acids
  Mass balance effects between diet protein and predator protein
(2) Perturbation of food chain by the ecological factors:
  Difference in apparent α among fauna by changes in feeding habit (e.g., normal type and $\Delta\delta^{13}C/TL$ = zero group)
  Difference in vegetation such as C3 plant and C4 plant food base (two-source diet model). Physiological variable C/N isotope ratios of primary producers in time and space
  Differences in lipid content along the life cycles
  Activity of homogenizer animal such as livestock in the grasslands and recycle of N as like a microbial loop in the sea and detritus food chain

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Fig. 4 The $\delta^{13}C$ and $\delta^{15}N$ map for Baikal seals (*Phoca sibirica*). The *open circles* denotes juvenile seals (< 2 years old), and *filled circles* denotes mature seals (older than 2 years)

The effect of multiple source isotope mixing

Is the linear relationship robust against trophic interactions within food chains? As long as a similar $\Delta\delta^{15}N/\Delta\delta^{13}C$ is maintained within a food chain, any two-source (or even more) isotope mixing in the food chain could not disturb the linear relationship on the $\delta^{15}N$–$\delta^{13}C$ map. A typical example is the case for omnivorous animals whose position on the $\delta^{15}N$–$\delta^{13}C$ map could be found on the same line. Besides, provided the C/N fractionation factor of the amino acid synthesis is kept similar throughout the food chain, the linear relationship must be kept constant by so-called mass-balance effect. Under these conditions, for example, the recycling of amino acids derived from protein degradation in animal bodies would not cause disorder to the linearity of the isotopic map, either.

Mammal-specific isotopic fractionation

We here point out two possible factors that might cause alteration in the signals of C/N isotope fractionation specifically in mammals. In Fig. 2, we could identify several points greatly deviated from the virtual regression lines to the higher $\delta^{15}N$ for low $\delta^{13}C$. For the data of cattle (Fig. 2), two of the five samples (bolded) are revealed to be from individuals at infant stage, the main diet of which is milk although the ages of the others could not be identified (Kohzu, unpublished data). This dietary effect is consistent with the Baikal seals. Figure 4 shows individual data on Baikal seals. Here, seals younger than 1 year old (including some fetus, open circles) exhibited significantly higher $\delta^{15}N$ values than older individuals (filled circles), possibly because they take their nutrition through the placenta or milk. It is generally recognized that adult individuals of Baikal seal feed on fish such as *Comephorus dybowskii* (Yoshii et al. 1999) and thus, the trophic level of infant or prenatal stages of mammals might look even higher than that of the adult individuals. This is consistent with previous reports about the $\delta^{15}N$ enrichment observed in infant stage of mammals (Balasse et al. 2001; Balasse and
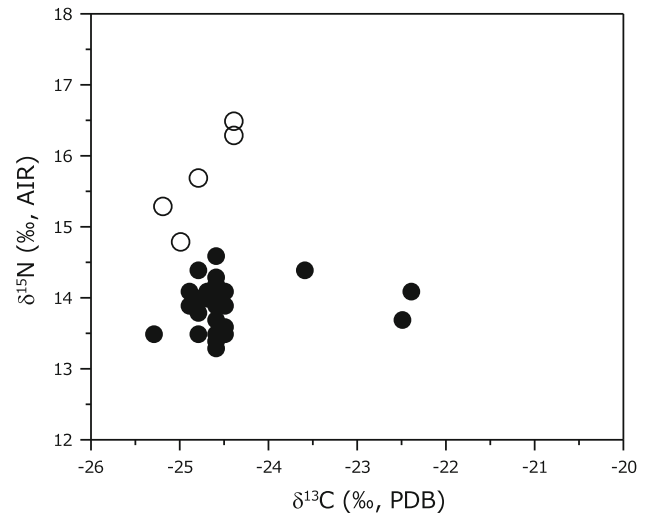
Tresset 2002; Dalerum et al. 2007; Makarewicz and Tuross 2006). We consider their quasi-high trophic level is due to their nutrition taken via milk or placenta of their mothers. This is a notable finding, and makes us expect the same kinds of phenomena, TL deviation, to be observed when the decomposed amino acids are partially re-used in amino acid metabolism especially in mammals, although details are still unknown at this point.

Another possible important feature of mammals is that they forage in wider area over various topography and other physical conditions. In general, livestock in Mongolia, by moving and grazing both in lowlands and highlands, are considered to homogenize the spatial heterogeneity in δ values of grasses. However, in case of cattle, their foraging area is mostly limited to lower ground and do not expand uphill. Thus, the cattle samples might exhibit higher $\delta^{15}N$ values reflecting the denitrification effect during the downslope water movement (Kohzu et al. 2009). Note that this homogenizing effect by foraging behavior of sheep and goats looks very similar to that of the vertically migrating zooplankton in Lake Baikal.

Fluctuation of isotope data from the corresponding linearity

Finally, we can consider some ecological phenomena that may cause the fluctuation of the linear relationships of a food chain more in detail. Spatial and temporal variation of C/N isotope ratios in primary producers must be one of the principal factors causing the fluctuation in question. For example, blooming of Melosira, the dominant diatom in the Baikal, exhibits typical oscillation (11 years) that causes variations in $\delta^{15}N$ and $\delta^{13}C$ in Lake Baikal along its dynamics in late spring

and autumn. Decadal fluctuations in carbon and nitrogen isotope ratios reported for omul scales might reflect the fluctuations occurring all through the food chain of the lake Baikal (Ogawa et al. 2000).

We believe that our hypotheses featuring a new aspect of the trophic fractionation of C/N isotopes would pave the way to elucidate the principal food chain structure of the $\delta^{15}N$–$\delta^{13}C$ map. In other words, the key factor for observing rather similar relationship of $\Delta\delta^{15}N/\Delta\delta^{13}C$ (e.g., $\delta^{15}N = 1.61 \, \delta^{13}C$ + constants, see above) for various ecosystems might lie in kinetic isotope effects at the level of metabolic processes such as the processes of amino acid syntheses. Based on our hypothesis, we therefore suspect that the ratios of $\Delta\delta^{15}N/\Delta\delta^{13}C$ observed for the three ecosystems might be quite close to that expected by the operation of common metabolic map, because of its generality irrespective of the site and kinds of animal.

In this report, we tried to suggest a possible mechanism to explain the changes in trophic fractionation of C/N isotopes over different kinds of ecosystems to promote future development in this scientific field. Detailed explanations of the variations in $\Delta\delta^{15}N/\Delta\delta^{13}C$ will be conducted by further field studies in terrestrial, freshwater, and marine ecosystems and laboratory experiments. The ecological significance and implications on food-chain and ecosystem sustainability can also be considered with emphasis on food-chain studies in the future.

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