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Article

Ecological application of compound-specific stable nitrogen isotope analysis of amino acids – A case study of captive and wild bears

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Abstract

Several recent studies have suggested an innovative method for estimating the food sources, feeding habits, and trophic position of consumers in food webs, based on the compound-specific stable isotope analysis (CSIA) of amino acids. In this study, we used CSIA to study terrestrial mammals in a controlled feeding experiment with captive Asiatic black bears (*Ursus thibetanus*). The trophic position is estimated to be 1.7–1.9 for the bears, which is consistent with their actual trophic position (approximately 2). We also investigated the feeding habits of six wild Asiatic black bears (the same species as the captive bears; three were test animals suspected of feeding on trout and the others were controls) in the area around a rainbow trout farm. The flux of trout-derived proteins in the bear diet was calculated from the observed isotopic composition of amino acids. The results show that of the three test animals, two clearly fed on rainbow trout from the farm but other did not. Although this study is the first simple and systematic investigation of the ecological application of CSIA of amino acids to modern terrestrial mammals, we conclude that this technique is potentially useful in estimating the trophic position, food sources, and feeding habits of wildlife in ecological and biological studies.

Key words: compound-specific stable isotope analysis (CSIA), nitrogen isotope, amino acid, wildlife, bear

1. Introduction

Over the past two decades, the stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic compositions of bulk animals (or their tissues) have been used as an important factor in estimating the food sources, feeding habits, and trophic position of animals in ecological food webs (DeNiro and Epstein 1981; Minagawa and Wada 1984; Kelly 2000). However, the nitrogen isotope analysis of bulk tissues has the following drawbacks: i) the ^{13}C and ^{15}N enrichment factors (i.e., the increase of ～0.5‰ for carbon and ～3.4‰ for nitrogen with each trophic

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level) vary significantly between samples and even between tissues within a single sample (DeNiro and Epstein 1981; Vander Zanden and Rasmussen 2001; McCutchan Jr et al. 2003); and ii) such analyses require characterization of the isotope values of the primary producers in the food web of interest. This latter requirement is difficult to fulfill in many cases because primary producers (such as phytoplankton in aquatic environments and higher plants in terrestrial environments) show great variation in their isotope values, both spatially and temporally (Yoshioka et al. 1989; Roff 2000; Craine et al. 2009). These drawbacks inevitably result in errors in the observed results and uncertainties in the related discussion (O'Reilly et al. 2002).

However, several recent studies have suggested an innovative method for estimating the food sources, feeding habits, and trophic levels of animals in food webs, based on the compound-specific stable isotope analysis (CSIA) of amino acids in the animals (McClelland and Montoya 2002; McCarthy et al. 2007; Chikaraishi et al. 2007, 2009; Naito et al. 2010). It has been proposed that (i) the nitrogen isotopic composition of glutamic acid increases by ～8.0‰ per trophic level and therefore provides trophic information, whereas that of phenylalanine increases only slightly, by ～0.4‰ per trophic level, and therefore provides the isotopic composition of primary producers in the food web; and (ii) the trophic position can be estimated by comparing the nitrogen isotopic composition of these two amino acids in the animal samples of interest (Chikaraishi et al. 2007, 2009). Therefore, unlike isotope analyses of bulk tissues, this novel method (CSIA of amino acids) does not require characterization of the isotope values of the primary producers to estimate the trophic positions of animals in the food web. To further illustrate the potential of this method, Naito et al. (2010) quantified the fraction of marine proteins in archeological human diets based on the clear distinction in the isotopic composition of amino acids between aquatic and terrestrial animals.

More recently, Chikaraishi et al. (2009, 2010, 2011) investigated the isotopic signatures of amino acids in terrestrial C_3 and C_4 plants, and in several insects, including the caterpillar, aphid, bee, wasp, and hornet, and established the following equation with which to estimate the trophic position:

$$\text{Trophic position} = (\delta^{15}N_{\text{glutamic acid}} - \delta^{15}N_{\text{phenylalanine}} + \beta)/7.6 + 1 \quad (1)$$

where β represents the isotopic difference between glutamic acid and phenylalanine in the primary producers (-3.4 ± 0.9‰ for aquatic cyanobacteria and algae, $+8.4 \pm 1.6$‰ for terrestrial C_3 plants, and -0.4 ± 1.7‰ for terrestrial C_4 plants). The ^{15}N-enrichment factor (7.6 ± 1.2‰) is commonly useful in both aquatic and terrestrial food webs (Chikaraishi et al. 2010).

In this study, we tested the applicability of this method to terrestrial mammal studies (i) in controlled feeding experiments with captive Asiatic black bears (*Ursus thibetanus*), and (ii) by investigating the feeding habits of wild Asiatic black bears (the same species as the captive bears) in the area around a rainbow trout farm. We anticipated that CSIA of amino acids would be useful in studies of mammals and would provide more detailed information about their feeding habits than that obtained from isotope analysis of bulk tissues, which should enable a better assessment of the conflict between humans and wildlife.

Wild Asiatic black bears may have diversified their feeding habits in response to habitat environment, and the trophic position of the bears in the food web is not always clear, although a variety of approaches (i.e. direct observations of scat and analyses of stomach contents) have been developed to study animal diets. Accurate information on the food of bears is critical in understanding the ecological role of bears and in developing effective management and conservation strategies for these animals.

2. Samples and methods

2.1. Samples

Blood samples were collected from three captive bears (bears 1–3) at Ani Bear Park, Akita, Japan, in September 1999. These bears were mature females and had been reared on controlled diets consisting almost

solely of corn (more than 95% of the total food) during their active season (April to November) for 8–12 years, whereas they were given no food during their hibernation season (December to March). Before sampling, the bears were immobilized by a blow dart or spear injection of either a combination of ketamine HCl (Ketaral, Sankyo, Japan) and medetomidine HCl (Domitor, Meiji, Japan) at doses of 5 mg/kg and 0.04 mg/kg body weight, respectively, or a mixture of zolazepam and tiletamine HCl (Zoletil, Virbac, France) at a dose of 9 mg/kg body weight. After the bears were immobilized, the blood samples were collected from their jugular veins into vacuum tubes. The bears were not fed on the night before sampling. Blood samples were also collected from six wild bears (bears 4–9) in the Central Japan Alps, Nagano, Japan. Bears 4–6 were captured near a rainbow trout farm as possible perpetrators of damage at the farm. Bears 7–9 were collected from the same region but were used as controls, having had no access to the trout farm, as confirmed by a radio tracking system. The blood was centrifuged and the separated serum was used for analysis. We also analyzed the corn used in the controlled feeding experiment and the fish at the trout farm as potential bear food.

2.2. Isotope analyses

The samples described above were prepared for CSIA analysis of their amino acids by HCl hydrolysis and *N*-pivaloyl/isopropyl ester (Pv/iPr) derivatization, according to the method described by Chikaraishi et al. (2007). In brief, each sample was hydrolyzed with 12 M HCl at 100 ℃, and the hydrolysate was washed with *n*-hexane/dichloromethane (6:5, v/v) to remove hydrophobic constituents such as lipids. After derivatization with thionyl chloride/2-propanol (1:4, v/v) and subsequently with pivaloyl chloride/dichloromethane (1:4, v/v), the Pv/iPr derivatives of the amino acids were extracted with n-hexane/dichloromethane (6:5, v/v). The nitrogen isotopic composition of individual amino acids was determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using a trace GC Ultra (Thermo Fisher Scientific) coupled to a Delta V (Thermo Fisher Scientific) IRMS via a GC/C/TC III interface (Hayes et al. 1990; Brand et al. 1994). For details of the analytical conditions for GC/C/IRMS, see Chikaraishi et al. (2009).

To obtain the isotopic composition of amino acid, admixtures of eight reference amino acids (alanine, glycine, valine, leucine, aspartic acid, serine, glutamic acid, and phenylalanine) with a known isotopic composition were analyzed every four or five GC/C/IRMS runs. The nitrogen isotopic composition is expressed in the conventional δ notation against atmospheric N_2 (air) on scales normalized to the known isotopic composition of the reference amino acids. To confirm the accuracy of the isotope measurements, the isotopic compositions of representative samples were also independently determined by GC/C/IRMS at Japan Agency for Marine-Earth Science and Technology (by Yoshito Chikaraishi and Naohiko Ohkouchi). The precision and accuracy of the measured reference amino acids were 0.5 (1σ) and 0.0‰, respectively, for a minimum sample amount of 50 ng N. The δ^{15}N values for the amino acids glutamic acid and phenylalanine are reported for the samples, for which the precision was better than 0.5‰.

3. Results and discussion

3.1. Controlled feeding experiment in captive Asiatic black bears

The δ^{15}N values of glutamic acid and phenylalanine in the captive bears were $+15.8 \pm 0.4$‰ and $+9.3 \pm 0.8$‰, respectively (Fig. 1). Because these bears were mainly fed corn (a C_4 plant), their trophic position should be calculated using Eq. 1 with a β value of −0.4‰ (Chikaraishi et al. 2010). This yields an estimated trophic position of 1.7–1.9. In contrast, the δ^{15}N values for glutamic acid and phenylalanine in corn are 6.5‰ and 6.6‰, respectively, indicating a trophic position of 0.9. Considering the propagation error in Eq. 1 (～0.3 for bears and ～0.2 for corn), the estimated trophic position is consistent with the actual trophic position of the bears (approximately 2) and with that of corn (1.0; Fig. 1).

The slight underestimate of the trophic position of the bears may reflect the effects of isotopic fractionation,

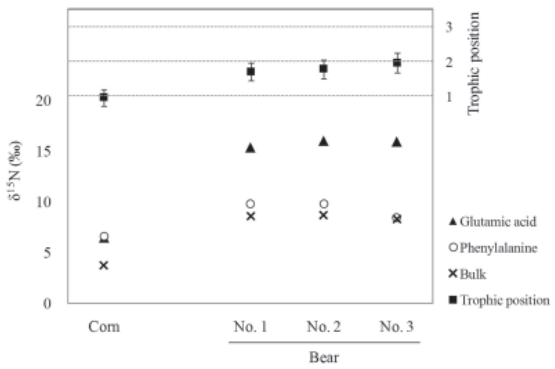

Fig. 1. δ^{15}N values of glutamic acid, phenylalanine, and bulk, and the estimated trophic position of three captive bears (Nos 1–3) and their food (corn). The error bars for trophic position indicate the propagation error.

which may occur if amino acid is measured in blood. Recently, Lorrain et al. (2009) reported that the trophic position determined by CSIA of amino acids in the plasma of penguin blood is approximately 1 unit lower than the trophic position expected from biological observations. They suggested that the ^{15}N-enrichment factor for glutamic acid in blood is smaller than that observed in muscle samples. Because blood contains a high abundance of food-derived amino acids and peptides, the explanation proposed by Lorrain et al. (2009) is reasonable (Yoshito Chikaraishi, pers. comm.). In fact, in the present study, although the bears had been fasted for half a day before the sample collection, the residual contaminants from food-derived amino acids in the blood may have caused a slight reduction in the enrichment factor from the value of 7.6 ± 1.2 reported previously (Chikaraishi et al., 2010). For bears with a trophic position of 2.0 (based on the fact that the bears were fed corn in this study), the enrichment factor is calculated to be 6.0 ± 1.0. Further studies may be required that compare the ^{15}N-enrichment factors between different tissues and between serum and blood cells within the blood. Conversely, the slight underestimate of the trophic position may reflect the fact that the diet of these bears also contained small amounts of C_3-plant-derived foods. In C_3 plants, the δ^{15}N value of glutamic acid is much lower (by 8.4‰) than that of phenylalanine, indicating that the δ^{15}N value of glutamic acid in bears would decrease if the bears were fed on C_3-plant-derived materials. Because there is no substantial difference between the trophic position estimated by CSIA of amino acids and that expected from biological or experimental observations (i.e., the difference is less than 0.3 units), we conclude that the estimation of trophic position by CSIA of amino acids is applicable to terrestrial mammal studies.

3.2. Assessment of rainbow trout consumption by wild Asiatic black bears

Table 1 lists the δ^{15}N values of glutamic acid and phenylalanine in six wild bears, which range from 10.4‰ to 26.0‰ and from 5.4‰ to 13.5‰, respectively; the values for rainbow trout are 31.4‰ and 10.4‰, respectively. The similar δ^{15}N values between bear 4 and rainbow trout suggest that this bear fed extensively on trout from the farm.

Following Naito et al. (2010) and the principle that underlies this method (Chikaraishi et al., 2009), the δ^{15}N values of glutamic acid and phenylalanine for bear ($\delta^{15}N_{bear_Glu}$ and $\delta^{15}N_{bear\_Phe}$, respectively), rainbow trout ($\delta^{15}N_{trout\_Glu}$ and $\delta^{15}N_{trout\_Phe}$, respectively), other food of the bear ($\delta^{15}N_{food\_Glu}$ and $\delta^{15}N_{food\_Phe}$, respectively), and the primary producer at the base of the food web ($\delta^{15}N_{PP\_Glu}$ and $\delta^{15}N_{PP\_Phe}$, respectively) are defined respectively by the following equations:

$$\delta^{15}N_{bear\_Glu} = (1-f) \times \delta^{15}N_{food\_Glu} + f \times \delta^{15}N_{trout\_Glu} + 8.0 \quad (2)$$

$$\delta^{15}N_{bear\_Phe} = (1-f) \times \delta^{15}N_{food\_Phe} + f \times \delta^{15}N_{trout\_Phe} + 0.4 \quad (3)$$

$$\delta^{15}N_{food\_Glu} = \delta^{15}N_{PP\_Glu} + 8.0 \times (TP - 1) \quad (4)$$

$$\delta^{15}N_{food\_Phe} = \delta^{15}N_{PP\_Phe} + 0.4 \times (TP - 1) \quad (5)$$

$$\delta^{15}N_{PP\_Glu} = \delta^{15}N_{PP\_Phe} - 8.4(R_{C3}) + 0.4(R_{C4}) + 3.4(R_{aquatic}) \quad (6)$$

$$R_{C3} + R_{C4} + R_{aquatic} = 1 \quad (7)$$

where f represents the flux of trout-derived proteins in the food of the bears ($0 < f < 1$), TP indicates the trophic position of bear food, and R indicates the relative contribution ratio of the primary producers (i.e., C_3, C_4, and aquatic plants) as potential food. Focusing on f,

Table 1. $\delta^{15}N$ values of glutamic acid ($\delta^{15}N_{bear\_glu}$) and phenylalanine ($\delta^{15}N_{bear\_phe}$) in six wild bears, estimated $\delta^{15}N$ values of their diet ($\delta^{15}N_{food\text{-}\_glu}$ and $\delta^{15}N_{food\_phe}$), and the contribution to their diets of trout (f)

	Bears suspected of feeding on farmed trout			Control bears		
	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
$\delta^{15}N_{bear\_glu}$	26.0	17.6	13.3	12.1	11.5	10.4
$\delta^{15}N_{bear\_phe}$	13.5	8.3	8.8	7.7	8.8	5.4
$\delta^{15}N_{food\_glu}$	10.8	3.4	4.8	3.5	4.8	1.2
$\delta^{15}N_{food\_phe}$	14.5	7.2	8.4	7.2	8.5	4.8
f	0.35	0.22	0.02	0.02	-0.05	0.04

equations (2–7) are rewritten as follows:

$$f = (\delta^{15}N_{bear\_Glu} - \delta^{15}N_{bear\_Phe} - X) / (\delta^{15}N_{trout\_Glu} - \delta^{15}N_{trout\_Phe} - X + 7.6) \quad (8)$$

$$X = 7.6TP - 8.4\,(R_{C3}) + 0.4\,(R_{C4}) + 3.4\,(R_{aquatic}) \quad (9)$$

Because the isotopic composition of glutamic acids and phenylalanine for bears and trout has been determined above, f is a simple function of four factors: TP and the relative contribution ratios of the three primary producers.

Because the f value should be zero for the control bears (Nos 7–9), the background X value is calculated to have a mean value of 4.0, as obtained using equation (8) and considering the isotopic composition of glutamic acids and phenylalanine of the bears. Therefore, equation (8) becomes

$$f = (\delta^{15}N_{bear\_Glu} - \delta^{15}N_{bear\_Phe} - 4.0) / (\delta^{15}N_{trout\_Glu} - \delta^{15}N_{trout\_Phe} + 3.6) \quad (10)$$

Using equation (10), the calculated f values for bears 4 and 5 are 0.35 and 0.22, respectively (Table 1), indicating that rainbow trout account for 35% and 22% of the total food intake of these bears, respectively. In contrast, the f value of bear 6 is 0.0, indicating that it does not feed on rainbow trout, even though it was captured near the fish farm and is therefore a candidate trout hunter at the farm. Thus, CSIA of amino acids provides information on not only the trophic position but also the feeding habits of mammals in the food web.

There exist several limitations with CSIA that should be addressed in future studies to improve the accuracy and precision of the results. In this study, we used a value of 4.0 for X in equation (8), given that the f value for the control bears should be zero; however, this value may be inaccurate or may vary among different bears. In fact, the $\delta^{15}N$ values for glutamic acid and phenylalanine in the food of bear No. 4 ($\delta^{15}N_{food\_glu}$ and $\delta^{15}N_{food\_phe}$) are estimated to be +10.8‰ and +14.5‰, respectively, which is much higher than the values calculated for the other bears (from 1.2‰ to 4.8‰ for glutamic acid and from +4.8‰ to +8.5‰ for phenylalanine) (Table 1). These results may indicate that the background X value is consistently 4.0 (1σ 1.2) in this study but that its composition (i.e., relative contribution ratio of C_3, C_4, and aquatic plants, and trophic position) varies among individuals. For example, it is simply expected that bear No. 4 consumed large amounts of agricultural plants, such as crops, because such plants sometimes show relatively positive isotopic values, reflecting the artificial input of isotopically heavy nutrients. Although it is currently difficult to solve this problem, a combined approach based on carbon isotope analyses of bulk tissues and/or amino acids may be useful in future studies.

Another limitation is that little is known of the effects of pregnancy, lactation, and hibernation on the isotopic compositions and trophic levels of bears, although such effects are likely to have been minimal in the present study because we collected the samples in September, during autumn, whereas these effects are expected to occur during winter and spring. In fact, the isotopic compositions of amino acids are closely related to the metabolic system of an organism (e.g., Chikaraishi et al., 2007), and many species of mammals have a unique metabolism system. This is clearly different from aquatic organisms such as plankton and fish, and from terrestrial insects, as reported in previous studies. These effects should also be clarified in future studies.

4. Summary and conclusion

We evaluated the applicability of CSIA of amino acids to ecological and biological studies of terrestrial mammals. In the controlled feeding experiment, the

trophic position estimated by CSIA was 1.7–1.9 for captive bears, which is consistent with their actual trophic position (approximately 2). In an investigation of the feeding habits of three wild bears in an area close to a trout farm (making them candidate consumers of farmed trout), we estimated the fluxes of trout-derived proteins in their diets. The results show that two of the bears fed on rainbow trout from the farm, whereas the third bear did not. Thus, CSIA of amino acids is useful in estimating the trophic position, food sources, and feeding habits of mammals.

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References

Brand W.A., Tegtmeter A. and Hilkert A. (1994) Compound-specific isotope analysis: Extending toward $^{15}N/^{14}N$ and $^{18}O/^{16}O$. *Org. Geochem*. **21**, 585-594.

Chikaraishi Y., Kashiyama Y., Ogawa N.O., Kitazato H. and Ohkouchi N. (2007) Biosynthetic and metabolic controls of nitrogen isotopic composition of amino acids in marine macroalgae and gastropods: Implications for aquatic food web studies. *Mar. Ecol-Prog. ser.* **342**, 85-90.

Chikaraishi Y., Ogawa N.O., Kashiyama Y., Takano Y., Suga H., Tomitani A., Miyashita H., Kitazato H. and Ohkouchi N. (2009) Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. *Limnol. Oceanogr. Methods* **7**, 740-750.

Chikaraishi Y., Ogawa N.O. and Ohkouchi N. (2010) Further evaluation of the trophic level estimation based on nitrogen isotopic composition of amino acid. In: Earth, Life, and Isotopes (Ohkouchi N., Tayasu I., Koba K., Eds), pp. 37-51, Kyoto: Kyoto University Press.

Chikaraishi Y., Ogawa N.O., Doi H. and Ohkouchi N. (2011) $^{15}N/^{14}N$ ratios of amino acids as a tool for studying terrestrial food webs: a case study of terrestrial insects (bees, wasps, and hornets). *Ecol. Res*. **26**, 835-844.

Craine J.M., Elmore A.J., Aidar M.P.M., Bustamante M., Dawson T.E., Hobbie E.A., KahmenA., Mack M.C., McLauchlan K.K., Michelsen A., Nardoto G.B., Pardo L.H., Peñuelas J., Reich P.B., Schuur E.A.G., Stock W.D., Templer P.H., Virginia R.A., Welker J.M. and Wright I.J. (2009) Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytologist* **183**, 980-992.

DeNiro M.J. and Epstein S. (1981) Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* **45**, 341-351.

Hayes J.M., Freeman K.H., Popp B.N. and Hoham C.H. (1990) Compound-specific isotopic analyses: A novel tool reconstruction of ancient biogeochemical processes. *Org. Geochem*. **16**, 1115-1128.

Kelly J.F. (2000) Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. *Can. J. Zool*. **78**, 1-27.

Lorrain A., Graham B., Menard F., Popp B., Bouillon S., Breugel P. and Cherel Y. (2009) Nitrogen and carbon isotope values of individual amino acids: a

tool to study foraging ecology of penguins in the Southern Ocean. *Mar. Ecol. Prog. Ser*. **391**, 293-306.

McCarthy M.D., Benner R., Lee C. and Fogel M.L. (2007) Amino acid nitrogen isotopic fractionation patterns as indicators of heterotrophy in plankton, particulate and dissolved organic matter. *Geochim. Cosmochim. Acta* **71**, 4727-4744.

McClelland J.W. and Montoya J.P. (2002) Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. *Ecology* **83**, 2173-2180.

McCutcha Jr J.H., Lews Jr W.M., Kendall C. and McGrth C.C. (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. OIKOS **201**, 378-390.

Minagawa M. and Wada E. (1984) Stepwise enrichment of ^{15}N along food chains: Further evidences and the relation between δ^{15}N and animal age. *Geochim. Cosmochim. Acta* **48**, 1135-1140.

Naito Y.I., Noah V., Honch N.Y., Chikaraishi Y., Ohkouchi N. and Yoneda M. (2010) Quantitative evaluation of marine protein contribution in ancient diets based on nitrogen isotope ratios of individual amino acids in bone collagen: An investigation at the Kitakogane Jomon site. *Am. J. Phy. Anthropol*. **143**, 31-40.

O'Reilly C.M., Hecky R.E., Cohen A.S. and Plisnier P.-D. (2002) Interpreting stable isotopes in food webs: Recognizing the role of time averaging at different trophic levels. *Limnol. Oceanogr*. **47**, 306-309.

Roff C. (2000) Seasonal variation in δ^{13}C and δ^{15}N of size-fractionated plankton at a coastal station in the northern Baltic proper. *Mar. Ecol. Prog. Ser*. **203**, 47-65.

Vander Zanden J. and Rasmussen J.B. (2001) Variation in δ^{15}N and δ^{13}C trophic fractionation: Implications for aquatic food web studies. *Limnol. Oceanogr.* **46**, 2061-2066.

Yoshioka T., Hayashi H. and Wada E. (1989) Seasonal variations of carbon and nitrogen isotope ratios of plankton and sinking particles in Lake Kizaki. *Jpn J. Limnol*. **50**, 313-320.