Intra-trophic isotopic discrimination of $^{15}$N/$^{14}$N for amino acids in autotrophs: Implications for nitrogen dynamics in ecological studies

Yuko Takizawa$^{1,2,3}$ | Prarthana S. Dharampal$^4$ | Shawn A. Steffan$^{4,5}$ | Yoshinori Takano$^3$ | Naohiko Ohkouchi$^3$ | Yoshito Chikaraishi$^{2,3}$

Abstract

The differential discrimination of nitrogen isotopes ($^{15}$N/$^{14}$N) within amino acids in consumers and their diets has been routinely used to estimate organismal trophic position (TP). Analogous isotopic discrimination can occur within plants, particularly in organs lacking chloroplasts. Such discrimination likely arises from the catabolic deamination of amino acids, resulting in a numerical elevation of estimated TP, within newly synthesized biomass. To investigate this phenomenon, we examined the $^{15}$N/$^{14}$N of amino acids ($\delta^{15}$N$_{AA}$) in spring leaves and flowers from eight deciduous and two annual plants. These plants were classified on the basis of their time of bloom, plants that bloomed when their leaves were absent (Type I) versus plants that bloomed while leaves were already present (Type II). Based on the $\delta^{15}$N$_{AA}$ values from leaves, both plant types occupied comparable and ecologically realistic mean TPs (=1.0 ± 0.1, mean ± 1σ). However, the estimated TPs of flowers varied significantly (Type I: 2.2 ± 0.2; Type II: 1.0 ± 0.1). We hypothesize that these results can be interpreted by the following sequence of events: (1) Type I floral biomass is synthesized in absence of active photosynthesis; (2) the catabolic deamination of amino acids in particular, leaves behind $^{15}$N in the residual pool of amino acids; and (3) the incorporation of these $^{15}$N-enriched amino acids within the biomass of Type I flowers results in the numerical elevation of the TPs. In contrast, the actively photosynthesizing Type II leaves energetically sustain the synthesis of Type II flower biomass, precluding any reliance on catabolic deamination of amino acids. Amino acids within Type II flowers are therefore isotopically comparable to the Type II leaves. These findings demonstrate the idiosyncratic nature of the $\delta^{15}$N$_{AA}$ values within autotrophic organs and have implications for interpreting trophic hierarchies using primary producers and their consumers.

Keywords

food web, isotopic fractionation, plant phenology, trophic position, winter dormancy, $\delta^{15}$N

1 Introduction

Amino acid metabolism within consumers causes predictable enrichment in their $^{15}$N compared to their diets. By quantifying the magnitude of this isotopic discrimination among unique amino acids through compound-specific isotope analysis (CSIA), ecologists have achieved unprecedented insights into complex food webs (e.g., Batista, Ravelo, Crusius, Casso, & McCarthy, 2014; Chikaraishi, Kashiya, Ogawa, ...
and storage biomass (after Buchanan et al., 2000; McClelland & Monteoya, 2002; Naito et al., 2016; Popp et al., 2007; Sackett, Drazen, Choy, Popp, & Pitz, 2015; Sherwood, lehmann, Schuber, Scott, & McCarthy, 2011; Steffan, Chikaraishi, Currie, et al., 2015). Chikaraishi et al. (2009) measured this "inter"-trophic isotopic discrimination between consumers and their diets and established the following equation (1) to estimate the trophic position (TP<sub>Tr/Src</sub>) of organisms in food webs:

\[
TP_{\text{Tr/Src}} = \left( \frac{\delta^{15}N_{\text{Tr}} - \delta^{15}N_{\text{Src}} - \beta_{\text{Tr/Src}}}{\text{TDF}_{\text{Tr/Src}}} \right) + 1
\]

where \(\delta^{15}N_{\text{Tr}}\) and \(\delta^{15}N_{\text{Src}}\) denote stable nitrogen isotopic composition of trophic (Tr, including alanine, valine, isoleucine, proline, and glutamic acid) and source (Src, including methionine and phenylalanine) amino acids in a single organism examined, respectively; \(\beta_{\text{Tr/Src}}\) denotes the isotopic difference between Tr and Src amino acids in primary producers at the base of food webs; and TDF<sub>Tr/Src</sub> (=\(\Delta\delta^{15}N_{\text{Tr}} - \Delta\delta^{15}N_{\text{Src}}\)) stands for the net intertrophic discrimination factor of Tr and Src amino acids between a consumer and its diet. By investigating several pairs of Tr and Src amino acids, Chikaraishi et al. (2009) identified glutamic acid and phenylalanine as the best combination to return the most accurate estimation of the trophic position of consumers (TP<sub>Glu/Prol</sub>). Since then, several studies further demonstrated significant variation in the isotopic discrimination of amino acids (Blanke et al., 2017; Chikaraishi, Steffan, Takano, & Ohkouchi, 2015; McMahon, Thorrold, Elsdon, & McCarthy, 2015).

With the exception of photosynthesis, there are several metabolic parallels between plants and heterotrophs (Figure 1, cf: Buchanan, Grissem, & Jones, 2000). For example, plants can store photosynthetically fixed energy in form of carbohydrates, lipids, and/or amino acids (Buchanan et al., 2000; Chapin, Schulze, & Mooney, 1990; Kermode, 2011; Millard, 1996). During periods of lean photosynthesis, such as heterotrophs, the catabolism of these storage compounds releases energy (i.e., ATP) that is subsequently used for the anabolism of new constituents (Buchanan et al., 2000), particularly in organs without chloroplasts (e.g., flower and root). If this catabolism within autotrophic biomass involves the deamination of amino acids, the resulting residual pool of amino acids (particularly for Tr amino acids) will be more enriched in \(^{15}\text{N}\) than the original source pool. The mobilization and assimilation of these \(^{15}\text{N}\)-enriched amino acids may generate isotopic differences between the source pool and newly synthesized biomass. Unlike "inter"-trophic isotopic discrimination that involves two separate organisms with unique trophic identities (e.g., Chikaraishi et al., 2007), "intra"-trophic isotopic discrimination arises as a result of catabolic deamination of storage amino acids among different tissues.

**FIGURE 1** Schematic illustration of the catabolism and anabolism in (a) plants and (b) animals: The solar energy is fixed as organic molecules (e.g., amino acids, lipids, and carbohydrates) during photosynthesis in plants, and the fixed energy is released during metabolic breakdown of complex to simple molecules (i.e., catabolism) in both plants and animals; these energies are used during anabolism to construct of their body and storage biomass (after Buchanan et al., 2000)
Takizawa and Chikaraishi (2014) first reported that sweet potato sprout grown in the absence of light has an unusually high $\text{TP}_{\text{Glu}}/\text{Phe}$ value of 2.2. Given that spraying occurred in dark, and in absence of photosynthesis, sprout biomass likely recorded the $^{15}\text{N}$-enrichment derived from the amino acid deamination during catabolism.

We hypothesize that plant organs lacking chloroplasts may undergo intratrophic isotopic discrimination via the aforementioned mechanisms. This leads to an increased $\delta^{15}\text{N}_n$ values in these organs, and therefore to an ecologically erroneous overestimation ($\text{TP}_{\text{Glu}}/\text{Phe} > 1.0$) for plant trophic position. The objective of our study was to investigate whether indeed there was a measurable amount of intratrophic isotopic discrimination between chloroplast-bearing leaves and chloroplast-lacking flowers, and to assess its implication for trophic position calculation.

2 | Materials and Methods

2.1 | Leaf and flower samples

Flowers and mature leaves of eight deciduous trees and two annual plants were collected in their blooming season (February–May) from either a farm or a house garden in Yugawara, Japan (35°08′N, 139°07′E) (Table S1). These plants commonly begin to grow leaves in spring and completely lose their leaves in autumn. They were classified into Type I and Type II, with respect to the timing of their spring and complete leaf loss in autumn. They were classified into Type I and Type II, with respect to the timing of their bloom relative to leaf emergence (Figure 2). Type I plants included four stone fruit plants (Amygdalus persica, Cerasus lannesiana, Cerasus pseudocerasus, and Prunus mume) and one wisteria (Wisteria floribunda). These plants bloom for about 2–3 weeks prior to the emergence of their first leaves. Type II plants included three deciduous tree species (Akebia quinata, Benthamidia japonica, and Hydrangea macrophylla) and two annual plant species (Cucumis sativus and Solanum melongena). These plants bloom for 2–3 weeks (for deciduous plants), or continually for 1–2 months (for annual plants) only after their leaves have emerged. The flowers of the Type II plants were collected approximately halfway through the spring bloom. Both Type I and Type II plants were chosen as they are commonly found in agricultural area and/or house gardens in the temperate region of Japan. Approximately ten leaves and ten flowers were collected for each plant. The collected samples were cleaned with distilled water to remove surface contaminants, homogenized to a fine powder using a Tube-Mill (IKA, Staufen, Germany), freeze-dried, and then stored at −20°C.

2.2 | Analysis of the $\delta^{15}\text{N}_{\text{AA}}$ values

The samples were prepared for the $\delta^{15}\text{N}_{\text{AA}}$ analysis after HCl hydrolysis and N-pivaloyl/isopropyl (Pv/iPr) derivatization, according to the procedure in Chikaraishi et al. (2009). In brief, the homogenized samples were hydrolyzed using 12 M HCl at 110°C overnight (>12 hr). The hydrolysate was washed with n-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. The derivatization was performed sequentially with thionyl chloride/2-propanol (1/4, v/v) at 110°C for 2 hr, and pivaloyl chloride/dichloromethane (1/4, v/v) at 110°C for 2 hr. The $\delta^{15}\text{N}_{\text{AA}}$ values were determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) using a

![Figure 2](https://example.com/figure2.png)

**FIGURE 2** The periods of blooming and leafing for the plants induced in the present study. Flowers of the Type I plants (Amygdalus persica, Cerasus lannesiana, Cerasus pseudocerasus, Prunus mume, and Wisteria floribunda) bloomed for 2–3 weeks in the leafless period or just prior to the leafing period in spring. Flowers of the Type II plants bloomed for 2–3 weeks (for deciduous tree plants: Akebia quinata, Benthamidia japonica, and Hydrangea macrophylla) or continually for 1–2 months (for annual plants: Cucumis sativus and Solanum melongena) during the mature-leaf period. Diamond symbols approximately indicate the collection dates (Table S1) of the flowers and leaves in the present study.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Alanine</th>
<th>Glycine</th>
<th>Valine</th>
<th>Leucine</th>
<th>Isoleucine</th>
<th>Proline</th>
<th>Serine</th>
<th>Glutamic acid</th>
<th>Phenylalanine</th>
<th>TP&lt;sub&gt;Glut/Phe&lt;/sub&gt;</th>
<th>TDF&lt;sub&gt;Glut/Phe&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave.</td>
<td>SD</td>
<td>Ave.</td>
<td>SD</td>
<td>Ave.</td>
<td>SD</td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
</tr>
<tr>
<td>Type I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
</tr>
<tr>
<td>Amygdalus persica</td>
<td>-13.1</td>
<td>0.1</td>
<td>-16.7</td>
<td>0.2</td>
<td>-14.2</td>
<td>0.2</td>
<td>-13.1</td>
<td>0.1</td>
<td>-13.0</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Cerasus lannesiana</td>
<td>-3.1</td>
<td>0.2</td>
<td>-13.0</td>
<td>0.4</td>
<td>-0.1</td>
<td>0.4</td>
<td>-4.2</td>
<td>0.2</td>
<td>-5.7</td>
<td>0.5</td>
<td>6.9</td>
</tr>
<tr>
<td>Cerasus pseudocerasus</td>
<td>-4.5</td>
<td>0.4</td>
<td>-17.4</td>
<td>0.5</td>
<td>-3.5</td>
<td>0.5</td>
<td>-5.7</td>
<td>0.4</td>
<td>-4.4</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Prunus mume</td>
<td>3.5</td>
<td>0.4</td>
<td>-12.8</td>
<td>0.2</td>
<td>7.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0.6</td>
<td>1.3</td>
<td>0.3</td>
<td>10.0</td>
</tr>
<tr>
<td>Wisteria floribunda</td>
<td>2.1</td>
<td>0.6</td>
<td>1.1</td>
<td>0.8</td>
<td>3.8</td>
<td>0.3</td>
<td>5.1</td>
<td>0.3</td>
<td>13.3</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Amygdalus persica</td>
<td>-6.6</td>
<td>0.6</td>
<td>-15.2</td>
<td>0.3</td>
<td>-6.3</td>
<td>0.1</td>
<td>-10.0</td>
<td>0.4</td>
<td>-0.4</td>
<td>0.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Cerasus lannesiana</td>
<td>-1.7</td>
<td>0.1</td>
<td>-14.4</td>
<td>0.5</td>
<td>-0.3</td>
<td>0.2</td>
<td>-7.0</td>
<td>0.3</td>
<td>-2.8</td>
<td>0.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Cerasus pseudocerasus</td>
<td>-3.3</td>
<td>0.3</td>
<td>-7.0</td>
<td>0.4</td>
<td>2.7</td>
<td>0.1</td>
<td>-1.2</td>
<td>0.4</td>
<td>-0.4</td>
<td>0.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Prunus mume</td>
<td>-2.0</td>
<td>0.7</td>
<td>-8.2</td>
<td>0.7</td>
<td>0.5</td>
<td>0.6</td>
<td>7.3</td>
<td>0.3</td>
<td>5.8</td>
<td>0.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Wisteria floribunda</td>
<td>-3.4</td>
<td>0.3</td>
<td>-14.3</td>
<td>0.5</td>
<td>-4.0</td>
<td>0.4</td>
<td>-9.1</td>
<td>0.7</td>
<td>-3.3</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
<td>Ave.</td>
</tr>
<tr>
<td>Akebia quinata</td>
<td>-16.4</td>
<td>0.3</td>
<td>-25.6</td>
<td>0.2</td>
<td>-20.2</td>
<td>0.3</td>
<td>-17.5</td>
<td>0.2</td>
<td>-18.7</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Benthamidia japonica</td>
<td>-12.1</td>
<td>0.2</td>
<td>-22.9</td>
<td>0.3</td>
<td>-10.4</td>
<td>0.4</td>
<td>-10.0</td>
<td>0.4</td>
<td>-12.4</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Cucumis sativus</td>
<td>-1.3</td>
<td>0.5</td>
<td>-15.7</td>
<td>0.4</td>
<td>-2.1</td>
<td>0.2</td>
<td>-2.3</td>
<td>0.1</td>
<td>-2.7</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Hydrangea macrophylla</td>
<td>-3.7</td>
<td>0.4</td>
<td>-12.6</td>
<td>0.4</td>
<td>-2.1</td>
<td>0.2</td>
<td>-0.7</td>
<td>0.6</td>
<td>-1.7</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Solanum melongena</td>
<td>-0.2</td>
<td>0.4</td>
<td>-18.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>0.2</td>
<td>2.5</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Akebia quinata</td>
<td>-24.9</td>
<td>0.3</td>
<td>-16.8</td>
<td>0.2</td>
<td>-16.6</td>
<td>0.2</td>
<td>-7.2</td>
<td>0.6</td>
<td>0.9</td>
<td>0.12</td>
<td>-0.9</td>
</tr>
<tr>
<td>Benthamidia japonica</td>
<td>-14.9</td>
<td>0.7</td>
<td>-30.8</td>
<td>0.5</td>
<td>-9.7</td>
<td>0.2</td>
<td>-13.2</td>
<td>0.2</td>
<td>-4.9</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Cucumis sativus</td>
<td>-1.1</td>
<td>0.4</td>
<td>-16.6</td>
<td>0.1</td>
<td>-0.9</td>
<td>0.2</td>
<td>-1.2</td>
<td>0.1</td>
<td>-1.6</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Hydrangea macrophylla</td>
<td>-3.1</td>
<td>0.5</td>
<td>-11.3</td>
<td>0.6</td>
<td>-1.4</td>
<td>0.3</td>
<td>-1.0</td>
<td>0.1</td>
<td>-0.5</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Solanum melongena</td>
<td>-2.2</td>
<td>0.2</td>
<td>-14.9</td>
<td>0.0</td>
<td>-2.2</td>
<td>0.3</td>
<td>-4.5</td>
<td>0.1</td>
<td>-7.1</td>
<td>0.2</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

The $\delta^{15}N$ value was determined by triplicate analysis for each sample.

$TP_{Glut/Phe} = \left[\delta^{15}N_{Glut} - \delta^{15}N_{Phe} + 8.4\right]/7.6 + 1$.

$TDF_{Glut/Phe} = \left[\delta^{15}N_{Flower,Glut} - \delta^{15}N_{Flower,Phe}\right] - \beta$. 
6890N GC (Agilent Technologies, Palo Alto, USA) instrument coupled to a DeltaplusXP IRMS instrument through combustion (950°C) and reduction (550°C) furnaces, a countercurrent dryer (Permeable membrane, Nafion™), and a liquid nitrogen CO₂ trap via a GC-C/TC III interface (Thermo Fisher Scientific, Bremen, Germany). The Py/iPr derivatives were injected using a programmable temperature vaporizing (PTV) injector (Gerstel, Mülheim, Germany) into an HP Ultra-2 capillary column (50 m; i.d. 0.32 mm; film thickness 0.52 μm; Agilent Technologies). The carrier gas (He) flow rate was maintained at 1.4 ml/min. To assess the reproducibility of the isotope measurement, a standard amino acid reference mixture (Indiana University, Bloomington, USA; Si science co., Sugito-machi, Japan) was analyzed after every five or six sample runs, with three pulses of reference N₂ gas discharged at the beginning and end of each run. The δ¹⁵N values were expressed relative to the isotopic composition of atmospheric nitrogen (AIR) on scales normalized to known δ¹⁵N values of the reference amino acids. The accuracy and precision for the reference mixtures were 0.0‰ (mean of Δ) and 0.3–0.5‰ (mean of 1σ) for sample sizes of ≥0.5 nmol N, respectively. The δ¹⁵N values of alanine, glycine, valine, leucine, isoleucine, proline, serine, glutamic acid, and phenylalanine were determined for sample leaves and flowers (Table 1), based on the S/N ratio of ≥20 with baseline separation on the chromatogram. All analyses were performed in triplicate, and the precision (1σ) for the δ¹⁵N values in the sample amino acids was 0.0–0.8‰ (mean: 0.3 ± 0.2‰).

2.3 Calculation of the TP Glu/Phe values

The TP Glu/Phe value was calculated from the observed δ¹⁵N values of glutamic acid (δ¹⁵N Glu) and phenylalanine (δ¹⁵N Phe), using equation (1) with the βTP Glu/Phe and TDFGlu/Phe (inter-TDF Glu/Phe) being -8.4 ± 1.6‰ and +7.6 ± 1.2‰, respectively, values commonly applied for terrestrial samples from previous studies (Chikaraishi, Ogawa, Doi, & Ohkouchi, 2011; Chikaraishi, Ogawa, & Ohkouchi, 2010; Chikaraishi et al., 2014).

2.4 Statistical analysis

Independent samples Mann–Whitney U test was used to compare the TP Glu/Phe values of Type I leaves versus Type I flowers, and Type II leaves versus Type II flowers. Here, the null hypothesis was that TP Glu/Phe values of leaves and flowers from any particular plant type would be indistinguishable. Independent samples Mann–Whitney U test was used to compare the TP Glu/Phe values between Type I and Type II leaves, and independent samples t test was used to compare the TP Glu/Phe values between Type I and Type II flowers. The null hypothesis assumed that the TP Glu/Phe values of both leaves type would be comparable, as would both flower types. One-sample Wilcoxon signed rank test was used to compare the TP Glu/Phe values of Type I and Type II leaves, and one-sample t test was used to compare Type I and Type II flowers to a test value of 1.0. The null hypothesis underlying these tests was that any plant sample whether leaves or flowers would have TP Glu/Phe = 1.0.

3 Results and Discussion

3.1 The δ¹⁵NAA and TP Glu/Phe values in leaves and flowers

Leaves and flowers fell within a similar but wide range in the δ¹⁵NAA value within Type I and Type II plants (Type I leaves = −2.5 ± 8.2‰; Type I flowers = −3.5 ± 7.9‰; Type II leaves = −6.3 ± 9.2‰; Type II flowers = −5.9 ± 9.2‰; mean ± 1σ, Table 1). As expected, TP Glu/Phe value for both Type I and Type II leaves reported a mean of 1.0 ± 0.1 (Figure 3), consistent with previously reported values (1.0 ± 0.2) of plant samples such as leaves, nuts, and sap (Chikaraishi et al., 2011, 2014; Steffan et al., 2013). However, the TP Glu/Phe value of Type I flowers (2.2 ± 0.2) was significantly higher than that of Type II flowers (1.0 ± 0.1) (tₜ = 10.63, p < .001). Additionally, the TP Glu/Phe value of Type I flowers was significantly higher than the functional trophic position of autotrophs (TP = 1.0) in any ecosystem (tₜ = 11.05, p < .001).
Conversely, the TP\textsubscript{Glu/Phe} value of Type II flowers was virtually identical to the expected trophic position values (Figure 3). The TP\textsubscript{Glu/Phe} value of Type I flowers was significantly higher than that of Type I leaves (TP\textsubscript{Flower} − TP\textsubscript{Leaf} = 1.2 ± 0.3; U = 25.0, \( p = .008 \)). However, there was no such difference between the TP\textsubscript{Glu/Phe} value of Type II flowers and leaves (TP\textsubscript{Flower} − TP\textsubscript{Leaf} = 0.0 ± 0.1).

### 3.2 Energy resources for blooming

As primary producers, plants occupy TP = 1.0 (Elton, 1927; Lindeman, 1942), a value that has been validated using the CSIA method (Chikaraishi et al., 2011; McCarthy et al., 2013; Steffan et al., 2013). However, whether this well-documented trophic identity applies to all organs within an individual plant has not been fully investigated (Takizawa & Chikaraishi, 2014). Our results indicate that even within a single plant, the \( \Delta \delta^{15}N\textsubscript{Glu−Phe} \) value (and therefore the TP\textsubscript{Glu/Phe} value) of different organs can vary significantly (Figures 3 and 4). We propose possible physiological scenarios that could contribute to this isotopic heterogeneity between leaves and flowers of Type I and Type II plants, potentially skewing their trophic identities.

Flowers have limited/no photosynthetic capacity and must therefore rely on other sources of energy to support their bloom. The elevation in the TP\textsubscript{Glu/Phe} value (>1.0) in Type I flowers indicates that the Tr amino acids used to synthesize the flower biomass were enriched in \( ^{15}N \). One possible mechanism to explain this enrichment is that in Type I plants, bloom occurs before the appearance of leaves (i.e., in the absence of photosynthetically fixed energy). During this time, over-wintered storage compounds are broken down to liberate metabolic energy (i.e., ATP) required to sustain Type I bloom. In case of stored proteins, the deamination of amino acids can preferentially eliminate the \( ^{14}N \) amino group as ammonia, leaving behind the enriched \( ^{15}N \) in the residual pool of Tr amino acids (Figure 5). If the enriched end products of deamination are used to assimilate Type I flowers, it would explain the enrichment of \( ^{15}N \) in floral Tr amino acids. Previous studies have shown that antifreeze protein helps deciduous trees to survive through winter dormancy (Arora et al., 1992; Hon, Griffith, Mlynarz, Kwok, & Yang, 1995). Because these proteins are not required during spring, they may be subsequently deaminated (Arora et al., 1992). The residual pool of \( ^{15}N \)-enriched amino acids generates “intra”-trophic discrimination, especially in Tr amino acids (e.g., glutamic acid), and when incorporated in newly synthesized Type I floral tissue, inflates their TP\textsubscript{Glu/Phe} value (Figure 5).
Type II plants represent a different phenology where bloom occurs in the presence of actively photosynthesizing leaves. As energy fixed by active photosynthesis is sufficient to support bloom, this may preclude the necessity of deamination of amino acids (Figure 5), which can explain the negligible intra-trophic isotopic discrimination of amino acids in Type II plants. Therefore, the $TDF_{\text{Glu/Phe}}$ value of Type II leaves and Type II flowers remains comparable with each other, and to the expected value of 1.0.

### 3.3 Intra-trophic isotopic discrimination

We suggest the following equation (2) to illustrate the intra-TDF of glutamic acid and phenylalanine ($TDF_{\text{Glu/Phe}}$) in plant organs (e.g., flowers):

$$TDF_{\text{Glu/Phe}} = \left( \delta^{15}N_{\text{Organ,Glu}} - \delta^{15}N_{\text{Organ,Phe}} \right) - \beta$$

where the subscript Organ indicates the plant organ of interest. The $\beta$ should be derived from the $\delta^{15}N$ value offset between glutamic acid and phenylalanine that has never undergone the deamination via plant catabolism. As our data show no substantial deamination in both Type I and Type II leaves, the standard $\beta$ value (-8.4 ± 1.6‰, Chikaraishi et al., 2010) reported for primary producers was incorporated in equation (2).

Our data show that the $TDF_{\text{Glu/Phe}}$ for Type I flowers was 9.3 ± 1.9‰, elevating $TDF_{\text{Glu/Phe}}$ values of flowers correspondingly by 1.2 ± 0.3 trophic units than expected (Figure 3). This high $TDF_{\text{Glu/Phe}}$ value can be explained by a large $TDF_{\text{Glu/Phe}}$ probably as an outcome of the assimilation of deaminated amino acids. Type II flowers were most likely sustained by foliar photosynthesis during bloom, resulting in a low $TDF_{\text{Glu/Phe}}$ (0.0 ± 0.5‰), and a $TDF_{\text{Glu/Phe}}$ value ~1.0, as expected. Moreover, when equation (2) is applied to the previously reported data for sprout and sweet potato ($TDF_{\text{Glu/Phe}} = 2.2$ and 1.4, respectively) (Takizawa & Chikaraishi, 2014), the $TDF_{\text{Glu/Phe}}$ is 9.1‰ for the sprout, and 2.8‰ for the sweet potato, indicative of strong reliance on deamination of amino acids for sprouting under dark conditions.

Interestingly, three of the five Type I plants (A. persica, C. lannesiana, and W. floribunda) included in this study started sprouting leaves from middle to end of the blooming period (Figure 2). It is plausible that these newly sprouted leaves are initially catabolically supported and later sustain themselves as photosynthesis ramps up. If so, the first few leaves to sprout could also present with higher TDF, elevating their $TDF_{\text{Glu/Phe}}$ value temporarily, before returning to ecologically realistic values as the season progresses. Although we currently do not have sufficient data to support this assumption, further research is required to investigate intratrophic discrimination in leaves sprouting early in the season.

The $TDF_{\text{Glu/Phe}}$ will be a useful parameter to assess the magnitude of deamination of amino acids, with respect to the energy consumption in specific phenology, within plants. Several factors could result in variation of the $TDF_{\text{Glu/Phe}}$ including length of dormancy relative to growing season (short versus long), flower biomass relative to storage amino acids (small versus large), and availability of storage lipids and/or carbohydrates as alternative catabolic energy sources (low versus high). With further research, the $TDF$ parameter could potentially be extended and used to investigate unusually high trophic positions of herbivores ($TDF_{\text{Glu/Phe}} > 2.0$).

#### 3.4 The $\delta^{15}N$ values of phenylalanine in leaves and flowers

There was a large variation in the $\delta^{15}N$ value of phenylalanine within Type I and Type II plants (Type I leaves = 6.5 ± 7.1‰; Type II leaves = 0.7 ± 3.6‰ and Type II leaves = 2.8 ± 9.3‰; Type II flowers = 2.3 ± 7.8‰), as well as a difference in phenylalanine value between leaves and flowers ($\Delta\delta^{15}N_{\text{Leaf,Phe}} = \delta^{15}N_{\text{Flower,Phe}} - \delta^{15}N_{\text{Leaf,Phe}}$) within a plant (Type I $\Delta\delta^{15}N_{\text{Phe}} = -5.8 ± 5.3‰, and Type II $\Delta\delta^{15}N_{\text{Phe}} = -0.4 ± 2.8‰$). Such large variability in the $\delta^{15}N_{\text{Phe}}$ and $\Delta\delta^{15}N_{\text{Phe}}$ values is uncharacteristic, given that it is a Src amino acid. However, this variation is consistent with data published in previous studies using leaves collected from the same farm (10.6 ± 3.8‰) (Chikaraishi et al., 2011, 2014). One likely source of such variability is the temporal and spatial heterogeneity in the abundance and $\delta^{15}N$ values of organic and inorganic nitrogen sources (NH$_4^+$, NO$_3^-$, and N$_2$) in soils. The timing of bloom was different among the plants examined (Figure 2), and this could have introduced temporal variability in their $\delta^{15}N$ values of phenylalanine. Additionally, the rate of incorporation of this isotopically variable phenylalanine may vary between leaves and flowers within a plant. However, evaluating the difference in the $\delta^{15}N$ value between phenylalanine and other amino acids ($\Delta\delta^{15}N_{\text{X-Phe}} = \delta^{15}N_X - \delta^{15}N_{\text{Phe}}$) can control for this background heterogeneity. Our data show that, notwithstanding this background noise, there was a significantly large difference between leaves and flowers for all examined amino acids in Type I plants ($\Delta\delta^{15}N_{\text{X-Phe}} = 9.0 ± 2.1‰), but was negligible in Type II plants ($\Delta\delta^{15}N_{\text{X-Phe}} = 0.3 ± 0.8‰) (Figure 4). Therefore, the background heterogeneity in the $\delta^{15}N$ value of phenylalanine does not sufficiently explain the high $\delta^{15}N_{\text{Phe}}$ value for Type I plants (Figure 4) and likely did not affect the numeral inflation of the $TDF_{\text{Glu/Phe}}$ values of Type I flowers (Figure 3). While our data show a clear pattern of differential isotopic discrimination in amino acids among plant tissues, we recommend further investigation using additional representative organs from Type I and Type II plants to evaluate the covariances in the $TDF_{\text{Glu/Phe}}$ value and $TDF_{\text{Glu/Phe}}$.  

#### 4 Implications

CSIA has expanded the ecologists’ toolbox by allowing high-resolution insights into trophic interactions. However, little information is available about the factors controlling inter- and/or intratrophic isotopic discrimination of amino acids in plants, animals, fungi, and bacteria (Chikaraishi et al., 2015; Gutiérrez-Rodríguez, Décima, Popp, & Landry, 2014; McMahon et al., 2015; Steffan, Chikaraishi, Currie, et al., 2015). Our findings reveal unique isotopic heterogeneity among wild plant organs, which can confound trophic estimations of these plants and the consumers that they support. For example, we will feeding
on Type I pollen in early spring elevate the TP\textsubscript{Glu/Phe} of pollinators and nectarivores above that expected of herbivores (>2.0)? How will the preferential feeding of herbivores on early spring flowers or new leaves imprint on the trophic positions of higher order consumers? What will be the trophic identity of the detritus derived from Type I flowers as they become a basal resource in the brown food web?

Ideally, the TP\textsubscript{Glu/Phe} (or TP\textsubscript{Tr/Sr}) value enables isotopic ecologists to deduce the ecological function (e.g., primary producer, herbivore, omnivore, and carnivore) of organisms (Bradley et al., 2015; Chikaraishi et al., 2014; Nielsen et al., 2015; Steffan, Chikaraishi, Horton, et al., 2015). However, our data indicate that the TP\textsubscript{Glu/Phe} value does not always reflect an organism’s functional trophic position in the food web. For instance, although Type I flowers returned a TP\textsubscript{Glu/Phe} value of 2.2, such value is typical of omnivores (TP > 2.0) and certainly does not represent the “functional” trophic identity of plants and their organs. Therefore, it appears that during sum of intra- and intertrophic isotopic discriminations, organismal TP\textsubscript{Glu/Phe} values may represent “energetic” tendencies rather than the organism’s true functional trophic position in food webs. These differences between the energetic and functional trophic positions arising from intratrophic isotopic discrimination can complicate food web studies. We therefore encourage continued investigations to reevaluate how CSIA-derived trophic position correlates with the $\delta^{15}$N\textsubscript{AA} values of organisms in food webs.

**ACKNOWLEDGMENTS**

We thank Prof. Masanobu Yamamoto and Dr. Eitaro Wada for expert advice and constructive discussion. Three anonymous reviewers and handing editors are greatly acknowledged for improving the manuscript. This work was supported by JSPS KAKENHI Grant Number JP 16J01704 to Y. Takizawa, Grant-in-Aid for Scientific Research of the JSPS to Y. Chikaraishi, Y. Takano, and N. Ohkouchi, funds from Silicone Industry Association of Japan to Y. Chikaraishi, and USDA-ARS appropriated funds to S.A. Steffan.

**CONFLICT OF INTEREST**

None declared.

**REFERENCES**


**SUPPORTING INFORMATION**

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

**How to cite this article:** Takizawa Y, Dharampal PS, Steffan SA, Takano Y, Ohkouchi N, Chikaraishi Y. Intra-trophic isotopic discrimination of $^{15}$N/$^{14}$N for amino acids in autotrophs: Implications for nitrogen dynamics in ecological studies. *Ecol Evol*. 2017;7:2916–2924. https://doi.org/10.1002/ece3.2866