Vol. 727: 81–90, 2024
https://doi.org/10.3354/meps14503

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published January 25

Amino acid nitrogen isotopic compositions show seep copepods gain nutrition from host animals

Naoto F. Ishikawa[1,*], Chong Chen[2], Rina Hashimoto[3], Nanako O. Ogawa[1], Daisuke Uyeno[3], Hidetaka Nomaki[2]

[1]Biogeochemistry Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

[2]X-STAR, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

[3]Graduate School of Science and Engineering, Kagoshima University, 1-21-35 Korimoto, Kagoshima 890-0065, Japan

ABSTRACT: Deep-sea chemosynthetic ecosystems harbour high biomass centred around animals with chemoautotrophic symbionts. Despite being intensively studied over the last 4 decades, microscopic animals associated with and/or parasitic on dominating holobionts remain understudied. Here, we combine bulk tissue isotope analysis for carbon and nitrogen and compound-specific isotope analysis of amino acids (CSIA-AA) for nitrogen to elucidate the relationship between 2 copepod—host pairs from the Off Hatsushima hydrothermal seep in Sagami Bay, Japan: the vesicomyid clam *Phreagena okutanii* and *Hyphalion sagamiense* living on its mantle, and the tubeworm *Lamellibrachia columna* and *Dirivultus kaiko* found on its plume. Bulk tissue isotope analyses found overall large variations in $\delta^{13}C$ and $\delta^{15}N$ values across both associations, and did not allow conclusions on the trophic relationship between each pair. In contrast, CSIA-AA for *Phreagena—Hyphalion* clearly showed trophic positions ($TP_{Glu/Phe}$) increasing from gill (symbiont) to adductor muscle (host tissue) to *H. sagamiense*. In the case of *Lamellibrachia—Dirivultus*, a similar increase in $TP_{Glu/Phe}$ was found from the plume to *D. kaiko*. These results show that both *H. sagamiense* and *D. kaiko* are nutritionally dependent on their respective hosts and therefore should be considered parasites despite being from copepod families that are typically not recognised as parasitic. Our CSIA-AA results represent the first use of this technique to document host—parasite relationships in chemosynthetic ecosystems. Understanding the role of parasites is of great importance in reconstructing energy flow in ecosystems, and our results underscore the promising nature of CSIA-AA in revealing their otherwise hidden relationships.

KEY WORDS: Chemosynthesis · Copepods · CSIA-AA · Deep sea · Parasitism

1. INTRODUCTION

Chemosynthesis-based ecosystems in the deep sea consist of dense communities mostly centred around specially adapted symbiotic fauna, first discovered in hydrothermal vents in the Galápagos Rift (Corliss et al. 1979) and subsequently in hydrocarbon seeps and organic falls (Levin 2005). Chemoendosymbiotic animals like vesicomyid clams and siboglinid tubeworms are powered by dense bacteria hosted in specific tissues of their body (gill and trophosome, respectively). Though faunal diversity is now relatively well-characterised in many chemosynthetic systems around the globe (Chapman et al. 2019), the 'hidden diversity' of meiofauna, and especially parasites, has been largely ignored and overlooked (de Buron & Morand 2004).

*Corresponding author: ishikawan@jamstec.go.jp

This is despite parasitic and epibiotic fauna being extremely important in understanding biodiversity and energy flow, as they may account for over half of total animal diversity (Weinstein & Kuris 2016). Recently, it was realised that copepods associated with charismatic symbiotic animals often occur at vents and seeps (Uyeno et al. 2020). Quantifying energetic contributions from hosts to associated fauna can discern the nature of the association between them (e.g. parasitic or not), and is vital in understanding the ecosystem-level energy flow because parasitism is believed to represent ca. 75% of all trophic links in food webs (Dobson et al. 2008, Lafferty et al. 2008).

Stable carbon and nitrogen isotope ($\delta^{13}C$ and $\delta^{15}N$) analysis has been used to assess the energetic dependence of associates on their hosts for over 2 decades in a variety of ecosystems (Doucett et al. 1999, Deudero et al. 2002, Stapp & Salkeld 2009). In chemosynthetic ecosystems, stable isotope analyses have almost exclusively used bulk tissue (e.g. Alfaro-Lucas et al. 2018, Ke et al. 2022). The only parasite–host relationship characterised using this method at vents and seeps is between the scale worm *Branchipolynoe* and its bathymodioline mussel host (Ke et al. 2022), though in addition to host tissue this kleptoparasitic worm also feeds on suspended organic matter (Britayev et al. 2007). However, there is a growing body of evidence that the $\delta^{13}C$ and $\delta^{15}N$ values of bulk tissues give only a limited clue to the entire picture of parasitic interactions (e.g. Pinnegar et al. 2001). This is because (1) trophic discrimination factors (TDFs) for $\delta^{13}C$ and $\delta^{15}N$ typically found in prey–predator relationships (on average 0.4 and 3.4‰, respectively, both with large variations; McCutchan et al. 2003) may not necessarily be identical to those found through parasitism (Thieltges et al. 2019), and/or (2) bulk tissue isotope values often show high variability among individuals which obscures their true relationship (Ohkouchi et al. 2015), especially at chemosynthetic systems where great variance in $\delta^{15}N$ is typical (Van Dover 2002).

Compound-specific isotope analysis of amino acids (CSIA-AA) for nitrogen ($\delta^{15}N$) is a more precise method for estimating trophic positions (TPs) of organisms (Chikaraishi et al. 2009, Ohkouchi 2023). By using a pair of trophic and source amino acids, such as glutamic acid and phenylalanine with large and small TDFs, respectively, the difference between the amino acid pair can clearly show the TP (i.e. $TP_{Glu/Phe}$), even in systems where the nitrogen isotope baseline is variable. Steffan et al. (2013) successfully elucidated a 4-level host–parasite hierarchy in insects consisting of apple leaves, apple aphid, hover fly, parasitoid, and hyperparasitoid using this method. Riekenberg et al. (2021) applied CSIA-AA to a host oyster species, *Crassostrea gigas*, infected by a parasite copepod, *Mytilicola orientalis*, and showed that the TP value of the parasite *M. orientalis* estimated from CSIA-AA was significantly different from that estimated from the conventional bulk tissue $\delta^{13}C$ and $\delta^{15}N$ analysis. Though these exemplify that the CSIA-AA method is excellent for disentangling relationships between hosts and associated fauna as well as hosts and endosymbionts in chemosynthetic ecosystems (Vokhshoori et al. 2021), such applications remain limited to date.

Here, we aimed to apply the CSIA-AA method in conjunction with conventional bulk $\delta^{13}C$ and $\delta^{15}N$ analysis to shed light on the true relationship between symbiotic hosts and their associated copepods in a chemosynthetic setting. The Off Hatsushima site in Sagami Bay, where a chemosynthetic system was first discovered in Japan, is a cool hydrothermal seep sustained by a warm end-member fluid predicted to be between 34 and 54°C (Tsunogai et al. 1996). This site is richly inhabited by tubeworms, mussels, and clams that exhibit endosymbiosis with chemoautotrophic bacteria. Among these, the clausidiid copepod *Hyphalion* has been found to be associated with the tubeworm genus *Lamellibrachia* and the dirivultid copepod in the genus *Dirivultus* with the vesicomyid clam genus *Phreagena* (Toda et al. 1992, Uyeno et al. 2020). As these copepods are members of families that are not obligatorily parasitic, it has been unclear whether they are parasites or simply using the hosts as substrates. We hypothesized that if the copepods are parasites, then (1) the $\delta^{15}N$ values of phenylalanine (source amino acid) should be consistent between host and copepod and (2) the $TP_{Glu/Phe}$ values of copepods should be one unit higher than those of their respective hosts.

2. MATERIALS AND METHODS

We sampled the Off Hatsushima site during a research cruise on board the RV 'Yokosuka' using the human-occupied vehicle (HOV) 'Shinkai 6500'. Individuals of the vesicomyid clam *Phreagena okutanii* were collected from a clam bed (35° 0.9589' N, 139° 13.3308' E at 856 m depth; Fig. 1A) using a scoop sampler, and individuals of the tubeworm *Lamellibrachia columna* were plucked from the seafloor using the manipulator (35° 0.9396' N, 139° 13.4104' E at 921 m depth; Fig. 1D). Upon recovery of samples on deck, host animals were first examined for the presence of associ-

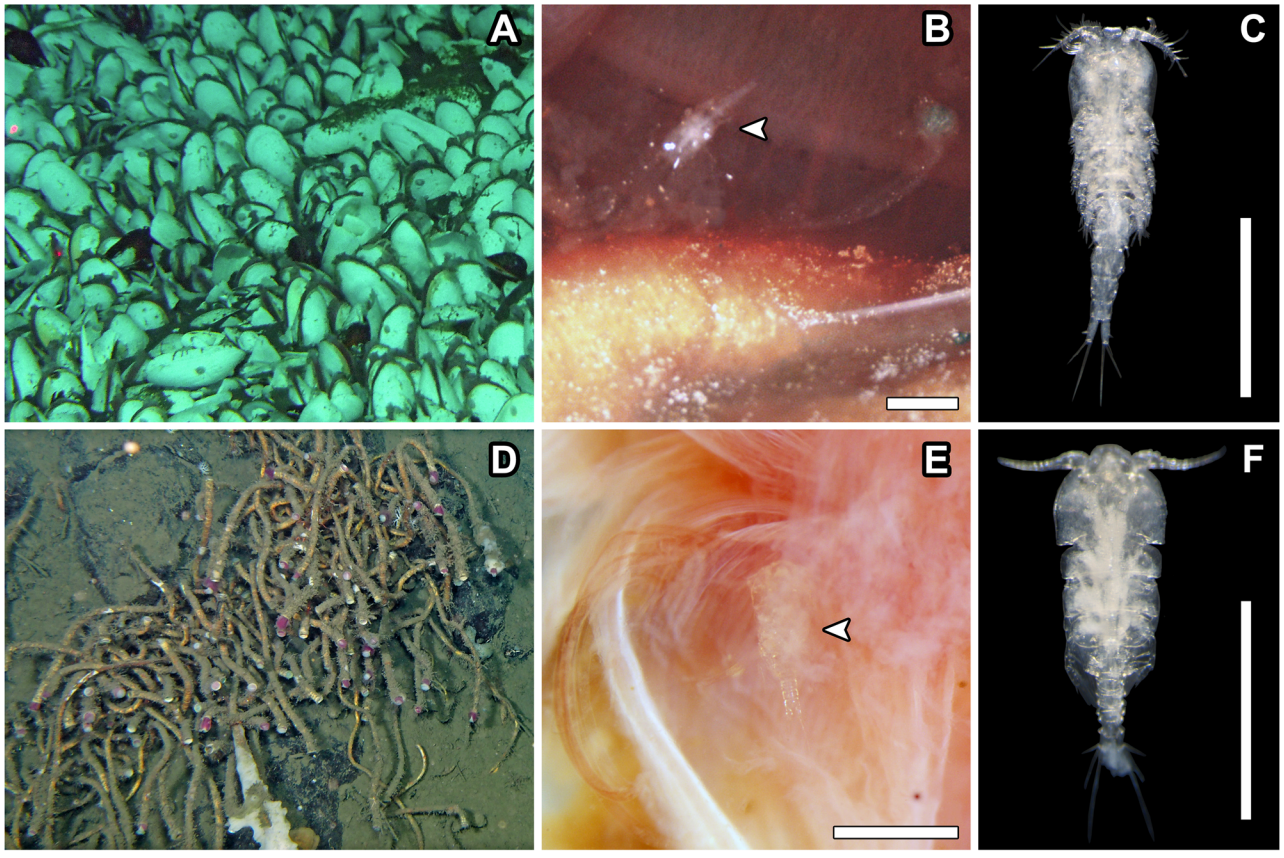

Fig. 1. Samples collected from the Off Hatsushima seep. (A) Vesicomyid clam colony; (B) the copepod *Hyphalion sagamiense* inside the mantle cavity of the vesicomyid clam *Phreagena okutanii*; (C) an individual of *H. sagamiense*; (D) a bouquet of the tubeworm *Lamellibrachia columna*; (E) the copepod *Dirivultus kaiko* on the plume of *L. columna*; (F) an individual of *D. kaiko*. Scale bars = 1 mm

ated copepods. Between 3 and 55 individuals of associated copepods per host individual were manually extracted under a microscope (Fig. 1). Host animal tissues were then rinsed, cleaned, and dissected into the following parts: for vesicomyid clams, the gill containing the sulfur-oxidising symbiont and the adductor muscle representing the host tissue; for tubeworms, the trophosome containing the sulfur-oxidising symbionts and the plume. From another 3 individuals of *P. okutanii*, the adductor muscle and the mantle were also sampled in order to compare the isotopic differences between these 2 non-symbiotic tissues (Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m727p081_supp1.pdf). All samples were placed in plastic containers and stored at −20°C before they were freeze-dried, ground into fine powders, and defatted with methanol and dichloromethane, with the exception of copepods for bulk isotope analysis (Ohkouchi et al. 1997).

Bulk tissue $\delta^{13}C$ and $\delta^{15}N$ in the samples were measured with a Flash EA1112 elemental analyzer, modified for an ultra-small-scale measurement (>1.8 µgC and >0.2 µgN), connected to a Delta Plus XP isotope ratio mass spectrometer (IRMS) with a Conflo III interface (Thermo Finnigan) (Ogawa et al. 2010). From the host tissue, 5 replicate individuals for the clam *P. okutanii* and 3 individuals for the tubeworm *L. columna* were sampled (Table S1 in Supplement 2 at www.int-res.com/articles/suppl/m727p081_supp2.xlsx). From 2 vesicomyid clams, 3 copepod individuals per host were analysed for bulk tissue isotope analyses; from 3 tubeworms, 2 or 3 copepod individuals were selected per host. The $\delta^{13}C$ and $\delta^{15}N$ values reported herein are relative to those of the international standards — Vienna Pee Dee Belemnite and atmospheric N_2, respectively — as follows:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 10^3 \quad (1)$$

where $R = \frac{^{13}C}{^{12}C}$ or $\frac{^{15}N}{^{14}N}$

The data were calibrated using 3 interlaboratory-consensus reference materials (standard name, $\delta^{13}C$,

and $\delta^{15}N$ as follows: BG-A, −26.9‰, and −1.7‰; BG-P, −10.3‰, and +13.5‰; and BG-T, −20.8‰, and +8.7‰) (Tayasu et al. 2011) and 3 in-house reference materials (BG-LC-G, −13.4‰, and −5.4‰; BG-GC-G, −13.4‰, and −5.7‰; and SK-GC-Vm, +0.2‰, and +60.4‰). The analytical errors of the $\delta^{13}C$ and $\delta^{15}N$ measurements obtained by the repeated analyses were less than ±0.13‰ for $\delta^{13}C$ (n = 19, 1.8–18.6 µgC) and less than ±0.15‰ for $\delta^{15}N$ (n = 19, 0.23–4.32 µgN).

Another set of samples was prepared for CSIA-AA according to published methods (Ishikawa et al. 2022). In brief, the samples were hydrolysed with 12 mol l^{-1} hydrochloric acid at 110°C for 12 h, defatted with *n*-hexane/dichloromethane (3/2, v/v), and derivatized sequentially with thionyl chloride/isopropanol (1/4, v/v) and pivaloyl chloride/dichloromethane (1/4, v/v). Three host individuals associated with abundant (over 20) copepods were used for both vesicomyid clams and tubeworms (the same 3 individuals of *P. okutanii* were included in the bulk tissue analyses). From each host individual, 20–55 copepods were pooled together for CSIA-AA. Prior to $\delta^{15}N$ measurements, all samples were injected into a gas chromatograph (GC) (6890N, Agilent Technologies) with a nitrogen and phosphorous detector to determine molar compositions of amino acids (Fig. S2 in Supplement 1). The molar ratios of glycine relative to glutamic acid (Gly/Glu) were calculated to estimate contributions from chemosynthetic bacteria to the host tissues (Vokhshoori et al. 2021). The $\delta^{15}N$ values of glutamic acid and phenylalanine ($\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$) of samples were determined using the Delta Plus XP IRMS connected to another GC (6890N, Agilent Technologies) via an interface (GC Combustion III, Thermo Finnigan) with combustion and reduction furnaces (Thermo Finnigan). The data were calibrated by a linear regression line using the working standard consisting of 9 amino acids between measured and known $\delta^{15}N$ values: alanine, +43.3‰; glycine, −26.6‰; leucine, +6.2‰; norleucine, +19.0‰; aspartic acid, +35.2‰; methionine, −3.8‰; glutamic acid, −5.7‰; phenylalanine, +1.7‰; and hydroxyproline, −9.2‰ (Ohkouchi et al. 2017). This standard mixture covered the entire variation in the amino acid $\delta^{15}N$ in our samples (from −14.0‰ to +16.8‰). Valine, isoleucine, and proline are not included in the working standard; however, their $\delta^{15}N$ values in the samples were corrected using the linear regression line common to all amino acids, and their corrected $\delta^{15}N$ values were reported unless the data did not meet our analytical criteria. We set these stringent criteria for data reporting because each amino acid was combusted and reduced after GC separation, and the $\delta^{15}N$ of ionized N_2 in which the original compound is no longer identifiable was measured by IRMS. In our samples, some unidentified impurities co-eluted with target compounds and the target peak was not necessarily separated from another peak on the chromatogram. Therefore, $\delta^{15}N$ data that did not meet our peak-definition criteria (Ishikawa et al. 2022) were strictly rejected. The TPs of samples were estimated using $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values (i.e. $TP_{Glu/Phe}$) as follows:

$$TP_{Glu/Phe} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta}{TDF_{Glu} - TDF_{Phe}} + 1 \quad (2)$$

where β is the initial difference (‰) between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ found in marine primary producers (i.e. −3.4 ± 0.9‰) and TDF_{Glu} and TDF_{Phe} are the offset of the trophic discrimination factors (‰) for $\delta^{15}N_{Glu}$ (+8.0 ± 1.2‰) and $\delta^{15}N_{Phe}$ (+0.4 ± 0.5‰), respectively (Chikaraishi et al. 2009).

The following parametric tests were conducted after normality and homogeneity of variances were examined using Shapiro-Wilk and Bartlett's tests, respectively. To test whether the Gly/Glu, $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$, and $TP_{Glu/Phe}$ are significantly different among gill, adductor muscle, and *H. sagamiense* in *P. okutanii* as well as among trophosome, plume, and *D. kaiko* in *L. columna*, ANOVA was performed. Between the groups that showed significant differences, post hoc multiple comparisons were conducted using Tukey's HSD. The paired *t*-test was used to test isotopic differences between the adductor muscle and mantle of *P. okutanii*. All statistics and graphing were conducted using MATLAB version 2023a with the statistics and machine-learning toolbox (Mathworks). The significance level was set at $\alpha = 0.05$.

3. RESULTS

All copepods from the vesicomyid clam *Phreagena okutanii* were identified as *Hyphalion sagamiense* Toda, Miura & Nemoto, 1992 based on morphology (Toda et al. 1992); the copepods were found exclusively in the mantle cavity of the clams, where they were mostly attached on the mantle while none were found in the gill. On the other hand, all copepods from the siboglinid tubeworm *Lamellibrachia columna* were a different species of copepod, *Dirivultus kaiko* Uyeno, Kakui, Watanabe & Fujiwara, 2020 (Uyeno et al. 2020). The vast majority of the *D. kaiko* individuals (111 out of 123) were found attached to the plume of *L. columna*.

Bulk $\delta^{13}C$ values for *P. okutanii* clams were consistent between gill (n = 3, mean ± SD: −37.6 ± 0.2‰) and adductor muscle (n = 3, −37.0 ± 0.2‰), while bulk $\delta^{15}N$ values were higher in adductor muscle (n = 3, −7.1 ± 1.1‰) than in gill (n = 3, −10.9 ± 1.2‰) (Fig. 2A). The associated copepod *H. sagamiense* bulk isotope values were much more variable ($\delta^{13}C$: n = 6, −37.6 ± 2.0‰; and $\delta^{15}N$: n = 6, −5.8 ± 0.8‰) than the host, especially in carbon. By contrast, bulk $\delta^{13}C$ and $\delta^{15}N$ values for *L. columna* tubeworms were vastly more variable for all types of analysed tissues than *P. okutanii*: trophosome (n = 3, $\delta^{13}C$: −24.6 ± 3.5‰; $\delta^{15}N$: −0.1 ± 1.3‰); plume (n = 3, $\delta^{13}C$: −25.2 ± 4.1‰; $\delta^{15}N$: 1.8 ± 1.6‰); and *D. kaiko* (n = 8, $\delta^{13}C$: −25.8 ± 3.7‰; $\delta^{15}N$: 2.6 ± 1.4‰) (Fig. 2B). In both cases, the copepods generally tended to have higher $\delta^{15}N$ values than the host animals.

The differences in Gly/Glu as an indicator for chemosynthesis between sample types were not significant for either the *Phreagena*—*Hyphalion* association or the *Lamellibrachia*—*Dirivultus* association (Table 1; Table S2 in Supplement 2). Regarding the difference between the adductor muscle (used for isotope measurements and the mantle tissue where *H. sagamiense* was found in *P. okutanii* clams; Fig. S1), none of $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$, or $TP_{Glu/Phe}$ showed significant differences between these 2 tissue types (Table S3 in Supplement 2).

Table 1. Parametric tests for Gly/Glu, $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$ and $TP_{Glu/Phe}$ data of the *Phreagena*–*Hyphalion* and *Lamellibrachia*–*Dirivultus* associations. TP: trophic position

Association	Predictor variable	Response variable	Shapiro-Wilk test		Bartlett's test		ANOVA				
			W	p	χ^2	p	Sum of Squares (SS)	df	Mean of SS	*F*	p
Phreagena–*Hyphalion*	Gill, adductor muscle and *H. sagamiense*	Gly/Glu	>0.77	>0.05	0.23	0.89	0.61	2	0.30	3.97	0.11
		$\delta^{15}N_{Glu}$	>0.94	>0.54	4.71	0.09	375	2	187	2150	<0.001
		$\delta^{15}N_{Phe}$	>0.83	>0.18	3.60	0.17	4.69	2	2.35	2.74	0.18
		$TP_{Glu/Phe}$	>0.89	>0.37	1.83	0.40	5.14	2	2.57	208	<0.001
Lamellibrachia–*Dirivultus*	Trophosome, Plume and *D. kaiko*	Gly/Glu	>0.78	>0.07	1.69	0.43	0.73	2	0.37	3.00	0.16
		$\delta^{15}N_{Glu}$	>0.79	>0.09	0.89	0.64	76.3	2	38.1	62.4	0.001
		$\delta^{15}N_{Phe}$	>0.82	>0.22	1.16	0.56	1.19	2	0.60	0.88	0.48
		$TP_{Glu/Phe}$	>0.87	>0.29	1.89	0.39	1.39	2	0.69	20.5	<0.001

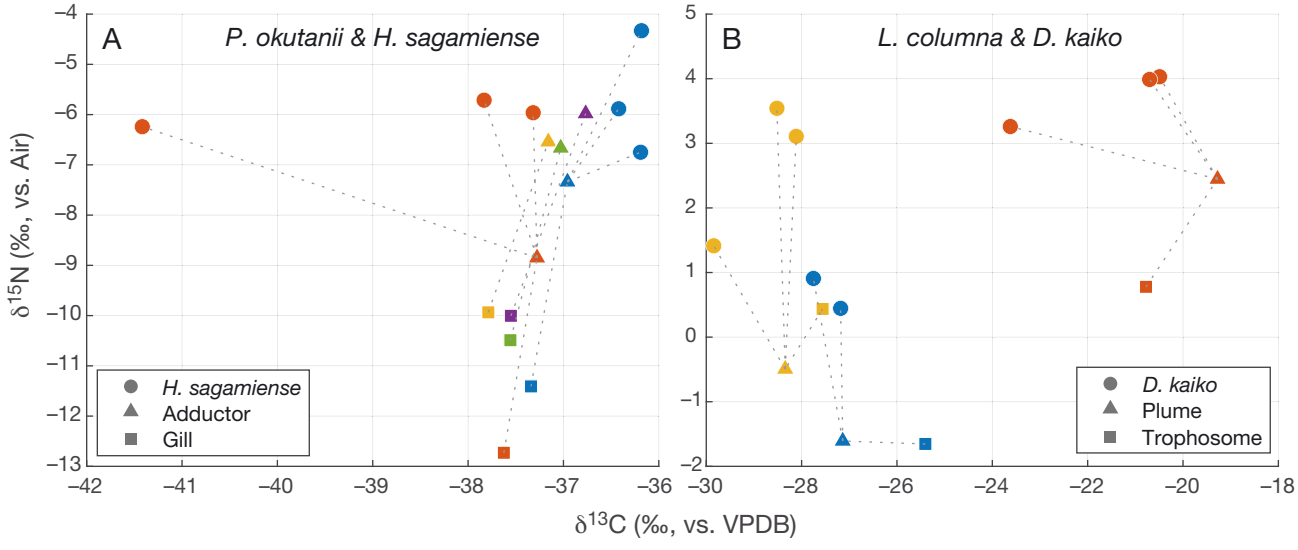

Fig. 2. Bulk $\delta^{13}C$ and $\delta^{15}N$ values for (A) gill and adductor muscle of *Phreagena okutanii* and the associated copepod *Hyphalion sagamiense*, and (B) trophosome and plume of *Lamellibrachia columna* and the associated copepod *Dirivultus kaiko*. Dotted lines link gill (or plume) and copepod samples to the same host individual. Different colours indicate different host—copepod individual sets. VPDB: Vienna Pee Dee Belemnite

The $\delta^{15}N_{Phe}$ values were consistent between gill (n = 3, −12.4 ± 1.4‰) and adductor muscle (n = 3, −12.1 ± 1.2‰) for *P. okutanii* (Fig. 3) as well as the copepod *H. sagamiense* (n = 3, −10.8 ± 0.2‰). The difference in $\delta^{15}N_{Phe}$ values between the 3 types of samples was not significant (Table 1). By contrast, the $\delta^{15}N_{Glu}$ values increased substantially in the order of gill (n = 3, −5.8 ± 0.6‰) and adductor muscle (n = 3, −0.5 ± 0.1‰) of the host and *H. sagamiense* (n = 3, +9.7 ± 0.2‰) (Fig. 3), which were significantly different from each other (Table 1; Tukey's HSD, $p < 0.001$). Their resulting $TP_{Glu/Phe}$ values were 1.4 ± 0.12, 2.1 ± 0.14, and 3.3 ± 0.04, respectively (Fig. 3), which were also significantly different from each other (Table 1; Tukey's HSD, $p < 0.005$).

The $\delta^{15}N_{Phe}$ values for the tubeworm *L. columna* were consistent between trophosome (n = 3, −3.8 ± 1.7‰), plume (n = 3, −2.9 ± 0.8‰), and the copepod *D. kaiko* (n = 3, −3.2 ± 2.1‰). The difference in $\delta^{15}N_{Phe}$ values between the 3 types of samples was not significant (Table 1). The $\delta^{15}N_{Glu}$ values of *L. columna* individuals increased from plume (n = 3, +8.8 ± 2.8‰) to trophosome (n = 3, +11.5 ± 2.1‰) and to *D. kaiko* (n = 3, +15.9 ± 1.3‰), all being significantly different from each other (Table 1; Tukey's HSD, $p < 0.03$). Their resulting $TP_{Glu/Phe}$ values were 2.1 ± 0.26, 2.6 ± 0.10, and 3.1 ± 0.10, respectively (Fig. 3); a significant difference was found only between plume and *D. kaiko* (Table 1; Tukey's HSD, $p = 0.007$).

4. DISCUSSION

4.1. Copepods associated with clams and tubeworms

The clausidiid copepod *Hyphalion sagamiense* was originally described from Off Hatsushima as an associate collected from the mantle cavity of the vesicomyid clam *Phreagena soyoae*, the sister species of

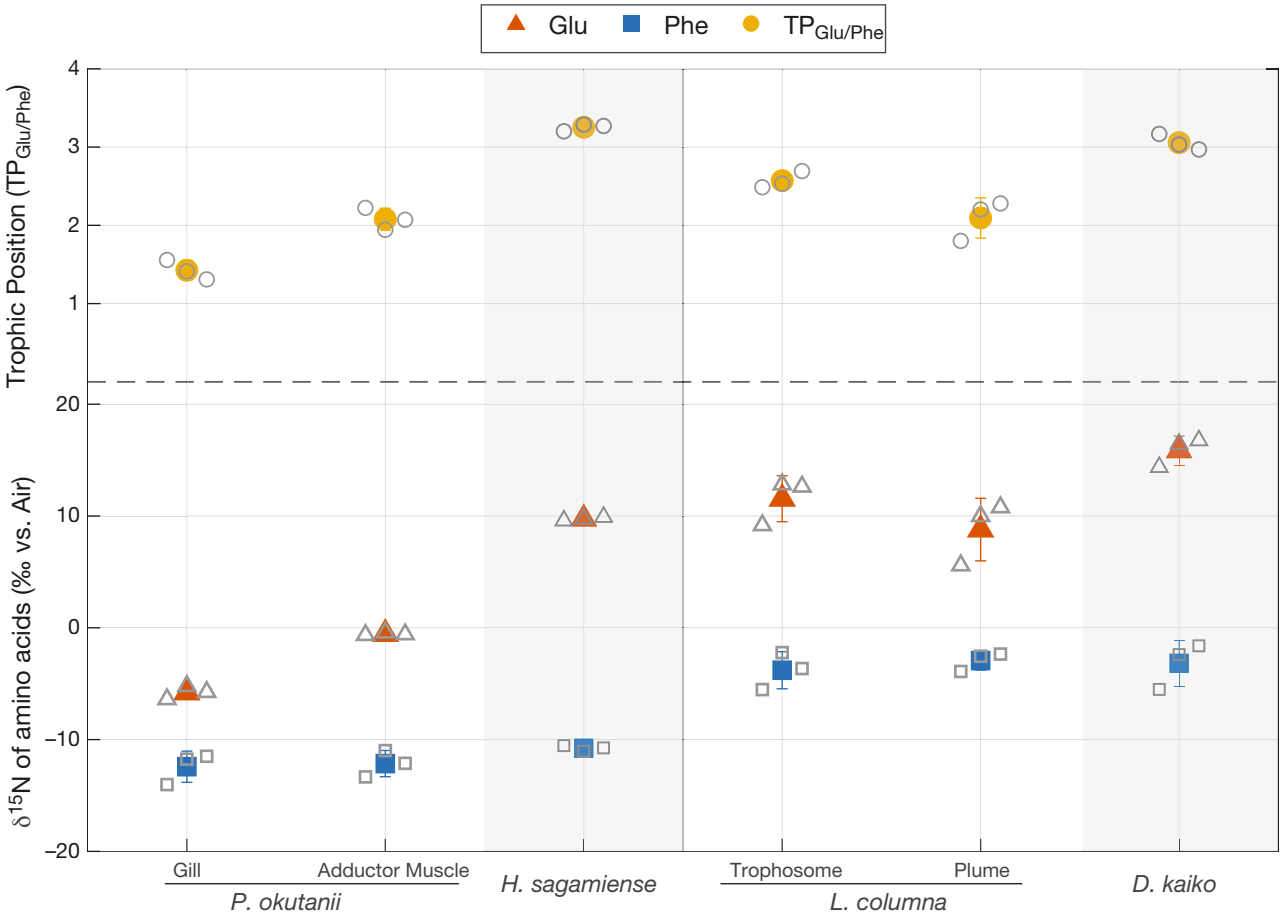

Fig. 3. Nitrogen stable isotope of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), and trophic position ($TP_{Glu/Phe}$) for gill and adductor muscle of *Phreagena okutanii* and the associated copepod *Hyphalion sagamiense*, as well as trophosome and plume of *Lamellibrachia columna* and the associated copepod *Dirivultus kaiko*. Mean and standard deviation are shown; individual data points are shown with open shapes

P. okutanii (Toda et al. 1992). Our discovery of *H. sagamiense* associated with *P. okutanii* confirms a second host species for this copepod. *Dirivultus kaiko* was named from specimens taken from the plume region of *Lamellibrachia columna* inhabiting a hydrothermal vent in Okinawa Trough (Uyeno et al. 2020). Although we found this same host-associate pair in Off Hatsushima, this is the first record for *D. kaiko* from a seep environment and also a range extension of the species. As *L. columna* is very widely distributed across the western Pacific, it is likely that *D. kaiko* also shares a similarly wide range and has simply been overlooked at other localities. Regarding the anatomical feeding mode of the 2 copepod species studied, dirivultids use their mouthparts (e.g. mandible, maxillule and maxilla) for feeding (Humes & Dojiri 1980, Heptner & Ivanenko 2002), and *Hyphalion* seems to use the same mouthparts. Morphological transformations, such as the fusing of segments, lack of setae, and reduction of size, which are common features in the mouthparts of parasitic species (Gollner et al. 2010), are observed in both copepod species studied herein. As such, there is no contradiction from morphological evidence that these copepods are parasitic. The insignificant differences in Gly/Glu between sample types indicate that the contributions of chemosynthetic bacteria to hosts and copepods are statistically indistinguishable.

4.2. *Phreagena–Hyphalion* association

Our bulk $\delta^{13}C$ and $\delta^{15}N$ data exhibit large variations, as previously reported from other chemosynthesis-based communities in the north-western Pacific (Yorisue et al. 2012, Yamanaka et al. 2015). The isotopic difference, with a small discrimination in $\delta^{13}C$ and 4‰ in $\delta^{15}N$ (Fig. 2), between the gill and adductor muscle of *P. okutanii* indicates a trophic step from the symbiotic bacteria to host tissue. This pattern is shared with published data on vesicomyid clams (Onishi et al. 2018) but contradicts a recent study which found that the difference in $\delta^{15}N$ values between the bathymodioline mussel *Bathymodiolus childressi* and its symbionts (~1‰) (Vokhshoori et al. 2021) was smaller than expected from the typical trophic discrimination in bulk $\delta^{15}N$ (3–4‰). From the bulk tissue results, the highly variable $\delta^{13}C$ and $\delta^{15}N$ values for *H. sagamiense* do not allow conclusions on the trophic relationship with the host clam. In contrast, the CSIA-AA data show consistent $\delta^{15}N_{Phe}$ values between all 3 sample types, and the stepwise increases in $\delta^{15}N_{Glu}$ values indicate that most of the nitrogen in the adductor muscle (i.e. host tissue) originates from the gill (i.e. symbionts) and the copepod *H. sagamiense* gains virtually all of its nitrogen from the host clam tissue. This is congruent with the fact that the mouthparts of *H. sagamiense* are compatible with parasitism, that most of them were found on the mantle (i.e. host tissue), and is further supported by the results that the adductor muscle was isotopically identical to the mantle tissue where *H. sagamiense* was found (Fig. S1). As such, our results show that *H. sagamiense* is a parasitic copepod feeding on *Phreagena* clams, an unusual ecology for the family Clausidiidae which seems to be typically non-parasitic. It should be noted that the consistent $\delta^{15}N_{Phe}$ values found in the *Phreagena–Hyphalion* association appear to be different from what was reported in Vokhshoori et al. (2021), although it is less reasonable that the $\delta^{15}N$ value of the source amino acid phenylalanine would change in such a semi-closed system with limited nitrogen sources. This is because most heterotrophs are unable to biosynthesize phenylalanine by themselves. Therefore, the $\delta^{15}N_{Phe}$ values of heterotrophs are expected to be identical to those of autotrophs in the closed system. The mean $TP_{Glu/Phe}$ value of the clam gill, which accommodates symbiotic bacteria, was slightly higher than 1 (i.e. autotrophic), but this can be explained either by the contribution from the host gill tissue or by a chemosynthesis-specific factor affecting β and TDF values in Eq. (2) (see also Section 4.4).

4.3. *Lamellibrachia–Dirivultus* association

The bulk $\delta^{15}N$ value for the copepod *D. kaiko* was on average ~3‰ higher than the plume and trophosome of the *L. columna* host, but large variations were found in both $\delta^{13}C$ and $\delta^{15}N$ values. The results might be caused by an isotopic heterogeneity in the inorganic carbon and nitrogen utilized by bacteria among the associations or by unknown factors specific to the chemosymbiosis controlling isotopic discriminations within the association. Unfortunately, this remains speculative in the present study, as more replications would be needed to determine whether this represents undesired noise or a real signal. In either case, these results preclude any conclusive interpretations about the trophic relationships between the host and the copepods. The CSIA-AA results illuminated a clear host–parasite trophic relationship between *D. kaiko* ($TP_{Glu/Phe}$ = 3.1 ± 0.10) and the plume ($TP_{Glu/Phe}$ = 2.1 ± 0.26) where the copepods were mostly found. This is again a rare case of a parasitic copepod in the family Dirivultidae.

However, Dirivultidae belongs to the order Siphonostomatoidea, which are predominantly parasitic, and *D. kaiko* likely represents a case of evolutionarily regaining parasitism following a loss. The $TP_{Glu/Phe}$ value of the trophosome (2.6 ± 0.10) was much higher than expected from being densely occupied by symbiotic sulfur-oxidizing bacteria. It is possible that the symbiont, which provides chemosynthetic energy to the host (*L. columna*), may in reverse exploit amino acid nitrogen from the host, or that glutamic acid in the host may be directly routed from the symbiont across a membrane with less ^{15}N fractionation. A recent study found that the chemosymbiotic bathymodioline mussel *Bathymodiolus japonicus* switches between receiving nutrients from symbionts directly and digesting the symbionts according to the availability of energy sources for the symbionts in the environment (Tame et al. 2023), and *L. columna* may exhibit similar mechanisms.

4.4. Using CSIA-AA to determine nutritional relationships

The working hypothesis of the present study, that *H. sagamiense* and *D. kaiko* are parasites of their respective clam and tubeworm hosts, was supported by the anatomical structures of these copepod species, the $TP_{Glu/Phe}$ results, and the consistent $\delta^{15}N_{Phe}$ values between hosts and copepods. The clam *P. okutanii* and the tubeworm *L. columna* occur sympatrically in our study site; however, their $\delta^{15}N_{Phe}$ values differed by 10‰, indicating that there is a large $\delta^{15}N$ variation at this cold seep site. It is not uncommon for the chemosynthesis-based ecosystems in the deep sea to have such a large variation in $\delta^{15}N$. For example, the bulk $\delta^{15}N$ values of invertebrates at the Kairei hydrothermal vent field in the Central Indian Ridge varied by 20‰, suggesting that the inorganic nitrogen utilized by their symbiotic bacteria have variable $\delta^{15}N$ values (Van Dover 2002). Even though the ambient environment is isotopically heterogeneous, the insignificant differences in $\delta^{15}N_{Phe}$ values between host and copepod in the present study suggest that they are likely to share the nitrogen source in the respective association. In addition to the anatomical structures and the $TP_{Glu/Phe}$ results, this isotopic homogeneity within the association provides another piece of evidence that the 2 copepods studied are parasitic.

Although our CSIA-AA results were able to clearly demonstrate the parasite–host relationships between the symbiotic seep animals and their associated copepods, bulk tissue $\delta^{13}C$ and $\delta^{15}N$ analyses were unable to do so. Large isotopic variations were seen in the bulk tissue data of both parasitic copepod species investigated, suggesting that the bulk $\delta^{13}C$ and $\delta^{15}N$ analysis does not offer sufficient resolution to detect trophic relationships between host and parasite. Our understanding of carbon and nitrogen dynamics during parasitic feeding is still limited, however. It is possible that our sample included copepod individuals with different times since settlement on the host, with varying amounts of lipid storage from the planktic larval stage on the surface layer (Nomaki et al. 2023), which potentially affects isotopic variations among the individuals. On the other hand, CSIA-AA eliminates this factor, as lipid and amino acid biosyntheses are decoupled in terms of nitrogen. Another possibility is that the baseline inorganic nitrogen source fluctuates both spatially and temporally in such hydrothermally influenced systems (Tsunogai et al. 1996), which would impact the $\delta^{15}N$ values seen. Furthermore, the parasitic copepods may move from one host individual to another, especially the species investigated herein which are not from typically parasitic families and are not physically embedded in host tissue. Even in such cases, using CSIA-AA can effectively clarify the $TP_{Glu/Phe}$ values since the differences between the pair of trophic and source amino acids would stay consistent. It should be noted that the β and TDF values in Eq. (2) are set at constants that have been empirically derived from marine photosynthetic ecosystems (Chikaraishi et al. 2009), although some previous studies reported variations in both β and TDF values (McMahon & McCarthy 2016, Ramirez et al. 2021). Since there is insufficient knowledge about these constants for chemosynthetic ecosystems, this uncertainty may be another reason that the $TP_{Glu/Phe}$ values herein for the adductor muscle of *P. okutanii* and the trophosome of *L. columna*, where endosymbiotic bacteria live, were higher than expected. The results provide an important benchmark for future studies in exploring β and TDF values in chemosynthetic ecosystems.

Our study is the first to use CSIA-AA to investigate host–parasite relationships in chemosynthetic ecosystems and adds to the evidence from the few studies to date in other systems (Steffan et al. 2013, Riekenberg et al. 2021) that the CSIA-AA is a valuable technique for unveiling otherwise clandestine host–parasite interactions. This is of paramount importance for quantifying the energetic contribution of parasitism to the entire trophic flow in natural ecosystems (Lafferty et al. 2008). Many copepods inhabiting the body

surface of larger animals have been traditionally considered to be commensals, but the host-associated copepods studied herein are nutritionally dependent on their hosts and highlight the possibility that many 'commensal' species may actually be parasites — warranting future research. Conversely, other parasites such as *Branchipolynoe* scale worms with only partial reliance have also been found in vents and seeps (Van Dover 2002, Britayev et al. 2007). The 'hidden diversity' of associated and parasitic fauna in chemosynthetic habitats is likely much greater than previously thought, as is their diversity in relationship to their hosts — and thus their roles in the energy flow of the ecosystem. We conclude that exploring more host–associate relationships in a broad range of environments is a promising focus target for future CSIA-AA studies.

Data archive. All data and MATLAB codes used in this study are available on Figshare (doi:10.6084/m9.figshare.22671004).

Acknowledgements. We thank the captain and crew of RV 'Yokosuka' as well as the HOV 'Shinkai 6500' team for their great help in our sample collection, Yoko Sasaki for her assistance with isotope measurements, and Naohiko Ohkouchi for insightful discussion. Valuable comments from 2 anonymous reviewers and Loïc N. Michel (University of Liège) improved an earlier version of the manuscript. This study was supported by JSPS KAKENHI grants (22K19857 and 23H02541).

LITERATURE CITED

Alfaro-Lucas JM, Shimabukuro M, Ogata IV, Fujiwara Y, Sumida PYG (2018) Trophic structure and chemosynthesis contributions to heterotrophic fauna inhabiting an abyssal whale carcass. Mar Ecol Prog Ser 596:1–12

Britayev TA, Martin D, Krylova EM, Von Cosel R, Aksiuk TS (2007) Life-history traits of the symbiotic scale-worm *Branchipolynoe seepensis* and its relationships with host mussels of the genus *Bathymodiolus* from hydrothermal vents. Mar Ecol 28:36–48

Chapman ASA, Beaulieu SE, Colaço A, Gebruk AV and others (2019) sFDvent: a global trait database for deep-sea hydrothermal-vent fauna. Glob Ecol Biogeogr 28: 1538–1551

Chikaraishi Y, Ogawa NO, Kashiyama Y, Takano Y and others (2009) Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. Limnol Oceanogr Methods 7: 740–750

Corliss JB, Dymond J, Gordon LI, Edmond JM and others (1979) Submarine thermal springs on the Galapagos Rift. Science 203:1073–1083

de Buron I, Morand S (2004) Deep-sea hydrothermal vent parasites: Why do we not find more? Parasitology 128: 1–6

Deudero S, Pinnegar JK, Polunin NVC (2002) Insights into fish host-parasite trophic relationships revealed by stable isotope analysis. Dis Aquat Org 52:77–86

Dobson A, Lafferty KD, Kuris AM, Hechinger RF, Jetz W (2008) Homage to Linnaeus: How many parasites? How many hosts? Proc Natl Acad Sci USA 105:11482–11489

Doucett RR, Giberson DJ, Power G (1999) Parasitic association of *Nanocladius* (Diptera: Chironomidae) and *Pteronarcys biloba* (Plecoptera: Pteronarcyidae): insights from stable-isotope analysis. J N Am Benthol Soc 18: 514–523

Gollner S, Ivanenko VN, Arbizu PM, Bright M (2010) Advances in taxonomy, ecology, and biogeography of Dirivultidae (Copepoda) associated with chemosynthetic environments in the deep sea. PLOS ONE 5:e9801

Heptner MV, Ivanenko VN (2002) Copepoda (Crustacea) of hydrothermal ecosystems of the World Ocean. Arthropoda Sel 11:117–134

Humes AG, Dojiri M (1980) A new siphonostome family (Copepoda) associated with a vestimentiferan in deep water off California. Pac Sci 34:143–151

Ishikawa NF, Ogawa NO, Sun Y, Chikaraishi Y, Takano Y, Ohkouchi N (2022) Integrative assessment of amino acid nitrogen isotopic composition in biological tissue samples determined by GC/C/IRMS, LC × EA/IRMS, and LC × GC/C/IRMS. Limnol Oceanogr Methods 20: 531–542

Ke Z, Li R, Chen Y, Chen D, Chen Z, Lian X, Tan Y (2022) A preliminary study of macrofaunal communities and their carbon and nitrogen stable isotopes in the Haima cold seeps, South China Sea. Deep Sea Res I 184:103774

Lafferty KD, Allesina S, Arim M, Briggs CJ and others (2008) Parasites in food webs: the ultimate missing links. Ecol Lett 11:533–546

Levin LA (2005) Ecology of cold seep sediments: interactions of fauna with flow, chemistry and microbes. Oceanogr Mar Biol 43:1–46

McCutchan JH Jr, Lewis WM Jr, Kendall C, McGrath CC (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. Oikos 102:378–390

McMahon KW, McCarthy MD (2016) Embracing variability in amino acid $\delta^{15}N$ fractionation: mechanisms, implications, and applications for trophic ecology. Ecosphere 7: e01511

Nomaki H, Kawatani K, Motomura Y, Tame A and others (2023) Bacterivory of the hydrothermal-vent-specific copepod *Stygiopontius senokuchiae* (Dirivultidae, Siphonostomatoida) from copepodite through adult stages. J Mar Biol Assoc UK 103:e21

Ogawa NO, Nagata T, Kitazato H, Ohkouchi N (2010) Ultra-sensitive elemental analyzer/isotope ratio mass spectrometer for stable nitrogen and carbon isotope analyses. In: Ohkouchi N, Tayasu I, Koba K (eds) Earth, life, and isotopes. Kyoto University Press, Kyoto, p 339–353

Ohkouchi N (2023) A new era of isotope ecology: nitrogen isotope ratio of amino acids as an approach for unraveling modern and ancient food web. Proc Jpn Acad, Ser B, Phys Biol Sci 99:131–154

Ohkouchi N, Kawamura K, Kawahata H, Taira A (1997) Latitudinal distributions of terrestrial biomarkers in the sediments from the Central Pacific. Geochim Cosmochim Acta 61:1911–1918

Ohkouchi N, Ogawa NO, Chikaraishi Y, Tanaka H, Wada E (2015) Biochemical and physiological bases for the use of

carbon and nitrogen isotopes in environmental and ecological studies. Prog Earth Planet Sci 2:1

Ohkouchi N, Chikaraishi Y, Close H, Fry B and others (2017) Advances in the application of amino acid nitrogen isotopic analysis in ecological and biogeochemical studies. Org Geochem 113:150–174

Onishi Y, Yamanaka T, Okumura T, Kawagucci S, Watanabe HK, Ohara Y (2018) Evaluation of nutrient and energy sources of the deepest known serpentinite-hosted ecosystem using stable carbon, nitrogen, and sulfur isotopes. PLOS ONE 13:e0199000

Pinnegar JK, Campbell N, Polunin NVC (2001) Unusual stable isotope fractionation patterns observed for fish host–parasite trophic relationships. J Fish Biol 59: 494–503

Ramirez MD, Besser AC, Newsome SD, McMahon KW (2021) Meta-analysis of primary producer amino acid $\delta^{15}N$ values and their influence on trophic position estimation. Methods Ecol Evol 12:1750–1767

Riekenberg PM, Joling T, IJsseldijk LL, Waser AM, van der Meer MTJ, Thieltges DW (2021) Stable nitrogen isotope analysis of amino acids as a new tool to clarify complex parasite–host interactions within food webs. Oikos 130: 1650–1664

Stapp P, Salkeld DJ (2009) Inferring host–parasite relationships using stable isotopes: implications for disease transmission and host specificity. Ecology 90:3268–3273

Steffan SA, Chikaraishi Y, Horton DR, Ohkouchi N and others (2013) Trophic hierarchies illuminated via amino acid isotopic analysis. PLOS ONE 8:e76152

Tame A, Maruyama T, Ikuta T, Chikaraishi Y and others (2023) mTORC1 regulates phagosome digestion of symbiotic bacteria for intracellular nutritional symbiosis in a deep-sea mussel. Sci Adv 9:eadg8364

Tayasu I, Hirasawa R, Ogawa NO, Ohkouchi N, Yamada K (2011) New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine–Earth Science and Technology. Limnology 12: 261–266

Thieltges DW, Goedknegt MA, O'Dwyer K, Senior AM, Kamiya T (2019) Parasites and stable isotopes: a comparative analysis of isotopic discrimination in parasitic trophic interactions. Oikos 128:1329–1339

Toda T, Miura T, Nemoto T (1992) *Hyphalion sagamiense*, a new species of Clausidiidae (Copepoda: Poecilostomatoida) associated with a vesicomyid bivalve from the Hatsushima cold-seep site in Sagami Bay, Japan. Proc Biol Soc Wash 105:102–111

Tsunogai U, Ishibashi J, Wakita H, Gamo T and others (1996) Fresh water seepage and pore water recycling on the seafloor: Sagami Trough subduction zone, Japan. Earth Planet Sci Lett 138:157–168

Uyeno D, Kakui K, Watanabe HK, Fujiwara Y (2020) Dirivultidae (Copepoda: Siphonostomatoida) from hydrothermal vent fields in the Okinawa Trough, North Pacific Ocean, with description of one new species. J Mar Biol Assoc UK 100:1283–1298

Van Dover C (2002) Trophic relationships among invertebrates at the Kairei hydrothermal vent field (Central Indian Ridge). Mar Biol 141:761–772

Vokhshoori NL, McCarthy MD, Close HG, Demopoulos AWJ, Prouty NG (2021) New geochemical tools for investigating resource and energy functions at deep-sea cold seeps using amino acid $\delta^{15}N$ in chemosymbiotic mussels (*Bathymodiolus childressi*). Geobiology 19:601–617

Weinstein SB, Kuris AM (2016) Independent origins of parasitism in Animalia. Biol Lett 12:20160324

Yamanaka T, Shimamura S, Nagashio H, Yamagami S and others (2015) A compilation of the stable isotopic compositions of carbon, nitrogen, and sulfur in soft body parts of animals collected from deep-sea hydrothermal vent and methane seep fields: variations in energy source and importance of subsurface microbial processes in the sediment-hosted systems. In: Ishibashi JI, Okino K, Sunamura M (eds) Subseafloor biosphere linked to hydrothermal systems: TAIGA concept. Springer, Tokyo, p 105–129

Yorisue T, Inoue K, Miyake H, Kojima S (2012) Trophic structure of hydrothermal vent communities at Myojin Knoll and Nikko Seamount in the northwestern Pacific: implications for photosynthesis-derived food supply. Plankton Benthos Res 7:35–40

Editorial responsibility: Sigrun Jónasdóttir, Charlottenlund, Denmark
Reviewed by: L. N. Michel and 1 anonymous referee, and previous version reviewed in MEPS by 2 anonymous referees

Submitted: September 7, 2023
Accepted: December 5, 2023
Proofs received from author(s): January 23, 2024