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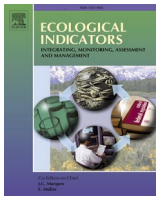

Isotopes of amino acids give novel insights on nitrogen sources partitioning and trophic position of invertebrates in a subtropical mangrove

Benoit Thibodeau [a,*], Laetitia Allais [b], Laura Elisabeth Agusto [c], Mandy Wing Kwan So [a,b], Stefano Cannicci [d,c,*]

[a] School of Life Sciences & Earth and Environmental Sciences Programme, The Chinese University of Hong Kong, Hong Kong SAR
[b] Department of Earth Sciences & Swire Institute of Marine Science, The University of Hong Kong, Hong Kong SAR
[c] Swire Institute of Marine Science, The University of Hong Kong, Hong Kong SAR
[d] Department of Biology, The University of Florence, Florence, Italy

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ABSTRACT

Mangrove forests are characterised by a specialised fauna with low ecological redundancy. Their faunal diversity is also decreasing across the tropics, with cascading effects on the ecosystem services provided by these forests. Traditional tools often failed to assess the importance of different sources of nutrients and a reliable trophic position estimate in mangroves, although this information is crucial to better advise on effective conservation strategies. Here, we present nitrogen and carbon isotope data of individual amino acids measured in primary producers and fauna inhabiting a subtropical mangrove. We quantified the relative importance of vascular and non-vascular source of nitrogen in the mangrove food web. We found that most gastropods mostly exclusively rely on nitrogen originating from the marine environment (non-vascular) while most brachyuran crabs integrate between 8 and 50% of their nitrogen from vascular sources, most probably by processing decaying mangrove leaves. This highlights the unique role of crabs in processing the low-nutritional mangrove leaves into the food web. Moreover, we estimated the trophic position for 17 invertebrate species inhabiting the mangrove. We highlighted previously unreported difference in term of trophic position and source of food for several species, which were previously thought to have overlapping feeding preferences. Our data thus suggest that the inherent lack of ecological redundancy present in mangrove may even be more severe than predicted by recent estimates.

1. Introduction

Integrating diversity within and across trophic levels is important to produce new knowledge about how biodiversity drives the functioning of ecosystems (Duffy et al., 2007). Determining the vertical (i.e., number of trophic levels) and horizontal (i.e., species per trophic level) diversity of a food web -including omnivory- can unravel how ecosystem functioning might be affected by consumptive interactions across trophic levels and/or competition within levels. For the past decades, stable isotope composition of carbon and nitrogen have been used for food web determination (e.g., Post, 2002). The utility of stable isotope lies on the isotopic enrichment of nitrogen of about 3 to 4 ‰ for each trophic level along the food web (De Niro and Epstein, 1981; Minagawa and Wada, 1984). However, bulk analysis of nitrogen isotopic composition is sensitive to variations in the isotopic signature of the nitrogen at the base of the food web. Thus, food web reconstruction based on bulk isotope analysis can become complex in ecosystems where primary producers can access different sources of nitrogen and carbon or when the food web is heavily tangled and includes omnivores with large trophic spectra (reviewed in Chikaraishi et al., 2014). Analysis of compound-specific nitrogen and carbon isotopes of amino acids ($\delta^{15}N_{AA}$ and $\delta^{13}C_{AA}$) can improve the determination of sources of nitrogen and the estimation of trophic position of consumers (McClelland and Montoya, 2002; McCarthy et al., 2007; Chikaraishi et al., 2009). This method is based on contrasting isotopic fractionation for selected amino acids during the metabolic process (Chikaraishi et al., 2014; McMahon and McCarthy, 2016). As an example, during the transfer of biomass from one trophic level to another, glutamic acid goes through transamination/deamination, which breaks carbon–nitrogen bonds and thus produce a strong fractionation in nitrogen. On the other hand, the

* Corresponding authors at: School of Life Sciences & Earth and Sciences Programme, The Chinese University of Hong Kong, Hong Kong SAR (B. Thibodeau). Department of Biology, The University of Florence, I-50019 Sesto Fiorentino, Italy (S. Cannicci).
E-mail addresses: benoit.thibodeau@cuhk.edu.hk (B. Thibodeau), stefano.cannicci@unifi.it (S. Cannicci).

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metabolism of amino acids like phenylalanine includes the formation of tyrosine without cleaving carbon–nitrogen bonds and thus preserving the original isotopic signature of the nitrogen source (reviewed in McMahon and McCarthy, 2016). These specific amino acids offer a way to disentangle potential change in the source identification of nitrogen from changes in the trophic position of the consumer. Furthermore, the $\delta^{13}C_{AA}$ value can help us distinguish between the origin of the amino acids among plant, fungal and bacterial sources and help identifying detritivores from herbivores (Larsen et al., 2009). Therefore, the use compound-specific isotopes of amino acid can contribute to improve our understanding of complex food webs with multiple energy sources.

Mangrove forests are intertidal ecosystems that colonise the soft-bottom shores of tropical and subtropical estuaries and sheltered bays (Giri et al., 2011; Friess et al., 2019). Although they provide invaluable services to mankind, such as carbon sequestration (Donato et al., 2011) and shoreline protection (Lee et al., 2014), they are threatened worldwide by deforestation and coastal development (Friess et al., 2019, 2020; Cannicci et al., 2021). Mangroves forests are characterised by a highly specialised and distinctive flora, mainly composed of woody plants (Duke et al., 1998), and a specialised invertebrate fauna dominated by gastropods, bivalves and decapod crustaceans, but characterised by a scarce functional redundancy (Cannicci et al., 2008, 2021). Among mangrove resident invertebrates, omnivorous crabs are the dominant and most diverse macrofaunal group (Cannicci et al., 2021) and, although their crucial role in the food chain has been demonstrated (Lee et al., 2014; Kristensen et al., 2017), it is still difficult to ascertain their tropic niche. It has been shown that crabs rely on a wide range of potential food resources, including animal prey, mangrove litter, epiphytic algae, microphytobenthos (MPB), bacteria and fungi, as well as macroalgae and a mixture of organic sources imported from adjacent aquatic environments by tidal currents (Dahdouh-Guebas et al., 1999; Bouillon et al., 2008). While mangrove leaf litter is rich in carbon, it is fairly poor in nitrogen and represents a nutrient poor diet for mangrove associated macrofauna (Micheli, 1993b; Nordhaus et al., 2017). Although some sesarmid crabs proved to gather enough nutrients from such a nitrogen poor food source to grow and reproduce (Micheli, 1993a; Herbon and Nordhaus, 2013), this diet has not been considered sustainable for many crabs (Skov and Hartnoll, 2002; Kristensen et al., 2010). Attempts to ascertain alternative sources of nitrogen available to mangrove fauna using bulk isotopes were diminished due to the large range of trophic discrimination factors and the variable partitioning of nitrogen source encountered in mangroves forest (Herbon and Nordhaus, 2013; Bui and Lee, 2014; Kristensen et al., 2017). This is one of the main reason why the determination of a reliable food web has proven challenging for mangrove ecosystems since these sources of food are difficult to discriminate with tradition stable isotope measurements (e.g., Bui and Lee, 2014; Harada et al., 2022). Therefore, the use of $\delta^{15}N_{AA}$ and $\delta^{13}C_{AA}$ can contribute to resolve long standing questions about the relative importance of the different source of energy (i.e., vascular plant, microphytobenthos, fungi and bacteria) and help clarify the position of certain omnivorous species of crabs, a critical lack of knowledge that affects our understanding about the transfer of energy from the mangrove vegetation to the food web (Dahdouh-Guebas et al., 2022).

Here, we propose to use compound-specific isotopes of amino acids to 1) quantify the relative importance of vascular plants (i.e., mangroves) in the food web, 2) ascertain the trophic position for the dominant faunal taxa using a mixed-source model and 3) investigate the presence of detritivory (consumption of fungi and bacteria) in the dominant faunal taxa.

2. Material and methods

2.1. Study site, sampling, and pre-treatment methods

Sampling was performed during the wet season, June to September 2018, at the mangrove forest of Tung Chung, Lantau Island, Hong Kong (22.290148, 113.923710), which is one of the ten largest mangrove areas in the Hong Kong territory (Tam et al., 1997; Tam and Wong, 2002). This forest is influenced by freshwater from the Pearl River, with salinity ranging between 26 and 35 in winter and 14 and 17 in summer (e.g., Archana et al., 2018; Geeraert et al., 2021) and hosts seven of the eight true mangrove trees native to Hong Kong, including one of the last pure stands of *Bruguiera gymnorrhiza* in Southern China (Tam et al., 1997; Wang et al., 2020). Most of the mangrove resident gastropods species present in Hong Kong are found in Tung Chung mangroves (Bravo et al., 2021), together with a wide variety of brachyuran crabs (Agusto et al., 2021) and two Asian horseshoe crab species (Kwan et al., 2016).

We collected 23 different categories of samples identified as: microphytobenthos (MPB), macroalgae, eight species of crabs, nine species of gastropods, and fallen mangrove leaves (decaying brown leaves) samples from the four most common tree species present in the forest (Table 1). For decaying leaves, two to three identifiable leaves of each species were gathered from the sediment in close vicinity to the tree trunk. A distance of at least 3 m was maintained between each tree. Leaf samples were rinsed and freeze dried and small sized leaves from the same species were pooled together, to obtain sufficient material. Samples were homogenized with a pestle and mortar into a fine powder. Brachyuran crabs and gastropods were collected by hand in randomly selected quadrats and brought to the lab, where they were rinsed with deionized water to remove all sediment and frozen at −20 °C. Samples of muscle tissue were extracted from the claws and from the foot, for crabs and gastropod, respectively. After the extraction, samples were freeze dried for 24 h. All samples were weighed to the nearest 0.001 mg. Microphytobenthos was collected by scraping the top 1 cm of sediment using a spatula. Sediment samples were transported to the lab, refrigerated, and processed the same or the following day. The MPB was isolated from the sediment when adding the colloidal silica until a density gradient formed and allowed the separation of MPB (Hamilton et al., 2005). Surface sediment was sieved to remove large grains, detritus and larger nematodes using Milli-Q water. The filtrate was then run through a pre-combusted glass filter to concentrate the sample. The residue was removed from the filter and divided into aliquots of around 5 mL in individual centrifuge tubes. The aliquots were lightly shaken to prevent clumping when adding the colloidal silica. Colloidal silica was then added until a density of 1.27 g cm^{-3} was reached (Hamilton et al., 2005). The mixture was centrifuged at 10,000 rpm for 10 min. The presence of MPB was confirmed by microscopic examination prior to pipetting off the distinct supernatant layer. Pre-combusted glass filters were loaded with the supernatant layer while adding Milli-Q water to remove as much of the silica as possible. Each filter was loaded with the suspension until it was clogged to a maximum amount. Then, filters were freeze dried in their entirety for 24 h after which the dried MPB was scraped off.

2.2. Preparation and isotopic analysis

All samples were prepared for isotope analysis following Chikaraishi et al. (2014). For marine invertebrates, a small sample of muscle tissue (~10 mg) was taken. For other sample types (i.e., macroalgae, leaves) the whole sample was used for isotopic analysis (~30 mg). Derivatization protocol from UC-Davis was used (Corr et al., 2007; Walsh et al., 2014; Yarnes and Herszage, 2017). Dry, homogenized sample materials were placed in borosilicate vials with heat- and acid-resistant caps. Hydrochloric acid (6 M) was added (0.5 mL for animal tissues and 2 mL for plant tissues) and vial threads wrapped with PTFE-tape. Vials were lushed with N_2, sealed, and placed in an oven at 150 °C for 70 min. After cooling, 200 μL of heptane:chloroform (6:5, v:v) was added to the acid hydrolysates of sample materials, the vials briefly vortexed, and the organic layer discarded in order to remove any remaining lipophilic compounds prior to drying. Samples were then dried in a heating block at 60 °C under a gentle stream of N_2. Sample acid hydrolysate was

Table 1
Species collected and their abbreviations used in this study.

Species	Abbreviation	Grouping	Other info/Family	Type of sample for isotope
Parasesarma bidens	Pb	Sesarmid Crab	Sesarmidae	Muscle
Episesarma versicolor	Ev	Sesarmid Crab	Sesarmidae	Muscle
Parasesarma affine	Pa	Sesarmid Crab	Sesaemidae	Muscle
Metopograpsus quadridentatus	Mq	Crab	Grapsidae	Muscle
Gelasimus borealis	Gb	Fiddler Crab	Ocypodidae	Muscle
Paraleptuca splendida	Ps	Fiddler Crab	Ocypodidae	Muscle
Metaplax longipes	Ml	Crab	Varunidae	Muscle
Macrophthalmus tomentosus	Mt	Crab	Macrophthalmidae	Muscle
Littoraria ardouiniana	La	Gastropod	MPB browser	Muscle
Nerita yoldii	Ny	Gastropod	MPB browser	Muscle
Terebralia sulcata	Ts	Gastropod	MPB browser	Muscle
Pirenella alata	Pala	Gastropod	MPB browser	Muscle
Pirenella asiatica	Pa	Gastropod	MPB browser	Muscle
Batillaria attramentaria	Ba	Gastropod	MPB browser	Muscle
Batillaria zonalis	Bz	Gastropod	MPB browser	Muscle
Batillaria cumingii	Bc	Gastropod	MPB browser	Muscle
Cerithidea moerchii	*Cm*	Gastropod	MPB browser	Muscle
Kandelia obovata	Ko	Mangrove	Tree	Leave
Bruiguiera gymnorrhiza	Bg	Mangrove	Tree	Leave
Aegiceras corniculatum	Ac	Mangrove	Tree	Leave
Avincennia marina	Am	Mangrove	Tree	Leave
Macroalgae	M.A	Algae	Algae	Algae
Microphytobenthos	MPB	Algae	Algae	Algae

combined with 10 μL of UC-Davis internal reference solution and then dried under a stream of N_2. One mL of Acidified methanol 1.85 M was added to each reaction vial and heated at 100 °C for 60 min. The remaining methanol was evaporated under nitrogen over ice. Chloroform was added (250 μL) and evaporated under N_2 at room temperature to remove excess reagents. The partial derivatives were acetylated with a mixture of acetic anhydride, trimethylamine, and acetone (1 mL; 1:2:5, v/v/v; 10 min., 60 °C) and the reagents evaporated under nitrogen gas in a cold block (0 °C). Once dry, ethyl acetate was added (2 mL), along with a saturated NaCl solution (1 mL), and the solution vortexed. Following phase separation, the aqueous phase was discarded and the ethyl acetate removed under nitrogen gas in a cold block (0 °C). Trace water was removed with two additions of chloroform (1 mL). Finally, ethyl acetate was added (100 μL) and the *N*-acetyl methyl esters transferred to a GC vial with insert. The amino acid derivatives were separated by a Thermo Trace GC 1310 gas chromatograph using DB1301 column (Agilent, 60 m * 0.25 mm, 1 μm film thickness) for $\delta^{15}N$ measurements and DB-23 column (Agilent, 30 m * 0.25 mm, 0.25 mm film thickness) for $\delta^{13}C$ measurements at the UC-Davis stable isotope facility. The gas chromatograph was interfaced with a Thermo Scientific Delta V Advantage isotope-ratio mass spectrometer via a CG IsoLink II combustion interface. All samples were analysed in duplicate, the average is presented. Two mixtures of pure amino acids of calibrated isotopic value (UCD AA1 and 2) were measured along the samples and used for the calibration and normalization of the data. A third mixture (UCD AA 3) was used as quality assurance standard with two natural samples of well know isotopic value (baleen and fish muscle). The mean standard deviation of duplicate was ± 0.49 ‰ and ± 0.71 ‰ for samples and standard respectively. The $\delta^{15}N$ was determined for Alanine (Ala), Aspartic acid (Asp), Glutamic acid (Glu), Glycine (Gly), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), and Valine (Val). The $\delta^{13}C$ was determined for the same amino acids except Met and Pro. Results are reported following delta notation against the atmospheric N_2 and Vienna Pee Dee Belemnite (VPDB) for nitrogen and carbon respectively:

$$\delta^{15}N = \frac{R(^{15}N/^{14}N)_{sample} - R(^{15}N/^{14}N)_{standard}}{R(^{15}N/^{14}N)_{standard}} \quad (1)$$

$$\delta^{13}C = \frac{R(^{13}C/^{12}C)_{sample} - R(^{13}C/^{12}C)_{standard}}{R(^{13}C/^{12}C)_{standard}} \quad (2)$$

2.3. Trophic position calculation

The trophic position (TP) was calculated based on the nitrogen isotopic composition of Glu, which fractionate strongly during transfer of biomass from one trophic level to another, and Phe, which show little isotopic enrichment, using the formula:

$$TP_{Glu/Phe} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta_{Glu/Phe}}{TEF_{Glu} - TEF_{Phe}} + 1 \quad (3)$$

where $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ represent respectively the $\delta^{15}N$ of Glue and Phe of the consumer and $\beta_{Glu/Phe}$ represent $^{15}N_{Glu} - {}^{15}N_{Phe}$ in the primary producer considered (Chikaraishi et al., 2009). Here, we measured $\beta_{Glu/Phe}$ locally in the different potential primary producers to align with recent recommendations (Ramirez et al., 2021). The trophic enrichment factor (TEF) is the trophic discrimination factor for each shift of trophic level which were traditionally considered to be around 8.0 ± 1.2 ‰ for Glu and 0.4 ± 0.5 ‰ for Phe (reviewed in Chikaraishi et al., 2014; McMahon and McCarthy, 2016). However, a *meta*-analysis displayed that the potential variability in $\delta^{15}N$ across diverse consumer-resource relationship is probably linked to diet quality and nitrogen excretion mode (McMahon and McCarthy, 2016) and thus this potential variability in the TEF will be explored in the discussion.

Possible statistical differences among the calculated TP for specimens belonging to the sampled brachyuran crab species were analysed by means of a one-way Permutational Analysis of Variance design (PERMANOVA: Anderson, 2001). We used only the data collected on crabs due to the low replicates of TP we could calculate for gastropods. The PERMANOVA univariate test was based on a matrix built on Euclidean distances and 9999 permutations and type I sum of square calculation. Since the factor species proved to be statistically significant, we then applied post-hoc PERMANOVA tests to check from differences in TP across each species.

2.4. Nitrogen sources partitioning calculation

In ecosystem like mangroves, where the base of the food web can include both marine and terrestrial resources, we correct the β-value for each consumer based on the relative proportion of marine primary producers and vascular plants. We thus calculated the relative proportion of these resources into the diet of each consumer using a 2-source

mixing model (Ishikawa et al., 2014; Harada et al., 2022):

$$f = \frac{\delta^{15}N_{Glu}[C] - \delta^{15}N_{Glu}[M]/TEF_{Glu} - \delta^{15}N_{Phe}[C] - \delta^{15}N_{Phe}[M]/TEF_{Glu}}{\delta^{15}N_{Glu}[V] - \delta^{15}N_{Glu}[M]/TEF_{Glu} - \delta^{15}N_{Phe}[V] - \delta^{15}N_{Phe}[M]/TEF_{Phe}} \quad (4)$$

where f represent the relative contribution of vascular plant in the consumer diet (from 0 to 1) and [C], [V] and [M] represents consumers, vascular plants, and marine primary producers respectively. The $\delta^{15}N_{Glu}$[V] and $\delta^{15}N_{Phe}$[V] where calculated using the average of eight different mangrove samples and yielded values of 9.9 ± 2.1 ‰ and 19.4 ± 2.5 ‰ respectively. The $\delta^{15}N_{Glu}$[M] and $\delta^{15}N_{Phe}$[M] where calculated using the average of three sample of microphytobenthos and macroalgae that represent marine primary producers and yielded values of 11.6 ± 0.8 ‰ and 7.3 ± 1.2 ‰, respectively. The f value was then used to calculate a specific $\beta_{Glu/Phe}$ for each consumer using this equation:

$$\beta_{Glu/Phe} = f \times TEF_{Glu} + (1-f) \times TEF_{Phe} \quad (5)$$

Equation (4) was used to replace $\beta_{Glu/Phe}$ in equation (1) for consumer that relies on both vascular and marine primary producers as their source of nitrogen.

The errors associated with the TEF, $\beta_{Glu/Phe}$, and consumer $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$, were propagated using the following equation:

$$\sigma_{TP} = \sqrt{\frac{\left(\sigma^2_{TEFGlu} + \sigma^2_{TEFPhe}\right)\cdot\left(-\beta_{\frac{Glu}{Phe}} - \delta^{15}N_{Glu} + \delta^{15}N_{Phe}\right)^2 + \left(TEF_{Glu} - TEF_{Phe}\right)^2\cdot\left(\sigma^2_{\beta_{\frac{Glu}{Phe}}} + \sigma^2_{\delta^{15}N_{Glu}} + \sigma^2_{\delta^{15}N_{Phe}}\right)}{\left(TEF_{Glu} - TEF_{Phe}\right)^4}} \quad (6)$$

2.5. Biosynthetic origin of amino acids in consumers

Differences in amino acid metabolisms between major prokaryotic and eukaryotic lineages generate unique amino acid carbon isotopic signature (Larsen et al., 2009, 2013, 2022). Plants and fungi have different pathway to synthesize Lys and their pathways for biosynthesizing Leu are compartmentalized differently (Kohlhaw, 2003; Hudson et al., 2006). The difference between Ile and Leu was found to be valuable in distinguishing plants and fungi from bacteria while the difference between Ile and Lys can be used to separate plants from fungi (Larsen et al., 2009). The $\delta^{13}C_{AA}$ of Ile, Lys and Leu was therefore used to assess the biosynthetic origin of our sample. This method was proven useful in an Australian mangrove to highlight the relative absence of detritivory (Harada et al., 2022). We plotted the difference in $\delta^{13}C$ between Ile and Leu against the difference between Ile and Lys alongside available data from the literature including vascular plants, microalgae, fungi, and bacteria.

3. Results

3.1. Nitrogen isotope of individual amino acids, source partitioning and trophic position

Both mangrove leaves and algae had a similar, relatively low, $\delta^{15}N$ of Glu ($\delta^{15}N_{Glu}$ = 11.7 and 11.9 ‰, respectively, Fig. 1), while gastropods and crabs had a higher $\delta^{15}N_{Glu}$, at 16.7 ‰ and 19.4 ‰, on average, respectively. The $\delta^{15}N$ of Phe for gastropods ($\delta^{15}N_{Phe}$ = 7.6 ‰) was the same as the algae (7.7 ‰). However, the $\delta^{15}N_{Phe}$ from crabs (10.8 ‰) was in between the $\delta^{15}N_{Phe}$ of algae (7.7 ‰) and mangroves (22.3 ‰). The relative contribution of vascular plant in the nitrogen pool (f) was estimated to be between 8 and 50 % for the different crab species and between 0 and 7 % for the gastropods (Table 2). Using this newly calculated specie-specific f, the $\beta_{Glu/Phe}$ calculated from equation (4) ranged from −3.4 to 2 for the crabs, which yielded TPs between 1.6 and 2.4 according to equation (3) (Table 2 and Fig. 2). The PERMANOVA test confirmed significant differences across the TP values of the different species (Pseudo-F = 10.282 $_7$; P less than 0.0001. The post-hoc tests revealed significant differences between the TP calculated for the sesarmid crabs and all other crabs species (Table 3). Moreover, the TP of the two species of fiddler crabs analysed significantly differed, as well as the TP values calculated for the two species of crabs known from the literature and deposit feeders, *M. tomentusus* and *M. longipes* (Table 3).

3.2. Carbon isotope of individual amino acids

Mangroves leaves $\delta^{13}C_{Ile\text{-}Leu}$ plotted against $\delta^{13}C_{Ile\text{-}Lys}$ were within the range of literature data for plants, while some samples were within the average values of fungi (Fig. 3). Crab samples were characterised by value close to microalgae, but some samples seem to also be influenced by bacteria or fungi (Fig. 3). Gastropods plotted within the range of microalgae as well, except for two species that shown signature closer to bacteria and fungi respectively (Fig. 3).

4. Discussion

4.1. Limitations

While the use of $\delta^{15}N_{AA}$ and $\delta^{13}C_{AA}$ offers a wide range of advantages compared to bulk isotope, it comes with some limitations. These limitations are notably due to variability in isotopic fractionation between trophic levels and in the difference between the isotopic composition of trophic and source amino acids in diverse primary producers (e.g., McMahon and McCarthy, 2016; Ramirez et al., 2021). To overcome the latter, we used a multi-sources approach to calculate a $\beta_{Glu/Phe}$ of each individual species to consider the variable integration of primary producers (vascular vs non-vascular) in the food web (eq. (4) and (5)). Another source of variability is the potential difference in TEF depending on quality of diet and nitrogen excretion mode (McMahon and McCarthy, 2016). Therefore, the use of a global means (8.0 ± 1.2 ‰ for Glu and 0.4 ± 0.5 ‰ for Phe, discussed in section 2.3) is not applicable in all ecosystems and therefore it is crucial to evaluate potential variability in our system. Available data on marine gastropods and crustaceans are relatively sparse, but displayed variable $TEF_{Glu\text{-}Phe}$ between ~6.5 and ~8.5 (Chikaraishi et al., 2007) and ~8 (Nakatomi et al., 2014), respectively. Therefore, while we are conscious that TEF may be affected by variability within our ecosystem, previous data suggest the global mean discussed above may be applicable to our ecosystem as most published data fall into this range.

4.2. Sources of nitrogen and carbon for brachyuran crabs and gastropods

Our analytical approach provides a quantitative partitioning of N sources in leaf-eating crabs and mangrove gastropods. Our results showed that from 8 to 50 % of the crabs' diet was based on vascular N supplied by the mangrove leaves (Table 2) and confirmed that most mangrove associated crabs integrate a nitrogen-poor diet that includes both leaf litter and marine-derived N, probably mainly from microphytobenthos or their consumers. Particularly interesting are the results

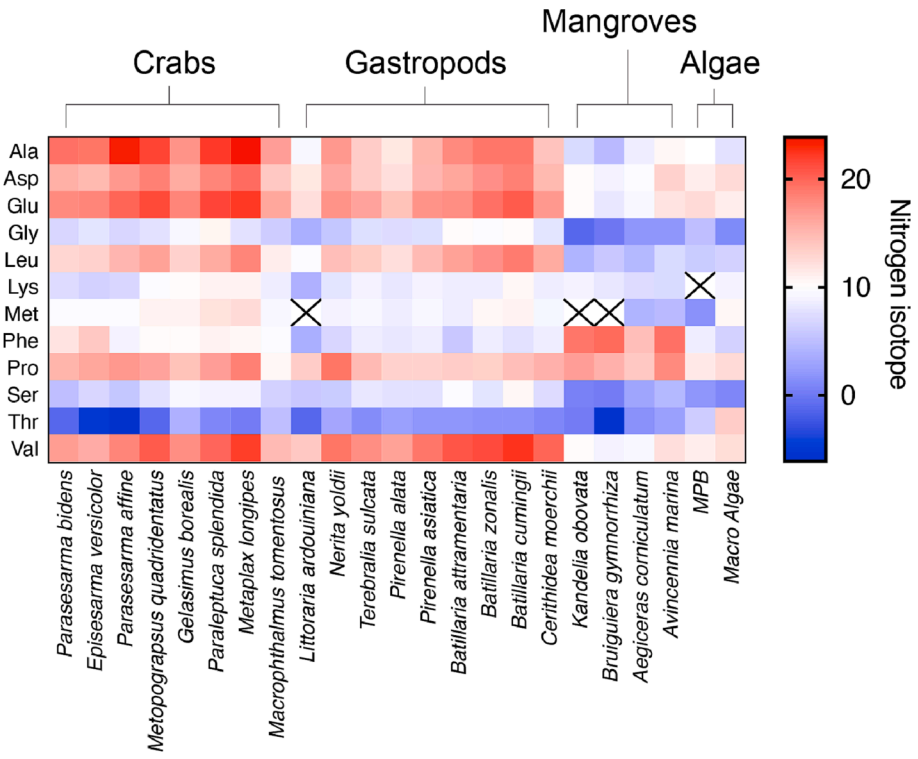

Fig. 1. Heat map of nitrogen isotope of individual amino acid for each species. Darker red represents higher value of $\delta^{15}N$ of selected amino acids while dark blue represents the lower value. The crosses represent under range values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

obtained for the sesarmid crabs, with the largest specie of Hong Kong, *Episesarma versicolor*, showing the largest amount of N supplied by the leaves and the sympatric *Parasesarma affine* showing very limited N intake of vascular plants origin. These data show a sharp niche partitioning between two closely related sesarmids that were thought to rely on a very similar, and rather overlapping, diet (Kwok and Lee, 1995; Herbon and Nordhaus, 2013). The data on the brachyuran species not belonging to the family Sesarmidae, confirmed that no less than 75 % of their N intake is coming from marine sources, probably microphytobenthos or marine organisms. For what concerns potential marine preys, many species belonging to the genus *Metopograpsus* and to the family Sesarmidae are known to predate on other species of crabs, small gastropods and bivalves, such as oysters (Cannicci et al., 1996; Dahdouh-Guebas et al., 1999; Fratini et al., 2000; Poon et al., 2010). Gastropods appeared to gather their nitrogen almost exclusively from the marine components of the food web, according to their $\delta^{15}N_{Phe}$ value, which was similar to microphytobenthos in almost all species. This is coherent with the $\delta^{13}C_{AA}$, which suggest that microalgae (or their consumers) are in general the dominant biosynthetic origin of amino acids in both our crabs and gastropods with some degree of detritivory (i.e., fungi and bacteria) (Fig. 3). Ingestion of fungi, lichens and bacteria has been described in the tree-leaving gastropods of the genus *Littoraria* (Lee et al., 2001) and in fiddler crab species leaving under the canopy of mangrove forests (Wolff et al., 2000; Koch and Wolff, 2002). Interestingly, some part of this detritivory signal appears to originate from the ingestion of mangrove leaves that has been colonised by fungi, as illustrated by the position of multiple mangrove leaves that were analysed (Fig. 3).

Table 2
Percentage of nitrogen (N) originating from vascular primary producers calculated from equation (4) and trophic position (TP) and its propagated error calculated from equations (3) and (6).

Species	*f*	TP
Parasesarma bidens	0.32	1.8 ± 0.2
Episesarma versicolor	0.50	1.9 ± 0.2
Parasesarma affine	0.08	2.1 ± 0.3
Metopograpsus quadridentatus	0.22	2.3 ± 0.3
Gelasimus borealis	0.19	1.9 ± 0.2
Paraleptuca splendida	0.23	2.3 ± 0.3
Metaplax longipes	0.19	2.4 ± 0.3
Macrophthalmus tomentosus	0.15	1.6 ± 0.2
Littoraria ardouiniana	0.00	1.6 ± 0.2
Nerita yoldii	0.00	1.9 ± 0.2
Terebralia sulcata	0.05	1.7 ± 0.2
Pirenella alata	0.04	1.4 ± 0.1
Pirenella asiatica	0.05	1.8 ± 0.2
Batillaria attramentaria	0.00	2.1 ± 0.3
Batillaria zonalis	0.04	2.0 ± 0.3
Batillaria cumingii	0.00	2.2 ± 0.3
Cerithidea moerchii	0.07	1.7 ± 0.2

4.3. Trophic position and food web complexity

While our data support the hypothesis that decaying mangrove leaves can be part of the mangrove food web (Kwok and Lee, 1995; Lee, 1997; Thongtham and Kristensen, 2005; Herbon and Nordhaus, 2013), we highlight that brachyuran crabs mostly rely on grazing marine-derived food sources like microphytobenthos or their consumers (Skov and Hartnoll, 2002; Kristensen et al., 2010, 2017) compared to the nutrient-poor diet of mangrove leave. On the other hand, the relatively low TP estimated does not support a consistent integration of microbivory or animal prey, at least not on a frequent basis (Fig. 3). While sesarmids are known to prey on other crabs, dead or alive (Cannicci et al., 1996; Dahdouh-Guebas et al., 1999), these predation events are probably rare and uncommon, and the integration with animal tissue is not enough to significantly increase their isotopic signature. Similarly, microbivory would increase the TP of primary consumer as they integrate dead organic matter into their biomass, adding a trophic step (Steffan et al., 2017). On the contrary, our TP value are relatively lower

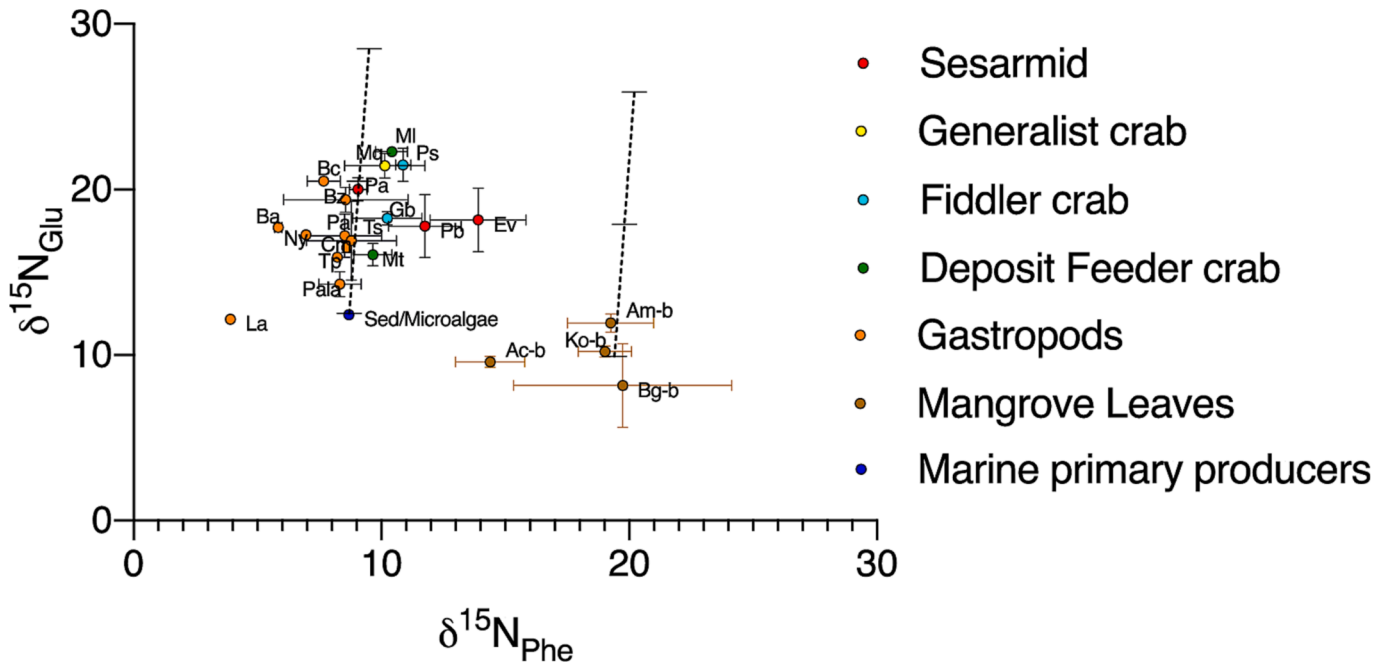

Fig. 2. Nitrogen isotope of Glutamic acid against Phenylalanine in gastropods and marine primary producers. The dashed lines represent trophic position calculated using marine primary producers (left) and mangrove leaves (right).

compared to expectation from field observations, with some known consumers having a TP lower than 2. This is also supported by the $\delta^{13}C_{AA}$ value of most crabs that does not display a strong bacteria signal.

We found a statistically significant niche partition between the two analysed fiddler crab species (subfamily Gelasiminae) with *Gelasimus borealis* (TP = 1.9), showing a lower TP with respect to *Paraleptuca splendida* (TP = 2.3). All fiddler crabs are known to sort the surface sediment with specialised mouth parts to gather food (Crane, 1975; Icely and Jones, 1978), but the nature of such food has been debated (Koch and Wolff, 2002). Our analysis suggests that *G. borealis* mainly relies on microphytobenthos and litter-decomposing bacteria while *P. splendida* probably includes a higher proportion of marine consumers (e.g., meiofauna) and is higher in the food web. Benthic microorganisms are a well-known food source for fiddler crabs (Icely and Jones, 1978; Koch and Wolff, 2002), but predation on mesofauna, mainly on nematodes, was also suggested for some South American fiddler crabs (Citadin et al., 2016, 2018). Therefore, our analyses were able to unveil a clear food niche partitioning in fiddler crabs living in the same forest. A similar situation was revealed by the statistical analyses performed on the trophic levels of *Macrophthalmus tomentosus* (Macrophthlmidae) (TP = 1.6) and *Metaplax longipes* (Varunidae) (TP = 2.4), which are both often classified as deposit feeders, since they crawl on the mangrove sediment scraping the surface with their chelipeds. Also in this case, a clear separation was found between the primary consumer habit of *M. tomentosus*, probably feeding on microphytobenthos, and *M. longipes*, which seems to rely on marine consumers. The only species of Grapsidae present in Tung Chung, the mangrove dwelling *Metopograpsus quadridentatus* (TP = 2.3), has one of the highest TP amongst other crabs in this mangrove, as confirmed by the PERMANOVA Post-hoc tests. The crabs of this genus are known to be very flexible generalists but they have also been observed to display peculiar hunting techniques to prey on a variety of invertebrates, which may tend to elevate their TP (Fratini et al., 2000; Giraldes et al., 2019).

Most of the mangrove dwelling gastropods are often assumed to depend on the bacterial and microalgal biofilms found on the sediment and mangrove surface. However, previous studies based on bulk isotopes suggested a broader diet for these gastropods, that could include also particulate mangrove matter and shredded leaves (Tue et al., 2012). As an example, plant cells were found in the guts of the arboreal gastropod of the genus *Littoraria* from Hong Kong (Lee et al., 2001) while some species of the genus *Terebralia* are known to feed on mangrove leaves (Fratini et al., 2001). Our data, however, rather tend to support a diet mostly based on marine N and thus microphytobenthos, rather than vascular plants. However, the $\delta^{13}C_{AA}$ value for one species seems to be influenced by fungi, which may be originating from the mangrove leaves as suggested earlier (Fig. 3).

Table 3
Results of the PERMANOVA post-hoc tests applied to the differences in TP values across species. The species compared, the value of the t parameter and the P level are shown.

Species	t	P
P. bidens, E. versicolor	0.30182	0.7774
P. bidens, P. affine	1.8614	0.1048
P. bidens, M. quadridentatus	3.9628	**0.0034**
P. bidens, G. borealis	0.39271	0.7054
P. bidens, P. splendida	3.047	**0.019**
P. bidens, M. longipes	3.8946	**0.006**
P. bidens, M. tomentosus	1.4912	0.1723
E. versicolor, P. affine	1.5239	0.1932
E. versicolor, M. quadridentatus	3.5095	**0.0105**
E. versicolor, G. borealis	0.068767	0.948
E. versicolor, P. splendida	2.663	**0.0464**
E. versicolor, M. longipes	3.5766	**0.0149**
E. versicolor, M. tomentosus	1.7884	0.1357
P. affine, M. quadridentatus	2.694	**0.0368**
P. affine, G. borealis	3.6823	**0.0208**
P. affine, P. splendida	2.0965	0.1079
P. affine, M. longipes	5.3769	**0.0062**
P. affine, M. tomentosus	6.9168	**0.0021**
M. quadridentatus, G. borealis	6.655	**0.0005**
M. quadridentatus, P. splendida	0.079167	0.9398
M. quadridentatus, M. longipes	1.8577	0.1102
M. quadridentatus, M. tomentosus	10.126	**0.0001**
G. borealis, P. splendida	5.1278	**0.007**
G. borealis, M. longipes	15.079	**0.0001**
G. borealis, M. tomentosus	4.7527	**0.0089**
P. splendida, M. longipes	1.334	0.2623
P. splendida, M. tomentosus	7.6949	**0.0019**
M. longipes, M. tomentosus	14.938	**0.0004**

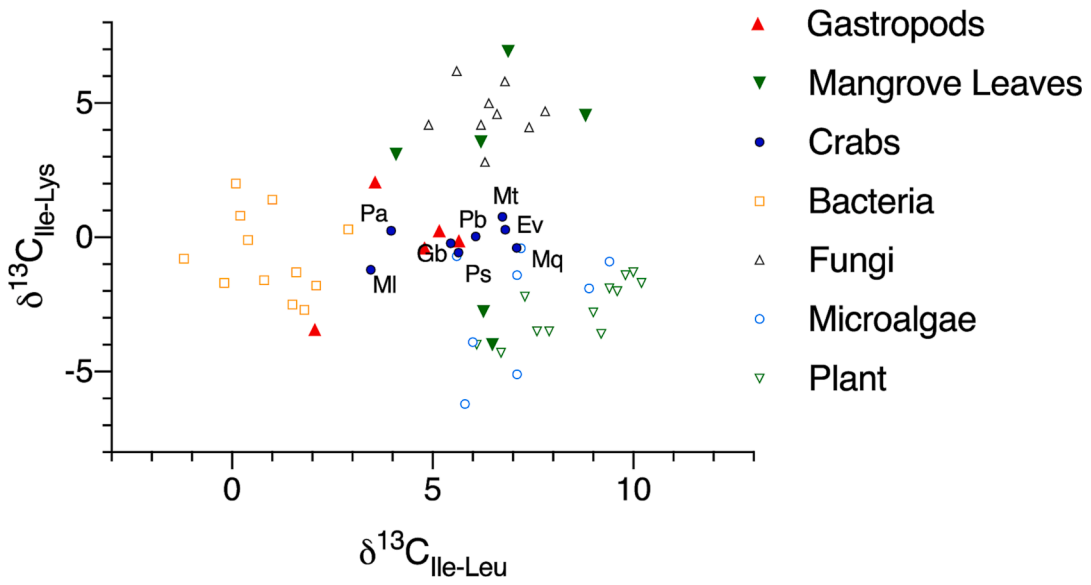

Fig. 3. Biosynthetic origins of essential amino acids in mangrove leaves and consumers. Empty dots represent literature data and full symbols originates from this study.

5. Conclusions

Our approach helped us highlight previously unreported differences of TP for several mangrove-associated crabs, which shed new light on their roles within the mangrove food web. We showed that species supposed to have overlapping niches, such as the various fiddler crabs and the 'deposit feeding' species of other families, show well separated feeding preferences and rely on different food sources. Such differences are probably associated with distinctive feeding behaviours, which are still not understood at present and should be ascertained. This partitioning of feeding niches could be the key to explain the abundance of crab populations, which often overlap with each other in the same microhabitat, and the high secondary production of mangroves. These new detailed data on feeding preferences could suggest that the inherent lack of ecological redundancy of mangrove faunal communities may be even more noticeable than recently determined by Cannicci et al. (2021), showing different feeding specialisations in congeneric, or closely phylogenetically related, species. Clearly, our understanding of the mangrove food web is far from being comprehensive and further studies aimed to understand the resilience of mangroves to anthropogenic and climatic changes will need to clarify in more details the actual link between ecological redundancy and feeding specialisations of their associated fauna.

Statement of Authorship

The research was designed by BT and SC, funding was acquired by BT and SC, fieldwork was performed by LA, LEA, MWKS and SC, sample preparation was done by LA, LEA and MWKS. Manuscript was written by BT and SC with comments from co-authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Link to data repository is within the paper.

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Data accessibility.

Data are available in the CUHK Research Data Repository https://doi.org/10.48668/DSXYYN.

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