Compound-specific isotope analysis of amino acids reveals dependency on grazing rather than detritivory in mangrove food webs

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ABSTRACT: Colonisation of decaying leaves fallen from mangrove trees by bacteria and fungi is thought to play an important role at the base of food webs in most tropical estuaries. Compound-specific isotope analysis of amino acids (CSIA-AA) has enabled the previously difficult methodological task of measuring plant, bacterial and fungal energy flows to food webs. Here, we assessed the biosynthetic origins of amino acids at the base of a mangrove food web using the CSIA-AA approach. Trophic positions of the 2 most common mangrove fauna — fiddler crabs and sesarmid crabs — estimated from nitrogen isotopes in phenylalanine and glutamic acid approached 2, suggesting that these species are herbivores rather than microbivores. Consistent with this finding, carbon isotope fingerprints in AAs did not support the importance of essential AAs derived from fungi and bacteria but rather suggested the importance of those originating from plants, especially microalgae. These results suggest that (1) microbial mineralization of decaying leaves supports the production of more easily assimilated microalgae and (2) bacteria and fungi, as intermediates, also routinely incorporate plant-derived AAs into their biomass.

KEY WORDS: Food web · Stable isotope analysis · Amino acids · Carbon · Nitrogen · Mangrove

1. INTRODUCTION

Mangrove forests are important coastal wetlands in most of the tropics and subtropics, supporting the base of the coastal food web and fisheries. To effectively restore and conserve the remaining mangrove forests in the world, a better understanding of the functional aspects of mangrove forests is required (Lee et al. 2019), e.g. food web dynamics. However, studying mangrove food webs that involve both plants and microbes (such as bacteria) and fungi is methodologically challenging. Stable carbon ($\delta^{13}$C), nitrogen ($\delta^{15}$N) and sulfur ($\delta^{34}$S) isotope analysis of total organic matter (bulk SIA) has generally been used to quantify the relative contributions of organic matter sources to food webs (Bouillon et al. 2008), but the variety of organic matter inputs in these ecosystems often results in complex mixtures that are not well resolved based on the isotopic separation of the sources (Geraldi et al. 2019).

A newer technique, compound-specific isotope analysis of amino acids (CSIA-AA), has shown greater potential to quantify the contribution of different organic matter sources to food webs because isotopic compositions in individual amino acids differ among organic matter sources, likely due to the difference in amino acid biosynthesis (Larsen et al. 2013, Chikaraishi et al. 2014). For example, $\delta^{15}$N values of phenylalanine and glutamic acid were used to indicate grazing dependency rather than detritivory in mangrove food webs.
lalanine (Phe) in vascular plants are relatively enriched in $^{15}$N compared to those of microalgae. This enrichment is likely associated with deamination of Phe during lignin biosynthesis, a process specific to vascular plants (Kendall et al. 2019). $^{15}$N values in AAs are also useful for estimating the trophic levels of organisms, as trophic fractionation of $^{15}$N between 2 groups of AAs (source AAs and trophic AAs) differ substantially. While the former group, including Phe, shows little to no enrichment during each trophic transfer (0.4 ± 0.5‰) and is useful for estimating the basal $^{15}$N values, the latter, including glutamic acid (Glu), shows substantial $^{15}$N enrichment during each trophic step (8 ± 1.2‰) and is useful for estimating trophic position (TP) (Chikaraishi et al. 2009, Ohkouchi et al. 2017). Furthermore, Larsen et al. (2009) demonstrated that a combination of $^{13}$C values in 3 AAs—leucine (Leu), isoleucine (Ile) and lysine (Lys)—can distinguish among plant, fungal and bacterial origins of AAs. These AAs clearly distinguish detritivores (e.g. enchytraeids and collembolans), which rely on AAs derived from bacteria and fungi, from herbivores (e.g. moths), which rely on plant-derived AAs (O’Brien et al. 2003, Larsen et al. 2013, Larsen et al. 2016, Fig S1 in Supplement 1 at www.int-res.com/articles/suppl/m681p013_supp1.pdf). Although our understanding of how biochemical processes control isotope variations in individual AAs is still limited, substantial isotope variations in AAs among different organic matter sources exist, so CSIA-AA can potentially help us understand complex food web interactions in estuaries (Larsen et al. 2012, Vane et al. 2018, Harada et al. 2020a).

Here, food sources at the base of a mangrove food web were explored using CSIA-AA of the 2 most common and functionally important components of the mangrove macrofauna—fiddler crabs and sesarmid crabs. Our specific aims were to (1) estimate the TPs of organisms and relative contributions of mangroves and microalgae to the consumers using $^{15}$N$_{\text{phe}}$ and $^{15}$N$_{\text{glu}}$ following the method of Ishikawa et al. (2014) and (2) evaluate feeding dependencies on fungi, bacteria and plants (mangrove and microalgae) using $^{13}$C values in 3 AAs (Leu, Ile, Lys) following the method of Larsen et al. (2009). We expected that increasing detritivory (higher consumption of fungi and bacteria) results in higher TP because microbes (fungi and bacteria) convert non-living organic matter into living microbial biomass, thus introducing an additional trophic step (Steffan et al. 2017).

2. MATERIALS AND METHODS

2.1. Field sampling

Two mangrove species and microphytobenthos (MPB) (as primary producers) and 3 crab species (as consumers; see Table 1) were collected from a mangrove forest at Tallebudgera Creek (28° 06’ 25.5” S, 153° 26’ 49.5” E), Queensland, Australia, between January and March 2017. The sampling site was located approximately 2 km from the river mouth. The maximum tidal range was about 1.5 m. Rhizophora stylosa (red mangrove) and Avicennia marina (grey mangrove) were the dominant mangrove species. All samples were collected within 150 m of each other at the location. Senescent yellow leaves of A. marina and R. stylosa were harvested from trees as being most representative of mangrove organic matter that enters food webs. MPB were collected from the surface sediment (top 0.5 cm) and later isolated in the laboratory. For consumers, 3 crab species were collected, including 2 putative leaf-eating sesarmid crab species (Neosarmatium trispinosum and Parasesarma erythodactyla) and a fiddler crab species (Gelasimus vomeris) that putatively feeds on MPB. After the collection, samples were thoroughly washed in distilled water and kept on ice until they were transported to the laboratory at Griffith University, Gold Coast, QLD, Australia. Immediately after arriv-

<table>
<thead>
<tr>
<th>Group</th>
<th>Common name</th>
<th>Putative food source</th>
<th>Species</th>
<th>Code</th>
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<tbody>
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<td>Leaves</td>
<td>Neosarmatium trispinosum</td>
<td>Sesarmid 1</td>
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<td></td>
<td>Sesarmid crab</td>
<td>Leaves</td>
<td>Parasesarma erythodactyla</td>
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<td></td>
<td>Fiddler crab</td>
<td>Sediment</td>
<td>Gelasimus vomeris</td>
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<td>Mangrove</td>
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<td>Avicennia marina</td>
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<td></td>
<td>Red mangrove</td>
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<td>Rhizophora stylosa</td>
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<td>Microalgae</td>
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Table 1. Field samples (n = 3 for all samples) collected for the present study. MPB: microphytobenthos
2.2. CSIA-AA

Dry, homogenised sample materials were weighed into 8 mg (animal tissues) or 30 mg (plant tissues) aliquots and transferred to borosilicate vials with heat- and acid-resistant caps. The samples were flushed with \( N_2 \) gas, sealed and hydrolysed in 0.5 ml (animal tissues) or 2 ml (plant tissues) of 6 M HCl at 150°C for 70 min. The samples were then dried in a heating block at 60°C under a stream of \( N_2 \) gas. The dried samples were derivatised by esterification-acetylation (N-acetyl amino acid isopropyl esters for \( \delta^{15}N_{AA} \) measurements) or methoxycarbonylation (methoxycarbonyl amino acid methyl esters for \( \delta^{13}C_{AA} \) measurements) as described elsewhere (Walsh et al. 2014, Yarnes & Herszage 2017). AA derivatives were separated by a Trace GC Ultra gas chromatograph (Thermo Scientific) using a DB-1301 (Agilent, 60 m × 0.25 mm, 1 μm film) for \( \delta^{15}N_{AA} \) measurements and using a DB-23 column (Agilent, 30 m × 0.25 mm, 0.25 μm film) for \( \delta^{13}C_{AA} \) measurements at the stable isotope facility at the University of California, Davis. The gas chromatograph was interfaced with a Delta V Plus isotope ratio mass spectrometer via a GC IsoLink (Thermo Scientific). For quality control and assurance, L-norleucine was used as an internal standard and to calculate provisional values for each sample. Mixtures composed of pure AAs with known \( \delta^{15}N \) and \( \delta^{13}C \) values were co-measured as secondary quality assurance materials. \( \delta^{15}N \) values were determined for alanine, aspartic acid, Glu, glycine, hydroxyproline, Ile, Leu, Lys, Phe, proline, serine, threonine and valine. \( \delta^{13}C \) values were determined for alanine, aspartic acid, Glu, glycine, Ile, Leu, Lys, Phe, proline and valine. Exogenous carbon from the derivatisation was accounted for using the procedure of Docherty et al. (2001). All samples were measured twice; the average values are reported. The isotope values are expressed as parts per thousand in delta (δ) notation: \( \delta = (R_{sample} / R_{standard}) - 1 \times 1000 \), where \( R \) is the ratio of the heavy to the light isotope; the standard is atmospheric air (AIR) for N and Vienna Pee Dee Belemnite (VPDB) for C. For \( \delta^{15}N \) values, mean SD for the reference AA materials was ±0.71‰. For \( \delta^{13}C \) values, mean SD for the reference AA materials was ±0.43‰.

2.3. Data analysis

Using the \( \delta^{15}N_{Glu} \) and \( \delta^{15}N_{Phe} \) values, the TPs of animals were estimated as follows:

\[
TP_{Glu/Phe} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta_{Glu/Phe}}{TEF_{Glu} - TEF_{Phe}} + 1
\]

where \( \delta^{15}N_{Glu} \) and \( \delta^{15}N_{Phe} \) are the \( \delta^{15}N \) of Glu and Phe of the animal studied, respectively, and \( \beta_{Glu/Phe} \) is the difference between \( \delta^{15}N_{Phe} \) and \( \delta^{15}N_{Glu} \) in the primary producer. Trophic enrichment factors (TEFs) of 8.0 ± 1.2‰ for \( \delta^{15}N_{Glu} \) and 0.4 ± 0.5‰ for \( \delta^{15}N_{Phe} \) were used (Chikaraishi et al. 2009, 2014, Ishikawa 2018). However, in ecosystems such as wetlands, rivers and estuaries where basal resources potentially come from both terrestrial and aquatic primary producers, Eq. (1) cannot simply be used to estimate the TP\(_{Glu/Phe}\) of animals, as the \( \beta_{Glu/Phe} \) value differs substantially between vascular plants (i.e. 8.4 ± 1.6‰) and microalgae (i.e. −3.4 ± 0.9‰) including eukaryotic and prokaryotic photoautotrophs (Chikaraishi et al. 2009, 2014). For this reason, \( \beta_{Glu/Phe} \) values must be corrected for animals that rely on both terrestrial and aquatic resources to accurately assess their TP\(_{Glu/Phe}\) (Hebert et al. 2016, Choi et al. 2017). For any given consumer, the relative contribution (\( f \)) of vascular plants (i.e. mangrove) (0 ≤ \( f \) ≤ 1) was obtained using a 2-source mixing model as follows (Ishikawa et al. 2014):

\[
f = \frac{\delta^{15}N_{Glu}[C] - \delta^{15}N_{Glu}[A]}{TEF_{Glu}} - \frac{\delta^{15}N_{Phe}[C] - \delta^{15}N_{Phe}[A]}{TEF_{Phe}}
\]

where 0 ≤ \( f \) ≤ 1 and [C], [V] and [A] represent levels in consumers, vascular plants and microalgae, respectively. TEF\(_{Glu}\) and TEF\(_{Phe}\) are TEFs for \( \delta^{15}N_{Glu} \) (8.0‰) and \( \delta^{15}N_{Phe} \) (0.4‰), respectively (Chikaraishi et al. 2009, Ishikawa et al. 2014). For the end-members, the mean (±SD) value of 6 mangrove samples (17.8 ± 3.6‰ for \( \delta^{15}N_{Phe} \), 7.0 ± 2.5‰ for \( \delta^{15}N_{Glu} \)) was used and the mean value of 3 MPB samples (3.2 ± 0.1‰ for...
Ranged from 0.1−0.4. To test the effect that the selected TEFs had on the $\sigma_{\text{TP}}$ values, $\sigma_{\text{TP}}$ values associated with the TEF error ranged from 0.1−0.2. To test the effect that the TEFs had on the $\sigma_{\text{TP}}$ values, $\sigma_{\text{TP}}$ values associated with the TEF error ranged from 0.1−0.2.

For the $\delta^{13}C_{\text{AA}}$ data set, in addition to the data from the present study, data from the literature on primary producers including mangroves and MPB and consumers from mangrove forests from 2 other geographic locations, including tropical northern Australia (Harada et al. 2020a) and Spanish Cayes Belize (Larsen et al. 2012), were included in the analysis. From Harada et al. (2020a), we used grey mangrove Avicennia marina (n = 6), MPB (n = 2), fiddler crab Tubuca signata (n = 6), sesarmid crab (Sesarmidae sp.; n = 5), gastropod Telescopium sp. (n = 6) and oyster Crassostrea sp. (n = 4). From Larsen et al. (2012), we used buttonwood mangrove Conocarpus erectus (n = 1), dwarf red mangrove Rhizophora mangle (n = 1), white mangrove Laguncularia racemosa (n = 1), MPB (n = 1), oyster C. rhizophorae (n = 2), sesarmid crab Aratus pisonii (n = 1), gastropod Littoraria sp. (n = 1) and snapper Lutjanus griseus (n = 1). Because MPB generally consists of diatoms and cyanobacteria, 5 diatom species and 4 cyanobacteria species from Larsen et al. (2013) were included in the microalgae group. Following the methods described by Larsen et al. (2009), differences in $\delta^{13}C$ values between Ile and Leu ($\delta^{13}C_{\text{Ile-Leu}}$) and Ile and Lys ($\delta^{13}C_{\text{Ile-Lys}}$) were used to produce a scatterplot to distinguish 4 groups — vascular plants, microalgae, fungi and bacteria — and to assess the biosynthetic origins of AAs in mangrove consumers. For this analysis, 9 species of fungi and 12 species of bacteria from Larsen et al. (2013) were used. Additionally, the biosynthetic origins of AAs were compared among the mangrove consumers, terrestrial detritivores including enchytraeids and collembolans isolated from peatland soils of northern Alaska and reared enchytraeids (from Larsen et al. 2016) and terrestrial herbivores including eggs of reared hawkmots Amphinom florigenis that had been fed grape leaves as larvae and nectar (sucrose solution) as adults (from O’Brien et al. 2002; Figs. S1 & S2). A full list of samples and data used in this study is provided in Table S1 in Supplement 2 (www.int-res.com/articles/suppl/m681p013_supp2.xlsx). ANOVA was performed to explore the differences between group means. Before performing ANOVA, the assumptions of homogeneity of variance and normality in distribution were checked by performing Levene’s test and Shapiro-Wilks tests, respectively. All statistical analyses were conducted in R version 3.4.3 with RStudio interface version 1.1.414, with $\alpha$ set at 0.05.

3. RESULTS

The $\delta^{15}N_{\text{pfe}}$ values differed significantly between mangroves and microalgae, with mangroves having much higher $\delta^{15}N_{\text{pfe}}$ values than the microalgae (ANOVA $F_{1,7} = 46.5, p < 0.001$; Fig. 1). Relative contributions ($f$) of mangroves to consumers ranged from 0−0.7. TPs of consumers ranged from 1.5−1.9 (Fig. 1). The $\delta^{13}C$ values in 3 AAs (Ile, Lys and Leu) separated plants (mangroves and microalgae), fungi and bacteria (Fig. 2). The $\delta^{13}C_{\text{Ile-Leu}}$ and $\delta^{13}C_{\text{Ile-Leu}}$ values both differed significantly among the microalgae, bacteria, fungi, plant and consumer groups (ANOVA $F_{4,79} = 61.2, p < 0.001$ and $F_{4,79} = 62.4, p < 0.001$, respectively). While the fungi group was characterized by higher $\delta^{13}C_{\text{Ile-Leu}}$ values, the bacteria group had a lower $\delta^{13}C_{\text{Ile-Leu}}$ value, the mangrove group had a higher $\delta^{13}C_{\text{Ile-Leu}}$, value and the microalgae group had an intermediate $\delta^{13}C_{\text{Ile-Leu}}$ value. While the consumers did not overlap with the fungi and bacteria groups, there was a strong overlap between the consumers and the microalgae group.

4. DISCUSSION

Food webs are thought to be based either on energy obtained from the consumption of living biomass by herbivores or the consumption of dead biomass by detritivores (Moore et al. 2004, Holt 2006). However, most real food webs represent an interplay...
between the two, with many consumers feeding on food items from both living-autotroph and detritus-based webs (Wolkovich et al. 2014). Colonisation of decaying mangrove leaves by fungi and bacteria is thought to provide an important food resource to consumers at the base of estuarine food webs (Odum & Heald 1975); however, our results from the CSIA-AA approach did not suggest an importance of AAs derived from fungi and bacteria in 2 common mangrove macrofaunal groups—fiddler crabs and sesarmid crabs—suggesting that they are more grazers than microbivores. Microbivory can increase the TP of primary consumers in a detrital food web, as microbes convert nonliving organic matter into living microbial biomass, thus adding a trophic step (Steffan et al. 2017). However, the mangrove consumers from the present study that showed a TP approaching 2 (i.e. herbivores) did not support microbivory. Consistent with this finding, a combination of δ¹³C values in Leu, Ile and Lys showed that the mangrove consumers rather relied on AAs originating from plants, especially microalgae. The consumers were more similar to herbivores (hawkmoth) than detritivores (enchytraeids and collembolans; see Fig. S2). This trend was also consistent for other consumers from 2 other mangrove sites, including fish, bivalves and gastropods. This result also partially agrees with many other isotope studies of mangrove food webs that have shown the importance of microalgae (e.g. Larsen et al. 2012, Harada et al. 2020a,b). Similarly, studies of mangrove food webs using fatty acids generally support the importance of algal food sources (Meziane & Tsuchiya 2000, Guo et al. 2020).

In a quantitative sense, the 2-source mixing model using δ¹⁵N values of Phe and Glu and source contributions estimated from a 2-source mixing model. Dashed lines: endmember mixing lines for microalgae and mangroves, respectively. Vertical position indicates TP; horizontal position indicates dietary reliance on microalgae and mangrove; solid lines indicate TP prediction. Symbol colours as in Fig. 2 was possibly overestimated. However, consistent with our mixing model result, field observations showed that fiddler crabs feed on bulk sediment in canopy gaps of the mangrove forest where light availability promoted MPB abundance (Harada et al.
2020b). Strong reliance on microalgae with a minimal contribution from mangroves was therefore expected for this crab. On the other hand, the sesarmid crab *Neosarmatium trispinosum* (Sesarmid 1), which builds mounds and burrows under stilt roots of red mangroves and actively feeds on freshly fallen leaves, showed a considerable contribution from mangroves (70%). Consistent with this finding, the gut content analysis showed that vascular plant material was the dominant food item (83.3 ± 4.6%) (Harada & Lee 2016). A mixing model using δ13S values of red mangrove (−14.8‰), MPB (10.2‰) and *N. trispinosum* (−6.7‰) produced a similar result in that the mangrove contribution was 68% (Table S1). The other sesarmid crab, *Parasesarma erythodactyla* (Sesarmid 2), which also feeds on mangrove leaves, showed a stronger reliance on microalgae (80%) than on mangrove leaves (20%), suggesting that even when mangrove litter is ingested it is not necessarily assimilated by the animal. The mixing dynamics are relatively well resolved in the δ15N data set (Fig. 1) but not in the δ13C data set, with missing endmembers likely associated with microalgae (Fig. 2). This is probably because carbon isotopic compositions in AAAs of microalgae vary considerably in the marine environment (Larsen et al. 2020, Skinner et al. 2021). Overall, the assessment of feeding dependency generally shows that most mangrove consumers rely on more easily assimilable microalgae rather than refractory mangroves, and the use of mangrove leaves is limited for specialised consumers such as *N. trispinosum*. These findings partially support the assertion that the mangrove food web is a grazing food web rather than a detrital food web, with most consumers in the food webs being algivores.

Although our understanding of how and what biochemical processes determine isotope variations in individual AAAs is still limited, consistent with several other studies (e.g. Larsen et al. 2012), substantial isotope variations in AAAs among different source organic matter exist, and such variations are especially useful for disentangling complex food webs that involve microbes. The CSIA-AA approach provided molecular-level information, but similar to the conventional bulk SIA approach, obtaining the right endmembers was not easy. Sampling a wider range of consumers with different feeding types may identify missing but important food resources assimilated by the consumers in the mangrove food web (Then et al. 2021). The bacteria and fungi samples used in the study were not directly collected at the mangrove sites, and more detailed sampling and characterisation of bacterial and fungal endmembers in mangrove food webs are needed. Based on the assumption that δ13CAA fingerprints are associated with the difference in AA biosynthesis among major phyligenic groups and are consistent among different ecosystems (Larsen et al. 2009), the current data did not support the importance of AAAs derived from fungi and bacteria in the mangrove food web. Likely explanations for these results include that (1) mineralisation of decaying leaves by bacteria and fungi support the production of more easily assimilated microalgae by supplying nutrients such as N (Fry & Ewel 2003, Maie et al. 2008); and (2) bacteria and fungi as intermediates also incorporate plant-derived AAAs into their biomass, which are then passed to the consumers (Talbot et al. 2008, Steffan et al. 2015, Yamaguchi et al. 2017). The existence of such bacterial and/or fungal intermediates was partially evident from the δ15N data set that slightly underestimated the TP of the primary consumers (i.e. 1.5−1.9). For example, bacterial and/or fungal intermediates could possibly incorporate dissolved inorganic N available in the environment (such as ammonium) and then reset the nitrogen baseline and shift the trophic level of primary consumers downwards from the 2.0 value expected for herbivores. TP estimates are also sensitive to TEFs, which are thought to vary depending on protein intake and quality (McMahon & McCarthy 2016). In our case, the typical TEFs (8.0 ± 1.2‰ for δ15NGlu and 0.4 ± 0.5‰ for δ15NPhe) underestimated the TP of mangrove consumers, suggesting that the use of the typical TEFs for mangrove detritivores should be carefully evaluated in the future. Overall, the results suggest that the involvement of bacterial and fungal intermediates is possibly significant in the mangrove food web but was not apparent from the CSIA-AA approach. A more detailed characterisation of endmembers in future research is needed to improve mangrove food web analyses.

**Acknowledgements.** We acknowledge support from Hols-worth Wildlife Research Endowment—Equity Trustees Charitable Foundation and the Ecological Society of Australia. R.C. acknowledges the Global Wetlands Project, supported by a charitable organisation which neither seeks nor permits publicity for its efforts.

**LITERATURE CITED**


Harada Y, Connolly RM, Fry B, Maher DT and others (2020a) Stable isotopes track the ecological and biogeochemical legacy of mass mangrove forest dieback in the Gulf of Carpentaria, Australia. Biogeosciences 17: 5599–5613


Then AYH, Adame MF, Fry B, Chong VC, Riekensberg PM, Zakaria RM, Lee SY (2021) Stable isotopes clearly track


Editorial responsibility: Erik Kristensen, Odense, Denmark
Reviewed by: T. Larsen and 2 anonymous referees

Submitted: March 4, 2021
Accepted: October 7, 2021
Proofs received from author(s): December 26, 2021