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Quantifying terrestrial carbon in freshwater food webs using amino acid isotope analysis—case study with an endemic cave fish

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Abstract

1. Flow of terrestrial carbon though aquatic ecosystems (allochthony) is an important but underestimated component of the global carbon cycle. A lack of clear consensus about the importance of allochtonous (terrestrial) organic carbon is sometimes attributed to uncertainties associated with conventional 'bulk' isotope data, the most widely used ecological tracer.
2. Amino acid-specific isotope analysis is an emerging research method promising to address existing limitations of bulk C and N isotope analyses. We tested the efficacy of amino acid $\delta^{13}C$ data as a generalisable measure of allochthony by analysing an aggregated dataset (*n*=168) of primary and secondary data of carbon sources from disparate geographical locations across the globe.
3. We found the $\delta^{13}C$ fingerprints amino acids to be consistently distinct between allochthonous (terrestrial) and autochthonous (aquatic) carbon sources. We also found that our approach is most effective when we use only essential amino acid tracers (i.e., isoleucine, leucine, phenylalanine, threonine, and valine). Predictive trends in $\delta^{13}C$ fingerprints appear to be largely compatible across studies and/or laboratories.
4. As a case study, we used this approach to quantify the contribution of terrestrial carbon to an endemic cave fish, *Cryptotora thamicola*, and found that its biomass was comprised largely of autochthonous carbon (~75%).

Introduction

Allochthonous flow of terrestrial carbon into aquatic food webs (allochthony) is thought to be an underestimated component of the global carbon cycle (Boyero et al. 2011; Hanson et al. 2014). Allochthony is commonly studied using naturally occurring carbon and nitrogen isotopes (Jardine et al. 2017; Brett et al. 2017; Tanentzap et al. 2017), yet methodological constraints have thus far

hindered the formulation of a clear consensus about the role of terrestrial organic carbon in aquatic ecosystems (Brett et al. 2017).

A major limitation of conventional 'bulk tissue' stable isotope analysis (non-specific, whole-tissue analysis of all organic compounds in an organism) is that nitrogen and carbon isotope profiles of aquatic and terrestrial plants often overlap (Finlay 2001). Moreover, bulk isotope signals are susceptible to fluctuating rates of trophic discrimination—i.e., the difference in carbon isotope ratios ($\delta^{13}C$) of predators relative to their prey (Caut et al. 2009; Ohkouchi et al. 2015). This can differ between taxonomic groups (Vander Zanden & Rasmussen 2001) as well as across the spectrum of predator-prey biochemical compatibility (Bastos et al. 2017; Brett et al. 2017). Consequently, bulk isotope approaches are difficult to up-scale spatially and temporally because time- and location-specific sampling of primary producers are needed to account for uncertainties in primary producer $\delta^{13}C$ (Hadwen et al. 2010; Bowes & Thorp 2015). Relevant primary producers, however, can be difficult to collect (e.g., unidentifiable source of terrestrial detritus in cave rivers), isolate (e.g., biofilm mixed with fine substrate and microorganisms), or even find (e.g., time-appropriate producers in longitudinal studies using museum-preserved consumer specimens (Thorp & Bowes (2017)).

Uncertainty in bulk isotope data are sometimes attributed to differences in the biosynthetic pathways of the various biochemical compounds (e.g., protein, carbohydrate, lipid) comprising a heterotrophic organism's diet and tissue (McMahon et al. 2010; Ohkouchi et al. 2015). Advances in mass spectrometry offer a logical solution to this problem—increase data specificity by profiling individual biochemical compounds separately (compound specific isotope analysis or CSIA) (Larsen et al. 2009). Candidate compounds include fatty acids and amino acids, but interpretation of the former can be complicated by the yet unresolved unpredictability in trophic discrimination (Nielson et al. 2018). Moreover, pioneering publications show that amino acid $\delta^{13}C$ patterns are useful identifiers of potential carbon sources (Scott et al. 2006; Larsen et al. 2009; 2013). Since then,

variations of this approach have been successfully applied in terrestrial (Gomez et al. 2018), marine (Arthur et al. 2014; McMahon et al. 2015), and freshwater ecosystems (Thorp & Bowes 2017). However, amino acid-specific $\delta^{13}C$ analysis has not been widely adopted, possibly because of the high analytical costs involved and the presently inadequate understanding of its ecological applications (Nielson et al. 2018).

In this paper, we use a comprehensive amino acid $\delta^{13}C$ dataset comprising both primary and secondary data to explore a new, widely-generalisable approach to tracing terrestrial organic carbon in aquatic food webs. While amino acid $\delta^{13}C$ patterns of terrestrial plants are conserved across space (Larsen et al. 2012), data from marine predatory fish suggest that amino acid $\delta^{13}C$ may be more variable in aquatic primary producers (Wang et al. 2018). Therefore, we asked if amino acid $\delta^{13}C$ profiles can consistently identify terrestrial (allochthonous) and aquatic (autochthonous) organic carbon sources across disparate geographical regions. As a case study, we quantified the contribution of terrestrial carbon to the biomass of the endemic waterfall-climbing cave angel fish, *Cryptotora thamicola* (Kottelat 1989). Cave river ecosystems are often inaccessible to researchers, and unsurprisingly, published data are scarce and sometimes conflicting (see Simon et al. (2003) and Venarsky et al. (2014)). Considering its potential for generalised applications, amino acid $\delta^{13}C$ data may be well-suited for quantifying the degree of allochthony in cave river systems where carbon sources are not always apparent. Here, we test the assumption that subterranean food webs are largely supported by allochthonous organic carbon subsidies (Culver 1982; Polis et al. 1997).

Methods

Data collection

We collected a total of 47 primary carbon source data points (Appendix A; Supplementary Data) by sampling producers from tropical forest stream ecosystems and associated riparian zones in

Singapore (1º N, 103 E), Peninsular Malaysia (5º N, 103º E), and north-western Thailand (19º N, 99º E). These comprised periphyton (autochthonous, *n*=9), macrophytes (autochthonous, *n*=5), peat (allochthonous, *n*=3), C3 plants (allochthonous, *n*=18), and C4 grasses (allochthonous, *n*=12). All producer samples were preserved in salt (Arrington & Winemiller 2002) before subsequent processing in the laboratory. Samples were dried at 70ºC for 48 hours and homogenized. Approximately 20mg worth of ground samples were packed in 12 ml Borosilicate Extainer Vials and shipped to the University of California, Davis (UC Davis) Stable Isotope Facility for amino acid $\delta^{13}C$ isotope analysis (details of protocol in Appendix B).

We compiled secondary data via a systematic literature search on *Web of Science* (search term=[("compound specific isotope analysis" OR "stable isotope analysis" OR "stable isotope" OR "isotope analysis") AND ("amino acid") AND ("carbon" OR "d13C' OR "carbon-13")]). We examined returned studies to ascertain suitability and extracted all $\delta^{13}C$ data associated with strictly autotrophic taxa (photoautotrophs and chemolitotrophs) through which inorganic carbon (e.g., CO_2) enters biological systems. Using this search protocol, we aggregated 121 data points (Appendix A; Supplementary Data) comprising 78 allochthonous carbon sources (Fogel & Tuross 2003; Larsen et al. 2013; Paolini et al. 2015; Jarman et al. 2017; Thorp & Bowes 2017) and 43 autochthonous carbon sources (Scott et al. 2006; Larsen et al. 2013; Thorp & Bowes 2017). Geographically, origins of non-cultured producer samples include Africa [i.e., Nigeria (9 N, 9º E)], Australia (25º S, 133º E), continental USA [i.e., Alaska (61º N, 156º W), Kansas (39º N, 98º W), and Maryland (39º N, 77º W)], the Caribbean [i.e., Puerto Rico (18 N, 67º E)], Europe [i.e., Denmark (56º N, 10º E); Germany (51º N, 10º E); Italy (42º N, 13º E); Spain (40º N, 94 W)], South America [i.e., Ecuador (2º N, 78º E)], and South Asia [i.e., Sri Lanka (8º N, 81º E)].

Study design

We consolidated both primary and secondary data for subsequent analyses (Liew et al. 2019). The combined dataset comprised $\delta^{13}C$ values of 12 amino acids (AA) (isoleucine, leucine, phenylalanine, threonine, valine, alanine, aspartate, glutamate, glycine, methionine, proline, and tyrosine) from 168 primary producers from locations listed above. However, measurements for less abundant AA (e.g., methionine) often fell below detection limits. We excluded lysine in consideration of potential co-elution with tyrosine (McMahon et al. 2010).

Amino acid selection can influence the effectiveness of $\delta^{13}C$ data in quantifying assimilated terrestrial carbon. The selection process should seek a balance between maximising the number of tracers (i.e., AAs) and maximising replication, the latter necessitating exclusion of AAs with missing data. Further, tracer selection should also consider the potential confounding impacts of trophic discrimination sometimes observed in AAs which are non-essential to vertebrates (e.g., alanine, glutamate) (McMahon et al. 2010). Therefore, we tested four selection strategies:

1. "All AAs"—maximise retention of tracers (12 amino acids, *n*=47);
2. "Abundant AAs"—increase replication by excluding AAs with more than 5% missing value (i.e., methionine, proline, tyrosine, and threonine) (8 amino acids, *n*=162);
3. "All essential AAs"—maximise essential AA retention (5 amino acids, *n*=156); and
4. "Abundant essential AAs"—maximise replication by excluding essential AAs with missing values (4 amino acids, *n*=168).

We were interested in relative carbon isotope fractionation between AAs, rather than absolute $\delta^{13}C$, so our data were normalized to corresponding sample means using the following formula:

$$\delta^{13}C_{n,ij} = \delta^{13}C_{ij} - \frac{\sum_{k=1}^{P} \delta^{13}C_{kj}}{P} \quad \text{.....(1),}$$

where $\delta^{13}C_{n,ij}$ represents normalized $\delta^{13}C$ of the *i*-th AA of the *j*-th data point, while $\delta^{13}C_{ij}$ represents the raw $\delta^{13}C$ value of the *i*-th AA of the *j*-th data point, and *P* represents the number of AAs retained for analyses under strategies 1–4 listed above, respectively.

Statistical analyses

We visualized potential differences in $\delta^{13}C_n$ profile (henceforth referred to as $\delta^{13}C$ fingerprints (*sensu* Larsen et al. 2013)) between allochthonous and autochthonous carbon sources by running Principal Components Analyses (PCA) for each set of AAs corresponding to respective selection strategies.

We assessed the cogency of $\delta^{13}C$ fingerprints in predicting probability of terrestrial origin for all AAs using Bayesian logistic regressions on the *rjags**4.6 statistical package (Plummer 2006). This was chosen over a frequentist approach to maintain philosophical consistency with subsequent Bayesian mixing models (see *Case study*) commonly used for isotopic diet quantification (Parnell et al. 2013). We analysed our data with a combination of multivariate and univariate approaches. In our multivariate approach, we tested the relationship between our binary response variable (i.e., allochthonous or autochthonous) and two continuous predictor variables (i.e., PC1 and PC2) by parameterising the posterior distributions of model coefficients with 100,000 iterations of the following model (burn-in = 5,000) on four parallel chains (thinning interval = 1):

$$Log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1(PC1) + \beta_2(PC2) \quad \text{..... (2)},$$

where *p* represents the probability that a carbon source is allochthonous, while PC1 and PC2 represent the first two principal components from PCA outputs corresponding to the respective selection strategies. Priors for all parameters were weakly informative. Here, non-zero β_1 and/or β_2 coefficients (95% credible interval does not overlap with zero) suggest that a set of $\delta^{13}C_n$ profiles (derived with the respective selection strategy) are distinct between allochthonous and autochthonous carbon sources and *vice versa*.

In our univariate approach, we parameterised the posterior distributions of model coefficients describing the relationship between our binary response variable (i.e., allochthonous or autochthonous) and continuous predictor variables (i.e., $\delta^{13}C_n$ values) with the following model using the same procedure as in equation (2):

$$Log\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_3(\delta^{13}C_{n,i}) \quad \text{..... (3).}$$

Here, p_i represents the probability that a carbon source is allochthonous given a set of normalized $\delta^{13}C$ values of the *i*-th AA, while $\delta^{13}C_{n,i}$ represents normalized $\delta^{13}C$ of the *i*-th AA. Non-zero β_3 coefficients suggest that the respective set of $\delta^{13}C$ fingerprints associated with the *i*-th AA are predictive of the probability that a carbon source is allochthonous.

Both multivariate and univariate analyses were repeated for each selection strategy because $\delta^{13}C$ fingerprints of respective AAs may fluctuate with differing $\delta^{13}C$ means calculated for corresponding AA pools. For example, the $\delta^{13}C$ mean across twelve AAs in Strategy 1 is likely to differ from the $\delta^{13}C$ mean across five AAs in Strategy 3.

We also compared AA selection strategies quantitatively using outputs from Linear Discriminant Analyses (LDA) of $\delta^{13}C$ fingerprints. Here, data subsets from each selection strategy were randomly allocated for model training (75%) and validation (25%). We then estimated model performance in distinguishing between allochthonous and autochthonous carbon sources by computing AUC (Area Under the Curve) scores. An AUC score of 1.0 reflects a perfectly predictive model, while a score of 0.5 suggests that model predictions are not statistically different from chance classifications. We repeated this over 1,000 iterations.

Inter-laboratory data compatibility

The utility of our approach as a generalisable measure of allochthonous carbon assumes that AA $\delta^{13}C$ data are compatible across laboratories. However differences in CSIA methodology (e.g., derivatization protocol) can produce measurement discrepancies which are difficult to control for, given the scarcity of pairwise inter-laboratory comparisons (Roberts et al. 2018). We checked that our results are robust to potential inter-laboratory measurement discrepancies using two separate tests.

First, we assessed the generalisability of predictive relationship (determined via equation (3)) across studies (or laboratories) by introducing an interaction term with *study* as a covariate (where *study* is a categorical variable with three levels: i) Liew et al. (present study, i.e., primary data from this study); ii) Larsen et al. (2013); and iii) Thorp & Bowes (2017)). We excluded data from Fogel & Tuross (2003), Paolini et al. (2015) and Jarman et al. (2017) because autochthonous carbon source data were not reported, while Scott et al. (2006) was excluded because allochthonous carbon source data were lacking. We fit one multivariate model (modified from equation 2) and one univariate model (modified from equation 3) to our data:

$$Log\left(\frac{p_k}{1-p_k}\right) = \beta_0 + \beta_1(PC1) + \beta_2(PC2) + \beta_4(study_k) + \beta_5[(PC1)(study_k)] + \beta_6[(PC2)(study_k)] \quad \text{..... (4);}$$

$$Log\left(\frac{p_{ik}}{1-p_{ik}}\right) = \beta_0 + \beta_3(\delta^{13}C_{n,i}) + \beta_4(study_k) + \beta_7\left[(\delta^{13}C_{n,i})(study_k)\right] \quad \text{.....(5).}$$

Here, p_k and p_{ik} represents the probability that a carbon source is allochthonous, PC1 and PC2 are principal components of PCAs conducted for each selection strategy, $\delta^{13}C_{n,i}$ represents normalized $\delta^{13}C$ of the *i*-th AA, and $study_k$ represents studies 1–3 listed above. We were interested in the coefficients describing the interaction terms (i.e., $\beta_{5–7}$) because if these were not statistically different from zero, differences in $\delta^{13}C$ fingerprints between allochthonous and autochthonous

carbon sources (described by coefficients β_{1-3}) can be generalised between studies. Conversely, we were not interested in the main effect of *study* because a non-zero β_4 would simply suggests that the likelihood of a carbon source being allochthonous differ between studies/datasets. This would imply that the proportion of allochthonous data points were greater in some studies/datasets than in others. We fit the model in equation (5) and (6) to data subsets in every selection strategy, except Strategy 1 where only one dataset (from the present study) was retained given the selection criteria.

Second, we generated 1,000 simulated datasets in which random error in both directions (i.e., positive and negative) were added to our data. Simulated random error were specific to each AA and was drawn from a uniform distribution bounded by ± mean inter-laboratory discrepancy reported by Arthur et al (2014) and Gomez et al. (2018), respectively (Appendix H). Here, we reduce the accuracy of measurements by increasing the difference between simulated and measured values. We fit Bayesian logistic regressions of the statistical model described in equation (3) to each of our 1,000 simulated datasets. Resulting coefficient estimates (i.e., 1,000 mean β_3 values) were then compared to those derived from observed data. In view of the computation demands and data availability (Arthur et al. (2014) only compared essential AAs), we restricted error simulation to Strategy 3.

Case study

Study site

We tested our approach to quantifying allochthony in an oligotrophic cave river ecosystem in Tham Susa of the Maehongsorn region of northwest Thailand (19°28'N; 98°08'E). The endemic, highly threatened (possibly critically endangered (Vidthayanon 2011)) cave fish, *Cryptotora thamicola* (Family Balitoridae), is a rheophile that has only been recorded from a single series of rapids (spanning a 50 metre reach) within this cave (Trajano et al. 2002). To date, nothing is known about the feeding ecology of *C. thamicola*. While other cave fishes are presumed generalists (Trajano et al.

2002), surface-dwelling confamilials of *C. thamicola* subsist primarily on biofilm (Yang & Dudgeon 2010; Sheldon 2011). The only other metazoa known to occur in the Tham Susa cave system are terrestrial cockroaches and small, unidentified flying insects.

As with many subterranean communities, little is known about the carbon sources supporting the food web at our study site (Trajano et al. 2002). This system is well-suited for testing our approach because there are two main sources of organic carbon: particulate organic matter from terrestrial C_3 and/or C_4 plants outside the cave (allochthonous) and biofilm growing on submerged cave walls (autochthonous) and instream rock surfaces (Trajano et al. 2002). In cave ecosystems, strict definitions of allochthonous input would include organic matter from aquatic producers (e.g., periphyton) outside the cave. However, we do not make a distinction between surface- and cave-aquatic carbon sources because we were mainly interested in identifying terrestrial primary production. For convenience, all aquatic (or submerged) primary producers will henceforth be equivalently referred to as "autochthonous" carbon sources.

Data collection and analyses

We collected one adult *C. thamicola* (standard length ca. 25 mm) from the downstream face of boulders at the rapids in the Tham Susa cave system. Greater sample sizes for AA $\delta^{13}C$ analysis were unavailable given the species' relative scarcity, low population size (possibly 10^2–10^3 individuals) and highly restricted distribution (Trajano et al. 2002). This specimen, which constituted one of three individuals of the species ever collected (two are in museum collections), was salt-preserved (Arrington & Winemiller 2002) for subsequent processing. We visualised the $\delta^{13}C$ fingerprints of *C. thamicola* in relation to allochthonous and autochthonous producers using a PCA.

Relative carbon contributions from allocthonous and autochthonous carbon were estimated using Bayesian mixing models on the *simmr**0.3 (Parnell 2016) statistical package on the *R* statistical

platform. We ran 100,000 iterations (burn-in = 5,000) of a two-source (i.e., allochthonous and autochthonous) mixing model on four parallel chains (thinning interval = 1). We did this with twelve, eight, five, and four tracers (corresponding to AA selection strategies 1–4), respectively. Source information (allochthonous and autochthonous $\delta^{13}C_n$ mean and standard deviation) were derived from data subsets associated with each AA selection strategy. We conducted all analyses on the R*3.4.1 statistical platform (R Core Team 2017).

Results

Ordination outputs (PCA and LDA) of different combinations of AAs across all our four selection strategies suggest that $\delta^{13}C$ fingerprints are consistently distinct between allochthonous and autochthonous carbon sources (Fig. 1) (Table 1). Strategy 3, where we all essential AAs were retained, accounted for the greatest proportion of variance in the data between its two major principal components (60% by PC1 and 26% by PC2). Strategy 3 also returned the joint-highest mean AUC score of 0.89 (Table 1).

Multivariate and univariate logistic regression output show that Strategy 3 produced generally (Table 1) and individually (Table 2) (Appendix E) predictive $\delta^{13}C$ fingerprints. In contrast, Strategy 1 was relatively inefficient, in that only five of its twelve AAs (Table 2) were predictive of allochthony. Direction of predictions were generally consistent across selection strategies. For example, $\delta^{13}C$ fingerprints of leucine and valine were mostly lower in allochthonous carbon sources while the opposite was true for phenylalanine.

Inter-laboratory data compatibility

Overall, $\delta^{13}C$ fingerprints produced by Strategy 2 and Strategy 3 were generalisable across studies (Table 1). Selection strategy 3 also produced $\delta^{13}C$ fingerprints which were mostly generalisable—phenylalanine $\delta^{13}C$ being the only exception. However, in all selection strategies, at least one AA was associated with a non-zero interaction term (β_7) when analysed individually (Table 1). Moreover, we

found that $\delta^{13}C$ fingerprints retained their predictive efficacy after statistically controlling for discrepancies in measurements between studies (Appendix C;D).

Bayesian logistic regression of 1,000 simulated datasets produced mean β_3 coefficient ranges which overlap with the mean β_3 coefficients estimated from observed data (Fig. 2). This was true for all five essential AAs, suggesting that differences in $\delta^{13}C$ fingerprints between allochthonous and autochthonous carbon sources were not significantly confounded by the artificial introduction of additional uncertainty in the accuracy of isotope measurements.

Case study

The $\delta^{13}C$ fingerprint of *C. thamicola* overlapped with allochthonous producers in ordination space for selection strategies 1 and 2, where non-essential AAs were retained as source tracers (Fig 1; Fig S2). Conversely, autochthonous signals were clear when we retained only essential AAs (Strategies 3 and 4) (Fig 1; Fig S2). This trend is mostly conserved in our mixing model output, with one notable exception—only Strategy 1 produced a source contribution estimate indicative of allochthonous carbon dominance (~95%) (Fig. 3). Source contribution estimates from Strategies 2, 3, and 4 suggest autochthonous carbon dominance, ranging from ~74% to ~81%. We also modified Strategy 3 by selecting only AAs with statistically generalisable trends in $\delta^{13}C$ fingerprints (isoleucine, leucine, threonine, valine (Table 1)) as source tracers. This had little effect on source contribution estimates (Fig. 3).

Discussion

We show in this paper that amino acid $\delta^{13}C$ fingerprints are consistently distinct between allochthonous and autochthonous carbon sources collected globally, making them reliable tracers of terrestrial organic carbon assimilation in freshwater food webs (Fig. 1; Table 1; Appendix I). It is important to note, however, that this is contingent upon AA selection. When analyses were

restricted to essential AAs (Strategy 3), all associated $\delta^{13}C$ fingerprints were cogent predictors of organic carbon source. Moreover, differences in $\delta^{13}C$ fingerprints between carbon sources were also mostly generalisable across studies (or laboratories).

When non-essential AAs were retained (e.g., Strategy 1), mixing model output presented a skewed perception of allochthonous carbon contribution (Fig. 1; Fig 3; Appendix I). These erroneous observations were consistent with our understanding of trophic discrimination patterns (see McMahon et al. 2010). For example, *C. thamicola* $\delta^{13}C$ fingerprints derived using selection strategy 1 would likely be placed in an ordination space associated with autochthonous carbon sources if its data point was shifted in a direction which 'corrects' for known trophic discrimination values—i.e., by inflating alanine, glutamate, and glycine $\delta^{13}C$ (higher on both *x*- and *y*-axes on Fig. 1) (McMahon et al. 2010). Notably, the confounding influence of trophic-discrimination was minimised (Fig. 3) when source tracers used in mixing models comprised a reduced proportion of non-essential AAs (i.e., Strategy 2).

With the right combination of AA tracers (i.e., Strategy 3), our approach to quantifying allochthony addresses two common shortfalls associated with conventional bulk isotope approaches. First, consistent differences in $\delta^{13}C$ fingerprints between allochthonous and autochthonous carbon sources contrasts the unpredictable fluctuations in bulk tissue $\delta^{13}C$ data Bowes & Thorp 2015). Second, the availability of non-fractionating tracers (i.e., essential AAs) (McMahon et al. 2010) eliminates the need for explicit knowledge about the exact number of trophic steps between consumers and relevant primary producers at the base of the food web. This will be particularly useful in complex food webs with near ubiquitous omnivory (e.g., tropical lake food webs (Liew et al. 2018)) where consumer trophic levels may be difficult to determine. Moreover, $\delta^{13}C$ fingerprints are mostly invariant across different growing conditions (Larsen et al. 2015)—strengthening allochthony estimates.

When studying rare/endangered taxa, ecologists commonly work with small sample sizes. Without adequate replication, mixing models using bulk isotope data can fail to resolve source contribution (Phillips et al. 2014). Issues with source identification are further exacerbated when source isotope profiles overlap (Brett 2014), especially if analyses are reliant on the small number of tracers available with bulk tissue approaches (Fry 2006; Fry 2013). By using five tracers (i.e., Strategy 3), we show that carbon source contributions can be resolved despite sample size limitations. Moreover, the exclusion of AAs with non-generalisable patterns in $\delta^{13}C$ fingerprints did not appear to impact the efficacy of carbon source resolution (Fig. 3).

A major strength of this approach is the ability to measure allochthonous input using an aggregated source dataset in lieu of collecting time- and site-specific primary producer data. This, however, assumes cross-laboratory data compatibility. Using two independent lines of evidence, our findings support the assumption of data compatibility when using our recommended AA selection strategy. First, we show that the differences in $\delta^{13}C$ fingerprints between allochthonous and autochthonous carbon sources were mostly generalisable across datasets produced by two different labs (Table 1,2; Appendix G)—the UC Davis Stable Isotope Facility (i.e., primary data for this paper and Thorp & Bowes (2017)) and the Liebniz Laboratory for Radiometric Dating and Stable Isotope Research (i.e., Larsen et al., 2013). Second, we also found that parameters estimated from our logistic regressions remained consistent, even with the introduction of simulated measurement errors (Fig. 2). These suggest that $\delta^{13}C$ fingerprints of essential AA are reliable tracers of carbon source despite possible measurement discrepancies in multi-laboratory datasets.

Another observation of note was that $\delta^{13}C$ fingerprints were more conserved in some functional groups than in others (Appendix F). For example, C_3 and C_4 terrestrial plant $\delta^{13}C$ were less dispersed in ordination space than autotrophic bacteria. We postulate that these trends may be attributable to lower phylogenetic diversity in terrestrial plants relative to autotrophic bacteria (Hug et al. 2016), where the latter comprise taxa ranging from photoautotrophic cyanobacteria to chemolithotrophic

bacteria (e.g., *Aquifax* sp.). This is congruent with documented links between primary metabolic pathways (e.g., functional groups involved, number of reaction steps) and the relative isotopic enrichment/depletion between AAs (Macko et al. 1987; Ohkuichi et al. 2015) which determine $\delta^{13}C$ fingerprints.

Case study

Our findings suggest that carbon in *C. thamicola* biomass is mostly derived from autochthonous (i.e., internal aquatic) sources. As cave fishes are often apex predators in the subterranean river ecosystem they occupy (Parzefall & Trajano 2010), the autochthonous dominance we observed in *C. thamicola* is likely applicable to the entire food web as apex predators reflect overall assimilation of energy from all trophic pathways (Rooney et al. 2008; Wolkovich et al. 2014).

Cave river ecosystems were thought to be dependent on allochthonous organic carbon subsidies (Culver 1982; Polis et al. 1997). However, recent data from studies using ecological tracers (e.g., stable isotopes) show that the contribution of autochthonous production have been hitherto underestimated (Simon et al. 2003; Caroll et al. 2016)—as substantiated by our findings. In our case study, autochthonous organic carbon sources are likely derived from epilithic biofilm. Given the absence of light sources, chemoautotrophy is a potential biochemical pathway (Sarbu et al. 1996; Dattagupta et al. 2009). Alternatively, the cave food web may be supported by aquatic production in upstream surface habitats, although we are not able to distinguish them from subterranean autochthonous production.

Recommendations and conclusions

We show here that $\delta^{13}C$ profiles of five essential AAs (Strategy 3)—isoleucine, leucine, phenylalanine, threonine, and leucine—normalised to the mean ($\delta^{13}C$ fingerprints), are generalisable tracers of allochthonous carbon in aquatic food webs across trophic levels. This addresses one of the major gaps in our understanding of CSIA (Whiteman et al. 2019), at least when used to answer broad

ecological questions. In the absence of contextual data from site-specific producers, we propose the use of aggregated source information in mixing models (Table 2). If a more conservative approach is preferred, non-generalisable AAs (i.e., phenylalanine) can be removed from mixing models without significantly impacting carbon source estimates (Fig. 3). With greater adoption, amino acid $\delta^{13}C$ fingerprints may contribute to the resolution of debates about the importance of allochthonous carbon in fresh waters (Brett et al. 2017) and facilitate assessments of its place in the global carbon cycle.

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Data accessibility

Aggregated data, metadata, and R script: DRYAD entry doi:10.5061/dryad.7gc968r

Author Contributions

LJH and KCWJ developed the idea, formulated study design, and conducted sample preparations. LJH, KCWJ, EA, and AS conducted data collections. LJH analysed the data and took the lead in writing the manuscript. All authors provided important feedback which helped shape the research and manuscript.

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Table 1 Summary of posterior distributions of coefficients describing the multivariate relationship between amino acid $\delta^{13}C$ fingerprints (represented by PC1 and PC2) and the probability that a carbon source is allochthonous across four selection strategies. Posterior distributions of interaction coefficients indicative of the predictive generalisability of a set of $\delta^{13}C$ fingerprints across studies/data sources are also summarised. Asterisks denote all coefficients which are statistically different from zero, that is, the coefficients' 95% credible intervals do not overlap with zero.

Selection strategy	Mean β_1 coefficient (β_1 95% Credible Interval lower limit, upper limit)	Mean β_2 coefficient (β_2 95% Credible Interval lower limit, upper limit)	PC1 generalisable across studies (β_5 95% Credible Interval lower limit, upper limit)	PC2 generalisable across studies (β_6 95% Credible Interval lower limit, upper limit)	Mean AUC for LDA predictions (± Standard Deviation)
Selection Strategy 1 (7 non-essential amino acids, 5 essential amino acids)	−0.58 (−1.01, −0.26)*	−0.17 (−0.44, 0.04)	NA	NA	0.89 (±0.11)
Selection Strategy 2 (4 non-essential amino acids, 4 essential amino acids)	−0.10 (−0.18, −0.03)*	0.40 (0.27, 0.55)*	Yes (−0.05, 0.04)	Yes (−0.01, 0.13)	0.89 (±0.05)
Selection Strategy 3 (5 essential amino acids)	−0.35 (−0.52, −0.21)*	0.65 (0.44, 0.88)*	Yes (−0.13, 0.07)	Yes (−0.12, 0.23)	0.89 (±0.05)
Selection Strategy 4 (4 essential amino acids)	−0.57 (−0.77, −0.39)*	−0.41 (−0.69, −0.15)*	No (0.04, 0.32)	No (0.14, 0.94)	0.85 (±0.06)

Table 2 Summary of posterior distributions of coefficients describing the univariate relationship between amino acid $\delta^{13}C$ fingerprints and the probability that a carbon source is allochthonous across four selection strategies. Posterior distributions of interaction coefficients indicative of the predictive generalisability of a set of $\delta^{13}C$ fingerprints across studies/data sources are also summarised. Mean effect sizes represent the odds that a carbon source is allochthonous for every unit increase in $\delta^{13}C_n$. Here an odds-ratio value of 1 suggests no difference in $\delta^{13}C$ fingerprints between carbon sources, while odds-ratio>1 suggests that $\delta^{13}C_n$ is more enriched in allochthonous carbon sources and *vice versa*. Asterisks denote all coefficients which are statistically different from zero, that is, the coefficients' 95% credible intervals do not overlap with zero. Essential amino acids are marked with the *E* superscript.

Amino acid	Mean β_3 coefficient (β_3 95% Credible Interval lower limit, upper limit)	Mean effect size (odds-ratio)	Generalisable across studies (β_7 95% Credible Interval lower limit, upper limit)
Selection Strategy 1 (7 non-essential amino acids, 5 essential amino acids)			
Alanine	−1.28 (−2.20, −0.57)*	0.28	NA
Aspartate	−0.08 (−0.37, 0.16)	0.92	NA
Glutamate	−0.48 (−1.33, 0.05)	0.62	NA
Glycine	−0.07 (−0.30, 0.12)	0.93	NA
Isoleucine^E	1.06 (0.18, 1.98)*	2.89	NA
Leucine^E	−0.24 (−0.68, 0.07)	0.79	NA
Methionine	−0.12 (−0.36, 0.06)	0.89	NA
Phenylalanine^E	0.41 (0.04, 0.83)*	1.51	NA
Proline	0.84 (0.21, 1.61)*	2.32	NA
Threonine^E	0.41 (0.14, 0.74)*	1.51	NA
Tyrosine	−0.14 (−0.61, 0.19)	0.87	NA
Valine^E	−0.14 (−0.62, 0.19)	0.87	NA
Selection Strategy 2 (4 non-essential amino acids, 4 essential amino acids)			
Alanine	−0.80 (−1.07, −0.55)*	0.45	No (0.07, 0.33)*
Aspartate	−0.18 (−0.29, −0.07)*	0.84	Yes (−0.13, 0.01)
Glutamate	−0.01 (−0.14, 0.12)	0.99	Yes (−0.12, 0.03)
Glycine	0.12 (0.04, 0.20)*	1.13	Yes (−0.03, 0.03)

Isoleucine^{E}	0.11 (−0.04, 0.28)	1.12	No (0.04, 0.52)*
Leucine^{E}	−0.35 (−0.56, −0.15)*	0.70	Yes (−0.08, 0.02)
Phenylalanine^{E}	0.54 (0.36, 0.73)*	1.72	No (−0.33, −0.08)*
Valine^{E}	−0.06 (−0.23, 0.08)	0.94	Yes (−0.04, 0.13)
Selection Strategy 3 (5 essential amino acids)			
Isoleucine^{E}	−0.29 (−0.56, −0.04)*	0.75	Yes (−0.05, 0.19)
Leucine^{E}	−0.93 (−1.21, −0.61)*	0.39	Yes (−0.46, ~0)
Phenylalanine^{E}	0.34 (0.17, 0.51)*	1.40	No (−0.29, −0.06)*
Threonine^{E}	0.26 (0.14, 0.40)*	1.30	Yes (−0.02, 0.05)
Valine^{E}	−0.85 (−1.19, − 0.53)*	0.43	Yes (−0.12, 0.24)
Selection Strategy 4 (4 essential amino acids)			
Isoleucine^{E}	−0.01 (−0.21, 0.18)	0.99	No (0.04, 0.37)*
Leucine^{E}	−0.92 (−1.27, −0.59)*	0.40	Yes (−0.27, 0.02)
Phenylalanine^{E}	0.61 (0.41, 0.83)*	1.84	No (−0.37, −0.11)*
Valine^{E}	−0.41 (−0.70, −0.14)*	0.66	No (0.18, 0.62)*

Table 3 Summary of aggregated source information calculated by normalising isoleucine, leucine, phenylalanine, threonine, and valine $\delta^{13}C$ to the mean of all five essential amino acids (i.e., $\delta^{13}C$ fingerprints *sensu* Larsen et al. (2013)).

Tracer	**Autochthonous carbon source (mean ± std. dev.)**	**Allochthonous carbon source (mean ± std. dev.)**
Isoleucine	1.03 (±1.54)	0.35 (±1.30)
Leucine	−5.15 (±2.01)	−7.46 (±1.22)
Phenylalanine	−2.86 (±2.08)	−1.13 (±2.24)
Threonine	10.00 (±3.00)	12.72 (±3.25)
Valine	−3.02 (±1.06)	−4.49 (±1.37)

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Figure 1 Ordination plot of $\delta^{13}C$ fingerprints associated with allochthonous and autochthonous carbon sources and *C. thamicola* across four selection strategies (top-left: Strategy 1; top-right: Strategy 2; bottom-left: Strategy 3; bottom-right: Strategy 4). Arrows represent the direction and magnitude of eigenvectors associated with each amino acid, while values in parentheses reflect percentage of variance accounted for by PC1 (x-axis) and PC2 (y-axis), respectively.

Figure 2 Posterior distribution of source contributions from allochthonous and autochthonous carbon sources to *C. thamicola* using $\delta^{13}C$ fingerprints derived across four selection strategies (and one modified strategy) as source tracers. Numbers in parentheses correspond to selection strategies while asterisks denote strategy modification involving the removal of phenylalanine from mixing models.

Figure 3 Density curves of mean β_3 coefficients estimated from Bayesian logistic regression of simulated datasets in which random measurement errors were introduced. Vertical dashed lines represent mean β_3 values estimated from Bayesian logistic regression of observed data.

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