Carbon isotopes of essential amino acids highlight greater contribution of far-field vs near-field subsidies to predators on oceanic coral reefs

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Abstract

Reef predators are partly sustained by oceanic production sources, but the pathways through which this occurs remain poorly understood. Studies exploring reef-pelagic linkages have used bulk stable isotopes, yet these have limited power to discriminate between major source types. We used δ13C values of essential amino acids (δ13CEAA), which can better resolve different modes of carbon acquisition, to trace the origin of the carbon sources sustaining reef predator biomass in the Maldives. White muscle tissue was sampled from four key fishery target groupers and eight primary consumer species (representing six energy pathways). Primary consumer δ13CEAA values separated into four distinct clusters: 1) algae/detritus, 2) coral, 3) reef plankton, and 4) pelagic plankton. Bayesian stable isotope mixing models identified pelagic plankton as primarily sustaining all four groupers across the atoll, indicating that oceanic nutrients are available throughout and that these reefs may be more resilient to bleaching-induced loss of live coral.

Introduction

Coral reefs are traditionally considered to be productive hotspots in oligotrophic deserts (Darwin 1842) but their food webs are complex (Bierwagen *et al.* 2018) and the mechanisms through which they maintain exceptionally high diversity and biomass remain poorly understood. There is increasing evidence that oceanic production sources are fundamentally important in sustaining reef fish communities (McCauley *et al.* 2012; Frisch *et al.* 2014; Frisch *et al.* 2016; Matley *et al.* 2018; Skinner *et al.* 2019), particularly on degraded forereef slopes (Morais & Bellwood 2019).

Bulk stable isotope data have helped elucidate these reef-pelagic linkages but they lack resolution, for example co-occurring sources may not be isotopically distinct (Skinner *et al.* 2019; Whiteman*et al.* 2019), preventing accurate separation. The isotopic data that characterise food-web baselines will also vary with environmental conditions (Boecklen *et al.* 2011; Larsen *et al.* 2013), requiring robust sampling of dietary sources to compare data across spatial and temporal scales (Hadwen *et al.* 2010; Liew *et al.* 2019). Furthermore, as macromolecules are often not directly routed to consumer tissue, there is a trophic fractionation factor between consumer and diet (DeNiro & Epstein 1978) which varies substantially among species (Wyatt *et al.* 2010).

The profiling of specific biochemical compounds, such as amino acids is now feasible (compound-specific stable isotope analysis; CSIA). As the building blocks of proteins, amino acids can be categorised as: essential

(EAA, organisms cannot synthesize them *de novo*), conditionally essential (*de novo* synthesis requires specific physiological conditions), or non-essential (organism can synthesise them *de novo*) (Whiteman *et al.* 2019). Theδ ^{13}C values of individual amino acids ("^{13}C fingerprints") help reveal modes of carbon acquisition; the δ ^{13}C derives from the specific synthesis pathways involved (Larsen *et al.* 2009). As organisms cannot synthesize EAAs *de novo* , fractionation between diet and consumer is minimal and the δ ^{13}C values of consumer amino acids represent the primary producer sources of carbon (McMahon *et al.* 2010). Even when bulk values vary,δ ^{13}C primary producer fingerprints are robust to differing growth and environmental conditions (Vokhshoori *et al.* 2014; Larsen *et al.* 2015; McMahon *et al.* 2015a) and broad patterns are consistent across studies and labs (Liew *et al.* 2019).

In both terrestrial and aquatic systems (Larsen *et al.* 2009; Larsen *et al.* 2013; McMahon *et al.* 2015a; McMahon *et al.* 2016), EAAs help distinguish primary producers with different carbon origins; amino acid δ ^{13}C values of aquatic types of primary producers are especially distinct (Arthur*et al.* 2014; Wang *et al.* 2018). Bayesian mixing models using bulk stable isotope data indicate that reef predators are predominantly sustained by planktonic production sources, even inside atoll lagoons (Skinner *et al.* 2019). However, due to the methodological constraints associated with the lower resolution of bulk stable isotopes and the inability to separate isotopically similar planktonic sources, the origin of this pelagic production remains unclear. Phytoplankton are primarily composed of protein (Nguyen & Harvey 1997; Hedges *et al.* 2002) and different plankton communities have been separated using δ ^{13}C amino acid values (McMahon *et al.* 2015a). This suggests that planktonic sources with different origins may have distinctδ ^{13}C EAA values, providing additional resolution to disentangle the sources of planktonic carbon sustaining predators on reefs.

Bulk stable isotope data vary in their resolution of relationships between body size and trophic ecology (Layman *et al.* 2005; Ou*et al.* 2017; Dalponti *et al.* 2018). Organisms may change their diets over time; larger body size allows a wider range of prey to be exploited (Scharf *et al.* 2000), which would lead to changes in stable isotope values. Few studies to date (e.g. McMahon *et al.*2012; Vane *et al.* 2018) have used the greater power of EAAδ ^{13}C data to investigate how resource use might change with increasing body size and how this might affect isotope values. To determine how consumers will respond to environmental change and fluctuations in resource availability, knowledge of their resource use and how it varies with increasing body size or spatially is needed.

Here, δ ^{13}C values of EAAs were used to help trace the origin of the organic carbon sustaining predator biomass across an oceanic atoll. The main questions addressed were: 1) Doδ $^{13}C_{EAA}$ values vary spatially or with body size? 2) Do primary consumers have distinctδ $^{13}C_{EAA}$ values? 3) If so, are there differences in predator planktonic resource usage spatially?

Material and methods

Tissue sampling procedure

Sampling occurred across both inner and outer atoll areas of North Malé atoll, Maldives (Figure S1). All tissue samples were collected during the NE monsoonal period (January – April 2017 and December 2018) to avoid any seasonal fluctuations in production sources and their signatures.

Groupers were chosen as representative reef predators as they are relatively site-attached, while other reef predators, e.g. snappers, have larger home ranges involving long-distance movements (Sluka & Reichenbach 1995; Farmer & Ault 2011; Green *et al.* 2015). Four grouper species were selected as they were the most abundant upper trophic level (assumed TL [?] 4) groupers in both inner and outer atoll (Skinner *et al.* 2019), reach a range of sizes allowing for comparison of resource use at different lengths, and are a key component of the local reef fishery (Sattar *et al.* 2014). Samples of white dorsal muscle tissue (˜1g wet mass) were removed from*Aethaloperca rogaa* (redmouth), *Anyperodon leucogrammicus*(slender), *Cephalopholis argus* (peacock), and*Cephalopholis miniata* (coral hind). Fish were sampled from both inner and outer atoll using a pole spear and across a large size range (˜150 mm) relative to their maximum body size. Care was taken not to sample juveniles (< 15 cm) to control for dietary changes related to ontogeny. All tissue sampling was carried out in compliance with UK Home Office Scientific Procedures (Animals) Act Requirements.

Primary consumer species were used to represent food sources. Six energy pathways were identified: 1) benthic algae: powderblue surgeonfish,*Acanthurus leucosternon* (samples n = 7 inner, 6 outer) (Robertson*et al.* 1979); 2) detritus: bristletooth surgeonfish,*Ctenochaetus striatus* (n = 7 inner, 6 outer) (McMahon*et al.* 2016); 3) coral: scrawled butterflyfish, *Chaetodon meyeri* (n = 3 inner, 6 outer) (Sano 1989), 4) diurnal reef plankton: variable-lined fusilier, *Caesio varilineata* (n = 2 inner, 3 outer), yellowback fusilier, *Caesio xanthonota,* (n = 1 inner, 7 outer) (Bellwood 1988; Hamner *et al.* 1988; Russ *et al.*2017); 5) nocturnal reef plankton: lattice soldierfish,*Myripristis violacea* (n = 6 inner, 6 outer) (Hobson 1991); 6) pelagic plankton: mackerel scad, *Decapterus macarellus* (n = 7 inner) (Smith-Vaniz 1995), Indian Ocean squid, *Uroteuthis duvauceli* (n = 7 outer) (Islam *et al.* 2018).

Amino acid (AA) derivatisation and stable isotope analysis

Muscle tissue was oven dried at 50degC for 48 hours and then ground to a fine powder using a pestle and mortar. N-Acetyl Isopropyl Ester (NAIP) derivatives of amino acids were prepared by following the protocol described by Corr *et al.* (2007). Briefly, this entailed hydrolysis of individual aliquots (1.5mg) of dried powdered muscle tissue with internal standard norleucine (400 µg/mL), followed by isolation of the amino acid fraction using ion exchange chromatography with Dowex® 50WX8 hydrogen form resin (200 - 400 mesh). Isopropyl esters were prepared by addition of a 4:1 mixture of isopropanol and acetyl chloride and heating for 1 hour (100°C). After removal of excess reagents by re-dissolving in dichloromethane then drying with N_2 (40°C), acetylation was achieved by adding a mixture of acetone:triethylamine:acetic anhydride (5:2:1) and heating for 10 minutes (60°C). Isolation of the NAIP derivatives was achieved using liquid-liquid separation with NaCl solution (saturated) and ethyl acetate. All organic phases were combined and dried under a very gentle stream of N_2 (room temperature). Any residual water was removed with two successive 1 ml aliquots of DCM and evaporated under a very gentle stream of N_2 (ice bath). Samples were then stored in a freezer until they could be screened.

For screening, the derivatised AAs were resuspended in ethyl acetate and analysed using gas chromatography with an Agilent 7890 gas chromatograph with flame ionization detection (GC/FID), fitted with a DB-35 column 30m x 0.32mm x 0.5µm (Agilent), and an Agilent G4513A autosampler (Agilent Technologies, Santa Clara, CA, USA). The GC oven temperature was set to the following program: 70°C (hold 2 minutes) to 150°C at 15°C min^{-1}, then to 210°C at 2°C min^{-1}, then to 270°C at 8°C min^{-1}. The injection mode was Cold on Column (COC) and the injection volume was 1 µl with helium carrier gas at a flow rate of 2.00 ml/minute.

The $\delta\ ^{13}C$ isotopic compositions of the AAs were analysed using a GC/IRMS. A Thermo Scientific (Bremen, Germany) Delta V Plus isotope-ratio mass spectrometer (IRMS) was fitted with a Trace GC Ultra Oven, GC Isolink, and a ConFlo 4 for interface. The GC was fitted with a DB-35 column 30m x 0.32mm x 0.5µm (Agilent). The oven was set as follows: 40°C (hold 5 minutes) to 120°C at 15°C min^{-1}, then to 180°C at 3°C min^{-1}, then to 210°C at 1.5°C min^{-1}, then to 270°C at 5°C (hold 7 minutes).

Pulses of reference gas (CO_2) were introduced into the IRMS instrument during the analysis giving rise to peaks with known$\delta\ ^{13}C$ values (^{13}C:^{12}C ratio relative to Pee Dee Belemnite). These reference pulses were used to calculate the analyte peaks in each chromatogram. Identification of the derivatised amino acids was achieved by matching the peak elution times with those from a mixed amino acid standard (derivatised) containing alanine [Ala], glycine [Gly], valine [Val], leucine [Leu], norleucine [Nle], threonine [Thr], serine [Ser], proline [Pro], aspartic acid [Asp], glutamic acid [Glu], hydroxyproline [Hyd], phenylalanine [Phe], lysine [Lys] and tyrosine [Tyr].

To account for the change in measured values arising from the addition of carbon atoms during the derivatisation process, a correction factor was determined for each amino acid (Table S1). The correction factor calculation was:

$$1)\ \frac{((\text{cd x measured value of standard}) - (c\ x\ underivatised\ 13C\ value))}{d}$$

where c is the number of carbon atoms in the amino acid, $d$is the number of carbons added during the derivatisation process, andcd is the total number of carbon atoms in the derivative group. The correction

factor for each amino acid was then applied to the raw measured values of the samples using the following equation:

2) $\frac{((\text{cd x measured value of standard}) - (d\ x\ underivatised\ 13C\ value))}{c}$

All samples were derivatised at Newcastle University, UK, and all GC/FID work and GC/IRMS work was carried out at the Bristol Node of the NERC Life Sciences Mass Spectrometry Facility, UK. All primary consumer samples (except for the pelagic primary consumers *D. macarellus*and *U. duvauceli*) were derivatised and analysed in 2018, while all predators and the pelagic primary consumers were derivatised and analysed in 2019 (Table S1).

Data analysis

Isotopic signatures were derived from the five EAAs: leucine (Leu), lysine (Lys), phenylalanine (Phe), threonine (Thr), and valine (Val). Stable isotope ratios are reported using the delta (δ) notation with measured values expressed in per mil ($[(R_{sample} - R_{standard})/R_{standard}]$ and R is the ratio of heavy to light isotope (e.g.$^{13}C/^{12}C$). Analyses were carried out in R 3.5.2 (R Core Team 2017) interfaced with RStudio 1.1.463 (RStudio Team 2012).

Essential amino acid $\delta\ ^{13}C$ values were normalised to their respective sample means (denoted as$\delta\ ^{13}C_{EAAn}$). For each sample, the mean value of all five EAAs was calculated and then subtracted from the absolute EAA $\delta\ ^{13}C$ values (denoted as$\delta\ ^{13}C_{EAAa}$). Normalising the individual $\delta\ ^{13}C_{EAA}$ values to the mean removes natural variability in $\delta\ ^{13}C$ values of the individual amino acids arising from differing environmental (Larsen *et al.* 2013; Larsen *et al.* 2015; McMahon *et al.* 2015a), laboratory or study conditions (Liew*et al.* 2019). Using this method, trends in$\delta\ ^{13}C$ fingerprints are consistent and data across studies are compatible, allowing the major carbon sources of the predators to be investigated.

As groupers were sampled during two different time periods, differences in their $\delta\ ^{13}C_{EAAn}$ values were investigated using linear mixed effects models with the R package lme4 (Bates *et al.* 2015). Separate models were run for groupers in each atoll area (inner/outer), with the$\delta\ ^{13}C_{EAAn}$ value as the response variable, sampling year as a fixed effect and grouper species as a random effect. Following this, linear mixed effects models were run to investigate spatial and body size effects on grouper$\delta\ ^{13}C_{EAAn}$ values. The$\delta\ ^{13}C_{EAAn}$ value was the response variable with grouper species as a random effect and area (inner/outer) and body size (mm) as fixed effects. All model assumptions were checked by plotting the model residuals using histograms and qqplots, and plotting residuals vs fitted values. Wald tests were used to determine significant effects.

Primary consumers were collected from both inner and outer atoll so spatial differences in their$\delta\ ^{13}C_{EAAn}$ values were investigated using two sample t-tests. Where two primary consumer species were collected to represent the same food source, two sample t-tests were used to determine whether there were any differences in their $\delta\ ^{13}C_{EAAn}$ values. When data did not conform to normality, a non-parametric Mann-Whitney-Wilcoxon test was used instead.

Multivariate signatures of the$\delta\ ^{13}C_{EAAn}$ values were visualised with principal component analysis (PCA) for the a) groupers, b) primary consumers, and c) groupers and primary consumers using the covariance matrices.

To quantify the contribution of the different food sources to the four grouper species in both inner and outer atoll, a Bayesian stable isotope mixing model was run for each species using the MixSIAR package (Stock & Semmens 2016a). Primary consumer$\delta\ ^{13}C_{EAAn}$ values were separated into representative source groups using k-medoids clustering analysis based on the PAM (partitioning around medoids) algorithm (Kaufman & Rousseeuw 1990). A medoid is a point in the cluster for which the average dissimilarity between it and all the other points in the cluster is minimal. K-medoids clustering is thus more sensitive to outliers than k-means clustering, which uses the mean of points in the cluster. Clustering was carried out using the cluster package (Maechler *et al.* 2018) and the factoextra package (Kassambara & Mundt 2017). The optimal number of clusters was determined using the gap statistic which compares output values of clustering with different numbers of groups to output values from clustering under a reference null distribution of the data (Tibshirani

et al. 2001). The optimal number of clusters is that with the largest gap statistic, meaning the clustering structure is far from a random distribution of points. Mean and s.d. values were calculated for each cluster to represent source means in the mixing models.

The trophic discrimination factor was set to 0.1 ± 1.0 ‰ as essential AAs undergo minimal fractionation up the food chain (McMahon *et al.* 2016). A larger standard deviation value was included to provide the model with additional parameter space. Consumer data were individual grouper $\delta^{13}C_{EAAn}$ values. For each model, area (inner/outer) was included as a fixed factor and body size (mm) was included as a continuous variable. Each model was run with process x residual error terms to incorporate any variation in consumer digestibility or variation related to the sampling process (Stock & Semmens 2016b). Model MCMC parameters were set to short (chain length = 50000, burn = 25000, thin = 25, chains = 3). The models were considered converged when no variables had a Gelman-Rubin diagnostic > 1.05 and based on the Geweke diagnostic less than 5% of the variables were outside the 95% CI. Differences in the relative contribution of the dominant food source to groupers between atoll areas was tested for using a two-sample t-test.

Results

In total, $\delta^{13}C_{EAAn}$ values from 72 samples of four species of grouper and 67 samples of eight primary consumer species from both inner and outer atoll were analysed (Table 1). The range in Thr and Phe $\delta^{13}C_n$ values was greatest (12.86 and 12.07 respectively), followed by Leu (6.45), Val (6.43) and Lys (6.37).

Groupers in the inner atoll did not differ significantly in any of the $\delta^{13}C_{EAAn}$ values between sampling years (Table S2). Among the outer atoll groupers, only the Val $\delta^{13}C_n$ differed significantly between sampling years; all groupers from both sampling years were combined for all subsequent analyses. Atoll area and body size had no significant effect on any of the grouper $\delta^{13}C_{EAAn}$ values (Table 2).

None of the $\delta^{13}C_{EAAn}$ values differed between inner and outer atoll for *Acanthurus leucosternon* , *Chaetodon meyeri* or *Myripristis violacea* (t-test or Mann-Whitney-Wilcoxon test, $p > 0.05$) (Table S3). For each of these species all samples from both areas were combined and plotted as one group. The Phe $\delta^{13}C_n$ values of *Ctenochaetus striatus* differed significantly between areas (Mann-Whitney-Wilcoxon test, $W = 3$, $p = 0.02$) but the other $\delta^{13}C_{EAAn}$ values did not (Table S3) so this species was not combined. The caesionids *Caesio varilineata* and *C. xanthonota* showed differences in the $\delta^{13}C_n$ values of Phe (t-test, $t = 2.62$, $df = 10.78$, $p = 0.02$) and Val (t-test, $t = -2.43$, $df = 8.67$, $p = 0.04$) so these two species were not combined (Table S3). None of the $\delta^{13}C_{EAAn}$ values distinguished the two pelagic consumers *Decapterus macarellus* and *Uroteuthis duvauceli* (two-sample *t* -test, $p > 0.05$; Table S3), so these were considered as one "pelagic plankton" source group. The first two principal component axes of the PCA of the grouper $\delta^{13}C_{EAAn}$ values explained 69.2% of the variation and showed no clear grouping of species or atoll areas (Figure 1; Table S4). The PCA of the primary consumers showed clear separation of the different food source groups, particularly axis one, which explained 68.1% of the variation, while the second principal component axis explained 21.4% of the variation (Figure 2A; Table S4). The separation along PC1 indicated three broad groups of primary consumers: 1) pelagic plankton, 2) reef plankton, nocturnal plankton, and coral, and 3) benthic algae and detritus. A third PCA visualised the associations between the groupers and the primary consumers (Figure 2B; Table S4). The first two axes explained 89.4% of the variation and the groupers were closest in position to the pelagic plankton sources.

Based on the gap statistic and the cluster analysis the primary consumers were split into four source groups, which represented: 1) algae/detritus, 2) coral, 3) reef plankton, and 4) pelagic plankton (Figure 3). When running Bayesian isotope mixing models, sources can be combined *a posteriori* based on biological knowledge (Phillips *et al.* 2005; Phillips *et al.* 2014). Here, after the mixing models had run, the source groups representing coral and algae/detritus were combined into one group named "Reef Benthic".

The mixing models indicated that all four groupers derived the majority (95-99%) of their food from pelagic production sources in both inner and outer atoll (Figure 4). Median pelagic source reliance was significantly greater in the outer atoll (98-99%) than in the inner atoll (95-97%) (Two-sample *t* -test, $t = -5.06$, $df = 4.53$, $p = 0.005$). Patterns in pelagic reliance were consistent between atoll areas among the groupers. Of all four

groupers, *Aethaloperca rogaa* consistently had the highest median pelagic reliance, followed by *Cephalopholis miniata* , *Anyperodon leucogrammicus* and lastly *C. argus* . Median reliance on benthic reef and reef plankton sources was higher in the inner atoll (1.5-3% and 1-1.6% respectively) than in the outer atoll (0.4-1% and 0.3-0.6% respectively). Credible intervals were consistently larger for groupers in the inner atoll than in the outer atoll. *C. argus* and *A. leucogrammicus* had the largest overall credible intervals.

Discussion

Primary consumer $\delta\,^{13}C$ values of essential amino acids showed good discrimination among clusters broadly representing benthic algae/detritus, coral, reef plankton (diurnal and nocturnal) and pelagic plankton. The proximity of benthic algae and detritus to each other is not surprising. Although the powderblue surgeonfish, *Acanthurus leucrosternon* , is classified as a herbivore (Robertson *et al.* 1979) and the lined bristletooth, *Ctenochaetus striatus* , is classified as a detritivore (McMahon *et al.* 2016), much of the material they are feeding on originates from what is referred to as the epilithic algal matrix (Wilson *et al.* 2003). Consequently, they are not strictly feeding on a single homogenous production source. Diurnal and nocturnal reef plankton and coral were also isotopically similar to each other, perhaps indicative of dinoflagellate origins, while the fusiliers (*Caesio varilineata* and *C. xanthonota*) and soldierfish (*Myripristis vittata*) are likely feeding on localised reef-based plankton that is supported by the same phytoplankton sources (Hamner *et al.* 1988; Hobson 1991; Alldredge & King 2009). A novel finding here is that the $\delta\,^{13}C_{EAAn}$ values of the reef plankton and the pelagic plankton primary consumers were distinct from one another. Mackerel scad, *Decapterus macarellus* , and Indian Ocean squid, *Uroteuthis duvauceli* , are found in deeper oceanic waters and *U. duvauceli* come to the surface to feed at night (Smith-Vaniz 1986; Islam *et al.* 2018). Their $\delta\,^{13}C_{EAA}$ values may be a proxy for a pelagic, deep-water vertically migrating plankton community (Hays 2003) that is distinct from the localised reef plankton community comprised predominantly of copepods (Alldredge & King 2009). Further work on identifying the sources supporting caesionids is the recommended next step for this work.

Pelagic plankton, rather than reef plankton, primarily sustained all groupers. Oceanic atolls, like those in the Maldives, have an enhanced biomass of mesopelagic prey such as lanternfish and euphausiids (Bradbury *et al.* 1970; Letessier *et al.* 2016) which migrate to the surface waters to feed at night. Furthermore, particularly in the Indian Ocean, small benthic reef fish larvae are a key component of the ichthyoplankton and an abundant and continuous supply connect the reef-pelagic interface. Small juveniles and adults (< 50 mm in length) of these larvae provide 60% of consumed biomass on reefs, a contribution until now overlooked, and one thought to drive reef productivity (Brandl *et al.* 2019). The combination of enhanced mesopelagic prey and consistently available cryptobenthic fauna suggests these reefs may be a sink of pelagic energy (Letessier *et al.* 2016; Brandl *et al.* 2019). Conversely, on the Great Barrier Reef, open ocean water-column pathways supported only 57% of reef fish productivity on forereef slopes, however this contribution was expected to be higher on oceanic reefs (Morais & Bellwood 2019). Currently, little information exists on $\delta\,^{13}C_{EAA}$ incorporation rates or the timeframe that they may represent, however we hypothesize that the predominantly pelagic $\delta\,^{13}C_{EAA}$ values of the groupers is indicative of an atoll-wide food web fuelled by pelagic subsidies.

Even inside the atoll lagoons, groupers were almost exclusively reliant on pelagic production sources. Extensive mixing of oceanic waters renders lagoonal conditions in the Maldives reefs akin to the open ocean (Rogers *et al.* 2017), contributing to the consistently high pelagic reliance across the atoll. Furthermore, the Maldives are unique in that they lie across the equator and are therefore subject to equatorial currents that bring allochthonous materials to the archipelago from further afield (Sasamal 2007). These findings correlate with the bulk isotope data (Skinner *et al.* 2019) and previous research that found no difference in coral host and POM $\delta\,^{13}C$ and $\delta\,^{15}N$ between inner and outer reefs in the Maldives (Radice *et al.* 2019), providing further evidence of a well-mixed system where oceanic nutrients are available throughout. In contrast, several studies have found an increasing reliance of consumers on oceanic nutrients with proximity to the open ocean (Wyatt *et al.* 2012; Gajdzik *et al.* 2016). In the Red Sea, foraging by the snapper *Lutjanus ehrenbergii* was more benthic on shelf reefs and more planktonic on oceanic reefs (as identified by δ

$^{13}C_{EAA}$ values), but it was unclear whether this difference arose from a reliance on different food items in each location or from differing levels of planktonic inputs to the same food webs (McMahon *et al.* 2016). Similarly, here, while oceanic nutrients are clearly available throughout, it is uncertain whether the groupers are consistently selecting pelagic-derived prey or all the food webs across the atoll are supported by pelagic inputs.

Grouper $\delta\ ^{13}C_{EAAn}$ suggest all four species derive their carbon from the same pathways regardless of size. Previous research indicates$\delta\ ^{13}C_{EAA}$ values remain consistent across taxa (McMahon *et al.* 2016) and differing growth rates (Larsen *et al.* 2015), although the latter study investigated this for the marine diatom, *Thalassiosira weissflogii* only, so how this varies among upper level consumers is unknown. While growth rate is partly dependent on food availability, pelagic reef fish have higher growth rates as they exploit adjacent pelagic prey (Morais & Bellwood 2018). *A. rogaa,* which had the greatest pelagic reliance, also has the highest reported growth rate, while *C. argus,* which had the lowest pelagic reliance, also has the lowest reported growth rate (Mapleston *et al.* 2009). Although sampling was substantial, future work would benefit from including more samples across all sizes for greater statistical power. However, the lengthy derivatisation process and high cost of processing samples for CSIA meant this was beyond the scope of this study. Although the number of studies utilising $\delta\ ^{13}C$ of amino acids is increasing, the incorporation rates of AA from diet to consumer are scarcely known; there is substantial variation among amino acids (Bradley *et al.* 2014; Downs *et al.* 2014; Whiteman *et al.* 2018) and how this varies among taxa is uncertain (Whiteman*et al.* 2019). Consequently, the dietary timeframe represented by these values is unclear.

This is the first study to hypothesise multiple planktonic sources for reefs using $\delta\ ^{13}C_{EAA}$ values and distinguish them in a complex food web. In the Red Sea, calanoid copepods have been used to represent pelagic plankton signatures (McMahon *et al.* 2016), but this is a relatively enclosed, oligotrophic body of water, with limited exchanges with the adjacent Indian Ocean (Racault *et al.* 2015), yet planktonic primary productivity and N_2 fixation differ between open water and nearshore reef settings (Tilstra *et al.* 2018). Furthermore, POM increases to the South with increased proximity to nutrient-rich Indian Ocean water (Kürten *et al.* 2016). Consequently, the pelagic plankton signature derived from reef-based calanoid copepods further North (McMahon *et al.* 2016) may have been similar to that of the nearshore reef plankton of this study. Additional sampling of plankton from the open water and further South may have resulted in a distinct and separate pelagic plankton isotopic signature such as that found here.

As with all emerging technologies, there is still much that is unknown about $\delta\ ^{13}C_{EAA}$ data. Firstly, minimal fractionation of EAA stable isotopes between diet and consumer (McMahon *et al.* 2010) may not be the case. EAAs may not be directly routed from dietary material but instead might be assimilated from symbiotic gut microbes (Newsome *et al.* 2011). Alternatively, EAAs may undergo extensive catabolism when absorbed by cells lining the gut (Metges 2000). Both of these phenomena would lead to non-zero fractionation factors but are as yet relatively unexplored (Whiteman*et al.* 2019). Here, despite using a small fractionation factor for the mixing models, a larger standard deviation value was used to provide additional model parameter space (0.1 ± 1.0 %) in the absence of accurate fractionation factors. However, if the$\delta\ ^{13}C_{EAA}$ fractionation values are similar to that for non-essential amino acids (-0.5 – 2.4*et al.* 2015b), the mixing model may have been too constrained to find an appropriate solution. Consequently, this may explain the rigidity and lack of variation in the food source contribution estimates presented here. As CSIA becomes more routine, greater understanding of the mechanisms through which EAAs are integrated by consumers will be required, and is the recommended next step for future work.

Secondly, all the primary consumer samples (with the exception of the pelagic *U. duvauceli* and *D. macarellus*) were derivatised and analysed prior to the pelagic primary consumers and the groupers. The strong pelagic reliance of the groupers may be influenced by differences in $\delta\ ^{13}C_{EAA}$ values between studies arising from: 1) derivatising with different batches of reagents, and 2) the calibration settings of the GC/IRMS at different times (Zhang *et al.* 2012), causing the groupers to be closest isotopically to the pelagic primary consumers run at the same time as them. However, by using consistent laboratory standards and normalising the $\delta\ ^{13}C_{EAA}$ data to the mean, values should be comparable between studies (Larsen *et al.* 2013; Larsen *et al.*

2015; McMahon *et al.* 2015a; Liew *et al.* 2019), especially when samples were collected in the field at the same time and run on the same GC/IRMS. As such, it is unlikely that the strong pelagic signature arises solely from methodological discrepancies in $\delta\,^{13}C_{EAA}$ values, but future research should focus on how varying lab or GC/IRMS conditions may influence $\delta\,^{13}C_{EAA}$ values and their ecological interpretation. In addition, several grouper samples were collected at a different time to the others. However, they were caught during the same monsoonal season in the same location and no significant differences in values were identified. Furthermore,$\delta\,^{13}C_{EAA}$ values are thought to be robust to seasonal fluctuations (Larsen *et al.* 2015) and$\delta\,^{13}C_{EAAn}$ values show even less variability (McMahon *et al.* 2015a).

While fusiliers are classic reef planktivores, due to their highly mobile nature (Russ *et al.* 2017), they may not have been the most appropriate proxy for localised reef plankton in this context. Moreover, as groupers are typically more reef-associated ambush predators it is uncertain to what degree they would predate on them, perhaps explaining the lack of reliance on reef plankton sources. Sampling of other diurnal planktivores such as balistids (*Odonus niger*), pomacentrids (*Chromis* spp) and serranids (*Pseudanthias* spp), all frequently found in grouper stomach contents (Shpigel & Fishelson 1989; St John 1999; Dierking *et al.* 2011; Meyer & Dierking 2011), is the recommended next step for this work.

While coral reefs worldwide are experiencing unprecedented losses of live coral cover (Hughes *et al.* 2017), fish productivity on those that rely on pelagic subsidies may be more resilient to coral bleaching than previously thought (Morais & Bellwood 2019). Groupers are a fundamental component of the Maldivian reef fishery (Sattar *et al.* 2014) and their exceptionally high pelagic reliance found here suggests that fishery predictions based solely on habitat loss may be misleading (Robinson *et al.* 2019).

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