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Stable isotope and fatty acid biomarkers of seagrass, epiphytic, and algal organic matter to consumers in a pristine seagrass ecosystem

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Abstract. The relative importance of the identity and abundance of primary producers in structuring trophic ecology, particularly in seagrass-dominated ecosystems, remains unclear. We assessed the contributions of seagrass, epiphytes, macroalgae, and other primary producers to the diets of resident animals in the nearly pristine seagrass-dominated environment of Shark Bay, Australia, by combining fatty acid composition with carbon, nitrogen, and sulfur stable isotopes of primary producers and consumers. Overall, mixed inputs of these primary producers fuel secondary production, with tropical detrital seagrass inputs supporting most fish species, likely through benthic intermediates. Epiphytic organic matter inputs were most closely associated with snails, whereas seagrass detritus, macroalgae, gelatinous zooplankton, and/or phytoplankton may all contribute to higher trophic levels including sea turtles and sharks. The fatty acid and isotope data suggest that diets of large-bodied consumers were highly variable – future food web studies need to incorporate large sample sizes to account for this variability.

Additional keywords: epiphytes, fatty acids, food webs, seagrass, Shark Bay, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$.

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Introduction

Seagrass meadows are highly productive communities that serve a structural role by providing protection from predation and a trophic role by contributing to ecosystem primary productivity (Duarte 1989; Lee *et al.* 2001). The trophic importance of seagrass is debated, but generally thought to be minor compared with epiphytic algae, macroalgae, and benthic microalgae (Moncreiff and Sullivan 2001; Jaschinski *et al.* 2008; Doropoulos *et al.* 2009; Lebreton *et al.* 2011). Grazers of diverse body sizes, however, forage directly on seagrass and can remove considerable proportions of biomass (Lal *et al.* 2010; Douglass *et al.* 2011; Unabia 2011). Large grazers such as the dugong, *Dugong dugon*, have been shown to reduce seagrass shoot density, above ground biomass, and below ground biomass (DeIongh *et al.* 1995; Preen 1995), and green sea turtles, *Chelonia mydas*, may be responsible for recent declines in seagrass meadows in Bermuda (Fourqurean *et al.* 2010). Particularly in low latitudes, large grazers have played important roles historically in determining life-history characteristics of seagrasses and resident animals (Heck and Valentine 2006). More recently, some nearshore food webs have undergone a shift from large herbivores that can strongly impact seagrass biomass to smaller herbivores (for example the bucktooth parrotfish, *Sparisoma radians* and the Salema porgy, *Sarpa salpa* (Kirsch *et al.* 2002; Tomas *et al.* 2005)), which can consume large quantities of seagrass with lower impacts on seagrass density (Heck and Valentine 2006). Seagrass organic matter can also enter food webs via heterotrophic bacteria – in Florida Bay, seagrass-derived organic carbon accounted for 13–67% of bacterial $\delta^{13}C$ signatures (Williams *et al.* 2009). A complexity in determining the relative abundance of seagrass vs other inputs to food webs is that approaches such as stable isotope analysis often cannot provide a great enough distinction between seagrass and epiphyte and/or macroalgal organic matter and may suggest substantial seagrass consumption that is not supported by other methods such as gut content analysis or fatty acid analysis (Jaschinski *et al.* 2008; Crawley *et al.* 2009; Douglass *et al.* 2011).

Fatty acid fingerprinting can be a particularly useful tool for dietary studies where stable isotope composition alone may not provide the necessary resolution between discrete organic matter substrates or trophic levels. Unique, source-specific fatty acids or the general distribution of fatty acids can provide detailed tracking of carbon substrates in food webs because fatty acids from triacylglycerol storage lipids in a food item are taken up into consumer tissue with relatively minor or predictable modifications (Dalsgaard *et al.* 2003; Iverson *et al.* 2004; Budge *et al.* 2006).

The coupling of fatty acid analysis with stable isotope composition has provided additional information on the trophic importance of seagrass, yet results seem to vary greatly with habitat. For example, Kharlamenko *et al.* (2001) determined that seagrass was an important organic matter substrate, particularly for gastropods and some surface deposit-feeding bivalves in an eelgrass meadow in the Sea of Japan, although the link between seagrass and consumer relied on both detrital and bacterial pathways. Conversely, in south-western Australia, Crawley *et al.* (2009) determined that brown algae, as opposed to seagrass, disproportionally contributed to consumer food webs in surf-zones. Although brown algae were present in far lower abundances in surf-zone wrack than seagrass, fatty acid analysis demonstrated that allochthonous brown algae subsidised secondary production in these regions (Crawley *et al.* 2009). Similarly, Doropoulos *et al.* (2009) determined through laboratory feeding experiments that gastropods, particularly *Pyrene bidentata* snails, from coastal south-western Australia consumed only minimal amounts of seagrass, preferring instead kelp, red macroalage, and seagrass periphyton.

Here, we explore food web relationships in the relatively undisturbed subtropical seagrass ecosystem of Shark Bay, Western Australia. We couple stable isotope composition with fatty acid analysis to determine the relative inputs of seagrass, epiphytes, and other primary producers to a selected subset of consumers in higher trophic levels as a preliminary study to determine if these molecular approaches can provide sufficient separation of common primary producers in Shark Bay. There are limited studies of the trophic ecology in Shark Bay; however, previous studies using bulk stable isotopes suggest that mangrove-derived productivity contributes relatively little to food webs (Heithaus *et al.* 2011) and that some individual green sea turtles, often considered to be highly reliant on seagrasses, may in fact rely on other non-seagrass food sources even in extensive seagrass meadows (Burkholder *et al.* 2011). Furthermore, seagrass productivity is important to food webs in which batoids (rays) and small sharks feed (Vaudo and Heithaus 2011). Additionally, a large body of work from south-western Australia suggests that allochthonous organic matter sources, such as kelp and other macroalgae derived from reefs, can subsidise secondary production in seagrass meadows (Wernberg *et al.* 2006; Doropoulos *et al.* 2009; Hyndes *et al.* 2012), surf-zones (Crawley *et al.* 2009), and beach environments (Ince *et al.* 2007). Here, we provide what we believe to be the first study to integrate data from fatty acid and stable isotope compositions (particularly sulfur isotopes) of consumers in Shark Bay to gain more detailed insights into trophic structure. Because Shark Bay is characterised by limited anthropogenic influences this study provides a baseline for comparison to more impacted seagrass systems.

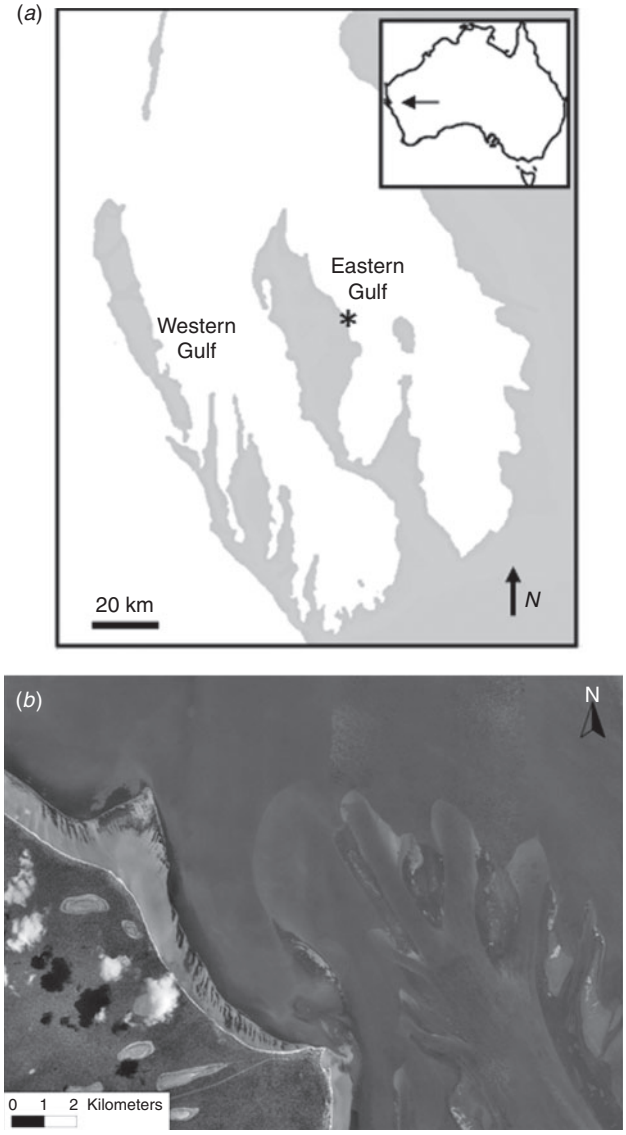

Fig. 1. (*a*) Shark Bay, Australia. (*b*) Sample collections occurred on offshore seagrass banks, nearshore seagrass and sand habitats, and in deeper channels. Asterisk indicates the location of the study area detailed in Fig. 1b.

Materials and methods

Study area and sampling

Shark Bay is a shallow (average depth 9 m) subtropical embayment, subdivided into Eastern and Western Gulfs by peninsulas and barrier islands (Heithaus 2004). Together with its designation as a United Nations Educational, Scientific and Cultural Organisation (UNESCO) World Heritage Site, the low population of the region and low fishing pressure have preserved Shark Bay as one of the World's most pristine seagrass ecosystems (Heithaus 2004; Heithaus *et al.* 2011). This study focussed on the Eastern Gulf of Shark Bay (Fig. 1) where shallow offshore banks (<4 m) are dominated by stands of *Amphibolis antarctica* or occasionally *Posidonia australis*, and deeper regions are sandy or silty with isolated patches of

Table 1. Summary of basal resources and consumers, sample type and number for both fatty acid stable isotope analysis, and lipid-extraction status for stable isotope analysis

	Sample type	*n*	Lipid extracted for isotope analysis
Amphibolis antarctica	Seagrass blades	3	X
Halodule uninervis	Seagrass blades	3	X
Cymodocea angustata	Seagrass blades	3	X
Epiphytes		3	X
Sediments		2	X
Sargassum spp.		2	
Ctenophores	Whole-body Composite	2	
Pyrene spp.	Whole-body	3	X
Pinctada spp.	Muscle	3	X
Tarwhine (*Rhabdosargus sarba*)	Muscle	3	X
Western butterfish (*Pentapodus vitta*)	Muscle	3	X
Yellowtail trumpeter (*Amniataba caudavittata*)	Muscle	3	X
Western school whiting (*Sillago vittata*)	Muscle	3	X
Striped trumpeter (*Pelates octolineatus*)	Muscle	3	X
Green sea turtle (*Chelonia mydas*)	Skin	2	
Loggerhead sea turtle (*Caretta caretta*)	Skin	2	
Tiger shark (*Galeocerdo cuvier*)	Fin Clip	3	

seagrass, with more tropical species like *Cymodocea angustata*, *Halophila ovalis*, *Halophila spinulosa*, and *Halodule uninervis*, among others (Heithaus 2004). In addition there are large expanses of nearshore shallows (<3 m) that are largely covered by sand with a strip of seagrass along the deeper portions of the flats (Vaudo and Heithaus 2009).

Samples were collected in March 2011 from nearshore shallows and offshore seagrass banks. We collected three species of seagrass, *A. antarctica*, *H. uninervis*, and *C. angustata* by hand on SCUBA. At least five plants were collected at each site, and multiple leaves were combined for composite samples. Three composites were analysed for each seagrass species. Seagrass was rinsed in DI water and gently scraped with a razor blade to remove epiphytes. Two sediment samples, SOM 63A (25°56′29.94″S, 114°13′39.6″E) and SOM 10 B (25°44′52.98″S, 113°45′18.65″E) were collected using a PVC coring tube. The core was pushed into the sediment 10 cm, recovered, and brought back to the boat. A subsample from the surface of the core (0–2 cm) was collected for analysis. All seagrass, epiphyte, and sediment samples were stored on ice in the field and immediately frozen in the laboratory until processing.

To determine if fatty acids and sulfur isotopes can resolve trophic dynamics in Shark Bay, we sampled a subset of the most common consumers in the study area, rather than comprehensively sampling the consumer community (Table 1). Details of methods for the capture and collection of tissues from sharks can be found in found in Matich *et al.* (2011) and for sea turtles in Burkholder *et al.* (2011). Fish were captured in fish traps (see Heithaus 2004) or by handline and small consumers were collected by hand. Samples were rinsed in ultrapure water, then either frozen intact (*Pyrene* spp. snails, seagrass, epiphytes) or dissected to isolate muscle tissue (fish and oysters), then frozen. Because of the long travel times to the laboratory in the United States, all samples were preserved in a small aliquot of isopropanol and butylated hydroxytoluene (BHT; 100 μg mL^{-1}) was added as an antioxidant (Christie 1989). On return to the laboratory, snails were removed from their shells.

Stable isotope and bulk parameter analysis

Lipid extracted residues (see below) of all seagrass, snail, oyster, fish, and sediment samples were rinsed with ultrapure water and allowed to dry overnight at 45°C for stable isotope analysis. Tissue was then homogenised with a mortar and pestle. Subsamples of snail and oyster tissue were treated with 10% HCl for 24 h to remove inorganic carbon then rinsed to neutral pH with ultrapure H_2O. Stable isotopes of *Sargassum* spp., the ctenophore composite, green sea turtles (*Chelonia mydas*), loggerhead sea turtles (*Caretta caretta*), and tiger sharks (*Galeocerdo cuvier*) were measured on non-lipid extracted tissues from split samples (Table 1). Although lipid extraction has been shown to result in a small (~1‰) enrichment in $\delta^{13}C$ and $\delta^{15}N$, these changes have not proven ecologically significant for food web analyses (Sotiropoulos *et al.* 2004; Murry *et al.* 2006). Because the compositions of primary producers and consumers vary much more than 1‰ in this study, particularly for $\delta^{15}N$, we feel confident that we can compare the relative isotope compositions between our lipid-extracted and non-lipid extracted samples. Stable carbon and nitrogen isotope composition were measured concurrently on the samples with an NA 1500 Elemental Analyzer (Carlo Erba, Milan, Italy) coupled to a Finnigan MAT Delta isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany) at Florida International University's Stable Isotope Laboratory. Samples were converted to SO_2 and ^{34}S determinations were performed at the University of Virginia using a Carlo Erba elemental analyzer coupled to an OPTIMA stable isotope ratio mass spectrometer (Isoprime, Inc., Manchester, UK) (see MacAvoy *et al.* 2000; for method description). The reproducibility of the measurement is typically better than ±0.2‰ for the sulfur isotope abundances and

Table 2. Groups of fatty acids used to partition bacterial, seagrass, diatom, and flagellate organic matter in this study, and their source assignments reported in the literature

Carbon source	References
Bacterial	
C_{13}-C_{19} odd-chain iso and anteiso branched fatty acids; C_{13}-C_{23} odd-chain *n*-alkanoic acids; C_{15}, C_{17}, and C_{19} monounsaturated fatty acids, 14:0i, 18:1ω7	Perry *et al.* (1979); Volkman *et al.* (1980); Sargent *et al.* (1987); Findlay and Dobbs (1993); Rajendran *et al.* (1993); Meziane and Tsuchiya (2000)
Seagrass/Vascular plants	
18:2ω6, 18:3ω3	Viso *et al.* (1993)
C_{22}-C_{32} saturated *n*-fatty acids (vascular plants only)	Eglinton and Hamilton (1967); Viso *et al.* (1993)
Diatoms	
14:0n, 16:1ω7	Volkman *et al.* (1989); Dunstan *et al.* (1994); Napolitano (1999)
16:2ω4, 16:3ω4, 16:4ω1, 20:5ω3	Volkman *et al.* (1989); Dunstan *et al.* (1994); Napolitano (1999); Dalsgaard *et al.* (2003); Ramos *et al.* (2003)
Flagellates	
18:4ω3, 22:5ω3, 22:6ω3	Volkman *et al.* (1989); Viso and Marty (1993)

the average isotope laboratory error for replicate glycine internal standards treated identically as the samples was 0.11‰ for $\delta^{13}C$ and 0.11‰ for $\delta^{15}N$. Due to low sample replication, we have not attempted stable isotope mixing models to constrain consumer food sources.

Lipid extraction and analysis

Lipids were extracted from seagrass, epiphytes, and muscle and skin tissues following a modification of Folch *et al.* (1957) and details are provided in Belicka *et al.* (2012). Briefly, samples were extracted with a 2 : 1 mixture (v/v) of methylene chloride : methanol (CH_2Cl_2 : MeOH), with trace amounts of isopropanol remaining from the preservation process. The original storage vials were rinsed three times with the 2 : 1 CH_2Cl_2 : MeOH solvent mixture, and the rinse was transferred to the extraction tube to ensure no sample material or isopropanol was left behind in the initial storage vial. Ultrapure (milli-Q) water was added to achieve a final ratio of 2 : 1 : 0.7 CH_2Cl_2 : MeOH : H_2O, the samples were strongly agitated, and the lower organic phase was removed to an evaporation flask. The extraction was repeated two more times, extracts were combined, and excess solvent was removed by rotary evaporation. Total lipid extracts were flushed with nitrogen and stored in CH_2Cl_2 at −20°C. Saponification and derivatisation follow the methods described in Belicka *et al.* (2012).

Fatty acids were identified and relative abundances were determined using gas-chromatography-mass spectrometry (GC/MS) with an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5973 mass spectrometer using a Restek FAMEWAX crossbond polyethylene glycol GC column (Restek Corporation, Bellefonte, PA, USA) (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness). GC and MS parameters are as described in Belicka *et al.* (2012). Identification of fatty acids was performed by comparison of chromatographic retention times with authentic standards (Supelco PUFA No. 3 (from menhaden oil), PUFA No. 1 (marine source), Bacterial Acid Methyl Ester (BAME) Mix, and C_4-C_{24} FAME Mix) and mass spectra of standard and previously reported compounds. Fatty acids are expressed here as a percentage of the total identified. The most abundant five fatty acids in all samples excluding sediments, resulting in a total of 12 fatty acids, are shown in figures here for brevity. Additionally, specific fatty acids were grouped into bacterial, seagrass and/or vascular plant, diatom, and flagellate fatty acids on the basis of source assignments in the literature (Table 2; see also Lebreton *et al.* 2011).

Statistical analyses applied for fatty acid fingerprinting used average-linkage hierarchical cluster analysis on Bray–Curtis dissimilarity coefficients of untransformed fatty acid data using the statistical package R (R Foundation, for Statistical Computing, Vienna, Austria). The dataset for the cluster analysis consisted of a total of 27 fatty acids, which included the 10 most abundant fatty acids in each sample, and represented between 84–99% of the total fatty acids in all samples. Overall, ~100 different fatty acids were identified in the sample set (see Supplementary information), and cluster analyses using the reduced versus full datasets resulted in very similar sample associations. Fatty acids are named here as A:BωC, where A refers to the number of carbon atoms in the molecule, B refers to the number of double bonds present, and C, listed where confirmed, indicates the position of the first double bond counted from the terminal methyl group, with additional double bonds separated by a single methylene group.

Results

Stable carbon, nitrogen, and sulfur isotope composition of basal resources and consumers

Stable carbon and nitrogen isotope compositions for seagrasses, epiphytes, *Pinctada* spp. oysters, turtles, and tiger sharks measured here all fall within previously reported ranges of much larger sample sizes (Fig. 2; see Burkholder *et al.* 2011 and Matich *et al.* 2011). Seagrasses were enriched in ^{13}C compared with most consumers, with $\delta^{13}C$ values averaging −11.1‰ for *A. antarctica*, -11.4 ± 0.5‰ s.d. for *H. uninervis*, and -9.8 ± 0.3‰ s.d. for *C. angustata* (Fig. 2). Epiphytes and sediments were more depleted in ^{13}C compared with seagrasses, with average $\delta^{13}C$ values of −12.9‰ and −14.9‰, respectively. Note that due to sample loss, epiphyte replicates 1 and 3 were combined so as to obtain one $\delta^{13}C$ measurement. The three

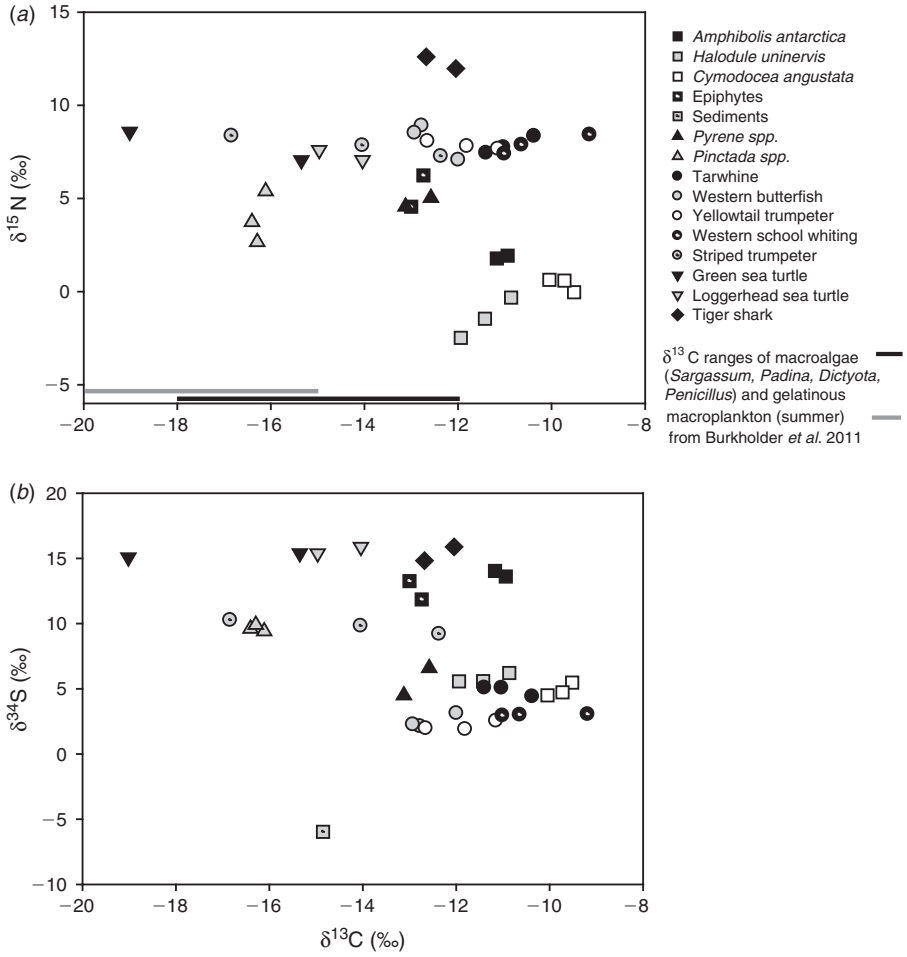

Fig. 2. (*a*) Stable carbon isotope composition versus stable nitrogen isotope composition and (*b*) stable carbon isotope composition versus stable sulfur isotope composition in primary producers and consumers from Shark Bay, Australia.

replicates were run individually for other isotope analyses. Values of $\delta^{13}C$ in *Pyrene* spp. snails were similar to those found in epiphytes, averaging −12.9‰ (Fig. 2). The other invertebrates investigated in this study, *Pinctada* spp. oysters, had much more depleted $\delta^{13}C$ values, which averaged -16.3 ± 0.2‰ s.d. Fish species measured here showed a wide range in $\delta^{13}C$ values, with the western school whiting (*Sillago vittata*) having the most enriched values which averaged -10.3 ± 1.0‰ s.d., whereas the striped trumpeter (*Pelates octolineatus*) had the most depleted average values (-14.4 ± 2.3‰ s.d.) (Fig. 2). Values of $\delta^{13}C$ for green sea turtles and loggerhead sea turtles averaged −17.2‰ and −14.5‰, whereas tiger sharks were more enriched, averaging −12.4‰ (Fig. 2).

Stable nitrogen isotope values increased with trophic level, with seagrasses having the most depleted $\delta^{15}N$ values which ranged from −2.5‰ to +1.9‰ (Fig. 2). *Halodule uninervis* was most depleted in ^{15}N, and *A. antarctica* was most enriched. Epiphytes were more enriched than seagrasses, and the three replicates had a wide range of values from 4.6‰ to 7.1‰. The invertebrates investigated here were also more enriched than seagrasses, with $\delta^{15}N$ for snails averaging 4.8‰, whereas oysters averaged 3.9‰ (Fig. 2). Fish species and both species of turtles also had similar ranges of $\delta^{15}N$ values, which were intermediate between the invertebrates and tiger sharks (Fig. 2). Values of $\delta^{15}N$ for turtles ranged from 7.1‰ to 8.6‰, whereas all fish species had $\delta^{15}N$ values between 7.1‰ and 8.9‰. Tiger sharks had the most enriched $\delta^{15}N$ values near 12‰ (Fig. 2).

Regarding S isotopes, the sediments were quite depleted in ^{34}S, with average values of −6.3‰ (Fig. 2*b*). The tropical seagrasses *H. uninervis* and *C. angustata* had intermediate $\delta^{34}S$ values which averaged 5.8 ± 0.4‰ s.d. and 4.9 ± 0.5‰ s.d., respectively, whereas the temperate seagrass *A. antarctica* was more enriched, averaging 14.2 ± 0.6‰ s.d. (Fig. 2*b*). All species of fish excepting striped trumpeter had similar $\delta^{34}S$ values, which ranged from 1.9‰ to 5.1‰ (Fig. 2*b*). Striped trumpeters were more enriched than other fish species, with $\delta^{34}S$ values averaging 9.8 ± 0.5‰ s.d. *Pyrene* spp. had $\delta^{34}S$ values similar to the tropical seagrasses, averaging 6.0 ± 1.3‰ s.d. (Fig. 2*b*). Average $\delta^{34}S$ values for oysters (9.6 ± 0.3‰ s.d.) and

epiphytes (12.3 ± 1.4‰ s.d.) were similar to $\delta^{34}S$ values found for the striped trumpeters. Tiger sharks and turtles were, on average, more enriched in ^{34}S than fish, with $\delta^{34}S$ values averaging 15.4 ± 0.6‰ s.d. for tiger sharks and 15.4‰ for the turtles. The samples most enriched in ^{34}S in this dataset were *Sargassum* spp. (19.7‰) and ctenophores (20.6‰).

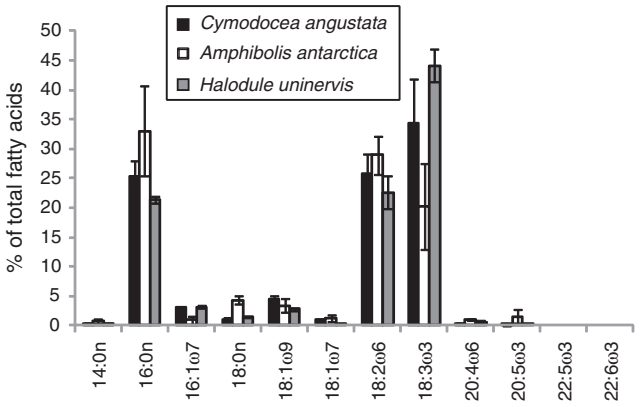

Fig. 3. Average relative abundance and standard deviation ($n = 3$) of predominant fatty acids in three species of seagrass from Shark Bay, Australia.

Fatty acid composition of basal resources and consumers

Each seagrass species was dominated by the same three fatty acids: 18:2ω6 (linoleic acid) and 18:3ω3 (α-linolenic acid), and 16:0n (palmitic acid), which is common in all organisms (Table 2, Fig. 3). Smaller contributions from 18:0n (stearic acid) and 18:1ω9 (oleic acid) were also present in all three species. Only minor amounts of 20:4ω6 (arachidonic acid) and 20:5ω3 (eicosapentaenoic acid) were present, and no C_{22} polyunsaturated fatty acids were present in any of the seagrass samples (Fig. 3). Unlike seagrass, epiphytes and *Sargassum* spp. were instead dominated by C_{20} polyunsaturated fatty acids, particularly 20:5ω3 and 20:4ω6 (Fig. 4*a*). The relative abundance of two of the dominant fatty acids in seagrasses, 18:2ω6 and 18:3ω3, were substantially lower in epiphytes and *Sargassum* spp., although abundances of 16:0n were similar to levels present in seagrass. *Sargassum* spp. contained higher abundances of 18:1ω9 and 14:0n compared with epiphytes and seagrass (Fig. 4*a*).

The fatty acid composition of *Pyrene* spp. snails was dominated by the following fatty acids: 16:0n, 20:4ω6, 20:5ω3, 22:5ω3, 16:1ω7, 18:0n, and 14:0n (Fig. 4*b*). *Pinctada* spp. oysters contained abundant 22:6ω3 (docosahexaenoic acid), as well as the fatty acids that dominated in *Pyrene* spp., with the exception of 16:1ω7. In general, all fish species contained similar fatty acid compositions, with abundant 16:0n, 22:6ω3, 20:4ω6, 18:0n, 18:1ω9, and 20:5ω3 (Fig. 4*c*). Striped trumpeter

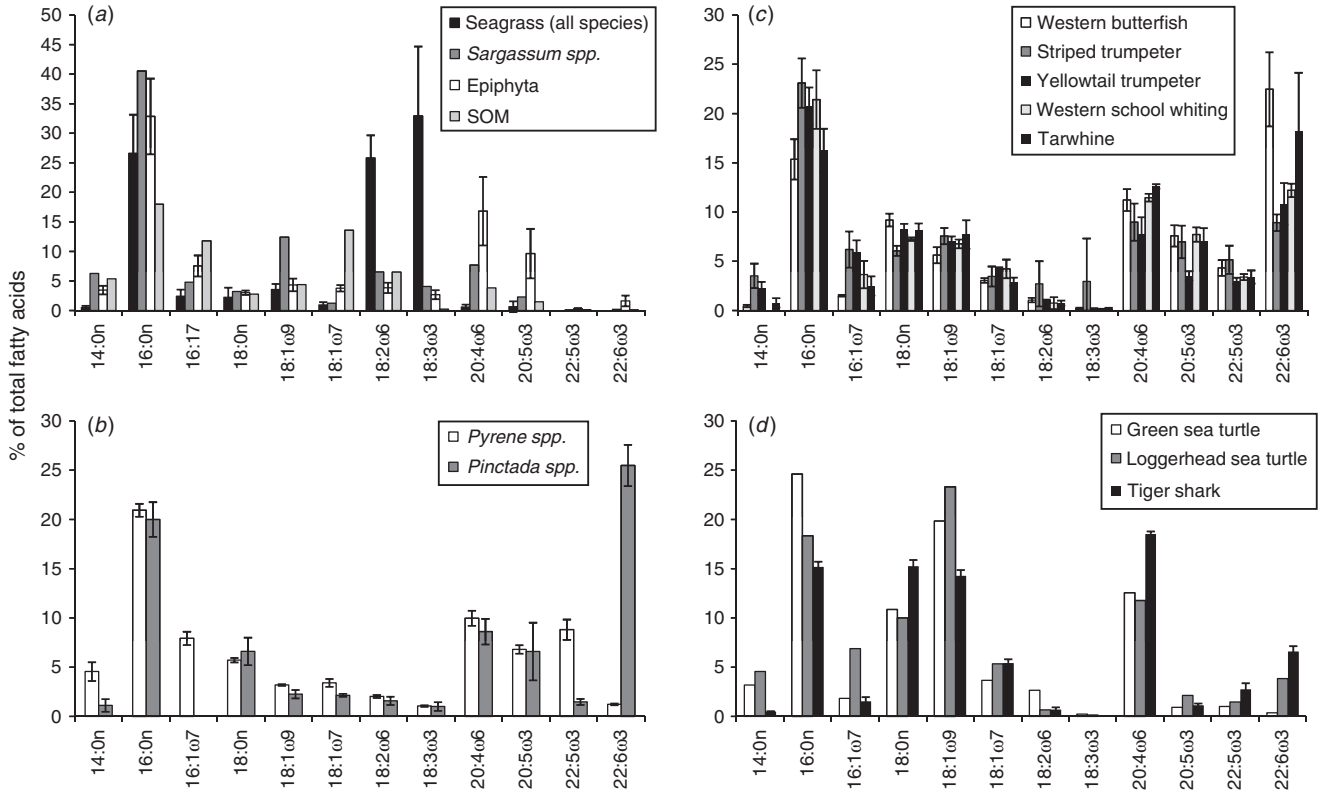

Fig. 4. Average relative abundance and standard deviation (shown where $n = 3$) of predominant fatty acids in (*a*) primary producers and sedimentary organic matter (SOM), (*b*) invertebrates, (*c*) fish, and (*d*) turtles and sharks from Shark Bay, Australia.

contained the highest levels of 18:2ω6 and 18:3ω3 out of all fish species, whereas western butterfish (*Pentapodus vitta*) and tarwhine (*Rhabdosargus sarba*) instead had high abundances of 22:6ω3 (Fig. 4*c*). Loggerhead sea turtles, green sea turtles, and tiger sharks all contained similar compositions of fatty acids, with high levels of the ubiquitous fatty acids 16:0n, 18:0n, and 18:1ω9, plus 20:4ω6 (Fig. 4*d*). Tiger sharks and loggerhead sea turtles contained higher levels of 22:6ω3 compared with green sea turtles; however, green sea turtles contained higher abundances of 18:2ω6.

When grouped by source (Fig. 5), bacterial fatty acids, especially 15:0i (iso branched) and 15:0a (anteiso branched), were abundant in the sedimentary organic matter, particularly for the sandy sediments that were not collected in the seagrass meadow. Flagellate fatty acids (Table 2) were present in low quantities in sediments and epiphytes, but were high in oysters, suggesting an important planktonic component of flagellates to these filter feeders, as well as western butterfish and tarwhine (Fig. 5*b*). Fatty acids indicative of diatoms (Table 2) were abundant in sediments and epiphytes, demonstrating the importance of these microalgae to benthic and epiphytic organic matter substrates (Fig. 5*c*), and also abundant in *Pinctada* spp., *Pyrene* spp., and striped trumpeter. The fatty acids most characteristic of seagrass, including 18:2ω6 and 18:3ω3, were present in only low abundances in sediments, striped trumpeter, and green sea turtles (Fig. 5*d*).

Hierarchical cluster analysis performed on the basis of the Bray–Curtis dissimilarities of fatty acid composition resolved most species into individual clusters (Fig. 6), suggesting low intraspecific variation relative to interspecific variation in fatty acid composition for these sampled organisms. Tarwhine was the only fish to not show this tight clustering, with two individuals clustering with western butterfish, and one individual clustering with western school whiting (Fig. 6). Seagrass, also, was not entirely resolved by species, yet formed its own cluster which was not closely related to any of the other basal resources or consumers (Fig. 6). As expected, the fatty acid composition of *Pyrene* spp. was closely related to that of epiphytic material (Fig. 6). Fish formed two clusters at the 20% dissimilarity level; the first including western butterfish, the majority of the tarwhine samples, and *Pinctada* spp. oysters, whereas the second cluster contained western school whiting, one individual tarwhine, yellowtail trumpeter (*Amniataba caudavittata*), and striped trumpeter. Striped trumpeter were less closely associated with western school whiting and yellowtail trumpeter (Fig. 6).

Discussion

The role of seagrass as a basal resource for consumers in Shark Bay

Evidence from fatty acids, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ provides conflicting insights into the potential importance of seagrass to consumers in Shark Bay. The fatty acid composition of seagrasses, similar in the three species analysed here and characterised by abundant 18:2ω6 and 18:3ω3 with low abundances of saturated and monounsaturated C_{18} fatty acids, was not strongly represented in the fatty acid compositions of any

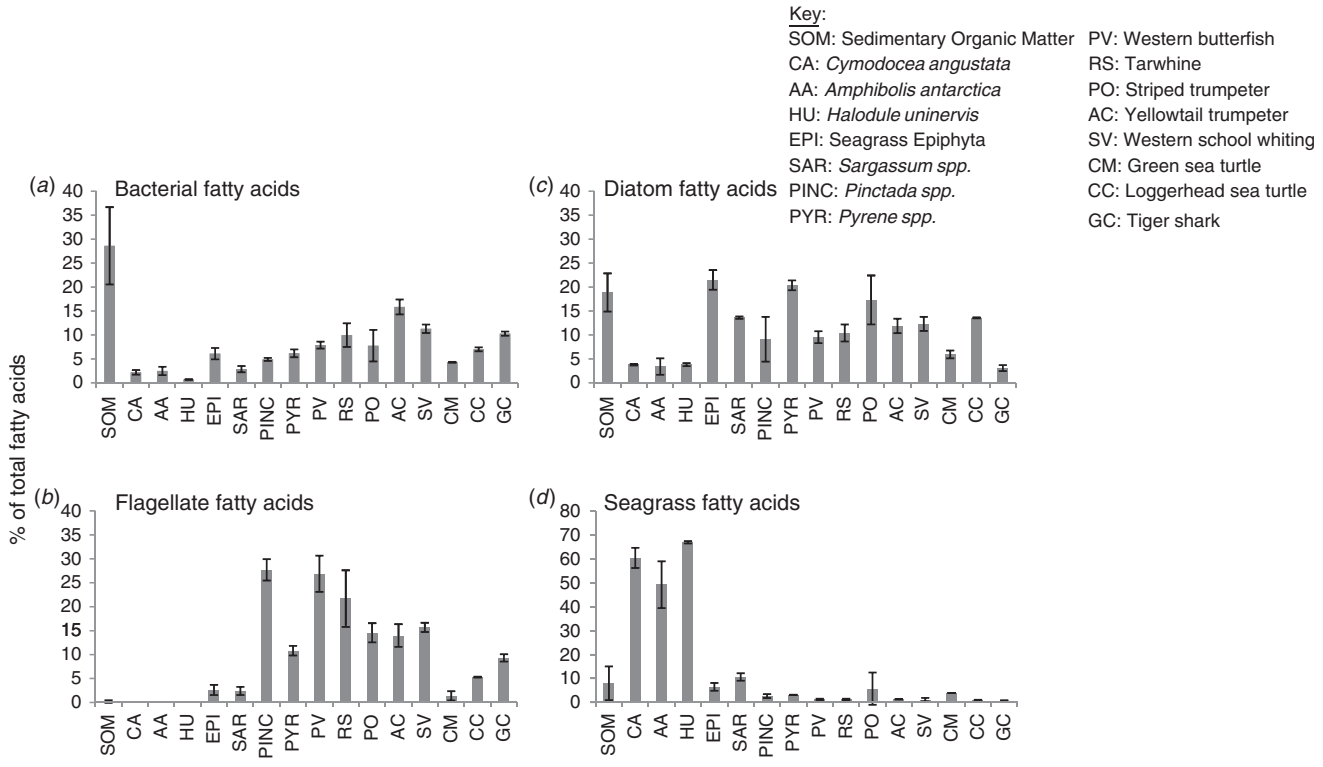

Fig. 5. Average relative abundance and standard deviation (shown where $n = 3$) of selected groups of fatty acids from typically identified sources. See Table 1 for fatty acid source assignments.

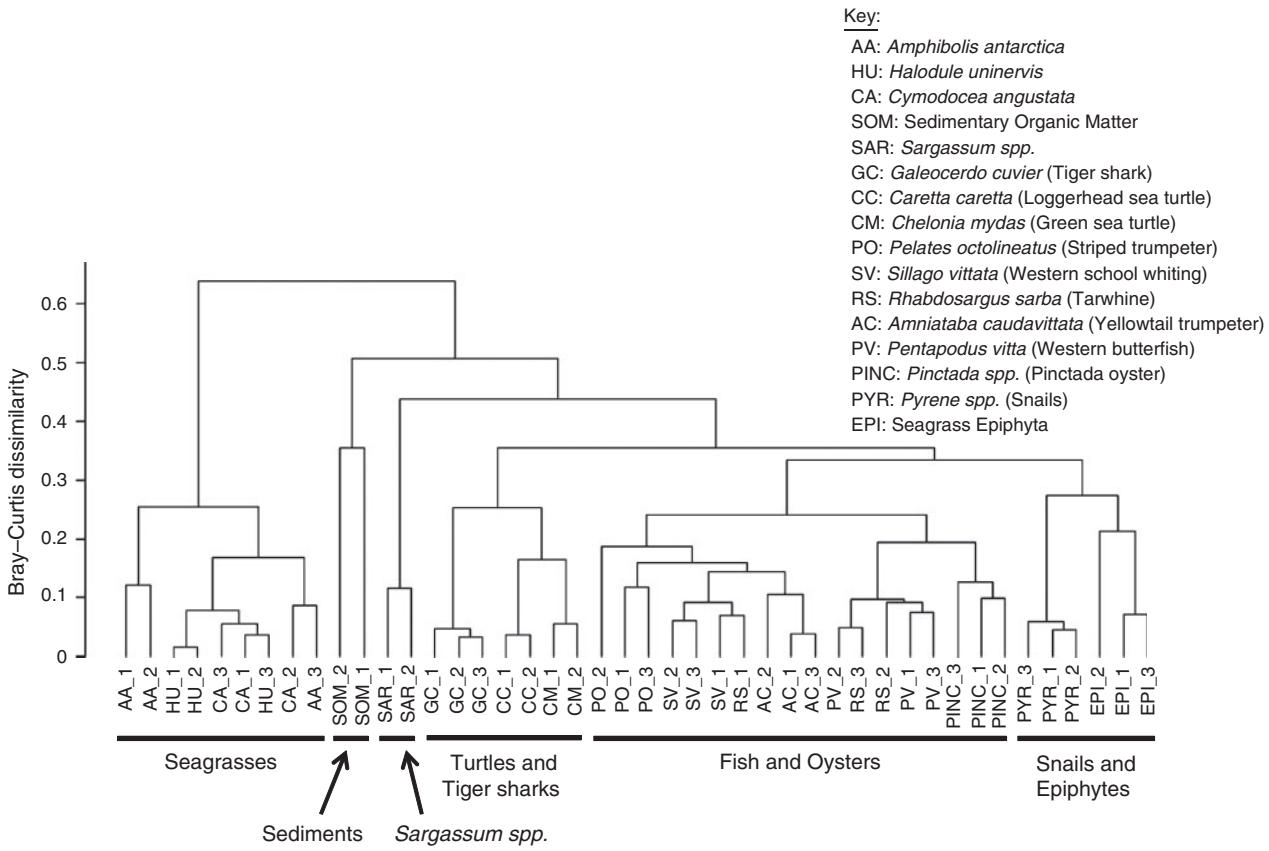

Fig. 6. Average-linkage hierarchical cluster analysis of Bray–Curtis dissimilarities identified on the basis of relative abundances of fatty acids in primary producers and consumers from Shark Bay, Australia.

consumers, suggesting that seagrass biomass was not an important component of the diet of any of the consumers. Hierarchical cluster analysis, using a suite of the most abundant fatty acids in all samples, further demonstrated that seagrasses were most dissimilar to all other consumers in terms of fatty acid composition (Fig. 6). Because dietary fatty acids are transferred relatively unchanged from food to consumer tissues with minor or predictable modifications (Dalsgaard *et al.* 2003; Budge *et al.* 2006; Jaschinski *et al.* 2011), the stark contrast between seagrass and consumer fatty acid composition as a whole and the negligible abundances of the predominant seagrass fatty acids in consumers implies little use of living seagrass as a food source. This is similar to findings from Crawley *et al.* (2009), who demonstrated using fatty acids that despite abundant seagrass in wrack biomass in surf-zones from south-western Australia, amphipods and two species of predatory fish relied disproportionately on macroalgal inputs (brown algae) compared with seagrass.

Stable isotope data suggested a more important role of seagrass organic matter for some of the organisms in food webs of Shark Bay seagrass beds. The strongest evidence for the transfer of seagrass organic matter to higher trophic levels is found in the $\delta^{13}C$ and $\delta^{34}S$ data of selected fish species. For example, western school whiting and tarwhine have relatively enriched $\delta^{13}C$ values (Fig. 2*a*) similar to those found for the three seagrass species studied here, and snails and all fish excepting striped trumpeter had $\delta^{34}S$ values similar to the two tropical seagrass species *H. uninervis* and *C. angustata* (Fig. 2*b*). In contrast, snails and oysters are substantially more deplete in ^{13}C than seagrass, instead appearing to utilise epiphytic and planktonic resources, respectively (Fig. 2*a*), and cluster analysis of the fatty acid data also highlighted strong associations between snails and epiphytes (Fig. 6). Our $\delta^{13}C$ and fatty acid data for the snails closely follows feeding experiments of *P. bidentata* in coastal south-western Australia that found that epiphytes and macroalgae were preferred relative to seagrass for this species (Doropoulos *et al.* 2009). Similarly, higher consumers such as striped trumpeter, green sea turtles, and loggerhead sea turtles also have more deplete stable carbon isotope signatures than seagrass, implying minimal usage of this food source in concordance with fatty acid data (Fig. 6).

One possible explanation for the presence of a carbon and sulfur isotope signal with a corresponding lack of fatty acid signature of tropical seagrass in some consumers is the relative importance of a detrital seagrass pathway. Two of the most abundant fatty acids in seagrass were 18:2ω6 and 18:3ω3. Their negligible presence in consumers tends to discount direct seagrass grazing, because these markers have been shown to be transferred to lipid depots in green sea turtles grazing on *Halophila hawaiiensis* (Seaborn *et al.* 2005). However,

polyunsaturated fatty acids such as 18:2ω6 and 18:3ω3 degrade faster than saturated fatty acids (Parker and Leo 1965; Nichols *et al.* 1982), suggesting that their presence in detrital seagrass materials may be significantly less compared with live seagrass. Although we were unable to obtain degraded seagrass samples from this study site for fatty acid and stable isotope analysis, both Kharlamenko *et al.* (2001) and Harbeson (2010) found that in *Zostera marina* detritus, these two fatty acids are present in amounts about an order of magnitude less compared with abundances in fresh material. Whether $\delta^{13}C$ of seagrass detritus is generally distinct from the $\delta^{13}C$ of living seagrass leaves is unclear. Because the more refractory lignocelluloses are more depleted than bulk material (Benner *et al.* 1987), one would expect detritus to be more depleted than the original seagrass. Indeed, during the first year of decomposition, seagrass detritus became more depleted by 2‰ than the starting material in Florida Bay seagrass meadows dominated by *Thalassia testudinum* (Fourqurean and Schrlau 2003). However, other studies have found no change in $\delta^{13}C$ during decomposition (e.g. Zieman *et al.* 1984), or even that dead seagrass leaves were more enriched than the living leaves (Harbeson 2010). Thus, it appears that tropical seagrass detritus, and presumably the microbial fauna colonising the detritus, contribute organic matter to fish species in Shark Bay food webs. Interestingly, the temperate seagrass species *A. antarctica* had considerably more enriched $\delta^{34}S$ values compared with *H. uninervis* and *C. angustata*, and is not likely to have a major contribution to fish or invertebrate species in Shark Bay food webs, although sulfur isotopes do suggest some contribution to loggerhead and green sea turtles and tiger sharks (Fig. 2*b*). Although both fish (Burkholder *et al.* 2012) and dugongs (Wirsing *et al.* 2007) forage on *Amphibolis*, experimental studies show that grazers in Shark Bay prefer tropical species greatly over this low-nutrient species (Burkholder *et al.* 2012).

On the basis of $\delta^{13}C$ values, Connolly *et al.* (2005) found seagrass and epiphytes, as detritus in seston, to be important food sources for the yellowfin whiting *Sillago schomburgkii* in southern Australia through detritivorous and carnivorous intermediaries (polychaetes). Similarly, Vaudo and Heithaus (2011) demonstrated with $\delta^{13}C$ the importance of seagrass organic matter, through crustacean intermediates, to elasmobranchs (primarily batoids) in Shark Bay. In our study, the ~8‰ enrichment in ^{15}N between seagrass and fish also supports the possibility of an invertebrate trophic intermediary because the average enrichment of ^{15}N between trophic levels is 3–4‰ (McCutchan *et al.* 2003). The use of detrital seagrass as a carbon source by invertebrate prey of fish such as western school whiting and tarwhine can explain the apparent discrepancy between the fatty acid and isotope data, and corresponds to conclusions drawn by Vaudo and Heithaus (2011) discussed above since western school whiting tend to feed in similar sand-flat environments as batoids. Western school whiting and tarwhine contained substantial inputs of microbial fatty acids (Fig. 5*a*), suggesting that detrital organic matter does contribute to their diet, but stable isotope and fatty acid analysis of degraded seagrass material and benthic invertebrates are necessary for further evaluation of detrital energy pathways and should be included in more comprehensive food web analyses of this region.

The role of epiphytes, plankton, and macroalgae as basal resources for consumers in Shark Bay

Epiphytic organic matter, scraped from the leaves of seagrass, was an important food source for snails, as shown by similarities in their $\delta^{13}C$ values (Fig. 2*a*) and fatty acid compositions (Fig. 6). On the basis of their fatty acid composition (i.e. abundant 16:1ω7 and 20:5ω3; (Volkman *et al.* 1989; Dunstan *et al.* 1993), epiphytes in this region have a large diatom component (Fig. 5*c*). Flagellate fatty acids were rare in epiphytes (Fig. 5*b*). Seagrass detritus may also be a dietary component for the snails, as evidenced by the similar $\delta^{34}S$ values between snails and the tropical seagrasses *H. uninervis* and *C. angustata* (Fig. 2*b*), although as mentioned above, feeding studies on *P. bidentata* suggested minimal seagrass consumption (Doropoulos *et al.* 2009). In contrast to the epiphytes, oysters contained abundant flagellate fatty acids, especially the 22:6ω3 fatty acid typically attributed to dinoflagellates (Volkman *et al.* 1989; Viso and Marty 1993), with additional, but more variable, inputs from diatom fatty acids (Fig. 5*b*, *c*). As filter feeders, the fatty acid composition of the *Pinctada* spp. oysters can be used as a proxy for the plankton community. Although there have been few studies characterising phytoplanktonic communities in Shark Bay, diatoms and dinoflagellates were reported to be the main contributors, with proportions varying with salinity (Kimmerer *et al.* 1985). Diverse communities of heterotrophic dinoflagellates have also been found in Shark Bay plankton (Tong 1997), and could be an important food source for filter-feeding bivalves, like *Pinctada* spp. oysters, in the study area.

Although not nearly as abundant as seagrasses, macroalgae are common primary producers in the Shark Bay ecosystem, and have been recently shown to be important organic matter sources for green sea turtles (Burkholder *et al.* 2011). Additionally, multiple studies from coastal south-western Australia have also highlighted the importance of macroalgal subsidy for seagrass and other coastal foodwebs (Wernberg *et al.* 2006; Ince *et al.* 2007; Crawley *et al.* 2009; Doropoulos *et al.* 2009; Hyndes *et al.* 2012). Macroalgae of the genera *Sargassum*, *Padina*, *Dictyota*, and *Penicillus*, ranged in $\delta^{13}C$ from −12.04‰ to −17.88‰ (Fig. 2*b*) in the study area (Burkholder *et al.* 2011). The most enriched macroalgae overlap $\delta^{13}C$ values for epiphytes, making stable carbon isotope composition alone difficult to distinguish between these two basal resources. The similarities in inputs of fatty acids typically attributed to diatoms (Figs 5*c*) also make further separation of these two basal resources challenging. In the hierarchical cluster analysis (Fig. 6), *Sargassum* spp. was not closely associated with a specific consumer, unlike the epiphytes, which were strongly linked to *Pyrene* spp., but instead was weakly linked to the groups of turtles and sharks, fish and oysters, and snails and epiphytes. *Sargassum* spp. was more enriched in ^{34}S than epiphytes, with an average value of the two samples analysed here of 19.7‰. The intermediate $\delta^{34}S$ values of higher consumers (tiger sharks, green sea turtles, and loggerhead sea turtles) between the values for epiphytes and *Sargassum* spp. imply that both sources of organic matter ultimately contribute to the highest trophic levels in Shark Bay – Matich *et al.* (2011) also suggested such trophic coupling for tiger sharks. Chemical characterisation of a larger number of epiphyte and macroalgal

samples will be necessary for a more rigorous separation of these two sources to higher trophic levels here.

The values of $\delta^{13}C$ suggest that most of the higher trophic level consumers we sampled likely rely on mixed detrital seagrass, epiphytic, macroalgal, and/or planktonic organic matter (Fig. 2*a*), and serve to integrate multiple trophic pathways, as is common for upper trophic level predators (Rooney *et al.* 2006). Fatty acid composition suggested that epiphytes are important to all fish in this study, on the basis of abundant 20:4ω6 and 20:5ω3. Fatty acids and stable isotopes also provided more specific information on food preferences for certain fish species. In particular, tarwhine, western school whiting, western butterfish, and yellowtail trumpeter likely utilise seagrass detrital organic matter, as discussed above, likely through benthic intermediates, as indicated by their depleted $\delta^{34}S$ signal compared with striped trumpeter, *Pinctada* oysters, turtles, and sharks (Fig. 2*b*). This is not surprising because tarwhine, western school whiting, and western butterfish are typically described as benthic carnivores, whereas yellowtail trumpeter is a benthic omnivore. In addition to epiphyte inputs, tarwhine and western butterfish use planktonic organic matter sources, as evidenced by the strong signal from dinoflagellate markers present in their muscle tissue (Fig. 5) and their association with *Pinctada* spp. oysters in the cluster analysis (Fig. 6). In contrast, the striped trumpeter most likely relies on benthic omnivory to a much lesser degree than other fish.

Values of $\delta^{34}S$ in striped trumpeter were more enriched than all other fish in the study, were similar to oysters and epiphytes, and were intermediate between the temperate and tropical seagrass species, whereas $\delta^{13}C$ was highly variable among the three individual striped trumpeter studied here (Fig. 2). Few published studies exist on the dietary preferences of striped trumpeter; however, Sanchez-Jerez *et al.* (2002), found a stronger link between a similar species of *Pelates* (*P. sexlineatus*) and mobile epifauna, including copepods, ostracods, and amphipods, than with benthic epifauna in seagrass beds of southeastern Australia. Spatial variability in the diets of *P. sexlineatus* was high though, with little inputs of macroalgae, but higher inputs of plankton to individuals near the entrances of estuaries (Sanchez-Jerez *et al.* 2002). This contrasts with the findings of Edgar and Shaw (1995), who determined that macroalgae were a predominant food source for *P. sexlineatus* along coastal southern Australia, as well as the findings of Crawley *et al.* (2009), who determined that brown algae as opposed to seagrass were a major food source for two predatory fish species (*Cnidoglanis macrocephalus* and *Pelsartia humeralis*). These prior studies and the fatty acid and stable carbon isotope data suggest that striped trumpeter diets may vary more among individuals compared with other teleost species we sampled. For example, seagrass inputs were important to the striped trumpeter individual with the most enriched $\delta^{13}C$ value (Fig. 2), as concentrations of the 18:3ω3 seagrass fatty acid in this fish were 15–23 times higher in abundance than in the other two individuals. In contrast, the individual striped trumpeter with the most deplete $\delta^{13}C$ value contained higher levels of diatomaceous and flagellate fatty acids, suggesting that epiphytes and/or macroalgae and planktonic energy pathways were more important. For this species, including mobile epifauna and a much larger number of individuals in the analysis would be necessary to fully characterise its diet.

Despite many reports suggesting an ontogenetic shift from carnivory to seagrass herbivory in green sea turtles at an approximate curved carapace length of 40–44 cm (Chaloupka and Limpus 2001; Arthur *et al.* 2008), recent studies have suggested that macroalgae and gelatinous zooplankton are much more important dietary items than previously believed (Heithaus *et al.* 2002; Arthur *et al.* 2007; Burkholder *et al.* 2011). For the two individuals studied here, $\delta^{13}C$ and $\delta^{34}S$ values and fatty acids suggested little to no seagrass inputs (Figs 2*a* and 5*d*). Composition of $\delta^{13}C$ was very different for each individual, although in general values spanned the range of isotope compositions of macroalgae and gelatinous zooplankton reported by Burkholder *et al.* (2011). Both species of turtles were less enriched in ^{34}S compared with *Sargassum* ($\delta^{34}S = 19.7‰$) and ctenophores ($\delta^{34}S = 20.6‰$), suggesting that mixed inputs from macroalgae and gelatinous zooplankton, along with a source of organic matter more deplete in ^{34}S, possibly seagrass. Our fatty acid analysis of *Sargassum* did not find a unique biomarker that could be used to solely trace macroalgae inputs through trophic levels. In general, *Sargassum* fatty acid distribution was similar to epiphtyes, with *Sargassum* containing more 18:1ω9 and less 20:4ω6 and 20:5ω3, on average, compared with epiphytes. The abundant levels of 18:1ω9 in both species of turtles and in tiger sharks could be indicative of *Sargassum* inputs to their diets; however, this association should be interpreted with caution as 18:1ω9 is ubiquitous with high relative abundances also found in some species of green algae, cryptophytes and dinoflagellates (Napolitano 1999).

Fatty acids in a composite sample of ctenophores were measured to determine if inputs from gelatinous zooplankton contributed to the diet of the two green sea turtles analysed here. The low molecular diversity of fatty acids (20 fatty acids in ctenophores compared with ~60 in epiphytes) and the fact that three ubiquitous fatty acids (14:0n, 16:0n, and 18:0n) accounted for >75% of the total fatty acids in the ctenophores (see Supplementary information) did not allow for an adequate analysis of the impact of gelatinous zooplankton on consumer diets. Total fatty acid composition of loggerhead sea turtles was most similar to that of green sea turtles (Fig. 6), although loggerheads contained higher inputs of diatom and flagellate fatty acids, suggesting planktonic and epiphytic algae are the basal resources for these consumers. Similarly, tiger sharks were most closely associated with both green sea turtles and loggerhead sea turtles on the basis of fatty acid composition (Fig. 6). A complicating factor for dietary analysis of green sea turtles lies in their ability for hindgut bacterial fermentation (Seaborn *et al.* 2005). In a typical monogastric consumer, dietary fatty acids are deposited into lipid depots with minor or predictable changes (Iverson *et al.* 2004; Budge *et al.* 2006); however, gut fermentation may cause substantial modification of dietary fatty acids before deposition in turtle lipid reserves (Joseph *et al.* 1985; Seaborn *et al.* 2005). Fatty acid compositions of green sea turtles must be interpreted with care until further studies on the effect of hindgut fermentation on fatty acid modification are undertaken.

Overall, combined fatty acid and carbon, nitrogen, and sulfur isotope data suggest that detrital, as opposed to live, *H. uninervis*

and *C. angustata* inputs likely contribute to the diets of tarwhine, western school whiting, western butterfish, and yellowtail trumpeter, through benthic intermediates, in the Shark Bay ecosystem. Planktonic, epiphytic, and macroalgal organic matter are all important basal resources for turtles and tiger sharks in Shark Bay, Australia. Future comprehensive food web studies should focus on additional characterisations of phytoplankton, zooplankton, and benthic invertebrates to form clearer associations between trophic levels. Stable sulfur isotopes should be included in future food web studies because they provided additional food source information not present from carbon isotopes or fatty acid compositions. Finally, because many species, especially large-bodied consumers, in Shark Bay show considerable variation in stable isotope values (e.g. green sea turtles, Burkholder *et al.* (2011); loggerhead sea turtles, Thomson *et al.* (2011); rays, Vaudo and Heithaus (2011); and tiger sharks, Matich *et al.* (2011), Heithaus *et al.* (2012)) future studies should incorporate a greater sample size of individuals to fully understand trophic structure in Shark Bay.

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