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Intraspecific variability in diet and implied foraging ranges of whale sharks at Ningaloo Reef, Western Australia, from signature fatty acid analysis

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ABSTRACT: We examined the feeding ecology of whale sharks by analyzing the signature fatty acids of their sub-dermal tissue and those of an extensive set of potential prey collected at Ningaloo Reef, Western Australia, in 2013, 2014 and 2015. Sub-dermal tissue of whale sharks was low in lipid content (4.0 mg g^{-1} dry mass) and dominated by phospholipids (72% of total lipids), with a calculated energy density of 18.7 kJ g^{-1} dry mass. There was significant intraspecific variability in fatty acid profiles of whale sharks, with cluster analysis identifying 4 distinct groups in 2013 and 5 groups in 2014. As this variability was not related to sex or size-class, we suggest that it may be attributed to differences in the feeding habitats used by these groups of whale sharks. Variation in dietary patterns was also observed between years, likely due to changes in the primary and secondary producers. Examination of food web interactions showed that fatty acid profiles of whale sharks and their presumed prey were significantly different, suggesting that sharks fed over a wide range of habitats, including deep waters. Our findings show that signature fatty acids of sub-dermal tissue can be used to examine broad trophic pathways and to identify spatial and temporal changes in the diet of these large and wide-ranging animals.

KEY WORDS: Biochemical analysis · Chondricthyans · Elasmobranchs · Feeding ecology · Fatty acids · Lipids

INTRODUCTION

The whale shark *Rhincodon typus* is the largest of the filter-feeding sharks and inhabits tropical and sub-tropical oceans worldwide (Ebert et al. 2013). As adults, these animals mostly reside in the open ocean where they are thought to follow zooplankton prey that undergoes diel vertical migrations between depths of 200 and 500 m during the day and in the surface (0 to 100 m) at night (Meekan et al. 2015). Because of the remoteness of these oceanic habitats, studies of the feeding behavior of these sharks are very challenging. However, predictable aggregations of whale sharks sometimes occur on tropical coasts, offering an opportunity to examine their ecology and diet in habitats more easily accessible to researchers.

Evidence from a number of studies at aggregation sites such as Ningaloo Reef, Western Australia (Meekan et al. 2006), Belize (Heyman et al. 2001) and Christmas Island (Meekan et al. 2009) suggest that whale sharks congregate in coastal habitats to target local pulses of prey availability (Colman 1997, Compagno 2001). A wide range of planktonic and nektonic organisms including copepods, gelatinous zooplankton (such as salps, siphonophores and jellyfish),

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chaetognaths, krill, mysids, amphipods, sergestids, fish eggs, small fish, shrimp and crab larvae have been identified as whale shark prey (see review Rowat & Brooks 2012). Most reports of diet are based on either anecdotal observations of whale sharks feeding in coastal surface waters during the day, plankton tows (Clark & Nelson 1997, Heyman et al. 2001, Rohner et al. 2015) or stomach (e.g. Silas & Rajagopalan 1963) and faecal (Jarman & Wilson 2004, Meekan et al. 2009) analyses. However, these methods have some well-recognized drawbacks as they can overestimate the importance of some prey and only represent 'snapshots' of recent feeding events (Iverson 2009). For these reasons, a more holistic approach to examine temporal and spatial patterns in the feeding habits of these sharks is required (Iverson 2009).

In the last decade, signature fatty acid (FA) analysis has proved to be a useful tool to investigate the feeding ecology of elasmobranchs (rays, skates and sharks) (Pethybridge et al. 2011, 2014, McMeans et al. 2012, Wai et al. 2012, Couturier et al. 2013a), and other free-ranging marine animals (e.g. Budge et al. 2008). When compared to other methods such as stomach content analyses, description of signature FA can provide longer-term (weekly to monthly) dietary information (Budge et al. 2006, Iverson 2009, Beckmann et al. 2014). This is possible because some FA in animal tissues (e.g. long-chain, $\geq C_{20}$, polyunsaturated FA, LC-PUFA) can be used as biomarkers as they pass relatively unchanged from the low trophic levels, where they are biosynthesized, up the food chain (Dalsgaard et al. 2003). Analysis of FA not only allows food web interactions to be described, but also provides an assessment of spatial and temporal changes in the diet of predators, both among and within individuals or populations (Iverson 2009). The technique also has the advantage that only a small amount of tissue (<0.5 g) is required for analysis, which can be removed as tissue biopsies from live animals without causing serious harm (Budge et al. 2006, Couturier et al. 2013a). This makes the technique ideally suited for examining the diets of protected species, such as whale sharks, which are not the subject of industrial fisheries and which strand only very occasionally (Speed et al. 2009).

Recently, Couturier et al. (2013b) and Rohner et al. (2013) used signature FA of sub-dermal tissue to examine diets of whale sharks from an aggregation in Mozambique, western Indian Ocean. These studies indicated that whale sharks had a wider foraging range than previously suggested, with important contributions from meso- and bathypelagic sources including deep-sea fish, macrozooplankton and demersal zooplankton. However, it remains unknown if these results characterize the diet of whale sharks over the wider Indian Ocean. Here, we investigate the feeding ecology of whale sharks sampled in the eastern Indian Ocean at Ningaloo Reef, Western Australia. We analyzed FA profiles of sub-dermal tissue of whale sharks collected in 2013 and 2014, and an extensive range of potential prey including zooplankton, small fishes and fish larvae, cephalopods, annelids, crab larvae, decapods, isopods, krill, mysids, amphipods and algae collected from 2013 to 2015. Fatty acid profiles were then used to assess and describe likely food web linkages and to investigate intrapopulation differences in diet associated with collection time, sex or size class of whale sharks from Ningaloo Reef. We also further examined the chemical composition (water and lipid content and lipid class determination) of whale shark sub-dermal tissue in an attempt to elucidate energy uptake, nutritional condition and resource usage patterns.

MATERIALS AND METHODS

Collection of samples

Whale shark tissue samples were collected in May 2013 and 2014, and potential prey samples in May 2013, 2014 and 2015 at Ningaloo Reef, Western Australia (22° 36′ S, 113° 39′ E; Fig. 1), a time that coincided with the annual aggregation of whale sharks at this locality and which is estimated to last from March to July every year. This study was conducted under approval by the University of Tasmania Animal Ethics Committee (A13102). Field work was carried out under permits and exemptions from the Department of Parks and Wildlife (SF009814, SF009227) and the Western Australian Department of Fisheries (2255, 2307).

Whale shark sub-dermal tissue

A total of 52 biopsies (19 in 2013 and 33 in 2014), were collected from different individual whale sharks. The presence or absence of claspers on the pelvic fins was used to identify the sex of an individual. Estimated total length (TL) of whale sharks ranged from 3 to 8.5 m and sharks were categorized into 4 groups according to size class: <4, – 6, >6–8 and >8 m. A snorkeler extracted a sub-dermal tissue sample using a modified hand spear with a biopsy probe tip. A

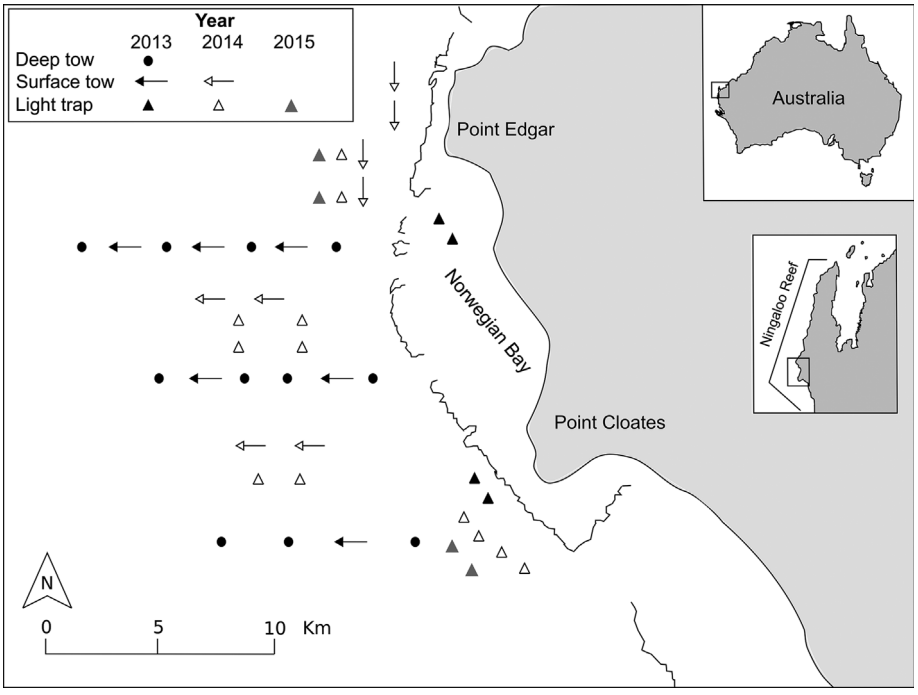

Fig. 1. Map showing collection of potential prey at Ningaloo Reef (Western Australia) in May 2013, 2014 and 2015

small core of ca. 2 cm length of sub-dermal tissue was taken from the left side of each shark behind the 1st dorsal fin. Immediately after collection, biopsies were cut into 3 equal parts in a transverse section in 2013 and in a longitudinal section in 2014. A third of the biopsy was stored frozen in liquid nitrogen for lipid class and FA analyses. In order to assess the distribution of FA along the sample of sub-dermal tissue, 5 whole biopsies were also collected in 2014 and divided into 2 equal parts (outer, which was closest to the skin, and inner, closest to the muscle) prior to freezing.

Potential prey

Zooplankton

In 2013, a zooplankton survey was carried out offshore from the reef break of Norwegian Bay, Ningaloo Reef, between Point Edgar and Point Cloates, to examine any selective feeding by whale sharks related to depth (Fig. 1). This survey consisted of 3 cross-shelf transects (north, middle and south). Four to 7 stations were sampled along each transect. Depths of stations varied according to the bathymetry of the area and were divided into: surface (from 0 to 2 m), upper (from surface to a maximum depth of 50 m) and bottom (from mid-water column to seafloor at a maximum depth of 90 m) layer. In 2014 only the surface layer was occasionally sampled (Fig. 1). Two types of nets were used to collect zooplankton. Firstly, a ring net with 200 µm mesh was used in surface tows. The second net, used to sample deeper water layers, was a specially designed 300 µm mesh net that closed and opened at the desired depth by a 'choking' system. During all hauls, nets were towed for 10 min at a speed of ~2 knots. When retrieved, nets were rinsed with seawater and the plankton concentrated in the cod-end were transferred to 500 ml plastic jars and kept in insulated containers of seawater for transportation to shore. Once onshore, zooplankton samples were split in 2 using a Folsom's Sample Divider. Half of each sample was kept frozen for lipid class and FA analyses. Of the remaining half, a quarter of the sample was fixed with 70% ethanol in filtered seawater for identification of component plankton and a quarter for stable isotope analysis (results to be reported elsewhere). In addition, all samples were fractionated by filtering through 100, 300, 500 and 1000 µm sieves to assess possible selective feeding behaviour by whale sharks related to prey size. When large (>1000 µm) zooplankton was abundant in collections (for example cladocerans, salps, pyrosomes, chaetognaths, siphonophores, ctenophores [beroe] and other jellyfish), a few representatives of each species were frozen. Individuals of these taxa were pooled between years into the category of larger zooplankton due to low sample size.

Other invertebrates, small fish and algae

We deployed single-chamber light traps (see Meekan et al. 2001 for design) to target mobile organisms (e.g. krill, cephalopods and small fishes) that were likely to avoid nets (Wilson et al. 2003). In 2013, we deployed 2 light traps (max. water column depth 7 m) in the inner part of the fringing reef just in front of Norwegian Bay (Fig. 1). Two traps were also deployed in the outer side close to the crest of the reef (max. water column depth 27 m). In 2014 and 2015, a total of 12 and 4 traps were deployed seawards of the outer side of the reef, respectively (max. water column depth 70 m) (Fig. 1). Light traps were deployed overnight and were suspended by floats so that the entrance to the light chamber was approx. 1 m below the surface. Samples collected by the traps were retrieved the next morning and transported to shore in insulated containers of seawater. Samples were then immediately sorted to the highest taxonomic resolution possible and frozen. Small fish (0–15 cm TL) were frozen whole once euthanized, whereas large fish (>15 cm TL) were sliced and blended. A homogenized subsample was used for lipid class and FA analyses.

Both phytoplankton and the macroalgae *Sargassum* sp. were also collected for lipid class and FA analyses in 2013. For phytoplankton, surface water samples were collected with clean plastic buckets, and at a depth of 15 m with Niskin bottles. Samples were transported to shore and immediately filtered onto 0.22 µm Whatman GF/F glass fiber filters and frozen. *Sargassum* sp. was sampled opportunistically from seaweed that was collected by zooplankton tows, transported to shore in insulated containers of seawater, then sorted and frozen.

Lipid class and fatty acid analyses

Lipid extraction

Lipid class and FA signature analyses were conducted at the CSIRO Marine Laboratories in Hobart, Tasmania, Australia. All samples were freeze-dried and weighed prior to analysis. Lipids were extracted following a modified Bligh & Dyer (1959) method using a one-phase methanol:chloroform:Milli-Q water (2:1:0.8 v/v/v) overnight extraction. In some instances chloroform was substituted with dichloro methane. The following morning, the phases were separated by adding 10 ml of chloroform and 10 ml of saline Milli-Q water to reach a final ratio of chloroform:methanol:water of 1:1:0.9 v/v/v. The lower layer was retained and lipids recovered by the removal of solvents *in vacuo* using a rotary evaporator at ~40°C. The total lipid extract (TLE) was concentrated to dryness in 1.5 ml glass vials under a stream of inert nitrogen gas and weighed. Samples were re-dissolved in chloroform and stored at −20°C for further analysis.

Lipid class determination

TLE samples were spotted in duplicate onto silica gel SIII Chromarods (5 µm particle size) using 1 µl disposable micropipettes along with standard solutions containing known quantities of common lipid classes including wax esters (WE), hydrocarbons (HC), triacylglycerols (TAG), free fatty acids (FFA), sterols (ST) and phospholipids (PL). Chromarods were developed in a polar solvent system (60:1:0.1 v/v/v, hexane:diethylether:acetic acid) for 25 min and then dried in an oven for 10 min at 100°C. After drying, samples were immediately analysed with an Iatrascan Mark V TH10 thin layer chromatograph (TLC) with a flame ionization detector (FID). Peaks were identified by comparison of sample retention times in relation to the standards and peak areas quantified using SIC-480II Iatroscan™ Integrating Software v.7.0-E (System Instruments, Mitsubishi Chemical Medicine). The FID was calibrated for each lipid class (phosphatidylcholine, cholesterol, cholesteryl oleate, oleic acid, squalene, TAG (derived from fish oil), WE (derived from orange roughy *Hoplostethus atlanticus* oil) and diacylglyceryl ether (DAGE; derived from shark liver oil; 0.1-10 µg range). Using predetermined linear regressions, peak areas were transformed to mass per µl spotted.

Fatty acid analyses

An aliquot of the TLE was trans-methylated to produce fatty acid methyl esters (FAME). FAME were obtained by adding 3 ml of methanol:hydrochloridric acid:chloroform (10:1:1, v/v/v) to an aliquot of the TLE and heated for 2 h at ~100°C. After cooling, 1 ml of Milli-Q water was added and FAME were extracted 3 times with 1.8 ml of hexane:dichloromethane (4:1, v/v). The FAME-extracted fraction was blown down under a gentle stream of nitrogen gas to dryness and an internal injection standard (C_{19} FAME or C_{23} FAME) added. FAME were analysed by gas chromatography (GC) using an Agilent Technologies 7890B GC equipped with a non-polar Equity™-1 fused silica capillary column (15 m × 0.1 mm internal diameter, 0.1 µl film thickness), an FID, a split/splitless injector

and an Agilent Technologies 7683B Series auto sampler. Helium was the carrier gas. Samples were injected in split-less mode at an oven temperature of 120°C, which was raised to 270°C after injection at 10°C min^{-1} and finally to 300°C at 5°C min^{1}. Agilent Technologies ChemStation software was used to quantify FAME peaks. Identification of selected FA samples was further confirmed by GC-mass spectrometry (GC-MS) using a Finnigan ThermoQuest GCQ GC-MS system fitted with an on-column injector and using Thermoquest Xcalibur software.

Water content and energy densities of whale shark sub-dermal tissue

Whale shark sub-dermal tissue was freeze-dried for 48 h, and the water fraction (WF) and wet mass:dry mass ratio determined by taking weights before and after drying. From lipid fraction values (LF) of dry mass (dm) and wet mass (wm), we calculated the fraction (F) that consisted of protein (P) and carbohydrate (C) according to:

$$PCF_{dm} = 1 - LF_{dm} \text{ and } PCF_{wm} = 1 - LF_{wm} - WF \quad (1)$$

Then, energy density (ED, kJ g^{-1} wm) of whale shark tissue was determined with the following equations and using published calorific values of 39.9 kJ g^{-1} for lipids and 17.8 kJ g^{-1} for protein and carbohydrates (Schmidt-Nielsen 1997), after Pethybridge et al. (2014):

$$ED = (1 - WF)\,[(LFwm \times 39.9) + (PCFwm \times 17.8)] \quad (2)$$

$$ED = (LFdm \times 39.9) + (PCFdm \times 17.8) \quad (3)$$

Statistical analyses

Fatty acids were expressed as area percentage of total FA (%TFA) and plotted as mean ± SE. A total of 59 and 56 FA were detected in 2013 and 2014 samples, respectively. Only those FA detected in both years were used for statistical analyses. FA in concentrations >1.0 in each group (whale sharks, zooplankton and other invertebrates and small fish) were used for within-group comparisons. For among-group comparisons (potential prey groups and prey–predator comparisons), FA > 1.0 in whale shark profiles were used in analyses. Inter-annual analyses were only conducted with whale shark and zooplankton samples. The other prey collected in either 2013, 2014 or 2015 (e.g. larger zooplankton, cephalopods, krill and mysid) were pooled between years of sampling due to low sample size.

PERMANOVA (permutational multivariate analysis of variance, based on 9999 permutations) was used to test for factorial (collection time, sex and size class) differences in FA profiles and post-hoc pairwise comparisons were used to test for differences within levels or combinations of levels. Comparison of homogeneity dispersion between groups was performed using PERMDISP. Similarity percentage analysis (SIMPER) was used to identify the contribution of each FA to similarities or dissimilarities within and among groups. Non-parametric multi-dimensional scaling (MDS) was employed to visually explore relationships of groupings within and between whale sharks and potential prey items. Hierarchical cluster analysis based on group averages was applied in the MDS plot to show clustering of similar groups. All analyses used PRIMER v6 software (Primer-E) on untransformed data with a nonparametric Bray-Curtis similarity matrix. All results are expressed as mean ± SE.

RESULTS

Whale shark chemical composition

Whale shark sub-dermal tissue was low in lipid (4.0 ± 0.9 mg g^{-1} dm) and high in water content (91.3 ± 2.6%) with a wm:dm ratio of 18.0 ± 5.2. Energy density for this tissue was estimated at 18.7 kJ g^{-1} dm (Table 1). Tissues collected in 2013 and 2014 were

Table 1. Biochemical data (mean ± SE) for whale shark sub-dermal tissue and zooplankton collected at Ningaloo Reef in May 2013 and May 2014. dm: dry mass; wm: wet mass. For comparative studies, 1 kCal = 4.184 kJ

Parameter	Unit	Whale sharks (n = 52)	Zooplankton (n = 56)
Water content	%	91.3 ± 2.6	–
wm:dm ratio	–	18.0 ± 5.1	–
Lipid content	mg g^{-1} dm	4.0 ± 0.9	42.7 ± 2.6
	mg g^{-1} wm	0.4 ± 0.0	–
Protein and carbohydrates	% dm	95.0 ± 0.0	–
Energy density	kJ g^{-1} dm	18.7 ± 0.2	–
	kJ g^{-1} wm	0.1 ± 0.1	–
Lipid class composition			
Wax esters	%	3.7 ± 1.3	6.5 ± 1.1
Triacylglycerols	%	7.7 ± 2.3	11.5 ± 1.6
Free fatty acids	%	2.0 ± 0.6	7.8 ± 1.4
Sterols	%	14.6 ± 1.3	5.2 ± 0.8
Phospholipids	%	71.9 ± 3.0	69.0 ± 3.2

dominated by PL (mean relative values of 72%), with minor lipid classes in order of decreasing importance including ST, TAG, WE and FFA (Table 1).

There was no significant difference in the FA composition between the outer and inner layers of sub-dermal tissue of whale sharks (PERMANOVA pseudo-F = 0.519, p = 0.97). Both layers had similar percentages of polyunsaturated FA (PUFA, 35.8% in the outer and 35.4% in the inner layer), followed by saturated FA (SFA; 34.4%) in the outer layer and by monounsaturated FA (MUFA, 30.4%) in the inner layer (Table S2 in Supplement 1 at www.int-res.com/articles/suppl/m554p115_supp.pdf).

Profiles of whale shark FA differed significantly between years (pseudo-F = 12.057, p < 0.001). In 2013, profiles were largely dominated by SFA (48.5%), followed by PUFA (26.1%) and MUFA (25.4%). In contrast, 2014 biopsies were dominated by PUFA (36.2%) followed by SFA (33.2%), with slightly lower levels of MUFA (30.7%, Table 2). Heterogeneity in multivariate dispersion of FA was revealed between the 2 years (PERMDISP F = 13.62, p = 0.005) with 2013 samples showing lower within-group similarity (70.2%, SIMPER) than samples collected in 2014 (83.2%, SIMPER). Major FA for 2013 samples were 18:0, 18:1ω9, 20:4ω6 (ARA, arachidonic acid) and 16:0 and major FA for 2014 samples were 18:0, ARA, 18:1ω9 and 16:0 in decreasing order of relative abundance (Table 2). SIMPER analysis indicated that a slight increase of ARA and 18:1ω9 (1.5-fold) and a decrease of 18:0 (2-fold) between 2013 and 2014, contributed most to the separation between years. In both years, the mean ω3/ω6 PUFA ratios were <1 and dominated by ARA (Table 2).

Cluster analyses revealed distinct groups of whale shark FA profiles in both years: 4 in 2013 (Groups A, B, C, D) and 5 in 2014 (Groups E, F, G, H, I) (Fig. 2). Profiles of sharks in Group A (2013) and Group E (2014), which formed the central cluster of samples,

Table 2. Fatty acid (FA) composition (% of total FA, mean ± SE) of whale shark biopsies and zooplankton collected at Ningaloo Reef (Western Australia) in May 2013 and May 2014. Others (<0.2%): a15:0, i14:0, 14:1ω5c, 14:1ω7c, 15:1ω6c, 16:1ω7t, 16:3, 16:4, 16:0FALD, C_{18}PUFA, 18:1, 18:1FALD, 18:0FALD, 18:1ω5c, 19:1 (2 isomers), 19:0, 21:0, C_{22}PUFA, 23:0, 24:6ω3, 24:5ω3, 24:1ω7c. SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; t: trans-configured MUFA; c: cis-configured MUFA. The suffix i denotes branched FA from the iso-series. FALD: fatty aldehyde analysed as dimethyl acetal

Fatty acid	Whale shark		Zooplankton	
	2013 (n = 19)	2014 (n = 33)	2013 (n = 54)	2014 (n = 14)
14:0	0.1 ± 0.1	0.2 ± 0.0	6.9 ± 0.5	3.3 ± 0.5
i15:0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
15:0	0.1 ± 0.0	0.2 ± 0.0	1.4 ± 0.1	1.0 ± 0.1
i16:0	0.1 ± 0.0	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0
16:0	11.9 ± 1.4	9.7 ± 0.5	34.1 ± 1.7	19.4 ± 0.7
i17:0	0.5 ± 0.1	1.2 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
17:0	0.8 ± 0.1	1.0 ± 0.0	2.4 ± 0.1	1.8 ± 0.1
i18:0	0.5 ± 0.1	0.7 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
18:0	32.0 ± 3.4	18.0 ± 0.5	11.5 ± 0.8	7.6 ± 0.3
20:0	0.8 ± 0.1	0.8 ± 0.4	1.1 ± 0.2	0.5 ± 0.0
22:0	0.8 ± 0.2	0.5 ± 0.0	1.3 ± 0.3	0.5 ± 0.0
24:0	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
Total SFA	**48.5 ± 3.2**	**33.2 ± 0.8**	**61.3 ± 2.7**	**35.3 ± 1.0**
16:1ω9c	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
16:1ω7c	0.9 ± 0.3	1.3 ± 0.1	3.2 ± 0.3	3.3 ± 0.2
16:1ω5c	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
16:1ω13t	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
17:1ω8c+a17:0	0.6 ± 0.1	1.1 ± 0.0	0.5 ± 0.1	0.3 ± 0.0
17:1	1.7 ± 0.3	2.5 ± 0.2	0.1 ± 0.0	0.4 ± 0.1
18:1ω9c	13.1 ± 1.9	15.6 ± 0.7	6.1 ± 0.6	5.5 ± 0.4
18:1ω7c	3.5 ± 0.5	4.1 ± 0.4	1.9 ± 0.2	2.2 ± 0.2
18:1ω7t	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
20:1ω11c	0.4 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
20:1ω9c	1.2 ± 0.4	1.4 ± 0.2	0.6 ± 0.1	0.6 ± 0.1
20:1ω7c	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1ω5c	0.7 ± 0.2	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
22:1ω11c	0.9 ± 0.6	0.5 ± 0.2	1.1 ± 0.3	0.2 ± 0.1
22:1ω9c	0.7 ± 0.2	0.6 ± 0.3	0.2 ± 0.0	0.2 ± 0.0
22:1ω7c	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
24:1ω11c	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
24:1ω9c	0.8 ± 0.1	1.6 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
Total MUFA	**25.4 ± 2.6**	**30.7 ± 1.2**	**16.9 ± 0.9**	**15.8 ± 0.7**
18:3ω3	0.1 ± 0.0	0.6 ± 0.3	0.9 ± 0.3	1.2 ± 0.2
18:4ω3	0.0 ± 0.0	0.3 ± 0.1	0.8 ± 0.1	1.6 ± 0.3
18:3ω6	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.1
18:2ω6	0.7 ± 0.0	1.0 ± 0.1	1.3 ± 0.2	1.6 ± 0.1
20:4ω6 (ARA)	12.5 ± 1.7	16.4 ± 1.0	0.9 ± 0.1	2.7 ± 0.6
20:5ω3 (EPA)	1.7 ± 0.2	2.0 ± 0.3	4.6 ± 0.6	10.2 ± 0.9
20:3ω6	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
20:4ω3	0.2 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.1
20:2ω6	0.4 ± 0.2	0.2 ± 0.0	0.5 ± 0.1	0.4 ± 0.1
21:5ω3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
22:5ω6	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
22:6ω3 (DHA)	2.4 ± 0.2	3.5 ± 0.3	9.9 ± 1.2	27.2 ± 1.5
22:4ω6	5.3 ± 0.7	6.8 ± 0.5	0.2 ± 0.0	0.4 ± 0.0
22:5ω3	2.0 ± 0.2	3.2 ± 0.3	0.5 ± 0.1	0.9 ± 0.1
Total PUFA	**26.1 ± 2.9**	**36.2 ± 0.9**	**21.7 ± 2.1**	**49.0 ± 1.3**
ω3/ω6	**0.3 ± 0.1**	**0.5 ± 0.1**	**3.7 ± 0.3**	**6.7 ± 0.6**
Others	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.0

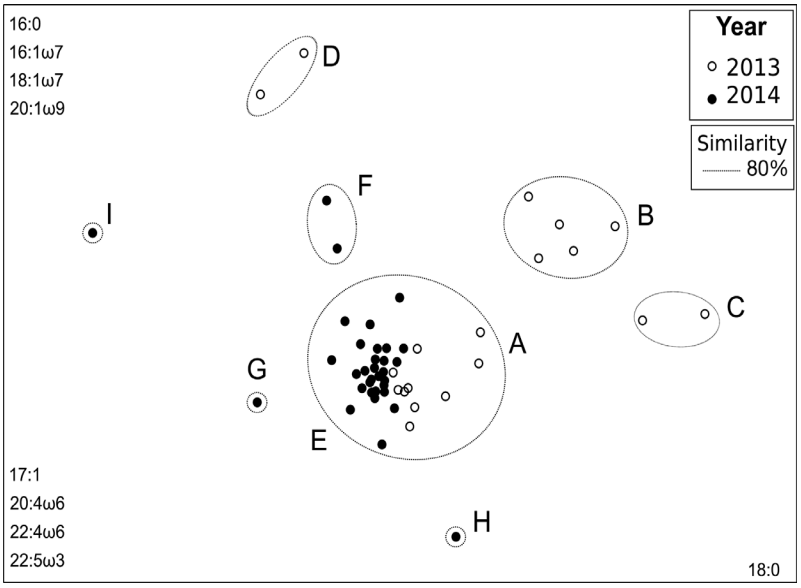

Fig. 2. Multi-dimensional scaling ordinations of whale shark clusters (Groups A, B, C, D, E, F, G, H and I) for 2013 and 2014. Fatty acid labels represent the main coefficients (>0.6) contributing to each axis

were significantly different (*t*-test, $t = 2.947$, $p < 0.001$), although all were characterized by moderate levels of the 4 major FA (16:0, 18:0, 18:1ω9 and ARA) (SIMPER). The FAs that separated each group from the main cluster of samples in each year are summarized in Table 3. SIMPER analyses revealed that whale shark Groups B and C (23.5%) and Groups E and F (24.7%) were the most similar in 2013 and 2014, respectively. In contrast, the highest dissimilarities occurred between Groups D and C (66.6%) and Groups H and I (54.3%) in 2013 and 2014, respectively. Between years, Groups C and I were the most dissimilar (63.1%, SIMPER; Fig. 2). No significant differences were detected in FA profiles of whale sharks according to sex (pseudo-$F = 0.672$, $p = 0.707$) or size class (pseudo-$F = 0.51$, $p = 0.875$).

Table 3. Collection and biological information of whale sharks analysed and grouped in this study by 80% similarity clusters of fatty acid profiles. Fatty acid indicators according to differences between each group to main cluster groups: Group A in 2013 and Group E in 2014 (SIMPER). DHA: docosahexaenoic acid; ARA: arachidonic acid

Cluster	Sex	Size class (m)	Number	Fatty acid indicators
2013				
Group A (n = 10)	Female	>6–8	1	Moderate levels of 18:0, ARA, 18:1ω9, 16:0
	Male	<4	2	Slightly higher 18:0 than Group E
		4–6	2	
		>8	1	
	Unknown	4–6	3	
		>8	1	
Group B (n = 5)	Male	4–6	3	Higher 16:0 and 18:0, lower ARA than Group A and E
	Unknown	4–6	2	
Group C (n = 2)	Male	<4	1	Much higher 18:0 and 16:0, lower ARA and 18:1ω9 than Group A and E
		>6–8	1	
Group D (n = 2)	Male	<4	2	Higher 18:1ω9 and 16:0, lower ARA than Group A and E
2014				
Group E (n = 28)	Female	<4	1	Moderate levels of 18:0, ARA, 18:1ω9, 16:0
		4–6	6	Slightly higher 18:1ω9 than Group A
	Male	<4	1	
		4–6	15	
		>6–8	4	
	Unknown	4–6	1	
Group F (n = 2)	Male	4–6	1	Lower ARA and 18:0, higher 18:1ω9 than Group A and E
		>6–8	1	
Group G (n = 1)	Male	>6–8	1	Much lower ARA, higher 18:0 than Group A and E
Group H (n = 1)	Male	4–6	1	Higher 22:4ω6, lower 18:1ω9 than Group A and E
Group I (n = 1)	Female	>6–8	1	Lower ARA, 18:0 and 18:1ω9 than Group A and E

Potential prey chemical composition

Zooplankton

Zooplankton samples were dominated by copepods, mainly from the genus *Paracalanus*, *Oncaea* and *Farranula*, followed by decapod larvae and foraminifers. The mean lipid content for all zooplankton samples was 42.7 ± 2.6 mg g^{-1} dm and was dominated by PL followed by TAG, FFA, WE and ST (Table 1).

There were no significant differences in FA profiles of zooplankton sampled from different parts of the water column (surface, upper and bottom) in 2013 (pseudo-F = 2.343, p = 0. 065) or of different size fractions (100, 300, 500 and 1000 µm) (pseudo-F = 0.511, p = 0.904). Samples from different depths and size fractions were thus pooled for further analysis.

Overall, the FA composition of zooplankton differed significantly between years (pseudo-F = 17.654, p < 0.001; Fig. 3A). FA profiles for zooplankton collected in 2013 showed lower within-group similarity (68%, SIMPER) and were dominated by SFA (61.3%) compared to the 2014 profiles that were PUFA-dominated (49%, Table 2). Major FA for 2013 samples were 16:0, 18:0, 22:6ω3 (DHA, docosahexaenoic acid), 18:1ω9 and 20:5ω3 (EPA, eicosapentaenoic acid) and major FA for the 2014 samples were DHA, 16:0, EPA, 18:0 and 18:1ω9, in decreasing order of abundance (Table 2). SIMPER analysis revealed that an increase of DHA (3-

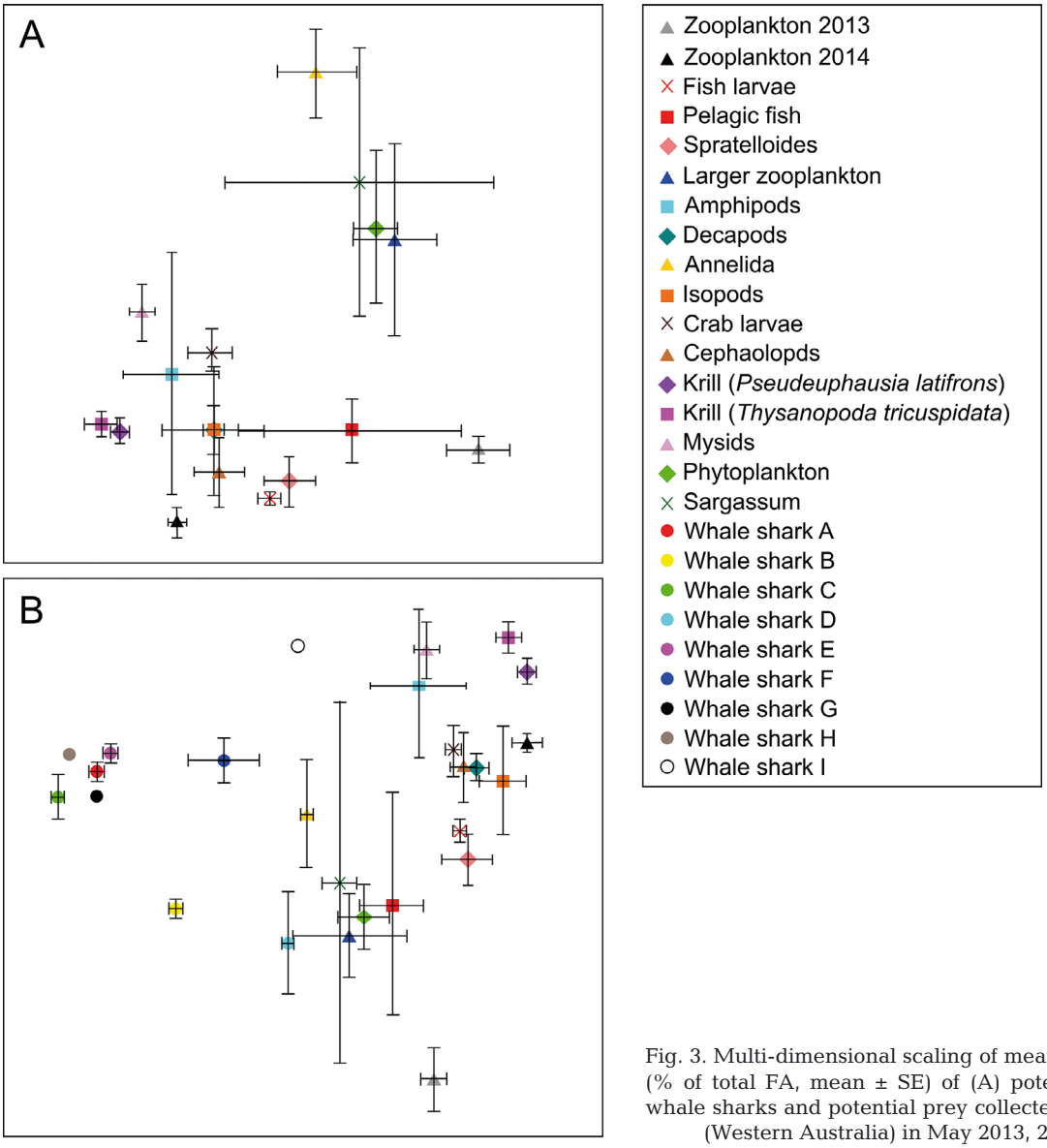

Fig. 3. Multi-dimensional scaling of mean fatty acid profiles (% of total FA, mean ± SE) of (A) potential prey and (B) whale sharks and potential prey collected at Ningaloo Reef (Western Australia) in May 2013, 2014 and 2015

fold) and EPA (2-fold) and a decrease of 16:0 (2-fold) between 2013 and 2014, contributed most to the separation between years. FA profiles of larger zooplankton were dominated by SFA (50.4%, Table S3 in Supplement 2 at www.int-res.com/articles/suppl/m554p115_supp.xlsx). SIMPER showed a higher influence of 18:0 and 18:1ω9 and a lower influence of DHA in these taxa than in overall zooplankton profiles (Table S3). The mean ω3/ω6 PUFA ratio, dominated by DHA, was consistently >1 for all zooplankton and almost doubled in 2014 compared to 2013 (Table 2).

Other invertebrates, small fish and algae

In 2013, we collected a wide range of small pelagic fish [*Spratelloides* sp., myctophids, reef fish juveniles (genera *Lethrinus*, *Chromis*, *Stegastes*, families Mullidae and Synodontidae)] cephalopods, annelids, crab larvae, decapods and isopods. In addition to these same taxa, krill (*Thysanopoda tricuspidata*), mysids and amphipods were collected in 2014. In 2015, an additional species of krill (*Pseudeuphausia latifrons*) was collected and included in the study.

The lipid content of all these organisms ranged from 9.7 (larger zooplankton) to 117.7 (cephalopods) mg g^{-1} dm (Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m554p115_supp.pdf). While most groups were dominated by PL, crab larvae and isopods also had high levels of TAG (Table S1).

The FA profiles of most taxa were dominated by PUFA (32.4 to 56.4%), with the exception of pelagic and reef fish larvae and phytoplankton samples, which had higher contributions of SFA (40.7 to 49.7%) than the remaining taxa. Overall, most of the taxa including cephalopods, crab larvae, decapods, isopods and both species of krill were dominated by 16:0 and 18:0, whereas the PUFA fraction was composed of high levels of DHA, lower levels of EPA and low levels of ARA, grouping closer in the MDS plot (Fig. 3A). The MUFA 18:1ω9 also occurred at high levels, with a mean value of 10% (Table S3 in Supplement 2). Fish were characterized by higher levels of 18:0 and lower levels of EPA and ARA, whereas mysids and amphipods showed higher contributions of ARA than any other taxa (Table S3). The FA profile of annelids was the most dissimilar to other prey taxa, with relatively high levels of 18:0 and EPA and lower levels of DHA (SIMPER; Fig. 3A, Table S3). Among all prey taxa, SIMPER analysis revealed high abundances of 18:1ω9, 18:0 and DHA in phytoplankton, whereas levels of EPA, ARA and 16:0 were higher in *Sargassum* sp. All prey taxa had ω3/ω6 PUFA ratios >1 dominated by DHA, with the exception of the annelids and *Sargassum* sp., which were EPA-dominated (Table S3).

Potential prey–predator fatty acid comparisons

FA profiles of whale sharks sampled in 2013 and 2014 were significantly different to those of all potential prey we sampled (*t*-test; $t = 2.162$ to 9.198, $p < 0.019$ Fig. 3B). SIMPER revealed that high levels of 18:0, 18:1ω9 and ARA in whale sharks and 16:0, DHA and EPA in potential prey were the main cause of these differences. Profiles of whale shark Groups C, H and G were the most different to those of prey taxa, whereas Groups F, D and B clustered towards the center of the MDS plot closest to the large grouping of a variety of different prey types (SIMPER, dissimilarity 41 to 67%; Fig. 3B).

Despite differences between profiles of whale sharks and prey groups, both whale sharks and zooplankton displayed similar inter-annual trends, with SFA dominating profiles in 2013 and PUFA in 2014 (Table 2). High levels of 16:0 and 18:0 in annelids, and high levels of ARA in mysids and amphipods, made these groups the most similar to whale sharks than any other prey category (SIMPER, dissimilarity 44 to 47%; Fig. 3B). In contrast, both species of krill and isopods clustered the furthest from whale sharks due to low levels of 18:0 and ARA and higher levels of DHA and EPA (SIMPER, dissimilarity 55%; Fig. 3B). Profiles of larger zooplankton were more similar to whale sharks than overall zooplankton samples in both years, mainly due to higher levels of 18:0 and 18:1ω9 (SIMPER, dissimilarity 47%; Fig. 3B). Although a higher influence of ARA was found in *Sargassum* sp. samples, phytoplankton was slightly more similar to whale sharks, mostly due to higher levels of 18:0 and 18:1ω9 (SIMPER, dissimilarity 46 to 49%, Fig. 3B).

DISCUSSION

Chemical composition of whale shark sub-dermal tissue

The sub-dermal tissue of whale sharks collected at Ningaloo Reef was high in water content (91.3%) and had chemical characteristics typical of a structural tissue with low lipid (4.0 mg g^{-1} dm or 0.4 mg g^{-1} wm) and energy content (18.7 kJ g^{-1} dm or 0.1 kJ g^{-1} wm), only low to moderate levels of TAG (energy storage lipid, 7.7% of total lipid) and high levels of PL (struc-

tural lipid, 72% of total lipid). Sub-dermal tissue had a much lower lipid content than that reported for the livers of other shark species (10 to 75% wm; Sargent et al. 1973, Nichols et al. 2001, Jayasinghe et al. 2003, Reme et al. 2006, Pethybridge et al. 2010, 2014, Davidson et al. 2014), a result that reflects the function of liver as an organ for lipid storage (Sheridan 1988). Additionally, sub-dermal tissue also tended to be slightly lower in lipid content than muscle tissues reported in other elasmobranchs (0.2 to 3.6% wm; Sargent et al. 1973, Pethybridge et al. 2010, 2014, Couturier et al. 2013a, Davidson et al. 2014). Despite the superficial resemblance of the sub-dermal layer of whale sharks to the blubber layer of marine mammals (see Meekan et al. 2015), our results suggest that this tissue is unlikely to play a role in lipid storage.

The lipid content of sub-dermal tissue of the whale sharks we sampled in the eastern Indian Ocean at Ningaloo Reef was lower (4.5-fold) than the values recorded for the same species in coastal waters off Mozambique in the western Indian Ocean (lipid content of 1.8 mg g^{-1} wm; Couturier et al. 2013b, Rohner et al. 2013). This variation in lipid content could reflect the different nutritional conditions (e.g. primary productivity) in each of these locations.

Although FA signatures of tissues, including sub-dermal tissue, liver and muscle, have been used as indicators of diet in both whale sharks and other elasmobranchs (Pethybridge et al. 2011, McMeans et al. 2012, Couturier et al. 2013a,b, Rohner et al. 2013, Beckmann et al. 2014, Pethybridge et al. 2014), the interpretation of FA analyses presently remains hindered by our limited knowledge of lipid metabolism and FA biosynthesis by elasmobranchs. For example, previous studies of deep-sea (squaliformes, chimaeriformes, hexanchiformes and carcharhiniformes) and Greenland sharks (*Somniosus microcephalus*) have demonstrated that dietary FAs are selectively incorporated into different tissues according to their metabolic and functional role (Pethybridge et al. 2010, McMeans et al. 2012). In addition, the time frame over which dietary FA are incorporated is likely to differ among tissues (Beckmann et al. 2014), since laboratory studies based on stable isotopes have shown that structural tissues such as muscle, fin and cartilage had slower turnover rates than more metabolically active tissues such as liver or blood (e.g. Logan & Lutcavage 2010, Hussey et al. 2011, Kim et al. 2012). However, the fibrous nature of the sub-dermal tissue coupled with poor vascularization (we recorded no signs of bleeding when biopsies were removed) suggests low metabolic activity and thus slower turnover rates of FA than occurring in tissues such as muscle or blood. Profiles of sub-dermal tissues may thus reflect longer-term (up to several months) dietary signatures. Taking into account that biopsies were collected in the middle of the whale shark season, comparison of FA profiles between tissues with low and high rates of turnover will help better elucidate whether sub-dermal tissue signatures are representative of feeding events while at Ningaloo Reef or before arriving at the aggregation.

Several studies have reported vertical stratification of FA profiles within tissues (see review by Budge et al. 2006). For example, the blubber layer of marine mammals has marked stratification in FA composition, with the more metabolically active inner layer reflecting more recent diet than the outer layer closer to the skin surface (Best et al. 2003, Budge et al. 2008). In contrast, we found no stratification in total lipid content or FA profiles in whale shark biopsies. This finding may indicate that all of the sub-dermal tissue has a slow rate of turnover, and hence further supports the idea that this tissue provides a medium- to longer-term indicator of diet.

Temporal and intraspecific variation in whale shark fatty acids

At Ningaloo Reef, there was high variability in the FA profiles of whale sharks both within and between years, with the MDS analysis identifying 4 different groups of whale sharks in 2013 and 5 groups in 2014. In other wide-ranging animals, such as seals, whales and seabirds, variability in FA profiles among individuals or groups of individuals has been linked to spatial variation in diet (e.g. Bradshaw et al. 2003, Iverson et al. 2007, Budge et al. 2008). Because these large, filter-feeding sharks face the challenge of meeting high energetic demands while consuming prey that are many orders of magnitude smaller than their own body size, similar to basking sharks *Cetorhinus maximus*, whale sharks target dense aggregations of prey (Sims & Quayle 1998, Rohner et al. 2015) in order to enhance cost-efficiency when foraging. As food is patchy in both space and time in oligotrophic tropical oceans, horizontal movements of whale sharks in search of prey are thus likely to show considerable variation among individuals. This idea is consistent with satellite-tagging studies that show that after departing Ningaloo Reef, individual whale sharks travel north towards the equator using many different routes (Wilson et al. 2006, Sleeman et al. 2010). Similarly, Hueter et al. (2013) showed that

individual whale sharks took different paths when travelling towards the Caribbean Sea and the South Atlantic after departing an aggregation off the Yucatan Peninsula in Mexico. Thus, the high variability in the FA profiles of whale sharks both within and between years at Ningaloo Reef may reflect variation in the prey consumed by individuals or groups of sharks, when travelling both to and from Ningaloo Reef, and while resident at the aggregation.

Although changes in feeding habits according to sex or size class are well documented in elasmobranchs (Wetherbee & Cortés 2004), neither of these factors accounted for the variability in FA analyses we observed. Similar to many other localities (e.g. Belize, Maldives and Mozambique; Rowat & Brooks 2012), the Ningaloo Reef aggregation comprised mostly juvenile males (Meekan et al. 2006) and our sampling reflected this skewed composition of the population. The low sample sizes of adults and females probably reduced the ability of our study to discern differences in FA analyses based on size or sex, given that such differences have been recorded in localities where aggregations consist of a wider range of sizes and a more balanced sex ratio, such as in the Gulf of California (Ketchum et al. 2013) and off the coast of India (Borrell et al. 2011). Broadening the sampling of sizes and increasing sampling of females should be an aim of future sampling.

The inter-annual shift from SFA to PUFA in FA profiles of whale sharks and zooplankton between 2013 and 2014 at Ningaloo Reef is likely a reflection of seasonal and/or interannual changes in the composition of primary producers. Oceanographic and environmental variations, such as changes in temperature, salinity, nutrients and light, can alter the FA composition of phytoplankton (Dalsgaard et al. 2003), which can in turn alter the composition of animals at higher trophic levels (Budge et al. 2008, Pethybridge et al. 2015). For example, a higher amount of unsaturation of FA, as we observed in 2014, could be due to the lower sea surface temperatures that occurred in this year (IMOS 2013, 2014), since cooler waters are associated with an increase in the membrane fluidity of cells facilitated by unsaturated FA (Parrish 2013).

Food web inferences

Although observational studies have suggested that whale sharks feed on pelagic zooplankton (see review Rowat & Brooks 2012), results of the FA profiles presented here and those of earlier studies of both whale sharks off the coast of Mozambique and manta rays *Manta alfredi* (Couturier et al. 2013a,b, Rohner et al. 2013) suggest a wider foraging range for these tropical large filter-feeders. Rather than profiles dominated by the ω3 FA, DHA and EPA, which are typical of pelagic systems (Dalsgaard et al. 2003) and are also found in high concentrations in the potential prey collected in this study (Table S3 in Supplement 2), we found that FA profiles of the subdermal tissue of whale sharks were dominated by the ω6 FA, ARA. Couturier et al. (2013a,b) and Rohner et al. (2013) have recently suggested that the high ω6 LC-PUFA signatures observed in whale sharks and manta rays might be linked to deep water foraging that included demersal zooplankton such as mysids that emerge nocturnally from the sediment into the water column, and deeper living fish and macrozooplankton.

FA profiles that include high levels of ARA are characteristic of benthic or benthopelagic organisms, including echinoderms, amphipods, fish and demersal zooplankton (e.g. Copeman & Parrish 2003, Connelly et al. 2014). High ARA levels are also found in some macroalgae (e.g. Johns et al. 1979, Virtue & Nichols 1994). Although not fully understood, the trophic path of this ω6 PUFA in the marine environment might originate from microheterotrophs and protists such as thraustochytrids, which are ubiquitous in oceanic environments including sediment (Nichols 2003, Lee Chang et al. 2012). Of the potential prey collected in this study, amphipods and mysids, which are known to be part of the demersal zooplankton in coastal habitats (Alldredge & King 1985), and *Sargassum* sp. showed high levels of ARA. Although fragments of macroalgae and mysids have been reported in whale shark stomachs in Mozambique and South Africa (Rohner et al. 2013), the ingestion of the former by whale sharks is likely to only be incidental. Satellite tagging has revealed that whale sharks in the open ocean dive to meso- bathypelagic depths (200 to 1000 m) (Graham et al. 2006, Wilson et al. 2006, Brunnschweiler & Sims 2011). These deep dives are thought to be foraging-related (Meekan et al. 2015). In addition, stable isotope reports from India also indicated benthic foraging by whale sharks larger than 4 m, which showed enriched $\delta^{13}C$ values ranging from −17.4 to −15.1‰ (Borrell et al. 2011), i.e. values associated with marine benthic algae (France 1995). Therefore, the link to high ARA in whale shark FA profiles is likely to come from direct ingestion of demersal zooplankton when close to the coast or other organisms in the deep scattering layer when off the continental shelf.

Although deep foraging is likely to account for FA profiles high in ARA, it is also possible that the higher relative levels of this FA is derived from other sources. For example, Wyatt et al. (2012) found that fish in the Ningaloo Reef area had profiles with ARA that they argued might be reef-derived from coralline algae and coral mucus (van Duyl et al. 2011). In addition, ARA in whale shark sub-dermal tissue could also have a metabolic origin. Whereas fishes are not able to biosynthesis LC-PUFA de novo, it is known that this group has metabolic pathways to elongate shorter chain ($\leq C_{18}$) PUFA to LC-PUFA (Monroig et al. 2013), an ability that could confound FA signatures derived from foraging. However, this idea is inconsistent with the high variability of ARA levels we found among individual sharks, indicated by high SE (Table 2), suggesting that such differences are more likely to be related to diet rather than physiology. Our results highlight the need to analyze tissues with faster rates of metabolic turnover, such as liver or blood, in order to differentiate between feeding and metabolic pathways for the accumulation of ARA in the sub-dermal tissues of whale sharks.

CONCLUSIONS

Our results are consistent with recent studies which suggest that a significant component of whale shark diets originates from benthic and deep-water habitats. Intraspecific variability in FA profiles implies differences in the diet of sharks aggregating at Ningaloo Reef that ultimately may reflect the patchy and unpredictable nature of foraging in oligotrophic tropical waters. Future studies need to target the sampling of tissues with higher rates of turnover so that the relative inputs of past and recent feeding events on FA profiles can be assessed. Combining signature FA analysis with other techniques such as stable isotope analysis, genetic data and long-term tagging data will also help better elucidate the feeding ecology and movements of this iconic species.

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