Unusually High Levels of n-6 Polyunsaturated Fatty Acids in Whale Sharks and Reef Manta Rays

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Received: 14 March 2013 / Accepted: 2 August 2013 / Published online: 22 August 2013 © The Author(s) 2013. This article is published with open access at Springerlink.com

Abstract  Fatty acid (FA) signature analysis has been increasingly used to assess dietary preferences and trophodynamics in marine animals. We investigated FA signatures of connective tissue of the whale shark *Rhincodon typus* and muscle tissue of the reef manta ray *Manta alfredi*. We found high levels of n-6 polyunsaturated fatty acids (PUFA), dominated by arachidonic acid (20:4n-6; 12–17 % of total FA), and comparatively lower levels of the essential n-3 PUFA—eicosapentaenoic acid (20:5n-3; ~1 %) and docosahexaenoic acid (22:6n-3; 3–10 %). Whale sharks and reef manta rays are regularly observed feeding on surface aggregations of coastal crustacean zooplankton during the day, which generally have FA profiles dominated by n-3 PUFA. The high levels of n-6 PUFA in both giant elasmobranchs raise new questions about the origin of their main food source.

Keywords  n-3 Fatty acids · Arachidonic acid · Planktivores · Zooplankton · Elasmobranch

Abbreviations
ARA  Arachidonic acid
DHA  Docosahexaenoic acid
EPA  Eicosapentaenoic acid
FA  Fatty acid(s)
GC  Gas chromatography
LA  Linoleic acid
LC-PUFA  Long chain- polyunsaturated fatty acid(s)
MUFA  Monounsaturated fatty acid(s)
PUFA  Polyunsaturated fatty acid(s)
SEM  Standard error of the mean
SFA  Saturated fatty acid(s)

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Introduction

The whale shark *Rhincodon typus* and the reef manta ray *Manta alfredi* are giant planktivorous elasmobranchs that are presumed to feed predominantly on aggregations of zooplankton in highly productive areas [1, 2]. Direct studies on the diet of these elasmobranchs are limited to examination of a few stomach contents, faecal material and stable isotope analyses [3–6], while recent field observations suggest that their diets are mostly composed of crustacean zooplankton [1, 7]. It is unknown, however, whether near-surface zooplankton are a major or only a minor part of their diets, whether these large elasmobranchs target other prey, or whether they feed in areas other than surface waters along productive coastlines.

Here we used signature fatty acid (FA) analysis to assess dietary preferences of *R. typus* and *M. alfredi*. The essential long-chain (≥C₂₀) polyunsaturated fatty acids (LC-PUFA) of fishes are most likely derived directly from the diet, as higher consumers generally lack the ability to biosynthesise these FA de novo [8, 9]. The fatty acid profile of zooplankton is usually dominated by PUFA with a high n-3/n-6 ratio, while generally contains high levels of eicosapentaenoic acid (EPA, 20:5n-3) and/or docosahexaenoic acid (DHA, 22:6n-3) [8, 10, 11]. Considering this, it was expected that FA profiles of *R. typus* and *M. alfredi* tissues would be similarly n-3 PUFA dominated.

Materials and Methods

Tissue samples were collected from live, unrestrained specimens in southern Mozambique (14 *R. typus* and 12 *M. alfredi*) and eastern Australia (9 *M. alfredi*) using a modified Hawaiian hand-sling with a fitted biopsy needle tip between June–August 2011. Biopsies of *R. typus* were extracted laterally between the 1st and 2nd dorsal fin and penetrated ~20 mm deep from the skin into the underlying connective tissue. Biopsies of *M. alfredi* were of similar size, but were mainly muscle tissue, extracted from the ventro-posterior area of the pectoral fins away from the body cavity. Biopsies were immediately put on ice in the field and then stored at −20 °C for up to 3 months before analysis.

Lipids were extracted overnight using the modified Bligh and Dyer [12] method with a one-phase methanol:chloroform:water (2:1:0.8 by volume) mixture. Phases were separated by adding water and chloroform, followed by rotary evaporation of the chloroform in vacuo at ~40 °C. Total lipid extracts were concentrated by application of a stream of inert nitrogen gas and samples were stored in chloroform at −20 °C before FA analysis.

The total lipid extract from each sample was spotted on chromarods that were developed for 25 min in a polar solvent system (hexane:diethyl-ether:acetic acid, 60:17:0.1 by volume). The chromarods were then dried in an oven for 10 min at 100 °C and analysed immediately. Lipid class composition was determined for each sample using an Iatroscan Mark V TH10 thin layer chromatograph combined with a flame ionisation detector. A standard solution containing wax esters, triacylglycerol, free FA, sterols and phospholipids (Nu-Chek Prep. Inc., MN, USA) was run with the samples. Each peak was identified by comparison of RI with the standard chromatogram. Peak areas were measured using SIC-480II Iatroscan™ Integrating Software v.7.0-E (System Instruments Co., Mitsubishi Chemical Medicine Corp., Japan) and quantified to mass per µL spotted using predetermined linear regressions.

An aliquot of the total extracted lipids was treated with methanol:hydrochloric acid:chloroform (10:1:1), heated at ~80 °C for 2 h and the resulting fatty acid methyl esters were extracted into hexane:chloroform (4:1). Samples were analysed using an Agilent Technologies 7890 B gas chromatography (GC) (Palo Alto, California, USA) equipped with a non-polar Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness), a flame ionisation detector, a split/split-less injector and an Agilent Technologies ChemStation software (Palo Alto, California, USA). Sterols were also separated under the GC conditions used, and largely comprised cholesterol. GC results typically have an error of up to ±5 % of individual component peak identities were confirmed with a Finnigan ThermoQuest GCQ GC mass-spectrometer (GC-MS) system (Finnigan, San Jose,CA) [13]. Percentage FA data were calculated from the areas of chromatogram peaks. All FA are expressed as mole percentage of total FA.

Results and Discussion

Fatty acids of both *M. alfredi* muscle tissue and *R. typus* connective tissue were predominantly derived from phospholipids (Table 1). The classes of phospholipids were not distinguished in this study, but should be examined in future studies where phospholipids are found to be the dominant lipid class of these two giant elasmobranchs. The FA profile of *M. alfredi* was dominated by PUFA (34.9 % of total FA), while saturated FA were most abundant in *R. typus* (39.1 % of total FA) (Table 2). The main FA in both species included 18:0, 18:1n-9, 16:0 and 20:4n-6.
Table 1 Means ± SE (standard error) lipid class compositions of whale shark (n = 14) and reef manta ray (n = 15) tissue samples, expressed as % of total lipid

| Lipid class | Whale shark (n = 14) % Total lipid ± SE | Reef manta ray (n = 15) % Total lipid ± SE |
|-------------|----------------------------------------|------------------------------------------|
| WE          | 2.8 ± 1.3                              | 0.6 ± 0.4                                |
| TAG         | 3.3 ± 1.4                              | 3.4 ± 0.7                                |
| FFA         | 5.3 ± 1.0                              | 2.1 ± 0.3                                |
| ST          | 20.5 ± 0.8                             | 10.8 ± 1.1                               |
| PL          | 68.1 ± 3.5                             | 83.0 ± 1.5                               |

Total lipid content (mg g⁻¹) 1.8 ± 1.1 3.8 ± 0.3

Total lipid content is expressed as mg g⁻¹ of tissue wet mass

WE wax esters, TAG triacylglycerols, FFA free fatty acids, ST sterols (comprising mostly cholesterol), PL phospholipids

Arachidonic acid (AA; 20:4n-6) was the most abundant FA in R. typus (16.9 %) whereas 18:0 was most abundant in M. alfredi (16.8 %). Both species had a relatively low level of EPA (1.1 and 1.2 %) and M. alfredi had a relatively high level of DHA (10.0 %) compared to R. typus (2.5 %). Fatty acid signatures of R. typus and M. alfredi were different to expected profiles of species that feed predominantly on crustacean zooplankton, which are typically dominated by n-3 PUFA and have high levels of EPA and/or DHA [8, 10, 11]. Instead, profiles of both large elasmobranchs were dominated by n-6 PUFA (>20 % total FA), with an n-3/n-6 ratio <1 and markedly high levels of AA (Table 2). The FA profiles of M. alfredi were broadly similar between the two locations, although some differences were observed that are likely due to dietary differences. Future research should aim to look more closely at these differences and potential dietary contributions.

The n-6-dominated FA profiles are rare among marine fishes. Most other large pelagic animals and other marine planktivores have an n-3-dominated FA profile and no other chondrichthyes investigated to date has an n-3/n-6 ratio <1 [14–16] (Table 3, literature data are expressed as wt%). The only other pelagic planktivore with a similar n-3/n-6 ratio (i.e. 0.9) is the leatherback turtle, that feeds on gelatinous zooplankton [17]. Only a few other marine species, such as several species of dolphins [18], benthic echinoderms and the bottom-dwelling rabbitfish Siganus nebulosus [19], have relatively high levels of AA, similar to those found in whale sharks and reef manta rays (Table 3).

The trophic pathway for n-6-dominated FA profiles in the marine environment is not fully understood. Although most animal species can, to some extent, convert linoleic acid (LA, 18:2n-6) to AA [8], only traces of LA (<1 %) were present in the two filter-feeders here. Only marine plant species are capable of biosynthesising long-chain n-3 and n-6 PUFA de novo, as most animals do not possess the enzymes necessary to produce these LC-PUFA [8, 9]. These findings suggest that the origin of AA in R. typus and M. alfredi is most likely directly related to their diet.

Although FA are selectively incorporated into different elasmobranch tissues, little is known on which tissue would best reflect the diet FA profile. McMeans et al. [14] recently showed that FA profile of muscle in the Greenland shark is the most representative of its prey FA profiles. It is thus assumed here that the muscle tissue of M. alfredi is representative of its diet, but the extent to which the FA profile of the subdermal connective tissue of R. typus reflects its diet is unknown.

Certain species of phytoplankton including diatoms, and some macro algae such as Rhodophyta can biosynthesise n-6 PUFA, with levels of over 40 % (as wt%) of AA recorded [20, 21]. Although phytoplankton and macro algae have been reported in R. typus stomach contents, they

Table 2 FA composition (mol% of total FA) of the whale shark R. typus (n = 14) and the reef manta ray M. alfredi (n = 21) [minor fatty acids (≤1 %) are not shown]

| FA          | R. typus Mean (±SEM) | M. alfredi Mean (±SEM) |
|-------------|----------------------|------------------------|
| ∑SFA        | 39.1 (0.7)           | 35.1 (0.7)             |
| 16:0        | 13.8 (0.5)           | 14.7 (0.4)             |
| 17:0        | 1.6 (0.1)            | 0                      |
| 18:0        | 11.1 (0.1)           | 0.3 (0.1)              |
| 18:1        | 17.8 (0.5)           | 16.8 (0.4)             |
| ∑MUFA       | 31.0 (0.9)           | 29.9 (0.7)             |
| 16:1n-7c    | 2.1 (0.3)            | 2.7 (0.3)              |
| 17:1n-8c    | 1.8 (0.3)            | 0.7 (0.1)              |
| 18:1n-9c    | 16.7 (0.7)           | 15.7 (0.4)             |
| 18:1n-9c    | 4.6 (0.5)            | 6.1 (0.2)              |
| 20:1n-9c    | 0.7 (0.02)           | 1.0 (0.03)             |
| 24:1n-9c    | 1.9 (0.1)            | 1.1 (0.1)              |
| ∑n-3        | 6.1 (0.3)            | 13.4 (0.6)             |
| 20:5n-3 (EPA)| 1.1 (0.1)           | 1.2 (0.1)              |
| 22:6n-3 (DHA)| 2.5 (0.2)           | 10.0 (0.5)             |
| 22:5n-3    | 2.1 (0.1)            | 2.0 (0.1)              |
| ∑n-6        | 23.8 (0.8)           | 21.0 (1.4)             |
| 20:4n-6 (AA)| 16.9 (0.6)           | 11.7 (0.8)             |
| 22:5n-6    | 0.9 (0.1)            | 3.3 (0.3)              |
| 22:4n-6    | 5.5 (0.3)            | 5.1 (0.5)              |
| n-3n-6      | 0.3 (0.02)           | 0.7 (0.1)              |

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, AA arachidonic acid

* Includes a17:0 coeluting
are assumed to be incidentally ingested [22]. The feeding apparatus and feeding strategy of *R. typus* and *M. alfredi* are adapted for targeting larger prey [23, 24]. There is no observational evidence of either species targeting phytoplankton, but there are frequent observations of feeding on zooplankton patches. More plausibly, n-6 LC-PUFA from phytoplankton could enter the food chain when consumed by zooplankton and subsequently be transferred to higher-level consumers. It is unclear what type of zooplankton is likely to feed on AA-rich algae. To date, only a few jellyfish species are known to contain high levels of AA (2.8–9.9 % of total FA as wt%), but they also have high levels of EPA, which are low in *R. typus* and *M. alfredi* [17, 25, 26].

### Table 3 Polyunsaturated fatty acid composition of chondrichthyan, planktivore, large pelagic and detrivore species

| Species                     | Feeding habitat          | Tissue | $\sum n$-3 | $\sum n$-6 | AA   | EPA | DHA | n-3/n-6 | Reference |
|-----------------------------|--------------------------|--------|------------|------------|------|-----|-----|---------|-----------|
| Whale shark—*R. typus* (mol%) | Epipelagic—planktivore   | Skin   | 6.1        | 23.8       | 16.9 | 1.1 | 2.5 | 0.3     | This study |
| Whale shark—*R. typus* (wt%) | Epipelagic—planktivore   | Skin   | 6.7        | 25.4       | 17.8 | 1.2 | 2.8 | 0.3     | This study |
| Reef manta ray—*M. alfredi* (mol%) | Epipelagic—planktivore | Muscle | 13.4       | 21.0       | 11.7 | 1.2 | 10.0 | 0.7     | This study |
| Reef manta ray—*M. alfredi* (wt%) | Epipelagic—planktivore | Muscle | 14.9       | 21.6       | 11.8 | 1.2 | 11.3 | 0.7     | This study |

Other chondrichthyans

| Species                       | Feeding habitat          | Tissue | $\sum n$-3 | $\sum n$-6 | AA   | EPA | DHA | n-3/n-6 | Reference |
|-------------------------------|--------------------------|--------|------------|------------|------|-----|-----|---------|-----------|
| Port Jackson shark—*Heterodontus portusjacksoni* | Demersal—carnivore | Muscle | 23.6       | 19.4       | 13.8 | 3.7 | 15.4 | 1.2     | [45]       |
| Sandy-backed stingaree—*Urolophus bucculentus* | Demersal—carnivore | Muscle | 32.9       | 16.5       | 12.6 | 3.1 | 27.9 | 2.0     | [45]       |
| Southern chimaera—*Chimaera fulva* | Deep sea—carnivore | Muscle | 30.4       | 11.2       | 4.7  | 3.4 | 23.3 | 2.7     | [46]       |
| Angel shark—*Squatina australis* | Demersal—carnivore | Muscle | 45.2       | 10.5       | 7.6  | 6.1 | 36.5 | 4.3     | [45]       |
| Longnose velvet dogfish—*Centroscelachus crepidater* | Deep sea—carnivore | Muscle | 39.1       | 6.6        | 4.4  | 2.3 | 32.2 | 5.9     | [46]       |
| Shortnose spurdog—*Squalus megalops* | Deep sea—carnivore | Muscle | 37.5       | 6.4        | 3.6  | 1.2 | 32.3 | 5.9     | [46]       |
| South China catshark—*Apristurus sinensis* | Deep sea—carnivore | Muscle | 38.5       | 6.4        | 3.4  | 2.9 | 28.9 | 6       | [46]       |
| Broadnose sevengill shark—*Notorynchus cepedianus* | Deep sea—carnivore | Liver  | 23.2       | 3.2        | 1.7  | 3.4 | 16.6 | 7.2     | [46]       |

Planktivores

| Species                       | Feeding habitat          | Tissue | $\sum n$-3 | $\sum n$-6 | AA   | EPA | DHA | n-3/n-6 | Reference |
|-------------------------------|--------------------------|--------|------------|------------|------|-----|-----|---------|-----------|
| Leatherback turtle—*Dermochelys coriacea* | Epipelagic—planktivore | Muscle | 15.5       | 17.3       | 15.5 | 6.1 | 5.7  | 0.9     | [17]       |
| Jellyfish—*Aurelia sp.* | Epipelagic—planktivore | Whole  | 34.5       | 12.2       | 9.9  | 14.1| 9.8  | 2.8     | [25]       |
| Finwhale—*Balaenoptera physalus* | Pelagic—planktivore | Blubber oil | 6.7        | 2.3        | 0.3  | 1.8 | 2.74 | 2.9     | [47]       |
| Anchovies—*Engraulis mordax mordax* | Pelagic—planktivore | Whole  | 22.9       | 4.9        | 0.4  | 13.5| 8.8  | 27.8    | [48]       |

Large pelagics

| Species                       | Feeding habitat          | Tissue | $\sum n$-3 | $\sum n$-6 | AA   | EPA | DHA | n-3/n-6 | Reference |
|-------------------------------|--------------------------|--------|------------|------------|------|-----|-----|---------|-----------|
| Dolphin—mixed species | Epipelagic—carnivore | Muscle | 16.3       | 18.6       | 14.2 | 6.4 | 7.6  | 0.9     | [18]       |
| Gray whale—*E. robustus* | Pelagic—planktivore | Muscle | 4.7        | 7.5        | 1.2  | ~1.8|       |         | [49]       |
| Ocean sunfish—*Mola mola*  | Pelagic—carnivore | Muscle | 29.4       | 10.8       | 7.73 | 8.8 | 17.0 | 2.7     | [50]       |

Benthic feeders

| Species                       | Feeding habitat          | Tissue | $\sum n$-3 | $\sum n$-6 | AA   | EPA | DHA | n-3/n-6 | Reference |
|-------------------------------|--------------------------|--------|------------|------------|------|-----|-----|---------|-----------|
| Sea cucumber—*Holothuria scabra* | Benthic—deposit feeder | Whole  | 10.7       | 22.6       | 19.1 | 8.2 | 1.5  | 0.5     | [19]       |
| Sea urchin—*Heliocidaris erythrogramma* | Benthic—deposit feeder | Whole  | 10.7       | 14.6       | 6.1  | 8.3 | 0.4  | 0.7     | [19]       |
| Dusky rabbitfish—*Siganus nebulosus* | Benthic—deposit feeder | Muscle | 18.5       | 20.5       | 12.4 | 1.3 | 14.6 | 0.9     | [19]       |

Data from this study for *Rhincodon typus* and *Manta alfredi* are expressed in both mol% and wt% format, with all literature data as wt% EPA eicosapentaeonoic acid, DHA docosahexaenoic acid, AA arachidonic acid.
Some protozoans and microeukaryotes, including heterotrophic thraustochytrids in marine sediments are rich in AA [27–30] and could be linked with high n-6 LC-PUFA and AA levels in benthic feeders (n-3/n-6 = 0.5–0.9; AA = 6.1–19.1 % as wt%; Table 3), such as echinoderms, stingrays and other benthic fishes. However, the pathway of utilisation of AA from these micro-organisms remains unresolved. \textit{R. typus} and \textit{M. alfredi} may feed close to the sea floor and could ingest sediment with associated protozoan and microeukaryotes suspended in the water column; however, they are unlikely to target such small sediment-associated benthos. The link to \textit{R. typus} and \textit{M. alfredi} could be through benthic zooplankton, which potentially feed within the sediment on these AA-rich organisms and then emerge in high numbers out of the sediment during their diel vertical migration [31, 32]. It is unknown to what extent \textit{R. typus} and \textit{M. alfredi} feed at night when zooplankton in shallow coastal habitats emerges from the sediment.

The subtropical/tropical distribution of \textit{R. typus} and \textit{M. alfredi} is likely to partly contribute to their n-6-rich PUFA profiles. Although still strongly n-3-dominated, the n-3/n-6 ratio in fish tissue noticeably decreases from high to low latitudes, largely due to an increase in n-6 PUFA, particularly AA (Table 3) [33–35]. This latitudinal effect alone does not, however, explain the unusual FA signatures of \textit{R. typus} and \textit{M. alfredi}.

We found that \textit{M. alfredi} contained more DHA than EPA, while \textit{R. typus} had low levels of both these n-3 LC-PUFA, and there was less of either n-3 LC-PUFA than AA in both species. As DHA is considered a photosynthetic biomarker of a flagellate-based food chain [8, 10], high levels of DHA in \textit{M. alfredi} could be attributed to crustacean zooplankton in the diet, as some zooplankton species feed largely on flagellates [36]. By contrast, \textit{R. typus} had low levels of EPA and DHA, and the FA profile showed AA as the major component.

Our results suggest that the main food source of \textit{R. typus} and \textit{M. alfredi} is dominated by n-6 LC-PUFA that may have several origins. Large, pelagic filter-feeders in tropical and subtropical seas, where plankton is scarce and patchily distributed [37], are likely to have a variable diet. At least for the better-studied \textit{R. typus}, observational evidence supports this hypothesis [38–43]. While their prey varies among different aggregation sites [44], the FA profiles shown here suggest that their feeding ecology is more complex than simply targeting a variety of prey when feeding at the surface in coastal waters. Trophic interactions and food web pathways for these large filter-feeders and their potential prey remain intriguingly unresolved. Further studies are needed to clarify the disparity between observed coastal feeding events and the unusual FA signatures reported here, and to identify and compare FA signatures of a range of potential prey, including demersal and deep-water zooplankton.

**Acknowledgments** We thank P. Mansour for his assistance with laboratory techniques and equipment, D. Holdsworth for management of the CSIRO GC-MS facility and C. F. (Rick) Phleger for early comments on this study. We thank E. Murphy, the Associate Editor and two anonymous reviewers for providing constructive comments that improved the quality of the manuscript. This study was supported by the ARC Linkage Grant LP110100712, Earthwatch Institute Australia and Sibelco Pty Ltd. Field work was supported by Casa Barry Lodge, Peri-Peri Divers, Lady Elliot Island Eco Resort and Manta Lodge and Scuba Centre and was conducted under Great Barrier Reef Marine Park permit (G09/29853.1) and Ethics approval (SBMS/071/08/SEAWORLD).

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