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Fatty acids and stable isotopes as indicators of early-life feeding and potential maternal resource dependency in the bull shark *Carcharhinus leucas*

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ABSTRACT: The degree of reliance of newborn sharks on energy reserves from maternal resource allocation and the timescales over which these animals develop foraging skills are critical factors towards understanding the ecological role of top predators in marine ecosystems. We used muscle tissue stable carbon isotopic composition and fatty acid analysis of bull sharks *Carcharhinus leucas* to investigate early-life feeding ecology in conjunction with maternal resource dependency. Values of $\delta^{13}C$ of some young-of-the-year sharks were highly enriched, reflecting inputs from the marine-based diet and foraging locations of their mothers. This group of sharks also contained high levels of the 20:3ω9 fatty acid, which accumulates during periods of essential fatty acid deficiency, suggesting inadequate or undeveloped foraging skills and possible reliance on maternal provisioning. A loss of maternal signal in $\delta^{13}C$ values occurred at a length of approximately 100 cm, with muscle tissue $\delta^{13}C$ values reflecting a transition from more freshwater/estuarine-based diets to marine-based diets with increasing length. Similarly, fatty acids from sharks >100 cm indicated no signs of essential fatty acid deficiency, implying adequate foraging. By combining stable carbon isotopes and fatty acids, our results provided important constraints on the timing of the loss of maternal isotopic signal and the development of foraging skills in relation to shark size and imply that molecular markers such as fatty acids are useful for the determination of maternal resource dependency.

KEY WORDS: Essential fatty acid deficiency · Fatty acids · Food webs · Maternal investment · Stable isotopes · Sharks

INTRODUCTION

The potential importance of sharks in marine ecosystems coupled with declines in populations of many species around the world (see Ferretti et al. 2010 for a review) has led to great interest in their ecological roles and factors that might impact population sizes. Uncertainties remain in our understanding of shark community dynamics, especially with respect to the trophic dynamics of these apex predators. In particular, few studies have addressed the early-life feeding ecology of sharks and the timescales over which newborn sharks are dependent on maternal provisioning. Recently it was suggested that newborn sharks may rely on energy reserves from maternal allocation, in the form of an enlarged liver, during the first weeks to months of life (Hussey et al. 2010). This maternal head-start is advantageous while newborn sharks develop foraging skills, and may be particularly crucial in densely-populated nursery areas, where prey availability may also impact the condition of juvenile sharks (Duncan & Holland 2006, Hussey et al. 2010).

Attempts to clarify feeding ecology relationships have used methods ranging from more traditional approaches, such as stomach content analysis (New-

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man et al. 2010, Torres-Rojas et al. 2010), to molecular tools including stable isotope and lipid biomarker analysis (MacNeil et al. 2005, Pethybridge et al. 2010, Wai et al. 2011). Although stomach content analysis can provide evidence for specific dietary items, biochemical approaches have the advantage of providing information on assimilated organic substrates and can be especially helpful in complex ecosystems with multiple organic carbon substrates (Dalsgaard et al. 2003, Iverson et al. 2004, Whiles et al. 2010). Bulk $\delta^{13}C$ has been widely used for food web studies, providing information on the source of organic matter in food webs (Fry et al. 1978, Fry & Sherr 1984, McCutchan et al. 2003). Furthermore, stable isotopic composition of neonatal animals has been demonstrated to reflect maternal diet and foraging location instead of neonatal diet, and this complication must be considered when interpreting isotope values in neonates and juveniles, especially for tissues with slow isotopic turnover times (e.g. muscle tissue in elasmobranchs; MacNeil et al. 2005, Olin et al. 2011). Fatty acid signatures are particularly useful for dietary studies because fatty acids from a prey item are taken up into consumer adipose and muscle tissue with relatively minor or predictable modifications (Iverson et al. 2004). Thus, a predator's fatty acid signature closely matches the fatty acid signature of its prey.

Fatty acid composition can also provide information on the nutritional status of an organism (Holman 1960, Barbarich et al. 2006, Briend et al. 2011). For example, certain fatty acids, in particular 18:3ω3 (α-linolenic acid) and 18:2ω6 (linoleic acid), are essential for growth, development, and cellular function and furthermore cannot be synthesized by mammals and most other animals, but instead must be obtained through the diet (Le et al. 2009). If these fatty acids are not obtained through the diet, ω9 unsaturated fatty acids, such as 20:3ω9, are synthesized by the animal instead to take the place of polyunsaturated fatty acids derived from the essential fatty acids in biochemical reactions. Thus, the presence of 20:3ω9 in plasma and tissues has long been used as a nutritional status marker not only in humans (Holman 1960, Siguel et al. 1987, Jeppesen et al. 1998, Le et al. 2009) but also in animals such as carp *Cyprinus carpio* (Farkas et al. 1977, Csengeri 1996), mice *Mus musculus* (Duffin et al. 2000), and the green sea urchin *Strongylocentrotus droebachiensis* (Gonzalez-Duran et al. 2008).

Here, we investigated maternal resource dependency and early-life feeding ecology of bull sharks *Carcharhinus leucas* in the Florida Coastal Everglades, USA. Specifically, we used stable carbon isotopes together with fatty acid composition in muscle tissue to understand variation in the diets of individ-

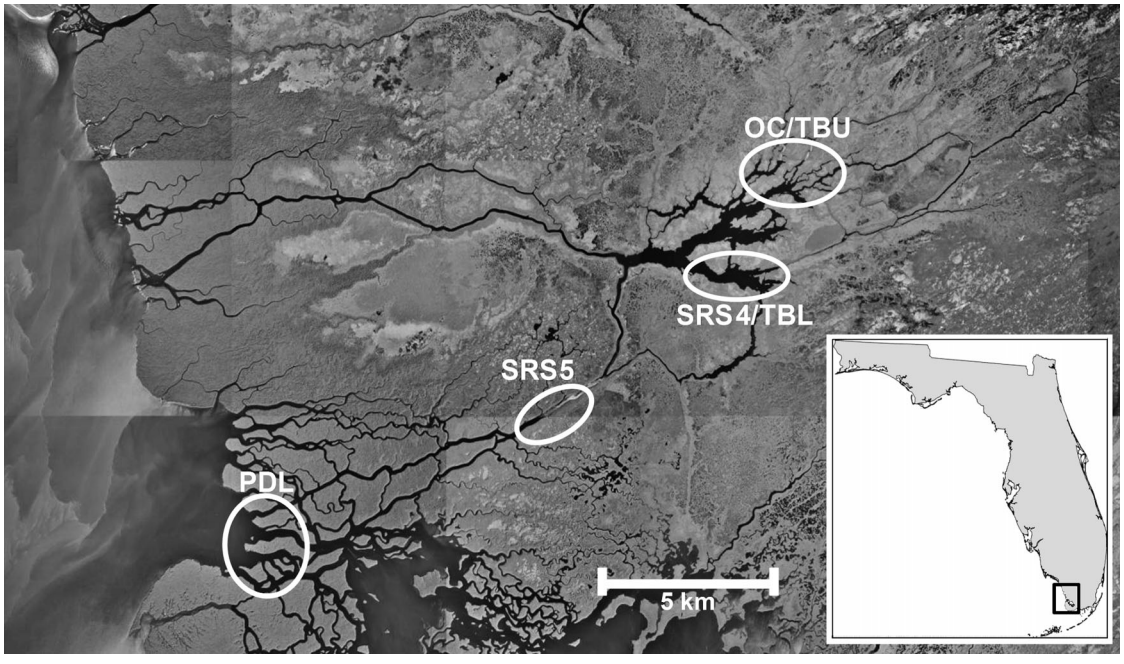

Fig. 1. Sampling locations along a salinity gradient in the Shark River Estuary of the Florida Everglades, USA. OC/TBU: Otter Creek/Tarpon Bay (upper); SRS4/TBL: Shark River Slough Site 4/Tarpon Bay (lower); SRS5: Shark River Slough Site 5; PDL: Ponce de Leon Bay. For geographical reference, the position of SRS5 is 25.377°N, 81.032°W

ual bull sharks and the possibility that young-of-the-year (YOY) sharks might rely on maternal provisions. Abundances of 20:3ω9 and the ratio of 20:3ω9 to 20:4ω6 were used as indicators of essential fatty acid deficiency, which infers non-feeding or poorly-developed foraging skills, with potential reliance on maternal resources.

MATERIALS AND METHODS

In total, 28 bull sharks were captured in the Shark River Estuary of Everglades National Park, Florida, USA, with longlines as described by Heithaus et al. (2009) and Matich et al. (2011). Bull sharks in this sample collection ranged from 66 to 200 cm total length (TL) and were evenly split between males and females. Sharks were captured along a salinity gradient reaching from Otter Creek (OC) near the upper portion of Tarpon Bay (TBU) to Ponce de Leon Bay (PDL) in the Gulf of Mexico (Fig. 1). Sampling at mid- and downstream sites (SRS4 and SRS5; Fig. 1) occurred near long-term environmental sampling platforms maintained by the Florida Coastal Everglades Long-Term Ecological Research program (http://fce.lternet.edu). All sharks <100 cm TL analyzed for fatty acids were captured in TB, either in the upper portion near OC or in the lower portion of the bay near SRS4 (Table 1, Fig. 1). All sharks >150 cm TL were captured from downstream portions of the estuary or coastal waters of PDL (Table 1, Fig. 1). An 8 mm sterile biopsy punch (Acuderm) was used to collect a 0.5 cm^3 sample of muscle tissue, which was subsequently placed on ice in the field and frozen upon return to the laboratory. Skin was removed from muscle samples, and a portion of the remaining tissue was dried and pulverized for stable isotope analysis.

Oven-dried, homogenized tissue was analyzed for stable carbon isotopic composition with a Carlo Erba

Table 1. *Carcharhinus leucas.* Characteristics and capture information for the bull sharks in this study. OC/TBU: Otter Creek/Tarpon Bay Upper; SRS4/TBL: Shark River Slough Site 4/Tarpon Bay Lower; PDL: Ponce de Leon Bay; SRS5: Shark River Slough Site 5; YOY: young of the year; TL: total length; NT: not tagged

Tag no.	Capture date (mm/dd/yy)	Capture location	Sex	Maturity	TL (cm)	$\delta^{13}C$ (‰)
J10043	06/24/09	OC/TBU	M	YOY	69	−13.255
J10633	06/23/10	SRS4/TBL	F	YOY	78	−14.080
J10058	07/25/09	SRS4/TBL	F	YOY	66	−15.016
J10053	07/25/09	SRS4/TBL	M	YOY	69	−23.776
J10091	09/17/09	SRS4/TBL	F	YOY	71	−16.820
D1[a]	07/25/09	SRS4/TBL	F	YOY	72	−16.700
D2[a]	07/30/09	SRS4/TBL	F	YOY	72	−17.420
J10063	07/30/09	OC/TBU	F	YOY	73	−14.786
D3[a]	08/13/10	SRS4/TBL	M	YOY	74	−14.950
J10020	06/12/09	OC/TBU	M	YOY	75	−19.308
J10006	08/04/09	SRS4/TBL	F	YOY	76	−19.510
NT	09/17/09	SRS4/TBL	F	YOY	77	−14.478
J10014	06/12/09	SRS4/TBL	M	YOY	79	−20.120
NT[a]	07/30/09	OC/TBU	F	YOY	79	−15.395
J10046	06/24/09	SRS4/TBL	M	YOY	81	−22.308
J10673	10/28/10	SRS4/TBL	M	Immature	81	−23.310
J10076	08/04/09	OC/TBU	M	Immature	106	−25.884
J10054	08/01/09	SRS5	M	Immature	108	−24.738
J10031	05/30/09	OC/TBU	M	Immature	132	−20.868
J10056	11/14/09	SRS5	M	Immature	132	−21.989
J10089	11/19/09	PDL	M	Immature	175	−19.672
J10657	02/05/11	SRS5	F	Immature	116	−15.880
J10652	11/19/10	PDL	M	Immature	161	−16.950
J10041	06/17/09	PDL	F	Immature	182	−17.764
J10659	11/19/10	PDL	F	Immature	185	−17.740
J10635	10/27/10	PDL	M	Immature	189	−17.510
J10072	07/14/09	PDL	F	Immature	200	−17.707
J10061	06/17/09	PDL	F	Immature	212	−15.558

[a]Deceased

NA 1500 Elemental Analyzer coupled to a Finnigan MAT Delta isotope ratio mass spectrometer at the Stable Isotope Laboratory of Florida International University. All isotopic values are presented using the standard δ notation with Pee Dee Belemnite as a standard for $\delta^{13}C$. The average isotopic lab error for replicate glycine internal standards treated identically as the samples was 0.29‰ for $\delta^{13}C$. Lipid extraction of the tissue was not performed prior to isotopic analysis because C:N ratios were generally below those suggested as necessary for extraction or mathematical correction (mean = 3.09 ± 0.06 SE, Post et al. 2007).

Lipids were extracted from dried, homogenized muscle tissue following a modified method of Folch et al. (1957). Briefly, homogenized samples were ultrasonically extracted with a 2:1 mixture (v/v) of methylene chloride:methanol (CH_2Cl_2:MeOH). A saline solution (0.9% NaCl) 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. Fresh organic solvent was added to the remaining tissue and the extraction was repeated 2 more times. The 3 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.

Total lipid extracts were then saponified with 0.5 N methanolic KOH at 70°C for 30 to 60 min (Ju et al. 2009). Neutral lipids were partitioned 3 times with a mixture of hexane:diethyl ether (9:1) after addition of water and archived for subsequent analysis. Samples were then acidified to pH <2 with HCl, and free fatty acids were partitioned into 9:1 hexane:diethyl ether 3 times and combined. Fatty acids were methylated to corresponding methyl esters (FAMEs) with freshly distilled diazomethane.

Fatty acids were identified and relative abundances were determined using gas-chromatography-mass spectrometry (GC-MS) with an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer operating in electron ionization (EI) mode at 70 eV. Although many studies of quantitative analyses of fatty acids have been carried out by GC-flame ionization detection (FID), Dodds et al. (2005) have shown that the GC-MS technique used here compares satisfactorily to GC-FID. We used relative abundances of fatty acids, instead of absolute concentrations of fatty acids, to compare sharks of different size and mass. GC column and oven parameters were identical to those in Jaffé et al. (2001). Identification of fatty acids was performed by comparison of chromatographic retention times and mass spectral characteristics with authentic standards (Supelco PUFA No. 3 from Menhaden oil, Supelco FAME Mix, C_4–C_{24} Unsaturates, and Matreya Mead Acid Standard [Methyl 5,8,11-eicosatrienoic acid]) and previously reported mass spectra. Selected samples were analyzed with a Restek FAMEWAX column (Crossbond® polyethylene glycol stationary phase, 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness), with an oven program of 6°C min^{-1} from 100 to 230°C, followed by a 10 min hold, to confirm structural assignment because coelutions of polyunsaturated fatty acids were minimal with the FAMEWAX column. Following quantification of the FAMEs, double bond positions in predominant unsaturated fatty acids were further confirmed through analysis of picolinyl esters following the method of Dubois et al. (2006).

Overall, 64 distinct fatty acids were quantified in the shark sample set. For brevity, only a subset of the 10 most abundant fatty acids in each sample, resulting in a total of 21 fatty acids, are presented and discussed here. Unsaturated fatty acids are named here as A:BωC, where A is carbon chain length, B is number of double bonds, and C is position of first double bond counted from terminal methyl end. The full dataset is available on the Florida Coastal Everglades Long-Term Ecological Research program website (http://fce.lternet.edu). The subset of 21 fatty acids accounted for 91 to 97% of the total fatty acids in all samples, and this subset was used in average-linkage hierarchical cluster analysis based on Bray-Curtis similarity coefficients on untransformed data (Howell et al. 2003) using the statistical package R as an exploratory data analysis technique to determine relationships between individuals based on the fatty acid composition data. The Kruskal-Wallis non-parametric analysis of variance (ANOVA) was used to compare mean relative abundances of fatty acids between groups of sharks determined by the cluster analysis at the 95% significance level. Post hoc comparisons following the Kruskal-Wallis test were performed using kruskalmc in the package pgirmess in R to evaluate statistical differences in mean fatty acid concentration between shark groups.

RESULTS

Muscle tissue $\delta^{13}C$ values were highly variable, ranging from −13.3 to −25.9‰ (Fig. 2). This broad range in values closely follows the isotopic compositions of the 2 distinct basal resource pools located in the system: a freshwater/estuarine component with a

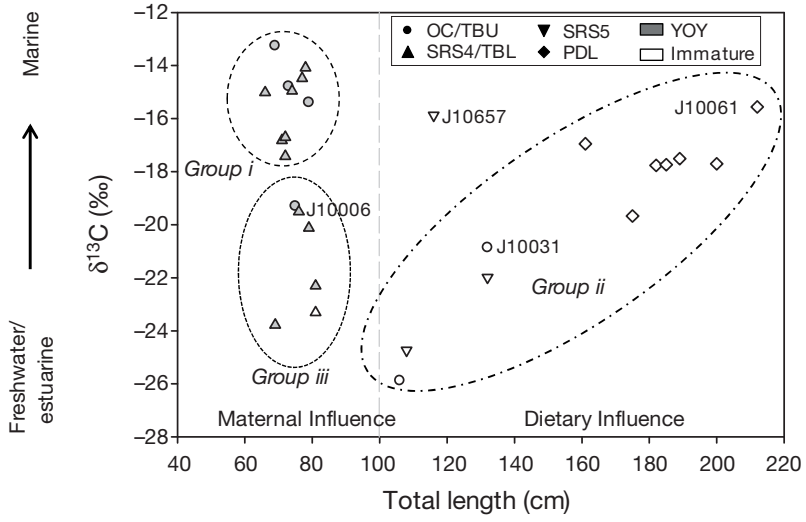

Fig. 2. *Carcharhinus leucas.* Stable carbon isotopic composition of shark muscle tissue as a function of total shark length. Groups of sharks defined by hierarchical cluster analysis as shown in Fig. 3. Note that shark J10006 clustered with group *ii* in Fig. 3, and an ungrouped sample, shark J10657, clustered with group *iii* in Fig. 3. The outliers in the cluster analysis, J10061 and J10031, are also labeled. Site abbreviations as in Fig. 1; YOY: young of the year

mean ± SE $\delta^{13}C$ of −29.7‰ ± 0.7 and a marine component, based largely on seagrass, with an average $\delta^{13}C$ of −14.5‰ ± 0.3 (Fry & Smith 2002, Chasar et al. 2005, Williams & Trexler 2006, Matich et al. 2011). Stable carbon isotopic composition was independent of location of capture, with some sharks from both the upper estuary (OC/TBU) and marine end-member (PDL) displaying highly enriched $\delta^{13}C$ (Fig. 2). Sharks with total lengths <100 cm displayed strong variation in $\delta^{13}C$ (Fig. 2).

We identified 64 fatty acids in the shark muscle tissue samples; however, a subset of 21 structures accounted for >90% of the total fatty acids in all samples (Table 2). A hierarchical cluster analysis based on these 21 fatty acids segregated the shark muscle tissue samples into 3 main groups at the 20% dissimilarity level (Fig. 3). Importantly, the 3 groups identified by this cluster analysis based on fatty acid composition, with the exception of a few outliers, match the clusters of sharks found when examining the relationships between $\delta^{13}C$ and length (Fig. 2), as well as the relationships between specific fatty acid ratios with total length and $\delta^{13}C$ (see Figs. 5 & 6, with further explanation below). The first cluster of sharks (group *i*) included a subset of shark muscle tissue samples that contained significantly higher total monounsaturated fatty acids compared to the sharks in groups *ii* or *iii* and significantly lower total satu-

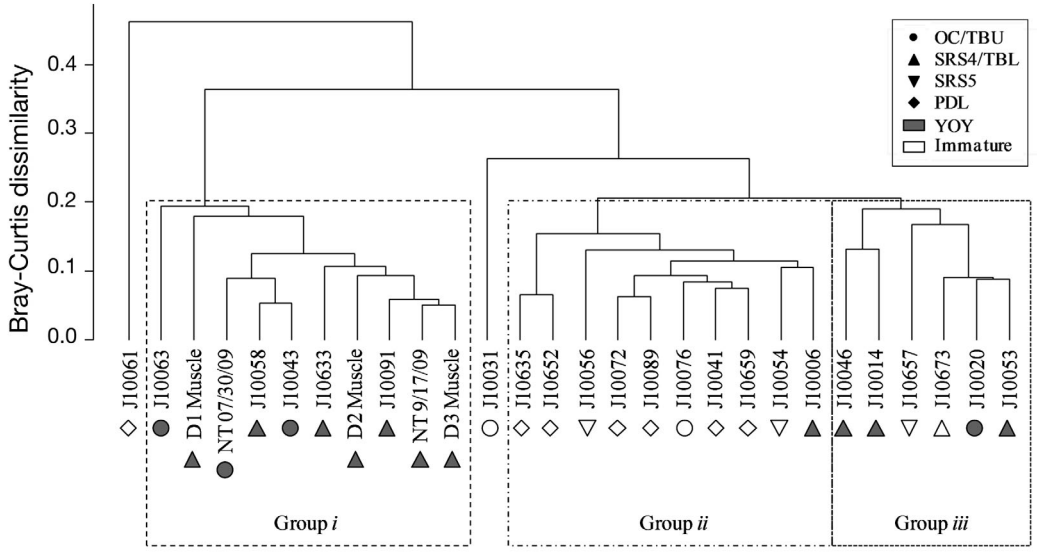

Fig. 3. *Carcharhinus leucas.* Average-linkage hierarchical cluster analysis of composition of predominant fatty acid composition of shark muscle tissue based on Bray-Curtis dissimilarity matrix. Muscle tissue clustered into 3 main groups (*i*, *ii*, and *iii*) at a 20% dissimilarity level, with the exception of outliers (J10061 and J10031). Site abbreviations as in Fig. 1; YOY: young of the year

Table 2. *Carcharhinus leucas.* Relative abundance (% of total fatty acids) in shark muscle tissue samples. Shark group number (*i*, *ii*, or *iii*) shown in parentheses. Unsaturated fatty acids named as A:BωC, where A is carbon chain length, B is number of double bonds, and C is position of first double bond counted from terminal methyl end (listed where confirmed through picolinyl ester method or comparison with standard compound). NT: not tagged; Tr: trace amounts detected (<0.1%); –: not detected. Sharks J10031 and J10061 were not assigned to groups based on the hierarchical cluster analysis (Fig. 3)

	J10043 (*i*)	J10633 (*i*)	J10058 (*i*)	J10053 (*iii*)	J10091 (*i*)	D1 (*i*)	D2 (*i*)	J10063 (*i*)	D3 (*i*)	J10020 (*iii*)	J10006 (*ii*)	NT 09/17/09 (*i*)	J10014 (*iii*)	NT 07/30/09 (*i*)
Saturated (SAT)														
14:0n	0.4	0.4	0.3	0.2	0.4	0.6	0.4	1.1	0.3	0.4	0.2	0.4	1.2	0.2
15:0n	0.1	0.2	0.1	0.1	0.1	0.2	0.2	0.8	0.1	0.2	0.2	0.1	0.6	0.1
16:0n	12.1	16.1	12.4	14.0	13.5	20.8	16.3	13.7	13.1	15.6	12.5	11.8	15.9	10.9
18:0n	13.7	9.7	13.0	14.2	11.3	9.6	11.3	20.5	9.4	14.1	15.1	10.8	20.3	12.1
Total SAT	26.3	26.4	25.8	28.5	25.4	31.1	28.1	36.2	23.0	30.2	27.9	23.1	38.0	23.3
Monounsaturated (MUFA)														
16:1ω9	0.3	2.5	2.4	–	2.2	0.6	0.6	–	2.5	0.2	0.2	1.9	–	2.4
16:1ω7	6.9	6.5	6.2	2.0	5.5	9.7	7.1	3.6	6.1	2.6	1.5	6.1	2.3	6.0
17:1ω8	0.4	–	0.3	0.2	0.4	1.0	0.8	0.2	0.6	0.4	0.6	0.4	–	0.3
18:1ω9	25.3	22.8	24.0	14.4	19.5	26.3	21.4	17.6	22.6	14.7	14.2	22.4	11.5	22.1
18:1ω7	9.2	9.5	8.5	8.6	8.2	5.2	5.1	6.5	8.5	7.5	6.8	7.6	7.0	8.6
Total MUFA	42.2	41.3	41.5	25.1	35.8	42.9	35.1	27.9	40.2	25.4	23.3	38.4	20.8	39.4
Polyunsaturated (PUFA)														
18:2	1.9	2.9	0.8	0.2	0.5	0.8	0.8	0.8	0.8	0.3	0.3	0.6	0.5	0.9
18:2	3.1	0.5	2.9	0.5	3.0	1.9	2.9	1.5	3.1	0.9	–	2.8	0.7	3.3
18:2ω6	–	0.1	0.1	1.5	0.3	0.3	0.4	0.3	0.3	0.8	1.1	0.3	0.8	0.2
20:2ω9	6.3	3.9	6.2	1.6	4.2	2.8	3.7	3.0	5.2	1.8	0.5	4.7	0.9	6.1
20:3ω9	10.4	7.8	11.8	4.8	12.1	7.0	10.2	14.5	10.0	8.4	3.0	11.8	5.9	13.3
20:4ω6	0.5	3.2	0.6	10.0	3.1	2.0	3.2	2.3	2.5	6.9	14.4	2.3	9.4	1.1
20:5ω3	0.2	0.8	0.2	0.7	0.8	0.5	1.1	0.5	0.7	1.1	1.2	0.5	1.1	–
22:3	1.9	1.7	1.9	1.2	2.3	1.3	1.9	1.7	2.5	1.4	0.4	2.8	1.2	0.5
22:4ω6	0.3	0.8	0.3	5.4	1.0	0.4	0.7	0.5	0.5	3.8	4.5	0.9	2.4	0.4
22:5ω6	0.1	0.7	0.2	2.8	0.8	0.6	0.8	0.4	0.6	1.6	2.8	0.7	1.4	0.3
22:5ω3	0.4	0.7	0.7	2.7	0.9	0.7	1.3	0.7	0.9	3.0	3.4	0.6	1.7	3.2
22:6ω3	1.3	4.0	2.0	9.5	4.3	3.1	4.9	3.7	4.0	9.2	11.1	4.4	7.4	2.9
Total PUFA	26.4	27.1	27.6	40.9	33.3	21.6	31.9	29.9	31.1	39.4	42.8	32.5	33.6	32.1

	J10046 (*iii*)	J10673 (*iii*)	J10076 (*ii*)	J10054 (*ii*)	J10031	J10056 (*ii*)	J10089 (*ii*)	J10657 (*iii*)	J10652 (*ii*)	J10041 (*ii*)	J10659 (*ii*)	J10635 (*ii*)	J10072 (*ii*)	J10061
Saturated														
14:0n	1.9	0.4	0.3	0.3	1.0	0.4	0.3	0.2	0.2	0.4	0.3	0.2	0.2	5.5
15:0n	1.0	0.4	0.2	0.2	1.6	0.2	0.3	0.2	0.2	0.2	0.2	0.1	0.2	5.8
16:0n	22.5	14.4	14.1	16.5	9.8	13.4	15.4	17.3	20.6	20.3	18.7	17.7	17.3	33.4
18:0n	20.8	11.2	16.9	16.0	33.2	15.1	13.6	11.0	14.0	17.6	15.8	13.5	14.4	27.2
Total SAT	46.2	26.3	31.5	33.0	45.6	29.1	29.6	28.7	35.0	38.5	35.0	31.4	32.1	71.9
Monounsaturated														
16:1ω9	–	1.0	tr	–	–	–	–	0.5	0.2	–	0.3	0.1	–	–
16:1ω7	1.8	2.1	1.3	1.2	1.4	1.3	1.4	4.1	1.7	1.2	3.6	0.5	1.7	0.7
17:1ω8	1.3	0.6	0.5	0.5	0.1	0.5	0.5	0.6	0.2	0.4	0.9	0.4	0.4	0.4
18:1ω9	13.4	15.2	16.1	13.6	11.4	15.3	13.8	17.9	11.0	16.3	16.6	10.9	14.6	8.0
18:1ω7	7.7	8.7	7.3	6.1	6.7	4.8	5.7	6.9	5.2	6.5	5.3	5.0	7.9	4.3
Total MUFA	24.2	27.7	25.2	21.3	19.6	21.9	21.5	30.0	18.2	24.5	26.6	16.8	24.6	13.4
Polyunsaturated														
18:2	0.4	0.3	0.2	0.1	0.2	0.2	0.1	0.4	0.1	0.1	0.1	tr	0.2	0.3
18:2	0.5	0.7	0.3	0.2	0.2	–	–	0.5	0.1	0.1	–	–	–	0.3
18:2ω6	1.0	1.1	1.6	0.8	1.4	1.8	1.1	0.4	0.5	0.6	0.6	0.6	0.6	0.5
20:2ω9	1.2	1.9	0.4	0.9	–	–	0.2	1.4	0.3	0.2	0.2	0.2	0.4	–
20:3ω9	3.4	4.1	1.1	2.5	0.6	0.8	1.1	2.8	0.3	1.2	0.2	0.1	0.9	–
20:4ω6	6.6	7.0	14.1	9.8	15.2	22.2	15.8	5.5	11.2	15.2	13.8	12.3	14.7	4.7
20:5ω3	–	1.0	0.5	0.4	0.5	0.5	0.7	2.6	1.2	0.6	1.1	1.3	0.5	–
22:3	0.7	3.2	tr	0.7	–	–	0.2	0.8	–	–	–	0.1	–	–
22:4ω6	2.0	2.9	6.3	9.0	2.7	7.3	9.3	1.8	9.5	6.2	6.7	11.6	8.3	0.5
22:5ω6	0.9	2.4	2.4	3.0	1.1	4.3	4.5	1.2	3.6	2.0	3.0	5.1	3.2	0.4
22:5ω3	1.3	3.3	2.8	3.1	1.5	1.6	2.3	5.0	3.9	1.5	1.9	3.2	2.0	–
22:6ω3	4.6	9.6	8.5	10.4	7.4	6.3	8.5	13.9	12.7	6.5	7.8	13.7	8.1	1.0
Total PUFA	22.7	37.4	38.1	40.9	30.7	44.9	43.8	36.4	43.3	34.2	35.3	48.2	38.9	7.7

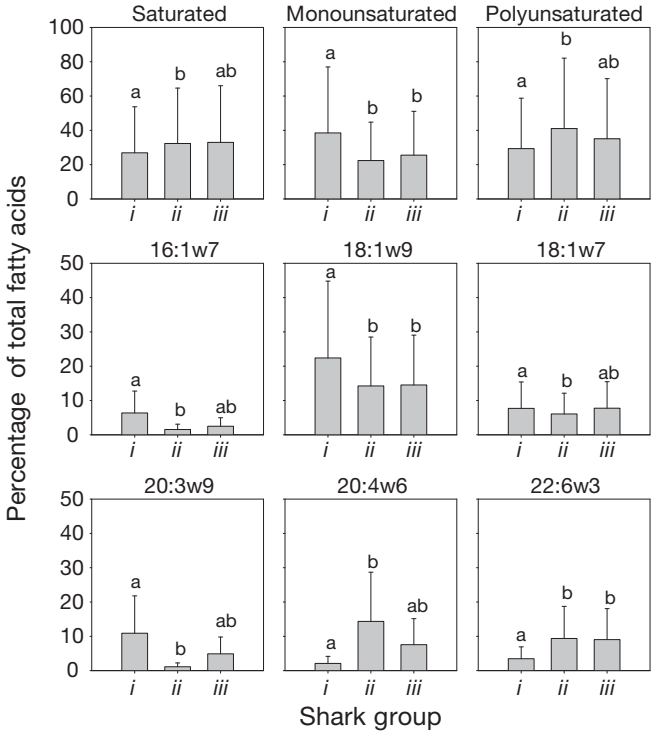

Fig. 4. *Carcharhinus leucas.* Average (±SD) relative abundance of grouped (saturated, monounsaturated, and polyunsaturated fatty acids) and selected individual fatty acids in group *i*, *ii*, and *iii* sharks. Different letters represent significant differences ($p < 0.05$)

rated and polyunsaturated fatty acids compared to group *ii* (Fig. 4). No significant differences (Kruskal-Wallis; $\alpha = 0.05$) were found for mean relative abundances of 14:0n, 15:0n, 16:0n, 17:1ω8, and 20:5ω3 among the 3 groups of sharks, although, on average, 16:0n, 17:1ω8, and 20:5ω3 were lower in group *i* sharks than in groups *ii* and *iii*. For all other fatty acids in the dataset, mean relative abundance was significantly different between group *i* and group *ii* sharks. Average (± SD) relative abundances of the 3 predominant monounsaturated fatty acids, 16:1ω7, 18:1ω9, and 18:1ω7, and the dominant representative polyunsaturated fatty acid from the ω9, ω6, and ω3 families, in each of the 3 groups of sharks are presented in Fig. 4. Group *i* sharks contained significantly higher abundances of 16:1ω7, 18:1ω7, and 20:3ω9 compared to group *ii*, and significantly higher 18:1ω9 compared to both groups *ii* and *iii* (Fig. 4). In contrast, group *i* contained significantly lower 20:4ω6 than group *ii* and significantly lower 22:6ω3 than both groups *ii* and *iii* (Fig. 4). No significant differences in these fatty acids were found between groups *ii* and *iii* (Fig. 4). The ratio of 20:3ω9 to 20:4ω6 ranged from 2.4 to 21 in group *i* sharks, which were also relatively small (66 to 79 cm TL) and isotopically enriched (−13.3 to −17.4‰; Figs. 5 & 6). Group *iii* sharks, which were also small (69 to 116 cm TL), had lower ratios of 20:3ω9/20:4ω6, ranging from 0.48 to 1.22, and more deplete isotopic values (−15.9 to −23.8‰) compared to the group *i* sharks (Figs. 4 & 5). Group *ii* sharks were in general the largest, but had the smallest 20:3ω9/20:4ω6 ratios (0.0 to 0.26) and wide ranges of carbon isotopic composition (−15.6 to −25.9‰; Figs. 5 & 6).

For a subset of sharks in which body mass was available (n = 17), the relationship between total length (cm) and body mass (kg) was linear and highly significant (body mass = 0.212 × [total length] − 12.432, $R^2 = 0.8816$, $p < 0.001$). From this relationship, expected body masses were determined. The difference between observed and expected body masses were used as estimates of body condition. No significant relationship was found between this estimate of body condition and the 20:3ω9/20:4ω6 ratio ($R^2 = 0.023$, $p = 0.558$).

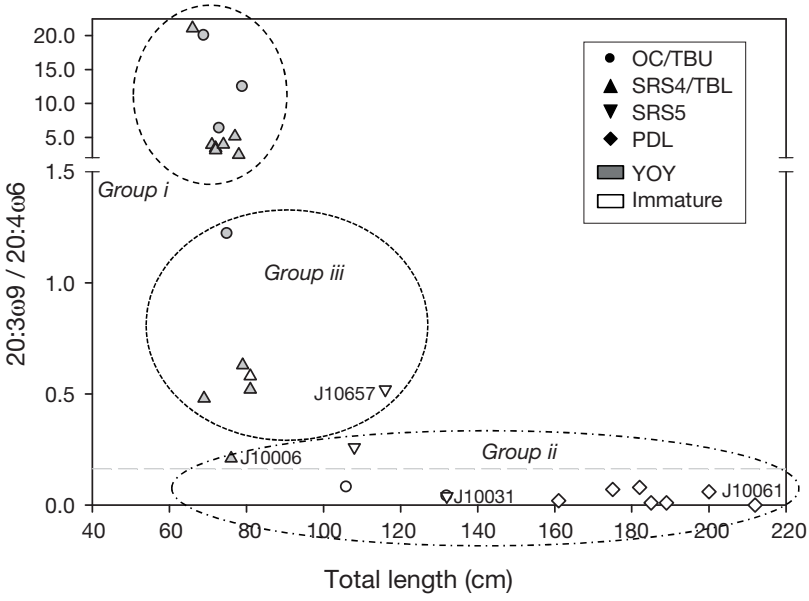

Fig. 5. *Carcharhinus leucas.* Ratio of 20:3ω9/20:4ω6 in muscle tissue as a function of shark length. Groups of sharks defined by hierarchical cluster analysis as shown in Fig. 3. The outliers in the cluster analysis, J10061 and J10031, are labeled. Note that sharks J10006 and J10657, atypical in Fig. 2, group as assigned by the cluster analysis. Site abbreviations as in Fig. 1; YOY: young of the year

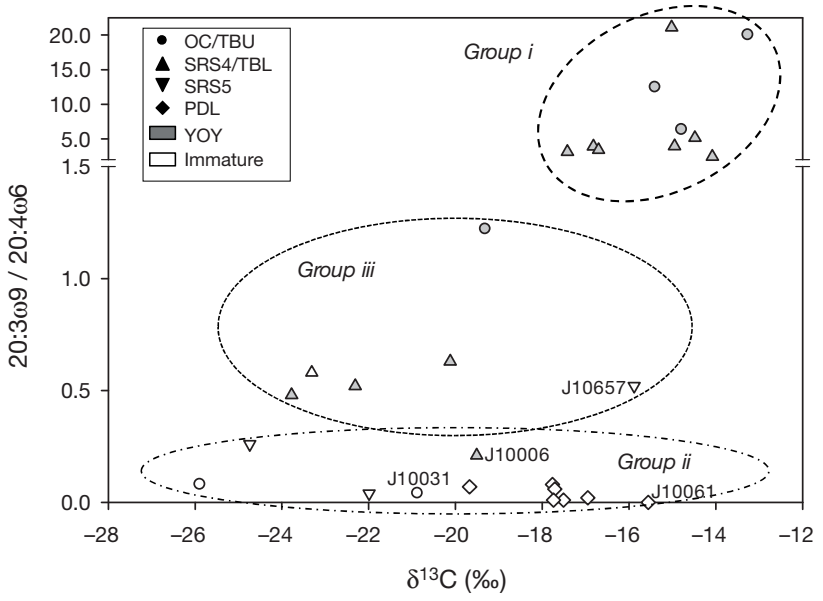

Fig. 6. *Carcharhinus leucas.* Ratio of 20:3ω9/20:4ω6 in muscle tissue as a function of $\delta^{13}C$. See Fig. 5 for details

DISCUSSION

Together, stable isotopic and fatty acid compositions revealed the presence of 3 groups of sharks in the Shark River Estuary of the Florida Everglades: (*i*) young, small (<100 cm) sharks with $\delta^{13}C$ values reflective of maternal diet and foraging locations and fatty acid compositions suggestive of poorly developed foraging skills with possible reliance on maternal resources; (*ii*) older, maternally-independent sharks (>100 cm) that appear to be foraging sufficiently from diverse food webs, with a shift in diet from estuarine-based to marine-based with increasing size; and (*iii*) small sharks (also <100 cm) with tissues displaying a mixture of maternal and dietary resource, or primarily dietary, signals. Alternatively, the isotopic and fatty acid composition of group *iii* sharks could be indicative of poor nutritional status, either through inadequate development of foraging skills or lack of available prey items leading to undernourishment.

The highly enriched 'marine-like' $\delta^{13}C$ values for group *i* sharks did not match their location of capture (near TB in the upper reaches of the estuary), nor life history considerations, which show that young sharks tend to remain in lower-salinity waters to avoid predation (Wiley & Simpfendorfer 2007, Heithaus et al. 2009). For these sharks, it is likely that the carbon isotopic signal in muscle tissue is not directly reflective of diet (see Matich et al. 2010, Olin et al. 2011). The highly enriched $\delta^{13}C$ values of these young sharks instead likely reflect maternal signals either from resources transferred during gestation or from maternal provisions used post-parturition (i.e. liver), as was shown for bull and Atlantic sharpnose sharks *Rhizoprionodon terraenovae* (McMeans et al. 2009, Matich et al. 2010, Olin et al. 2011), bottlenose dolphins *Tursiops truncatus* (Knoff et al. 2008), and elephant seals *Mirounga leonina* (Ducatez et al. 2008). Use of maternal provisioning, in the form of an enlarged liver, was also demonstrated for juvenile dusky sharks *Carcharhinus obscurus* based on declining liver mass and hepatosomatic index and a declining condition factor based on total body mass and length (Hussey et al. 2010).

Interestingly, differences in fatty acid compositions led to groupings of sharks similar to the groupings based on the relationship between stable isotopic composition and shark length (see Figs. 2 & 5), with group *i* sharks possessing larger relative abundances of 20:3ω9 and 18:1ω9 and lower relative abundances of 20:4ω6 and 22:6ω3 than group *ii* (and sometimes group *iii*) sharks (Fig. 4). This pattern of fatty acid composition, with higher ω9 fatty acids and lower ω6 and ω3 fatty acids, is notable, as it suggests that group *i* sharks are depleted in essential fatty acids.

Fatty acids are critical in living organisms because they play important roles in biochemical processes (including metabolism and growth), cell structure, and intracellular messaging (Das 2006). There are 3 main families of important unsaturated fatty acids: ω3, ω6, and ω9 (Le et al. 2009). Mammals and most other animals lack the enzymes to synthesize the building-block fatty acids of the ω3 and ω6 families, so these fatty acids—18:3ω3 (α-linolenic acid) and 18:2ω6 (linoleic acid)—must be obtained through dietary sources and are thus termed 'essential' (Le et al. 2009). Most animals lack the enzymes required for interconversion of fatty acids between families; however, unsaturated fatty acids can be converted within a family through enzymatic processes such as desaturation and elongation. This occurs in the reactions which produce 20:5ω3, 22:6ω3, and 20:4ω6, critical precursors for the eicosanoids and leukotrienes (Le et al. 2009). The ω3, ω6, and ω9 families compete for the same enzymes, with ω3 and ω6 at a competitive advantage over ω9. For this reason, during essential

fatty acid deficiency, ω9 unsaturated fatty acids such as 20:3ω9 are synthesized from ubiquitous C_{18} saturated and monounsaturated fats present in cells because of the almost complete lack of competitive effects of ω3 and ω6 fatty acids (Le et al. 2009). Although 20:3ω9 has been found in high abundances in cartilage (Adkisson et al. 1991), the samples analyzed here are purely muscle from the dorsal side of the animal near the first dorsal fin. Any cartilage or skin attached to the muscle tissue was removed, so we expect negligible inputs of 20:3ω9 from cartilaginous tissue. Therefore, the pattern of higher abundances of C_{18} monounsaturated fatty acids and 20:3ω9, combined with very low abundances of the ω6 and ω3 polyunsaturated fatty acids, clearly demonstrates that group *i* sharks are depleted in essential fatty acids.

The essential fatty acid deficiency present in group *i* sharks suggests that these animals are either not feeding, have inadequate foraging skills, or that prey abundance is limited. Limitation of prey is not likely, as other individuals captured in the same region as group *i* sharks showed little to no essential fatty acid deficiency (Fig. 5). The former 2 possibilities, starvation or inadequate foraging skills, both lend support for the hypothesis that group *i* sharks have been relying on maternal resources such as an enlarged liver, as was demonstrated for juvenile dusky sharks (Hussey et al. 2010), and may only recently have begun to develop foraging skills.

For humans, a plasma ratio of 20:3ω9/20:4ω6 (triene-tetraene ratio) >0.2 is considered diagnostic of essential fatty acid deficiency (Holman 1960, Holman et al. 1979, Siguel et al. 1987, Le et al. 2009). Although we cannot assume that the 0.2 cut-off value of the triene-tetraene ratio defined in humans applies exactly to bull sharks, the fact that all larger (>120 cm) bull sharks in this study possessed 20:3ω9/20:4ω6 ratios at or below this value (Fig. 4) does lend support to this assumption since they are replete in essential fatty acids. Additionally, the triene-tetraene ratio for group *i* sharks was 12 to 106 times the human cut-off value of 0.2, while the ratio for group *ii* sharks was only 0.05 to 1.3 times the human cut-off value (Fig. 4).

No significant relationship was found (Pearson r = 0.153; df = 16; p = 0.558) between this estimate of body condition and markers of essential fatty acid deficiency, even for group *i* sharks only (Pearson r = 0.429; df = 6; p = 0.336) in which muscle tissue suggested a high degree of essential fatty acid deficiency. This lack of relationship between body condition and essential fatty acid deficiency could stem from our small sample size (we only had body mass measurements for 17 sharks), but is more likely a reflection of the different timescales in which muscle tissue fatty acids and body condition based on total body mass respond to dietary change. Additional studies of the fatty acid composition of other tissues compared to morphometric measures, such as total body fat or liver mass, would be useful to clarify these dietary response timescales. The fatty acid composition of liver, the major lipid storage depot, can be challenging to link directly to dietary inputs because extensive lipid synthesis and oxidation and modification of lipids for buoyancy regulation occurs in livers (see Ballantyne 1997); however, assessment of liver fatty acid composition in conjunction with muscle tissue might provide further information on the timescales of transition from maternal resource dependency to foraging or information on the nutritional quality of maternal resources.

A distinct change in the relationship between shark total length and muscle tissue $\delta^{13}C$ occurred for sharks with lengths greater than approximately 100 cm compared to the smaller sharks (Fig. 2). Similarly, sharks >100 cm had very low triene-tetraene fatty acid ratios (Fig. 4). The positive relationship between shark length and $\delta^{13}C$ for sharks >100 cm implies firstly that sharks at this age/size are largely independent of maternal provisioning and their tissue composition is reflective of diet (see also Matich et al. 2010), and secondly, that some sharks transition from freshwater/estuarine habitats to marine waters, with a greater reliance on marine, seagrass-based food webs as they age (Fig. 2). Because we do not have many sharks in the range of 80 to 100 cm, and because individuals feed and mature at different rates, it is difficult to determine an exact size, or size range, where the shark tissue would transition from reflecting maternal influence to dietary influence. Olin et al. (2011) suggested that based on $\delta^{13}C$ and $\delta^{15}N$, bull shark muscle and liver tissue indicate a loss of maternal signal by umbilical scar stage 5 (faint scar visible), although they caution that categorizing this slow-growing species by umbilical scar stages may not be appropriate. Given that umbilical scars are no longer visible in bull sharks in the Shark River estuary by the time sharks reach 75 to 85 cm total length (M. Heithaus & P. Matich unpublished data), an estimate of 100 cm for the point at which maternal isotopic signal is lost is most likely conservative. This size estimate is presumably even more conservative when we consider that dietary changes become recorded in tissue fatty acids on timescales of <12 wk in juvenile iridescent sharks *Pangasius hypothalmus*

(Asdari et al. 2011), as opposed to stable isotopes, which turn over on the order of ~1 to 1.5 yr in elasmobranch muscle tissue (see MacNeil et al. 2006, Logan & Lutcavage 2010, Matich et al. 2010, and references therein).

Fatty acids in shark muscle tissue were not sufficient to distinguish between freshwater/estuarine versus seagrass-based food sources, as demonstrated by the similarity in fatty acid composition among group *ii* sharks, despite the substantial range of $\delta^{13}C$. Typical markers for terrestrial organic matter in freshwater/estuarine environments, such as long-chain saturated fatty acids characteristic of vascular plant debris (Eglinton & Hamilton 1967) or 18:3ω3 indicative of cyanobacterial inputs to the abundant periphyton in the freshwater prairies of the Everglades (Neto et al. 2006), were either absent or only present in trace levels in shark muscle tissue. Whether this absence reflects a lack of feeding on food sources that use vascular plant debris or periphyton as basal resources, or, alternatively, that inputs from these sources are not accumulating up the food chain to high trophic level consumers remains to be determined. Investigation into more specific biomarkers or techniques such as compound-specific isotopic analysis may help answer this question. Similarly, temporal and spatial variability in shark diet may also be affecting our ability to fully resolve the early-life feeding ecology of bull sharks. Our data encompassed individuals collected in both wet and dry seasons over multiple years, but was not large enough to adequately address changes in shark diet on spatial and temporal scales.

Because size at birth is quite variable in bull sharks (Neer et al. 2005) and growth rate among individuals is also variable, especially for smaller animals (Branstetter & Stiles 1987), it is possible that individuals that fall between 60 and 80 cm total length (in Fig. 4) may represent sharks whose isotopic compositions are both completely dependent and increasingly or completely independent of maternal composition (see Olin et al. 2011). This concept explains the presence of group *iii*, i.e. sharks with less enriched isotopic compositions and intermediate triene-tetraene ratios. If these sharks are substantially older than sharks of the same size with highly enriched isotopic signals, it is possible that their muscle tissues are losing their maternal signal and are becoming more indicative of diet. The lower triene-tetraene ratios support increasing dietary input of essential fatty acids, implying greater nutritional condition compared to their group *i* counterparts. However, the triene-tetraene ratio for group *iii* sharks was still elevated compared to group *ii* sharks and may suggest poor foraging skills leading to essential fatty acid deficiency, particularly for those sharks with more depleted isotopic compositions (approximately −20 to −24‰), if both fatty acid and isotopic composition have already fully lost any maternal influence (Fig. 5). Feeding studies with individuals of known age would greatly improve our understanding of these transitional animals.

CONCLUSIONS

The stable isotopic composition of YOY bull sharks is complicated by inputs from maternal biochemical makeup. As sharks grow and initiate foraging, the maternal signal is gradually lost with sharks slowly incorporating more dietary signals into their tissues. By a total shark length of approximately 100 cm, muscle tissues are wholly representative of diet, with an apparent transition from freshwater/estuarine to seagrass-based diets as shark length increases for some individuals. Fatty acid composition of muscle tissue of some YOY bull sharks shows severe essential fatty acid deficiency, indicating non-feeding or inadequate feeding, with the possibility of maternal resource dependence during this time. Further investigations should focus on the paired stable isotope and fatty acid compositions of multiple tissues with differing turnover times to further constrain the timescales of the loss of maternal signature from tissues and potential use of maternal reserves.

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