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Dietary and reproductive biomarkers in a generalist apex predator reveal differences in nutritional ecology across life stages

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ABSTRACT: Knowledge of the nutritional requirements of apex predators is key for determining ecological interactions. However, an understanding of how diet is influenced by reproduction, and the consequences of foraging variation on the nutritional status of a predator, is limited. Here, we used short-term dietary markers (plasma and whole-blood fatty acids) integrated with reproductive hormones (17β-estradiol and testosterone) and ultrasonography as a non-lethal approach to investigate the effect of life stage on nutritional status and trophic dynamics of female tiger sharks *Galeocerdo cuvier*. Despite their generalist feeding behavior, female tiger sharks fed on different food sources and/or modulated their fatty acid metabolism depending on the reproductive context. This suggests some adjustment in their nutritional requirements associated with changes in their reproductive state. Plasma and whole-blood fatty acids indicated distinct dietary sources across life stages, with a high dependence on coastal/benthic food resources during juvenile life stages, and on pelagic/oceanic and reef-associated food resources during adult life stages. Higher percentages of highly unsaturated omega-3 fatty acids found in females during their reproductive cycles suggest the dependency on these fatty acids as a source of metabolic energy during reproduction. A high percentage of arachidonic acid (ARA) found in plasma of gravid females suggests the possibility of a selective diet of ARA-rich prey species and/or selective mobilization of ARA from stored energy during gestation. Based on our findings, we propose a conceptual model of expected changes in nutritional and trophic markers across life stages of female tiger sharks.

KEY WORDS: Trophic markers · Nutritional condition · Dietary patterns · Tiger shark · *Galeocerdo cuvier* · Reproduction · Trophic ecology · Physiology

1. INTRODUCTION

Understanding dietary patterns of top predators and the factors influencing what is consumed are central issues in determining how they can impact ecosystem structure and function through top-down predation effects (Estes et al. 2016, Hammerschlag et al. 2019). Throughout the life cycle of a predator, energetic requirements can vary in response to internal biological processes, such as those related to somatic growth, reproduction and migration (e.g. Chaguaceda et al. 2020, Machovsky-Capuska & Raubenheimer 2020). Therefore, energetic requirements associated with these physiological processes

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are an important factor affecting food intake and foraging preferences (e.g. Kohl et al. 2015). Although studies have investigated the trophic roles and relationships among predators (e.g. Hussey et al. 2015, Shipley et al. 2019), our understanding of how diet and energy intake is influenced by life stage, and the consequences of foraging variation on the nutritional status of the predator, remains limited (Wai et al. 2012, Dicken et al. 2017, Hammerschlag et al. 2018).

Studies on the foraging ecology of predators have generally focused on food quantity rather than quality (i.e. nutritional composition) (Kohl et al. 2015, Flecker et al. 2019). However, food quality can have a significant impact on fitness, including the survival, development rate and growth, and reproductive success of an individual (e.g. Simpson et al. 2010, Twining et al. 2018). Food quality can also reveal insights into trophic interactions and nutrient-specific food preferences (Kohl et al. 2015, Pethybridge et al. 2018). Fatty acids have physiologically important roles in living organisms, such as providing metabolic energy for maintenance and growth (Sargent et al. 1995, Tocher 2003), and influencing reproductive processes through a variety of mechanisms (Wathes et al. 2007). For example, highly unsaturated fatty acids of n3 (n3 HUFAs) and n6 series (n6 HUFAs) influence fertilization rates, as they act as the main component of sperm and oocyte plasma membranes (Izquierdo et al. 2001, Wathes et al. 2007). Additionally, arachidonic acid (C20:4n6, ARA), an n6 HUFA, is directly involved in follicle maturation and steroidogenesis during reproduction through prostaglandins (eicosanoids) (Wathes et al. 2007). Because vertebrates are unable to synthesize de novo polyunsaturated fatty acids (PUFAs), high-quality diets play a key role in their reproduction (e.g. Tocher 2003, Parrish 2009, Colombo et al. 2017). When transferred from prey to predator, some fatty acids are subject to biosynthesis (through chain elongation, desaturation or catabolism via β-oxidation); however, since most fatty acids remain relatively unchanged, these molecules serve as dietary biomarkers (Dalsgaard et al. 2003, Budge et al. 2006, Iverson 2009).

Tiger sharks *Galeocerdo cuvier* are large, globally distributed marine apex predators in tropical and warm-temperate coastal and pelagic waters (Holland et al. 2019). Tiger sharks are highly migratory and exhibit considerable variability in their habitat use and movements (Hammerschlag et al. 2012, Papastamatiou et al. 2013, Lea et al. 2015, Ajemian et al. 2020). As generalist and opportunistic consumers, tiger sharks exploit a wide variety of prey, including invertebrates, teleosts, sea turtles, marine mammals, sea snakes, seabirds and other elasmobranchs (e.g. Gallagher et al. 2011, Hammerschlag et al. 2015, Dicken et al. 2017). The ontogenetic shifts in their diet are well reported across several ocean basins, in which large prey (e.g. turtles and elasmobranchs) become more important in their diet with increasing shark size (Dicken et al. 2017, Ferreira et al. 2017, Salinas-de-León et al. 2019). While ontogenetic diet expansion is well documented for this species, no studies have explicitly investigated for potential variation in foraging ecology across life stages. Female tiger sharks have a unique reproductive strategy (embryotrophy, i.e. a type of aplacental viviparity where embryos are nourished by an intracapsular fluid, Castro et al. 2016), a suggested triennial breeding cycle with a long gestation period (up to 16 mo, Whitney & Crow 2007), and comparatively large broods (18–70) of large embryos (~75 cm, Whitney & Crow 2007, Castro et al. 2016). Accordingly, a detailed description of how diet and nutritional condition vary throughout their life and life stages is valuable for understanding their energetic needs and functional roles.

Here, we combined analyses of short-term biomarkers with information on tiger shark life stages to non-lethally assess their nutritional ecology. Specifically, we compared plasma and whole-blood fatty acid profiles among female life stages (i.e. immature, adult non-gravid and gravid) and related these data to body size and reproductive hormones (17β-estradiol and testosterone) to evaluate variability in nutritional ecology across different life-history stages. Plasma is a good candidate fluid to investigate feeding patterns and nutritional status via fatty acid analysis because: (1) it has relatively fast turnover rates (i.e. days to weeks, Käkelä et al. 2009), and (2) it functions in transporting dietary and non-dietary fatty acids (e.g. inter-tissue routing of membrane lipids and for metabolic functions), and therefore, has high similarity with prey fatty acid profiles (e.g. McMeans et al. 2012, Beckmann et al. 2014, Bierwagen et al. 2019). Although fatty acid profiles in whole blood have not been explored in sharks, it is a convenient fluid to use in field-based studies where obtaining large blood samples and/or isolating plasma is challenging (Baylin et al. 2005, Tierney et al. 2008).

We considered the physiologically important fatty acids (n3 and n6 HUFAs) and known trophic markers (e.g. dinoflagellates and bacteria) to investigate 2 hypotheses related to reproduction and 1 hypothesis related to ontogeny and spatial variation. First, we hypothesized that females preparing to reproduce would have higher dietary quality (i.e. higher per-

centages of n3 and n6 HUFAs) than other life stages. This is because: (1) the importance of HUFAs in vertebrate reproduction is well established, e.g. through promoting egg viability and improving survival (e.g. Tocher 2003, Arts & Kohler 2009), and (2) non-gravid adult females have been found to exhibit higher energy demand (i.e. with higher corticosteroid levels) compared to immature females (B. Rangel, N. Hammerschlag, J. Sulikowski, R. Moreira unpubl. data). Secondly, given that a capital–income breeding strategy has been previously demonstrated for tiger sharks, i.e. they rely on both energy stores and opportunistic feeding to support reproduction (Hammerschlag et al. 2018), we anticipated that gravid females would not display a nutritional deficiency in essential fatty acids. In addition, given that smaller tiger sharks spend more time foraging in coastal and shallow waters, while larger tiger sharks spend more time foraging in offshore pelagic food webs (e.g. Dicken et al. 2017), our third hypothesis was that the diet of females would vary across life stages, demonstrating ontogenetic changes in trophic markers (i.e. basal resources, Fig. 1). Based on that hypothesis, we expected that smaller sharks would have higher coastal fatty acid trophic markers (i.e. C18:2n6, ARA), whereas large tiger sharks would have higher percentages of pelagic/oceanic markers (i.e. docosahexaenoic acid [DHA, C22:6n3], C18:1n9, C16:1n7).

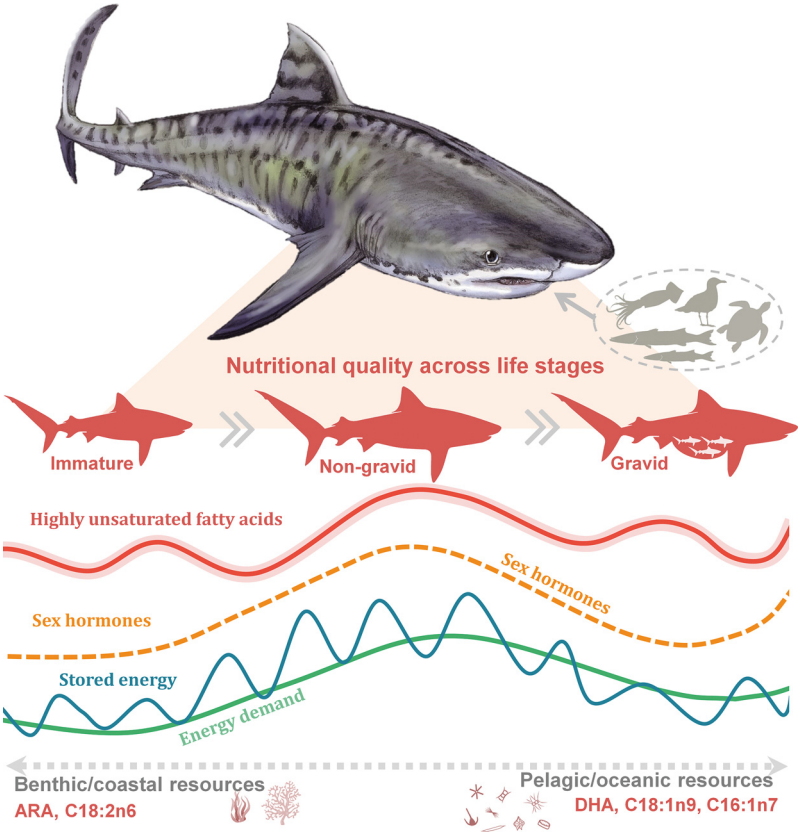

Fig. 1. Conceptual model of hypothesized changes in dietary quality (highly unsaturated fatty acids) and trophic markers across life stages (immature, adult non-gravid, gravid) of female tiger sharks *Galeocerdo cuvier*. We predicted that non-gravid adult females preparing to reproduce would exhibit both higher circulating sex hormones and higher percentages of highly unsaturated fatty acids (omega-3 and -6), in response to higher energetic demands to support gonad development and preparation for reproduction (B. Rangel, N. Hammerschlag, J. Sulikowski, R. Moreira unpubl. data). We further predicted that gravid females would not display nutritional deficiency in essential fatty acids (i.e. highly unsaturated fatty acids) due to a capital–income breeding strategy, where females rely on both energy stores and opportunistic feeding to support reproduction. Smaller tiger sharks should rely more on coastal/benthic prey sources, exhibiting higher coastal fatty acid trophic markers, while larger tiger sharks should rely more on offshore food sources, exhibiting a higher percentage of pelagic/oceanic trophic markers

2. MATERIALS AND METHODS

2.1. Field procedures

Tiger shark blood samples were collected on week-long expeditions to Tiger Beach during December 2011, July 2012, October 2013, May 2014, November 2014, April 2018 and January 2019, at the northwestern edge of little Bahama Bank, off the west end of Grand Bahama Island, Bahamas (~26.6° N, 79.1° W). Sharks were passively captured using circle-hook drumlines (details in Gallagher et al. 2014), which were deployed (10–40 m deep) and left to soak for 1 h before being checked for shark presence. On capture, sharks were secured by hand on the side of the boat or on a partially submerged platform, and a water pump was inserted into the shark's mouth to facilitate ventilation. Once sharks were secured, blood samples were obtained, sex was identified (based on the absence of claspers for females and presence for males), and morphological measurements were taken (e.g. total length, cm). Finally, sharks were tagged for identification and released.

Blood (~20 ml) was collected from the caudal vein using 18-gauge needles and 10 ml heparinized syringes and immediately centrifuged (410 × *g*, 2 min). Plasma was then removed, placed in a cooler on the boat and then stored frozen at −20°C for future analyses.

2.2. Hormone analysis and reproductive status

The plasma concentrations of gonadal steroids (17β-estradiol and testosterone) and life stages of tiger sharks were based on those determined by Sulikowski et al. (2016). In brief, both 17b-estradiol and testosterone were measured by non-radiolabelled steroid hormone (Steraloids) by radioimmunoassay, following the procedure of Sulikowski et al. (2004). A Tri-Carb 2900TR liquid scintillation analyzer (PerkinElmer) was used to measure radioactivity (see Sulikowski et al. 2016 for details). The mean intra-assay coefficients of variation for testosterone and estradiol were 10 and 6%, respectively, and the inter-assay coefficients of variation were 10% for both hormones.

Following Sulikowski et al. (2016), we considered length at maturity for tiger sharks in the studied region to be >300 cm total length (Branstetter et al. 1987, Whitney & Crow 2007) to distinguish immature from adult/non-gravid females. For sharks captured in 2011 in the Bahamas, the reproductive statuses of adult females were determined using the gravidity predicting model, which uses testosterone (mean ~250 pg ml^{-1} for non-gravid and ~145 pg ml^{-1} for gravid females) and estradiol concentrations (mean ~200 pg ml^{-1} for non-gravid and ~30 pg ml^{-1} for gravid females) (see details in Sulikowski et al. 2016).

For females sampled between 2012 and 2019, pregnancy status were assessed through ultrasonography (Ibex Pro portable ultrasound, EI Medical Imaging; with a 60 mm curved linear array 2.5 to 5 MHz transducer [model 290470]) of the reproductive tract of each female shark (see Sulikowski et al. 2016). The presence of follicles or pups in the uterus was used to classify an individual as gravid or non-gravid.

2.3. Fatty acid analysis

Fatty acid profiles were analyzed in plasma and whole blood (100 µl) by direct transmethylation, as described by Parrish et al. (2015a). The samples were homogenized and directly transmethylated in 3 ml of methanol:dichloromethane:concentrated hydrochloric acid (10:1:1 v/v) solution for 2 h at 80–85°C. After cooling, 1.5 ml of Milli-Q® water and 1.8 ml of hexane and dichloromethane (4:1 v:v) were added to the test tubes, and the tubes were then mixed and centrifuged at 655.2 × *g* (5 min). The upper layer was then removed and transferred to 2 ml injection vials, and the volume was reduced under a nitrogen stream. Fatty acid analysis was carried out in a Varian gas chromatograph (GC, Model 3900, www.varian.com) coupled with a flame ionization detector and a CP-8410 autosampler, as described by Rangel et al. (2019). The data are presented as % of total fatty acid methyl esters based on peak area analyses.

2.4. Fatty acid trophic markers and nutritional indicators

Fatty acids that accounted for <0.5% were excluded from statistical analyses. The essential fatty acids, i.e. DHA, ARA and C20:5n3 (eicosapentaenoic acid, EPA), and ARA/EPA and n3/n6 ratios were used to compare the indices of shark nutritional status (Tocher 2003, Arts & Kohler 2009) and to infer physiological responses of eicosanoids (Tocher 2003). In terms of trophic markers, DHA was used as a marker of dinoflagellates, while C16:1n7/C16:0 was indicative of diatoms (Budge et al. 2006). The DHA/EPA ratio was used as a marker of trophic position, as it has been significantly correlated with $\delta^{15}N$ (El-Sabaawi et al. 2009, Sardenne et al. 2017), and the C18:1n9/C18:1n7 ratio was a marker of the degree of carnivory (Dalsgaard et al. 2003, Parrish et al. 2015b). Additionally, ARA and C18:2n6 values are useful to indicate if a species inhabits coastal/benthic environments (Sardenne et al. 2017), and odd-chain fatty acids (OFAs) and branched-chain fatty acids (BFAs) are biomarkers of heterotrophic bacteria (Dalsgaard et al. 2003).

2.5. Data analysis

Linear regression was used to separately test for a relationship between fatty acids (%) and body size (i.e. total length) and between fatty acids (%) and reproductive hormones 17β-estradiol and testosterone (to assess changes during growth and reproduction). Fatty acids, hormones and total length values were log transformed before analysis to meet assumptions of normality. Linear regression graphics were used to show significant relationships between plasma and whole-blood fatty acids (essential fatty acids and trophic markers) with body size and hormones.

Secondly, we compared fatty acids among life stages (i.e. immature, non-gravid and gravid) to describe the stage-specific variation. Differences between fatty acid profiles across female life stages were tested comparing each fatty acid using 1-way ANOVA with a Tukey post hoc test to parametric data. All data were tested for normality using the Shapiro–Wilk test, and homogeneity of variance was tested using Levene's test. If one of the assumptions was violated, we used Kruskal-Wallis *H*-tests followed by Dunn's post hoc test for non-parametric data. Statistical significance was set at $\alpha = 0.05$. All analyses were conducted in SigmaStat 3.10 (Systat-Software) and PAST 3.12 (Hammer et al. 2001, www.essential-freebies.de). Linear discriminant analyses (LDAs) were performed separately for plasma and whole blood to determine which combination of fatty acids best discriminates between female life stages. Multivariate analyses were conducted in PAST 3.12 (Hammer et al. 2001).

3. RESULTS

A total of 71 female tiger sharks were analyzed in the present study: 17 females (255.5 ± 33.7 cm total length; mean ± SD), 20 adult/non-gravid (345.2 ± 26.5 cm), and 18 gravid females (340.56 ± 22.7 cm).

The largest proportion of fatty acids in blood plasma were the saturated fatty acids (SFAs), predominantly C16:0 and C18:0 during all life stages. PUFAs, consisting of largely DHA and ARA, were in the greatest percentages for non-gravid and gravid females. However, monounsaturated fatty acids

Table 1. Comparative fatty acid profile of plasma and whole blood (%, mean ± SD) among life stages (immature, adult non-gravid, gravid) of female tiger sharks *Galeocerdo cuvier*; p-values for 1-way ANOVA with Tukey post hoc test for parametric data and Kruskal-Wallis *H*-tests followed by Dunn's post hoc for non-parametric data. Significant ($p < 0.05$) results are shown in **bold**. EPA: eicosapentaenoic acid; ARA: arachidonic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid; n3 (n6) PUFA: omega-3 (-6) PUFA; BFA: branched-chain fatty acid; OFA: odd-chain fatty acid: NA: data not available (missing values). Superscript letters denote significant differences among female life stages: immature, non-gravid and gravid (ANOVA, $p < 0.05$). *Non-parametric data (Kruskal-Wallis *H*-test)

Fatty acids	Plasma				Whole blood			
	Immature (n = 17)	Non-gravid (n = 20)	Gravid (n = 18)	p	Immature (n = 9)	Non-gravid (n = 13)	Gravid (n = 12)	p
C15:0	2.4 ± 1.45	1.4 ± 0.63	2 ± 1.39	*0.053	NA	NA	NA	NA
C17:0	0.9 ± 0.26	0.8 ± 0.29	0.9 ± 0.27	*0.722	3.3 ± 0.81^{a}	2.3 ± 0.55^{b}	2.5 ± 0.81^{ab}	**0.011**
C15:1	NA	NA	NA	NA	2.1 ± 1.21	1.6 ± 0.65	1.7 ± 0.64	0.409
BFA-OFA	3.2 ± 1.50^{a}	2.1 ± 0.78^{b}	2.8 ± 1.27^{ab}	***0.032**	5.3 ± 1.88	4.2 ± 1.11	5.0 ± 1.18	0.157
C14:0	4.0 ± 1.21	3.6 ± 1.06	3.5 ± 0.85	0.722	1.9 ± 0.51	2.3 ± 1.05	2.7 ± 1.58	0.319
C16:0	31.0 ± 5.28	27.4 ± 5.66	26.9 ± 5.59	0.062	28.8 ± 2.32	29.6 ± 4.33	29.2 ± 3.92	0.529
C18:0	10.7 ± 1.81^{a}	9.6 ± 2.27^{b}	9.1 ± 1.67^{b}	***0.022**	17.6 ± 1.54^{a}	14.7 ± 2.28^{b}	15.7 ± 1.95^{ab}	**0.009**
ΣSFA	46.9 ± 6.09^{a}	41.7 ± 7.77^{b}	40.0 ± 6.98^{b}	***0.017**	49.8 ± 2.45	49.4 ± 7.05	49.2 ± 5.27	*0.887
C14:1	2.7 ± 0.89	3.0 ± 1.51	3.2 ± 1.31	*0.627	1.6 ± 0.77	2.0 ± 0.88	2.1 ± 0.79	0.484
C16:1n7	3.0 ± 0.89	3.2 ± 0.82	3.2 ± 0.68	0.792	2.0 ± 0.46	2.6 ± 0.64	2.3 ± 0.81	0.147
C18:1n9	18.3 ± 3.89	16.7 ± 4.37	16.9 ± 3.73	*0.321	22.4 ± 2.56	21.4 ± 2.90	22.7 ± 2.22	0.426
C18:1n7	2.5 ± 0.97^{a}	3.4 ± 0.96^{b}	3.3 ± 0.95^{b}	**0.014**	4.1 ± 1.00	4.3 ± 1.01	4.8 ± 0.65	0.141
ΣMUFA	27.0 ± 4.42	27.5 ± 4.68	27.3 ± 4.82	*0.916	30.4 ± 2.01	31.2 ± 3.71	32.8 ± 3.73	0.267
C18:2n6	3.3 ± 1.01^{a}	2.5 ± 0.86^{b}	3.0 ± 1.57^{ab}	***0.031**	2.5 ± 0.62	1.9 ± 0.35	2.7 ± 1.84	*0.085
C20:5n3 (EPA)	1.6 ± 0.66	2.1 ± 0.93	1.6 ± 0.77	0.220	1.0 ± 0.17	1.3 ± 1.03	0.7 ± 0.18	0.202
C22:5n3	2.8 ± 0.89	2.3 ± 0.78	2.5 ± 0.76	0.302	1.7 ± 0.99	1 ± 0.40	1.1 ± 0.60	0.174
C22:6n3 (DHA)	6.5 ± 4.20^{a}	11.1 ± 5.81^{b}	9.9 ± 4.36^{b}	**0.022**	2.5 ± 0.86	5.7 ± 6.23	3.3 ± 1.4	0.480
C20:4n6 (ARA)	7.7 ± 3.60^{a}	7.8 ± 3.79^{a}	10.7 ± 3.9^{b}	**0.012**	6.1 ± 2.09^{a}	4.2 ± 3.8^{b}	4.6 ± 3.57^{ab}	*0.109
C22:4n6	2.6 ± 1.34	1.6 ± 1.01	2.2 ± 1.11	0.056	NA	NA	NA	NA
C22:5n6	1.1 ± 0.30	0.9 ± 0.33	1.2 ± 0.49	0.052	NA	NA	NA	NA
ΣPUFA	24.0 ± 10.92^{a}	28.7 ± 10.51^{ab}	31.4 ± 8.68^{b}	**0.042**	13.7 ± 3.22	15.4 ± 10.59	12.0 ± 6.72	0.262
Σ n3 PUFA	10.6 ± 5.37^{a}	16.0 ± 8.11^{b}	15.2 ± 5.25^{b}	**0.038**	4.6 ± 2.33	8.3 ± 7.07	5.1 ± 1.45	0.383
Σ n6 PUFA	13.4 ± 5.82^{ab}	12.7 ± 4.49^{a}	16.1 ± 6.06^{b}	**0.043**	9.9 ± 2.51	6.8 ± 3.87	6.8 ± 5.65	*0.069
n3/n6	0.8 ± 0.27^{a}	1.3 ± 0.65^{b}	0.9 ± 0.41^{a}	***0.014**	0.6 ± 0.42	1.1 ± 0.52	1.1 ± 0.85	*0.105
DHA/EPA	7.2 ± 3.94	6.7 ± 2.44	6.7 ± 2.82	0.575	2.6 ± 1.40	4.6 ± 2.24	4.4 ± 1.59	0.297
C16:1n7/C16:0	0.1 ± 0.02^{a}	0.1 ± 0.03^{b}	0.1 ± 0.03^{b}	**0.013**	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.03	0.195
C18:1n9/C18:1n7	7.3 ± 2.15^{a}	5.3 ± 1.84^{b}	5.5 ± 2.17^{b}	***0.005**	6.0 ± 2.15	5.2 ± 0.91	4.7 ± 0.49	*0.463
ARA/EPA	6.6 ± 2.56	5.5 ± 4.03	8.1 ± 3.68	*0.065	6.9 ± 1.08	4.3 ± 2.51	8.1 ± 3.13	0.051

(MUFAs), largely C18:1n9, were found in the highest proportion for immature sharks (Table 1). Whole blood was also largely comprised of SFAs followed by MUFAs for all life stages (Table 1). Female size influenced the composition of fatty acids (Tables 2 & 3), with DHA and n3/n6 ratios increasing with body size in both plasma (Fig. 2c,e) and whole blood (Fig. 2j,k). In contrast, plasma SFA decreased with body size, including C16:0 and C18:0 (Fig. 2a,b,d), while just C18:0 decreased in whole blood (Tables 2 & 3, Fig. 2i). In the plasma, a negative relationship was also found between C15:0, C18:2n6, C22:4n6, the bacterial marker (BFA-OFA) and the marker for degree of carnivory (C18:1n9/C18:1n7 ratio) and body size, and positive between C18:1n7 and the diatom marker (C16:1n7/C16:0 ratio) and body size (Tables 2 & 3, Fig. 2f–h). In the whole blood, C18:2n6 also increased and the C16:1n7/C16:0 ratio decreased with body size (Tables 2 & 3).

The plasma concentration of the gonadal steroid hormone testosterone was negatively related with plasma SFA, including C16:0 and C18:0 (Fig. 3a,b,d), while C18:0 in whole blood was negatively related with testosterone (Table 2, Fig. 3g). DHA and n3/n6 ratios increased with increasing 17β-estradiol in plasma (Fig. 3c,e), while C18:2n6 decreased with increasing testosterone (Tables 2 & 3). PUFA n3, DHA, C14:1, C18:2n6 and the DHA/EPA ratio in whole blood were negatively related with 17β-estradiol, while C16:1n7 increased with 17β-estradiol and EPA decreased with increasing testosterone (Tables 2 & 3, Fig. 3g–j). Plasma bacterial marker (BFA-OFA), including C15:0, C18:1n9/C18:1n7 and ARA/EPA were negatively related with testosterone. Plasma BFA-OFA, including C15:0, also decreased with increasing 17β-estradiol, while whole blood BFA-OFA increased with testosterone (Tables 2 & 3).

Among life stages, plasma SFAs, including C18:0, were higher in immature than both non-gravid and gravid females (Table 1, Figs. 4 & 5). In whole blood, C18:0 was also higher in immature compared to non-gravid and gravid females (Table 1). Plasma MUFA C18:1n7 was lower in immature than both non-gravid and gravid females (Table 1, Fig. 4b). Plasma total PUFAs were higher in gravid compared to immature females, and n3 PUFAs, including DHA, were lower in immature than both non-gravid and gravid females (Table 1, Figs. 4 & 5). Plasma n6 PUFAs were higher in gravid compared to non-gravid females, while the coastal/benthic marker C18:2n6 was higher in immature than non-gravid sharks, and ARA was higher in gravid compared to both immature and non-gravid females (Table 1, Figs. 4 & 5).

With respect to trophic markers and nutritional indicators, the plasma n3/n6 ratio was higher in non-gravid compared to both immature and gravid females (Table 1, Fig. 5e). In

Table 2. Linear regression models between plasma and whole-blood fatty acids (%) and total length, and plasma levels of gonadal steroids (testosterone and 17β-estradiol) in female tiger sharks *Galeocerdo cuvier*. Corresponding *t*- and p-values are included. **Bold**: significant values ($p < 0.05$). EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid. NA: data not available (missing values)

Fatty acids		Total length		Testosterone		17β-estradiol	
		t	p	*t*	p	*t*	p
C15:0	Plasma	**–2.053**	**0.045**	**–2.137**	**0.039**	**–2.457**	**0.019**
C17:0	Plasma	0.241	0.811	–1.507	0.141	–0.559	0.580
	Whole blood	–1.675	0.103	0.436	0.668	–0.637	0.532
C14:0	Plasma	–0.697	0.489	–1.289	0.205	0.459	0.649
	Whole blood	0.141	0.889	0.285	0.779	1.174	0.256
C16:0	Plasma	**–2.705**	**0.009**	**–2.175**	**0.036**	–0.351	0.728
	Whole blood	–0.442	0.661	1.260	0.222	1.405	0.175
C18:0	Plasma	**–2.137**	**0.037**	**–3.335**	**0.002**	–0.240	0.811
	Whole blood	**–2.314**	**0.027**	**–2.431**	**0.025**	–0.439	0.666
C14:1c	Plasma	0.874	0.386	0.214	0.832	**–3.028**	**0.004**
	Whole blood	0.907	0.372	0.726	0.477	0.410	0.687
C16:1n7	Plasma	1.193	0.238	0.0238	0.981	0.884	0.382
	Whole blood	1.152	0.258	–0.577	0.570	**2.916**	**0.009**
C18:1n9	Plasma	–0.703	0.485	–0.835	0.409	–0.56	0.579
	Whole blood	–1.217	0.232	–0.250	0.805	–0.752	0.461
C18:1n7	Plasma	**4.021**	**<0.001**	1.116	0.271	0.023	0.982
	Whole blood	0.373	0.712	–0.721	0.479	0.352	0.728
C18:2n6	Plasma	**–2.643**	**0.011**	**–2.748**	**0.009**	–0.844	0.404
	Whole blood	**–2.763**	**0.01**	–1.050	0.308	**–2.464**	**0.024**
C20:5n3 (EPA)	Plasma	1.039	0.305	1.411	0.169	–0.027	0.979
	Whole blood	0.149	0.883	**–2.835**	**0.014**	0.802	0.437
C22:5n3	Plasma	–1.155	0.253	0.530	0.599	1.083	0.286
	Whole blood	–1.514	0.142	–0.292	0.775	–0.713	0.487
C22:6n3 (DHA)	Plasma	**2.350**	**0.023**	1.332	0.191	**2.103**	**0.042**
	Whole blood	**2.552**	**0.016**	1.134	0.272	**–2.127**	**0.048**
C20:4n6 (ARA)	Plasma	–0.084	0.933	–0.918	0.364	0.520	0.609
	Whole blood	–1.732	0.094	–1.487	0.156	–1.969	0.067
C22:4n6	Plasma	**–2.247**	**0.030**	–0.989	0.330	–1.408	0.169
	Whole blood	–1.427	0.190	NA	NA	NA	NA
C22:5n6	Plasma	–0.789	0.435	0.059	0.953	–1.203	0.239
	Whole blood	–0.517	0.624	NA	NA	NA	NA

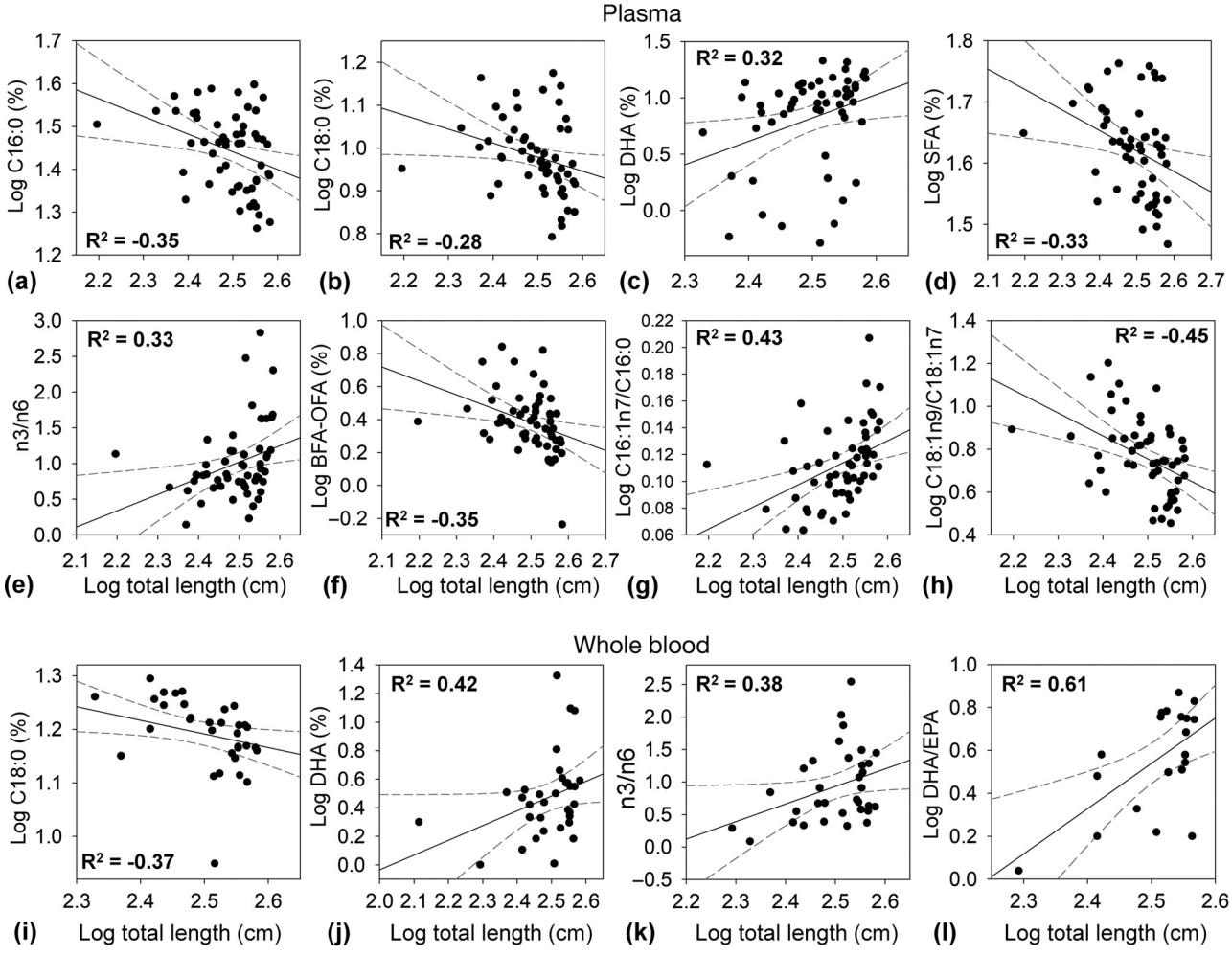

Fig. 2. Significant relationships between plasma and whole-blood fatty acids and total length for female tiger sharks *Galeocerdo cuvier*. Solid black line: regression; dashed lines: 95% confidence intervals. Numbers are log transformed. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acid; BFA: branched-chain fatty acid; OFA: odd-chain fatty acid

plasma, the bacterial marker (BFA-OFA) was higher in immature than non-gravid sharks (Fig. 6a), while the diatom marker (C16:1n7/C16:0 ratio) was lower in immature sharks compared to both non-gravid and gravid females (Fig. 5g). The carnivory index (C18:1n9/C18:1n7 ratio) was higher in immature than both non-gravid and gravid females (Fig. 5h).

The plasma LDA revealed that the first 2 discriminant functions distinguished the life stages (Table A1 in the Appendix, Fig. 6a,b). The first function separated immature and non-gravid females, mainly due to differences in percentages of DHA, C16:0, PUFAs, n3 PUFAs and SFA, while the second function separated non-gravid and gravid females, including their differences in ARA, C16:0, SFA, PUFAs and n6 PUFAs (Fig. 6a,b). From whole-blood fatty acids, LDA revealed that the first 2 discriminant functions distinguished the life stages (Table A1, Fig. 6c,d). The first function separated non-gravid and gravid sharks from immature females, mainly due to the differences in C18:0, DHA, n3 and n6 PUFAs and MUFAs (Fig. 6c,d). The second function separated non-gravid from gravid females, due to their differences in DHA, C18:1n9, PUFAs, n3 PUFAs and MUFAs (Table A1, Fig. 6c,d).

4. DISCUSSION

Through analysis of short-term dietary markers, our study revealed that the nutritional ecology of female tiger sharks, which are generalist apex predators, varied across life stages. Non-gravid and gravid females were characterized by higher percent-

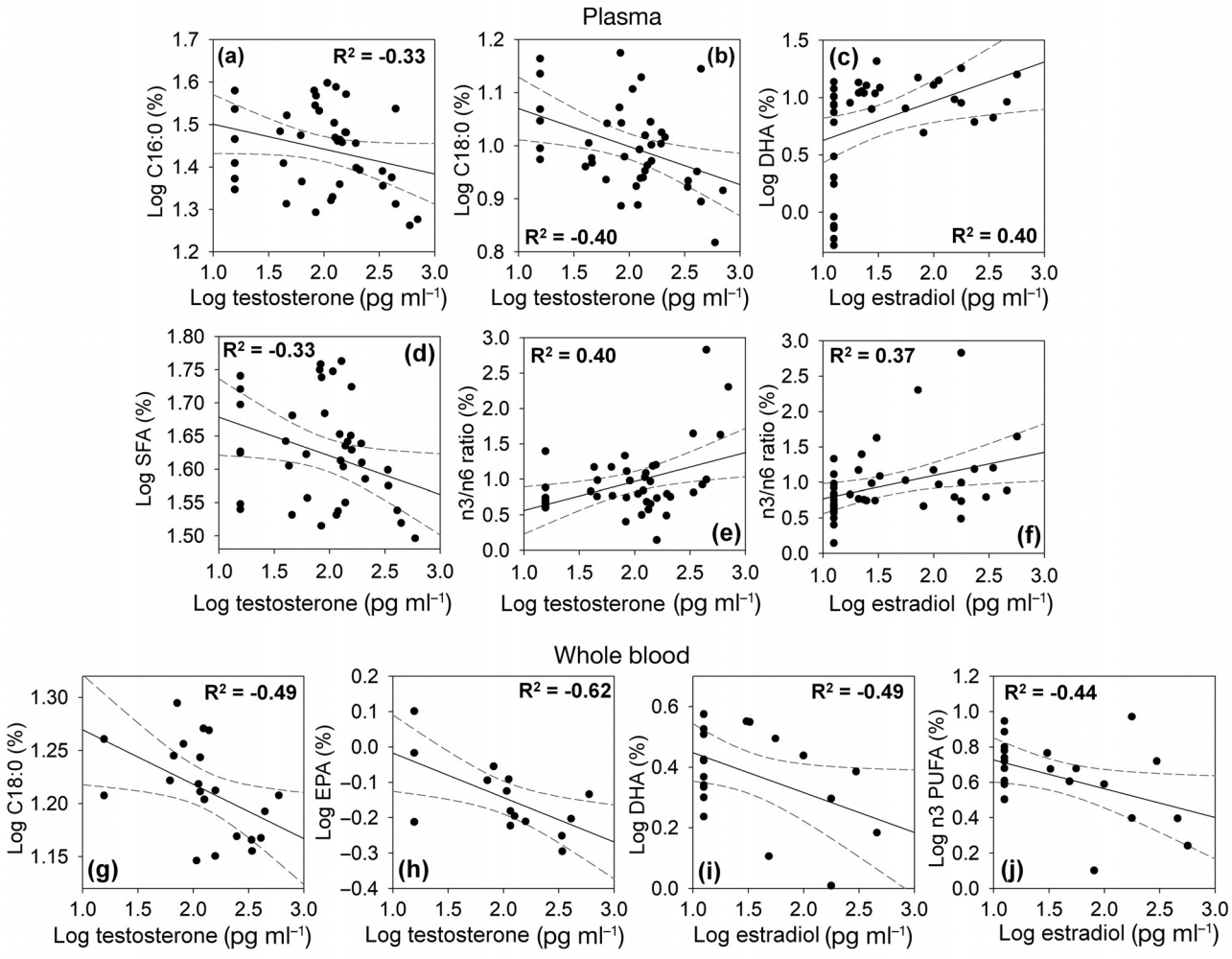

Fig. 3. Significant relationships between plasma and whole-blood fatty acids and reproductive hormones (testosterone and estradiol) in female tiger sharks *Galeocerdo cuvier*. The solid black line represents the regression line, and the dashed lines represent the 95% confidence intervals. Numbers are log transformed. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acid; PUFA: polyunsaturated fatty acid

ages of plasma n3 PUFAs, including DHA, than immature females, which exhibited higher plasma SFAs, including C18:0, than both non-gravid and gravid females. Additionally, gravid females exhibited higher percentages of plasma ARA compared to both immature and non-gravid individuals, demonstrating the importance of n6 HUFAs during gestation. These findings support our first hypothesis that females preparing to reproduce (i.e. adult, but non-gravid) would exhibit greater percentages of HUFAs, and thus better nutritional status (i.e. nutritional composition). While our second hypothesis predicted that gravid females would not be nutritionally deficient in essential fatty acids, gravid females had unexpectedly higher nutritional condition compared to immature females. Corroborating our third hypothesis, smaller females exhibited higher percentages of benthic/coastal and bacterial markers in the plasma, and differed in their trophic markers (e.g. degree of carnivory).

4.1. Nutritional status during reproduction

Increased plasma HUFAs found in adult females, together with a decrease in plasma SFAs, suggest high dependence on HUFAs as a source of metabolic energy for reproduction, whether through dietary and/or non-dietary origin (e.g. mobilized from storage tissues). Non-gravid and gravid females did not differ in plasma DHA and n3 PUFA percentages. However, higher values of the n3/n6 ratio found in non-gravid females, together with the positive relationship between DHA and the n3/n6 ratio with

Table 3. Linear regression models between plasma and whole-blood fatty acids (%) and total length, and plasma levels of gonadal steroids (testosterone and 17β-estradiol) in female tiger sharks *Galeocerdo cuvier*. Corresponding *t*- and p-values are included. **Bold**: significant values ($p < 0.05$). SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; BFA: branched-chain fatty acid; OFA: odd-chain fatty acid

Fatty acids		Total length		Testosterone		17β-estradiol	
		t	p	*t*	p	*t*	p
SFA	Plasma	**−2.561**	**0.013**	**−2.180**	**0.036**	−1.360	0.182
	Whole blood	−0.920	0.364	0.729	0.475	1.763	0.093
MUFA	Plasma	1.507	0.138	0.404	0.688	−0.737	0.465
	Whole blood	−0.065	0.948	−0.181	0.858	−0.126	0.901
PUFA	Plasma	1.210	0.232	0.822	0.416	1.315	0.196
	Whole blood	0.670	0.508	−0.931	0.363	−1.813	0.085
n3 PUFA	Plasma	1.053	0.297	1.087	0.284	1.870	0.069
	Whole blood	1.320	0.196	1.056	0.304	**−2.198**	**0.040**
n6 PUFA	Plasma	0.164	0.870	0.019	0.985	0.467	0.643
	Whole blood	1.057	0.298	−1.017	0.321	−0.608	0.550
n3/n6	Plasma	**2.539**	**0.014**	**2.694**	**0.010**	**2.464**	**0.018**
	Whole blood	**2.068**	**0.047**	0.399	0.694	−0.729	0.474
BFA-OFA	Plasma	**−2.672**	**0.010**	**−2.212**	**0.033**	**−2.123**	**0.040**
	Whole blood	0.028	0.978	**1.289**	**0.212**	0.082	0.936
DHA/EPA	Plasma	0.688	0.495	0.091	0.929	0.823	0.416
	Whole blood	**3.281**	**0.004**	1.618	0.134	**−2.302**	**0.042**
C16:1n7/C16:0	Plasma	**3.434**	**0.001**	1.252	0.218	1.000	0.324
	Whole blood	**2.371**	**0.024**	−0.973	0.342	1.544	0.138
C18:1n9/C18:1n7	Plasma	**−3.693**	**<0.001**	**−2.465**	**0.018**	−0.198	0.844
	Whole blood	−1.050	0.301	0.562	0.580	−0.762	0.455
ARA/EPA	Plasma	−0.957	0.344	**−2.258**	**0.032**	−1.208	0.237
	Whole blood	−1.147	0.267	−0.544	0.599	−0.701	0.501

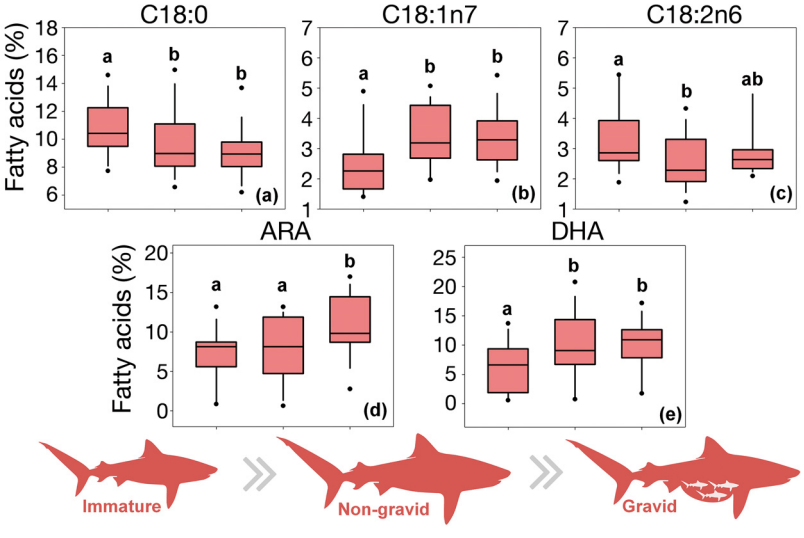

Fig. 4. Boxplots of plasma individual fatty acids (%) in female tiger sharks *Galeocerdo cuvier* at different life stages (left to right in panels: immature, adult non-gravid, gravid); black line indicates the median value and black dots indicate outliers. ARA: C20:4n6 (arachidonic acid); DHA: C22:6n3 (docosahexaenoic acid). Significant differences among life stages are denoted with different superscripts above bars (ANOVA, $p < 0.05$)

reproductive hormones, and the negative relationship between SFAs (including C16:0 and C18:0) and testosterone, suggest that females consume more omega-3-rich prey and/or allocate additional omega-3 from storage tissues during vitellogenesis. Additionally, we observed a negative relationship between reproductive hormones and whole-blood n3 PUFAs, including DHA and EPA, suggesting some allocation of n3 HUFAs from blood cells. Evidence from previous studies show that non-gravid females have higher relative corticosteroid levels than both immature and gravid individuals (B. Rangel, N. Hammerschlag, J. Sulikowski, R. Moreira unpubl. data), and a coincident elevation in body condition and plasma triglycerides (Hammerschlag et al. 2018). These findings suggest that non-gravid females may increase food intake and allocation of energy stored. During the energetically costly process of vitellogenesis, n3 HUFAs are allocated to the ovary through vitellogenin, a lipophosphoglycoprotein rich in DHA, synthesized in the liver under the control of 17β-estradiol (Reading et al. 2017). The importance and selective use of n3 HUFAs in the reproductive processes is well described in other vertebrates, as they affect many important physiological processes, such as brain and eye development and immune and inflammatory responses (Izquierdo et al. 2000, Tocher 2010, Gladyshev et al. 2017, Twining et al. 2018), but in sharks a comparable understanding relating to this process is still limited.

The high plasma ARA percentages found in gravid tiger sharks suggest the possibility of a selective diet on ARA-rich prey species and/or selective mobilization from stored energy. It is also possible that ARA is transferred from mother to offspring during gestation (Iverson et al. 1995), since this omega-6 plays a critical role in embryo development, including immune and inflammatory responses

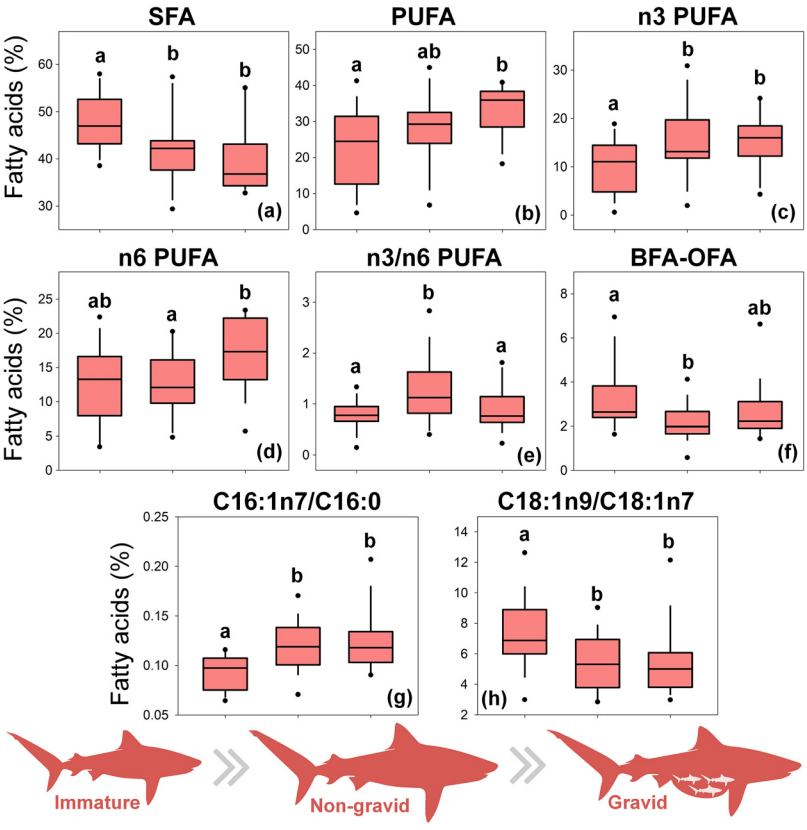

Fig. 5. Boxplots of plasma fatty acid sums (%) and ratios in female tiger sharks *Galeocerdo cuvier* at different life stages (left to right in panels: immature, adult non-gravid, gravid); black line indicates the median value and black dots indicate outliers. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; n3: omega 3; n6: omega 6; BFA: branched-chain fatty acid; OFA: odd-chain fatty acid. Significant differences among life stages are denoted with different superscripts above bars (ANOVA, $p < 0.05$)

(Arts & Kohler 2009, Gladyshev et al. 2017) and improving growth, survival and stress resistance (reviewed by Tocher 2010). Previous studies of marine migratory species have reported an increase in ARA (molecules that originate in coastal areas) found in tissues during reproduction (e.g. gray whale, Caraveo-Patiño et al. 2009; tuna, Sardenne et al. 2017), suggesting a relationship with migration patterns during seasonal breeding. Large female tiger sharks (>270 cm total length) in the study region exhibit seasonal migrations to coastal inshore areas of the subtropics, during cold months, including an area in the Bahamas nicknamed 'Tiger Beach,' which is utilized during gestation by female tiger sharks in the study region (Hammerschlag et al. 2012, Sulikowski et al. 2016). As this geographic area is rich in ARA, the primary component of mucus and algae in coral reefs in the Caribbean (van Duyl et al. 2011), it is possible that, in addition to offering refuge habitat in warm waters that facilitate gestation, Tiger Beach may also provide important nutrient sources to gravid females during embryo development.

Higher plasma SFAs and low DHA, and consequently low n3 PUFA percentages, found in immature sharks compared to other stages, may be a result of the maturation process, e.g. morphological changes in the reproductive tract, or shifts in diet during this period (discussed in Section 4.2). Similarly, decreasing n3 PUFA percentages during sexual maturation in teleost fish have been associated with selective mobilization of n3 PUFAs for gonadogenesis (Uysal & Aksoylar 2005, Manor et al. 2012). Previous findings comparing energetic hormones across life stages in female tiger sharks suggest increased catabolism related to growth and reproductive maturation in immature female tiger sharks (B. Rangel, N. Hammerschlag, J. Sulikowski, R. Moreira unpubl. data), corroborating this hypothesis, as SFAs and MUFAs are the main fatty acids catabolized for energy (Tocher 2003).

Collectively, our results demonstrated that dietary quality of females differed across life stages, likely either by consuming or by selectively storing and allocating specific fatty acids, and that this variation can be related to growth and reproductive processes. If tiger sharks relied only on energy stored for reproduction, we would have expected to find high percentages of SFAs in gravid females, as SFAs tend to be catabolized for energy and PUFAs are normally conserved (Tocher 2003), but this was not the case. Consistent with our hypotheses, our data suggest that gravid females likely require dietary n3 and n6 HUFAs, corroborating a previous hypothesis of a mixed capital–income breeding strategy for tiger sharks, in which females forage during gestation (Hammerschlag et al. 2018). Future research should investigate the diet preferences and fatty acid profiles of potential prey items across all life stages of female tiger sharks to confirm our findings (Fig. 1). The reproductive cycle for tiger sharks in our study region remains unclear, whereas it was found to be biennial in the North Atlantic (Castro 2009) and triennial in Hawaiian tiger sharks

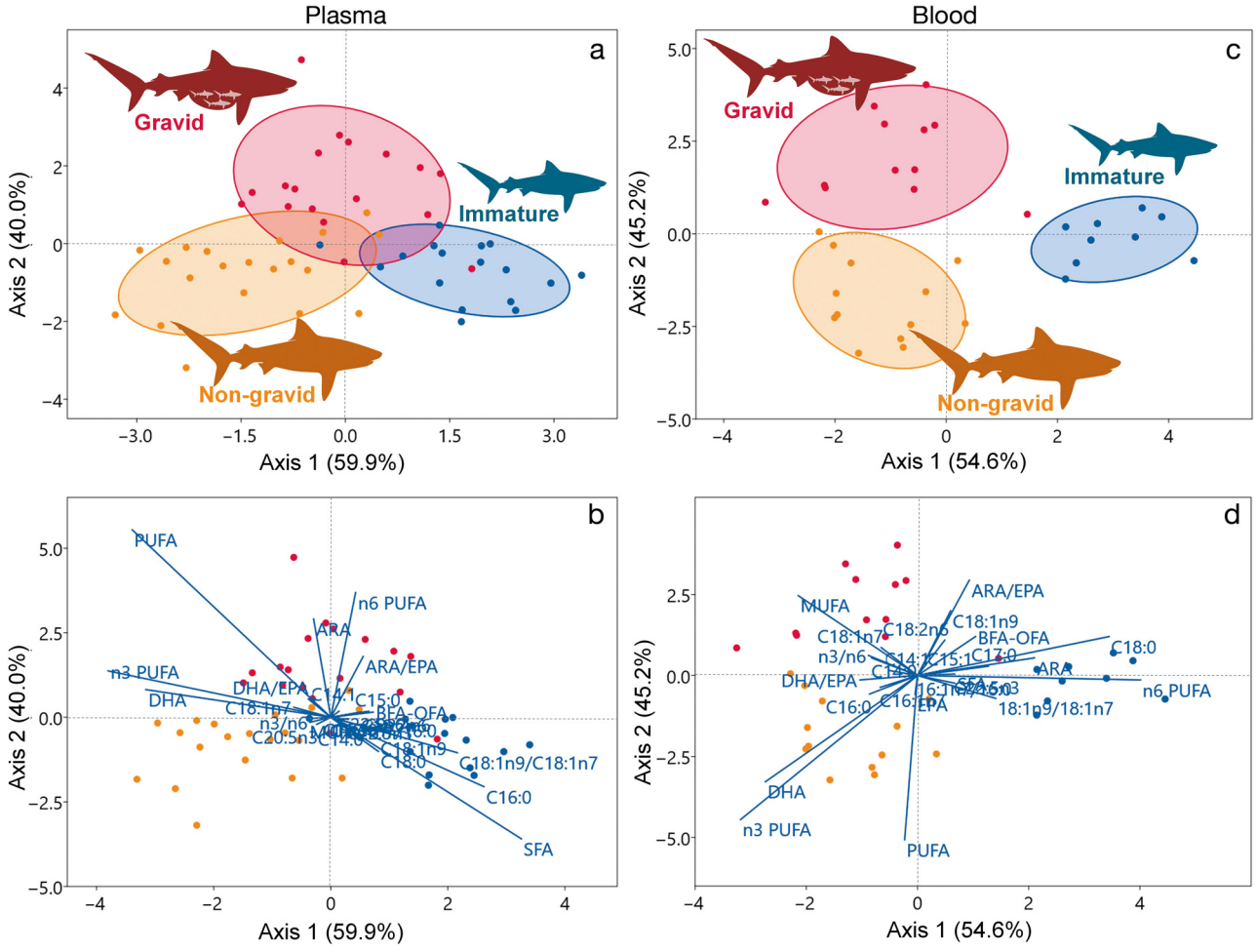

Fig. 6. Linear discriminant function analyses of all fatty acids and trophic markers for different life stages (immature, adult non-gravid, gravid) of female tiger sharks *Galeocerdo cuvier*. (a,b) plasma fatty acids (eigenvalues: Axis 1 = 1.73, Axis 2 = 1.15) and (c,d) whole-blood fatty acids (eigenvalues: Axis 1 = 3.62, Axis 2 = 3.00). The 70% ellipses similarity of each life stage are provided

(Whitney & Crow 2007); thus, additional studies on temporal changes in reproductive status will help to elucidate energetic requirements in each stage. Additionally, determining which fatty acids are transferred to offspring, e.g. by investigating neonates (Belicka et al. 2012, Wai et al. 2012, Rangel et al. 2020), would help to clarify the nutrients required for reproduction.

4.2. Trophic markers and ontogenetic shifts in the diet

The comparison of size-based fatty acid profiles indicated that larger tiger sharks spent more time foraging in offshore pelagic habitats, whereas immature females showed markers of benthic/coastal areas, corroborating our third hypothesis. This was evident in the decrease of plasma n6 PUFAs, including C18:2n6 and C22:4n6, and bacterial detrital markers (BFAs and OFAs), and an increase in DHA and n3/n6 ratio with body size. Additionally, whole-blood n6 PUFAs largely separated immature from both non-gravid and gravid females in the LDA. For example, C18:2n6 is characteristic of terrestrial and freshwater sources (mangroves and terrestrial plants, Kelly & Scheibling 2012), n3/n6 ratios are indicative of marine resources, and DHA is characteristic of marine food webs based on dinoflagellates (Parrish 2013, Meyer et al. 2019). Our result is further supported by other studies on the foraging ecology of tiger sharks, in which higher proportions of prey typical of inshore and shallow habitats, e.g. mollusks (Gulf of Mexico and Atlantic Ocean, Aines et al. 2018), batoids and benthic octopus species (South African waters, Dicken et al. 2017) indicated a high dependence of benthic/coastal nutrients at this life stage.

As tiger sharks grow, larger prey become more important in their diet, including reptiles, birds and marine mammals (e.g. Dicken et al. 2017, Aines et al. 2018, Salinas-de-León et al. 2019). Evidence for increasing trophic position was only evident in the whole-blood DHA/ARA ratio, which was positively correlated with body size. The DHA/ARA ratio is positively correlated with stable isotopes of nitrogen and trophic level in a variety of animals, including other marine predators and mesopredators (e.g. Cardona et al. 2015, Sardenne et al. 2017, Rangel et al. 2020). This is because DHA is biomagnified and preferentially retained at higher trophic levels (Dalsgaard et al. 2003). On the other hand, the C18:1n9/C18:1n7 ratio (carnivory/piscivory index), another typical trophic position marker (Dalsgaard et al. 2003), decreased with increasing body size and was higher in immature than in both non-gravid and gravid females. A lower carnivory index and higher values of the C16:1n7/C16:0 ratio (a diatom marker) found in larger females can be a result of their increased foraging on turtles and mammalian prey (e.g. mysticete whales that feed on small invertebrates situated at low trophic levels), as suggested for tiger sharks in South Africa (Dicken et al. 2017). For example, Cardona et al. (2015) found that loggerhead turtles and some marine bird species had a diatom-based diet; moreover, C16:1n7 is higher in coastal herbivores and is found in high levels in the blubber of marine mammals (Beck et al. 2005, Wai et al. 2011). As large tiger sharks consume highly mobile species (e.g. turtles and whales, Dicken et al. 2017), it is possible that differences found here among life stages may also be influenced by prey species habitat use. Therefore, future studies should consider the influence of the fatty acid profiles of potential prey species.

5. CONCLUSION

Our findings suggest that, despite their generalist and opportunistic feeding behavior, tiger sharks feed on different food sources and/or modulate their fatty acid metabolism differently across growth and reproductive periods, suggesting some adjustment in their nutritional requirement. Our results indicate that during life stages that carry high energetic demands (i.e. vitellogenesis and gestation), females require a diet consisting of n3 and n6 HUFAs. Our results also demonstrated that, although plasma seems to be better for distinguishing diet patterns and nutritional status, whole-blood fatty acids can provide valuable insights into aspects of feeding ecology. Taken together, plasma and whole-blood fatty acids suggest differences in trophic ecology across life stages and ontogenetic shifts in diet. Our results further confirm a high dependence of tiger sharks on coastal/benthic food resources during younger stages, and more pelagic/oceanic and reef-associated food resources during adult stages, especially during reproduction (vitellogenesis and gestation). Such knowledge is particularly important in areas highly used by tiger sharks, such as feeding areas or gestation and nursery grounds, where individual females depend on shared food resources (e.g. as reported in mammals, Stockley & Bro-Jørgensen 2011). This study expands our limited knowledge of the food quality and life stage variation in a generalist marine apex predator. The results also highlight the importance of considering specific life stage classifications when studying the trophic and functional ecology of sharks, as the energetic requirement and composition of fatty acids can vary substantially across life stages. Future studies should address if tiger sharks, despite being a generalist/opportunist species, can feed selectively according to the nutrient content of prey during reproduction and how prey quality can affect their reproductive performance. Finally, we present a conceptual model (Fig. 1) summarizing our findings into testable predictions to aid future investigations on the nutritional ecology of tiger sharks, as well as other apex predators.

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APPENDIX

Table A1. Additional data on linear discriminant functions of fatty acid profiles between life-stages (immature, non-gravid and gravid) for the 2 first axes (Fig. 6). **Bold** values indicate primary fatty acids contributing to dissimilarity. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; BFA: branched-chain fatty acid; OFA: odd-chain fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyun-saturated fatty acid; NA: data not available (missing values)

Fatty acids	Plasma		Whole blood	
	Axis 1	Axis 2	Axis 1	Axis 2
C14:0	0.12	−0.12	−0.14	0.09
C15:0	0.30	0.08	NA	NA
C15:1	NA	NA	0.07	0.03
C17:0	0.01	0.01	0.22	0.05
C16:0	**1.19**	**−0.93**	−0.15	−0.10
C18:0	0.37	−0.46	**0.58**	0.20
C14:1	−0.10	0.16	−0.09	0.03
C16:1n7	−0.06	0.01	−0.11	−0.06
C18:1n9	0.51	−0.25	0.10	**0.34**
C18:1n7	−0.29	0.14	−0.11	0.15
C18:2n6	0.24	0.07	0.08	0.19
C20:5n3 (EPA)	−0.11	−0.09	−0.01	−0.10
C22:5n3	0.13	−0.03	0.11	0.01
C22:6n3 (DHA)	**−1.44**	0.37	**−0.46**	**−0.56**
C20:4n6 (ARA)	−0.13	**1.32**	**0.36**	0.09
C22:4n6	0.27	0.07	NA	NA
C22:5n6	0.06	0.08	NA	NA
ΣBFA-OFA	0.33	0.07	0.18	0.20
ΣSFA	**1.48**	**−1.68**	0.11	−0.04
ΣMUFA	−0.16	−0.02	**−0.36**	**0.42**
ΣPUFA	**−1.54**	**2.52**	−0.04	**−0.86**
Σ n3 PUFA	**−1.73**	0.62	**−0.54**	**−0.75**
Σ n6 PUFA	0.19	**1.68**	**0.68**	−0.02
n3/n6	−0.16	−0.07	−0.15	0.11
DHA/EPA	−0.17	0.25	−0.18	−0.02
C16:1n7/C16:0	−0.01	0.01	0.00	0.00
C18:1n9/C18:1n7	0.98	−0.48	0.24	−0.12
ARA/EPA	0.25	0.82	0.16	0.50

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