BIANCA DE SOUSA RANGEL

Ecofisiologia e relações tróficas de elasmobrânquios: biomarcadores como ferramentas para conservação

Ecophysiology and trophic relationships of elasmobranchs: biomarkers as tools for conservation

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutora em Ciências, na Área de Fisiologia Geral.

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Coorientador: Prof. Dr. Neil Hammerschlag

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pelo amor e todo apoio e dedicação em todos os momentos da minha vida.
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“Nossas habilidades não nos pertencem.
Elas nos são emprestadas para que possam ser colocadas
a serviço dos outros seres”

Professor Fábio Hazin

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Índice

Resumo

Dada a elevada diversidade nas estratégias de história de vida, os elasmobrânquios (tubarões e raias) tornam-se interessantes modelos para o estudo de relações entre a fisiologia e interações ecológicas no ambiente marinho. Embora os esforços para a conservação dos elasmobrânquios, que é atualmente o segundo grupo de vertebrados mais ameaçado do planeta, tenha estimulado um aumento no número de estudos sobre os padrões ecológicos e impactos antrópicos, pouco ainda se sabe sobre sua fisiologia. Assim, nesta tese de doutorado foram investigadas as variações fisiológicas sazonais e espaciais associadas ao estágio de vida e comportamento de tubarões de diferentes histórias de vida, utilizando múltiplas ferramentas não-letais para fornecer uma melhor compreensão dos padrões energéticos e reprodutivos, além de uma base fisiológica que ajude a prever os efeitos de distúrbios ambientais nos tubarões. O capítulo 1 aborda as variações inter- e intraespecíficas na ecologia nutricional de tubarões de diferentes estratégias de história de vida em um sistema insular oceânico protegido, o Arquipélago de Fernando de Noronha. Foram abordados também as variações nos padrões de dieta e condição nutricional e metabólica relacionados à reprodução de fêmeas de tubarões-tigre *Galeocerdo cuvier* (capítulo 2) e machos de tubarões-lixa *Ginglymostoma cirratum* e tubarões-galha-preta *Carcharhinus limbatus* (capítulo 3). Os capítulos 4, 5 e 6 abordam os efeitos da vida urbana na condição nutricional e padrões alimentares de tubarões com diferentes estilos de vida, o tubarão-lixa, o tubarão-galha-preta e o tubarão-tigre, respectivamente. Os resultados mostraram que a influência da urbanização na qualidade da dieta dos tubarões parece ser mais pronunciada em espécies sedentárias, como o tubarão-lixa, quando comparado com espécies mais ativas. Por fim, o capítulo 7 trouxe uma abordagem inédita na pesquisa de tubarões, combinando múltiplos marcadores fisiológicos com informações obtidas através de ultrassonografia e da telemetria acústica passiva para entender relações entre os aspectos fisiológicos e comportamentais de tubarões-tigre expostos ao turismo de alimentação. Os resultados demonstraram que o estágio de vida, a regulação endócrina e a condição nutricional influenciam e/ou são influenciadas pelo tempo que os tubarões passam interagindo com o turismo de alimentação. Em conjunto, os resultados mostraram que os biomarcadores nutricionais, reprodutivos e metabólicos utilizados nesta tese fornecem uma poderosa ferramenta para descrever padrões ecológicos complexos dos tubarões, especialmente quando combinados com outras tecnologias para rastreamento da movimentação e identificação do estágio reprodutivo dos tubarões.

Palavras-chave: Tubarões; Biomarcadores nutricionais; Reprodução; Urbanização; Ecoturismo.

Abstract

Given the high diversity in life-history strategies, elasmobranchs (sharks and rays) become interesting models for the study of relationships between physiology and ecological interactions in the marine environment. Although efforts to conserve elasmobranchs, which are currently the second most endangered group among vertebrates on the planet, have stimulated an increase in the number of studies on ecological patterns and anthropic impacts, little is known about their physiology. Thus, in this Ph.D. thesis, seasonal and spatial physiological variations associated with the life-stage and behavior of sharks from different life histories were investigated. It was used multiple non-lethal tools to provide a better understanding of energetic and reproductive patterns, as well as a physiological basis that helps predict the effects of environmental disturbances on sharks. The chapter 1 addresses inter- and intraspecific variations in the nutritional ecology of sharks with different life-history strategies within a protected oceanic island system, the Fernando de Noronha Archipelago. Variations in dietary patterns and nutritional and metabolic status related to reproduction of female tiger sharks *Galeocerdo cuvier* (chapter 2) and males of nurse sharks *Ginglymostoma cirratum* and blacktip sharks *Carcharhinus limbatus* (chapter 3) were also addressed. Chapters 4, 5 and 6 address the effects of urban living on the nutritional status and diet patterns of sharks with different lifestyles, the nurse, blacktip and tiger shark, respectively. The results showed that the influence of urbanization on the quality of the sharks' diet seems to be more pronounced in sedentary species, such as the nurse shark, when compared to more active species. Finally, chapter 7 brought a new approach to shark research, combining multiple physiological markers with information obtained through ultrasound and passive acoustic telemetry to understand relationships between the physiological aspects and behavior of tiger sharks exposed to food provisioning tourism. The results showed that life-stage, endocrine regulation, and nutritional condition influence and/or are influenced by the time that sharks spend interacting with provisioning tourism. Taken together, the results showed that the nutritional, reproductive and metabolic biomarkers used in this thesis provide a powerful tool to describe complex ecological patterns in sharks, especially when combined with other technologies for tracking movement and identifying the reproductive stage of sharks.

Keywords: Sharks; Nutritional biomarkers; Reproduction; Urbanization; Ecotourism.

Introdução

Estudos descritivos de base ecológica e fisiológica são de grande valor para a conservação e identificação de áreas biologicamente importantes para espécies ameaçadas de extinção (Cooke *et al*., 2021; Hyde *et al*., 2022). Por exemplo, entender do que espécies ameaçadas se alimentam ao longo de seu ciclo de vida pode informar quais presas e nutrientes são mais importantes para seu crescimento e reprodução, além de auxiliar na identificação de áreas críticas durante as fases de vida (Rangel *et al*., 2021a,b; Shipley *et al*., 2022). Ainda, considerando as rápidas mudanças ambientais devido ao desenvolvimento humano, estudos de base ecofisiológica são especialmente úteis para prever como os indivíduos e populações irão responder a alterações no ecossistema. Por exemplo, quais espécies são mais suscetíveis à perturbação antrópica, quais períodos do ano seriam mais críticos, e quais medidas de gestão seriam as mais eficazes (e.g., Madliger e Love, 2015; Cooke *et al*., 2021).

Nesse contexto, as ferramentas fisiológicas têm sido aplicadas com sucesso para monitorar e prever o impacto de distúrbios antrópicos sobre espécies e populações (Wikelski e Cooke 2006). Entre essas ferramentas, os biomarcadores fisiológicos estão entre as ferramentas mais utilizadas, uma vez que essas substâncias podem ser utilizadas como indicadores de um estado ou condição biológica que pode ser medido em uma variedade de amostras, incluindo fluidos corporais, células, tecidos, urina e fezes (Biomarkers Definitions Working Group, 2001). Essas amostras podem ser obtidas de animais vivos (ou seja, vida livre e cativeiro; Hammerschlag e Sulikowski, 2011) por meio de métodos minimante invasivos (e.g., biópsia de tecido), pouco invasivos (e.g., clipes de barbatanas) e não invasivos (e.g., coleta de fezes e muco) (Madliger *et al*., 2018), e de animais mortos, seja por amostragens letais ou dependente da pesca comercial (Heupel e Simpfendorfer, 2010). Especificamente, o uso de biomarcadores é atraente para investigar características fisiológicas associadas à reprodução, alocação de energia, estresse, crescimento, sistema imunológico, desempenho, entre outros (Cooke *et al*., 2013; Madliger *et al*., 2018). Consequentemente, os biomarcadores fisiológicos fornecem informações integradas sobre os padrões, processos e mecanismos que ocorrem em várias escalas espaciais e temporais (Cooke *et al*., 2013; Petybridge *et al*., 2018).

Ao longo do ciclo de vida, os animais lidam com alterações no estado nutricional, relacionados a diferentes processos biológicos, incluindo a hiperfagia e uso dos estoques energéticos associados com a reprodução e migração (Sheridan, 1994; Williams e Buck,

2010). Deste modo, o estudo de animais em ambiente natural é essencial para a compreensão das estratégias alimentares adotadas, diversidade fisiológica e necessidades nutricionais de cada espécie (Wikelski e Cooke, 2006; Cooke *et al*., 2013; Gallagher *et al*., 2017). Dada a elevada diversidade nas estratégias de história de vida (e.g., Cortés, 2000), os elasmobrânquios (tubarões e raias) tornam-se interessantes modelos para o estudo de relações entre a fisiologia e interações ecológicas no ambiente marinho.

Os elasmobrânquios possuem uma fisiologia incomum, incluindo a estratégia osmorregulatória, organização do metabolismo energético e diversos modos reprodutivos, que são resultantes de sua longa história evolutiva de mais de 420 milhões de anos (Speers-Roesch e Treberg, 2010; Ballantyne, 2016). Dentre suas particularidades fisiológicas, destacam-se a retenção de ureia como estratégia osmótica, a ausência de albumina, limitada oxidação de ácidos graxos no músculo esquelético e cardíaco e uma grande dependência de corpos cetônicos, principalmente o ß-hidroxibutirato, e aminoácidos como combustíveis oxidativos (Speers-Roesch *et al*., 2006; Ballantyne, 2016). Além disso, os elasmobrânquios têm histórias de vida extremas, caracterizadas por crescimento lento, maturidade sexual tardia, baixa fecundidade, longo período de gestação e alguns dos mais altos níveis de investimento materno de todos os vertebrados (Cortés, 2000). Apesar de suas características únicas dentre os vertebrados, pouco tem sido investigado sobre a fisiologia de elasmobrânquios, considerando a alta diversidade deste grupo de mais de 1200 espécies (Ebert *et al*., 2021).

Devido ao crítico estado de conservação dos elasmobrânquios, a maioria dos estudos abrangendo sua fisiologia tem se concentrado no uso de abordagens não-letais e validação de ferramentas fisiológicas para estratégias de manejo e conservação do grupo (e.g., Hammerschlag e Sulikowski, 2011; Awruch *et al*., 2014). Isso porque os elasmobrânquios têm sofrido severas reduções populacionais nos últimos anos devido à sobrepesca (Pacoureau *et al*., 2021; Dulvy *et al*., 2021). Dados recentes mostram que desde a década de 70 a abundância de espécies oceânicas diminuiu em 71% (Pacoureau *et al*., 2021). Globalmente, estima-se que mais de 100 milhões de tubarões sejam mortos todos os anos pela pesca comercial e esportiva (Niedermueller *et al*., 2021), e que um terço (37,5%) das mais de 1200 espécies de elasmobrânquios conhecidas estejam ameaçadas de extinção (Dulvy *et al*., 2021). Como resultado, os elasmobrânquios estão entre os vertebrados mais ameaçados de extinção avaliados até o momento, ficando atrás apenas dos anfíbios (IUCN, 2021 - www.iucnredlist.org).

Sendo predadores de topo ou mesopredadores, os elasmobrânquios desempenham destacado papel funcional na dinâmica dos ecossistemas marinhos, estuarinos e dulcícolas (Stevens *et al*., 2000, Ferretti *et al*., 2010). Ecologicamente, eles influenciam diretamente as interações tróficas através da predação e, indiretamente, outros níveis tróficos através da facilitação comportamental, modificação de habitat e risco de predação (Heithaus *et al*., 2022). Além disso, uma vez que muitos tubarões são predadores altamente móveis, eles podem atuar como vetores de nutrientes e matéria orgânica, desempenhando um papel significativo, porém pouco conhecido, na ciclagem de nutrientes do ecossistema marinho (Allgeier *et al*., 2016; Hammerschlag *et al*., 2019). Essa ciclagem pode ocorrer via ingestão e excreção de nutrientes dentro dos mesmos habitats em que o alimento foi consumido ou via translocação espacial vertical e horizontal de nutrientes através do consumo de recursos em um local (e.g. zona epipelágica) e excreção ou ingestão em outro (e.g. zona demersal) (Schmitz *et al*., 2010; Kiszka *et al*., 2022). É importante mencionar ainda que a alimentação seletiva, impulsionada por demandas fisiológicas durante alguns processos do seu ciclo de vida, como por exemplo para a reprodução, pode alterar a qualidade de nutrientes excretados sazonalmente (Schmitz *et al*., 2010). Portanto, espécies com tais atributos funcionais podem afetar diretamente a produtividade local e o recrutamento de outros animais (Allgeier *et al*., 2016).

Embora relativamente bem estudada, a dinâmica alimentar de tubarões é complexa, uma vez que está diretamente relacionada com a: (1) disponibilidade sazonal de presas, (2) complexidade das interações tróficas de cada local, (3) estratégia alimentar da espécie (i.e., se são especialistas ou oportunistas), (4) tamanho dos indivíduos (i.e., variação ontogenética da dieta), e (5) da interação com outros tubarões através da competição ou particionamento de recursos alimentares (Hussey *et al*., 2015; Shipley *et al*., 2022). Além disso, a produção primária de cada local pode influenciar significativamente a dinâmica de nutrientes dentro das teias alimentares e consequentemente a condição nutricional de predadores (Rangel *et al*., 2021a,b; 2022; Shipley *et al*., 2022). Portanto, estudos em escala regional são de grande importância para entender o papel funcional e a ecologia nutricional dos tubarões.

Apesar do aumento de estudos descritivos nos últimos anos sobre os padrões ecológicos e diferenças fisiológicas entre espécies de elasmobrânquios, relacionados ao uso de habitat e filogenia (Pethybridge *et al*., 2010), pouco ainda se sabe sobre como as variações sazonais, espaciais e do estágio de vida afetam sua condição nutricional e interações tróficas. Desta forma, realizar estudos em condições naturais para descrição de padrões fisiológicos fornece a oportunidade de testar e refinar hipóteses para desenvolver quadros preditivos sobre os

recursos energéticos utilizados em determinados estágios da vida, co-ocorrência e declínio de espécies chaves nos ecossistemas marinhos (Gallagher *et al*., 2017; Matich *et al*., 2017). Tal conhecimento é fundamental para entender os efeitos de perturbações ambientais e antrópicas nos processos fisiológicos e na transferência de energia nas redes tróficas de mesopredadores e predadores marinhos.

Nesta tese foram investigados os efeitos da variação espacial, temporal e do estágio de vida na ecologia nutricional de tubarões de diferentes estratégias de história de vida, combinando traços fisiológicos e variáveis ecológicas para fornecer ferramentas úteis e uma base fisiológica que pode ajudar a prever os efeitos das mudanças ambientais. Especificamente, investigamos três perguntas principais (Fig. 1), divididas em sete capítulos que tiveram com objetivo:

Capítulo 1: Investigar a condição nutricional e relações tróficas em tubarões de diferentes estratégias de história de vida em um sistema insular oceânico protegido (**Ecologia nutricional de tubarões em um refúgio oceânico no Atlântico Equatorial, o Arquipélago de Fernando de Noronha**).

Capítulo 2: Avaliar a variabilidade na ecologia nutricional em diferentes fases da história de vida de fêmeas de tubarões-tigre *Galeocerdo cuvier* (i.e., imatura, adulta/não grávida e grávida) através da integração de ácidos graxos plasmáticos e hormônios reprodutivos (17β-estradiol e testosterona) (**Dietary and reproductive biomarkers in a generalist apex predator reveal differences in nutritional ecology across life stages**).

Capítulo 3: Analisar como o estado energético e nutricional varia em relação ao estágio reprodutivo em machos de duas espécies de tubarões com ciclo anual, porém com diferentes estilos de vida, o tubarão-lixa (*Ginglymostoma cirratum*), espécie sedentária e bentônica e o tubarão-galha-preta (*Carcharhinus limbatus*), espécie ativa e epipelágica (**Physiological markers suggest energetic and nutritional adjustments in male sharks linked to reproduction**).

Capítulo 4: Investigar a influência da urbanização nos padrões alimentares e na qualidade nutricional de tubarões-lixa (*Ginglymostoma cirratum*) juvenis entre áreas altamente urbanizadas e relativamente pristinas da Baia de Biscayne Bay, no sul da Flórida. (**Urban living influences the nutritional quality of a juvenile shark species**).

Capítulo 5: Investigar as relações entre a exposição à urbanização e a ecologia nutricional em um tubarão costeiro ativo, o tubarão-galha-preta (*Carcharhinus limbatus*),

através da análise comparativa de marcadores dietéticos de curto prazo (ácidos graxos plasmáticos) e médio prazo (isótopos estáveis no sangue total) entre tubarões amostrados em duas áreas expostas a diferentes graus de urbanização costeira no sul da Flórida (**Effects of urbanization on the nutritional ecology of a highly active coastal shark: preliminary insights from trophic markers and body condition**).

Capítulo 6: Analisar se tubarões altamente móveis, migratórios exibem variações associadas na condição metabólica e nutricional, usando como modelo o tubarões-tigre (*Galeocerdo cuvier*) juvenis entre regiões expostas a diferentes graus de desenvolvimento urbano (i.e., sul da Flórida (EUA) e nas Bahamas) (**Metabolic and nutritional condition of juvenile tiger sharks exposed to regional differences in coastal urbanization**).

Capítulo 7: Investigar a relação entre condição fisiológica e o comportamento espacial tubarões-tigre (*Galeocerdo cuvier*) fêmeas expostas ao turismo de alimentação nas Bahamas através da integração de múltiplos marcadores fisiológicos relacionados à condição reprodutiva, metabólica e nutricional, integrados com ultrassonografia e telemetria acústica passiva (**Physiological state predicts space use of sharks at a provisioning tourism site**).

Figura 1. Gráfico conceitual ilustrando as três perguntas principais da presente tese. Os números são correspondentes aos capítulos, apresentados a seguir. Ilustações dos tubarões de Kelly Quinn e Alexandre Huber.

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Capítulo 1

Ecologia nutricional de tubarões em um refúgio oceânico no Atlântico Equatorial, o Arquipélago de Fernando de Noronha

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Resumo

O entendimento da dinâmica de processos biológicos e interações ecológicas de animais com importantes atributos funcionais, como os tubarões, é crucial para subsidiar os planos de conservação e gestão de áreas marinhas protegidas. Neste estudo investigamos aspectos relacionados à ecologia nutricional de tubarões de diferentes estratégias de história de vida em diferentes estações (i.e., seca e chuvosa) em um sistema insular oceânico protegido, o Arquipélago de Fernando de Noronha. Usamos a concentração de substratos energéticos plasmático (i.e., proteínas totais, glicose [GLU], triglicérides [TAG] e colesterol [CHOL]) e perfil de ácidos graxos plasmático para avaliar a condição nutricional e relações tróficas dos tubarões-de-recife *Carcharhinus perezi*, tubarões-tigre *Galeocerdo cuvier*, tubarões-lixa *Ginglymostoma cirratum* e tubarões-limão *Negaprion brevirostris*. Os resultados mostraram que os tubarões mais ativos (i.e., *C. perezi, G.cuvier, C. falciformis* e *C. plumbeus*) apresentaram maiores concentrações de GLU enquanto que os menos ativos (i.e., *G. cirratum e N. brevirostris*) tiveram maiores concentrações de proteínas totais e TAG. O perfil de ácidos graxos dos tubarões revelou uma separação entre os tubarões *G. cuvier* e *G. cirratum* do *C. perezi* e *N. brevirostris*. Os ácidos graxos polinsaturados ômega-6 foram os principais responsáveis pela sobreposição dos tubarões *G. cirratum* e *G. cuvier*, sugerindo que as espécies compartilham os mesmos recursos alimentares e/ou que os tubarões *G. cuvier* estão se alimentando de *G. cirratum*. Além disso, as variações sazonais observadas nos substratos energéticos e ácidos graxos dos tubarões *G. cuvier* e *G. cirratum* entre a estação seca e úmida sugerem flutuações na dieta entre as duas estações e ou uma relação com o período reprodutivo das espécies. Através da integração de diferentes biomarcadores nutricionais, o presente estudo investigou, pela primeira vez, aspectos da ecologia nutricional de tubarões em um sistema insular do Oceano Atlântico Sul. Futuros estudos e monitoramento a longo prazo dos padrões ecofisiológicos são recomendados, especialmente porque Fernando de Noronha é um arquipélago oceânico exposto à crescente visitação turística.

Palavras-chave: área marinha protegida, condição nutricional, elasmobrâqnuios, interações tróficas, padrões de dieta.

1.1. Introdução

Devido ao seu isolamento geográfico, as ilhas oceânicas são ambientes únicos em termos de biodiversidade e interações ecológicas (Kosaki *et al*., 2017; Pinheiro *et al*., 2018). Ilhas e montes submarinos oceânicos são locais altamente produtivos em meio a águas oceânicas abertas e oligotróficas (i.e. efeito ilha), o que favorece a manutenção de uma complexa cadeia trófica (*e.g.*, Morato *et al*., 2010). Esses refúgios oceânicos são considerados áreas de grande importância biológica, por agregarem uma grande densidade e biomassa de espécies e servirem como áreas de alimentação, berçário, crescimento e reprodução para diversos animais residentes e migratórios, incluindo espécies sobrepescadas em regiões costeiras (*e.g.*, Fontes *et al.*, 2014; Cambra *et al*., 2021). Por essas características, esses locais se tornam alvo para criação de áreas marinhas protegidas (Hyde *et al*., 2022). Portanto, o entendimento da dinâmica de processos biológicos e interações ecológicas de animais com importantes atributos funcionais, como os tubarões, é crucial para subsidiar os planos de conservação e gestão de áreas marinhas protegidas nestes locais.

Ecologicamente, os tubarões ocupam posições tróficas de destaque nas teias alimentares marinhas e influenciam diretamente as interações tróficas através da predação e, indiretamente, através da facilitação comportamental, modificação de habitat e risco de predação (Fig. 1, Hammerschlag *et al*., 2019; Heithaus *et al*., 2022). Atuam desta forma como importantes vetores de nutrientes e matéria orgânica, desempenhando um papel significativo na ciclagem de nutrientes do ecossistema marinho (Allgeier *et al*., 2016; Hammerschlag *et al*., 2019). Essa ciclagem pode ocorrer via ingestão e excreção de nutrientes dentro dos mesmos habitats em que o alimento foi consumido ou via translocação espacial vertical e horizontal de nutrientes através do consumo de recursos em um local (e.g. zona epipelágica) e excreção ou ingestão em outro (*e.g.*, zona demersal) (Schmitz *et al*., 2010; Kiszka *et al*., 2022).

Não surpreendente, o Arquipélago Fernando de Noronha é uma região importante para conservação dos tubarões no Oceano Atlântico equatorial, servindo como área de alimentação, reprodução, berçário e para o crescimento durante a fase juvenil das espécies: tubarão-lixa (*Ginglymostoma cirratum*) (Garla *et al*., 2009; Afonso *et al*., 2016), atualmente listado como vulnerável em ambas as listas de espécies ameaçadas da IUCN - União Internacional para a Conservação da Natureza (2021-3), e na lista nacional de espécies ameaçadas (Resolução Comissão Nacional de Biodiversidade - CONABIO 08/2021); tubarão-limão (*Negaprion brevirostris*) (Garla *et al*., 2009), listado como vulnerável pela IUCN e em perigo na lista nacional; e do tubarão-de-recife (*Carcharhinus perezi*) (Garla *et al*., 2006; Rangel *et al*., 2022), listado como em perigo pela IUCN e vulnerável na lista nacional. Além

disso, existem indícios de que o icônico tubarão-tigre (*Galeocerdo cuvier*), listado como quase ameaçado na IUCN, também possa utilizar o arquipélago para fins reprodutivos, ou pelo menos durante a gestação (Soto, 2001; Rangel B.S. *et al*., em revisão). É importante ressaltar que o arquipélago é um *hotspot* para o tubarão-tigre no Atlântico, sendo a área com maior diversidade genética global já descrita para a espécie e, portanto, sugerido como um importante refúgio ecológico (Carmo *et al*., 2020). Já para o tubarão-lombo-preto (*Carcharhinus falciformis*), quinta espécie mais abundante no arquipélago e listado atualmente como vulnerável na IUCN e lista nacional, não existem informações biológicas para o local.

Figura 2. Modelo conceitual dos papéis ecológicos dos tubarões em teias alimentares marinhas baseado em um modelo para pequenos cetáceos de Kiszka *et al.* (2022) adaptado para algumas das espécies analisadas no presente estudo. Ilustrações dos tubarões cedidas pela Kelly Quinn e demais recursos gráficos utilizados do www.canva.com.

Embora existam relações claras entre a variação do uso de recursos alimentares e condição energética e sua influência na aptidão do indivíduo, raros estudos têm explorado como esses padrões ocorrem em tubarões (*e.g*., Shipley *et al*., 2022). Para preencher parte destas lacunas, o presente trabalho tem como objetivo investigar os aspectos fisiológicos

relacionados à condição nutricional e relações tróficas em tubarões de diferentes estratégias de história de vida, no sistema insular de Fernando de Noronha. Biomarcadores nutricionais foram utilizados como ferramentas para acessar: (1) a condição nutricional, através da análise de substratos energéticos (*i.e.*, triglicérides [TAG], colesterol [CHOL], glicose [GLU], proteínas totais) e perfil de ácidos graxos; e (2) a ecologia trófica e interação entre os tubarões, através da análise do perfil de ácidos graxos; (3) variação sazonal desdes biomarcadores nos tubarões *G. cirratum* e *G. cuvier*. Este é o primeiro estudo que investiga a ecologia nutricional de tubarões em um sistema insular do oceano Atlântico Sul, e servirá, portanto, de base para futuros estudos sobre a complexidade trófica de tubarões em sistemas insulares oceânicos nesta região, bem como para o monitoramento a longo prazo da saúde dos tubarões e do ecossistema local.

1.2. Material e Métodos

1.2.1. Área de estudo

O Arquipélago de Fernando de Noronha (03°51'S, 32°25'W) (Fig. 2) é um grupo isolado de ilhas vulcânicas localizadas a 345 km do nordeste do Brasil. Parte do arquipélago compreende uma Área Marinha Protegida, o Parque Nacional Marinho de Fernando de Noronha, que protege os ecossistemas costeiros até a isóbata de 50 m. A outra parte consiste em uma área de uso sustentável (ou seja, a Área de Proteção Ambiental Fernando de Noronha – Rocas – São Pedro e São Paulo), onde a pesca é permitida com algumas restrições e a pesca de tubarão é proibida (ICMBio 2017). A região está sob a influência da Corrente Sul Equatorial e apresenta um clima oceânico tropical quente, com estação chuvosa (março a julho) e estação seca (agosto a fevereiro) (Barcellos *et al*., 2011). A temperatura da água do mar e a salinidade são em média de 26°C e 36‰, respectivamente, e são relativamente constantes durante todo o ano.

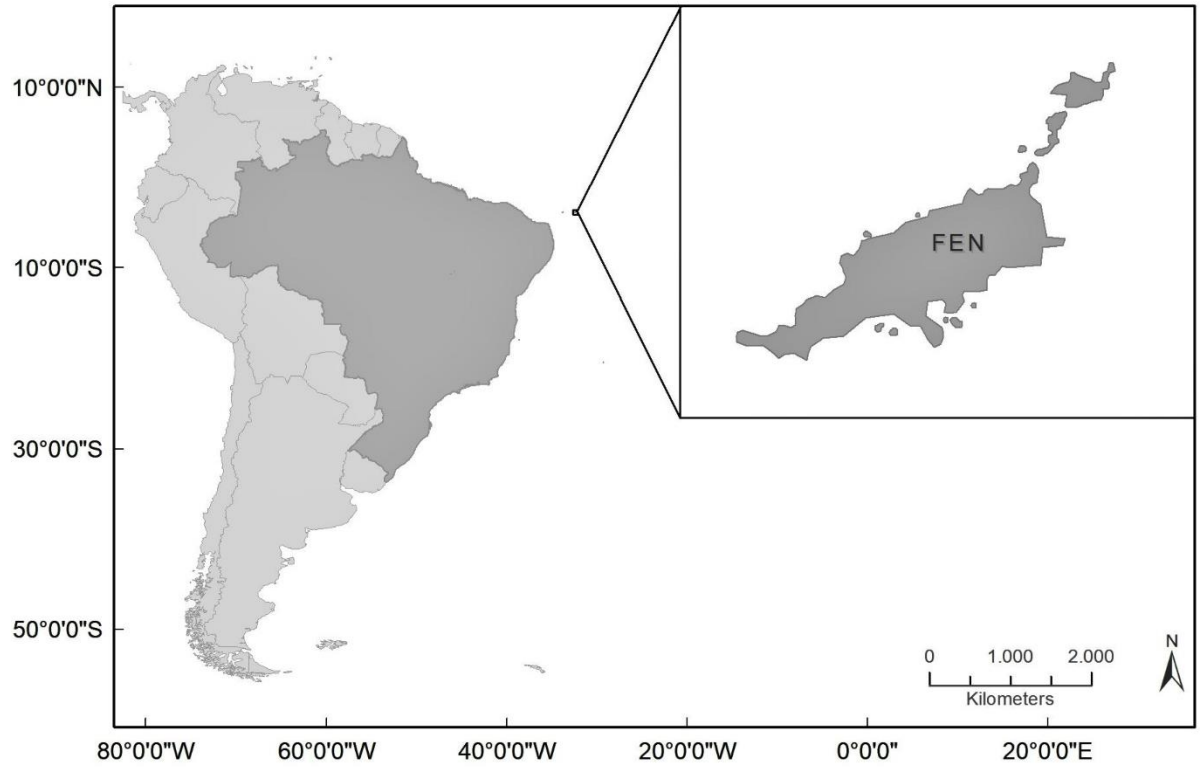

Figura 2. Mapa com a localização do Arquipélago de Fernando de Noronha, oceânico no Atlântico Equatorial. Feito por Nayara Bucair.

1.2.2. Captura, manejo e amostragem dos animais

Os tubarões foram capturados através do uso de linha de espera de fundo e de superfície e linha de mão (Fig. 3). A linha de espera e fundo foi o principal método utilizado, por ser um método padronizado e minimamente invasivo de pesca conforme descrito em Gallagher *et al.* (2014a). Em resumo, foram utilizadas de cinco a sete linhas de espera, cada uma delas consiste em uma poita de aproximadamente 15 kg amarrada a um cabo principal de polietileno torcido de 10 mm ou polipropileno trançado de 12 mm que vai à superfície por meio de uma bóia inflável (defensa náutica tamanho A2). Um chicote de ~20 m feito de monofilamento 300 (3 mm) é ligado a haste da poita por uma manilha reta de aço (10mm) e um Snap (15 cm), um anzol circular 18/0 sem *offset* é fixado na extremidade do chicote de monofilamento através de um girador 9/0 e cabo de aço (3mm). Como iscas foram utilizadas carcaças de teleósteos do gênero *Thunnus* e da espécie *Elagatis bipinnulata*. O tempo de imersão padrão estipulado do petrecho para a captura foi entre 60-90 minutos, cronometrado após o lançamento da primeira linha de espera. Decorrido este tempo, cada linha de espera foi sequencialmente verificada quanto à presença de tubarões.

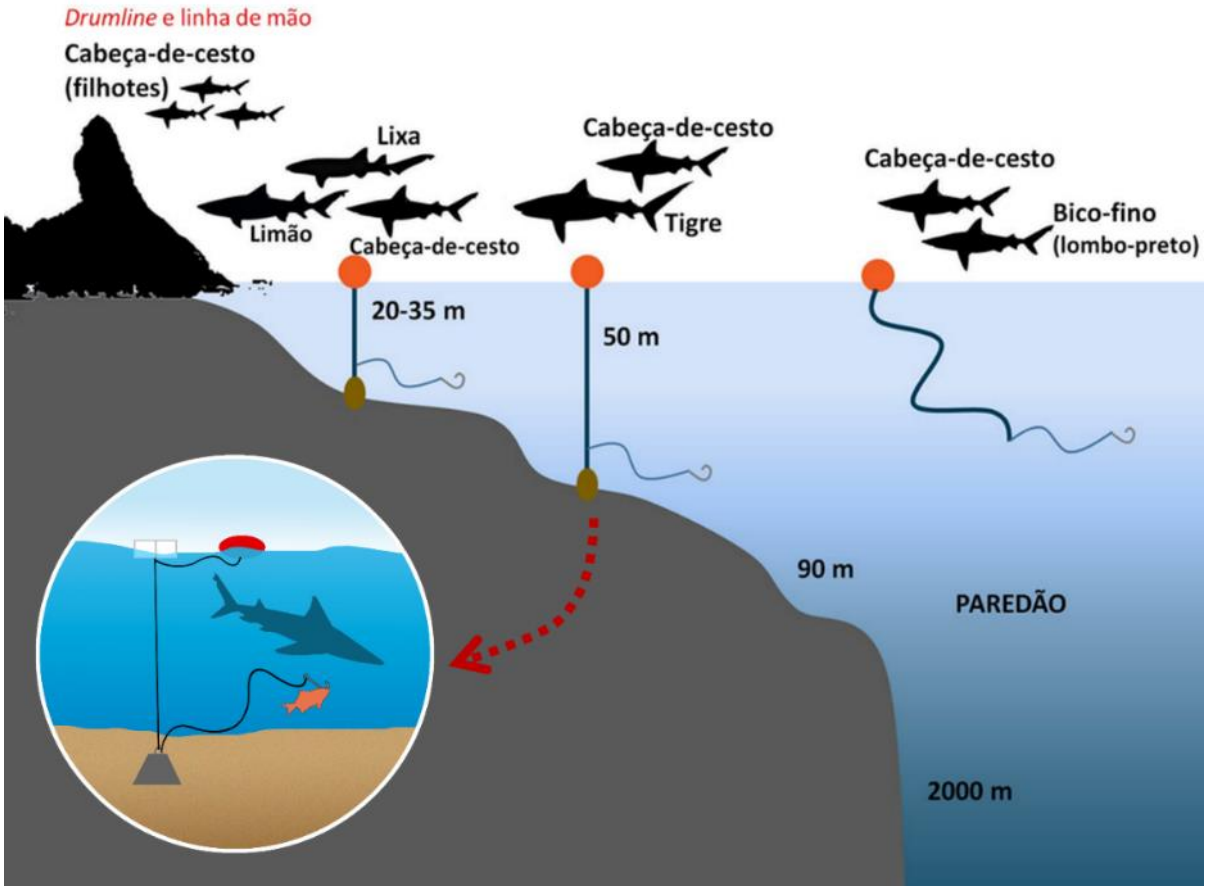

Figura 3. Detalhamentos dos métodos e locais de captura de tubarões no arquipélago.

O manejo pós captura dos tubarões seguiu protocolos distintos para animais de pequeno (>150 cm) e grande porte (<150 cm). Os de pequeno porte foram embarcados, e então uma mangueira com fluxo contínuo de água salgada foi inserida na boca do tubarão para bombear ativamente a água sobre as brânquias enquanto o animal ficava temporariamente imobilizado. Já os tubarões de grande porte foram mantidos dentro da água, contidos ao lado do barco para a realização de todos os procedimentos. Todos os tubarões e as raias capturados passaram pelos seguintes procedimentos: (1) foram identificados em nível de espécie com base em suas características morfológicas externas; (2) o sexo foi identificado através da presença/ausência dos cláspers; (3) receberam uma marca plástica do tipo *Stainless steel head dart tags* (SSD), identificados numericamente e com dados de contato em caso de recapturas, que foi inserida na inserção da primeira nadadeira dorsal e na região dorsal/posterior em raias; (4) foram obtidos dados biométricos (comprimento total, cm); (5) coletadas amostras de tecido dérmico da nadadeira com uma tesoura (~ 5mm) e tecido muscular com o auxílio de um *punch* para biópsia de 6 mm (para análise de ácidos graxos, resultados não apresentados aqui); (6) coletadas amostras de sangue, obtidas por punção da vasculatura caudal (~5 mL para animais de pequeno porte e 10 mL para animais de grande porte), utilizando seringas descartáveis e

heparinizadas. As amostras de tecido da nadadeira foram colocadas em etanol absoluto (para futuras colaborações em estudos de genética) e amostras de músculo e sangue foram acondicionadas em gelo no barco e posteriormente congeladas.

Todos os indivíduos foram marcados com marcas plásticas externas do tipo *Stainless steel head dart tags* (SSD). As marcas são confeccionadas na coloração amarela, com as inscrições de contato na cor preta. Três tubarões-tigre (fêmeas) receberam transmissores satélite, do tipo SPOT (*smart position and temperature transmitting tags*; SPOT5; Wildlife computers, USA). Na base de pesquisa, as subamostras de sangue foram então centrifugadas (3500 rpm, 410 × g) por 2 minutos em temperatura ambiente para separação do plasma. Todas as amostras de tecido, sangue total e plasma foram colocadas em tubos criogênicos, congeladas até serem acondicionadas em freezer em temperatura de -20°C, onde permaneceram até o período do transporte. No laboratório, as amostras estão mantidas em temperatura de -80°C até a realização das análises. Todos os métodos descritos acima estão previamente aprovados pelo ICMBio (SISBIO nº 80761) e pelo Comitê de Ética do Instituto de Biociências da Universidade de São Paulo (CEUA Protocolo no. 362/2020).

1.2.3. Substratos energéticos e ácidos graxos

Os substratos energéticos, incluindo os TAG, CHOL, glicose e proteínas totais foram analisados para avaliar a demanda energética e a variação da condição nutricional entre as espécies e estágios de vida. Os substratos energéticos foram medidos em amostras de plasma utilizando kits comerciais (Labtest®) e métodos colorimétricos correspondentes, utilizando-se um espectrofotômetro ELISA (Spectra Max 250, Molecular Devices).

O perfil de ácidos graxos foi utilizado para avaliar a variação da qualidade nutricional dos tubarões e para inferir sobre as interações tróficas intra e interespecíficas (*e.g.*, Rangel *et al.*, 2021a,b; 2022). Amostras plasma (100µL) foram analisadas por transmetilação direta descrita por Parrish *et al*. (2015a). Resumidamente, as amostras foram homogeneizadas em 3mL da solução metanol: diclorometano: ácido clorídrico concentrado (10:1:1 v:v:v) e mantidas em banho-maria por 2 horas a 80ºC. Após a retirada e resfriamento, foram adicionados 1 ml de água Milli-Q, seguido de 1.8 ml da solução hexano:diclorometano (4:1 v:v). Após a mistura completa, os tubos foram centrifugados a 2.000 rpm durante 5 min. A camada superior orgânica foi então removida e transferida para os *vials* de injeção e evaporados em nitrogênio. Os ácidos graxos foram analisados com o cromatógrafo a gás (Varian modelo 3900) acoplado a um ionizador de chama e auto injetor (CP8410). Os ácidos

graxos foram identificados com base no tempo de retenção, utilizando-se padrões conhecidos (Supelco, 37 *components* – Sigma – Aldrich, dentre outros).

Os ácidos graxos essenciais, ou seja, DHA (C20:6n3, ácido docosahexaenoico), ARA (20:4n6, ácido araquidônico) e EPA (C20:5n3, eicosapentaenoico), bem como a soma dos ácidos graxos polinsaturados (PUFA, do inglês *polyunsaturated fatty acids*) e saturados (SFA, do inglês *saturated fatty acids*), e razões PUFA ômega-3 / PUFA ômega-6 (n3/n6) e ARA/EPA foram usados para comparar os índices de qualidade nutricional do tubarão (Tocher 2003; Arts e Kohler 2009) e inferir respostas fisiológicas de eicosanoides (Tocher 2003). Apesar de estarem sujeitos à biossíntese quando transferidos da presa para o predador, os ácidos graxos permanecem inalterados em sua maioria relativamente, permitindo o seu uso como biomarcadores nutricionais (Dalsgaard *et al*., 2003; Budge *et al*., 2006; Iverson 2009). Em termos de marcadores tróficos, o DHA foi usado como indicador de dinoflagelados, enquanto C16:1n7/C16:0 como indicador de diatomáceas (Parrish *et al*., 2009, Budge *et al*., 2006). Além disso, os valores de ARA e C18:2n6 foram considerados úteis indicador de se uma espécie habita ambientes costeiros/bentônicos (Sardenne *et al*., 2017), e os ácidos graxos de cadeia ímpar (OFA, do inglês *odd chain fatty acids*) e ácidos graxos de cadeia ramificada (BFA, do inglês *branched chain fatty acids*) como biomarcadores de bactérias heterotróficas (Dalsgaard *et al*., 2003). Os ácidos graxos que representavam menos de 0,5% foram excluídos das análises estatísticas.

1.2.4. Análise de dados

Os substratos energéticos e razões dos ácidos graxos [n3/n6, EPA/ARA e DHA/ARA]) dos tubarões foram comparados entre as espécies utilizando-se Análise de Variância (*one-way* ANOVA) (Past 3.2). Para dados paramétricos, foi aplicado o teste post-hoc *Tukey*. Para os dados não paramétricos foi aplicado o teste Kruskal-Wallis seguido de Mann-Whitney. Análise discriminante linear e de variância multivariada permutacional (PERMANOVA) com e matriz de distância de Bray-Curtis foi usada para avaliar as potenciais diferenças nas concentrações de substratos energéticos e no perfil de ácidos graxos entre as espécies. Relações lineares entre os substratos energéticos, ácidos graxos essenciais [DHA, ARA e EPA], somatórias [SFA, PUFA, MUFA e BFA-OFA] e razões [n3/n6, EPA/ARA e DHA/ARA]) e CT foram explorados para cada espécie usando a matriz de correlação de Pearson. Para esta análise os dados foram log-transformados.

As diferenças sazonais entre as estações seca (janeiro e fevereiro) e chuvosa (maio) na porcentagem dos ácidos graxos mais abundantes (i.e., C16:0, C18:0, C18:1n9), os EFAs

(EPA, ARA e DHA) e somatórias (BFA-OFA, SFA, MUFA, PUFA, PUFA n3 e n6) foi testada nas espécies mais capturadas nas duas estações (i.e., *G. cuvier* e *G. cirratum*) usando Modelo Linear Generalizado Misto (GLMM). Foi usada a família Gaussiana de distribuição de erros, com o pacote mgcv (Wood, 2017). Para contabilizar os efeitos do tamanho corpóreo, a variável CT foi incluída como um efeito aleatório. Todas as análises foram realizadas no software R (versão 4.0.2) e Past 3.20 (Hammer *et al*., 2001). Foi considerada diferença estatisticamente significativa quando $p < 0,05$.

1.3. Resultados

Foram capturados um total de 87 tubarões (Tabela S1; Figs. 4 e 5) durante as três expedições realizadas (Fevereiro/2020, Janeiro/2022 e Maio/2022), destes 32 tubarões-bico-fino, *C. perezi* (média ± desvio padrão, 148,13 ± 50,23 cm CT, 14 fêmeas e 18 machos), 21 tubarões-tigre, *G. cuvier* (263,62 ± 66,08 cm CT, 15 fêmeas e 6 machos); 16 tubarões-lixa, *G. cirratum* (212,94 ± 37,98 cm CT; 5 fêmeas e 12 machos); 14 tubarões-limão, *N. brevirostris* (249,21 ± 17,49 cm CT, 8 fêmeas e 6 machos); 3 tubarões lombo-preto *C. falciformis* (234,33 ± 17,62 cm CT; 2 fêmeas e 1 macho), e 1 tubarão-galhudo *C. plumbeus* (196,00 cm CT, 1 macho).

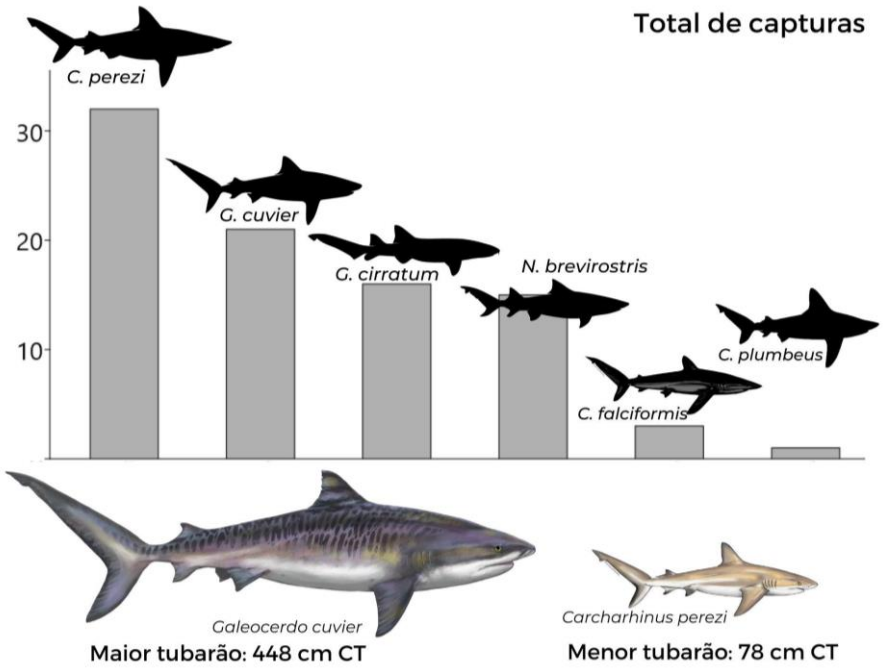

Figura 4. Quantidade total de tubarões capturados, em destaque o maior e menor tubarão medido.

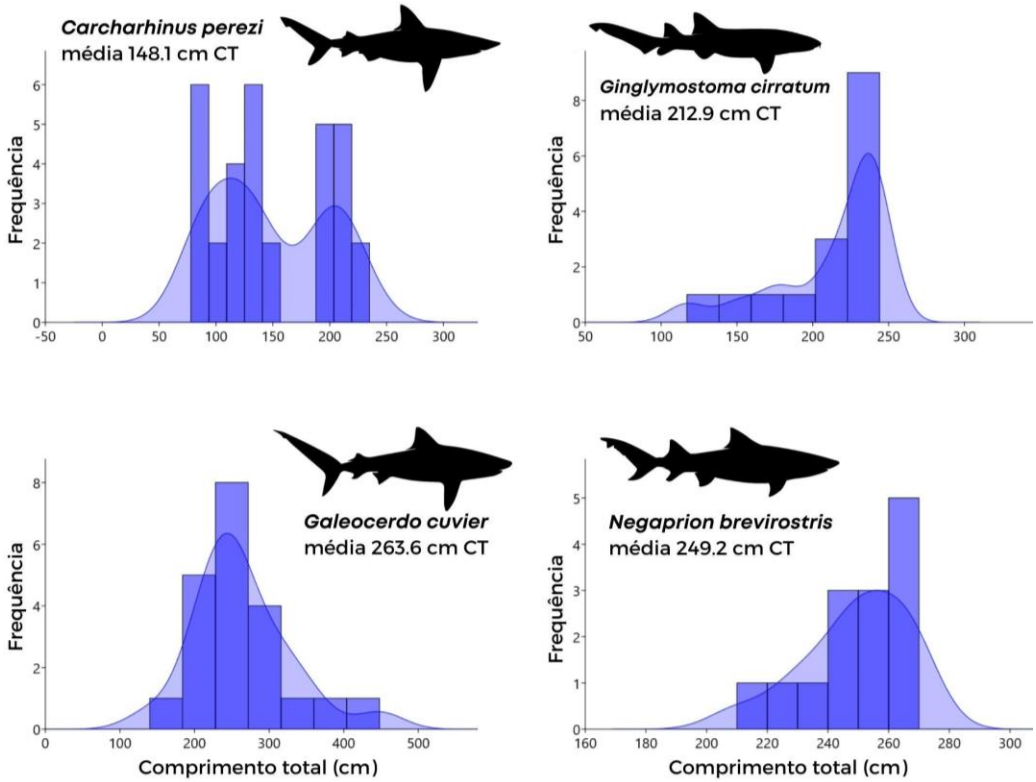

Figura 5. Frequência de captura por comprimento total das 4 espécies mais abundantes nas capturas.

1.3.1. Substratos energéticos plasmáticos

Substratos energéticos plasmáticos foram analisados em 80 tubarões e comparados entre os tubarões *C. perezi*, *G. cirratum*, *G. cuvier* e *N. brevirostris* (Tabela 2, Fig. 6). Os tubarões *G. cirratum* e *N. brevirostris* apresentaram maiores concentrações de proteínas totais plasmáticas comparados aos tubarões *C. perezi* e *G. cuvier* (Kruskal-Wallis test H= 23,75; p < 0,0001; Fig. 6a). O *G. cuvier* apresentou a maior concentração de GLU, seguido do *C. perezi* e *G. cirratum*, que não diferem do *N. brevirostris* (One way ANOVA F= 38,78; p < 0,0001; Fig. 6b). As concentrações de TAG foram menores no tubarão *C. perezi* comparado às outras três espécies que foram consideradas na análise estatística (Kruskal-Wallis test H= 23,04; p < 0,0001; Fig. 6c). Similarmente, as concentrações de CHOL foram menores no tubarão *C. perezi* que nos tubarões *G. cuvier* e *N. brevirostris* (Kruskal-Wallis test H= 16,63; p= 0,0008; Fig. 6d). Quando agrupados em "tubarões mais ativos" (*C. perezi*, *G. cuvier*, *C. falciformis* e *C. plumbeus*) e "tubarões menos ativos" (*G. cirratum* e *N. brevirostris*), encontramos maiores concentrações de proteínas totais (Mann-Whitney test, U= 301; p< 0,0001) e TAG (U= 478; p= 0,004) plasmáticos em espécies menos ativas (Fig. 7a,c), enquanto que o grupo de tubarões mais ativos apresentou maiores concentrações de GLU (U= 324; p< 0,0001; Fig. 7b).

Tabela 1. Valores métodos e desvio padrão para cada substrato energético plasmático medido (proteínas totais, glicose, triglicérides e colesterol) para os tubarão-de-recife *Carcharhinus perezi*, tubarão-lixa *Ginglymostoma cirratum*, tubarão-tigre *Galeocerdo cuvier*, tubarão-limão *Negaprion brevirostris*, tubarão-lombo-preto *Carcharhinus falciformis* e tubarão-galhudo *Carcharhinus plumbeus*.

Tubarões	N	**Proteínas totais (g/dL)**	**Glicose (mg/dL)**	**Triglicérides (mg/dL)**	**Colesterol (mg/dL)**
C. perezi	30	2,3 ± 0,66	62,6 ± 15,95	48,6 ± 17,65	40,4 ± 23,76
G. cuvier	19	1,9 ± 0,58	100,6 ± 18,11	74,1 ± 18,71	53,7 ± 16,63
G. cirratum	16	3,0 ± 0,53	40,4 ± 18,24	67,9 ± 18,16	45,8 ± 14,52
N. brevirostris	13	2,7 ± 0,9	55,6 ± 20,44	91,4 ± 43,0	54,8 ± 18,79
C. falciformis	2	2,3 ± 0,5	41,2 ± 14,51	52,7 ± 19,69	47,3 ± 2,46
C. plumbeus	1	3,1	84,7	79,2	88,5

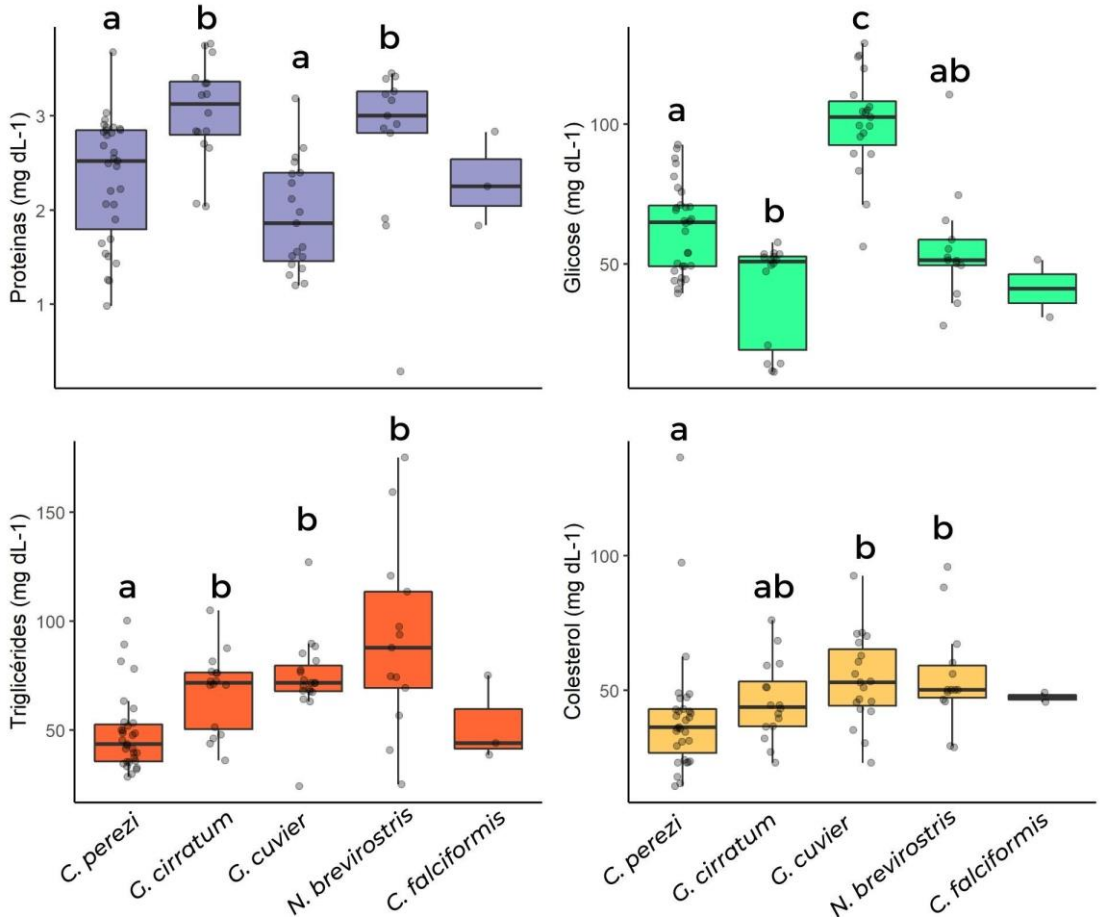

Figura 6. Comparativo dos substratos energéticos plasmáticos (proteínas totais, glicose, triglicérides e colesterol) entre o tubarão-de-recife *Carcharhinus perezi* (n = 31), tubarão-lixa *Ginglymostoma cirratum* (n= 16), tubarão-tigre *Galeocerdo cuvier* (n= 19), tubarão-limão *Negaprion brevirostris* (n= 14), tubarão-lombo-preto *Carcharhinus falciformis* (n= 3) (este não estatisticamente testato). Letras indicam diferença estatística entre os grupos (One way ANOVA ou Kruskal-Wallis *test*, $p < 0.05$).

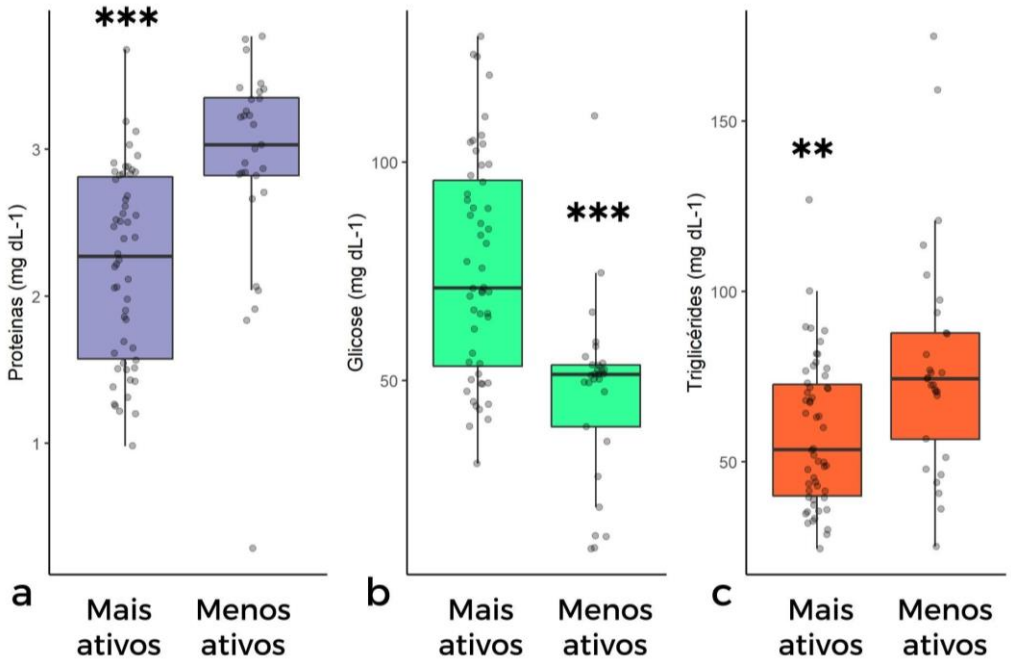

Figura 7. Comparativo dos substratos energéticos plasmáticos (proteínas totais, glicose, triglicérides) entre os tubarões mais ativos (tubarão-de-recife *Carcharhinus perezi* (n = 31), tubarão-tigre *Galeocerdo cuvier* (n= 19), tubarão-lombo-preto*Carcharhinus falciformis* (n= 3), tubarão-galhudo *Carcharhinus plumbeus* (n= 1)) e tubarões menos ativos (tubarão-lixa *Ginglymostoma cirratum* (n= 16), tubarão-limão *Negaprion brevirostris* (n= 14)). Diferenças significativas entre tubarões mais e menos ativos são indicadas por asteriscos (Mann-Whitney test *p < 0,05; **p < 0,01; ***p < 0,001).

Análise multivariada dos substratos energéticos plasmáticos revelou que o tubarão *G. cirratum* e *N. brevirostris* apresentam um perfil energético semelhante (PERMANOVA, F= 2.17; *p*= 0,697; Fig. 8). Na análise discriminante, o eixo 1 separou o *G. cuvier* do *N. brevirostris* e *C. perezi* principalmente devido às concentrações de GLU (Eixo 1 = 84,72%, eigenvalues = 2,42), enquanto que o eixo 2 separou o *G. cuvier* e *C. perezi* (Eixo 1 = 14,7%, eigenvalues = 0,42) principalmente devido às concentrações de TAG. A análise multivariada também revelou uma diferença estatística entre os tubarões *C. perezi* e *N. brevirostris* (PERMANOVA, F= 10,24; *p*= 0,0006), *G. cirratum* (F= 7.65; *p*= 0,0012) e *G. cuvier* (F= 22.89; *p*= 0,0006), e entre o *G. cuvier* e *N. brevirostris* (F= 9.561; *p*= 0,0006) e *G. cirratum* (F= 18.18; *p*= 0,0006; Fig. 8).

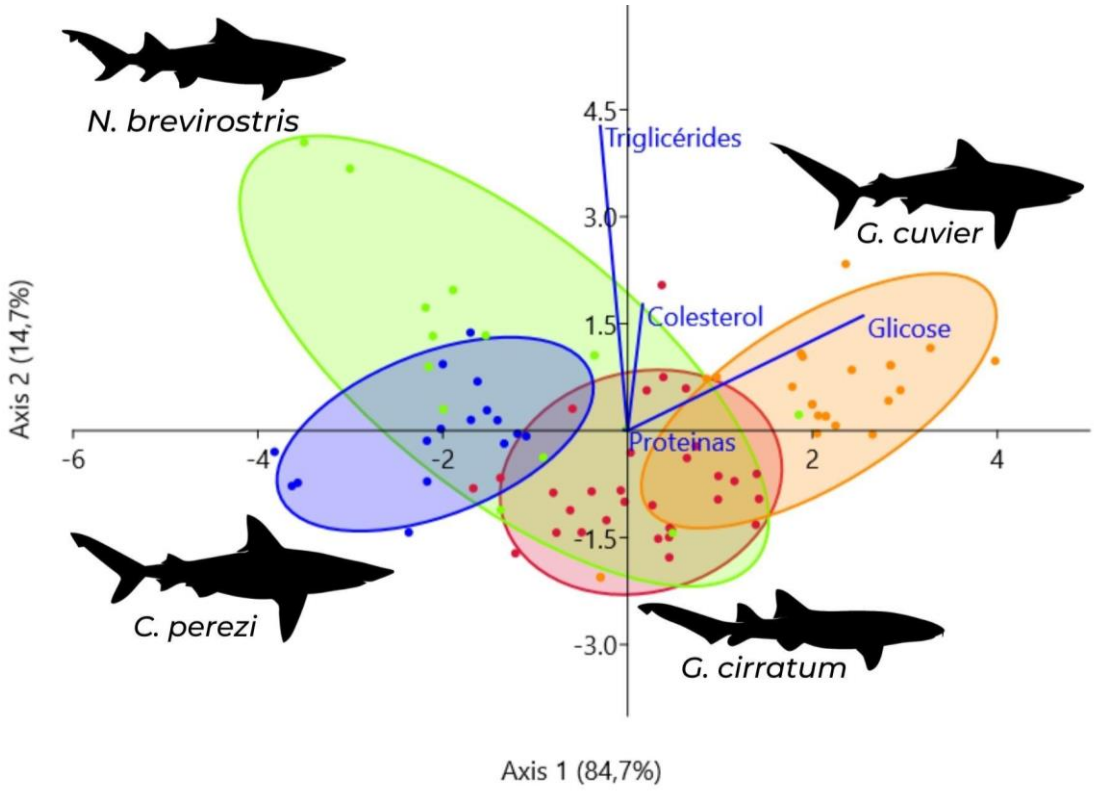

Figura 8. Análise da função discriminante linear das concentrações de substratos energéticos (proteínas totais, glicose, triglicérides e colesterol) das espécies tubarão-de-recife *Carcharhinus perezi* (n = 31), tubarão-lixa *Ginglymostoma cirratum* (n= 16), tubarão-tigre *Galeocerdo cuvier* (n= 19), tubarão-limão *Negaprion brevirostris* (n= 14).

Para o *C. perezi,* foram encontradas quatro relações significativas entre os substratos energéticos. A concentração de proteínas totais foi positivamente correlacionada com o CT (*t*= 3,39, df = 29, *p* = 0,002; Fig. 9a). Além disso, foi encontrado correlações positivas entre a GLU e TAG (*t*= 3,48, df= 28, *p*= 0,002; Fig. 9b), GLU e CHOL (*t*= 2,88, df= 28, *p*= 0,007; Fig. 9c), e TAG e CHOL (*t*= 2,57, df = 29, *p*= 0,015; Fig. 9d). Similarmente, para o *G. cuvier* as concentrações de proteínas totais foram positivamente correlacionadas com o CT (*t*= 2.36, df = 17, *p*= 0.031; Fig. 10a). As concentrações de GLU também foram positivamente correlacionadas com as de TAG (*t*= 3.84, df = 17, *p*= 0.001; Fig. 10b) e CHOL (*t*= 2.58, df = 17, *p*= 0.019; Fig. 10c). Para o *G. cirratum*, foi encontrada uma correlação positiva apenas entre as concentrações de GLU e TAG (*t*= 8.32, df= 14, p<0,001; Fig. 11a). Já para o *N. brevirostris* as concentrações de proteínas totais foram positivamente correlacionadas com as de TAG (*t*= 2.94, df = 11, *p*= 0.013; Fig. 11b) e CHOL (*t*= 2.94, df = 11, *p*= 0.013; Fig. 11c).

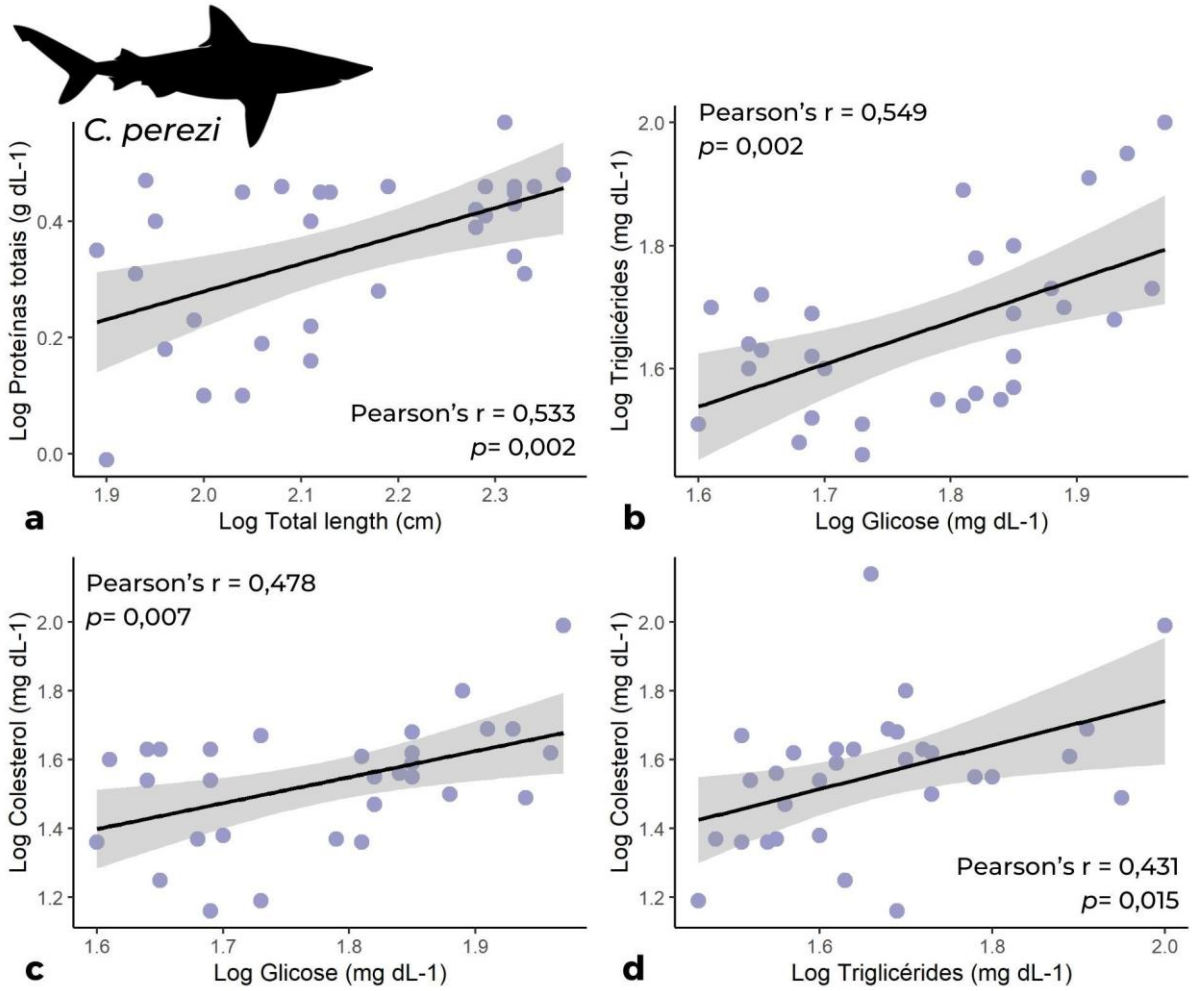

Figura 9. Gráficos de dispersão representando as correlações significativas entre os parâmetros detectados através da matriz de correlação de Pearson para o tubarão-de-recife *Carcharhinus perezi* (n = 31).

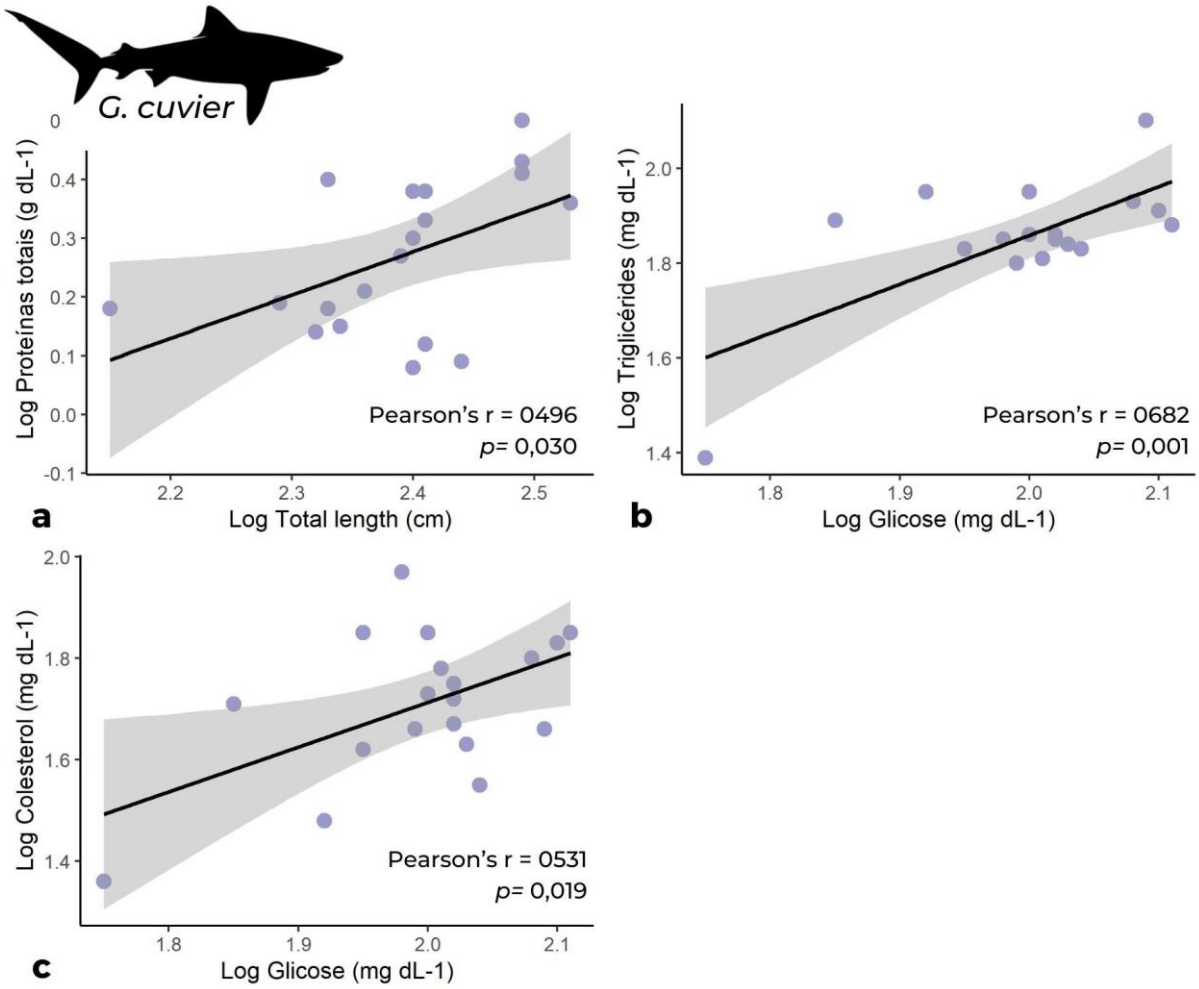

Figura 10. Gráficos de dispersão representando as correlações significativas entre os parâmetros detectados através da matriz de correlação de Pearson para o tubarão-tigre *Galeocerdo cuvier* (n = 19).

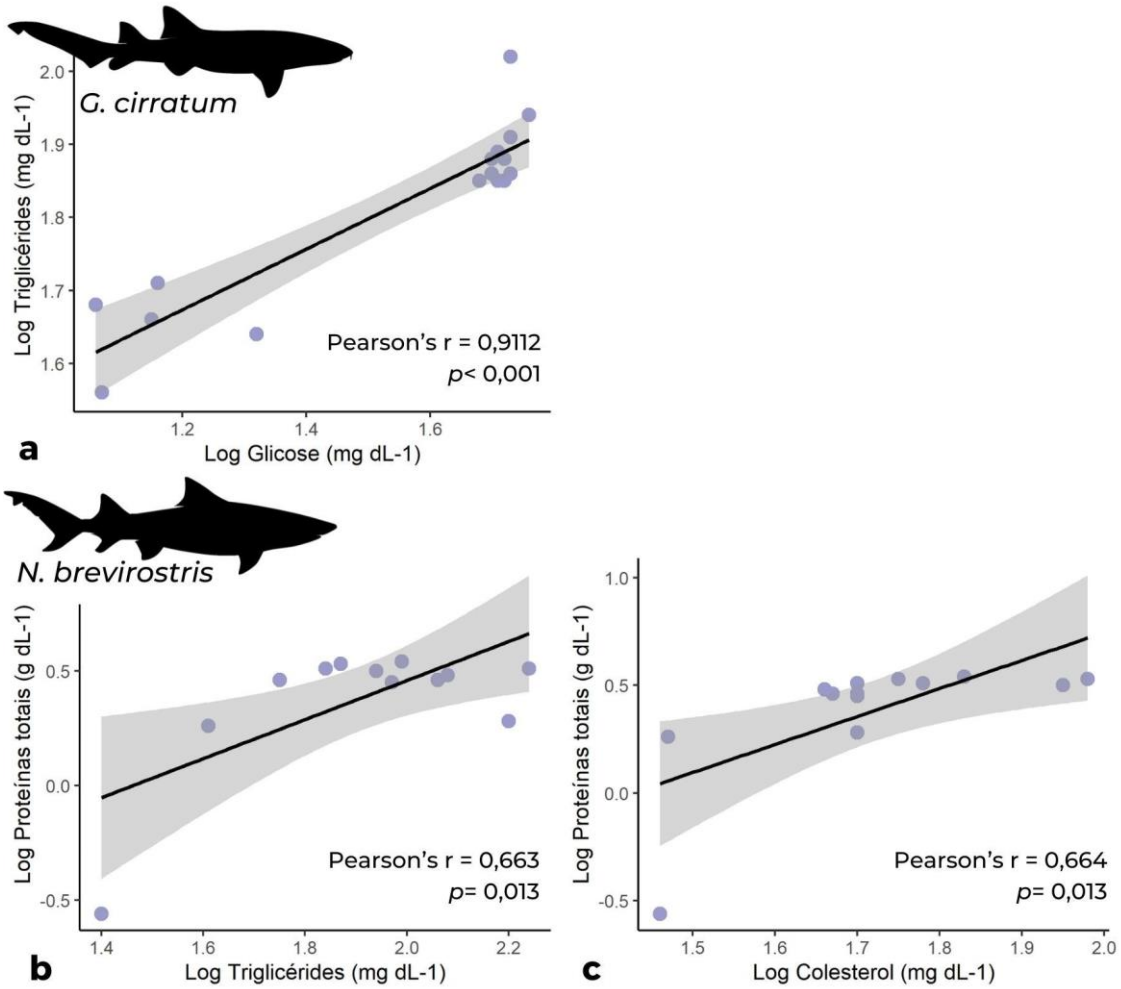

Figura 11. Gráficos de dispersão representando as correlações significativas entre os parâmetros detectados através da matriz de correlação de Pearson para os tubarões-lixa *Ginglymostoma cirratum* (n= 16) e tubarão-limão *Negaprion brevirostris* (n= 13).

1.3.2. Perfil de ácidos graxos

O perfil de ácidos graxos plasmático revelou que os SFAs (C16:0 e C18:0) foram dominantes no plasma de todas as espécies analisadas, seguidos de PUFAs (DHA, ARA, EPA) e MUFAs (C18:1n9), com exceção do *C. falciformis* que foi seguido de MUFAs e depois PUFAs (Tabela 2). Dentre os PUFAs, foi encontrado maior porcentagem de ômega-3 que ômega-6 em todas as espécies, principalmente devido às altas proporções de DHA.

Comparativamente, a análise multivariada dos perfis de ácidos graxos plasmáticos revelou diferença estatística entre as quatro espécies testadas (PERMANOVA, F= 2.96; *p*= 0,002; Fig. 12). Embora o tubarão *G. cirratum* e *G. cuvier* não diferiram estatisticamente (F= 1,13; *p*= 0,277), o perfil de ácidos graxos foi estatisticamente diferente entre o tubarão *C. perezi* e *N. brevirostris* (F= 3,96; *p*= 0,016), *C. perezi* e *G. cirratum* (F= 3,23; *p*= 0,028), *C. perezi* e *G. cuvier* (F= 2,87; *p*= 0,030), *G. cirratum* e *N. brevirostris* (F= 3,99; *p*= 0,009), e *G.*

cuvier e *N. brevirostris* (F= 2,68; *p*= 0,039). Na análise discriminante, o eixo 1 separou principalmente o *G. cuvier* e *G. cirratum* do *C. perezi* e *N. brevirostris* (Eixo 1 = 70,1%, eigenvalues = 2,71), principalmente devido às porcentagens de PUFA n6 (ARA), enquanto que o eixo 2 separou o *C. perezi* do *N. brevirostris* e *G. cirratum* (Eixo 1 = 19,1%, eigenvalues = 0,74), principalmente devido às porcentagens de PUFA n3 (DHA e EPA), SFA (C14:0 e C16:0) e C18:1n7 (Fig. 12). Comparando os valores médios das razões dos ácidos graxos, o tubarão *C. perezi* apresentou os maiores valores das razões n3/n6 (One way ANOVA F= 11,61; p<0,001; Fig. 13a), EPA/ARA (Kruskal-Wallis *test* H=29,55; p<0,0001; Fig. 13b) e DHA/ARA (One way ANOVA F= 12,06; p<0,001; Fig. 13c) comparado com os três outros tubarões.

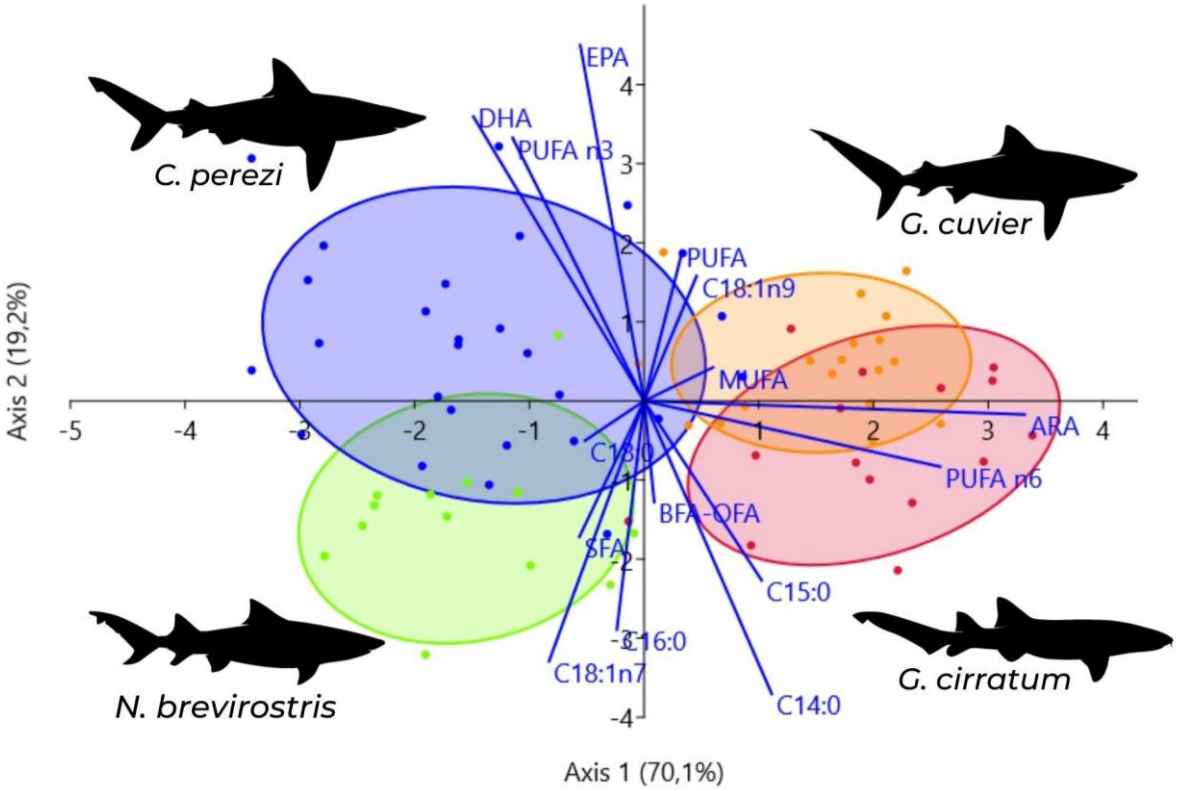

Figura 12. Análise da função discriminante linear dos perfis de ácidos graxos plasmáticos das espécies tubarão-de-recife *Carcharhinus perezi* (n = 27), tubarão-lixa *Ginglymostoma cirratum* (n= 15), tubarão-tigre *Galeocerdo cuvier* (n= 18), tubarão-limão *Negaprion brevirostris* (n= 13). SFA: ácidos graxos saturados; MUFA: ácidos graxos monoinsaturados; PUFA: ácidos graxos polinsaturados; n3: ômega-3; n6: ômega-6; EPA: ácido eicosapentaenoico; DHA: ácido docosahexaenoico; ARA: ácido araquidônico; BFA: ácidos graxos de cadeia ramificada; OFA: ácidos graxos de cadeia ímpar.

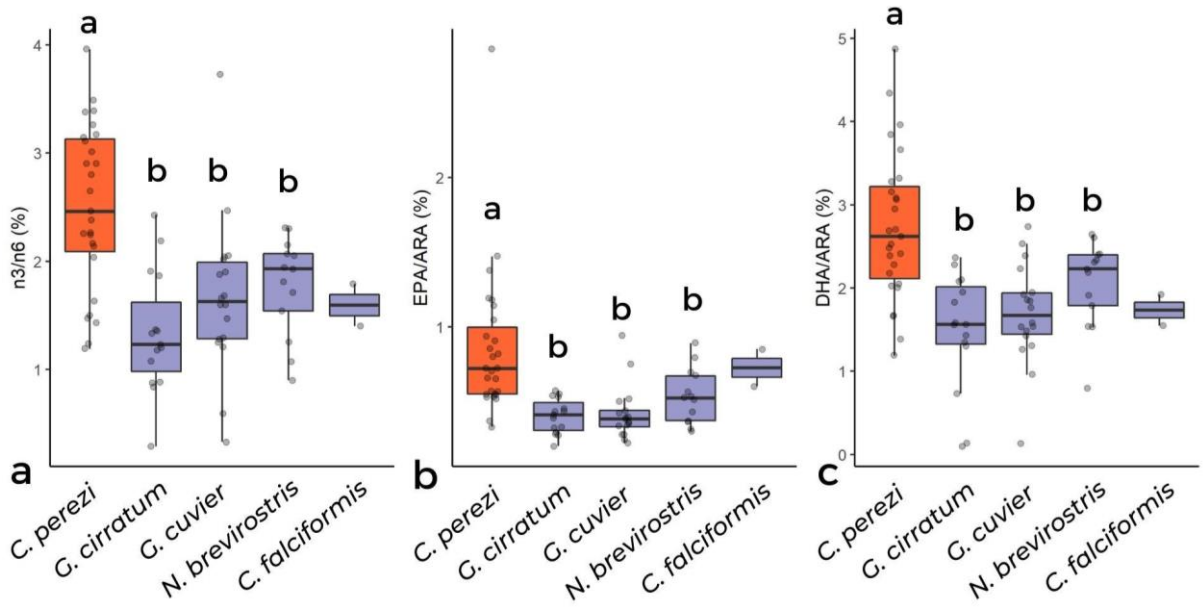

Figura 13. Comparativo das razões plasmáticas de ácidos graxos n3/n6, EPA/ARA e DHA/ARA entre o tubarão-de-recife *Carcharhinus perezi* (n = 27), tubarão-lixa *Ginglymostoma cirratum* (n= 15), tubarão-tigre *Galeocerdo cuvier* (n= 18), tubarão-limão *Negaprion brevirostris* (n= 13), tubarão-lombo-preto *Carcharhinus falciformis* (n= 3) (este não estatisticamente testado). n3: PUFA ômega-3; n6: PUFA ômega-6; EPA: ácido eicosapentaenoico; DHA: ácido docosahexaenoico; ARA: ácido araquidônico. Letras indicam diferença estatística entre os grupos (One way ANOVA ou Kruskal-Wallis *test*, $p < 0.05$).

Para o *G. cirratum*, foram encontradas três correlações negativas entre o CT e a porcentagem de PUFA (*t*= -2.56, df= 13, *p*= 0.023; Fig. 14a), PUFA n3 (*t*= -2.38, df= 13, *p*= 0.033; Fig. 14b) e razão n3/n6 (*t*= -3.14, df= 13, *p*= 0.007; Fig. 14c). O CT foi negativamente correlacionado com a porcentagem de DHA (*t*= -2.23, df= 16, *p*= 0.040; Fig. 14d) e razão n3/n6 (*t*= -2.47, df= 16, *p*= 0.025; Fig. 14e) no *G. cuvier*, enquanto que o CT foi negativamente correlacionado com MUFA no *C. perezi* (*t*= -2.78, df= 25, *p*= 0.010; Fig. 14f) e EPA no *N. brevirostris* (*t*= -2.34, df= 11, *p*= 0.038; Fig. 14g).

Tabela 2. Valores de média e desvio padrão (média ± DP) do perfil de ácidos graxos do plasma do tubarão-de-recife *Carcharhinus perezi* (n = 27), tubarão-lixa *Ginglymostoma cirratum* (n= 15), tubarão-tigre *Galeocerdo cuvier* (n= 18), tubarão-limão *Negaprion brevirostris* (n= 13), tubarão-lombo-preto *Carcharhinus falciformis* (n= 3), e tubarão-galhudo (n= 1).

Ácidos graxos	***C. perezi***	***G. cuvier***	***G. cirratum***	***N. brevirostris***	***C. falciformis***	***C. plumbeus***
C15:0	7,3 ± 7,35	5,2 ± 2,42	7,7 ± 4,45	5,5 ± 2,15	12,5 ± 2,98	4,6
C17:0	1,3 ± 0,69	1 ± 0,25	1 ± 0,39	1,2 ± 0,43	NA	0,9
C14:0	2,9 ± 1,34	3,3 ± 0,78	3,7 ± 0,96	3,7 ± 0,66	3,9 ± 0,95	4,7
C16:0	23,9 ± 6,67	24 ± 2,52	22,8 ± 3,64	29,5 ± 3,44	26,9 ± 1,50	25,8
C18:0	11,9 ± 2,56	11,6 ± 2,29	11,1 ± 1,92	12,5 ± 1,9	14,1 ± 1,31	12,5
C22:0	1,0 ± 0,21	0,8 ± 0,23	NA	0,9 ± 0,16	NA	NA
C24:0	1,5 ± 0,43	1,1 ± 0,37	1,3 ± 0,57	1,3 ± 0,59	1,1 ± 0,73	1,2
C14:1	2,2 ± 1,88	1,8 ± 0,74	2,2 ± 1,63	1,8 ± 1,04	3,6 ± 2,70	1,7
C16:1	2,0 ± 0,99	2,1 ± 0,54	1,6 ± 0,49	2,4 ± 1,48	1,0 ± 0,06	1,5
C18:1n9	13,8 ± 3,28	16,8 ± 8,69	13,1 ± 4,05	13,4 ± 5,52	13,7 ± 0,03	14,8
C18:1n7	2,2 ± 0,61	2,2 ± 0,54	2,3 ± 0,45	3 ± 0,65	1,8 ± 0,10	1,8
C20:1n9	NA	NA	0,7 ± 0,11	NA	NA	NA
C24:1	1,4 ± 0,5	2,5 ± 3,43	3,6 ± 6,14	1,6 ± 0,57	1,4 ± 0,67	1,5
C18:4n3	2,8 ± 1,59	2,9 ± 1,49	2,4 ± 1,02	1,4 ± 0,51	2,7 ± 0,66	NA
C18:2n6	1,3 ± 0,61	1,7 ± 1,22	1,7 ± 1,11	1,3 ± 0,31	0,9 ± 0,04	1,2
C18:3n6	NA	1 ± 0,68	0,8 ± 0,22	NA	NA	NA
C20:5n3 (EPA)	3,9 ± 1,77	3,2 ± 1,39	3,1 ± 1,29	2,4 ± 0,76	2,3 ± 0,27	2,7
C22:5n3	1,9 ± 0,75	1,5 ± 0,65	1,9 ± 0,64	2,4 ± 0,60	NA	1,0
C22:6n3 (DHA)	13,2 ± 5,33	12,0 ± 5,28	11,1 ± 6,53	9,9 ± 4,71	6,0 ± 2,98	15,5
C20:2n6	1,5 ± 1,37	NA	NA	0,7 ± 0,06	NA	NA
C20:3n6	1,2 ± 1,23	NA	NA	0,4 ± 0,12	NA	NA
C20:4n6 (ARA)	4,9 ± 1,54	7,2 ± 2,19	7,8 ± 2,43	4,9 ± 1,88	3,3 ± 1,22	7,1
C22:2n6	1,5 ± 0,96	1 ± 0,34	1,5 ± 0,43	0,9 ± 0,28	1,9 ± 0,53	NA
C22:4n6	1,2 ± 0,4	1,1 ± 0,44	1,6 ± 0,85	1,5 ± 0,62	NA	1,5
BFA-OFA	8,6 ± 7,46	6,2 ± 2,59	8,6 ± 4,76	7,0 ± 2,71	13,4 ± 1,66	5,6
SFA	40,3 ± 8,07	39,9 ± 4,32	38,9 ± 5,88	47,5 ± 5,34	46,0 ± 1,13	44,1
MUFA	21,2 ± 3,76	24,9 ± 10,34	22,5 ± 7,01	21,4 ± 6,06	21,5 ± 1,91	21,3
PUFA	29,5 ± 7,86	28,4 ± 9,36	29,8 ± 8,56	23,7 ± 7,84	18,6 ± 4,13	29,0
PUFA n3	20,7 ± 6,78	17,7 ± 7,56	16,9 ± 7,92	14,9 ± 5,81	11,5 ± 3,29	19,2
PUFA n6	8,6 ± 2,45	10,4 ± 2,53	12,8 ± 2,11	8,4 ± 2,48	7,1 ± 0,84	9,8
n3/n6	2,5 ± 0,76	1,7 ± 0,73	1,3 ± 0,56	1,8 ± 0,46	1,6 ± 0,27	1,9
EPA/ARA	0,9 ± 0,50	0,4 ± 0,18	0,4 ± 0,12	0,5 ± 0,19	0,7 ± 0,18	0,4
DHA/ARA	2,7 ± 0,89	1,7 ± 0,61	1,5 ± 0,70	2,1 ± 0,52	1,7 ± 0,26	2,2

SFA: ácidos graxos saturados; MUFA: ácidos graxos monoinsaturados; PUFA: ácidos graxos polinsaturados; n3: PUFA ômega-3; n6: PUFA ômega-6; EPA: ácido eicosapentaenoico; DHA: ácido docosahexaenoico; ARA: ácido araquidônico; BFA: ácidos graxos de cadeia ramificada; OFA: ácidos graxos de cadeia ímpar.

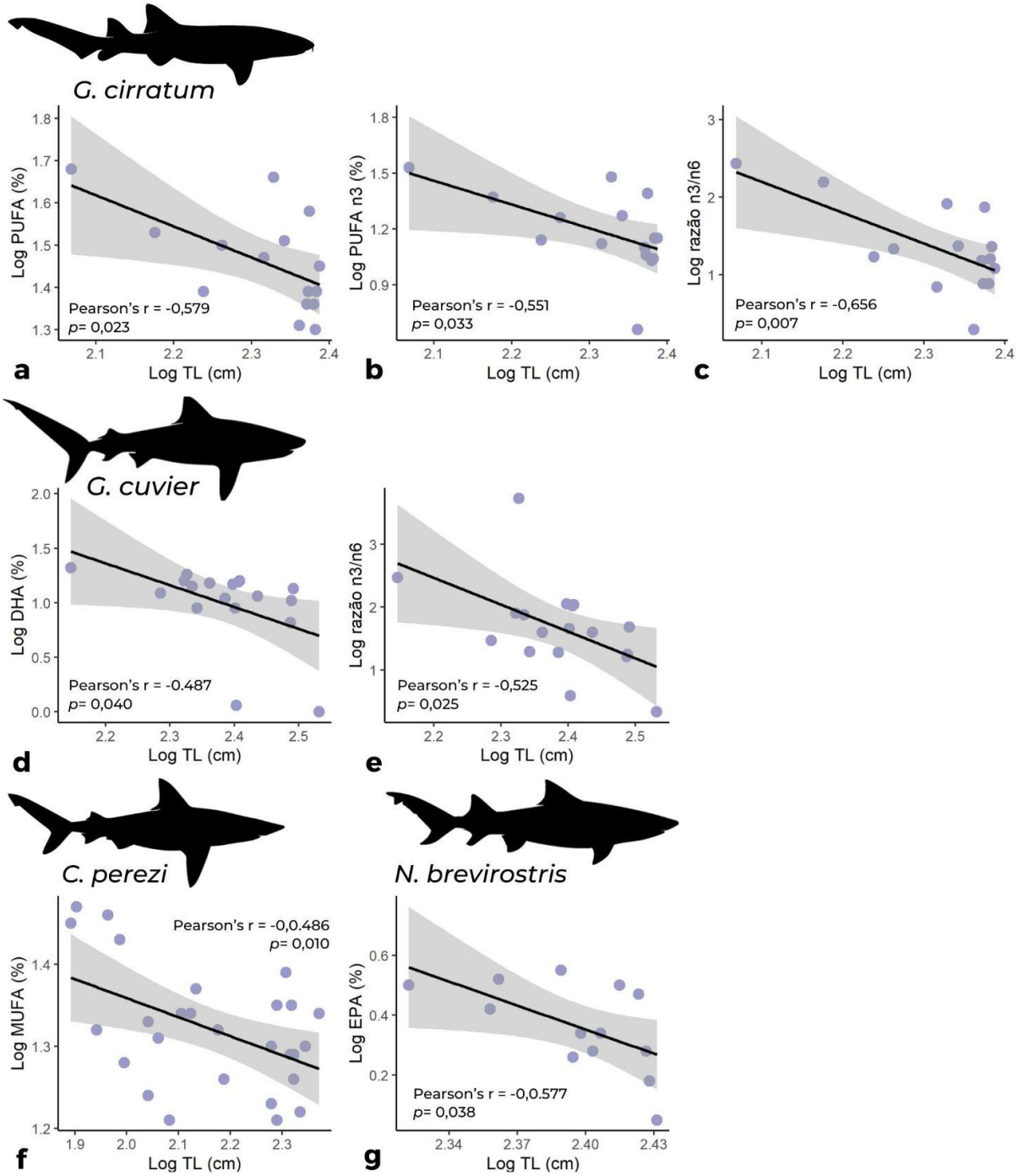

Figura 14- Gráficos de dispersão representando as correlações significativas entre os parâmetros plasmáticos detectados através da matriz de correlação de Pearson para o tubarão-lixa *Ginglymostoma cirratum* (n= 15), tubarão-tigre *Galeocerdo cuvier* (n= 18), tubarão-de-recife *Carcharhinus perezi* (n = 27), e tubarão-limão *Negaprion brevirostris* (n= 13). MUFA: ácidos graxos monoinsaturados; PUFA: ácidos graxos polinsaturados; n3: PUFA ômega-3; n6: PUFA ômega-6; EPA: ácido eicosapentaenoico; DHA: ácido docosahexaenoico; ARA: ácido araquidônico.

1.3.3. Variação sazonal nos substratos energéticos e ácidos graxos plasmáticos

A variação sazonal foi testada em tubarões *G. cuvier* e *G. cirratum*, uma vez que foram as únicas espécies amostradas em maior quantidade em ambas estações seca e chuvosa. Para o *G. cuvier*, as concentrações plasmáticas de proteínas totais (Fig. 15a), glicose (Fig. 15b) e porcentagem de C16:0 (Fig. 15c) foram maior na estação chuvosa, enquanto que as porcentagens de EPA (Fig. 13d), ARA (Fig. 15e), incluindo as somatórias PUFA (Fig. 15f), PUFA n3 (Fig. 15g) e PUFA n6 (Fig. 15h) foram maiores durante a estação seca (Tabela 3). Já o *G. cirratum* apresentou maiores concentrações de glicose (Fig. 16a) e triglicérides (Fig. 16b) e maiores porcentagens de SFA (Fig. 16g), incluindo o C16:0 (Fig. 16c) e C18:0 (Fig. 16d), durante a estação chuvosa, enquanto que apresentou maiores porcentagens de EPA (Fig. 16e), ARA (Fig. 16f) e PUFA n6 (Fig. 16h) durante a estação seca (Tabela 3).

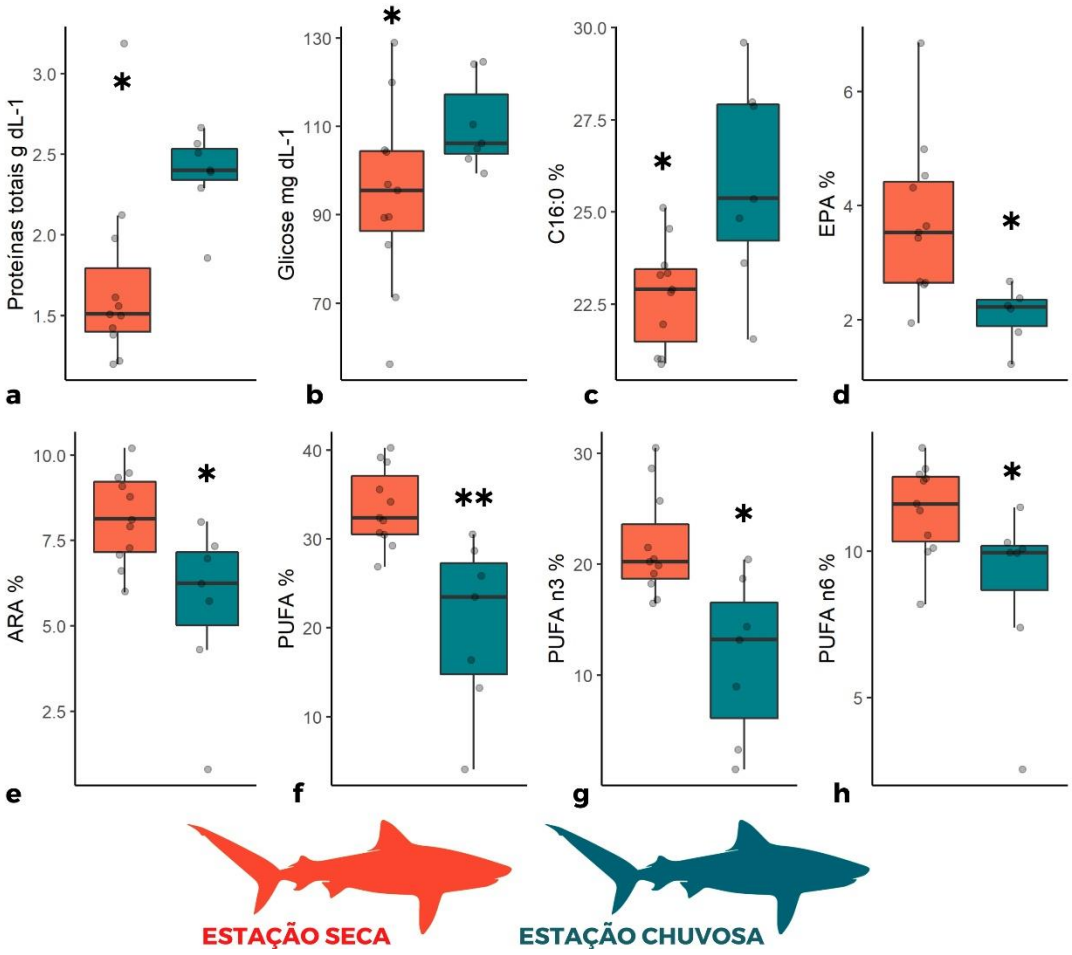

Figura 15- Comparativo entre as estações seca e chuvosa para substratos energéticos e ácidos graxos plasmáticos do tubarão-tigre, *Galeocerdo cuvier*. Legenda: EPA: ácido eicosapentaenoico; ARA: ácido araquidônico, PUFA: ácidos graxos polinsaturados; n3: PUFA ômega-3; n6: PUFA ômega-6. Diferenças significativas entre as estações seca e chuvosa são indicadas por asteriscos (Modelo Linear Generalizado Misto, *p < 0,05; **p < 0,01; ***p < 0,001).

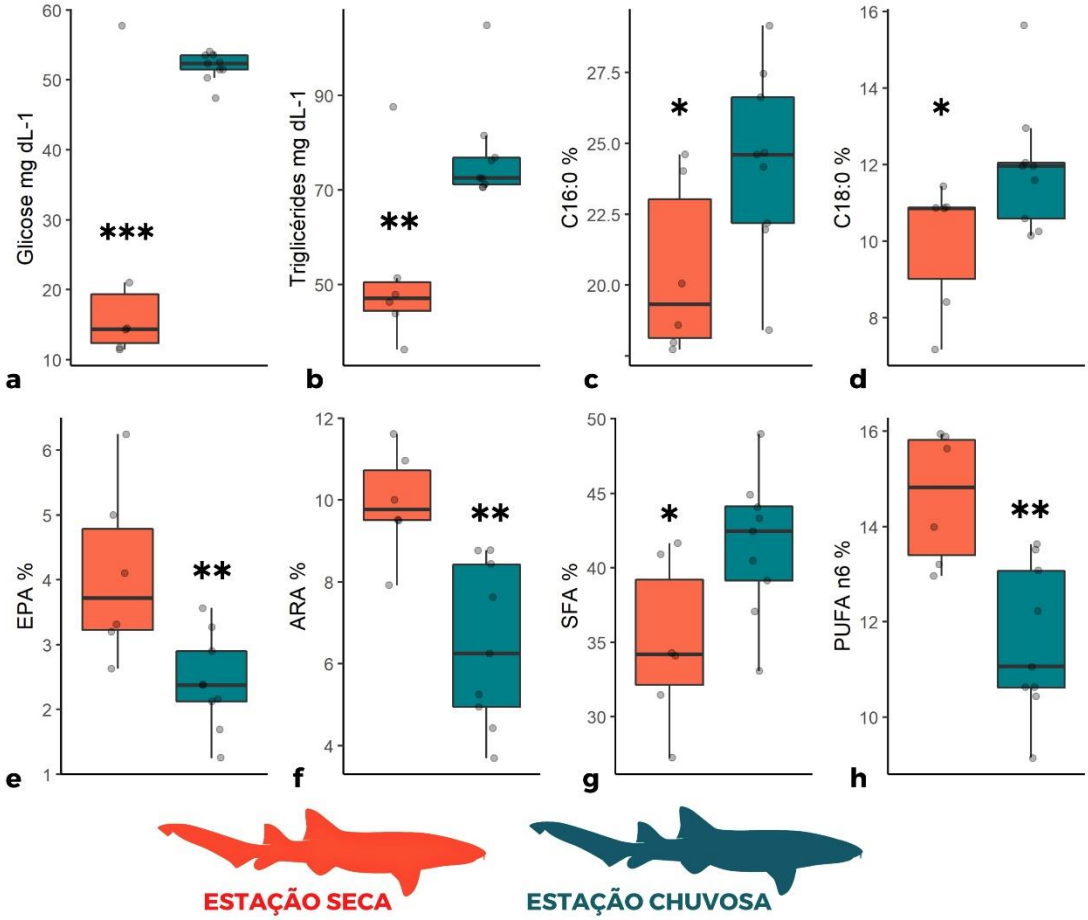

Figura 16- Comparativo entre as estações seca e chuvosa para substratos energéticos e ácidos graxos plasmáticos do tubarão-lixa, *Ginglymostoma cirratum*. Legenda: EPA: ácido eicosapentaenoico; ARA: ácido araquidônico, SFA: ácidos graxos saturados; PUFA n6: ácidos graxos polinsaturados ômega-6. Diferenças significativas entre as estações seca e chuvosa são indicadas por asteriscos (Modelo Linear Generalizado Misto, *p < 0,05; **p < 0,01; ***p < 0,001).

Tabela 3. Modelo Linear Generalizado Misto das concentrações de substratos energeticos e percentuais de ácidos graxos de tubarão-tigre (*Galeocerdo cuvier*) e tubarões-lixa (*Ginglymostoma cirratum*) em função da estação do ano (seca x chuvosa), apenas para aqueles foram significativos. Estão incluídos o tipo de biomarcador correspondente, variável de resposta (estação), estimativa de coeficiente (Est.), erro padrão (EP), valor t (t) e valor p (p) de cada modelo.

Galeocerdo cuvier					
Substratos energéticos		Est.	EP	t	p
Proteínas totais	(*Intercept*)	0,84	0,51	1,65	0,119
	Estação (chuvosa)	0,52	0,23	2,27	0,038
Glicose	(*Intercept*)	137,68	18,39	7,49	<0,0001
Ácidos graxos	Estação (chuvosa)	23.84	7,92	3,01	0,009
C16:0	(*Intercept*)	21,78	1,59	13,68	<0,0001
	Estação (chuvosa)	2,87	1,02	2,82	0,012
EPA	(*Intercept*)	3,74	0,35	10,61	<0,0001
	Estação (chuvosa)	-1,66	0,59	-2,79	0,014
ARA	(*Intercept*)	8,17	0,55	14,77	<0,0001
	Estação (chuvosa)	-2,54	0,89	-2,86	0,011
PUFA	(*Intercept*)	39,41	6,29	6,26	<0,0001
	Estação (chuvosa)	-12,19	3,37	-3,61	0,002
PUFA n3	(*Intercept*)	33,57	6,20	5,42	<0,0001
	Estação (chuvosa)	-7,86	2,74	-2,87	0,012
PUFA n6	(*Intercept*)	11,42	0,67	16,98	<0,0001
	Estação (chuvosa)	-2,60	1,08	-2,42	0,028
Ginglymostoma cirratum					
Substratos energéticos		Est.	EP	t	p
Glicose	(*Intercept*)	21,79	4,60	4,74	<0,001
	Estação (chuvosa)	30,05	5,93	5,06	<0,001
Triglicérides	(*Intercept*)	52,20	5,76	9,07	<0,0001
Ácidos graxos	Estação (chuvosa)	25.23	7,43	3,40	0,005
C16:0	(*Intercept*)	20,50	1,30	15,80	<0,0001
	Estação (chuvosa)	3,87	1,67	2,31	0,038
C18:0	(*Intercept*)	7,46	1,95	3,83	0,002
	Estação (chuvosa)	1,88	0,84	2,24	0,045
EPA	(*Intercept*)	6,17	1,20	5,15	<0,001
	Estação (chuvosa)	-1,60	0,48	-3,34	0,006
ARA	(*Intercept*)	10,19	1,08	9,46	<0,001
	Estação (chuvosa)	-3,44	0,92	-3,76	0,002
SFA	(*Intercept*)	30,15	5,26	5,73	<0,0001
	Estação (chuvosa)	6,40	2,56	2,50	0,027
PUFA n6	(*Intercept*)	14,60	0,61	23,76	<0,0001
	Estação (chuvosa)	-3,01	0,79	-3,80	0,002

SFA: ácidos graxos saturados; PUFA n3 e n6: ácidos graxos polinsaturados ômega-3 e ômega-6; EPA: ácido eicosapentaenoico; ARA: ácido araquidônico.

1.4. Discussão

Através da integração de diferentes biomarcadores nutricionais, o presente estudo investigou, pela primeira vez, aspectos da ecologia nutricional de tubarões em um sistema insular do Oceano Atlântico Sul. Os resultados revelaram que as espécies analisadas apresentam metabolismo energético e padrões alimentares distintos, o que é esperado considerando que apresentam diferentes histórias de vida. Além disso, os dados mostram a influência do tamanho corpóreo e variação sazonal para alguns biomarcadores nutricionais, que são discutidos em mais detalhes a seguir. Através das informações de captura, foi observado quatro espécies mais abundantes entre as águas rasas e os aproximados 60 metros de profundidade, o tubarão-de-recife *C. perezi*, tubarão-tigre *G. cuvier*, tubarão-lixa *G. cirratum* e tubarão-limão *N. brevirostris*. Essas informações corroboram estudos prévios na região, que descrevem estas mesmas espécies como as mais abundantes nesta faixa de profundidade do arquipélago (Soto, 2001; Garla *et al*., 2006; 2009; Afonso *et al*., 2016; 2017). O tubarão-lombo preto *C. falciformis* parece ser abundante na zona epipelágica de regiões mais profundas (i.e., abaixo dos 90 metros). Embora pouco esforço de captura tenha sido direcionado nesta região, existem relatos constantes de pescadores que os avistam diariamente (B. Rangel, observação pessoal). As capturas de neonatos e juvenis foram direcionadas para o *C. perezi* em regiões rasas próximo à ilha principal, sem praias, mas sabe-se que o arquipélago também é usado como área de berçário pelos tubarões *N. brevirostris* e *G. cirratum* (Garla *et al*., 2009).

Os resultados fisiológicos mostraram que os tubarões mais ativos (*i.e., C. perezi*, *G.cuvier*, *C. falciformis* e *C. plumbeus*) diferem no metabolismo energético daqueles menos ativos (*i.e., G. cirratum* e *N. brevirostris*). Similar ao encontrado em um estudo que comparou os lipídios plasmáticos em quatro espécies de tubarões mais ativos (*Carcharhinus leucas* e *Carcharhinus limbatus*) e menos ativos (*G. cirratum* e *G.cuvier*) (Gallagher *et al*., 2017), as concentrações de TAG foram menores em tubarões mais ativos, em destaque para o *C. perezi* que apresentou as menores concentrações de TAG e CHOL de todas as espécies analisadas. As altas concentrações de TAG e proteínas totais encontradas em tubarões menos ativos como *G. cirratum* e *N. brevirostris* podem estar relacionadas com as taxas metabólicas mais baixas destas espécies (*e.g.*, Whitney *et al*., 2016) e, portanto, sua mobilização poderia ser relacionada com movimentos de baixo custo em tubarões menos ativos, como observado por Gallagher *et al*. (2017). Os tubarões *G. cirratum* e *N. brevirostris* são frequentemente observados em repouso e conhecidos por terem comportamento mais residente (Garla *et al*., 2022). Diferente deste estudo prévio, classificamos aqui o *G. cuvier* como uma espécie ativa,

com base nos padrões de movimentação da espécie na região (Afonso *et al*., 2016; B. Rangel, dados não publicados). No entanto, as concentrações de TAG para o *G. cuvier* no presente estudo se assemelham às encontradas para os tubarões menos ativos, assim como observado por Gallagher *et al*. (2017).

Outra possível explicação para a concentração elevada de TAG pode estar relacionada diretamente ao teor calórico das presas consumidas pelos tubarões na região. Por exemplo, o tubarão *N. brevirostris* alimenta-se principalmente de peixes, e no arquipélago é frequentemente visto forrageando próximo à arrebentação onde há cardumes de sardinhas (Garla *et al*., 2017), peixe com alto teor de gordura (Bouriga *et al*., 2022). Isso poderia explicar também a alta porcentagem de SFAs circulante no plasma, principalmente C16:0, encontrada no tubarão *N. brevirostris*, uma vez que o C16:0 é o segundo ácido graxo mais abundante em sardinhas, depois do DHA (Bouriga *et al*., 2022). Os SFAs podem ser biossintetizados *de novo* a partir de precursores de carboidratos ou proteínas (Budge *et al*., 2006), no entanto, SFA plasmáticos de tubarões têm sido encontrados em proporções semelhantes ao observado em sua dieta (*e.g.,* McMeans *et al*., 2012; Bierwagen *et al*., 2019). Além disso, concentrações elevadas de TAG foram descritas em tubarões *Carcharias taurus* com maior dependência de recursos alimentares pelágicos, comparado aos indivíduos com maior dependência de recursos demersais (Shipley *et al*., 2022). É possível que algo semelhante esteja acontecendo com o *N. brevirostris* em Fernando de Noronha, uma vez que a sardinha é um peixe pelágico. No entanto, esta explicação não justifica as concentrações elevadas de TAG encontrada no *G. cirratum*, uma vez que a dieta desta espécie em outras regiões é baseada principalmente em presas demersais (e.g., pequenos teleósteos e polvos, Castro, 2000). Portanto, é possível que tanto a taxa metabólica quanto a preferência alimentar dos tubarões estejam relacionadas com as concentrações de TAG observadas no presente estudo (Gallagher *et al*., 2017; Shipley *et al*., 2022).

Além disso, é possível que as elevadas concentrações de TAG encontradas em *G. cirratum* seja impulsionado por um aumento sazonal durante a estação chuvosa. As concentrações de TAG durante a estação chuvosa pode indicar um aumento na condição corpórea (e.g., Gallagher *et al*., 2014b, 2017; Hammerschlag *et al*., 2018) talvez relacionado à reprodução da espécie que ocorre neste período (abril – setembro, Afonso *et al*., 2016; Rangel *et al*., 2021b). Em um estudo comparando substratos energéticos entre as estações chuvosa e seca para o *G. cirratum* no sul da Florida não encontrou diferença estatística no TAG entre as duas estações, mas encontrou a condição corpórea era superior em fêmeas durante a estação chuvosa (Moorhead *et al*., 2021).

A concentração de GLU é frequentemente utilizada para avaliar o estresse de captura em tubarões (*e.g.*, Marshall *et al*., 2012; Jerome *et al*., 2017), mas pouco explorada em estudos nutricionais. Apesar disso, os resultados demonstram que a concentração de GLU poderia ser um interessante indicador nutricional, uma vez que ela foi positivamente correlacionada com as concentrações de TAG e CHOL nos tubarões *C. perezi*, *G. cuvier* e *G. cirratum* (este apenas TAG), destacando uma possível organização comum dos substratos energéticos lipídicos e glicídicos. As elevadas concentrações de GLU encontradas em tubarões mais ativos corroboram com achados em estudos de estresse de captura, em que espécies mais ativas apresentaram elevados valores de GLU (e.g., Marshall *et al*., 2012; Jerome *et al*., 2017). Isso porque a GLU é liberada dos estoques de glicogênio no fígado durante o exercício, pelas catecolaminas (*e.g*., epinefrina e norepinefrina; DeRoos e DeRoos, 1978), e poderia explicar as elevadas concentrações em espécies com maior demanda energética. Além disso, maiores concentrações de GLU foram encontradas durante a estação chuvosa nos tubarões *G. cuvier* e *G. cirratum*, estação correspondente ao período reprodutivo de ambas as espécies (Afonso *et al*., 2016; Rangel *et al*., em revisão), quando é esperado que os tubarões tenham maior atividade muscular (Valls *et al*., 2016; Moorhead *et al*., 2021; Rangel *et al*., 2021).

As correlações positivas encontradas entre as proteínas totais e tamanho corpóreo nos tubarões *C. perezi* e *G. cuvier* pode ser um indicativo de maior uso das proteínas durante as fases juvenis, por exemplo para construção de tecido muscular, e consequente menor disponibilidade circulante no sangue (e.g., Dabrowski, 1986). É possível ainda que haja uma maior incorporação de dieta proteica em animais de maior tamanho corpóreo (Dabrowski, 1986). Ainda, encontramos maiores concentrações de proteínas totais durante a estação chuvosa para o *G. cuvier*, época que parece compreender o período reprodutivo da espécie na região, como mencionado acima (Rangel *et al*., em revisão), o que pode sugerir uma mudança sazonal da dieta ou na mobilização das proteínas totais para manter a reprodução. Embora poucos estudos têm utilizado as proteínas totais como biomarcador nutricional para acessar variações sazonais e ontogenéticas (e.g., Hoopes *et al*., 2022), as proteínas totais têm sido amplamente utilizadas para monitorar a saúde e a condição nutricional de tubarões de cativeiro (e.g., Parkinson *et al*., 2019; Hoopes *et al*., 2022), especialmente porque os tubarões dependem mais da oxidação de aminoácidos como combustível energético comparado a outros vertebrados (Speers-Roesch e Treberg, 2010).

Apesar dos tubarões compartilharem a mesma área (evidente nos dados de captura), a análise multivariada do perfil de ácidos graxos plasmáticos revelou que eles forrageiam

diferentes recursos alimentares, com exceção dos tubarões *G. cirratum* e *G. cuvier* que não diferiram estatisticamente. Uma possível explicação seria que os tubarões *G. cirratum* e *G. cuvier* compartilham alguns dos mesmos recursos alimentares ou presas com dietas semelhantes, ou ainda que o *G. cuvier* se alimenta com certa frequência de tubarões *G. cirratum* (e.g., Rada *et al*., 2015). Resultados similares foram observados em um estuário no norte da Austrália, em que grande similaridade no perfil de ácidos graxos foi encontrada entre os tubarões *Carcharhinus leucas* com outras duas espécies de tubarões (*Glyphis garricki* e *Rhizoprionodon taylori*), sugerindo que o *C. leucas* os está consumindo ou as três espécies estão consumindo presas semelhantes (Every *et al*., 2019).

As porcentagens de DHA e EPA, assim como a somatória PUFA n3 e as razões n3/n6, DHA/ARA e EPA/ARA foram maiores no tubarão *C. perezi* e responsáveis pela separação desta espécie das demais. Uma possível explicação é que os PUFAs n3, em particular o DHA, são sucessivamente enriquecidos através da cadeia alimentar (Parrish *et al*., 2015b; Meyer *et al*., 2019), indicando que o *C. perezi* esteja se alimentando de presas de maior nível trófico que as demais espécies, com maior grau de carnivoria. Isso porque o DHA é sintetizado principalmente por dinoflagelados e minimamente modificado durante os processos de ingestão e assimilação; consequentemente, é transferido e retido seletivamente nos consumidores (Dalsgaard *et al*., 2003). Além disso, estudos em outras regiões mostram que o DHA e EPA são encontrados em elevadas porcentagem em peixes recifais, planctívoros e cefalópodes (Belling *et al*., 1997; Bierwagen *et al*., 2019). Além disso, o DHA é característico em cadeias alimentares baseadas em dinoflagelados (Dalsgaard *et al*., 2003), o que indica uma interação trófica diferenciada desta espécie.

Com exceção do MUFA, que foi negativamente correlacionado com o tamanho corpóreo, surpreendente não foram encontradas outras correlações significativas entre os ácidos graxos e o tamanho corpóreo em *C. perezi*. Uma vez que foram analisados estágios de vida diferentes, ou seja, de tubarões neonatos (<90 cm CT) a adultos (> 200 cm CT), era esperado que o perfil de ácidos graxos variasse em relação ao tamanho corpóreo, especialmente entre neonatos e adultos. Isso porque em tubarões placentários, como é o caso do *C. perezi*, os filhotes nascem deficientes em ácidos graxos essenciais, adquirindo esses ácidos graxos apenas quando começam a se alimentar eficientemente (e.g., *Carcharhinus leucas*, Belicka *et al*., 2012). É possível que o maior investimento materno encontrado em tubarões *C. perezi* (3-6 filhotes, gestação de ~12 meses i.e., menos filhotes de maior tamanho) em comparação ao *C. leucas* (até 13 filhotes, gestação de ~12 meses, i.e., mais filhotes de menor tamanho) (Ebert *et al*., 2021) contribua para a diferença observada, uma vez que o

investimento materno durante a gestação tem grande influência no perfil de ácidos graxos neonatal (e.g., Rangel *et al*., 2020). Estudos adicionais focando na variação nutricional ontogenética da espécie são necessários para testar essa hipótese.

O ARA, e consequentemente a somatória PUFA n6, foram os principais responsáveis pela sobreposição dos tubarões *G. cirratum* e *G. cuvier* das demais espécies analisadas. Esses dados sugerem que ambos *G. cuvier* e *G. cirratum* sejam mais dependentes de recursos alimentares bentônicos como teleósteos e invertebrados, uma vez que o ARA é um dos ácidos graxos abundantes em algas marrons e corais (Kelly e Scheibling, 2012; van Duyl *et al*., 2011), e tem sido encontrado em altas proporções em animais bentívoros, como crustáceos e cefalópodes em recifes de corais (Bierwagen *et al*., 2019). Além disso, os valores de ARA para *G. cuvier* encontrados no presente estudo se assemelham ao observado nas Bahamas para a espécie (i.e., ~11,0 %, Rangel *et al*., 2021a), sugerindo que apesar da diferença entre os habitats, há uma certa semelhança nos padrões alimentares da espécie nestas regiões. Futuros estudos comparativos em grandes escalas espaciais ajudariam a revelar estes padrões nutricionais para a espécie.

Ambos *G. cuvier* e *G. cirratum* apresentaram diferença sazonal em alguns dos mesmos ácidos graxos, como C16:0, EPA, ARA, PUFA n6. Uma possível explicação é que os tubarões *G. cuvier* estão se alimentando de *G. cirratum* e/ou que as duas espécies compartilham os mesmos recursos alimentares (e.g., Every *et al*., 2019). Por exemplo, o EPA, ARA e a somatória PUFA n6 estava em maior proporção na estação seca em ambos tubarões *G. cuvier* e *G. cirratum*, também foram os principais responsáveis pela separação das duas espécies das demais, podendo sugerir uma flutuação sazonal no perfil nutricional das presas consumidas de ambas espécies. Ainda para o *G. cuvier*, foi detectada uma maior proporção de C18:1n9, que tem sido descrito como um marcador trófico de peixes mesopelágicos e cefalópodes de águas mais profundas (Phillips *et al*., 2001; Meyer *et al*., 2019; Xu *et al*., 2022).

Correlações negativas significativas encontradas entre o tamanho corpóreo e PUFA n3 e razão n3/n6 em *G. cirratum* e DHA e razão n3/n6 em *G. cuvier*, indicam para um menor consumo ou disponibilidade circulante de ácidos graxos ômega-3 com o crescimento corpóreo. Uma possível explicação é que as duas espécies apresentem uma variação na dieta com o crescimento corpóreo, ou seja, alimentando-se menos de peixes recifais e cefalópodes, ricos em ômega-3 (e.g., Belling *et al*., 1997; Bierwagen *et al*., 2019), mas os dados do presente estudo não permitem chegar a esta conclusão, pois não foram coletados animais suficientes durante as diferentes fases de crescimento. É possível ainda que quando adultos, os

tubarões *G. cirratum* e *G. cuvier* invistam ácidos graxos ômega-3 para na reprodução, ou seja, utilizem nos processos de vitelogênese (em fêmeas) e espermatogênese (machos) (e.g., tubarões, Rangel *et al*., 2021a,b; peixe-espada, Sardenne *et al*., 2022), diminuindo a disponibilidade desses ácidos graxos circulantes. As variações sazonais encontradas nas porcentagens de EPA e PUFA n3 para ambas as espécies parece corroborar essa hipótese. Menores porcentagens de EPA (*G. cuvier* e *G. cirratum*) e da somatória PUFA n3 (*G. cuvier*) foram encontradas durante a estação chuvosa, período no qual ambas espécies parecem estar na fase reprodutiva (i.e., cópula e início de gestação em fêmeas; Afonso *et al*., 2016; Rangel *et al*., em revisão).

Em resumo, os resultados revelaram que os tubarões mais abundantes ao redor do Arquipélago de Fernando de Noronha diferem no seu metabolismo energético e padrões de dieta, o que é consistente com o observado em outros estudos usando estes mesmos marcadores nutricionais (*e.g*., Gallagher *et al*., 2017; Every *et al*., 2019; Bierwagen *et al*., 2019). Embora tenha sido encontrado alguns padrões semelhantes entre algumas espécies, de forma geral os resultados indicam potenciais diferenças funcionais entre tubarões neste arquipélago oceânico. Além disso, as variações sazonais observadas nos substratos energéticos e ácidos graxos dos tubarões *G. cuvier* e *G. cirratum* entre a estação seca e chuvosa sugerem flutuações na dieta entre as duas estações e ou uma relação com o período reprodutivo das espécies. Futuros estudos e monitoramento a longo prazo dos padrões ecofisiológicos são recomendados, especialmente porque Fernando de Noronha é um arquipélago oceânico exposto à crescente visitação turística.

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Material Suplementar

Tabela S1. Lista com todos os tubarões capturados, com o respectivo número da marca plástica, data da captura, comprimento total (CT, cm) e sexo.

Espécie	Nº Tag	Método de pesca	Dia	Mês	Ano	CT (cm)	Sexo
Carcharhinus perezi	1061	Linha de espera	17	2	2020	136	Macho
Carcharhinus perezi	1055	Linha de espera	17	2	2020	137	Fêmea
Negaprion brevirostris	1004	Linha de espera	17	2	2020	245	Macho
Negaprion brevirostris	1067	Linha de espera	17	2	2020	260	Fêmea
Carcharhinus perezi	1068	Linha de espera	18	2	2020	221	Fêmea
Negaprion brevirostris	1056	Linha de espera	18	2	2020	210	Fêmea
Negaprion brevirostris	1066	Linha de espera	18	2	2020	228	Fêmea
Negaprion brevirostris	1052	Linha de espera	18	2	2020	248	Macho
Negaprion brevirostris	1057	Linha de espera	18	2	2020	250	Macho
Negaprion brevirostris	1063	Linha de espera	18	2	2020	253	Fêmea
Galeocerdo cuvier	00488	Linha de espera	21	2	2020	256	Fêmea
Negaprion brevirostris	00477	Linha de espera	21	2	2020	240	Macho
Negaprion brevirostris	00495	Linha de espera	21	2	2020	268	Fêmea
Carcharhinus falciformis	00483	Linha de espera	22	2	2020	220	Macho
Carcharhinus falciformis	00479	Linha de espera	22	2	2020	229	Fêmea
Carcharhinus falciformis	00501	Linha de espera	22	2	2020	254	Fêmea
Carcharhinus perezi	00514	Linha de espera	22	2	2020	129	Macho
Carcharhinus perezi	00496	Linha de espera	22	2	2020	216	Fêmea
Ginglymostoma cirratum	00486	Linha de espera	22	2	2020	236	Fêmea
Negaprion brevirostris	01067	Linha de espera	22	2	2020	260	Fêmea
Negaprion brevirostris	00505	Linha de espera	23	2	2020	265	Fêmea
Negaprion brevirostris	00503	Linha de espera	23	2	2020	267	Fêmea
Carcharhinus perezi	430	Linha de mão	24	2	2020	86	Fêmea
Carcharhinus perezi	426	Linha de mão	24	2	2020	90	Fêmea
Carcharhinus perezi	434	Linha de mão	24	2	2020	110	Fêmea
Carcharhinus perezi	443	Linha de mão	24	2	2020	110	Macho
Carcharhinus perezi	447	Linha de mão	24	2	2020	150	Macho
Carcharhinus perezi	00506	Linha de mão	24	2	2020	195	Fêmea
Carcharhinus perezi	441	Linha de mão	25	2	2020	78	Fêmea
Carcharhinus perezi	441	Linha de mão	25	2	2020	92	Fêmea
Carcharhinus perezi	437	Linha de mão	25	2	2020	97	Macho
Carcharhinus perezi	440	Linha de mão	25	2	2020	99	Macho
Carcharhinus perezi	432	Linha de mão	25	2	2020	115	Macho
Carcharhinus perezi	00494	Linha de espera	26	2	2020	235	Fêmea
Carcharhinus perezi	00480	Linha de espera	27	2	2020	203	Macho
Galeocerdo cuvier	00499	Linha de espera	27	2	2020	310	Macho
Ginglymostoma cirratum	00489	Linha de espera	27	2	2020	117	Macho
Carcharhinus perezi	446	Linha de espera	29	2	2020	87.5	Macho
Carcharhinus perezi	436	Linha de espera	29	2	2020	121	Macho
Carcharhinus perezi	439	Linha de espera	29	2	2020	127.5	Macho
Carcharhinus perezi	444	Linha de espera	29	2	2020	128	Fêmea
Carcharhinus perezi	442	Linha de espera	29	2	2020	133	Fêmea
Carcharhinus perezi	00435	Linha de espera	10	1	2022	190	Macho
Carcharhinus perezi	00525	Linha de espera	10	1	2022	207	Fêmea
Galeocerdo cuvier	00493	Linha de espera	10	1	2022	250	Fêmea
Negaprion brevirostris	00507	Linha de espera	10	1	2022	230	Macho
Carcharhinus perezi	00478	Linha de espera	11	1	2022	190	Macho
Carcharhinus perezi	00504	Linha de espera	11	1	2022	195	Macho

Galeocerdo cuvier	00517	Linha de espera	11	1	2022	212	Fêmea
Galeocerdo cuvier	00484	Linha de espera	11	1	2022	220	Macho
Galeocerdo cuvier	00515	Linha de espera	11	1	2022	230	Fêmea
Negaprion brevirostris	00491	Linha de espera	11	1	2022	270	Fêmea
Galeocerdo cuvier	408	Linha de espera	19	1	2022	140	Fêmea
Galeocerdo cuvier	00497	Linha de espera	19	1	2022	210	Fêmea
Galeocerdo cuvier	00521	Linha de espera	19	1	2022	252	Macho
Galeocerdo cuvier	00519	Linha de espera	19	1	2022	260	Fêmea
Galeocerdo cuvier	00582	Linha de espera	19	1	2022	260	Macho
Ginglymostoma cirratum	NA	Linha de espera	19	1	2022	207	Macho
Ginglymostoma cirratum	00518	Linha de espera	19	1	2022	230	Macho
Galeocerdo cuvier	00547	Linha de espera	22	1	2022	193	Fêmea
Galeocerdo cuvier	00524	Linha de espera	22	1	2022	448	Fêmea
Ginglymostoma cirratum	00512	Linha de espera	22	1	2022	237	Macho
Carcharhinus perezi	427	Linha de mão	23	1	2022	80	Macho
Carcharhinus perezi	00500	Linha de espera	23	1	2022	210	Macho
Galeocerdo cuvier	00476	Linha de espera	23	1	2022	273	Macho
Ginglymostoma cirratum	00508	Linha de espera	23	1	2022	213	Macho
Ginglymostoma cirratum	520	Linha de espera	10	5	2022	240	Macho
Ginglymostoma cirratum	492	Linha de espera	10	5	2022	241	Fêmea
Ginglymostoma cirratum	490	Linha de espera	10	5	2022	242	Macho
Ginglymostoma cirratum	511	Linha de espera	13	5	2022	235	Macho
Galeocerdo cuvier	522	Linha de espera	15	5	2022	243	Fêmea
Galeocerdo cuvier	598	Linha de espera	15	5	2022	253	Fêmea
Galeocerdo cuvier	596	Linha de espera	15	5	2022	308	Fêmea
Ginglymostoma cirratum	593	Linha de espera	15	5	2022	173	Macho
Ginglymostoma cirratum	546	Linha de espera	15	5	2022	183	Fêmea
Ginglymostoma cirratum	512	Linha de espera	15	5	2022	239	Macho
Ginglymostoma cirratum	595	Linha de espera	15	5	2022	244	Fêmea
Carcharhinus perezi	509	Linha de espera	16	5	2022	154	Macho
Carcharhinus perezi	NA	Linha de espera	16	5	2022	210	Fêmea
Ginglymostoma cirratum	531	Linha de espera	16	5	2022	150	Fêmea
Galeocerdo cuvier	585	Linha de espera	18	5	2022	255	Macho
Galeocerdo cuvier	599	Linha de espera	18	5	2022	340	Fêmea
Carcharhinus perezi	588	Linha de espera	19	5	2022	208	Macho
Galeocerdo cuvier	580	Linha de espera	19	5	2022	216	Fêmea
Negaprion brevirostris	986	Linha de espera	19	5	2022	255	Macho
Carcharhinus plumbeus	576	Linha de espera	21	5	2022	196	Macho
Galeocerdo cuvier	591	Linha de espera	21	5	2022	307	Fêmea
Ginglymostoma cirratum	600	Linha de espera	21	5	2022	220	Macho

*NA: animais que acabaram não sendo marcados. Receberam apenas identificação da sequência de captura, para individualizá-los para análises laboratoriais.

Capítulo 2

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 a predator's nutritional quality, 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 quality and trophic dynamics of female tiger sharks (*Galeocerdo cuvier*). Despite their generalist feeding behavior, female tiger sharks fed on different food sources and/or modulate 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 for coastal/benthic food resources during juvenile life stages, and pelagic/oceanic and reef-associated food resource 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 suggest the possibility of a selective diet on 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.

Keywords: trophic markers, nutritional condition, dietary patterns, tiger sharks, *Galeocerdo cuvier*, reproduction, trophic ecology, physiology.

2.1. 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). Across a predator's life cycle, 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 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 predator's nutritional condition remains limited (Dicken et al. 2017, Wai et al. 2012, 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. 1999, 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, the 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. 2016). 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 (Darlsgaard et al. 2003, Iverson 2009, Budge et al. 2006).

The tiger shark (*Galeocerdo cuvier*) is a large, globally distributed marine apex predator 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 (Ferreira et al. 2017, Dicken 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 months, Whitney & Crow 2007), and a comparatively large broods (18–70) of large embryos (~75 cm, Whitney & Crow 2007, Castro et al. 2016). Accordingly, detailed description of how diet and nutritional condition vary across their life and life stage 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 condition 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 getting 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 two hypotheses related to

reproduction and one hypothesis related to ontogeny and spatial variation. First, we hypothesized that females preparing to reproduce would have higher nutritional quality (i.e. higher percentages of n3 and n6 HUFAs) than other life-stages. This is because: (i) 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 (ii) adult, non-gravid females have been found to exhibit higher energy demand (i.e. with higher corticosterone and lower T_3 [triiodothyronine] hormone levels) compared to immature and gravid females (B. Rangel, N. Hammerschlag, J. Sulikowski and R. Moreira, unpubl. data). Secondly, given that 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 is that females would vary in diet across life stages, demonstrating ontogenetic changes in trophic markers (i.e. basal resources, Fig. 1). Based on that, we expected that smaller sharks would have higher coastal fatty acid trophic markers (i.e. C18:2n6, ARA), whilst large tiger sharks would have higher percentages of pelagic/oceanic markers (i.e. DHA [docosahexaenoic acid, C22:6n3], C18:1n9, C16:1n7).

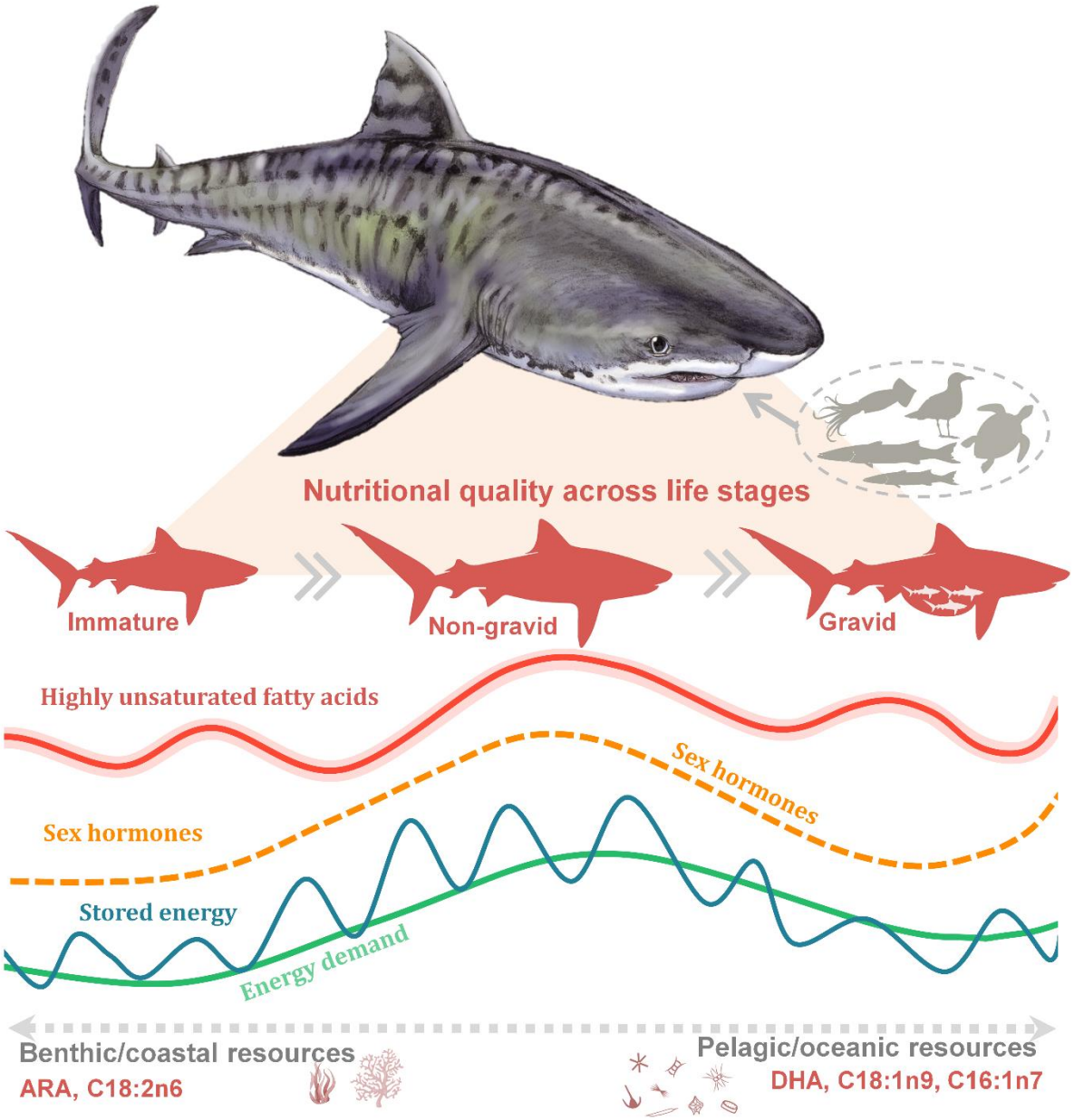

Figure 1- Conceptual model of hypothesized changes in nutritional quality (highly unsaturated fatty acids) and trophic markers across life stages (immature, adult, but non-gravid and gravid) of female tiger sharks (*Galeocerdo cuvier*). We predict that non-gravid adult females preparing to reproduce will 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 (Rangel et al. unpublished data). We further predict that gravid females will not display a 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 will rely more on coastal/benthic prey sources, exhibiting higher coastal fatty acid trophic markers, while larger tiger sharks will rely more on offshore food sources, exhibiting a higher percentages of pelagic/oceanic trophic markers.

2.2. Material & Methods

2.2.1. 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 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 in a partially submerged platform, and a water pump 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 (i.e. total length, cm). Finally, sharks were tagged for identification and released.

Phlebotomy (~20 ml) was conducted from the caudal vein using 18-gauge needles and 10-mL heparinized syringes and immediately centrifuged (3500 rpm, 410 × g) for 2 min. Plasma was then removed, placed within a cooler on the boat and then stored frozen at −20°C for future analyses.

2.2.2. Hormone analysis and reproductive status

The plasma concentration values of the gonadal steroids (17β-estradiol and testosterone) and life stages of tiger sharks were based on those determined by Sulikowski et al. (2016). In brief, the hormone levels were measured were from Steraloids (Newport, RI) by radioimmunoassay, following the procedure from Sulikowski et al. (2004). A Tri-Carb 2900TR liquid scintillation analyzer (PerkinElmer, Waltham, MA) 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 use 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 samples between 2012 and 2019, pregnancy status were assessed through ultrasonography (Ibex Pro portable ultrasound, EI Medical Imaging, Loveland, CO), 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 as gravid or non-gravid.

2.2.3. Fatty acid analysis

Fatty acid (FA) 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 3mL 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, then the tubes were mixed and centrifuged at 2,000 rpm for 5 min. The upper layer was then removed, transferred to 2 ml injection vials and the volume reduced under a nitrogen stream. FA analysis was carried out in a Varian gas chromatograph (GC, Model 3900, www.varian.com) coupled with a flame ionisation detector and a CP-8410 autosampler, as described by Rangel et al. (2019). The data are presented as % of total FA methyl-esters based on peak area analyses.

2.2.4. Fatty acid trophic markers and nutritional indicators

Fatty acids that accounted for less than 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 quality (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 as an indicative of diatoms (Parrish et al. 2000, Budge et al. 2006). The DHA/EPA ratio was used as marker of trophic position, as it has been significantly correlated with $\delta^{15}N$ (El-Sabaawi et al. 2009, Sardenne et al. 2017), and C18:1n9/C18:1n7 ratio as a marker of degree of carnivory (Dalsgaard et al. 2003, Parrish et al. 2015b). Additionally, ARA and C18:2n6 values have been found to be useful to indicate if a species inhabits coastal/benthic environments (Sardenne et al. 2017), and the odd chain fatty acids (OFA) and branched chain fatty acids (BFA) as biomarkers of heterotrophic bacteria (Dalsgaard et al. 2003).

2.2.5. Data analysis

Linear regression was used to separately test for a relationship between fatty acids (%) and body size (i.e. total length) and 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 (those physiologically 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. To determine the difference between fatty acid profiles across female life stages was tested comparing each fatty acid using one-way ANOVA with 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 assumptions were violated, Kruskal-Wallis H tests followed by Dunn's post hoc for non-parametric data. Statistical significance was based on $p<0.05$. All analyses were conducted in SigmaStat 3.10 (SystatSoftware, Inc., www.systat.com) and PAST 3.12 (EFB, www.essential-freebies.de). Discriminant analyses (LDA) 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).

2.3. Results

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

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. Polyunsaturated fatty acids (PUFAs), consisting of largely DHA and ARA, were in the greatest percentages for non-gravid and gravid. However, monounsaturated fatty acids (MUFAs), largely C18:1n9 were in the highest proportion for immature sharks (Table 1). Whole blood also largely comprised of SFAs followed by MUFAs for all life stages (Table 1). Female size influenced the composition of fatty acids (Tables 2 and 3), with DHA and n3/n6 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-d), while just C18:0 decreased in whole blood (Table 2 and 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 of degree of carnivory (C18:1n9/C18:1n7) and body size, and positive between C18:1n7 and the diatoms marker (C16:1n7/C16:0) and body size (Table 2 and 3, Fig. 2f-h). In the whole blood, C18:2n6 also increased and C16:1n7/C16:0 decreased with body size (Table 2 and 3).

The plasma concentration of the gonadal steroid hormones, 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 and 3, Fig. 3g). DHA and n3/n6 increased with increasing 17β-estradiol in plasma (Fig. 3c,e), while C18:2n6 decreased with increasing testosterone (Table 2 and 3). PUFA n3, DHA, C14:1, C18:2n6 and 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 (Table 2 and 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 (Table 2 and 3).

2.3.1. Fatty acids differences across life stages

Among life stages, plasma SFA, including C18:0, were higher in immature than both non-gravid and gravid females (Figs. 4 and 5, Table 1). 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 (Fig. 5b, Table 1). Plasma total PUFA was higher in gravid compared to immature females, and n3 PUFA, including DHA, was lower in immature than both non-gravid and gravid females (Table 1, Fig. 4 and 5). Plasma n6 PUFA was higher in gravid compared to non-gravid, 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, Fig. 4 and 5).

With respect to trophic markers and nutritional indicators, plasma n3/n6 ratio was higher in non-gravid compared to both immature and gravid females (Table 1, Fig. 5e). In plasma, the bacterial maker (BFA-OFA) was higher in immature than non-gravid sharks (Fig. 6a), whilst the diatoms marker (C16:1n7/C16:0) was lower in immature sharks compared to both non-gravid and gravid females (Fig. 6b). The carnivory index (C18:1n9/C18:1n7) was higher in immature than both non-gravid and gravid females (Fig. 6c).

The LDA plasma analyses revealed that the first two discriminant functions distinguished the life stages (Table S1, Fig. 7a,b). The first function separated immature and non-gravid females, mainly due to differences in DHA, C16:0, PUFA, n3 PUFA, and SFA percentages, while second function separated non-gravid and gravid females, including their differences in ARA, C16:0, SFA, PUFA and n6 PUFA (Fig. 7a,b). From whole blood fatty acids, LDA analyses revealed that the first two discriminant functions distinguished the life stages (Table S1, Fig. 7c,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 PUFA, and MUFA (Fig. 7c,d). The second function separated non-gravid from gravid females, due to their differences in DHA, C18:1n9, PUFA, n3 PUFA and MUFA (Fig. 7c,d, Table S1).

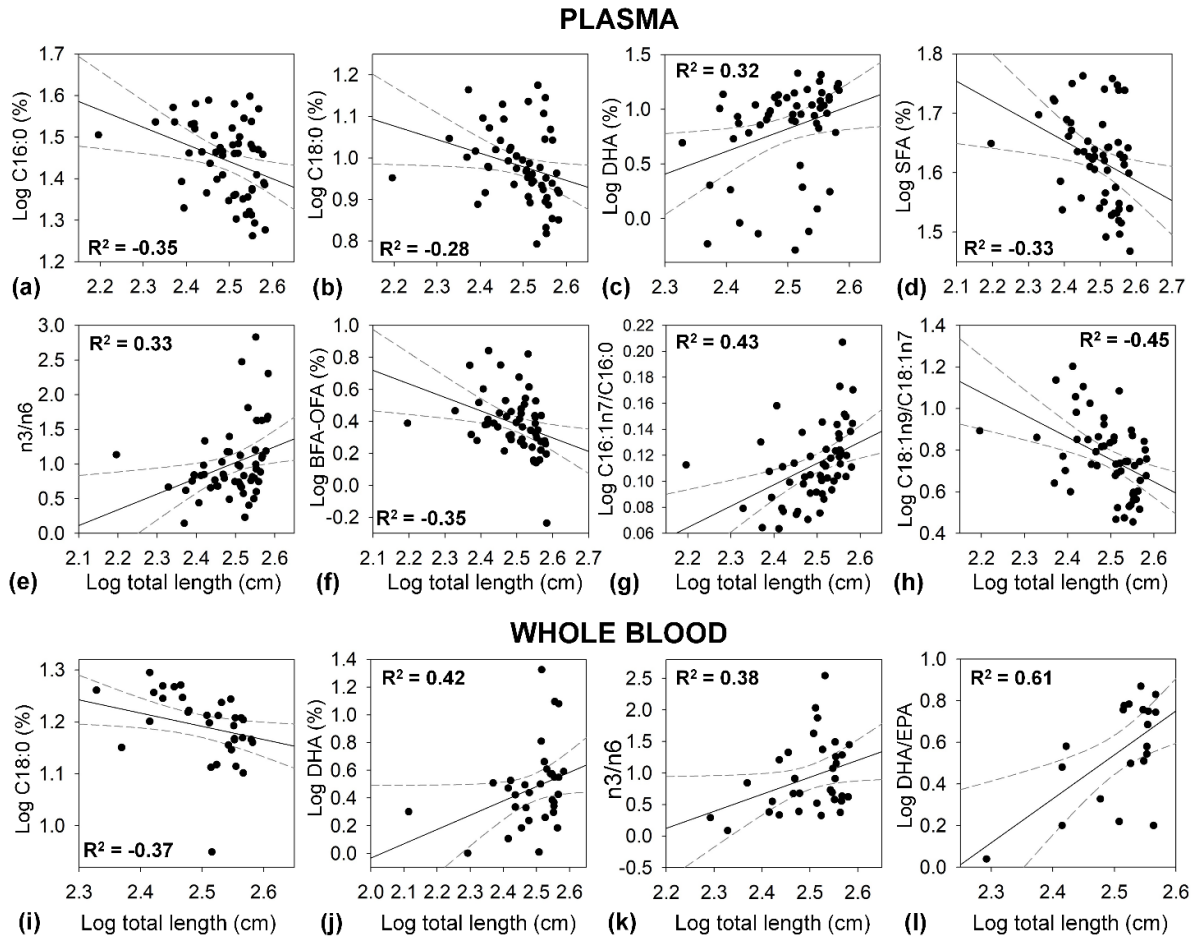

Figure 2- Significant relationships between plasma and whole blood fatty acids and total length (cm) for female tiger sharks (*Galeocerdo cuvier*). The solid black line represents the regression line, and the horizontal dashed lines represent the 95% confidence intervals. Numbers are log transformed. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, SFA: saturated fatty acid, BFA – OFA: branched chain and odd chain fatty acid.

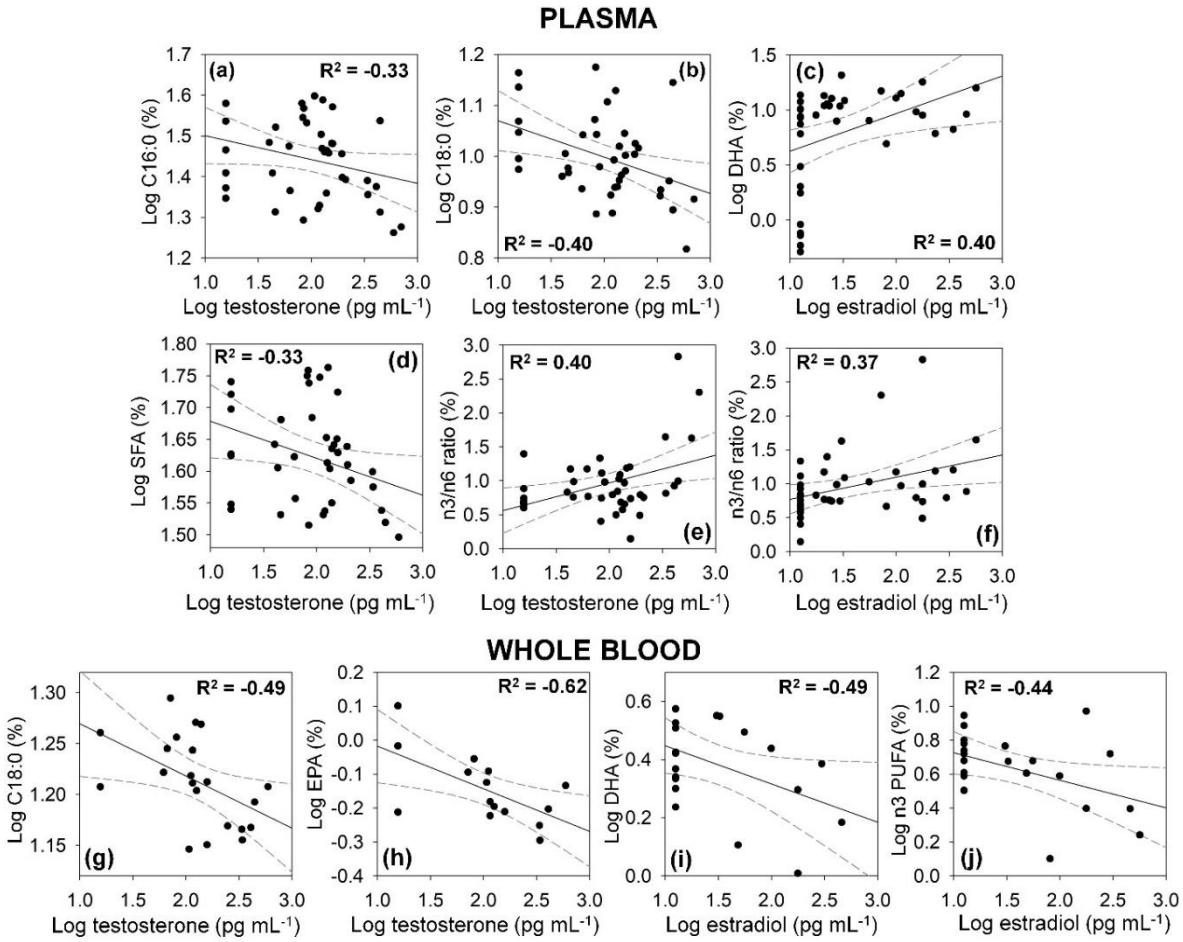

Figure 3- Significant relationships between plasma and whole blood fatty acids and reproductive hormones (testosterone and estradiol) for female tiger sharks (*Galeocerdo cuvier*). The solid black line represents the regression line, and the horizontal dashed lines represent the 95% confidence intervals.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

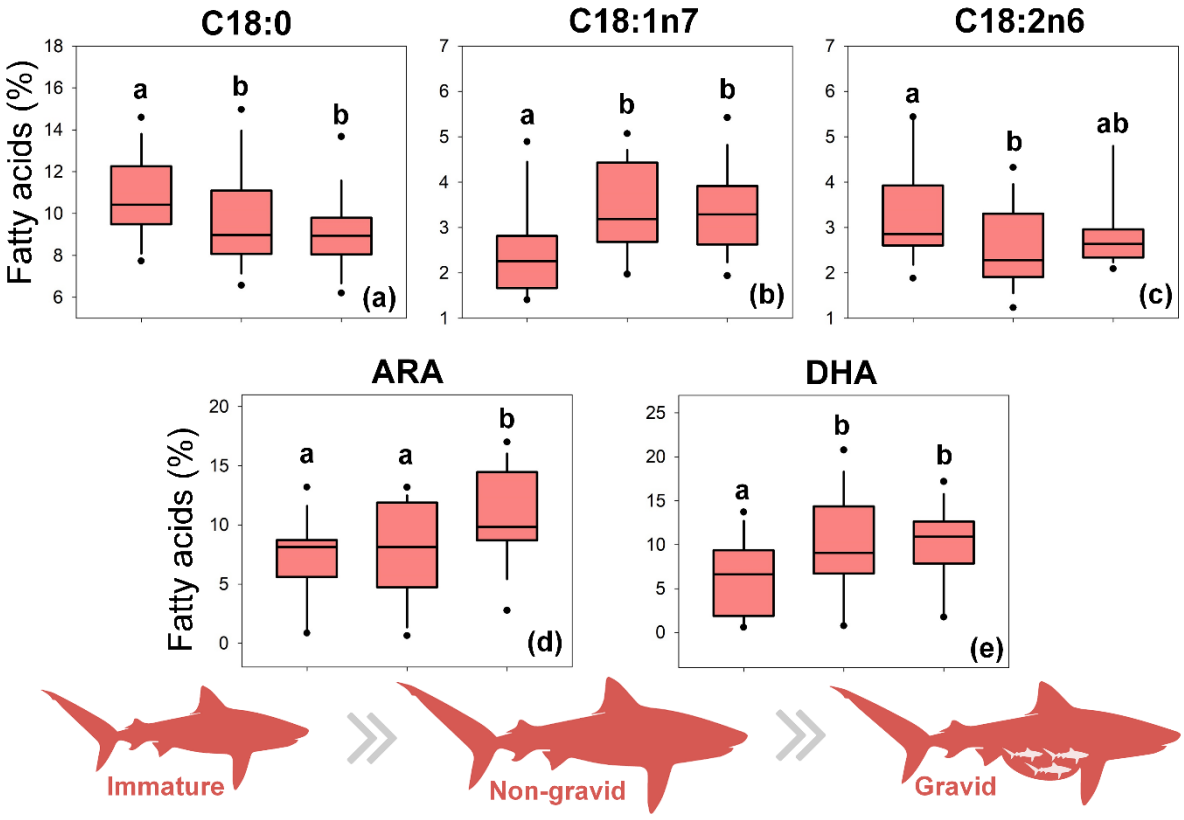

Figure 4- Boxplots of plasma fatty acids (%) in female tiger sharks (*Galeocerdo cuvier*) of different life stages (immature, adult, but non-gravid and gravid), black line indicates the median value. ARA**:** C20:4n6 (arachidonic acid), DHA: C22:6n3 (docosahexaenoic acid). Significant difference among life stages are denoted with different superscripts above bars (ANOVA, $p < 0.05$).

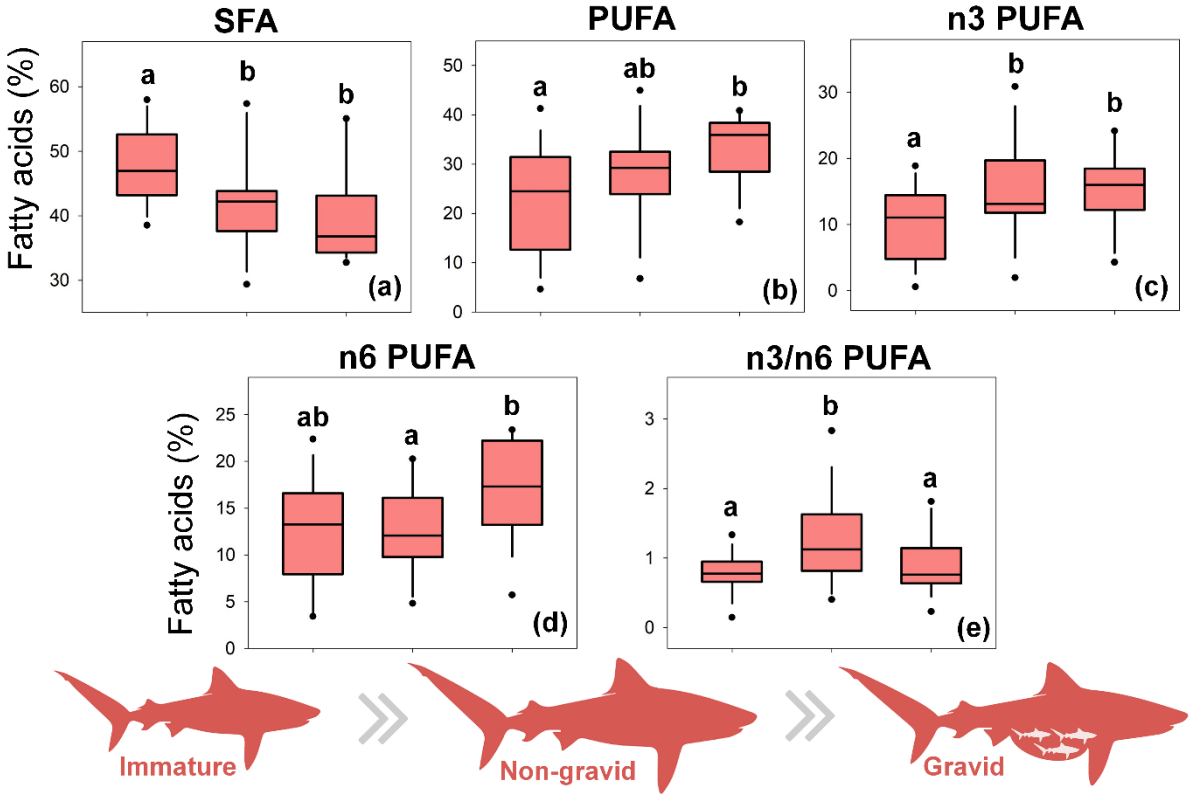

Figure 5- Boxplots of plasma fatty acids in female tiger sharks (*Galeocerdo cuvier*) of different life stages (immature, adult, but non-gravid and gravid), black line indicates the median value. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, n3: omega, n6: omega 6. Significant difference among life stages is denoted with different superscripts above bars (ANOVA, $p < 0.05$).

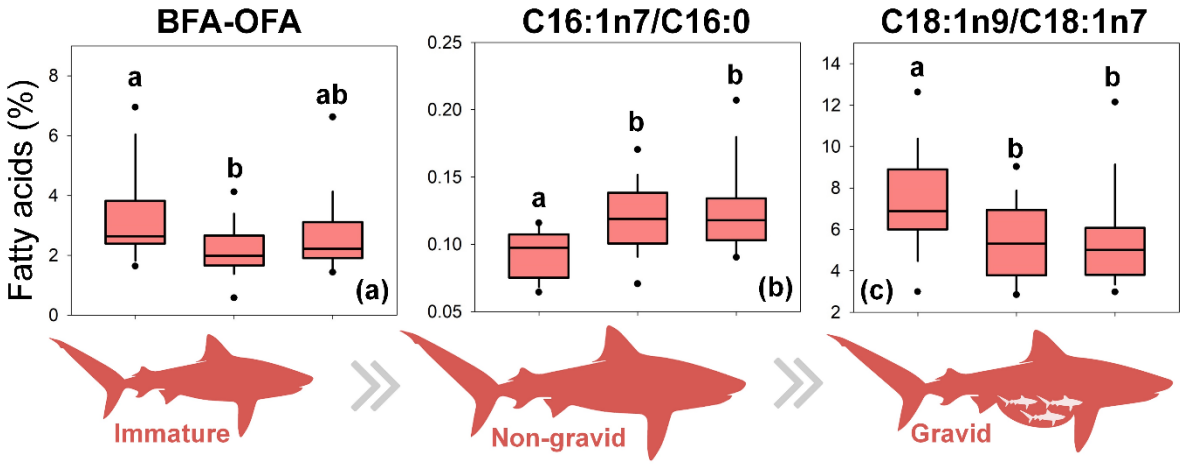

Figure 6- Boxplots of plasma fatty acid trophic markers in female tiger sharks (*Galeocerdo cuvier*) of different life stages (immature, adult, but non-gravid and gravid), black line indicates the median value. BFA-OFA: branched chain and odd chain fatty acid. Significant difference among life stages is denoted with different superscripts above bars (ANOVA, $p < 0.05$).

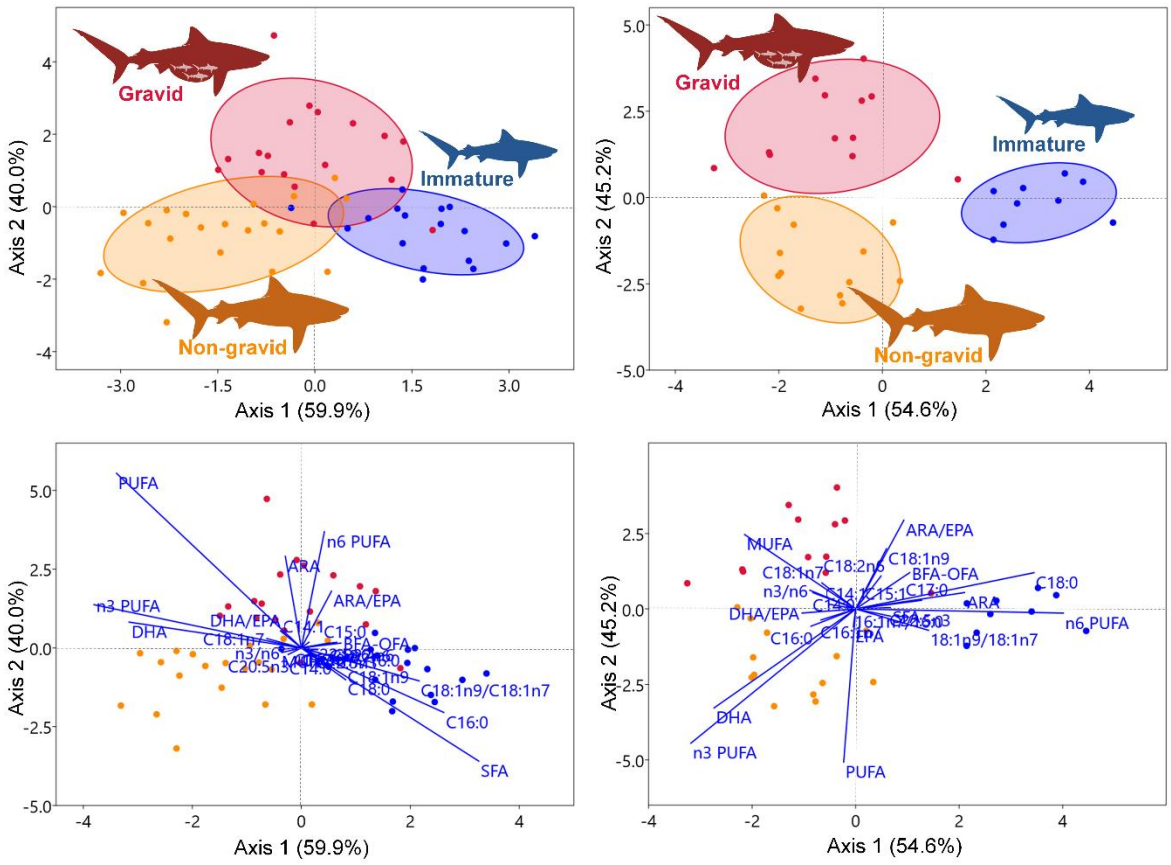

Figure 7- Linear discriminant function analyses of all fatty acids and trophic markers for life stages (immature, adult, but non-gravid and gravid) of female tiger sharks, *Galeocerdo cuvier*. **(a-b)** plasma fatty acids (eigenvalues: Axis 1= 1.73, Axis 2= 1.15) and **(c-d)** whole blood fatty acids (eigenvalues: Axis 1= 3.62 and Axis 2= 3.00). The 70% ellipses similarly of each life stage is provided.

Table 1- Comparative fatty acid profile of plasma and whole blood (mean % ± standard deviation) among life stages (immature, adult but non-gravid, and gravid) of tiger sharks (*Galeocerdo cuvier*). P values for one-way ANOVA with Tukey post-hoc test to 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 PUFA: omega 3 PUFA, n6 PUFA: omega 6 PUFA, BFA – OFA: branched chain and odd chain fatty acid. NA (missing value).

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

Σ n6 PUFA	13.4 ± 5.82[ab]	12.7 ± 4.49[a]	16.1 ± 6.06[b]	***p*= 0.043**	9.9 ± 2.51	6.8 ± 3.87	6.8 ± 5.65	**p*= 0.069
n3/n6	0.8 ± 0.27[a]	1.3 ± 0.65[b]	0.9 ± 0.41[a]	***p*= 0.014**	0.6 ± 0.42	1.1 ± 0.52	1.1 ± 0.85	**p*= 0.105
DHA/EPA	7.2 ± 3.94	6.7 ± 2.44	6.7 ± 2.82	*p*= 0.575	2.6 ± 1.40	4.6 ± 2.24	4.4 ± 1.59	*p*= 0.297
C16:1n7/C16:0	0.1 ± 0.02[a]	0.1 ± 0.03[b]	0.1 ± 0.03[b]	***p*= 0.013**	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.03	*p*= 0.195
C18:1n9/C18:1n7	7.3 ± 2.15[a]	5.3 ± 1.84[b]	5.5 ± 2.17[b]	***p*= 0.005**	6.0 ± 2.15	5.2 ± 0.91	4.7 ± 0.49	**p*= 0.463
ARA/EPA	6.6 ± 2.56	5.5 ± 4.03	8.1 ± 3.68	**p*= 0.065	6.9 ± 1.08	4.3 ± 2.51	8.1 ± 3.13	*p*= 0.051

[abc] 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).

Table 3- Linear regression models between plasma and whole blood fatty acids (%) and (1) total length, and plasma level of gonadal steroids (2) testosterone and (3) 17β-estradiol of female tiger sharks (*Galeocerdo cuvier*). Corresponding t-values and p-values are included. **Bold**: significant values ($p < 0.05$). EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, ARA: arachidonic acid. NA (missing value).

Fatty acids		**Total length**		**Testosterone**		**17β-estradiol**	
		t	*p-value*	*t*	*p-value*	*t*	*p-value*
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

Table 4- Linear regression models between plasma and whole blood fatty acids (%) and (1) total length, and plasma level of gonadal steroids (2) testosterone and (3) 17β-estradiol of female tiger sharks (*Galeocerdo cuvier*). Corresponding t-values and p-values are included. **Bold**: significant values (p<0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, ARA: arachidonic acid, BFA: branched-chain fatty acids, OFA: odd-chained fatty acids.

Fatty acids		**Total length**		**Testosterone**		**17β-estradiol**	
		t	*p-value*	*t*	*p-value*	*t*	*p-value*
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

2.4. Discussion

Through analysis of short-term dietary markers, our study revealed that the nutritional ecology of female tiger shark, a generalist apex predator, varied across life stages. Non-gravid and gravid females were characterized by higher percentages of plasma n3 PUFAs, including DHA, than immature, which exhibited higher plasma SFA, 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 quality (*i.e.* nutritional composition). While our second hypothesis predicted that gravid females would not be a nutritional deficient in essential fatty acids, gravid females had unexpectedly higher nutritional quality 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).

2.4.1. Nutritional quality during reproduction

Increased plasma HUFAs found in adult females, together with a decrease in plasma SFA, 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 n3/n6 ratio with reproductive hormones, and negative relationship between SFA (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 PUFA, including DHA and EPA, suggesting some allocation of n3 HUFAs from blood cells. Evidence from previous studies show that non-gravid females have higher corticosterone levels than both immature and gravid (B. Rangel, N. Hammerschlag, J. Sulikowski and 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 to 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 response (Izquierdo et al. 2000, Tocher 2010, Gladyshev et al. 2017, Twining et al. 2018), but in sharks a comparative understanding relating to this process is still limited.

The high plasma ARA percentages found in gravid females 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 has a critical role in embryo development, such as immune and

inflammatory responses (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, and tuna, Sardenne et al. 2017), which found evidence suggesting a relationship to 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 mucus and algae of coral reef 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 SFA and low DHA, and consequently low n3 PUFA percentages found in immature compared to other stages may be a result of maturing process, e.g. morphological changes in reproductive tract, or shifts in diet during this period (discussed below). Similarly, decreasing n3 PUFA percentages during sexual maturation in teleost fish have been associated with selective mobilization of n3 PUFA 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 and R. Moreira, unpubl. data), corroborating this hypothesis, as SFA and MUFA are the main fatty acids catabolized for energy (Tocher 2003).

Collectively, our results demonstrated that females nutritional quality differed across life stages, likely either by consumption 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 SFA in gravid females, as SFA 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 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). As the reproductive cycle

for tiger sharks in the region remains unclear, it was found to be biennial in the North Atlantic (Castro 2009) and triennial in Hawaiian tiger sharks (Whitney & Crow 2007), additional studies on temporal changes in the reproductive status will help to elucidate energetic requirements in each stage. Additionally, determining which fatty acids are transferred to offspring, e.g. investigating neonates (Belicka et al. 2012, Wai et al. 2012, Rangel et al. 2020) would help to clarify the nutrients required for reproduction.

2.4.2. 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 showed markers to benthic/coastal areas, corroborating our third hypothesis. This was evident in the decrease of plasma n6 PUFA, including C18:2n6 and C22:4n6, and bacterial detrital markers (BFA-OFA), and an increase in DHA and n3/n6 ratio with body size. Additionally, whole blood n6 PUFA largely separated immature from both non-gravid and gravid females in LDA analysis. For example, C18:2n6 is a characteristic of terrestrial and freshwater sources (mangroves and terrestrial plants, Kelly & Scheibling 2012), while high n3/n6 ratios are indicative of marine resources, whereas, 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 proportion of prey typical of inshore and shallow habitats, e.g. mollusks (Gulf of Mexico and Atlantic Ocean, Aines et al. 2017), batoids and benthic octopi species (South African waters, Dicken et al. 2017), indicating a high dependence of benthic/coastal nutrients at this life stage.

As tiger sharks grow, larger prey become more important in their diet, such as reptiles, birds and marine mammals (e.g. Aines et al. 2017, Dicken et al. 2017, 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. DHA/ARA ratio has been found to be positively correlated to stable isotopes of nitrogen and trophic level in a variety of animals, including other marine predators and mesopredators (e.g. Cardona et al. 2015, Rangel et al. 2020, Sardenne et al. 2017). This is because DHA is biomagnified and preferentially retained at higher trophic levels (Dalsgaard et al. 2003). On the other hand, 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 both, non-gravid and gravid females. Lower carnivory index and higher values of C16:1n7/C16:0 (diatoms marker) found in larger females can be a result of their increased

foraging on turtle and mammalian prey (e.g. mysticete whales that feed small invertebrates situated at low trophic levels), as suggested for tiger sharks from South Africa (Dicken et al. 2017). For example, Cardona et al. (2015) found that loggerhead turtles and some marine bird species were found to have a diatom-based diet, also C16:1n7 is higher in the coastal herbivores and found in high levels in the blubber of the 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 influenced by prey species habitat use. Therefore, future studies should consider the influence of the fatty acid profiles of potential prey species.

2.5. 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 quality, 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 high use areas by tiger sharks, such as feeding areas or gestation and nursery grounds, where individual females depend on shared food resources (e.g. 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 in to testable predictions to aid future investigations on the nutritional ecology of tiger sharks, as well as other apex predators.

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Supporting Information

Table S1. Linear discriminant functions for the 2 first axis. **Bold** values indicate primary fatty acids contribution to dissimilarity. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; BFA: branched-chain fatty acids; OFA: odd-chained fatty acids.

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

Capítulo 3

Physiological markers suggest energetic and nutritional adjustments in male sharks linked to reproduction

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Abstract

Energetic condition is one of the most important factors that influence fitness and reproductive performance in vertebrates. Yet, we lack evidence on how energetic states change in response to reproduction in large marine vertebrates. In the present study, we used a non-lethal approach to assess relationships among reproductive stage, circulating steroid hormones (testosterone and relative corticosteroid levels), plasma fatty acids and the ketone body β-hydroxybutyrate in male sharks of two species with divergent ecologies, the benthic nurse shark (*Ginglymostoma cirratum*) and the epipelagic blacktip shark (*Carcharhinus limbatus*). We found higher relative corticosteroid levels in adult nurse sharks during the pre-mating period and in blacktip sharks during the mating period. Higher levels of β-hydroxybutyrate were found in adult nurse sharks during the mating period, but concentrations of this ketone body did not significantly vary across reproductive stages in blacktip sharks. We also detected reduced percentages of essential fatty acids during the mating period of both nurse and blacktip sharks. Taken together, our findings suggest that nurse and blacktip sharks differ in their energetic strategy to support reproduction, however, they likely rely on physiologically important fatty acids during mating, such to support spermatogenesis.

Key words: dietary patterns, reproductive hormones, trophic markers, elasmobranchs, lipid metabolites.

3.1. Introduction

As long-lived and k-selected species, sharks as a group exhibit relatively slow growth, late sexual maturity, long gestation periods and reduced fecundity (Cortés 2000; Dulvy and Forrest 2010). The evolutionary success of sharks, at least in part, is due to their diverse female reproductive modes (e.g. lecithotrophy, placentotrophy, oophagy, and histotrophy; Hamlett et al. 2005) and mating strategies, with polyandry and polygyny, as well as sperm storage and multiple paternity being commonly reported (Pratt and Carrier, 2005). Despite their importance for reproductive performance, energetic investment are rarely considered in studies of shark reproduction (e.g. Dudley and Cliff 1993; Hammerschlag et al. 2018; Rangel et al., 2021a). Furthermore, there are no published studies investigating the nutritional quality and dietary patterns of free-living male sharks across life-stages.

In male sharks, energetic investments in reproduction is mainly associated with spermatogenesis, copulation, male-male competition, and where necessary, migration to access mates (e.g. Pratt and Carrier 2005). Like many other vertebrates, seasonal adjustments of sperm production and mating behavior are often driven by changes in hormones that coordinate timing of breeding and energy allocation (Awruch 2013). Sex hormones, including androgens, progestins and estrogens, are well-documented players in the regulation of reproductive morphological changes (e.g. testicular development and secondary sex characteristics) and associated behaviors (e.g. courtship and territoriality) (Awruch 2013; Becerril-Garcia et al. 2020; De Acevedo et al. 2020). Although studies investigating the role of glucocorticoids in sharks are lacking, these hormones are known to play an important role in regulating the acquisition and mobilization of resources in many vertebrates, and is expected to be higher during energetically expensive life history stages, such as reproduction (e.g. Romero 2002; Crespi et al. 2013; Romero and Wingfield 2016). As such, measuring hormones integrated with nutritional indicators can offer a great opportunity to non-lethally examine patterns of energy regulation across reproductive stages (e.g. Hammerschlag et al. 2018).

Nutritional quality is one of the most important factors that influence reproductive performance in vertebrates, including those related to gamete quality (both eggs and sperm), fecundity, and offspring survival (Izquierdo et al. 2001; Wathes et al. 2007; Bobe and Labbé 2010). In this context, fatty acids have been identified as major essential dietary nutrients that influence reproductive processes through a variety of mechanisms (Izquierdo et al. 2001; Tocher 2010). For example, arachidonic acid (C20:4n6, ARA), an omega-6 polyunsaturated fatty acid (n6 PUFA), is metabolized to form prostaglandins (eicosanoids) that are involved in

follicle maturation and steroid production during reproduction in females (.g. Izquierdo et al. 2001; Lund et al., 2008). Omega-3 polyunsaturated unsaturated fatty acids (n3 PUFAs), e.g. docosahexaenoic acid (C22:6n3, DHA) and eicosapentaenoic acid (C20:5n3, EPA), are well described as important nutrients in successful vertebrate reproduction, as they affect many important physiological processes, such as immune and inflammatory responses, as well as brain and eye development (Tocher 2010). PUFAs also act as the main component of the cell membranes, including sperm and oocyte, which is important during fertilization (Izquierdo et al. 2001; Bobe and Labbé 2010). Because these important fatty acids cannot be synthesized *de novo* (Parrish 2009; Colombo et al. 2016), high-quality diets play a key role in vertebrate reproduction.

In the present study, we integrated multiple physiological markers to non-lethally assess if and how energetic state changes in relation to reproductive stage in two species of male shark. For this, we investigated two annual breeders with seasonal sperm production, but with divergent ecologies, the relatively sedentary and benthic nurse shark (*Ginglymostoma cirratum*) and the relatively active and epipelagic blacktip shark (*Carcharhinus limbatus*). Based on previous studies demonstrating that males of both species exhibit temporal decoupling between sperm production and mating behavior (e.g. Dudley and Cliff 1993; Baremore and Passerotti 2013; Rêgo et al. 2014), we expected that energetic state would vary across different reproductive stages. As such, we hypothesized that male sharks of both species would exhibit higher energetic demand and mobilization during the mating period, given copulatory activities require high energetic investment (e.g. Manire et al. 2007; Valls et al. 2016). We also expected to measure higher nutritional quality during early stages of the breeding season (i.e. pre-mating period), when male sharks are investing in sperm production.

To test this hypothesis, we used plasma fatty acid profile as short-term dietary biomarkers (McMeans et al. 2012; Beckmann et al. 2014) as a proxy for nutritional quality (Rangel et al. 2020). Clasper measurements and testosterone concentrations (the main androgen in male sharks) were used to inform sexual maturity and reproductive stage (reviewed by Awruch 2013; Becerril-Garcia et al. 2020). Relative corticosteroid concentrations were used as a proxy for energetic demand, as the role of corticosteroids in regulating the acquisition and mobilization of resources is highly conserved across vertebrates (Romero and Wingfield 2016; Crespi et al. 2013). Finally, we used the ketone body β-hydroxybutyrate as a proxy for mobilization of lipid reserves, as sharks routinely utilize this ketone body as an alternate aerobic fuel source (Speers-Roesch ans Treberg 2010). β-hydroxybutyrate typically increases during fasting and starvation in sharks (Speers-Roesch

ans Treberg 2010; Wood et al. 2010) and has been related to intense physical activity and reproduction (Valls et al. 2016; Moorhead et al. 2021). We therefore expected to find increased circulating n3 and n6 PUFAs in adults during the spermatogenesis phase, coincident with increased testosterone levels (i.e. pre-mating) (Fig. 1). We also predicted increased saturated fatty acids (SFA), relative corticosteroid and β-hydroxybutyrate during the mating period, when sharks are believed to reduce feeding activity (Pratt and Carrier 2001) (Fig. 1).

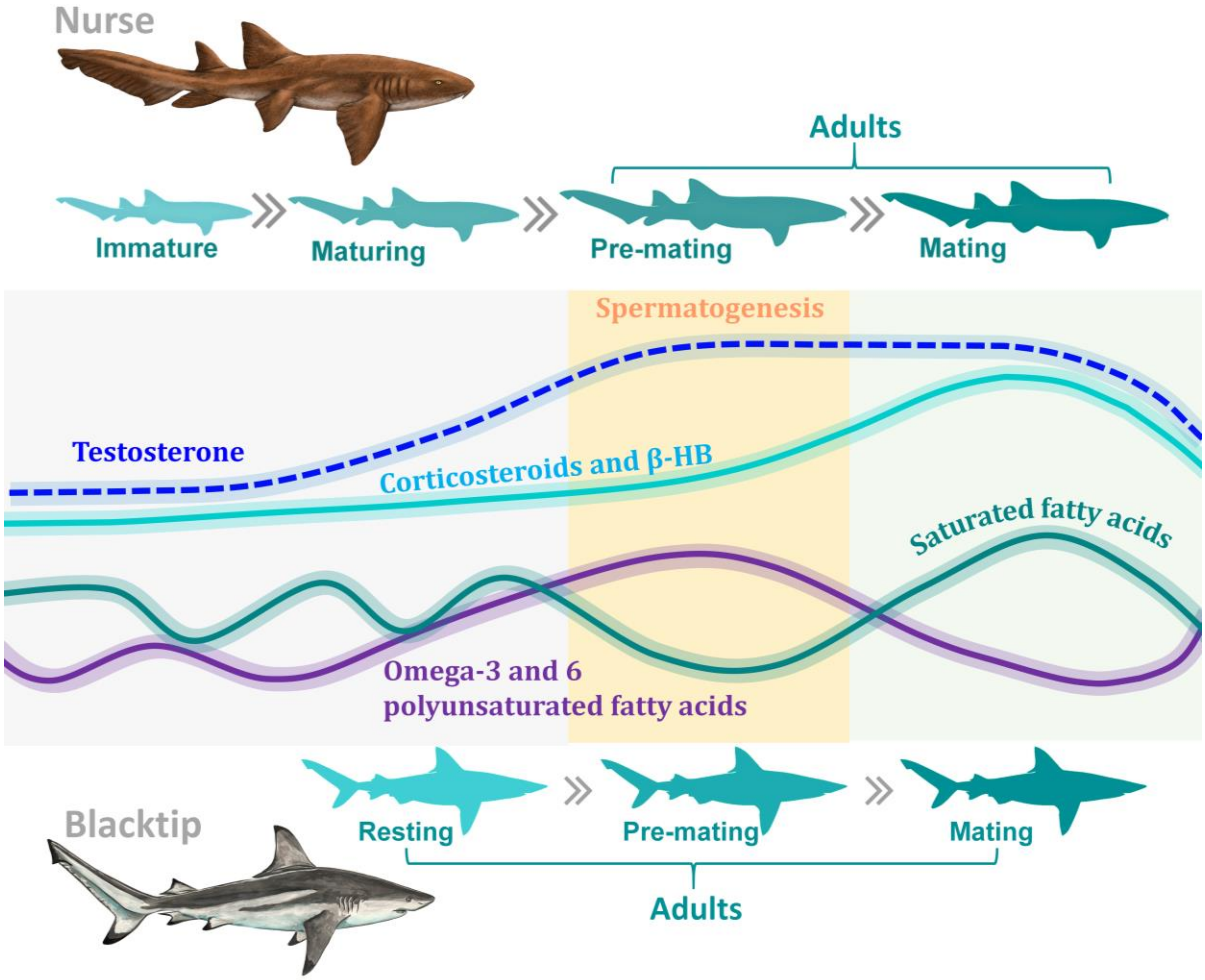

Fig. 1 Conceptual figure of predicted changes on profiles of plasma fatty acids, steroid hormones (testosterone and relative corticosteroid levels) and ketone body β-hydroxybutyrate (β-HB) across reproductive stages of male nurse sharks *Ginglymostoma cirratum* and blacktip sharks *Carcharhinus limbatus*. We predicted these results based on our hypothesis that male sharks of both species would have higher nutritional quality during early stages of the breeding season (i.e. pre-mating period), when they are investing in sperm production, and higher and energetic demand and energy mobilization during the mating period, since copulatory activities required high energetic investment. Illustration of nurse shark courtesy Kelly Quinn, and blacktip shark courtesy Alexandre Huber.

3.2. Materials and Methods

3.2.1. Study species

The nurse shark is a large-bodied (maximum 305 cm total length, TL), relatively sedentary shark, exhibiting the lowest metabolic rate measured in any shark species to date (Whitney et al., 2016). Nurse sharks inhabit tropical and subtropical coastal and insular areas within parts of the Atlantic Ocean (Rosa et al. 2006). Female nurse sharks are a yolk-sac viviparous species, exhibiting a biennial cycle but with a shorter gestation period (5 – 6 months). Males exhibit an annual cycle and mate in June-July (Castro 2000; Pratt et al. 2018). The nurse shark is currently listed as "Data Deficient" by the IUCN (Rosa et al. 2006). Although biological parameters are well understood in some populations (Castro 2000), there is a lack of information on population dynamics for this species throughout much of its range (Rosa et al. 2006).

The blacktip shark is a medium sized species, with a maximum length of approximately 200 cm TL (Compagno 1984). The blacktip shark is cosmopolitan in tropical and subtropical waters, found mainly on continental and insular shelves (Compagno 1984). Female blacktips are placental viviparous, with a reported annual reproductive cycle in populations inhabiting southern Florida (Verkamp 2019). In males, the breeding season extends from February to June and mating period from April to August (Castro 1996, Baremore and Passerotti 2013). While blacktips tagged off northeast Florida display seasonal migrations (Kajiura and Tellman 2016), the population off southeast Florida may be year round residents (Hammerschlag and Tinari, In Revision). The blacktip shark is harvested in both commercial and artisanal fisheries worldwide, and currently listed as Near Threatened by the International Union for the Conservation of Nature Red List (IUCN, Burgess and Branstetter 2009).

3.2.2. Sampling sites and capture

Sampling occurred opportunistically between 2015 and 2019 within Biscayne Bay, Florida, USA (25.61°N, 80.17°W). Sharks were captured using circle-hook drumlines, a passive fishing technique that allows the captured sharks to swim (as described by Gallagher et al. 2014). Briefly, gear consisted of a submerged weight with two attachment points: (1) a line running to the surface with buoy floats and (2) a swivel connecting a 23-m monofilament ganglion line that terminated with a baited 16/05° -offset circle hook. To access the time each shark has been on the line, a hook timer (Lindgren Pitman HT600) was connected between the proximal end of the monofilament line and the weight. Hooking duration was not guaranteed for all sharks captured due to gear malfunction and damage of hook times.

Drumlines were deployed (10 - 40 m deep, between 10:00 – 16:00 h) to soak for 1 h before being checked for shark presence. On capture, sharks were secured by hand to a platform. Once landed, a water pump moving fresh seawater was inserted into the shark's mouth to actively pump water over the shark's gills while temporarily immobilized. While sharks were secured, blood samples were obtained, sex was recorded and morphological measurements were taken (cm), including shark total length (TL) as well as inner clasper length, outer clasper length, and clasper width for a subset of males. Sharks were then tagged for identification (in the sulcus between the dorsal fin and the body) and released. The entire procedure from the moment of capture (removal of the drumline from the water) to release, varied from 5-10 minutes. No mortality was reported during the sampling period of the present study. Procedures and animal husbandry were approved by the University of Miami Institutional Animal Care and Use Committee (Protocol 15-238) and research permits from Florida Fish and Wildlife Conservation Commission, Biscayne National Park and National Marine Fisheries Service.

3.2.3. Blood collection and physiological analysis

Approximately 10 mL of blood was collected from the caudal vein of each shark and immediately centrifuged (3500 rpm, 410 × g) for 2 min. Plasma was then removed and stored frozen at −80°C for future hormonal analyses. Plasma levels of testosterone, corticosteroids and ketone body β-hydroxybutyrate were quantified in duplicate by hormone enzyme-linked immunoasorbent assays (ELISA) using commercial kits (Cayman Chemical Company, MI, USA), with colorimetric enzymatic reaction using a spectrophotometer ELISA (SpectraMax 250, Molecular Devices). For corticosterone assay the plasma dilution selected was 1:5 for both species, and for testosterone was 1:100 for nurse sharks and 1:1000 for blacktip sharks (diluted with Cayman Assays buffer). All hormone assays were validated for nurse and blacktip sharks by conducting tests of parallelism. The mean intra-assay coefficients of variation were 8% (nurse) and 15.1% (blacktip) for testosterone, and 12% (nurse) and 13.1% (blacktip) for corticosterone.

An assay for 1α-hydroxycorticosterone (1α-OH-B, the primary corticosteroid in elasmobranchs) is not commercially available, thus we measuring relative corticosteroid concentrations using a corticosterone ELISA assay. The corticosterone kit assay has been previously validated to quantify relative 1α-OH-B, by exploiting the cross-reactivity of the corticosterone antibody with 1α-OH-B concentrations (Evans et al., 2010) and by excluding other corticosteroids by mass spectrometry (Lyons et al., 2019). However, as this approach

will not deliver precision for concentrations of 1α-OH-B, and we did not identify other corticosteroids (e.g. cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone) as did by (Lyons et al., 2019), we assumed that the corticosterone ELISA would reflect corticosteroid concentrations. Therefore, the results are referred to as relative corticosteroid concentrations. Since corticosteroids are related to stress responses in sharks (e.g. Ruiz-Jarabo et al. 2019; Iki et al. 2020), a regression analysis was employed to first test for possible effects of hooking duration (i.e. hook time) on relative corticosteroid concentrations. However, no significant correlation was found between relative corticosteroid concentrations and hook time in both nurse ($n = 30$, $R = 0.23$, $p = 0.216$) and blacktip sharks ($n = 20$, $R = 0.13$, $p = 0.587$) (Fig. S1). Therefore, we were able to confirm that relatively corticosteroid concentrations measured were not reflective of hooking duration stress.

Fatty acid profiles were analyzed in plasma (100 μL) by direct transmethylation, without lipid extraction, as described by Parrish et al. (2015). Briefly, the samples were homogenized and directly transmethylated in 3mL of methanol: dichloromethane: concentrated hydrochloric acid (10:1:1 v/v) solution for 2 h at 80–85 °C. After this process, was added 1.5 ml of Milli-Q® water and 1.8 ml of hexane and dichloromethane (4:1 v:v), and then the tubes were mixed and centrifuged at 2,000 rpm for 5 min. The upper layer was removed, transferred to 2 ml injection vials and the volume reduced under a nitrogen stream. Fatty acid analysis was carried out in a Varian gas chromatograph (Scion 436) coupled with a flame ionisation detector and a CP 8410 auto-sampler. Hydrogen was used as a carrier gas at a linear velocity of 1.4 mL/min cm/s, and the capillary column used was CP Wax, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length. The column was programmed at 170 °C for 1 min, followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The injector and flame ionisation detector temperatures were 250 and 260 °C, respectively. Fatty acid methyl esters were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualmix, PUFA fish M, Menhaden Oil, Larodan). The data are presented as % of total fatty acid methyl-esters based on peak area analyses.

3.2.4. Reproductive status

We used clasper measurements, testosterone plasma levels, and published length at maturity data to classify the reproductive stage of both nurse and blacktip sharks. Based on published data for nurse sharks, 50% of males at 214 cm TL were sexual mature (Castro 2000), with the mating period occurring from June to July (Castro 2000; Pratt and Carrier

2001; Pratt et al. 2018). Given that the lowest testosterone plasma concentration found in mature nurse sharks during the mating period was 2.5 ng mL^{-1}, individuals with < 214 cm TL with testosterone level higher than 1.0 ng mL^{-1} were considered as maturing males. Mature male nurse sharks were obtained from March to July, corresponding the breeding season, i.e. pre-mating period (March - May) and mating period (June and July; Castro 2000; Pratt et al. 2018). Therefore, nurse sharks were classified as: (1) immature (< 214 cm TL with testosterone < 1.0 ng mL^{-1}), (2) maturing (< 214 cm TL with testosterone > 1.0 ng mL^{-1}), (3) mature (> 214 cm TL) during the pre-mating period (March - May) and (4) during the mating period (> 214 cm TL, between June - July).

For blacktip sharks, only mature males were analyzed. Based on published data, the breeding season extends from January to August, with the mating period occurring from May to August (Castro 1996, Baremore and Passerotti 2013). We classified mature male blacktip sharks as (1) non-breeders (i.e. resting period) those sampled from October to December, (2) breeders during the pre-mating period, sampled from January to April and (3) breeders during the mating period, sampled from May to August.

3.2.5. Fatty acid markers

The essential fatty acids, i.e. DHA, ARA and EPA, as well as the sum (PUFA, n3 and n6 PUFA), ARA/EPA, and n3/n6 ratios were used to compare the indices of shark nutritional quality (Tocher 2003, Arts & Kohler 2009) and to infer physiological responses of eicosanoids (Tocher 2003). Despite subject to biosynthesis when transferred from prey to predator, fatty acids mostly remain relatively unchanged, permitting use as nutritional biomarkers (Darlsgaard et al. 2003; Iverson 2009; Budge et al. 2006). In terms of trophic markers, DHA was used as a indicator of dinoflagellates, while C16:1n7/C16:0 as an indicator of diatoms (Parrish et al. 2000, Budge et al. 2006). Additionally, ARA and C18:2n6 values have been found to be a useful indicator of whether a species inhabits coastal/benthic environments (Sardenne et al. 2017), and the odd chain fatty acids (OFA) and branched chain fatty acids (BFA) as biomarkers of heterotrophic bacteria (Dalsgaard et al. 2003). Fatty acids that accounted for less than 0.5% were excluded from statistical analyses.

3.2.6. Data analysis

Linear regression was used to assess for relationships between testosterone plasma levels and clasper measurements across reproductive stages. Testosterone values were log transformed before analyses to meet assumptions of normality. Potential difference among

clasper measurements, hormones, fatty acid profiles and the ketone body β-hydroxybutyrate were statistically compared across reproductive stages of nurse (i.e. immature, maturing, and mature during the pre-mating period and mating period) and blacktip sharks (i.e. resting, pre-mating, and mating periods) using one-way Analysis of Variance (ANOVA) with Tukey post-hoc test for parametric data or Kruskal-Wallis H tests followed by Dunn post-hoc tests for non-parametric data. As nurse sharks have a well-defined mating period (June and July; Castro, 2000), all physiological variables were compared among breeding months for adults (i.e. May, June, and July).

Discriminant analyses were performed to identify which combination of fatty acids better discriminated between male reproductive stages in both species. Fatty acids that accounted for less than 0.5% of total fatty acids were excluded from statistical analyses. Statistical significance was declared at $p<0.05$, and all analyses were conducted in SigmaStat 3.10 (SystatSoftware, Inc.; www.systat.com), PAST 3.12 (EFB; www.essential-freebies.de; Hammer et al., 2001) and R software (version 4.0.2).

3.3. Results

3.3.1. Nurse shark

A total of 75 male nurse sharks were analyzed, comprising 21 immature (mean ± S.D., 122.8 ± 38.4 cm TL), 28 maturing (175.7 ± 30.7 cm TL), and 26 mature during the breeding season, including 14 during the pre-mating period (237.9 ± 9.6 cm TL) and 12 during the mating period (242.8 ± 8.8 cm TL). All clasper measurements were positively related with testosterone concentrations (Fig. 2a-c) and differed among reproductive stages, with higher values found in adult males during both the pre-mating and mating periods (Fig. 2d-f) (Supplemental Table S1). Testosterone concentrations also differed among all reproductive stages (Fig. 2g). Immature animals had the lowest testosterone concentrations followed by maturing males, then mature males during the mating season, followed by males during the pre-mating season (Fig. 2g). Testosterone concentrations also exhibited temporal shifts in adult males, decreasing from May to June to July (Fig. 4a).

Relative corticosteroid levels was higher in mature males during the pre-mating period compared to immature and maturing males. However, relative corticosteroid levels did not differ between the pre-mating and mating period (Fig. 2h), nor among months (Fig. 4b; Supplemental Table S1). Concentrations of the Ketone body β-hydroxybutyrate was higher in males during the mating period compared to all other reproductive stages (Fig. 2i), and gradually increased from May to July in adult males (Fig. 4c; Supplemental Table S1).

Blood plasma comprised mainly SFAs (C16:0 and C18:0) for all life-stages, followed by PUFAs, mainly DHA and ARA, and monounsaturated fatty acids (MUFAs, mainly C18:1n9) (Supplemental Table S2). Adults had higher proportions of C18:1n7 than immature males (Fig. 3b), while males during the pre-mating period had higher proportions of DPA compared to immature and maturing sharks (Figs. 3c). For fatty acids biomarkers, BFA-OFA, including C17:0, were lower in males during the pre-mating period compared to immature and maturing sharks (Fig. 3a and 3d) (Supplemental Table S2). A temporal shift in fatty acid profiles was detected in adult males (Fig. 5). Total PUFAs, total n3 PUFAs, EPA, DPA, DHA and the n6 PUFA ARA decreased from May to July (Fig. 4) (Supplemental Table S2).

Regarding the discriminant analyses, the first three discriminant functions distinguished the life and reproductive stages (Figs. 7a and b, Supplemental Table S4), with Axis 1 accounting for 59.2%, Axis 2 for 24.6% and Axis 3 for 16.2% of the variation (eigenvalues: Axis 1= 0.97; Axis 2= 0.41; and Axis 3= 0.27). The first function separated immature and maturing males from adults (pre-mating and mating periods), mainly due to differences in the proportions of DHA, ARA, DPA and C18:0. The second function, however, separated adults during the pre-mating period from those during the mating period, mainly due to differences in C18:1n9, C18:0 and DHA.

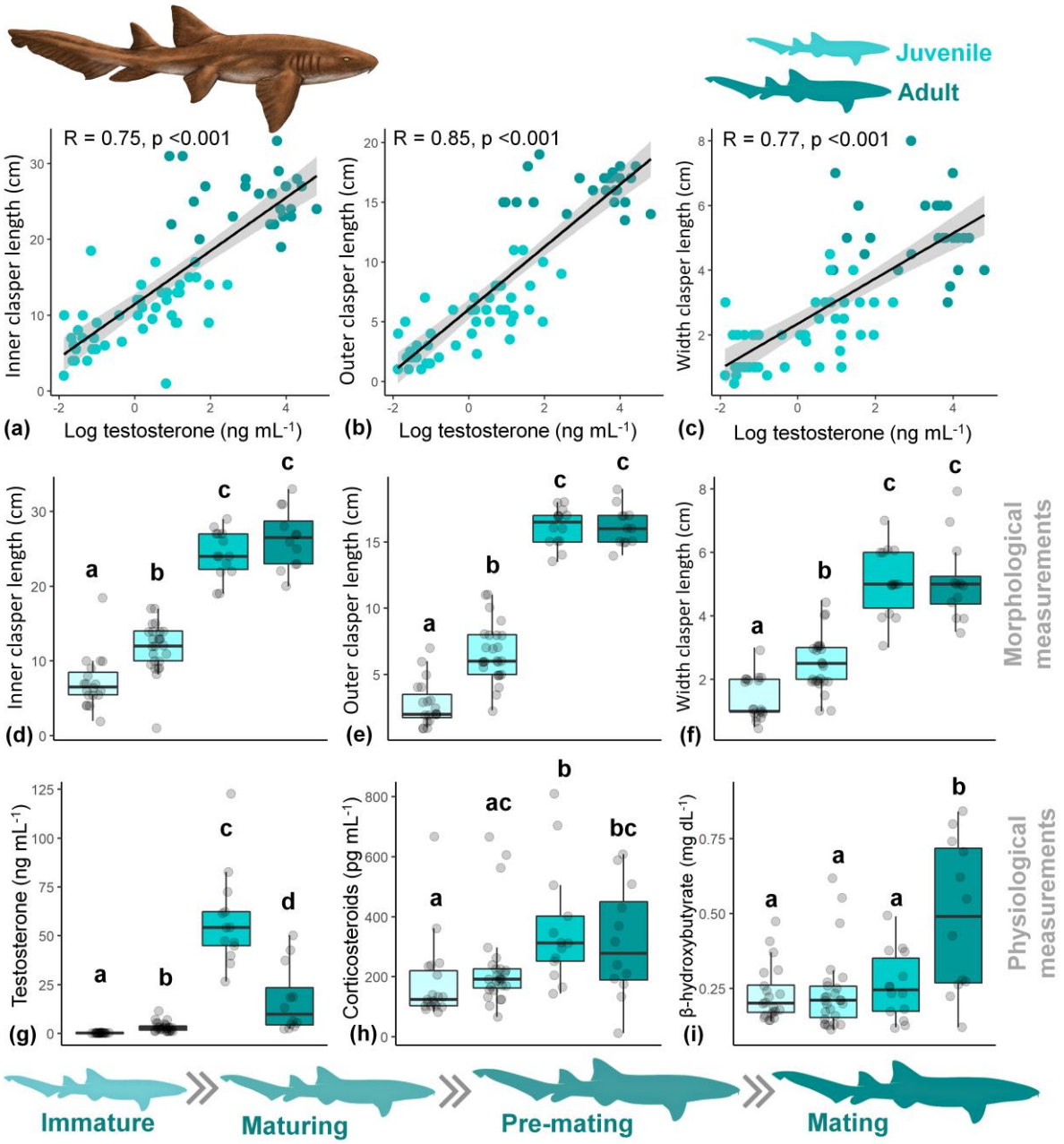

Fig. 2 Linear regression models and comparison across reproductive stages of nurse sharks (*Ginglymostoma cirratum*), including immature males (N=21), maturing males (N=28), mature males during the pre-mating period (N=14) and mating period (N=12). **(a-c)** relationship between inner, outer and width clasper length (cm) and testosterone (ng mL^{-1}). Comparison across reproductive stages of **(d)** inner clasper length (cm), **(e)** outer clasper length (cm), **(f)** width clasper length (cm), **(g)** testosterone levels, **(h)** relative corticosteroid levels and **(i)** ketone body β-hydroxybutyrate levels. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc).

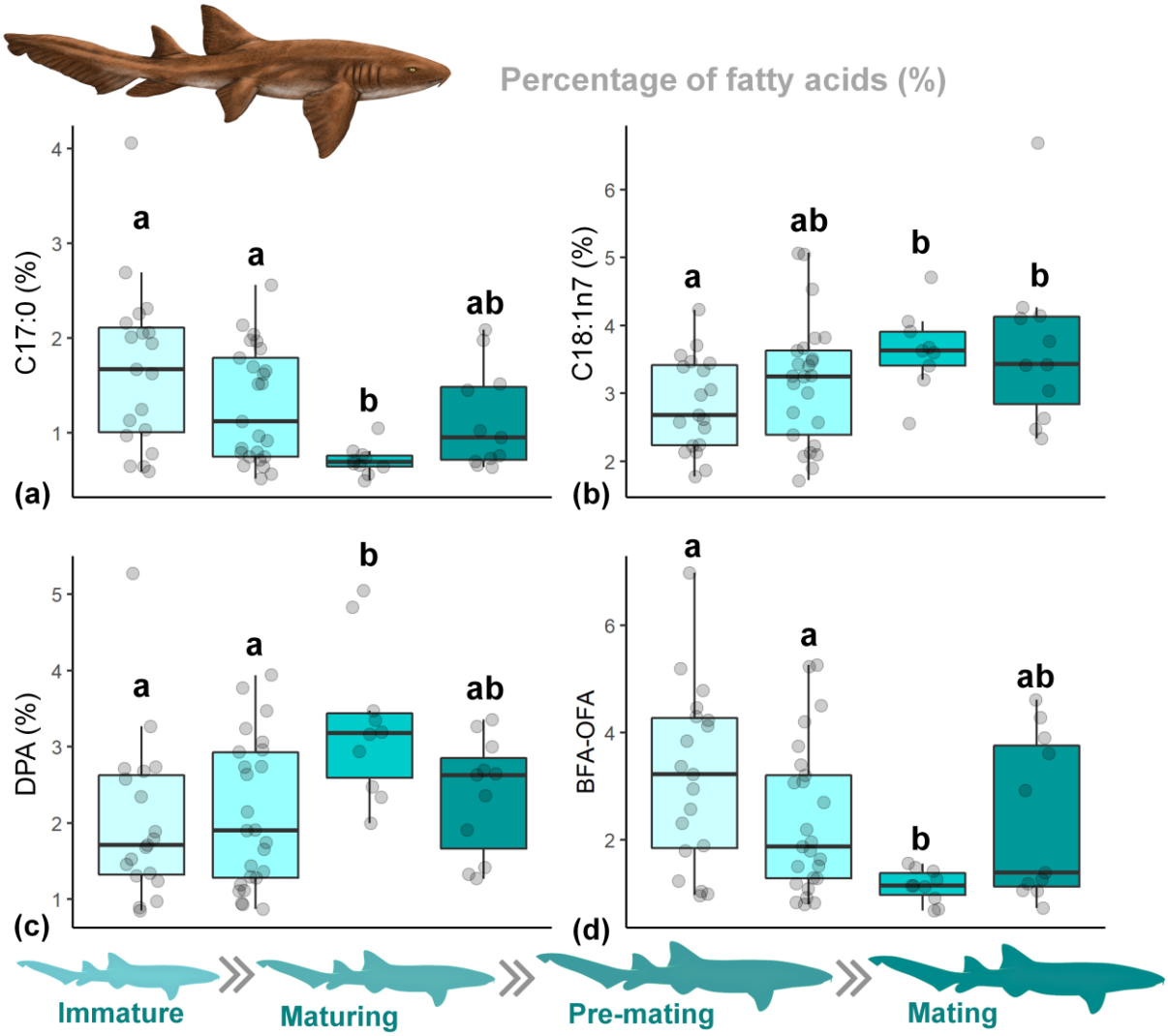

Fig. 3 Comparison of fatty acids throughout reproductive stages of nurse sharks (*Ginglymostoma cirratum*), including immature males (N=21), maturing males (N=28), mature males during the pre-mating period (N=14) and mating period (N=12). **(a)** C17:0, **(b)** C18:1n7, **(c)** DPA (C22:5n3, docosapentaenoic acid), **(d)** BFA: branched-chain fatty acids; OFA: odd fatty acids. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc).

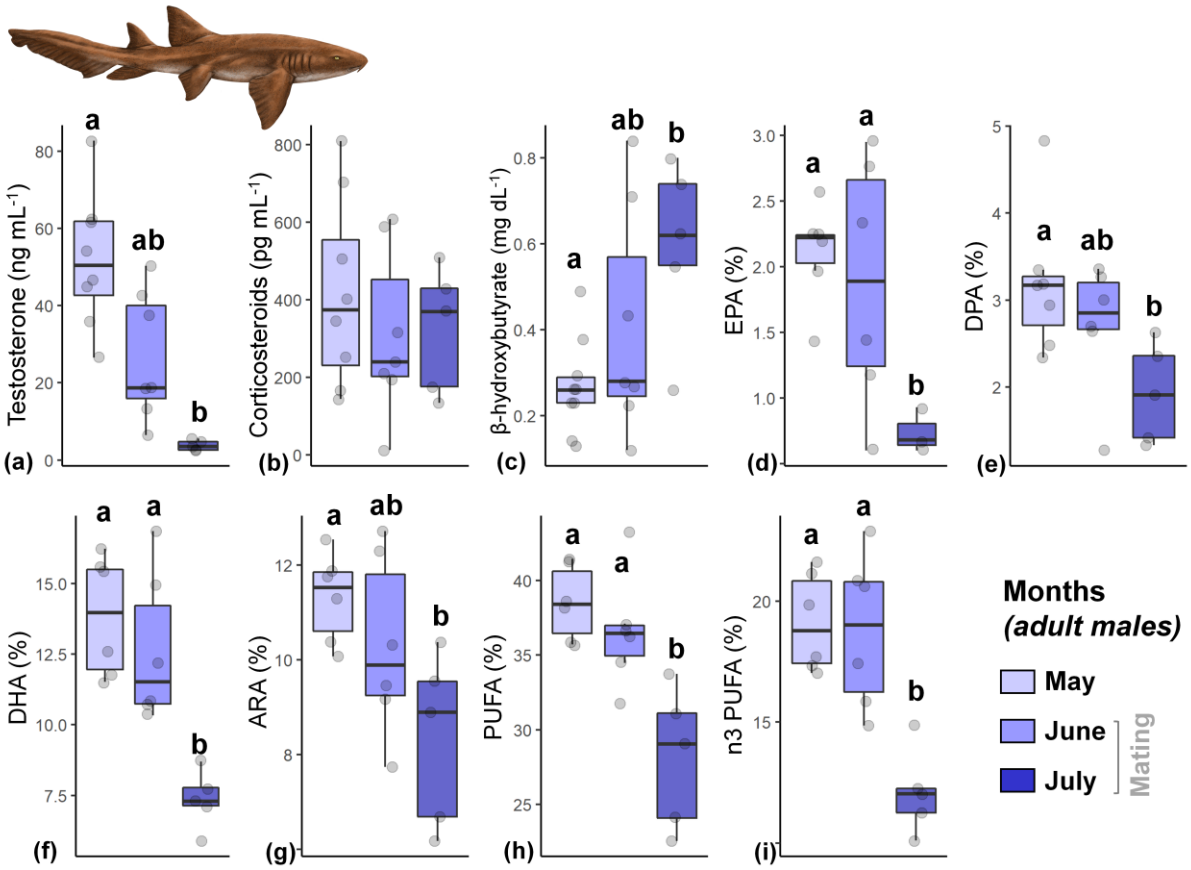

Fig. 4 Comparison of physiological variables throughout breeding months (May (N=9), June (N=7), and July (N=5)) of nurse sharks (*Ginglymostoma cirratum*), **(a)** testosterone, **(b)** relative corticosteroid levels, **(c)** ketone body β-hydroxybutyrate levels, fatty acids: **(d)** EPA (C20:5n3, eicosapentaenoic acid), **(e)** DPA (C22:5n3, docosapentaenoic acid), **(f)** DHA (C22:6n3, docosahexaenoic acid), **(g)** ARA (C20:4n6, arachidonic acid), **(h)** ΣPUFA (polyunsaturated fatty acids), and **(i)** Σ n3 PUFA (omega-3 polyunsaturated fatty acids). Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc).

3.3.2. Blacktip shark

A total of 41 adult male blacktip sharks were analyzed, comprising 16 during the resting period, and 25 during the breeding season, including 11 during the pre-mating period and 14 during the mating period (Supplemental Tables S1). While inner and outer clasper lengths did not differ among reproductive stages (Fig. 5a-b), clasper width values were higher in males during the pre-mating and mating period, compared with males during the resting period (Fig. 5c). None of the clasper measurement were significant related to testosterone concentrations (Supplemental Table S1). Adult males had higher concentrations of testosterone during the breeding season (pre-mating and mating periods) compared to males during the resting period (Fig. 5d; Supplemental Table S1). Relative corticosteroid levels were higher in males during

the mating period compared to sharks during the resting and pre-mating periods (Fig. 5d; Supplemental Table S1), while concentrations of the Ketone body β-hydroxybutyrate did not differ among reproductive stages (Fig. 5e; Supplemental Table S1).

Plasma fatty acid profiles of blacktip sharks were comprised mainly of PUFAs during the resting period, while SFAs were the most abundant fatty acids measured during the mating period (Supplemental Table S3). Males during the mating period exhibited higher proportions of total SFA (including C16:0 and C18:0) and total MUFA (including C18:1n9) compared to males during the resting period (Fig. 6). In contrast, males during the resting and pre-mating periods exhibited higher proportions of n3 PUFA (including EPA, DHA and the n3/n6 ratio) compared to those during the mating period (Fig. 6). In terms of biomarkers, BFA-OFA (including C17:0) were higher in resting males, whereas breeders during the mating period had the highest ARA/EPA ratio (Figs. 6).

Discriminant analyses revealed that the first two discriminant functions distinguished the reproductive stages (Figs. 7c and d; Supplemental Table S4), with Axis 1 accounting for 67.9% and Axis 2 for 32.0% of the variation (eigenvalues: Axis 1= 1.5 and Axis 2= 0.7). The first function separated males during the resting and pre-mating periods from those during the mating period, mainly due to differences in the proportions of C16:0, DHA and C18:1n9. The second function separated males during the resting period from breeders during the pre-mating period, mainly due to C17:0, C16:0, DHA and C18:1n9 (Figs. 7c and d, Supplemental Table S4).

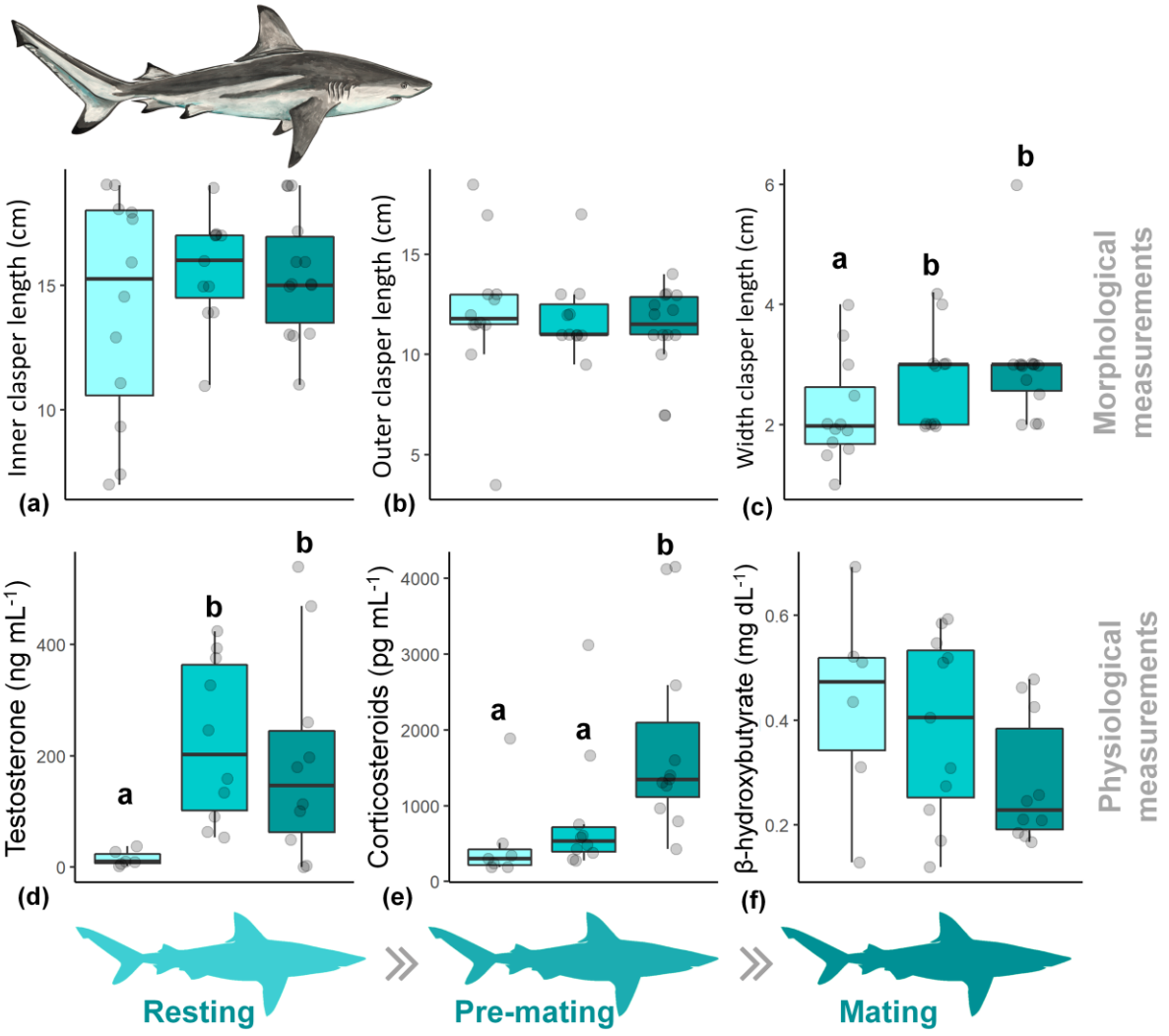

Fig. 5 Comparison throughout the reproductive stages of blacktip shark (*Carcharhinus limbatus*), including males during the resting period (N=16), pre-mating period (N=11), and mating period (N=14). **(a)** inner clasper length (cm), **(b)** outer clasper length (cm), **(c)** width clasper length (cm), **(d)** testosterone levels, **(e)** relative corticosteroid levels and **(f)** ketone body β-hydroxybutyrate levels. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc).

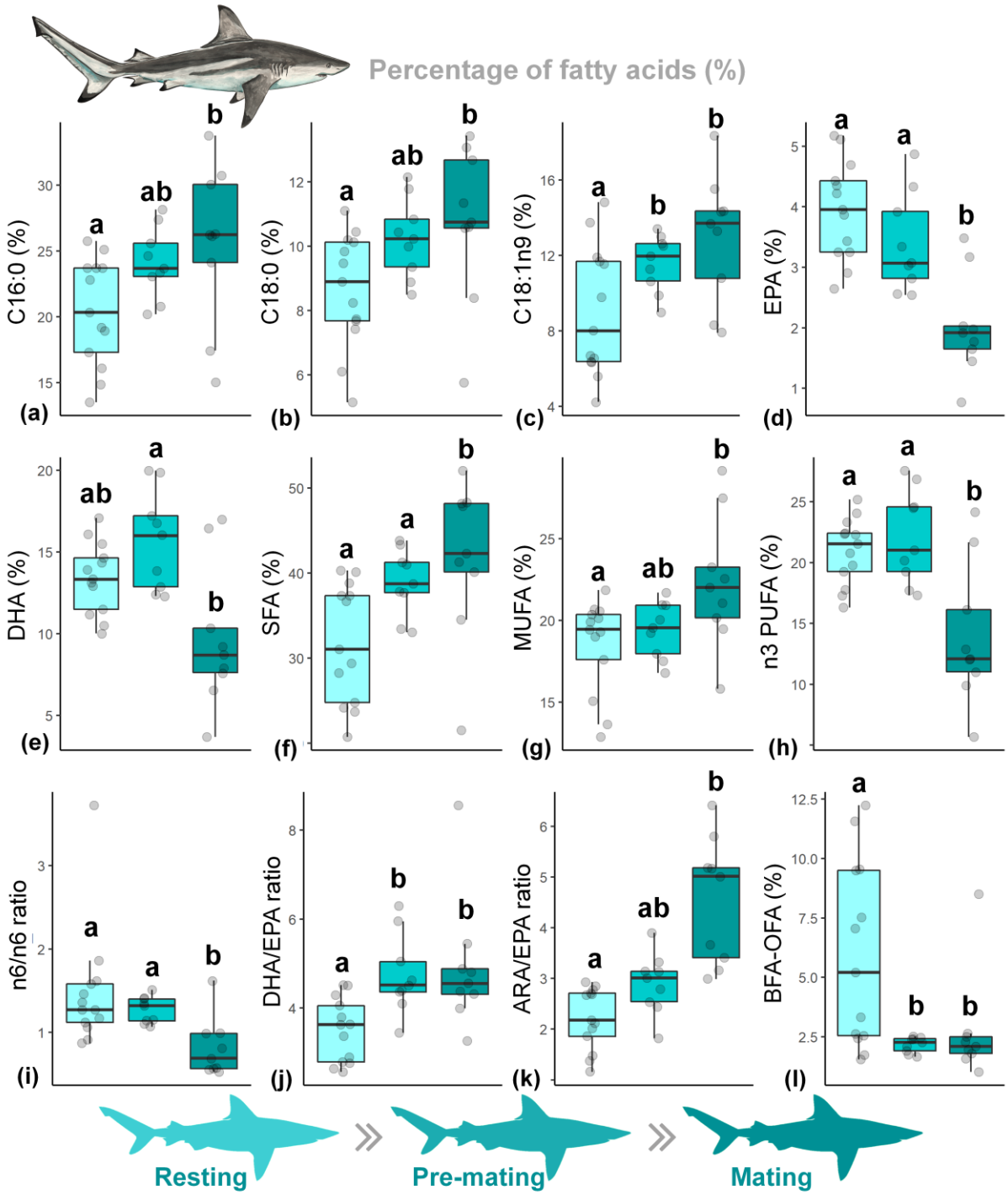

Fig. 6 Comparison of fatty acids throughout reproductive stages of blacktip shark (*Carcharhinus limbatus*), including males during the resting period (N=16), pre-mating period (N=11), and mating period (N=14). **(a)** C16:0, **(b)** C17:0, **(c)** C18:0, **(d)** C18:1n9, **(e)** EPA, **(f)** DHA (C22:6n3, docosahexaenoic acid), **(g)** ΣSFA (saturated fatty acids), **(h)** ΣMUFA (monounsaturated fatty acids), **(i)** Σn3 PUFA (omega-3 polyunsaturated fatty acids), **(j)** n3/n6 PUFA ratio, **(k)** ARA/EPA ratio, ARA (C20:4n6, arachidonic acid) and **(l)** BFA: branched-chain fatty acids; OFA: odd fatty acids. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc).

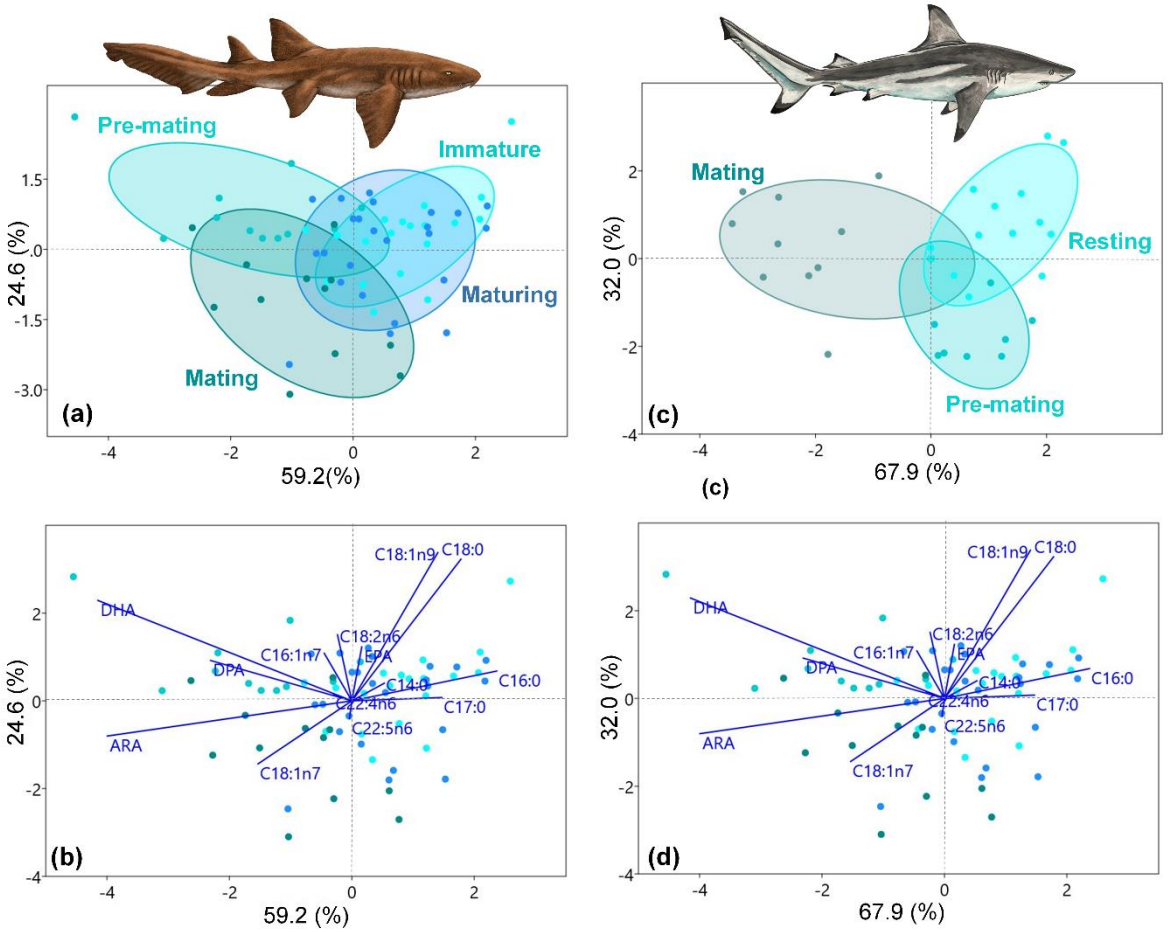

Fig. 7 Linear discriminant function analyses of selected fatty acids (based on their abundance in all samples) of **(a-b)** nurse sharks (*Ginglymostoma cirratum*), including immature males (N= 21), maturing males (N=28), mature males during the pre-mating period (N= 14) and mating period (N= 12); and **(c-d)** blacktip shark (*Carcharhinus limbatus*), including males during the resting period (N= 16), pre-mating period (N= 11), and mating period (N= 14). EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid. The 70% ellipses similarly of seasons in each site is provided.

3.4. Discussion

The integration of multiple physiological markers in this study shed new insights into how energetic state varies across reproductive stages in male sharks. Consistent with our predictions, nutritional state (in terms of fatty acids) decreased in both nurse and blacktip sharks during the mating period. Increased relative corticosteroid levels were found during the mating period in male blacktip sharks and during the pre-mating and mating periods in nurse sharks. While β-hydroxybutyrate levels did not differ across reproductive stages in blacktip sharks, higher levels of this energetic subtract were found in adult nurse sharks during the mating period as predicted. Understanding how energetic condition changes across

reproductive stages in wild animals may provide an improved capacity to detect breeding strategies (Soulsbury 2019) and monitor impacts from environmental disturbance (e.g. trophic mismatch vulnerability; Williams et al. 2017).

Patterns of testosterone concentration measured in both nurse and blacktip sharks were consistent with previous studies describing a pronounced seasonal change in their testicular development and sperm production (Castro 1996; 2000; Baremore and Passerotti 2013; Verkamp 2019). While the highest values of testosterone were found in mature nurse sharks (> 214 cm TL), intermediate values in maturing males (and associated smaller claspers) may correspond with puberty, when testosterone acts promoting testicular development and secondary sex characteristics (Gelsleichter et al. 2002). Mature individuals during the pre-mating period exhibited higher testosterone levels that declined during the mating period. Similar patterns have been observed in other shark species with temporal decoupling between sperm production and mating behavior, such as bonnethead shark *Sphyrna tiburo* (Manire and Rasmussen 1997) and Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Hoffmayer et al. 2010). In blacktip sharks, clasper width and testosterone levels increased during the breeding season (i.e. pre-mating and mating periods) and decreased during the resting period, a time of testicular regression, also consistent with previous studies (Castro 1996; Baremore and Passerotti 2013; Verkamp 2019). However, despite morphological evidences for temporal decoupling in sperm production and mating for this species (Dudley and Cliff 1993; Castro 1996; Baremore and Passerotti 2013), we did not find differences between pre-mating and mating periods. This suggests the possible role of testosterone in stimulating copulatory behaviors in blacktips as found in other shark species (e.g. Awruch 2013).

A temporal decoupling in sperm production and mating may allow males to direct energy investment to one process at a time, i.e. spermatogenesis and mating activity (male–male competition and courtship) (Hoffmayer et al. 2010; Awruch 2016). Although both nurse and blacktip sharks seemed to exhibit temporal decoupling in sperm production between the pre-mating and mating periods, the relative corticosteroid and β-hydroxybutyrate data suggest that they may have differed in their energetic strategy. Specifically, while nurse sharks exhibited elevated β-hydroxybutyrate levels during the pre-mating period, blacktip sharks exhibited elevated relative corticosteroid levels during the mating period. One possible explanation is that nurse sharks may utilize β-hydroxybutyrate as an energetic substrate to fuel copulatory activities, though mobilization of lipid storage as recently suggested (Moorhead et al. 2021). This corroborates previous observations in the field, where no evidence of feeding was observed during nurse shark mating (Pratt and Carrier 2001),

suggesting males may rely on stored endogenous resources to fuel mating behaviors. In contrast, blacktip sharks may utilize more carbohydrates during mating, as corticosteroids are associated with enhanced glycolysis and gluconeogenesis (e.g. Ruiz-Jarabo et al. 2019). Although not significant, possibly due to relatively low sample size, a decreasing trend in plasma β-hydroxybutyrate levels during the mating period of blacktips provides preliminary support for this hypothesis.

Based on our results, it is plausible that nurse sharks are capital breeders, i.e. using stored endogenous resources (typically lipids) to finance reproduction, whereas blacktip sharks are income breeders, utilizing exogenous resources (typically carbohydrates) to finance reproduction (Soulsbury 2019). Corroborating this hypothesis for blacktip sharks, increased plasma SFAs (including C16:0 and C18:0) and MUFAs (including C18:1n7 and C18:1n9) were found during the mating period, suggesting an increase of *de novo* biosynthesis of fatty acids in the liver (i.e. from carbohydrate or protein precursors; Budge et al. 2006). This is because *de novo* biosynthesis is inhibited during fasting (Budge et al. 2006). Also, increased biosynthesis of fatty acids may fuel increased activity levels associated with courtship and mating behaviors, since SFAs are the main fatty acids catabolized for energy (Tocher 2003).

Another possible explanation for the observed pattern of β-hydroxybutyrate and corticosteroid levels may be related to the intensity and overall duration of physical activity during the mating behavior (Clark 2012; Soulsbury 2019). For example, in other vertebrates, males performing sustained mating behavior typically use lipids, whereas those performing short bouts of intense activity more often use carbohydrates (Soulsbury 2019). Although mating behavior in blacktips has not been observed, it is well described for the nurse shark (Castro 2000; Pratt and Carrier 2001; Colbachini et al. 2020). During the mating period, male nurse sharks actively “patrol” for potential mates, frequently mating multiple times daily, while compete with other males for access to females (Pratt and Carrier 2001). Further research involving additional physiological markers coupled with behavior observations would be valuable for testing these hypotheses.

Male nurse sharks had relatively low testosterone and relative corticosteroid concentrations, at levels comparable to those found in benthic species, such as the demersal stingray *Hypanus sabinus*, in both androgens (0.0 – 36.0 pg ml^{-1}; Snelson et al. 1997) and corticosteroids (median maximum of 550.0 pg ml^{-1}; Manire et al. 2007). On the other hand, the concentrations of both testosterone and relative corticosteroids in male blacktip sharks were relatively high, comparable to levels found in other active and epipelagic shark species. For instance, a mean of 182.0 ng ml^{-1} of circulating testosterone (Marine and Rasmussen,

1997) and a median of 3000.0 pg ml^{-1} of circulating corticosteroid has been previously reported in the bonnethead shark (Manire et al. 2007). Accordingly, a relationship may exist between levels of these two hormones and a species ecology, however, comparative studies are needed to test this phenomenon.

Consistent with findings in other vertebrates (e.g. teleosts, Izquierdo et al. 2001; mammals, Wathes et al. 2007), males of both nurse and blacktip sharks exhibited a decrease of physiologically important omega-3 fatty acids during the mating period, suggesting use of these physiologically important fatty acids during mating, such to support spermatogenesis. Moreover, DHA was the main fatty acid responsible for separating breeders during the pre-mating period from other stages, when presumably males are investing in sperm production. This n3 PUFA has an important structural function, as it is found in high proportions in sperm of several teleost species (e.g. Pérez et al. 2000; Baeza et al. 2014; 2015).

Other important n3 PUFA, EPA, decreased during the mating period in both shark species, which resulted in an increased ARA/EPA ratio in blacktip sharks. EPA has a physiological role in modulating the synthesis of androgens and is related to volume of sperm produced in teleosts (Baeza et al. 2015). Additionally, in some species, e.g. in European eels (*Anguilla anguilla*), the proportions of n3 and n6 PUFAs remain constant in the testes during spermiation, suggesting some mobilization of these fatty acids from the liver for testicular maintenance (Baeza et al. 2015). If a similar phenomenon occurs in male sharks, it would indicate that species may allocate physiologically important fatty acids to support reproduction, whether through dietary and/or non-dietary origin (e.g. mobilized from storage tissues). Moreover, as both nurse and blacktip sharks store sperm prior to copulation (e.g. Dudley and Cliff 1993; Baremore and Passerotti 2013; Rêgo et al. 2014), these fatty acids may have some role in semen production or sperm maintenance and activation (e.g. Baeza et al. 2015).

Shifts found in proportions of ARA in nurse sharks, imply that this fatty acid is also critical to male reproduction. ARA was an important fatty acid in discriminating maturity in nurse sharks, exhibiting a gradual increase from immature to mature sharks during the pre-mating period. ARA has been demonstrated to stimulate testicular steroidogenesis in teleost fishes by regulating the cholesterol transfer within mitochondrial membrane (e.g. Hu et al. 2010). ARA is also directly involved in testosterone production through elevating cyclic adenosine monophosphate (cAMP) levels (Mercure and Van der Kraak 1995). Experimentally, increased dietary ARA has also been found to significantly increase androgen production in male teleosts (e.g. *Solea senegalensis*, Norambuena et al. 2013), and therefore,

it has several important implications for reproduction. One plausible reason for the lack of significance of ARA among reproductive stages in blacktip sharks might be related to high levels of testosterone maintained during the mating period. Consistent with these results, decreases in n3/n6 ratios were found in blacktip sharks during the mating period, while no differences were observed in n3/n6 ratio in nurse sharks across reproductive stages and breeding months.

Regarding trophic markers, the bacterial markers, including C17:0 and C18:1n7, were found in higher proportions in immature nurse sharks and resting blacktips. Increased bacterial marker contribution found during these stages is likely to be a result of greater intake of benthic/demersal prey, since these fatty acids are markers for heterotrophic bacteria associated with sediments and suspended organic material (Kelly and Scheibling 2012). Moreover, given that some of immature nurse sharks were captured close to urbanized areas, higher proportions of bacterial markers maybe related to anthropogenic influence, such as eutrophication process and increased production of organic materials (e.g. Le Moal et al. 2019; Rangel et al., 2021b). Other biomarkers, such as the DHA, were the most abundant PUFA in both nurse and blacktip sharks across all reproductive stages, suggesting a greater dependency on marine food webs based on dinoflagellates (Dalsgaard et al. 2003). Despite this, high proportions of ARA and relatively low ratio of n3/n6 found in both species imply they are utilizing food webs influenced by freshwater, commonly found in coastal shark species (e.g. Every et al. 2016). Future studies comparing these two sympatric shark species in relation to their diet and habitat use patterns are needed to characterize these possible trophic dynamics.

Our multiple physiological markers approach allowed us to better understand the relationship between nutritional and reproductive stages in male sharks. However, due to opportunistic sampling and because we used a non-lethal approach and correlative analyses, our study has several limitations. This includes the inability to identify dietary and/or non-dietary origin of fatty acids, seasonal patterns and specific mechanisms through breeding period. Since we used the corticosterone ELISA assay as a proxy for the potential effects of corticosteroids, further comparative studies are needed to identify which specific corticosteroids are present in nurse and blacktip sharks at physiologically relevant concentrations, including the adrenocorticotropic hormone levels. Another study limitation is that we did not consider other factors that may be influencing the physiological variables measured here, such as location, season, temperature, time of day, capture stress, and

urbanization. More extensive sampling integrated with other nutritional markers will help to clarify the changes observed in nutritional status across male reproduction.

3.5. Conclusion

Our study presents evidence of energetic and nutritional adjustments in male sharks linked to reproduction. Though more research is needed to identify specific mechanisms, decreased nutritional quality (in terms of essential fatty acids) found during the mating period of both blacktip and nurse sharks, indicating the possible use of physiologically important fatty acids to support reproduction, such as spermatogenesis and/or higher activity behaviors related to mating. In general, while both omega-3 and -6 seemed to be important for nurse shark reproduction, only omaga-3 differed across reproductive stages of blacktip sharks. Despite similar trends and shifts in fatty acid profile for both blacktip and nurse sharks, these species appeared to differ in their energetic strategy to finance reproduction, maybe a result of their divergent ecologies (sedentary and benthic = nurse shark versus active and epipelagic = blacktip) and energy resource-use strategies for reproduction. This knowledge is particularly important for predicting how these on other species may respond physiologically to environmental disturbance, such as trophic mismatch vulnerability.

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Supporting Information

Table S1 Summary information for nurse shark (*Ginglymostoma cirratum*) and blacktip sharks (*Carcharhinus limbatus*) data (mean % ± standard deviation), including the total length (cm), inner, outer and width clasper length (cm), testosterone (ng mL^{-1}), relative corticosteroid levels (pg mL^{-1}) ketone body β-hydroxybutyrate levels (mg dL^{-1}). Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc).

Nurse shark					
Measurements	**Immature (N=21)**	**Maturing (N=28)**	**Pre-mating (N=14)**	**Mating (N=12)**	**p-value**
Total length	111.3 ± 32.6	178.0 ± 23.9	238.6 ± 8.3	242.8 ± 8.8	***<0.001**
Inner clasper length	7.1 ± 3.5	11.8 ± 3.3	24.4 ± 3	26.3 ± 4	**<0.001**
Outer clasper length	2.8 ± 1.7	6.6 ± 2.2	16.1 ± 1.5	16.2 ± 1.5	**<0.001**
Width clasper length	1.4 ± 0.7	2.5 ± 0.9	5.1 ± 0.9	5.1 ± 1.3	***<0.001**
Testosterone	0.3 ± 0.2	3.2 ± 2.4	57.8 ± 24.6	17.2 ± 17.1	***<0.001**
Corticosteroids	179.7 ± 138.3	231.3 ± 145.9	362.9 ± 222.2	315.7 ± 188.1	***<0.001**
β-hydroxybutyrate	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.3	***0.021**
Blacktip shark					
Measurements		**Resting (N=16)**	**Pre-mating (N=11)**	**Mating (N=14)**	
Total length		154.9 ± 11.4	152.9 ± 12.4	154.1 ± 16.7	0.919
Inner clasper length		14.2 ± 4.5	15.6 ± 2.16	15.4 ± 2.48	*0.886
Outer clasper length		12.2 ± 3.65	12 ± 1.96	11.3 ± 2.11	*0.617
Width clasper length		2.2 ± 0.88	2.7 ± 0.82	2.9 ± 0.97	***0.050**
Testosterone		80.3 ± 123.9	248.6 ± 128.1	284.2 ± 170.6	***0.011**
Corticosteroids		701.6 ± 650.3	655.1 ± 347.5	2181.1 ± 1370.7	***0.010**
β-hydroxybutyrate		0.39 ± 0.18	0.42 ± 0.17	0.26 ± 0.12	0.106

* non-parametric data (Kruskal-Wallis H test).

Table S2 Plasma fatty acid profile of nurse sharks (*Ginglymostoma cirratum*) among reproductive stages (immature (N=21), maturing (N=28), mature during the pre-mating (N=14) and mating periods (N=12)) and among months (May (N=9), June (N=7), and July (N=5)) in adults (mean % ± standard deviation). P values for one-way ANOVA with Tukey post-hoc test to parametric data and Kruskal-Wallis H tests followed by Mann-Whitney hoc for non-parametric data. Significant ($p < 0.05$) results are shown in **bold**.

Fatty acids	Reproductive stages				p-value	Months			p-value
	Immature	Maturing	Pre-mating	Mating		May	June	July	
C17:0	1.7 ± 0.9	1.3 ± 0.6	0.7 ± 0.2	1.1 ± 0.5	***0.004**	0.7 ± 0.2	1.1 ± 0.5	1.2 ± 0.6	*0.436
C14:0	2.6 ± 0.7	2.4 ± 0.4	2.3 ± 0.5	2.3 ± 0.5	0.457	2.2 ± 0.5	2.1 ± 0.2	2.5 ± 0.7	*0.596
C16:0	26.9 ± 4.0	26.1 ± 3.5	25.3 ± 4.2	26.7 ± 3.1	0.679	25.4 ± 4.5	25.3 ± 1.9	28.3 ± 3.6	*0.185
C18:0	12.2 ± 2.2	11.7 ± 1.7	11.5 ± 3.2	11.2 ± 1.5	*0.259	11.6 ± 3.5	10.8 ± 1.6	11.6 ± 1.5	*0.575
C14:1	1.4 ± 0.6	1.7 ± 0.6	1.6 ± 0.3	1.2 ± 0.4	*0.145	1.5 ± 0.2	1.3 ± 0.3	1.1 ± 0.5	*0.354
C16:1n7	1.9 ± 0.5	2.1 ± 0.6	2.5 ± 0.3	1.9 ± 0.5	0.045	2.5 ± 0.3	2.0 ± 0.7	1.7 ± 0.3	*0.051
C18:1n9	16.7 ± 2.7	16.8 ± 2.2	16.5 ± 4.4	16.1 ± 2.4	*0.663	16.7 ± 4.7	14.9 ± 2.2	17.5 ± 2.1	*0.258
C18:1n7	2.8 ± 0.7	3.2 ± 0.9	3.6 ± 0.3	3.7 ± 1.2	***0.042**	3.7 ± 0.2	3.4 ± 0.8	4.0 ± 1.6	0.548
C18:2n6	2.8 ± 1.2	3.3 ± 0.7	3.5 ± 1.1	2.7 ± 1.1	*0.102	3.3 ± 1.0	2.5 ± 0.8	3.0 ± 1.5	0.459
EPA	1.8 ± 0.9	2.2 ± 0.8	2.2 ± 0.4	1.5 ± 0.9	0.161	2.1 ± 0.4	1.9 ± 0.9	0.7 ± 0.2	***0.039**
C22:5n3	2 ± 1.1	2.1 ± 1.0	3.3 ± 0.8	2.4 ± 0.8	***0.014**	3.2 ± 0.8	2.7 ± 0.8	1.9 ± 0.6	**0.035**
DHA	11.1 ± 4.6	9.9 ± 2.9	12.8 ± 2.3	10.2 ± 3.4	*0.172	13.8 ± 2.1	12.6 ± 2.7	7.4 ± 1.0	**<0.001**
ARA	8.3 ± 3.2	9.1 ± 2.5	10.7 ± 0.9	9.4 ± 2.1	0.133	11.3 ± 0.9	10.3 ± 1.9	8.3 ± 1.8	**0.026**
C22:4n6	3.1 ± 1.6	3.8 ± 1.4	3.7 ± 0.8	3.2 ± 1.0	*0.455	3.6 ± 0.5	3.4 ± 1.0	3.0 ± 1.2	0.582
C22:5n6	1.6 ± 0.5	1.6 ± 0.5	1.6 ± 0.2	1.6 ± 0.3	0.991	1.6 ± 0.3	1.7 ± 0.3	1.5 ± 0.3	0.764
BFA	3.2 ± 1.7	2.4 ± 1.4	1.1 ± 0.3	2.4 ± 1.5	***0.008**	1.2 ± 0.3	1.9 ± 1.2	2.9 ± 1.8	*0.485
SFA	42.8 ± 6.4	41.3 ± 4.9	40.1 ± 6.8	41.2 ± 4.1	*0.334	40.1 ± 7.3	39.3 ± 3.0	43.6 ± 4.4	*0.161
MUFA	22.6 ± 2.6	23.8 ± 2.3	23.7 ± 2.7	23.3 ± 2.6	*0.384	23.7 ± 2.8	21.8 ± 1.6	25.1 ± 2.5	0.103
PUFA	32.2 ± 7.0	32.3 ± 6.3	37.5 ± 2.3	32.7 ± 6.0	0.156	38.5 ± 2.5	36.6 ± 3.8	28.1 ± 4.7	**<0.001**
n3 PUFA	16.3 ± 4.7	14.4 ± 3.7	18.3 ± 2.2	15.7 ± 4.3	0.108	19.1 ± 2.0	18.8 ± 3.2	12.1 ± 1.8	**<0.001**
n6 PUFA	15.8 ± 4.8	17.8 ± 4.0	19.2 ± 1.2	17.0 ± 3.4	*0.117	19.4 ± 0.9	17.8 ± 2.6	15.9 ± 4.2	*0.378
n3/n6	1.2 ± 0.7	0.8 ± 0.3	1.0 ± 0.1	0.9 ± 0.3	*0.055	1.0 ± 0.1	1.1 ± 0.2	0.8 ± 0.2	0.095
ARA/EPA	5.1 ± 2.5	5.0 ± 2.9	5.3 ± 1.4	9.0 ± 5.1	*0.118	5.5 ± 1.0	12.2 ± 4.4	7.4 ± 4.9	*0.189

* non-parametric data (Kruskal-Wallis H test). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; BFA: branched-chain fatty acids; OFA: odd fatty acids.

Table S3 Plasma fatty acid profile of blacktip sharks (*Carcharhinus limbatus*) among reproductive stages (resting (N=16), pre-mating (N=11), and mating periods (N=14)) (mean % ± standard deviation). P values for one-way ANOVA with Tukey post-hoc test to parametric data and Kruskal-Wallis H tests followed by Mann-Whitney hoc for non-parametric data. Significant ($p < 0.05$) results are shown in **bold**.

Fatty acids	Resting	Pre-mating	Mating	p-value
C15:0	0.9 ± 0.28	0.8 ± 0.14	0.8 ± 0.17	*0.358
C17:0	4.8 ± 4.06	1 ± 0.12	1.9 ± 2.02	***0.001**
C17:1cis	0.6 ± 0.09	0.6 ± 0.08	NA	NT
C14:0	1.8 ± 0.49	2.2 ± 0.32	2.2 ± 0.68	0.201
C16:0	20.4 ± 4.08	24.1 ± 2.68	25.5 ± 6.07	**0.033**
C18:0	8.6 ± 1.78	10.2 ± 1.23	10.7 ± 2.43	**0.036**
C22:0	0.6 ± 0.04	0.8 ± 0.23	1.1 ± 0.42	***0.016**
C24:0	1.4 ± 0.29	1.9 ± 0.36	2.8 ± 1.34	***0.023**
C14:1	1.6 ± 0.32	1.6 ± 0.26	2.2 ± 0.38	**0.008**
C16:1n7	4 ± 2.42	2.7 ± 0.42	2.5 ± 1.29	*0.145
C18:1n9	9 ± 3.41	11.6 ± 1.5	13 ± 3.38	***0.034**
C18:1n7	4.8 ± 1.44	3.5 ± 0.4	4.7 ± 0.9	***0.008**
C18:2n6	2.2 ± 1.03	2.4 ± 0.55	2.4 ± 0.72	0.779
C20:5n3 (EPA)	3.9 ± 0.81	3.4 ± 0.82	2 ± 0.84	**<0.001**
C22:5n3	2.9 ± 0.37	2.9 ± 0.53	2.1 ± 0.81	***0.028**
C22:6n3 (DHA)	13.4 ± 2.17	15.7 ± 3.02	9.7 ± 4.39	***0.039**
C20:4n6 (ARA)	8.4 ± 1.88	9.4 ± 1	8.6 ± 2.59	*0.706
C22:4n6	3.3 ± 0.85	2.8 ± 0.79	4.3 ± 2	*0.231
C22:5n6	2 ± 0.38	2.5 ± 0.55	2.4 ± 1.14	*0.333
BFA	5.9 ± 3.87	2.2 ± 0.32	2.7 ± 2.23	***0.015**
SFA	31.7 ± 7.02	38.9 ± 3.89	41.8 ± 9.31	**0.007**
MUFA	18.5 ± 2.86	19.4 ± 1.69	22.3 ± 4.05	**0.029**
PUFA	35.8 ± 4.78	39.5 ± 5.55	31.6 ± 9.29	0.094
n3 PUFA	21 ± 2.72	22.1 ± 3.87	13.9 ± 5.82	***0.013**
n6 PUFA	15.8 ± 4.08	17.4 ± 1.93	17.6 ± 5.04	0.486
n3/n6	1.5 ± 0.73	1.3 ± 0.16	0.8 ± 0.35	***0.006**
ARA/EPA	2.2 ± 0.61	2.9 ± 0.59	4.5 ± 1.25	***<0.001**

* non-parametric data (Kruskal-Wallis H test). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; BFA: branched-chain fatty acids; OFA: odd fatty acids.

Table S4 Linear discriminant functions for the 3 first axis of nurse sharks (*Ginglymostoma cirratum*), including immature males (N= 21), maturing males (N=28), mature males during the pre-mating period (N= 14) and mating period (N= 12). Discriminant functions for the 2 first axis of blacktip sharks (*Carcharhinus limbatus*), including males during the resting period (N= 16), pre-mating period (N= 11), and mating period (N= 14). **Bold** values indicate primary fatty acid contribution to dissimilarity. EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid.

Fatty acids	Nurse shark			Blacktip shark	
	Axis 1	Axis 2	Axis 3	Axis 1	Axis 2
C14:0	0.10	0.08	0.16	NA	NA
C16:0	0.45	0.13	**1.12**	**-1.19**	**1.23**
C17:0	0.28	0.02	0.29	0.64	**1.36**
C18:0	**0.34**	**0.62**	**0.54**	-0.48	-0.53
C16:1n7	-0.09	0.21	-0.07	0.37	0.43
C18:1n9	0.27	**0.65**	0.23	**-0.93**	**-0.82**
C18:1n7	-0.29	-0.28	-0.23	-0.05	0.52
C18:2n6	-0.05	0.29	**-0.40**	-0.01	-0.09
C20:5n3 (EPA)	0.0	0.24	-0.31	0.51	0.08
C22:5n3 (DPA)	**-0.44**	0.18	-0.14	0.21	-0.06
C22:6n3 (DHA)	**-0.79**	**0.44**	0.32	**0.99**	**-1.07**
C20:4n6 (ARA)	**-0.76**	-0.15	**-0.88**	-0.02	-0.36
C22:4n6	-0.06	0.03	**-0.56**	-0.28	0.29
C22:5n6	-0.01	-0.08	-0.04	-0.10	-0.14

NA: data not available (missing values).

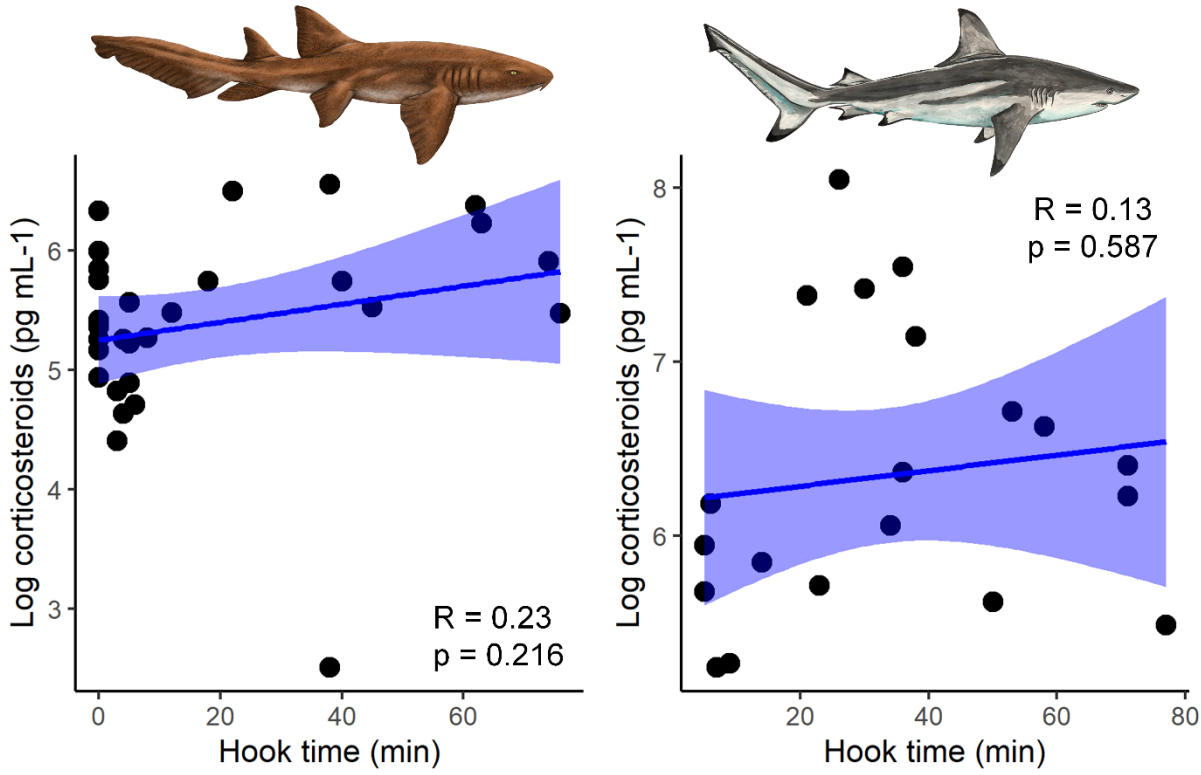

Figure S1. Linear regression models of the interaction between hook time (minutes) and relative corticosteroid levels in both male **(a)** nurse sharks (*Ginglymostoma cirratum*) (N=30) and **(b)** blacktip shark (*Carcharhinus limbatus*) (N=20).

Capítulo 4

Urban living influences the nutritional quality of a juvenile shark species

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Abstract

The field of marine urban ecology is a nascent, but growing area of research. An understanding of how urbanization may alter the diets and nutrition of marine species living in urbanized coastal habitats is limited. In the present study, we investigated the influence of urbanization on dietary patterns and nutritional quality of the nurse shark *Ginglymostoma cirratum*, a coastal epibenthic mesopredator. We tested the hypothesis that sharks sampled in urbanized areas (hereafter, 'urban sharks') would exhibit lower nutritional quality than individuals sampled in adjacent, but more pristine areas (hereafter 'non-urban sharks'). To accomplish this, we compared plasma fatty acid profiles of juvenile nurse sharks in proximity to Miami, a large coastal city, within Biscayne Bay, Florida. Results revealed that urban sharks contained higher levels of plasma saturated and bacterial fatty acids compared to non-urban sharks. Urban sharks also exhibited lower proportions of essential fatty acids (i.e., highly unsaturated fatty acids, HUFAs), mainly due to low contributions of omega-6 HUFAs. These results suggest that urban sharks consumed lower-quality food resources than conspecifics in less impacted areas. The apparent poor nutritional quality of prey consumed by shark living in urban areas may have several long-term consequences on their health and growth.

Keywords: anthropogenic impacts, *Ginglymostoma cirratum*, trophic ecology, trophic markers, marine predator, non-lethal methods.

4.1. Introduction

Urbanization can significantly alter ecosystems, for example, through habitat degradation, sewage effluent, chemical pollution (e.g. heavy metals, pesticides), and noise pollution (McKinney, 2002; Briceño et al., 2011). These environmental impacts have the potential to directly or indirectly alter organismal diet, increase their susceptibility to disease, cause increased competition for limited resources and alter trophic interactions (reviewed in Bradley and Altizer, 2006; Shochat et al., 2006; Grimm et al., 2008; Isaksson, 2015; El-Sabaawi, 2019). While the effects of urbanization on terrestrial species is relatively well known, marine urban ecology is a relatively nascent, but growing field (Todd et al., 2019). For example, the effects of coastal urbanization on the trophic ecology of marine animals remains poorly known (Puccinelli et al., 2016; Birnie-Gauvin et al., 2017). One of the most problematic processes in this context is eutrophication, resultant of an excess of nutrients derived from urban and agricultural wastewater (Paerl et al., 2014). Eutrophication can potentially trigger environmental changes, altering the phytoplankton community structure by stimulating plant growth, including harmful algal blooms, epiphytes, and invasive plants, consequently altering the entire food chain (Paerl et al., 2014; Todd et al., 2019).

Fatty acids are especially relevant biomarkers to study diet patterns and nutritional shifts, as they are transferred with little modification from prey to predator (Budge et al., 2006; Iverson, 2009). Additionally, fatty acids provide valuable information for identifying the food quality (Twining et al., 2018) and basal food chain dependencies (e.g., bacteria, diatoms, dinoflagellates; Dalsgaard et al., 2003). Because consumers are unable to produce *de novo* polyunsaturated fatty acids (PUFAs) and limited in converting them to highly unsaturated fatty acids (HUFAs), they rely on the diet and primary producers to obtain omega-3 and omega-6 PUFAs, which bioaccumulate up the food web (Darlsgaard et al., 2003; Budge et al., 2006). For example, changes in nutrient input or primary production, as a result of artificial eutrophication, can induce bottom-up processes, resulting in lower transfer rates of physiologically important fatty acids to higher trophic levels, affecting the nutritional status of consumers (Gladyshev et al., 2012; Gomes et al., 2016; Whorley et al., 2017). An inadequate dietary intake of PUFAs can compromise an individual's immunity and may also impair reproductive success due to the crucial function of essential fatty acids in a variety of physiological processes, such as immune and inflammatory responses, membrane fluidity, cardiac function, and brain development (Sargent et al., 1999; Tocher 2003, 2010; Birnie-Gauvin et al., 2017).

Marine predators, including many shark species, are relatively sensitive to human-induced environmental degradation because of their large body size, relatively low metabolic rate and slow population growth (Cortes, 2000; Conrath and Musick 2012; Gallagher et al., 2012). As high-level consumers, they tend to bioaccumulate and biomagnify contaminants (e.g., Hammerschlag et al., 2016, Merly et al., 2019), which can affect several physiological processes, which can result in feminization (Kidd et al., 2007), infertility (Gelsleichter et al., 2005), and behavioral alterations (e.g. increasing feeding rate (Brodin et al., 2014). Many shark species use nearshore and shallow waters as nursery grounds during their early life-stages, where juveniles can find abundant food, grow at faster rates, and receive increased refuge from predators (Heupel et al., 2007), however, it is also where they are typically more susceptible to the anthropogenic impacts (Knip et al., 2010), including coastal urbanization. Given that juvenile sharks living in urbanized habitats have been observed to spend significant time in human-altered areas (i.e. channels, marinas and dredged creeks; Curtis et al., 2013; Roemer, 2018), it is of conservation value to understand if and how urbanization affects the dietary patterns and nutritional quality of this critical life stage.

Among coastal sharks, the nurse shark, *Ginglymostoma cirratum* (Bonnaterre, 1788), is an appropriate model species to investigate the effect of urbanization on nutritional condition, because it is relatively sedentary and exhibits high residency and site fidelity to coastal areas, especially at the juvenile stage (Chapman et al., 2005; Garla et al., 2016; Roemer, 2018). This species is also an opportunistic predator, which primarily consumes small teleosts, crustaceans and mollusks (Castro, 2000). Previous findings suggest that juvenile nurse sharks living in urbanized area feed more frequently and/or consume more prey than sharks within adjacent less-impacted area (Moorhead, 2019), implying that they may be “urban exploiters” and take the advantage of resources from the urban environment (McKinney, 2002). However, as food sources in urban areas are often calorie-rich but nutrient-poor (Bateman and Fleming, 2012; El-Sabaawi, 2019), it is possible that nurse sharks are experiencing poor-quality diets in urbanized areas.

In the present study, we investigated the influence of urbanization on dietary patterns and nutritional quality of nurse sharks. Specifically, we compared short-term dietary markers (i.e. plasma fatty acid composition) of juvenile nurse sharks between high and low-altered areas in Biscayne Bay, South Florida. It is well known that the urbanization induces bottom-up regulation of food web quality though shifts in phytoplankton community composition, and consequently changes the diet quality of predators (e.g. Razavi et al., 2014; Whorley et al., 2017). Therefore, we expected that juvenile nurse sharks within highly urbanized areas

(hereafter ‘urban sharks’) would consume lower-quality diets compared to those in less-impacted areas nearby (hereafter ‘non-urban sharks’). Based on this hypothesis, we further predicted that urban sharks would exhibit a lower overall proportion of highly unsaturated fatty acids (HUFAs), given that high levels of nutrients are often associated with high-altered habitat due to coastal runoff, which can reduce proportions of HUFAs in the base of the food web, consequently decreasing trophic transfer (e.g. Gladyshev et al., 2012) (Fig. 1). We also expected to find higher proportions of saturated and bacterial fatty acids in urban sharks, as these biomarkers are highly correlated with urbanization, for example due to domestic sewage effluent (e.g. Boëchat et al., 2014; Jiménez-Martínez et al., 2019).

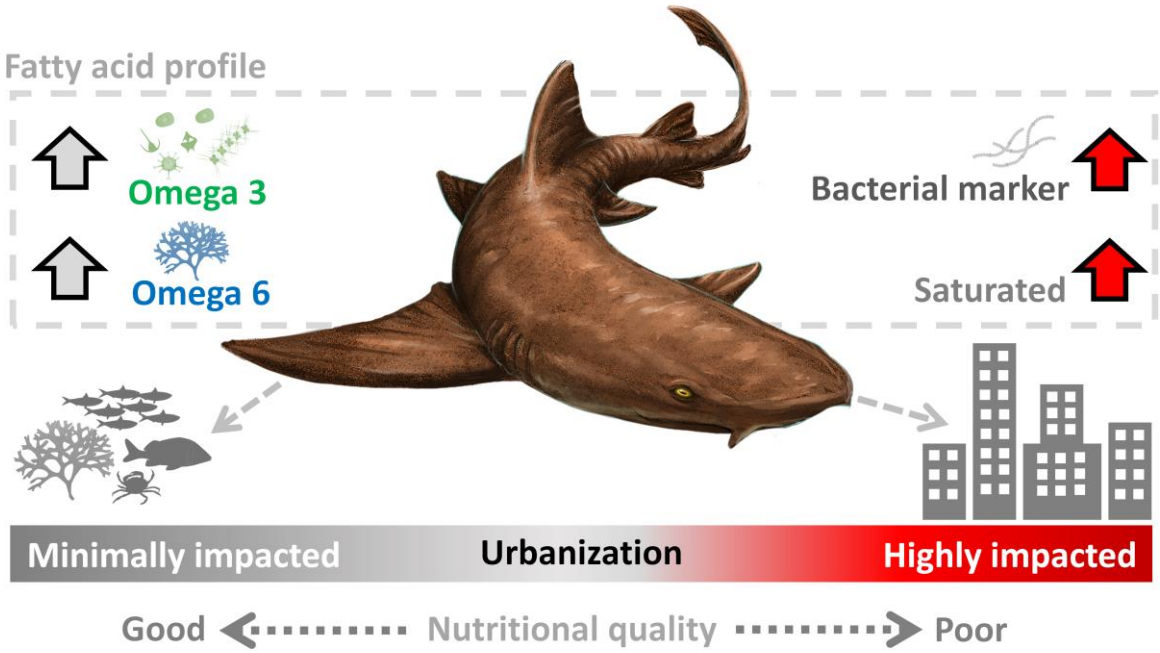

Figure 1. Conceptual illustration of expected differences in nutritional quality of juvenile nurse sharks (*Ginglymostoma cirratum*), between low-altered and high altered areas. It is expected that nurse sharks within high-altered area would have poorer nutritional quality, and consequently higher percentages of bacterial and saturated fatty acids, as these fatty acids are highly correlated to urbanization. In contrast, we expected that nurse sharks within low-altered area would have higher percentages of omega 3 and 6 highly unsaturated fatty acids, i.e. physiologically important fatty acids. Illustration of tiger shark is a courtesy of Kelly Quinn. Fishes, macroalgae, phytoplankton and bacteria images from IAN/UMCES symbol and image libraries (http://ian.umces.edu/imagelibrary/).

4.2. Material and methods

4.2.1. Study area

Biscayne Bay is a coastal lagoon located in subtropical southeast Florida (Fig. 2). The study is exposed to high variation in urbanization, with the city of Miami to the north of the Bay, and Biscayne National Park in the central and South. Bordering the north shoreline, Miami-Dade is the seventh most populous county of the United States, comprising a population of more than 2.7 million inhabitants (www.census.gov). Miami metropolitan region has a well-documented process of habitat alteration associated with the development, including diminished water quality, increased levels of pollutants and nutrients, and increased boat traffic (e.g. Serafy et al., 2003; Lirman et al., 2008; Briceño et al., 2011). Located in the northern portion of Biscayne Bay, this area has undergone extreme anthropogenic alteration, resulting in reduction of approximately 80% of mangrove forest (Serafy et al., 2003), and in direct impacts on corals (i.e. reducing grow rates, Hudson et al., 1994), fishes (e.g. deformities) and marine mammals (e.g. exposure to contaminants) (Browder et al., 2005; Briceño et al., 2011).

Biscayne National Park is located in the central and southern Biscayne Bay, comprising federal protection for 73,240 hectares under more natural conditions. The prevalence of less-altered habitat likely contributes to higher and more stable salinities and more natural benthic communities composed for example by seagrass, macroalgae, corals and sponges (Browder et al., 2005; Lirman et al., 2008), including higher fish abundance compared to the northern portion of Biscayne Bay (Serafy et al., 1997). Although to a lesser degree, the waters of Biscayne National Park are exposed anthropogenic influence including fishing as well as increased nutrient influx and chlorophyll-a near canal entrances and marinas along its western shoreline (Millette et al., 2019). Accordingly, only samples collected from the eastern shoreline areas of Biscayne National Park where considered as the low-impacted, non-urban, areas.

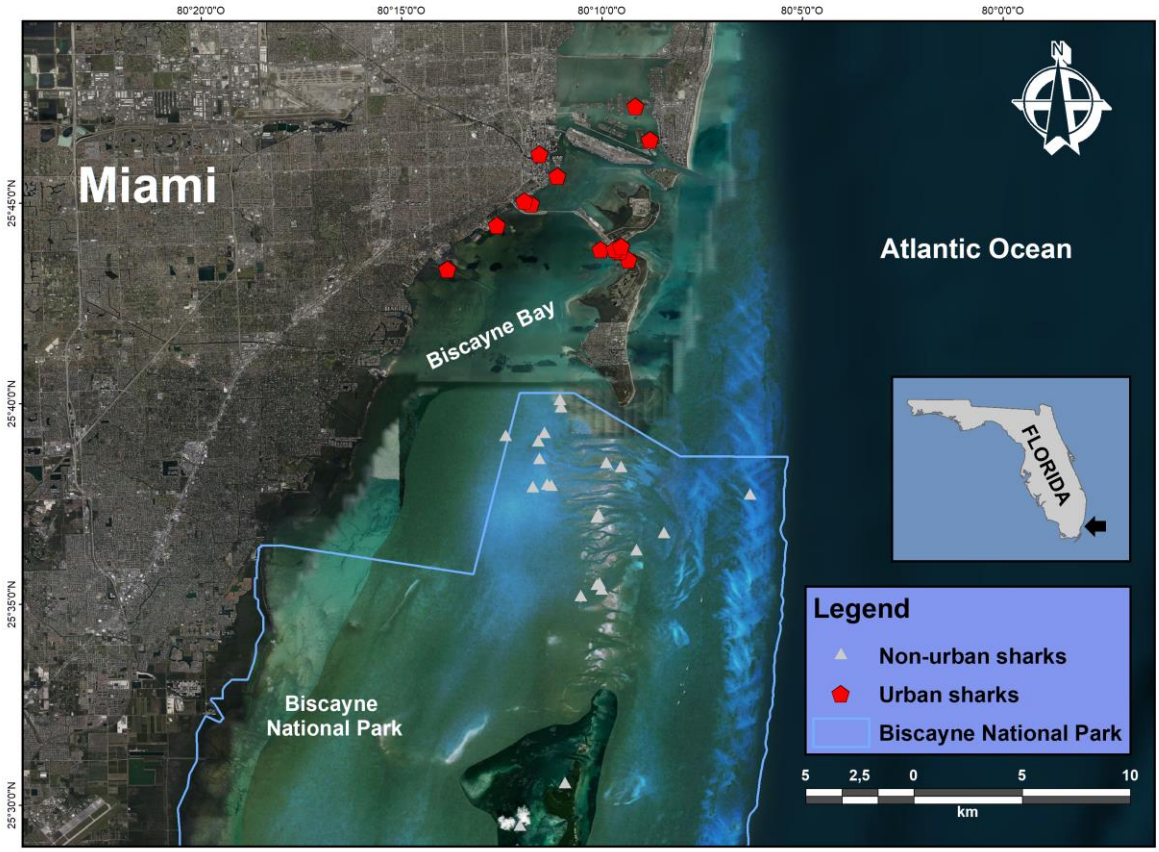

Figure 2. Sampling locations of juvenile nurse sharks (*Ginglymostoma cirratum*) within urbanized areas associated with Miami (n= 47; i.e., 'urban sharks') and relatively pristine areas of Biscayne National Park (n= 28; i.e., 'non-urban sharks').

There is no data on the shark diet in this study region. However, based on a previous study analyzing stomach contents of nurse sharks in South Florida, they predominantly feed on small teleosts (in 88% of the stomachs), including grunts (Haemulidae) and Porgies (Sparidae) (Castro, 2000). Besides, cephalopods, usually octopi, were found in 14% of the stomachs, while Crustacea, including spiny lobsters and small spider crabs, were found in 8% of the stomachs (Castro, 2000). All of these prey items are also abundant in the Biscayne Bay, for example, blue striped grunt (*Haemulon sciurus*), gray snapper (*Lutjanus griseus*) blue crab (*Callinectes sapidus*), and Caribbean spiny lobster (*Panulirus argus*) (e.g. Browder et al., 2005; Serafy et al., 2007; Hammerschlag and Serafy, 2010; Butler and Dolan et al., 2017).

4.2.2. Capture and sampling

Nurse sharks were sampled along the urban gradient of Biscayne Bay in 2015 (Apr, May, Dec), 2017 (Feb – Apr, Jun – Aug, and Oct - Dec), 2018 (from Feb to Nov) and 2019

(Jan), across both, dry (Nov – Apr) and wet seasons (May – Oct). All sharks were captured using circle-hook drumlines, a passive fishing technique that allows the captured sharks to swim (as described by Gallagher et al., 2014). In brief, drumlines were deployed (10 - 40 m deep) to soak for 1 h before being checked for shark presence. On capture, sharks were secured by hand to a partially submerged platform. Once landed, a water pump moving fresh seawater was inserted into the shark's mouth to actively pump water (94.5 liters per minute) over the shark's gills while temporarily immobilized. While sharks were secured, blood samples were obtained, sex was recorded and total length (TL, cm) were taken; sharks were then tagged for identification and released. Procedures and animal husbandry were approved by the University of Miami Institutional Animal Care and Use Committee (Protocol 15-238) and research permits from Florida Fish and Wildlife Conservation Commission, Biscayne National Park and National Marine Fisheries Service.

Blood (~10 ml) was collected from the caudal vein and immediately centrifuged (3500 rpm, 410 × g) for 2 min. Plasma was then removed and stored frozen at −80°C for analyzing fatty acid profiles.

4.2.3. Fatty acid analysis

Plasma fatty acid profiles were analyzed by direct transmethylation described by Parrish et al. (2015), using 100 μL of plasma without previous lipid extraction. Briefly, the samples were homogenized and directly transmethylated in 3mL of methanol: dichloromethane: concentrated hydrochloric acid (10:1:1 v/v) solution for 2 h at 80–85 °C. After this process, 1.5 mL of Milli-Q® water and 1.8 mL of hexane and dichloromethane (4:1 v:v) were added and, mixed and centrifuged at 2,000 rpm for 5 min. The upper layer was then removed, transferred to 2 ml-injection vials and reduced under a nitrogen stream. This process was repeated two times. Fatty acid analysis was carried out in a gas chromatograph Scion 436 equipped with a flame ionizer (FID) and CP 8410 auto-sampler. The capillary column used was CP Wax, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length. Hydrogen was used as a carrier gas at a linear velocity of 1.4 mL/min cm/s. The column was programmed at 170 °C for 1 min, followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The injector and FID temperatures were 250 and 260 °C, respectively. Fatty acids methyl esters (FAME) were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualmix, polyunsaturated fatty acids (PUFAs) fish M, Menhaden Oil, Larodan). The data are presented as % of FAME, based on peak area analyses.

4.2.4. Fatty acid nutritional indicators and trophic markers

Fatty acids that accounted for less than 0.5% were excluded from statistical analyses. The essential fatty acids, including eicosapentaenoic acid (EPA, C20:5n3), docosahexaenoic acid (DHA, C22:6n3) and arachidonic acid (ARA, C20:4n6) were used to compare the indices of shark nutritional quality, as they are the most physiologically important (Tocher, 2003, Arts and Kohler, 2009). The ARA, ARA/EPA and n3/n6 ratio were used to infer physiological responses of eicosanoids, i.e. inflammatory responses (Tocher, 2003). In terms of trophic markers, DHA/EPA ratio was used as marker of trophic position and C18:1n9/C18:1n7 ratio as degree of carnivory/piscivory (Dalsgaard et al., 2003, El-Sabaawi et al., 2009, Parrish et al., 2015). The C18:2n6 was used as an indicative for terrestrial resources, while ARA values have also been found to be a marker of species inhabiting coastal/benthic environments (Sardenne et al., 2017). For the relevant markers in the context of urbanization, the odd chain fatty acids (OFA), branched chain fatty acids (BFA), and C18:1n7 were used as biomarkers of heterotrophic bacteria (Dalsgaard et al., 2003; Kelly and Scheibling, 2012), which increase with decomposition of organic debris (Le Moal et al., 2019). Additionally, the C16:0 and C18:1n9 was used as indicators for domestic sewage (Jardé et al., 2005; Boëchat et al., 2014).

4.2.5. Data analysis

We used Student's *t*-test for independent samples for normally distributed data or a Mann-Whitney-Wilcoxon rank test for non-normally distributed data to compare fatty acid percentages between urban and non-urban sharks. Previous published length at maturity data for nurse sharks were used to distinguish juvenile (< 214.0 cm TL for males and < 223.0 cm TL for females; Castro, 2000). To explore differences on plasma fatty acid profile between seasons, discriminant analyses (LDA) were performed separating each season (dry and wet) and each site (non-urban and urban area).

Permutational multivariate analysis of variance (PERMANOVA) with Bonferroni correction was used to evaluate differences in fatty acid profile between season and between sites in each season. Each fatty acid was log transformed and tests were based on Euclidean distance matrix. Statistical significance was declared at $p < 0.05$, and all analyses were performed in Past 3.20 (Hammer et al., 2001) and SigmaStat software version 3.10 (Systat Software Inc., San Jose, CA, USA).

4.3. Results

A total of 75 juvenile nurse sharks were analyzed, 28 sampled within non-urban area (mean ± standard deviation, 169.7 ± 38.9 cm TL) and 47 sampled within urban area (133.72 ± 45.4 cm TL), including 42 females (147.81 ± 46.8 cm TL) and 33 males (145.1 ± 44.6 cm TL).

In general, blood plasma comprised mainly saturated fatty acids (SFAs) (C16:0 and C18:0) for juvenile nurse sharks in both areas, followed by PUFAs (ARA and DHA), and monounsaturated fatty acids (MUFAs) (C18:1n9 and C18:1n7) (Table 1; Fig. 3d, e). Plasma ΣSFA and ΣSFA/ΣPUFA ratios were significantly higher in urban versus non-urban sharks (Fig. 3a, d), including C14:0 and C16:0 (Table 1; Fig. 3a,b). Conversely, ΣPUFA was lower urban compared to non-urban sharks, mainly due to n6 PUFA (Fig. 4c), including ARA, C22:4n6 and C22:5n6 (Table 1; Fig. 3g, h, i) and the n3 PUFA C22:5n3 (Table 1, Fig. 3f). The lower proportions of ARA in urban sharks resulted in lower values of ARA/EPA and ARA/DHA ratios and higher n3/n6 ratio compared to non-urban sharks (Fig. 4e, g, h). Although ΣMUFA did not differ between areas, C14:1 and C18:1n7 was lower in urban, while C18:1n9 and C18:1n9/C18:1n7 ratio was lower in non-urban sharks (Table 1). The bacterial makers, ΣBFA-OFA, mainly due to C17:0, was higher in urban sharks, while there was no difference in other trophic markers (Figs. 3c and 4f).

The LDA analyses revealed that the first discriminant function discriminated the non-urban from urban sharks (Axis 1= 74.4%, eigenvalues= 3.13), mainly due to the contribution of C22:4n6, ARA, DPA and C17:0 (Table S1; Fig. 5). Whereas the second function separated the dry and wet seasons in non-urban sharks (Axis 2= 15.2%, eigenvalues= 0.64), mainly due to C17:0, C18:2n6 and C16:1n7 (Table S1; Fig. 5). Multivariate analyses also revealed a statistical difference between non-urban and urban sharks during both wet (PERMANOVA, F= 7.19; p = 0.006) and dry season (F= 14.13; p= 0.006). Fatty acid profiles of non-urban sharks differed between dry and wet season (F= 5.03; p= 0.018), while there was no difference in fatty acid profiles between seasons for urban sharks (F= 1.17; p= 1.000).

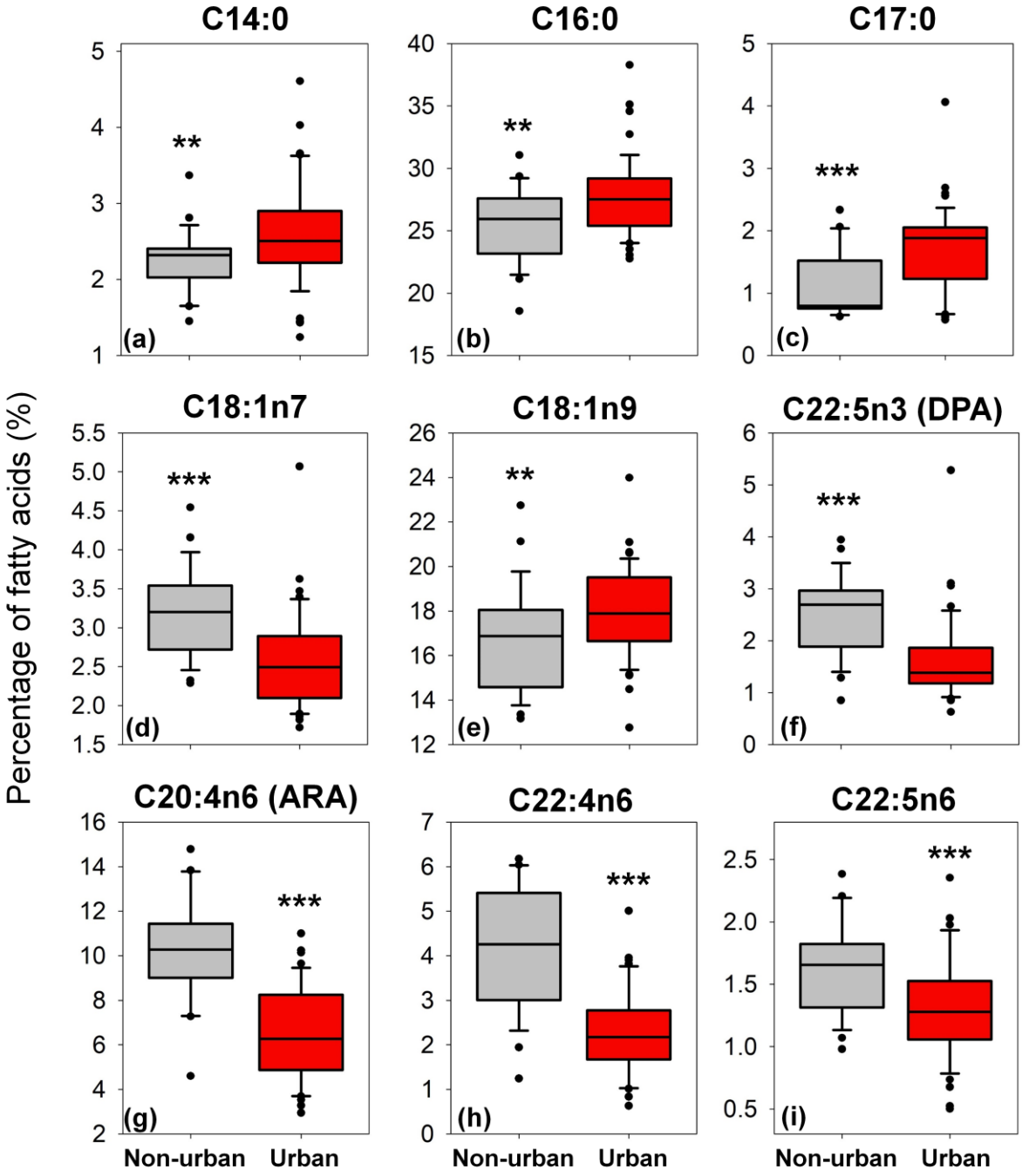

Figure 3- Boxplots of plasma fatty acids sum and ratios in juvenile nurse sharks (*Ginglymostoma cirratum*) sampled within urbanized areas associated with Miami (n= 47; i.e., 'urban sharks') and relatively pristine areas of Biscayne National Park (n= 28; i.e., 'non-urban sharks'). Black line indicates the median value. DPA: docosapentaenoic acid (C22:5n3); ARA: arachidonic acid (C20:4n6). Significant differences between urban and non-urban sharks are indicated by asterisks (Student's *t*-test *p<0.05; **p< 0.01; ***p< 0.001).

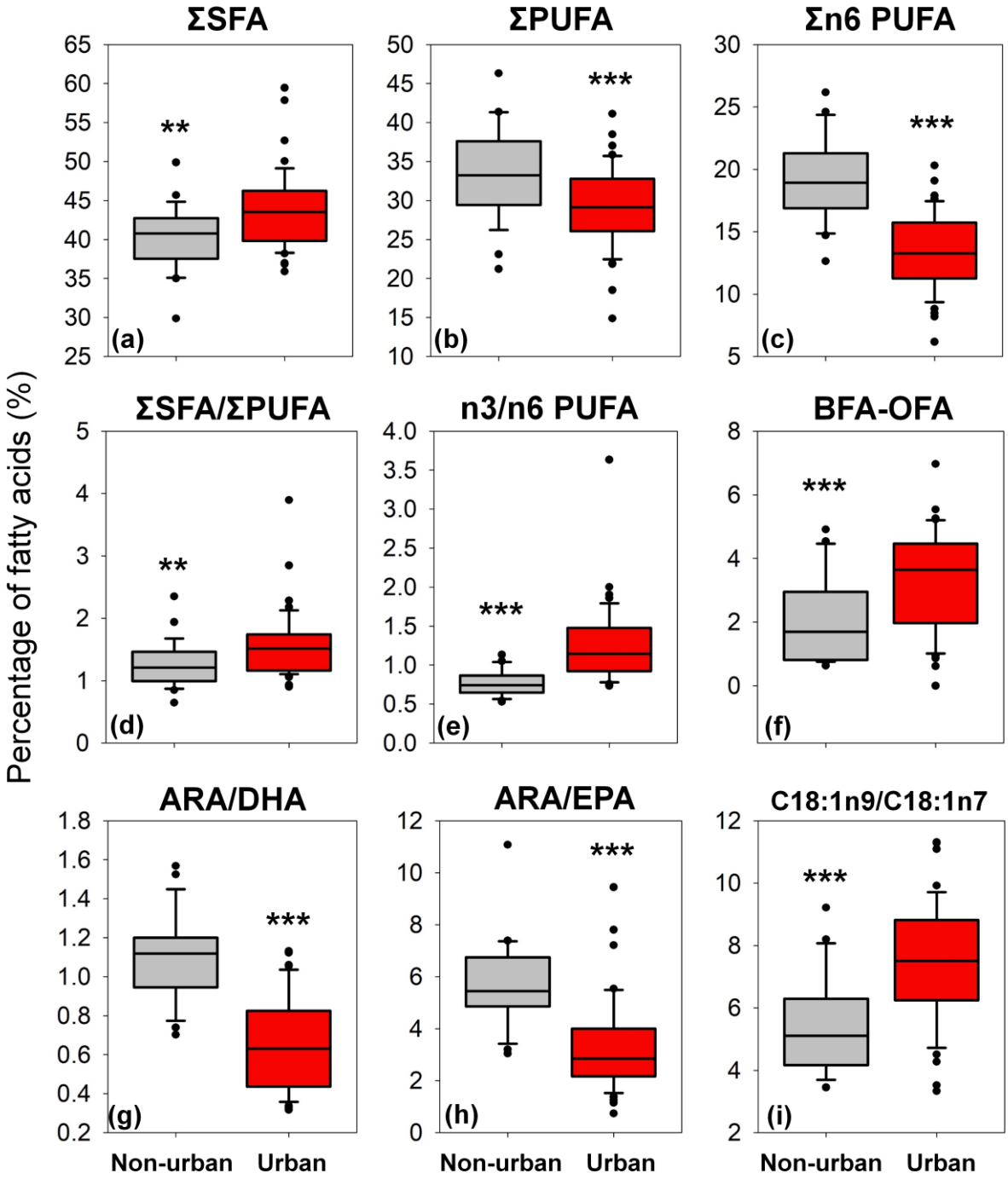

Figure 4- Boxplots of plasma fatty acids sum and ratios in juvenile nurse sharks (*Ginglymostoma cirratum*) sampled within within urbanized areas associated with Miami (n= 47; i.e., 'urban sharks') and relatively pristine areas of Biscayne National Park (n= 28; i.e., 'non-urban sharks'). Black line indicates the median value. SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids; BFA: branched-chain fatty acids; OFA: odd-chained fatty acids; ARA: arachidonic acid (C20:4n6); DHA: docosahexaenoic acid (C22:6n3); EPA: eicosapentaenoic acid (C20:5n3). Significant differences between urban and non-urban sharks are indicated by asterisks (Student's *t*-test *p<0.05; **p< 0.01; ***p< 0.001).

Table 1. Plasma fatty acid profile of juvenile nurse sharks (*Ginglymostoma cirratum*) sampled within urbanized areas associated with Miami (n= 47; i.e., 'urban sharks') and relatively pristine areasof Biscayne National Park (n= 28; i.e., 'non-urban sharks'). Data are mean % ± standard deviation. *t* Score values for Student's *t*-test, *z* values for Mann-Whitney-Wilcoxon rank test (non-normally data). Significant ($p < 0.05$) results are **bolded**.

Fatty acids	Non-urban	CV	Urban	CV	*t* or *z*	*p*-value
C15:0	0.5 ± 0.1	18.2	0.5 ± 0.2	32.1	-0.275	*p* = 0.788
C17:0	**1.1 ± 0.5**	**48.4**	**1.7 ± 0.6**	**32.8**	**-3.956**	***p* < 0.001**
ΣBFA-OFA	**2.0 ± 1.3**	**64.8**	**3.4 ± 1.2**	**34.9**	**-3.771**	***p* < 0.001**
C14:0	**2.3 ± 0.4**	**17.3**	**2.6 ± 0.6**	**23.4**	**-2.613**	****p* = 0.009**
C16:0	**25.4 ± 2.8**	**11.1**	**27.8 ± 3.4**	**12.2**	**-3.264**	***p* = 0.002**
C18:0	11.5 ± 1.6	14.1	12.2 ± 1.9	15.2	-1.647	*p* = 0.104
C24:0	1.0 ± 0.3	25.5	1.3 ± 0.5	37.9	1.705	*p* = 0.110
ΣSFA	**40.3 ± 4.0**	**9.9**	**43.6 ± 5.2**	**12.0**	**-3.075**	***p* = 0.003**
C14:1	**1.9 ± 0.4**	**22.7**	**1.4 ± 0.5**	**37.9**	**-4.329**	****p* < 0.001**
C16:1n7	1.9 ± 0.5	24.5	2.2 ± 0.8	38.4	-0.772	**p* = 0.439
C18:1n9	**16.8 ± 2.3**	**13.8**	**17.9 ± 2.1**	**11.5**	**-2.356**	***p* = 0.021**
C18:1n7	**3.2 ± 0.6**	**17.9**	**2.6 ± 0.7**	**25.1**	**4.160**	***p* < 0.001**
ΣMUFA	23.8 ± 2.3	9.6	24.0 ± 2.5	10.3	-0.592	**p* = 0.554
C18:2n6	2.9 ± 0.9	29.2	3.1 ± 0.9	32.4	-0.373	**p* = 0.709
C18:4n3	2.1 ± 2.1	101.3	1.8 ± 2.1	119.3	-0.345	*p* = 0.733
C20:5n3 (EPA)	2.0 ± 0.6	30.9	2.3 ± 0.9	40.7	-1.073	**p* = 0.283
C22:5n3 (DPA)	**2.5 ± 0.8**	**31.1**	**1.6 ± 0.8**	**50.1**	**-4.557**	****p* < 0.001**
C22:6n3 (DHA)	9.6 ± 2.7	27.7	10.6 ± 3.6	34.4	-1.267	*p* = 0.209
C20:4n6 (ARA)	**10.2 ± 2.2**	**21.2**	**6.5 ± 2.1**	**32.8**	**7.498**	***p* < 0.001**
C22:4n6	**4.2 ± 1.4**	**33.7**	**2.3 ± 0.9**	**39.8**	**-5.277**	****p* < 0.001**
C22:5n6	**1.6 ± 0.4**	**22.4**	**1.3 ± 0.4**	**29.6**	**3.447**	***p* < 0.001**
ΣPUFA	**33.6 ± 5.7**	**16.9**	**29.1 ± 5.4**	**18.6**	**3.472**	***p* < 0.001**
Σ n3 PUFA	14.6 ± 3.3	22.7	15.7 ± 3.9	24.2	-0.196	*p* = 0.196
Σ n6 PUFA	**19.1 ± 3.2**	**16.7**	**13.4 ± 3.0**	**22.5**	**7.815**	***p* < 0.001**
ΣSFA/ΣPUFA	**1.3 ± 0.4**	**28.4**	**1.6 ± 0.6**	**35.9**	**-3.032**	****p* = 0.002**
n3/n6	**0.8 ± 0.2**	**20.6**	**1.3 ± 0.5**	**41.6**	**-5.659**	****p* < 0.001**
DHA/EPA	5.2 ± 1.8	34.5	5.3 ± 2.6	49.0	-0.069	**p* = 0.945
ARA/DHA	**1.1 ± 0.2**	**19.9**	**0.7 ± 0.3**	**37.3**	**-7.886**	***p* < 0.001**
ARA/EPA	**5.7 ± 1.7**	**30.1**	**3.3 ± 1.8**	**55.7**	**-4.326**	****p* < 0.001**
C18:1n9/C18:1n7	**5.5 ± 1.5**	**25.6**	**7.4 ± 1.9**	**25.9**	**-4.259**	***p* < 0.001**
C16:1n7/C16:0	0.1 ± 0.0	27.7	0.1 ± 0.0	37.8	-0.489	**p* = 0.624

* Mann-Whitney-Wilcoxon rank test. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; BFA: branched-chain fatty acids; OFA: odd-chained fatty acids.

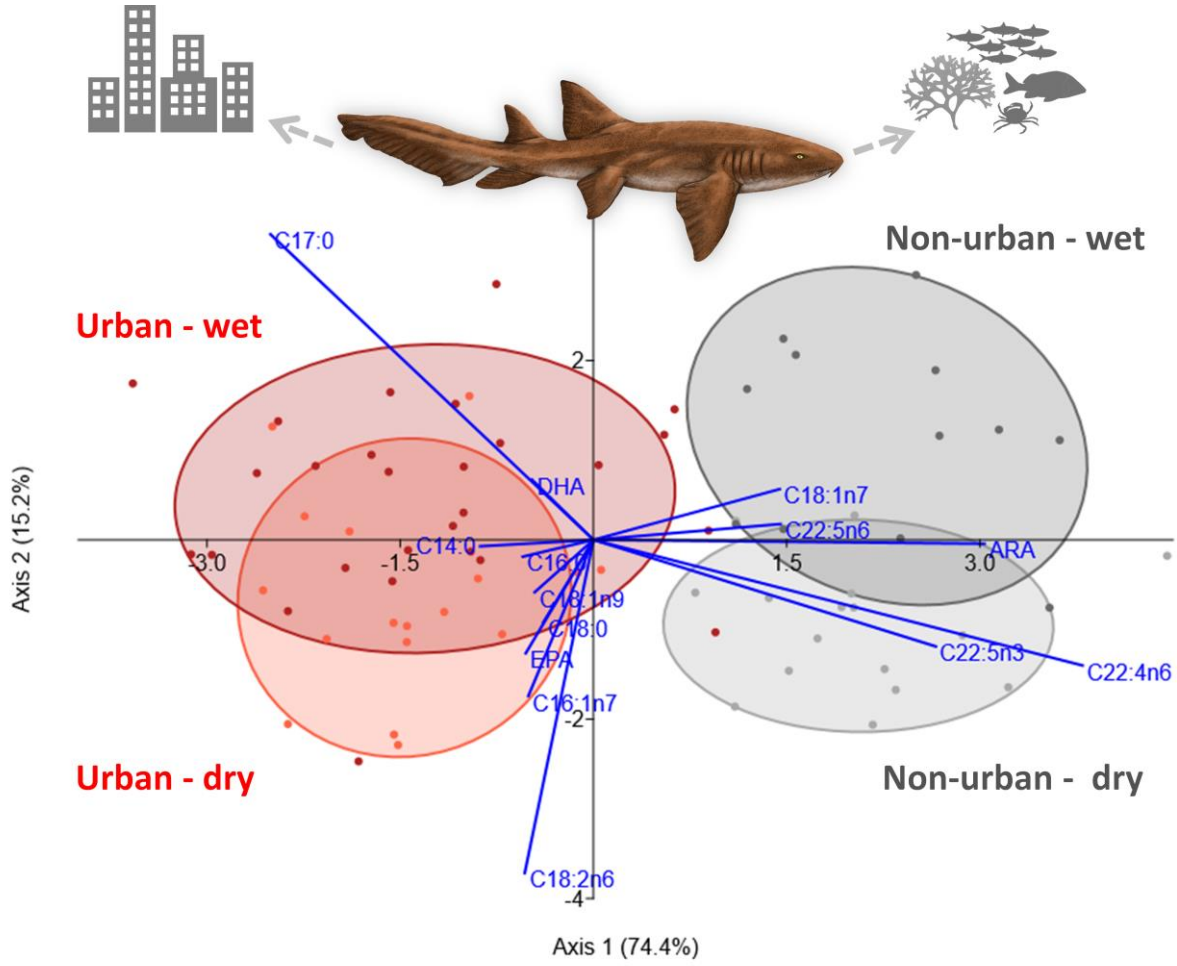

Figure 5- Linear discriminant function analyses of selected fatty acids (based on their abundance in all samples) of juvenile nurse sharks (*Ginglymostoma cirratum*) sampled within urbanized areas associated with Miami ('urban sharks') and relatively pristine areas of Biscayne National Park ('non-urban sharks') during the dry (November – April) and wet (May – October) season. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid. The 70% ellipses similarly of seasons in each site is provided. Fishes, macroalgae, phytoplankton and bacteria images from IAN/UMCES symbol and image libraries (http://ian.umces.edu/imagelibrary/).

4.4. Discussion

Here we compared short-term dietary markers in coastal sharks in adjacent areas that differ spatially in their exposure to urbanization. We found differences in dietary patterns between low and highly-urbanized areas. These data supported our hypothesis that urban sharks would exhibit lower nutritional quality than their non-urban counterparts. Specifically, urban sharks had lower proportions of ΣPUFA, mainly omega-6 PUFA, and higher ΣSFA and bacterial FA indicative of decomposition of organic debris and domestic sewage. However, omega-3 highly unsaturated fatty acids, including DHA and EPA, did not differ between

urban and non-urban sharks, suggesting that the urbanization does not affect the dietary intake of these fatty acids. Based on these findings, our study suggests that juvenile nurse sharks inhabiting urban areas seem to consume lower-quality food resources (in terms of essential fatty acids) during this critical life-history stage.

Findings from previous work in the study area on nurse sharks (Moorhead, 2019) found that urban sharks had higher plasma triglyceride and lower free-fatty acid levels than non-urban nurse sharks, suggesting that urban sharks may feed more frequently than non-urban nurse sharks (Moorhead, 2019). However, based on our results for multiple fatty acid biomarkers, including those which are known to be physiologically important, it appears that despite possible increased food quantity, urban sharks consumed lower-quality prey (e.g. Gladyshev et al., 2012; Gomes et al., 2016). Higher proportions of circulating ΣSFA (including C14:0 and C16:0), as well as higher ΣSFA/ΣPUFA ratio in urban sharks, indicate that they are consuming more SFA-rich prey compared to non-urban counterparts. Although SFAs can be *de novo* biosynthesized from carbohydrate or protein precursors (Budge et al, 2006), plasma SFAs of sharks have been found in similar proportions to those observed in their diet (e.g. McMeans et al., 2012; Bierwagen et al., 2019). Moreover, because urban nurse sharks had higher circulating triglycerides levels than conspecifics within low-altered area (Moorhead, 2019), it is unlikely that urban sharks are biosynthesizing FA, since they are probably consuming a diet containing adequate or excess fat (e.g. Budge et al., 2006). Higher energy reserves in nurse sharks could also explain their higher circulating C16:0 and C18:1n9 levels, given that these fatty acid are the main constituents of fat stores (e.g. Pethybridge et al., 2014; Guglielmo et al., 2018).

Other ecological and physiological phenomena may explain the difference found in nutritional quality between urban and non-urban sharks. First, it is possible that SFAs are transferred directly or indirectly from domestic sewage, since C16:0, as well as C18:1n9, have been found to be the main components of domestic sewage, and therefore, highly correlated to urbanization (Jardé et al., 2005; Boëchat et al., 2014; Jiménez-Martínez et al., 2019). Second, SFAs are resistant to peroxidation and PUFAs are highly damaged due to induced oxidative stress in urban animals living under environmental stress (Isaksson, 2015). Therefore, it is plausible to speculate that increased plasma SFA and decreased PUFAs may be a result of high peroxidation rate in nurse sharks and/or in their prey. Finally, another possible explanation may be related to pollutants, since increased SFAs and decreased PUFAs have been observed in several marine teleost species exposure to organic and inorganic pollutants (reviewed by Filimonova et al., 2016). Urban sharks were collected close to Miami

River, one of the most contaminated waterways in Florida, exhibiting higher sediment levels of organic contaminants, such as pesticides, polychlorinated biphenyls (PCBs), and trace metals (Browder et al., 2005; Briceño et al., 2011). Either by fat storage, trophic transfer or due to induced oxidative stress, increased plasma SFAs and decreased PUFAs can compromise different physiological processes of nurse sharks, including inflammatory response, cardiovascular tone, renal and neural function, and reproduction (e.g. Berry, 2009; Tocher, 2010). Further studies investigating these possible scenarios will help to improve our understanding of the drivers responsible for variation on nutritional quality of sharks within urbanized habitat. For example, it would be valuable to test if peripheral blood proteins, a proxy of health, differ between urban and non-urban sharks (Attalabenson et al., 2019).

The omega-6 HUFA ARA was the main fatty acid responsible for distinguishing urban from non-urban sharks. Proportion of ARA was approximately 64% higher in non-urban sharks, suggesting a significant decrease in the percentage of this physiologically important fatty acid from sharks sampled within high-altered areas. ARA is the most abundant fatty acid in brown algae, coralline algae or corals (Kelly and Scheibling, 2012, van Duyl et al., 2011), and therefore, can be transfer to sharks via primary consumers feeding on these producers (Sardenne et al., 2017; Bierwagen et al., 2019). This result is not surprising given non-urban sharks were sampled within relatively pristine area of Biscayne Bay, where the benthos is dominated by a mix of soft and hard corals, macroalgae, coral-algal bank fringes, sponges and several species of seagrasses (Browder et al., 2005). Additionally, two other omega-6 highly unsaturated fatty acid were higher in non-urban sharks, C22:4n6 and C22:5n6 (indicator for fish and cephalopods, Meyer et al., 2019), also suggesting a higher availability of these fatty acid in prey items within less impacted areas. Indeed, higher proportions of omega-6 HUFA have previously been observed in stomach contents and tissues of teleost fishes found in mesotrophic reservoirs, compared to those living in hypereutrophic reservoirs (Gomes et al., 2016), indicating the high influence of urbanization on the availability of omega-6 fatty acids to predators.

The observed decreases in omega-6 HUFAs found in urban sharks here may negatively influence their health and growth performance, especially during the development phases (e.g. Arts and Kohler, 2009; Beckmann et al., 2014; Araujo et al., 2019). Specifically, ARA is the most physiologically important omega-6 fatty acid, as it is metabolized to form bioactive eicosanoids such as two-series prostaglandins, leukotrienes and thromboxanes, which act as second messengers in the control of inflammation and immune responses, as well as promoting growth (Arts and Kohler, 2009; Calder, 2011). ARA-derived eicosanoids are

precursors for proinflammatory substances, and are more biologically active than those eicosanoids derived from EPA (Tocher, 2003; Arts and Kohler, 2009). As such, the lower ARA/EPA ratio found in sharks within urban influenced areas may significantly alter their physiological and inflammatory responses.

Contrary to our hypotheses that plasma omega-3 highly unsaturated fatty acids would differ between low- and highly-altered areas, we found no evidence to support this prediction. Only the omega-3 DPA significantly differed between urban and non-urban sharks, however, it was in very low proportions. We would have expected to find lower proportion of DHA in urban sharks, given that the urbanization can alter phytoplankton community composition (e.g. Razavi et al., 2014), but this was not observed. The lack of difference in DHA between urban and non-urban sharks may be because DHA is biomagnified and preferentially retained at higher trophic levels (Dalsgaard e.t al., 2003; Meyer et al., 2019). Despite the lower proportions of total PUFA found in urban sharks, our study did not detect any omega-3 difference in plasma profiles, indicating that dietary intake of DHA by nurse sharks occur in similar proportions in both low and highly-altered area. Given that DHA directly influences membrane fluidity and is the major structural lipid in neurological development (Izquierdo et al., 2001), we suggest that future work monitor DHA levels in urban sharks to detect critical environmental changes.

In terms of trophic markers, no differences were found in DHA/EPA ratios, suggesting that urban and non-urban sharks are foraging on prey at similar trophic position (Parrish et al., 2015; Bierwagen et al., 2019). However, it is important to note that urban sharks had higher C18:1n9/C18:1n7 ratios (indicator for carnivory/piscivory, Dalsgaard et al., 2003), suggesting that urban sharks could be primarily consuming fishes, while non-urban sharks could be consuming a broader diversity of prey types, including crustaceans and mollusks (Castro, 2000). Given that C18:1n9 is one of the main components of domestic sewage, it is possible that these could be transferred though the food web in urbanized habitat (Jiménez-Martínez et al., 2019). As predicted, urban sharks had higher percentages of heterotrophic bacterial markers (ΣBFA-OFA, including C17:0). Increased bacterial markers contribution in urban sharks is likely to be a result of anthropogenic induced processes, which can cause increased production of organic materials and consequently, increasing bacterial communities associated with organic detritus (Le Moal et al., 2019). Similarly, heterotrophic bacterial markers have been found in higher proportions in stomach content and tissues of teleost fishes within hypereutrophic reservoirs compared to conspecifics from mesotrophic reservoir (Gomes et al., 2016). Accordingly, our results may suggest a higher contribution of bacteria

in urbanized habitat. It is also likely that urban sharks are consuming different food items than their non-urban counterparts, e.g. feeding a higher proportion of demersal fish species rich in BFA-OFA (Käkelä et al., 2005; Kelly and Scheibling, 2012).

Evidences for seasonal variation in fatty acids was only observed in non-urban sharks. Though these patterns are preliminary, and our results for increased C17:0 during the wet season may be related to a higher consumption of suspension feeders by the sharks (e.g. Kelly and Scheibling, 2012) given seasonal variation in the prey base in Biscayne Bay (Serafy et al., 2003). It is also possible that increased freshwater flow and influx of organic sources during the wet season likely contribute for increasing bacterial community (Kelly and Scheibling, 2012). However, increased C16:1n7, another bacterial marker, was found in shark tissues during the dry season, showing the complex dynamics of benthic community within Biscayne Bay. Interesting, increased C18:2n6, a characteristic terrestrial marker for vascular plant debris (Every et al., 2016), was also observed during dry season. Altogether, these results provide evidence for seasonal diet shifts in juvenile nurse sharks living within non-urban areas. The lack of seasonal variation in diet patterns of urban sharks may suggests a more homogeneity of nutrients in the urbanized habitat, at least with respect to fatty acid sources.

Because we used a non-lethal approach through analyzing the percentage of plasma fatty acid profiles, our study presents limitations in terms of specifically identifying dietary and/or non-dietary origin (e.g. mobilized from storage tissues), and which fatty acid are incorporated in the nurse shark's tissues. However, plasma fatty acids have been extensively demonstrated as a promising method to assess short-term shifts in diet in the context of urbanization (e.g. Andersson et al., 2015; Isaksson, 2015; Toledo et al., 2016), and in elasmobranch's trophic ecology (e.g. Semeniuk et al., 2007; Beckman et al., 2014; McMeans et al., 2012; Bierwagen et al., 2020; Rangel et al., 2020). Moreover, considering the applicability of plasma fatty acids in the conservation physiology toolbox (Madliger et al., 2018), these short-term dietary markers provide useful information for detecting and monitor populations under threat (Cooke et al., 2013; Birnie-Gauvin et al., 2017).

4.5. Conclusion

Based on multiple fatty acid biomarkers, our findings demonstrated that juvenile nurse sharks differed markedly in their dietary patterns between low and highly urbanized areas within a subtropical bay. Though more research is needed to identify specific drivers for differences found between urban and non-urban sharks, our study suggest that urban sharks

consume lower-quality food resources in anthropogenically altered habitat. Urban sharks exhibited lower omega-6 highly unsaturated fatty acids and higher levels of both saturated and bacterial fatty acids, which has the potential to negatively affect their health and growth. We hypothesize these results are driven by bottom-up effects and altered trophic transfer of fatty acids through food web to nurse sharks. However, further studies investigating other physiological parameters integrated with foraging behavior are required. Taken together, previous and current work suggest several possibilities and hypothesis to explain the nutritional differences between urban and non-urban sharks, including ecological interactions that should be considered in future researches. The long-term impacts of low-quality diets to wildlife associated with living in urbanized landscapes is lacking for many organisms, especially in aquatic systems, presenting an area of opportunity for future research.

Acknowledgements

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Supporting Information

Table S1. Linear discriminant functions for the 3 first axis, **Bold** values indicate primary fatty acids contribution to dissimilarity. EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid.

Fatty acids	Axis 1	Axis 2	Axis 3
C17:0	**-0.052**	**0.071**	**-0.084**
C14:0	-0.018	-0.001	0.028
C16:0	-0.011	-0.004	0.014
C18:0	-0.008	-0.019	0.008
C16:1n7	-0.010	**-0.036**	0.022
C18:1n9	-0.009	-0.012	0.004
C18:1n7	0.030	0.012	0.017
C18:2n6	-0.011	**-0.077**	0.024
C20:5n3 (EPA)	-0.011	-0.026	-0.037
C22:5n3 (DPA)	**0.055**	-0.025	**0.052**
C22:6n3 (DHA)	-0.010	0.014	0.006
C20:4n6 (ARA)	**0.063**	-0.001	**-0.046**
C22:4n6	**0.079**	-0.029	**-0.045**
C22:5n6	0.030	0.004	-0.025

Capítulo 5

Effects of urbanization on the nutritional ecology of a highly active coastal shark: preliminary insights from trophic markers and body condition

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Abstract

The synergistic effects of coastal urbanization have dramatically impacted biological communities. Yet, few studies have investigated how urbanization can influence the diet quality and trophic ecology of coastal sharks. In a preliminary study, we examined for spatial variation in the nutritional ecology of a highly active marine predator, the blacktip sharks (*Carcharhinus limbatus*) exposed to regional differences in coastal urbanization in southeast Florida. We used medium-term nutritional indicators (i.e., body condition; whole blood stable isotopes [$\delta^{15}N$ and $\delta^{13}C$]) and short-term dietary markers (i.e., plasma fatty acid profiles) to test the hypothesis that the nutritional ecology of marine predators would differ in areas exposed to increased urbanization. Our initial results showed that blacktip sharks sampled in high urbanized area (hereafter, 'urban sharks') exhibited relatively higher body condition, blood $\delta^{15}N$ levels, and percentages of saturated fatty acids compared to sharks sampled in low urbanized area (hereafter 'non-urban sharks'). Collectively, these results suggest a possible positive alteration in the amount of food consumed by sharks and/or in the caloric value of their prey. We also found lower percentages of bacterial markers and higher values of dinoflagellate markers in urban sharks. Compared to more resident species evaluated in the region, we did not detect a reduction in diet quality (in terms of essential fatty acids) in this highly active species exposed to urbanization. Therefore, it is possible that the lifestyle and feeding behavior have an influence on the quality of food consumed by urban sharks, and maybe the impacts of urbanization are more pronounced in resident, sedentary and benthic species.

Keywords: Market gravity, fatty acids, stable isotopes, body condition, Florida, Miami-Dade.

5.1. Introduction

The world's population is rapidly growing and increasingly urbanizing, especially along coastlines where the population density is three times the global average (Small and Nicholls, 2003; Neumann et al., 2015). The modification of coastal marine environments by anthropogenic activities has dramatically impacted biological communities, with fitness consequences (Todd et al., 2019; Alter et al., 2020). Major threats to environments exposed to coastal urbanization include habitat degradation, sewage effluent, urban run-off, overfishing, increased shipping, and acoustic, light and chemical pollution (Halpern et al., 2008; Todd et al., 2019). Although the lethal and sublethal effects of urbanization on terrestrial species has been a burgeoning area of research (e.g. Bonier, 2012; Birnie-Gauvin et al., 2017), the effects of coastal urbanization on marine ecosystems remains relatively poorly studied (Todd et al., 2019). The synergistic effects of coastal urbanization can directly or indirectly affect the behavior and physiology of marine predators by reducing the availability and quality of their prey (e.g. Rangel et al., 2021a) and altering coastal food webs though the bottom-up (i.e. predators are impacted by alteration in prey and basal resources) and top-down controls (i.e. lower trophic levels are impacted by reductions in the abundance or diversity of predators) (Bradley and Altizer, 2007; Grimm et al., 2008).

Biochemical tracers, such as stable isotopes and fatty acids, can provide valuable indicators of resource use and individual nutritional ecology (e.g. Pethybridge et al., 2018; Meyer et al., 2019). For instance, stable isotopes of carbon ($\delta^{13}C$) have been used to inform individual foraging habitat (e.g., inland mangroves vs. coastal neritic, Shipley et al., 2019), while nitrogen isotopes ($\delta^{15}N$) have been used to infer the trophic position, as it is gradually enriched though trophic transfer up the food web (Gallagher et al., 2017; Shiffman et al., 2019). Fatty acids can also provide insights in to trophic ecology, since they remain relatively unchanged from prey to predator, and consequently reflect trophic interactions and basal food chain dependencies (dinoflagellates, bacteria, diatoms; Budge et al., 2006; Gomes et al., 2021). For example, eutrophication-induced loss of phytoplankton taxa rich in omega-3 fatty acids (n3 PUFAs, e.g. diatoms, cryptophytes and dinoflagellates) reduces the transfer of n3 PUFAs to higher trophic levels (e.g. Taipale et al., 2016). Additionally, as animals cannot synthesize n3 and n6 PUFAs, which are biochemical compounds essential for their survival, fitness and reproduction, fatty acids provide useful markers to assess nutritional quality in aquatic organisms (Arts and Kohler, 2009, Tocher, 2010; Meyer et al., 2019; Rangel et al., 2020).

As long-lived marine predators, sharks are particularly sensitive to anthropogenic stressors, mainly due to their relatively low metabolic rate, large body size, slow population growth and large home range requirements (Cortés, 2000; Gallagher et al., 2012). Although fishing is the main threat to sharks (e.g. Pacoureau et al., 2021), the sublethal effects of urbanization, including habitat loss and pollution, may have significant long-term impacts on coastal populations. For instance, plasma fatty acids measured in the mesopredatory nurse sharks (*Ginglymostoma cirratum*) suggest this species consumes lower-quality food resources in high urbanized areas as compared to conspecifics in intermediate locations (Rangel et al., 2021a). Also, previous studies have found higher infertility rates (Gelsleichter et al., 2005) and epigenetic modifications (Beal et al., 2021) in sharks exposed to high concentrations of contaminants associated with urbanization.

In the present study, we conducted a preliminary investigation into the potential relationships between exposure to coastal urbanization and aspects of nutritional ecology in a high active coastal shark, the blacktip shark (*Carcharhinus limbatus*). This species often occurs close inshore, including off river mouths, estuaries and shallow bays (Ebert et al., 2021), which makes them particularly susceptible to urbanization impacts. Here, we compared the short-term dietary markers (i.e. plasma fatty acids), medium-term trophic markers (i.e. whole blood stable isotopes), and medium-term nutritional indicator (i.e. body condition) between blacktip sharks sampled in two different areas exposed to varying degrees of coastal urbanization in South Florida. Based on previous findings in the study area, which found differences in the plasma lipid metabolites and fatty acid profiles of nurse sharks exposed to urbanization (Moorhead, 2019; Rangel et al., 2021a), we hypothesized that blacktip sharks sampled within high urbanized areas would exhibit higher body condition, but lower diet quality, compared to conspecifics sampled in relatively low urbanized areas nearby (Fig. 1). We therefore predicted that blacktip sharks exposed to greater market gravity would exhibit an enriched $\delta^{15}N$ and lower overall proportion of n3 PUFAs and of both diatoms and dinoflagellates fatty acid markers due to the increased contribution of anthropogenic nutrient enrichment (e.g. nitrogen and phosphorus) (Gladyshev et al., 2012; Mancinelli and Vizzini, 2014; Prado et al., 2020). We also expected to find that blacktip sharks sampled in high urbanized areas would exhibit higher proportions of saturated and bacterial fatty acids, which are markers for domestic sewage effluent (e.g. Boëchat et al., 2014; Jiménez-Martínez et al., 2019; Rangel et al., 2021a). Additionally, because sharks were sampled in areas exposed to different freshwater input, we expected to find spatial variation in omega-6 PUFA (Parrish et al., 2000), a terrestrial fatty acid marker (Fig. 1).

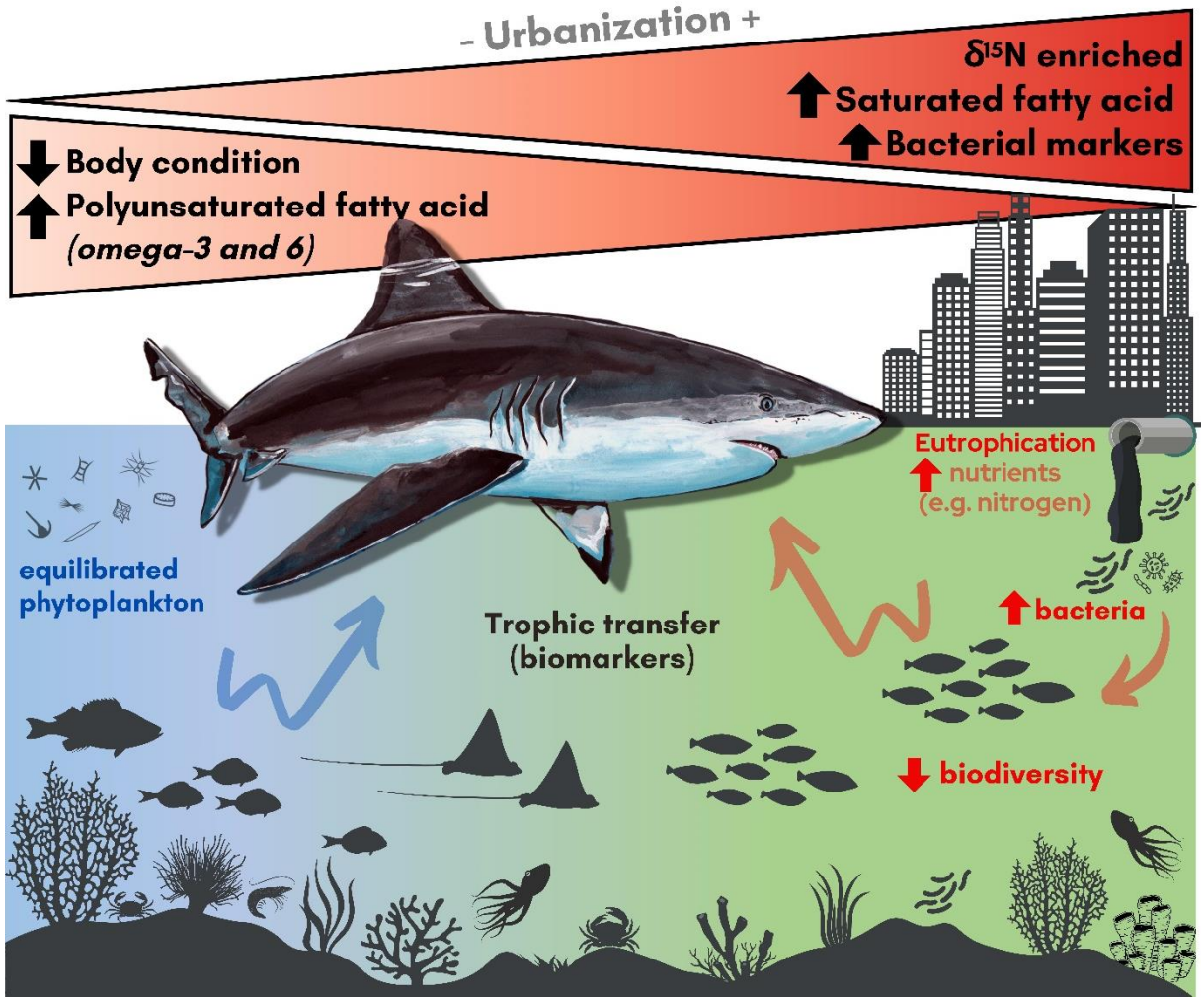

Fig. 1. Conceptual illustration of expected differences in nutritional ecology blacktip sharks (*Carcharhinus limbatus*) along a gradient of coastal urbanization, southeast Florida. We hypothesized that blacktip sharks sampled within high urbanized areas would exhibit a better body condition, but a lower dietary quality (in terms of fatty acids) compared to those sampled within low urbanized areas. We also expected blacktip sharks exposed to high urbanization would exhibit an enriched $\delta^{15}N$, higher percentages of saturated and bacterial markers and lower overall proportion of n3 polyunsaturated fatty acids because of contribution of anthropogenic nutrient enrichment. Illustration of blacktip shark is a courtesy of Alexandre Huber. Other imaginary sources are from Canva (www.canva.com).

5.2. Material and Methods

5.2.1. Study area

The study was conducted in two geographically proximate regions, but with different degree of urbanization in South Florida: (1) waters exposed to high urbanization, adjacent to the metropolis of Miami-Dade, in northern Biscayne Bay and (2) waters exposed to relatively low urbanization, encompassing Everglades National Park and Florida Bay (Fig. 2).

Biscayne Bay is a coastal lagoon exposed to high variation in urbanization, with the city of Miami to the north of the Bay (Fig. 2). Bordering the north shoreline of Biscayne Bay (Fig. 2), Miami-Dade is the seventh most populous county of the United States, comprising a population of more than 2.7 million inhabitants (www.census.gov). Miami metropolitan region has a well-documented process of habitat alteration associated with the development, including diminished water quality, increased levels of pollutants and nutrients, and increased boat traffic (e.g. Briceño et al., 2011; Lirman et al., 2008; Ng et al., 2021; Serafy et al., 2003). This area has undergone extreme anthropogenic alteration, resulting in reduction of approximately 80% of mangrove forest (Serafy et al., 2003), and in direct impacts on corals (i.e. reducing grow rates, Hudson et al., 1994), fishes (e.g. deformities) and marine mammals (e.g. exposure to contaminants) (Browder et al., 2005; Briceño et al., 2011).

Florida Bay is a high productive inlet of the western Atlantic Ocean (Fig. 2), which supports several marine ecotones dominated by seagrass, mangrove, and coral reef-derived primary production pathways (Vaslet et al., 2012). Florida Bay has high biomass of prey fishes (including planktivorous and low-level predatory fishes like Engraulidae, Blenniodei, Gobiidae and Clupeidae, and mesopredatory fishes from the family Sciaenidae, Thayer and Powell 1999). Due to high levels of productivity, this region supports a robust and diverse predatory community, composed of numerous large-bodied shark species (Tinari and Hammerschlag, 2021). The northern portion of the Bay is protected within Everglades National Park. Everglades National Park and Florida Bay waters are exposed to huge influxes of nutrients from land, including from agricultural runoff (Brand et al., 2010).

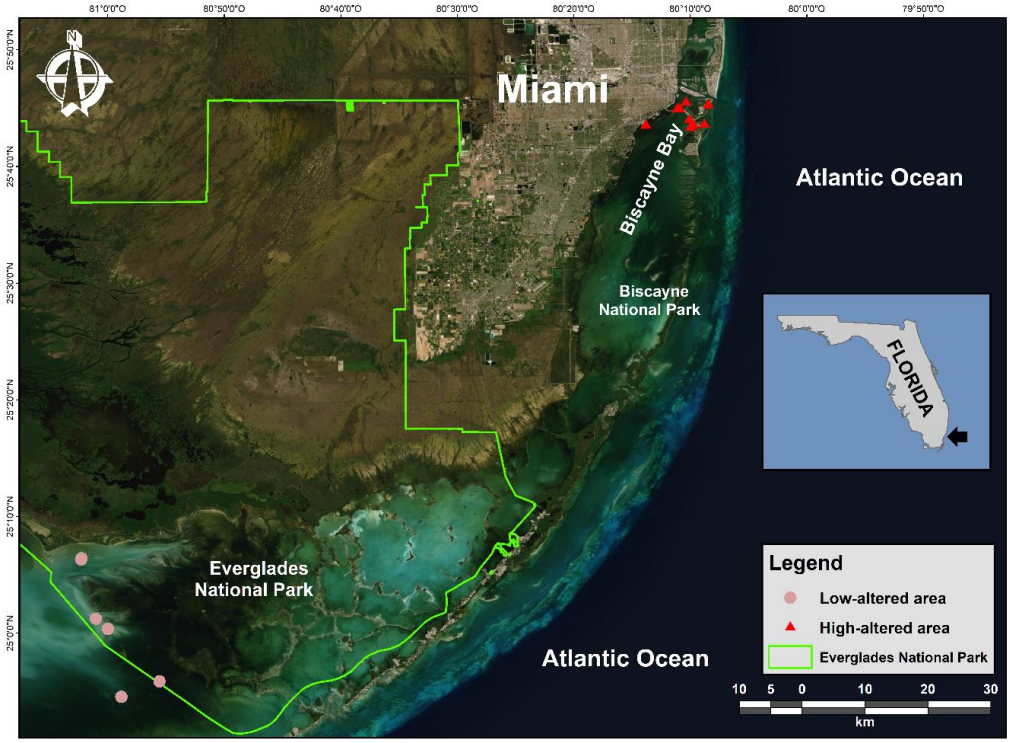

Fig. 2. Sampling locations of blacktip sharks (*Carcharhinus limbatus*) within low urbanized area (n= 32) and high urbanized areas (n= 20).

5.2.2. Capture and sampling

Sampling occurred during wet season (November - Abril) from 2011 to 2018 as part of ongoing coastal shark surveys (see Tinari and Hammerschlag, 2021). All sharks were captured using circle-hook drumlines, a minimally invasive technique that allows the captured sharks to swim (Gallagher et al., 2014). In brief, drumlines were deployed (10 - 40 m deep) to soak for 1 h before being checked for shark presence. On capture, sharks were secured by hand. Once landed, a water pump moving fresh seawater was inserted into the shark's mouth to actively pump water (94.5 liters per minute) over the shark's gills while temporarily immobilized. While sharks were secured, sex was recorded, various length and span measurements were taken, and blood samples were obtained; sharks were then tagged for identification and released (Gallagher et al., 2014). Blood (~10 ml) was collected from the caudal vein. A subset of whole blood was kept for stable isotope analysis, while the remaining blood was immediately centrifuged (3500 rpm, 410 × g) for 2 min to obtain plasma. Resulting plasma was collected. Both whole blood and plasma were stored in an ice slurry on the boat until returning to land where samples were stored in a −80°C freezer.

Procedures and animal husbandry were approved by the University of Miami Institutional Animal Care and Use Committee (Protocol 15-238) and research permits from

Florida Fish and Wildlife Conservation Commission, Biscayne National Park and National Marine Fisheries Service.

5.2.3. Body condition

The body condition was used as a proxy for overall organismal health (Hussey et al., 2009; Irschick and Hammerschlag, 2014). We used the span condition analysis (SCA) developed by Irschick and Hammerschlag (2014) to quantify the body condition of blacktip sharks. For each individual, SCA was calculated on the basis of five morphological measurements, including: (1) precaudal length (PCL, linear distance from the tip of the snout to the insertion of the caudal fin into the body); (2) lateral span (LS, distance spanning from the insertion point of the anterior edge of one pectoral fin to the same point on the other pectoral fin); (3) frontal span (FS, distance spanning from the insertion point of the anterior edge of the dorsal fin to a line oriented parallel to the horizontal plane of the pectoral fin); (4) proximal span (PS; distance spanning from the insertion point of the posterior edge of the dorsal fin to a line oriented parallel to the horizontal plane of the pectoral fin); and (5) caudal keel circumference (CKC, total circumference at the base of the tail as measured at the caudal keel). The body condition was then calculated using the following equation: SCA = (LS+FS+PS+CKC)/PCL.

5.2.4. Stable isotope analysis

$\delta^{15}N$ and $\delta^{13}C$ were analyzed from whole blood samples. This tissue was selected because of its intermediate isotopic turnover rate (as compared to muscle, fin, or skin), and thus reflecting diet over weeks to few months (MacNeil et al., 2005, Kim et al., 2012). Lipid and urea extraction were not undertaken for either tissue based on the recommendation of Kim et al. (2012) for whole blood. Also the mean of C/N ratio of our samples were less than 3.0 (2.15 ± 0.11, n= 83), indicating that there was little interference from the lipid and urea concentrations in the sample. Whole blood samples were freeze dried and ground to a fine powder, and 400 to 600 μg of material was weighed into tin capsules. The stable isotope values of carbon and nitrogen were then determined by combustion of samples by continuous flow-isotope ratio mass spectrometry, using an elemental analyzer (Model 1110, Carlo Erba) interfaced to an isotope ratio mass spectrometer (Finnigan, ThermoQuest; Delta Plus, Finnigan MAT). The isotopic composition of carbon and nitrogen was calculated as $\delta X = [(R\text{sample}/R\text{standard}) - 1] \times 1000$, where R is the molar ratio 13C/12C or 15N/14N in the sample and standard, expressed as delta (δ) per mil (‰). The standards used for nitrogen and

carbon were PDB and atmospheric nitrogen, respectively. Analytical precision was calculated as 0.3 and 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$ values, respectively.

5.2.5. Fatty acid analysis

Fatty acid profile was analyzed in plasma samples. Plasma was used because it has a relatively rapid turnover rate (i.e. days to weeks, Käkelä et al., 2009), and exhibits a high similarity with prey fatty acid profiles, as it functions in transporting fatty acids, e.g. inter-tissue routing of membrane lipids and for metabolic functions, and therefore, (e.g. McMeans et al., 2012, Beckmann et al., 2014, Bierwagen et al., 2019). Plasma fatty acid profile was analyzed by direct transmethylation described by Parrish et al. (2015), using 100 μL of fluid without previous lipid extraction. Briefly, the plasma samples were homogenized and directly transmethylated in 3mL of methanol: dichloromethane: concentrated hydrochloric acid (10:1:1 v/v) solution for 2 h at 80–85 °C. After this process, 1.5 mL of Milli-Q® water and 1.8 mL of hexane and dichloromethane (4:1 v:v) were added and, mixed and centrifuged at 2,000 rpm for 5 min. The upper layer was then removed, transferred to 2 ml-injection vials and reduced under a nitrogen stream (this process was repeated two times). Fatty acid analysis was then carried out in a gas chromatograph Scion 436 equipped with a flame ionizer (FID) and CP 8410 auto-sampler. The capillary column used was CP Wax, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length. Hydrogen was used as a carrier gas at a linear velocity of 1.4 mL/min cm/s. The column was programmed at 170 °C for 1 min, followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The injector and FID temperatures were 250 and 260 °C, respectively. Fatty acids methyl esters (FAME) were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualmix, polyunsaturated fatty acids (PUFAs) fish M, Menhaden Oil, Larodan). The data are presented as % of FAME, based on peak area analyses. Fatty acids that accounted for less than 0.5% were disregarded.

5.2.6. Fatty acid nutritional indicators and trophic markers

The saturated fatty acids (SFA) and the essential fatty acids, including eicosapentaenoic acid (EPA, C20:5n3), docosahexaenoic acid (DHA, C22:6n3) and arachidonic acid (ARA, C20:4n6) were used as indices of shark nutritional quality (Tocher, 2010, Arts and Kohler, 2009). These physiologically important fatty acids have previously been used to distinguish differences in the diet quality of sharks, including those exposed to urbanization (Rangel et

al., 2021a; b). The percentages of ARA, and ARA/EPA and n3/n6 ratios were used to infer physiological responses of eicosanoids, i.e. inflammatory responses (Tocher, 2010). The C16/C18 ratio as an indicator of diatoms and the sum of C16:0 + EPA + DHA was used as an indicator of the presence of dinoflagellates (Léveillé et al., 1997). Originally, the dinoflagellates marker is based on the specific ratio (C16:0 + C18:4n3 + EPA + DHA/C18:3n3 +ΣC16 PUFA) (Léveillé et al., 1997), however, the fatty acids C18:4n3, C18:3n3, and C16 PUFA were absent or in percentages less than 0.5 (which were disregarded in the present study) in the analyzed sharks. The C18:1n9/C18:1n7 ratio was used as degree of carnivory/piscivory (Dalsgaard et al., 2003; El-Sabaawi et al., 2009; Parrish et al., 2015). The C18:2n6 was used as an indicative for terrestrial resources, while ARA values have also been found to be a marker of species inhabiting coastal/benthic environments (Parrish et al., 2000; Sardenne et al., 2017). For the relevant markers in the context of urbanization, the odd chain fatty acids (OFA), branched chain fatty acids (BFA), and C18:1n7 were used as biomarkers of heterotrophic bacteria (Dalsgaard et al., 2003; Kelly and Scheibling, 2012), which increase with decomposition of organic debris (Le Moal et al., 2019). Additionally, the C16:0 and C18:1n9 was used as indicators for domestic sewage (Jardé et al., 2005; Boëchat et al., 2014).

5.2.7. Statistical analysis

Differences in body condition, stable isotopes and plasma fatty acid profile between sampling locations were investigated using Generalized Linear Mixed Models (GLMM) performed with the mgcv package (Wood, 2017). The percentage of each fatty acid was log transformed to meet normality assumptions. Models included the respective biomarker values as the response variables and used Gaussian families of error distribution. Biological variation was also accounted for by including shark total length (TL) as a continuous factor, to control for individual length in the analysis.

To explore for additional differences in plasma fatty acid profile among areas with different degree of urbanization, principal component analysis (PCA) and Permutational multivariate analysis of variance (PERMANOVA) based on a Bray-Curtis distance matrix and Bonferroni-corrected p values was then applied to each area. Statistical significance was declared at $p < 0.05$, and all analyses were performed in the R software (version 4.0.2) and in Past 3.20 (Hammer et al., 2001).

5.3. Results

A total of 52 blacktip sharks (41 females and 11 males) were analyzed in the present study, comprising 32 from Florida Bay/Everglades National Park (i.e., 'non-urban sharks') and 20 individuals sampled from northern Biscayne Bay (i.e., 'urban sharks'). Non-urban sharks measured 146.3 ± 21.2 cm TL (mean ± standard deviation) and urban sharks measured 159.8 ± 11.34 cm TL. Body condition (n= 25) did not vary significantly as a function of shark size (Table 1), however urban sharks were in better body condition (1.46 ± 0.1) than non-urban sharks (1.34 ± 0.1; Table 1, Fig. 3a). In terms of stable isotopes, $\delta^{15}N$ values (n= 52) were higher in urban sharks (12.7 ± 0.7 ‰) than non-urban sharks (12.1 ± 0.6 ‰; Table 1, Fig. 3b), while $\delta^{13}C$ values did not differ significantly between urban (-13.9 ± 0.8 ‰) and non-urban sharks (-14.4 ± 1.0 ‰; Table 1, Fig. 3c).

Blood plasma (n= 23) comprised mainly PUFAs for non-urban sharks, while SFAs were the dominant sum for urban sharks (Supplemental Table S1). While fatty acids did not vary significantly as a function of shark size, the sampling location effected fatty acid variation (Table 2). Urban sharks had higher percentages of C16:0 (Fig. 4a), C18:0 (Fig. 4d), including the ΣSFA (Fig. 5a) and SFA/PUFA ratio (Table 2; Fig. 5b) than non-urban sharks. Urban sharks also had higher values of C22:5n6 (Fig. 4f) and dinoflagellates marker (C16:0 + EPA + DHA) (Fig. 5c) than non-urban sharks. Sharks sampled in low urbanized areas had higher percentages of heterotrophic bacteria biomarkers, including the C17:0 (Fig. 4c), C18:1n7 (Fig. 4e) and BFA-OFA (Table 2; Fig. 5d).

Multivariate analyses also revealed a statistical difference in plasma fatty acid profiles between sampling locations (PERMANOVA, $F= 14.8$; $p < 0.001$). The PCA analyses revealed that the first discriminant function was primarily responsible for discriminating between urban and non-urban sharks (PC1= 34.6%), mainly due to the contribution of C17:0, BFA-OFA, C18:1n7, C18:2n6, and SFA (Supplemental Table S2; Fig. 6).

Table 1. Generalized Linear Model of body condition and stable isotopes of blacktip shark (*Carcharhinus limbatus*) as a function of sampling location (high urbanized, n= 20; low urbanized, n= 32) and total length (TL, cm). Included are the corresponding response variable, coefficient estimate (Est.), standard error (SE), t-value (t), p-value (p) and deviance explained (Dev. Exp.) of each model. Significant ($p < 0.05$) results are **bolded**.

Response	Variable	Est.	SE	t	p	Dev. Exp.
Body condition	Intercept	1.44	0.15	9.97	<0.001	29.9%
	(Location) Urban	0.15	0.05	2.74	**0.012**	
	TL	-0.00	0.00	-0.75	0.462	
Stable isotopes						
δ^{15}N	Intercept	11.85	0.75	15.83	<0.001	20.2%
	(Location) Urban	0.62	0.20	3.05	**0.004**	
	TL	0.00	0.01	0.29	0.776	
δ^{13}C	Intercept	-15.76	1.14	-13.85	<0.001	7.1%
	(Location) Urban	0.28	0.31	0.92	0.362	
	TL	0.01	0.01	1.23	0.224	

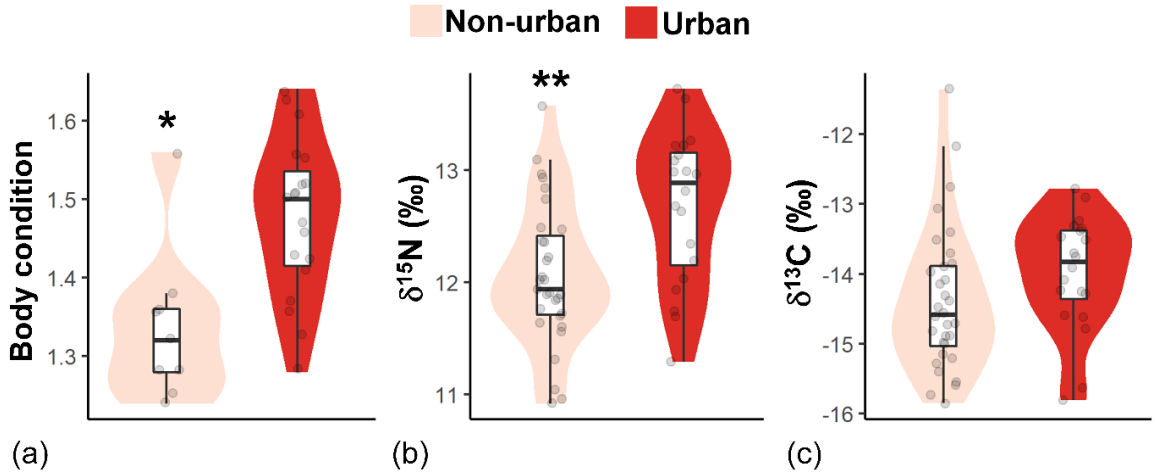

Fig 3. Differences in **(a)** body condition, **(b)** δ^{15}N, and **(c)** δ^{13}C of blacktip sharks (*Carcharhinus limbatus*) between low urbanized area (n= 32) and high urbanized areas (n= 20). Significant differences between urban and non-urban sharks are indicated by asterisks (Generalized Linear Mixed Model *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Table 2. Generalized Linear Model of fatty acid percentages of blacktip shark (*Carcharhinus limbatus*) as a function of sampling location (high urbanized, n= 13 and low urbanized, n=10) and total length (TL), only for those were significant. Included are the corresponding response variable, coefficient estimate (Est.), standard error (SE), t-value (t), p-value (p) and deviance explained (Dev. Exp.) of each model. Significant ($p < 0.05$) results are **bolded**.

Fatty acids	Variable	Est.	SE	t	p	Dev. Exp.
C14:0	Intercept	2.61	1.64	1.59	0.129	23.6%
	(Location) City	0.28	0.12	2.28	**0.036**	
	TL	-0.41	0.33	-1.22	0.241	
C16:0	Intercept	-8.15	16.19	-0.50	0.621	62.5%
	(Location) City	0.32	0.07	4.39	**<0.001**	
	TL	-0.38	0.18	-2.05	0.057	
C17:0	Intercept	386.04	82.94	8.27	<0.001	94.1%
	(Location) City	-0.68	0.20	-3.34	**0.004**	
	TL	0.19	0.42	0.46	0.649	
C18:0	Intercept	-15.70	20.39	-0.77	0.452	58.0%
	(Location) City	0.32	0.09	3.49	0.003	
	TL	-0.17	0.23	-0.72	0.482	
C18:1n7	Intercept	164.30	46.38	3.54	0.003	80.8%
	(Location) City	-0.31	0.11	-2.67	**0.017**	
	TL	0.19	0.24	0.79	0.438	
C22:5n6	Intercept	-0.52	2.16	-0.24	0.814	38.5%
	(Location) City	0.36	0.16	2.19	**0.042**	
	TL	0.23	0.44	0.51	0.614	
SFA	Intercept	-12.47	16.28	-0.77	0.454	70.4%
	(Location) City	0.37	0.07	4.99	**<0.001**	
	TL	-0.33	0.18	-1.81	0.088	
SFA/PUFA ratio	Intercept	1.59	1.54	1.03	0.316	23.7%
	(Location) City	0.27	0.12	2.27	**0.037**	
	TL	-0.36	0.31	-1.14	0.270	
Dinoflagellate marker	Intercept	-5.71	10.17	-0.56	0.581	
	(Location) City	0.19	0.05	4.31	**<0.001**	
	TL	0.12	0.16	-0.99	0.332	
Bacterial marker	Intercept	382.61	85.83	4.46	<0.001	85.3%
	(Location) City	-0.53	0.21	-2.49	**0.024**	
	TL	0.00	0.44	0.01	0.993	

SFA: saturated fatty acids; Bacterial marker: branched-chain fatty acids (BFA); odd-chained fatty acids (OFA); Dinoflagellates makers: EPA + DHA + C16:0 (DHA: docosahexaenoic acid).

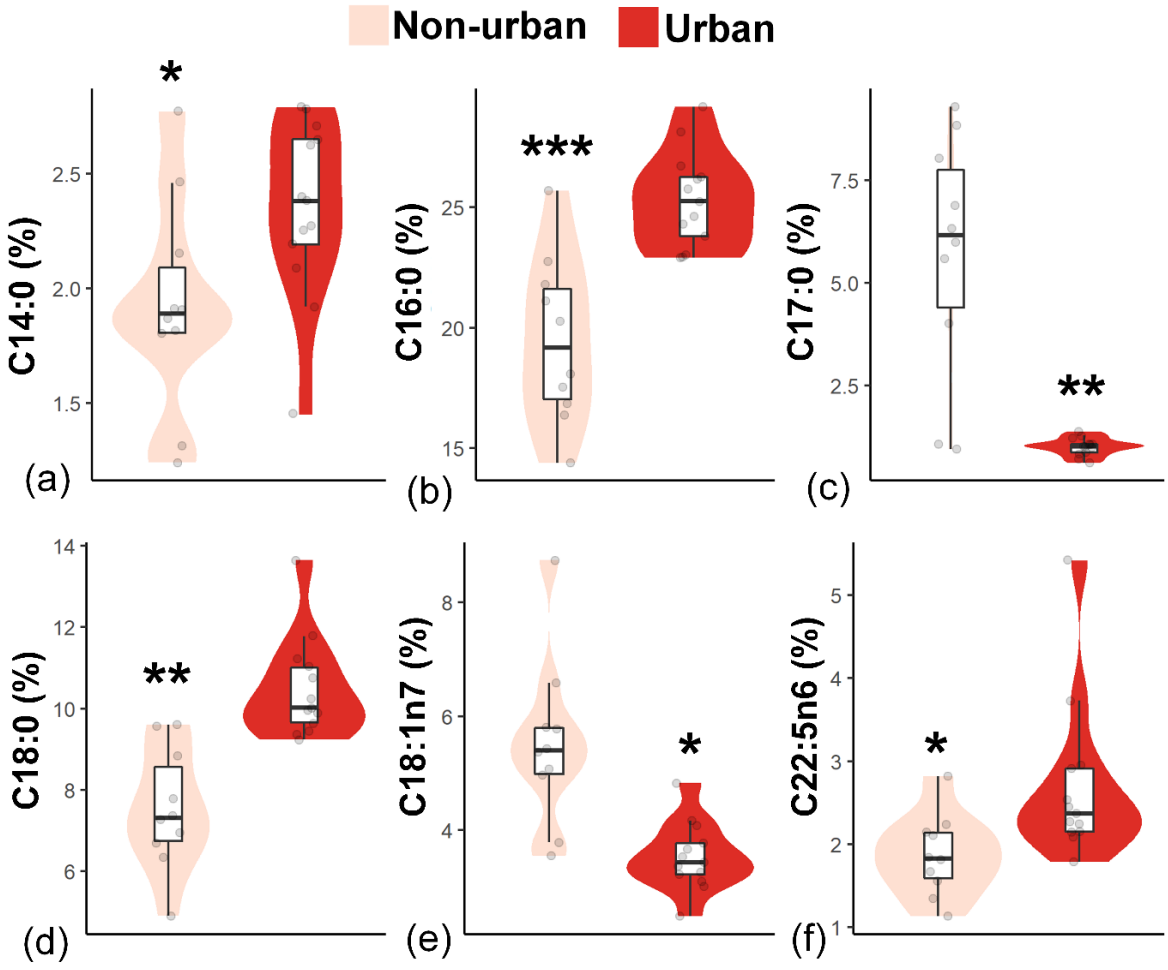

Fig 4. Differences in the fatty acids **(a)** C14:0, **(b)** C16:0, **(c)** 17:0, **(d)** C18:0, **(e)** C18:1n7, **(f)** C22:5n6 of blacktip sharks (*Carcharhinus limbatus*) between low urbanized area (n= 10) and high urbanized areas (n= 13). Significant differences between urban and non-urban sharks are indicated by asterisks (Generalized Linear Mixed Model *p < 0.05; **p < 0.01; ***p < 0.001).

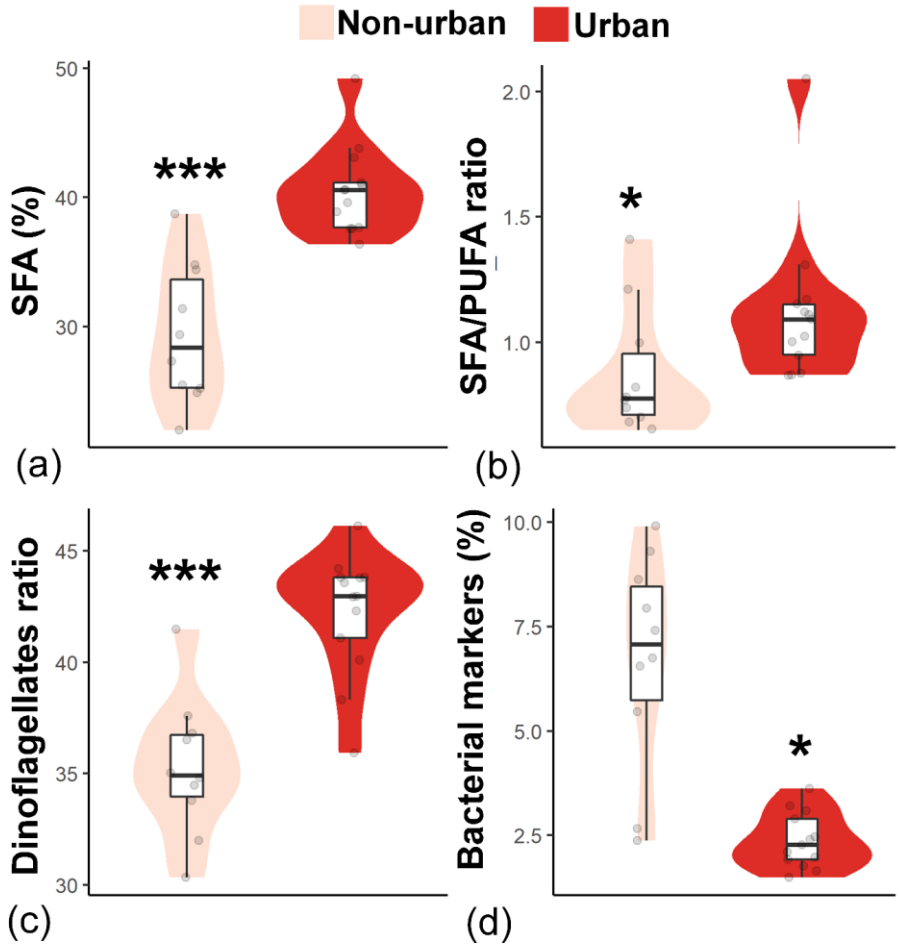

Fig 5. Differences in the fatty acids **(a)** saturated fatty acids (SFA), **(b)** saturated fatty acids/ polyunsaturated fatty acids (SFA/PUFA), **(c)** dinoflagellates marker (C16:0 + EPA + DHA), and **(d)** branched chain and odd chain fatty acid (BFA – OFA) of blacktip sharks (*Carcharhinus limbatus*) between low urbanized area (n= 10) and high urbanized areas (n= 13). Significant differences between urban and non-urban sharks are indicated by asterisks (Generalized Linear Mixed Model *p < 0.05; **p < 0.01; ***p < 0.001).

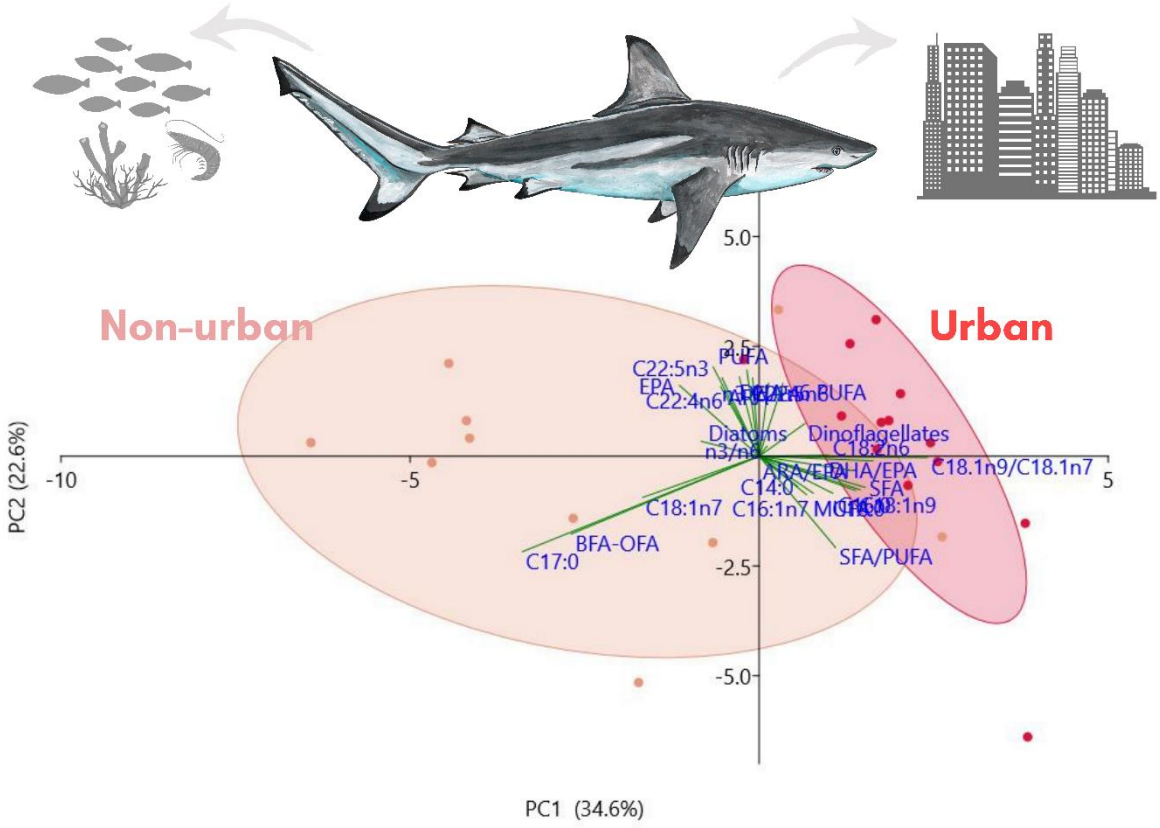

Fig. 6. Principal component (PC) analysis of fatty acid profile of blacktip shark (*Carcharhinus limbatus*) sampled within low urbanized area (n= 10) and high urbanized areas (n= 13). EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid, SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, n3 PUFA: omega 3 PUFA, n6 PUFA: omega 6 PUFA, BFA – OFA: branched chain and odd chain fatty acid, and dinoflagellates marker (C16:0 + EPA + DHA). The 70% ellipses similarly of each site is provided. Illustration of blacktip shark is a courtesy of Alexandre Huber.

5.4. Discussion

In this preliminary investigation, we used morphological and physiological parameters to test the hypothesis that exposure to coastal urbanization negatively effects the nutritional ecology of a highly active marine predator. Our initial results revealed significant spatial variation in body condition as well as short- and medium-term nutritional markers of blacktip sharks sampled between two areas that differ in their exposure to urbanization. As expected, urban sharks exhibited a better body condition and enriched $\delta^{15}N$ values compared to non-urban sharks. The fatty acid data partially corroborated our hypothesis that dietary quality would be lower in urban sharks. As expected, we found higher values of SFA in urban sharks; however, contrary to our initial prediction, we found lower percentages of bacterial markers

(i.e. C18:1n7 and BFA-OFA) and higher values of dinoflagellate markers and the omega-6 fatty acid C22:5n6 in non-urban sharks.

The enriched $\delta^{15}N$ values found in urban sharks could be associated with the contribution of anthropogenic nutrient enrichment caused by both domestic and industrial wastewater (e.g., Mancinelli and Vizzini, 2014; Prado et al., 2020). As a consequence, it is plausible that the nutrient enrichment is being transferred up the food web, from autotrophic organisms to consumers (e.g., Prado et al., 2020). Corroborating the possible influence of domestic and industrial wastewater on nutrients, higher percentages of SFA (C16:0 and C18:0) were found in sharks sampled in high urbanized area. These fatty acids are the main components of domestic sewage, and can be transferred directly or indirectly to consumers (Boëchat et al., 2014; Jardé et al., 2005; Jiménez-Martínez et al., 2019). Similarly, higher percentages of SFA were found in nurse sharks sampled within high urbanized area (Rangel et al., 2021a).

It is also possible that higher proportions of circulating SFA (including C16:0 and C18:0) and C18:1n9 in urban sharks may indicate they are consuming a diet containing an excess fat (Rangel et al., 2021a), and consequently accumulating more fat (e.g. Budge et al., 2006). Indeed, urban sharks had higher values for body condition than conspecifics in low urbanized areas. If body condition is a reasonable proxy for stored fat, our results imply that blacktip sharks sampled in high urbanized areas may are feeding more frequently and/or consuming more prey. Likewise, nurse sharks sampled in high urbanized areas off Miami, Florida, also exhibited higher values for body condition and circulating triglycerides levels (i.e. proxy for energy reserves) than non-urban sharks (Moorhead, 2019). Although blacktip and nurse sharks have distinct lifestyles and no niche overlap, i.e. likely foraging for different prey resources in Biscayne Bay (e.g., Shiffman et al., 2019), the same pattern of higher percentages of SFA and better body condition seem to maintain in both species when exposed to high degree of urbanization. Collectively, these results reinforce the hypothesis that sharks seem to have different feeding frequencies and diet patterns when occupying high altered environments (Moorhead, 2019; Rangel et al., 2021a).

We expected to find higher percentages of bacterial markers in urban sharks (Rangel et al., 2021a). This because increased anthropogenic-induced production of organic materials can cause an increase of bacterial communities associated with organic detritus (Le Moal et al., 2019). However, the fatty acid data suggested a higher contribution of heterotrophic bacterial detrital markers (C18:1n7 and BFA-OFA) in non-urban sharks, which is inconsistent with our initial prediction. One possible explanation could be the high productivity of Florida

Bay waters (e.g., Torres et al., 2006), where freshwater flow and influx of organic sources likely supports an increased heterotrophic bacterial community. It is also possible that sharks within low urbanized area may have a higher dependence of benthic nutrients, such as demersal fish species, with are rich in BFA-OFA (Käkelä et al., 2005; Kelly and Scheibling, 2012). Similarly, bacterial markers have been found in tiger sharks (*Galeocerdo cuvier*) sampled in the Bahamas (low coastal urbanization), attributed to a possible dependence on benthic nutrients (Rangel et al., 2021b). It is also important to consider that Florida Bay has experienced more frequent and persistent blooms of cyanobacteria, mainly due to increases in the flux of agricultural fertilizer, sewage, and animal wastes (e.g., Brand et al., 2010; Butler et al., 1995), which may be contributing to the higher percentages of bacterial markers in sharks in this area. Future studies in this area should consider the influence of agricultural runoff and its impacts on trophic ecology and physiology of sharks.

Also inconsistent with our initial predictions, we found higher values of dinoflagellates marker ratios (i.e., C16:0 + EPA + DHA) in urban sharks. It is likely that the higher percentages of C16:0 found in sharks sampled in highly urbanized areas are influencing this ratio, as neither the EPA nor the DHA differ between locations. Based on $\delta^{13}C$ values, blacktip sharks may rely on both inland mangroves and coastal neritic-derived prey resources in the two locations (e.g. Shiffman et al., 2019; Shipley et al., 2019). It is also worth considering that the study locations are geographically separated by several hundred kilometers, thus on a latitudinal gradient, which could influence the isotopic values.

Finally, while the findings for nurse sharks seem to match the predictions for urban environments, i.e. greater contributions of bacterial and saturated, and less of PUFAs (Rangel et al., 2021a), our study showed some contrasting results from the initial predictions, especially related to bacterial and polyunsaturated fatty acids. This is likely to be a result of their divergent lifestyle (resident, sedentary, and benthic = nurse shark versus transient, active, and epipelagic = blacktip shark), suggesting that the influence of urbanization on the nutritional ecology of sharks can be species-specific and lifestyle-dependent. Being more active, blacktip sharks can quickly move to other less urbanized areas to feed a better diet quality (in terms of essential fatty acids). In addition, blacktip shark feed in the pelagic zone (Castro, 1996; Shiffman et al., 2019), unlike nurse sharks which have a more demersal feeding behavior (Castro, 2000), where a great proliferation of heterotrophic bacteria can occur (Dalsgaard et al., 2003; Kelly and Scheibling, 2012). Therefore, it is possible that feeding behavior has a strong influence on the quality of food consumed by urban sharks, placing sharks with benthic feeding behavior as the most exposed to urban impacts.

While preliminary, this is the first study that integrated biochemical tracers of stable isotopes and fatty acids along with body condition to investigate the relationship between physiological condition and spatial variation in blacktip sharks. Although we used a non-lethal approach that has been successful used in ecophysiological studies with sharks (e.g. Moorhead et al., 2020; Rangel et al., 2021a,b; Shiffman et al., 2019), our study has some limitations. This includes the fact that we do not know if sharks are actually residents or transient in the study area, i.e. their residency patterns in South Florida remain unknown. Also, sharks were opportunistically sampled, in different years and months. Due to sample size limitations, we were not able to test possible seasonal and sex influences on nutritional condition, which should be considered in future studies. Additionally, given that plasma only reflects the individual's momentary metabolic state, plasma fatty acid results need to be interpreted with caution. Despite this, we emphasize that plasma fatty acids have been extensively demonstrated as a promising method to assess short-term shifts in shark diet (e.g. McMeans et al., 2012; Bierwagen et al., 2019; Rangel et al., 2020; 2021c) and in the context of urbanization (e.g. Andersson et al., 2015; Isaksson, 2015; Rangel et al., 2021a,b; Toledo et al., 2016). Finally, other nutritional markers (e.g., plasma triglycerides, cholesterol, amino acids, etc.) could help to elucidate the difference in nutritional status among areas with different degrees of urbanization.

5.5. Conclusion

Though using both short- and medium-term nutritional indicators our initial findings revealed a significant spatial variation in nutritional ecology of blacktip sharks along a gradient of coastal urbanization. Contrary to our hypothesis, we did not detect a reduction in diet quality (in terms of essential fatty acids), as found in the nurse shark (Rangel et al., 2021a). However, the higher percentages of SFA, SFA/PUFA ratio, and body condition found in both blacktip and nurse shark exposed to waters off Miami suggests that, at least in part, both species are being similarly influenced by urbanization. Collectively, these results reveal a possible alteration in the amount of food consumed by sharks and/or in the caloric value of their prey (i.e., amount of fat), indicating sharks possibly increase their foraging efficiency either by finding a greater prey abundance and/or fewer competitors in areas exposed to urbanization. Future studies should monitor the nutritional quality of both prey and predators, especially because increased plasma SFA and SFA/PUFA ratio can compromise different physiological processes, including cardiovascular tone, inflammatory response, reproduction, and renal and neural function (e.g. Berry, 2009; Tocher, 2010). Although preliminary, our

study expands our limited knowledge of the impact of urbanization on the dietary patterns and nutritional condition of marine predators.

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Supporting Information

Table S1- Plasma fatty acid profiles of blacktip shark (*Carcharhinus limbatus*) sampled within low urbanized area n= 10) and high urbanized areas (n = 13).

Fatty acids	Non-urban	Urban
C17:0	5.7 ± 2.9	1 ± 0.2
C14:0	1.9 ± 0.5	2.3 ± 0.4
C16:0	19.5 ± 3.4	25.3 ± 2
C18:0	7.5 ± 1.5	10.5 ± 1.2
C16:1n7	3.4 ± 1.8	2.7 ± 0.6
C18:1n9	10.3 ± 4.2	11.7 ± 1.2
C18:1n7	5.5 ± 1.5	3.5 ± 0.6
C18:2n6	1.8 ± 1.2	2.6 ± 0.5
C20:5n3 (EPA)	4.5 ± 1.3	3.3 ± 0.9
C22:5n3	3.5 ± 0.8	2.7 ± 0.7
C22:6n3 (DHA)	11.3 ± 2.7	13.6 ± 2.6
C20:4n6 (ARA)	8.4 ± 1.5	9.1 ± 1.2
C22:4n6	3.3 ± 0.9	3.1 ± 0.8
C22:5n6	1.9 ± 0.5	2.7 ± 1.0
SFA	29.3 ± 5.4	40.5 ± 3.4
MUFA	19.5 ± 4.6	19.7 ± 1.7
PUFA	34.4 ± 5.2	37.4 ± 4.9
n3 PUFA	20.0 ± 3.2	19.7 ± 3.9
n6 PUFA	15.3 ± 2.7	17.6 ± 2.8
n3/n6	1.3 ± 0.2	1.1 ± 0.3
DHA/EPA	2.7 ± 1.1	4.2 ± 0.6
ARA/EPA	2.0 ± 0.6	2.9 ± 0.9
SFA/PUFA	0.9 ± 0.3	1.1 ± 0.3
Diatoms marker	0.9 ± 0.1	1.0 ± 0.1
Dinoflagellates marker	35.3 ± 3.1	42.2 ± 2.7
BFA-OFA	6.7 ± 2.6	2.4 ± 0.7
C18.1n9/C18.1n7	2.1 ± 1.2	3.4 ± 0.6

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid, SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, n3 PUFA: omega 3 PUFA, n6 PUFA: omega 6 PUFA; bacterial marker: branched chain and odd chain fatty acid; diatoms (C16/C18); Carnivory/piscivory (C18:1n9/C18:1n7); dinoflagellates marker (C16:0 + EPA + DHA).

Table S2- Principal component (PC) results for the 2 component axis of fatty acid profile of blacktip shark (*Carcharhinus limbatus*) sampled within low urbanized area (n= 10) and high urbanized areas (n = 13). **Bold** values indicate primary fatty acids contribution to dissimilarity.

Fatty acids	**PC 1**	**PC 2**
C17:0	**-0.50**	**-0.32**
C14:0	0.08	-0.10
C16:0	0.20	-0.11
C18:0	0.15	-0.12
C16:1n7	0.10	-0.13
C18:1n9	0.21	-0.11
C18:1n7	**-0.24**	-0.14
C18:2n6	**0.24**	-0.02
C20:5n3 (EPA)	-0.17	0.24
C22:5n3	-0.10	**0.30**
C22:6n3 (DHA)	-0.04	0.26
C20:4n6 (ARA)	0.02	0.24
C22:4n6	-0.08	0.23
C22:5n6	-0.01	0.26
BFA-OFA	**-0.40**	-0.26
SFA	**0.22**	-0.10
MUFA	0.11	-0.12
PUFA	-0.03	**0.29**
n3 PUFA	-0.08	0.26
n6 PUFA	0.05	0.24
n3/n6	-0.12	0.05
DHA/EPA	0.15	0.00
ARA/EPA	0.20	-0.05
SFA/PUFA	0.16	**-0.31**
Diatoms marker	0.02	0.03
Dinoflagellates marker	0.10	0.11
C18.1n9/C18.1n7	**0.35**	0.00

Capítulo 6

Metabolic and nutritional condition of juvenile tiger sharks exposed to regional differences in coastal urbanization

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Abstract

How varying levels of human activity, such as proximity and size of the nearest market (i.e., market gravity), influence the nutritional ecology and physiological condition of highly migratory marine predators is poorly understood. In the present study, we used a non-lethal approach to compare the concentration of metabolic hormones (i.e. corticosteroids and thyroid hormones) and plasma fatty acids between juvenile female tiger sharks (*Galeocerdo cuvier*) sampled in two areas of the subtropical north Atlantic, which differed markedly in their levels of coastal urbanization, Florida and the Bahamas (high *versus* low, respectively). We hypothesized that juvenile female tiger sharks sampled in water surrounding high coastal urbanization (Florida), would exhibit evidence of lower prey quality and higher energetic demands as compared to individuals sampled in relatively less urbanized areas of Northern Bahamas. Results revealed that relative corticosteroid levels (a proxy for energy mobilization) were higher in juvenile female tiger sharks sampled in Florida; however, no differences were found in concentrations of thyroid hormones (proxies of energetic adjustments) between the two locations. We found higher percentages of omega-3 polyunsaturated fatty acids (indicative of high prey quality) in juvenile tiger sharks from Florida, whereas higher percentages of bacterial markers (often indicative of domestic sewage effluent) were detected in the individuals sampled in the Bahamas. Taken together, these findings do not suggest that the differences in nutritional quality and metabolic condition found between the two sampling locations can be fully attributed to foraging in areas exposed to differing levels of urbanization. We speculate that these patterns may be due to the highly migratory nature and generalist feeding strategy of this species, even at the juvenile life stage, as well as proximity of sampling locations from shore.

Keyword: *Galeocerdo cuvier,* urban ecology, marine predator, fatty acids, corticosteroids, thyroid hormones, nutritional ecology, market gravity

6.1. Introduction

Assessing the nutritional ecology of predators at relevant spatial scales is critical for understanding patterns of habitat use and their influence on food web structure and nutrient cycling (e.g. Estes et al., 2016; Hammerschlag et al., 2019). For instance, regional variation in dietary patterns of predators can be driven by ontogenetic variations (Aines et al., 2017; Dicken et al. 2017), food availability, prey preferences (Acuña-Marrero et al., 2017; Salinas-de-León et al., 2019), intra- and interspecific interactions (Every et al., 2018), as well as by urbanization (Rangel et al. 2021a) and tourism provisioning (Semeniuk et al., 2009; Meyer et al., 2019). Such knowledge is particularly relevant for highly migratory predators, such as large sharks, due to their wide areas of space use and high energetic requirements (e.g. Estes et al., 2016; Hammerschlag et al., 2019).

The tiger shark (*Galeocerdo cuvier*) is a large-bodied generalist predator (growing up to 5.5 m in length), which exhibits considerable variability in habitat use and movements patterns, making this species an interesting model for investigating the effects of spatial variation on marine predator nutritional ecology (Hammerschlag et al. 2012; Papastamatiou et al. 2013; Lea et al. 2015; 2018; Ajemian et al. 2020). Tiger sharks exploit a wide variety of prey, including invertebrates, teleosts, elasmobranchs, reptiles, seabirds and marine mammals, with prey diversity and size expanding with ontogeny (e.g. Aines et al., 2017; Dicken et al. 2017). Recent studies showed that despite being an opportunistic forager, their dietary patterns, nutritional and metabolic condition are life stage-dependent, suggesting female tiger shark can adjust their nutritional and metabolic requirement during reproduction (Hammerschlag et al., 2018; Rangel et al., 2021b).

At younger life stages, tiger sharks tend to occupy and forage more in coastal inshore waters, expanding their range to offshore waters as adults (e.g. Lea et al. 2018; Ajemian et al. 2020). While nearshore waters can offer abundant food and provide shelter from predators (e.g. Heupel et al., 2007), juvenile sharks using these environments are exposed to numerous anthropogenic disturbances associated with coastal development and urban sprawl, including habitat loss, fishing and pollution (Knip et al., 2010). Moreover, primary productivity is often dramatically modified in inshore areas exposed to such urbanization, thus altering food availability and quality for marine predators through bottom up processes (Faeth et al., 2005; El-Sabaawi, 2019). Indeed, numerous studies have demonstrated that size and proximity of the nearest market (i.e., market gravity) has a strong negative effect on the abundances of coral reef fishes, including sharks and their prey (Cinner et al., 2018, Valdivia et al., 2017,

Ruppert et al. 2018). Since energetic status can affect growth, survival and future reproductive performance in wild vertebrates (e.g. Birnie-Gauvin et al., 2017), a better understanding of how impacts from coastal urbanization and market gravity may affect the physiological condition of early life stages can help in prioritizing critical habitats and establishing appropriate mitigation efforts.

Here, we used a combination of physiological markers to test if juvenile tiger sharks sampled in neighboring regions of differing exposure to coastal development and human activity (i.e., South Florida (USA) versus the Bahamas) would exhibit associated variations in metabolic and nutritional condition. Because coastal urbanization can directly or indirectly reduce food availability (e.g. Faeth et al., 2005; El-Sabaawi, 2019), we hypothesized that juvenile tiger sharks sampled in a region exposed to high levels of urbanization and greater market gravity (South Florida), would exhibit a higher energetic demand and lower nutritional quality compared to tiger sharks sampled in relatively more pristine waters (the northern Bahamas, Fig. 1).

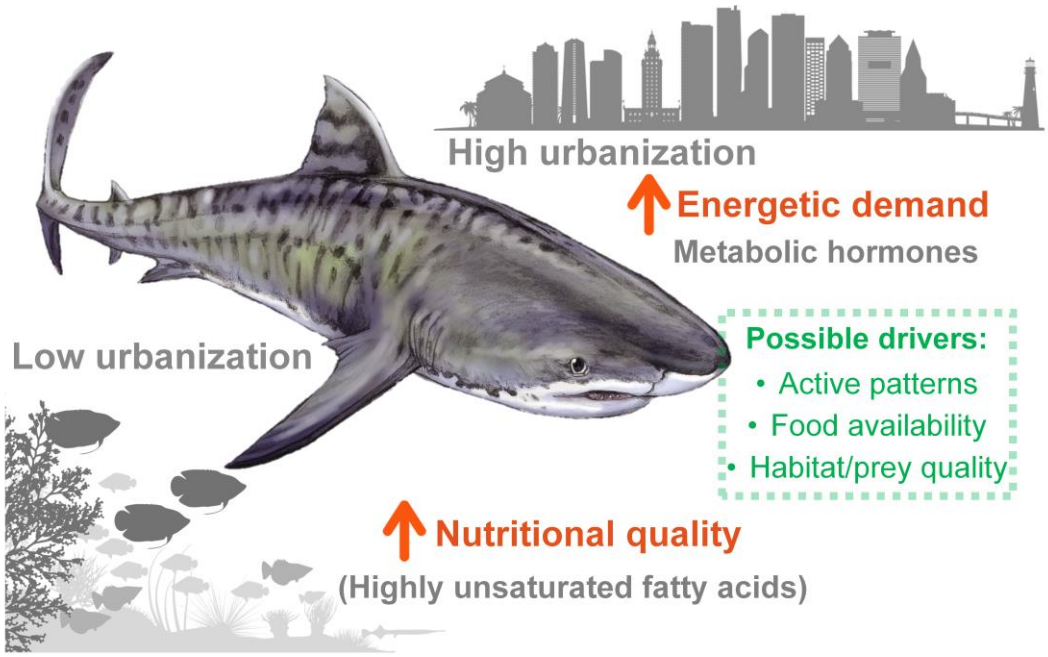

Figure 1. Conceptual illustration of expected differences in energetic demand and nutritional quality of juvenile female tiger sharks (*Galeocerdo cuvier*) sampled in waters adjacent to high levels of coastal urbanization (Florida, USA) versus from neighboring locations of low levels of coastal development (the Northern Bahamas). We hypothesized that juvenile tiger sharks sampled off Florida would have higher energetic demands (as measured by higher concentration of metabolic hormones), and poorer nutritional quality (as measured by higher percentages of bacterial and saturated fatty acids, and lower overall proportions of omega 3 and 6 highly unsaturated fatty acids). Illustration of tiger shark is a courtesy of Kelly Quinn. City and coral reef and fishes from Canva (www.canva.com).

To test this hypothesis, we compared metabolic hormones (i.e. relative corticosteroids and thyroid hormones) and fatty acid dietary markers in juvenile tiger sharks sampled in South Florida and the northern Bahamas. The relative corticosteroid concentrations were used as a proxy for energy mobilization. Glucocorticoid concentrations fluctuate according to energetic demands, increasing in response to anticipated or perceived environmental changes (Romero, 2002; McEwen and Wingfield, 2003), for example increasing during long-term food deprivation (e.g. Lynn et al., 2003; Iki et al., 2020). The thyroid hormones (thyroxine [T_4] and triiodothyronine [T_3]) are important mediators in the regulation of development and metabolic rate in vertebrates, and therefore, are attractive biomarkers to investigate energetic adjustments (e.g. Norris and Carr 2013; Behringer et al. 2018). Based on our hypothesis, we expected that juvenile tiger sharks occupying water adjacent to high levels of coastal urbanization would exhibit higher concentrations of these metabolic hormones, compared to individuals sampled in waters adjacent to low levels of coastal urbanization.

Plasma fatty acid profiles were used as short-term dietary markers to make inferences about prey quality (McMeans et al. 2012; Beckmann et al. 2014; Rangel et al. 2020; 2021a), and basal food chain dependencies (e.g., bacteria, diatoms, dinoflagellates; Dalsgaard et al., 2003). Because fatty acids are transferred with little modification from prey to predator, they are especially relevant biomarkers to study diet patterns and nutritional shifts in the urbanization context (Budge et al., 2006; Iverson, 2009; Gomes et al., 2016). Moreover, consumers are unable to produce *de novo* omega-3 and -6 polyunsaturated fatty acids (n3 and n6 PUFAs) and limited in their ability in converting them to highly unsaturated fatty acids. Therefore, the consumers rely on the diet to obtain PUFAs, such as docosahexaenoic acid (DHA, C22:6n3), arachidonic acid (ARA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3) (Darlsgaard et al., 2003; Budge et al., 2006). Because these PUFAs have crucial functions in a variety of physiological processes, an inadequate dietary intake of PUFAs can compromise the individual's health and survival (Izquierdo et al. 2001; Tocher 2010; Birnie-Gauvin et al., 2017). Based on our hypothesis, we predicted that juvenile tiger sharks exposed to greater market gravity would have higher proportions of saturated (SFA) and bacterial fatty acids, as these biomarkers are often highly correlated with domestic sewage effluent (e.g. Boëchat et al., 2014; Jiménez-Martínez et al., 2019). We also anticipated that juvenile tiger sharks sampled from more urbanized coastlines would exhibit a lower overall proportion of n3 and n6 PUFAs compared to those from the Bahamas, due to reduced production of PUFAs in the base of the food web in South Florida (e.g. Gladyshev et al., 2012).

6.2. Material and Methods

6.2.1. Sampling period and study sites

Juvenile female tiger shark were sampled in two areas of the subtropical north Atlantic, which differed markedly with respect to their levels of coastal urbanization: (i) a region relatively pristine (the northern Bahamas); and (ii) in a region exposed to high levels of urbanization (South Florida, USA; Fig. 2). In South Florida, blood samples were collected off Miami and Biscayne Bay, and inside Florida state waters within Everglades National Park (Fig. 2), in September 2013, November 2013, December 2013, July 2014, September 2014, October 2016, October 2017, and May through November 2018. In the northern Bahamas, blood samples were collected in December 2011, July 2012, October 2013, May 2014, November 2014, April 2018 and January 2019, from tiger sharks sampled at northwestern edge of little Bahama Bank, off the west end of Grand Bahama Island, Bahamas (Fig. 2).

Grand Bahama is exposed to low urbanization, comprising a human population of 51,368 inhabitants (Palgrave Macmillan, 2016). Grand Bahama is the northernmost of the islands of the Bahamas, lying approximately 180 km off study site, South Florida. The environment is a shallow (average 5 m deep), and mostly homogenous sand flat, with irregular seagrass patches and small patches of coral. The study site in South Florida is exposed to high urbanization. Miami-Dade is the seventh most populous county of the United States, comprising a population of more than 2.7 million inhabitants (www.census.gov). Some proportions of South Florida coastline have undergone extreme anthropogenic alteration, resulting in reduction of approximately 80% of mangrove forest (Serafy et al., 2003), with direct impacts on fishes (e.g. deformities) and marine mammals (e.g. exposure to contaminants) (Browder et al., 2005).

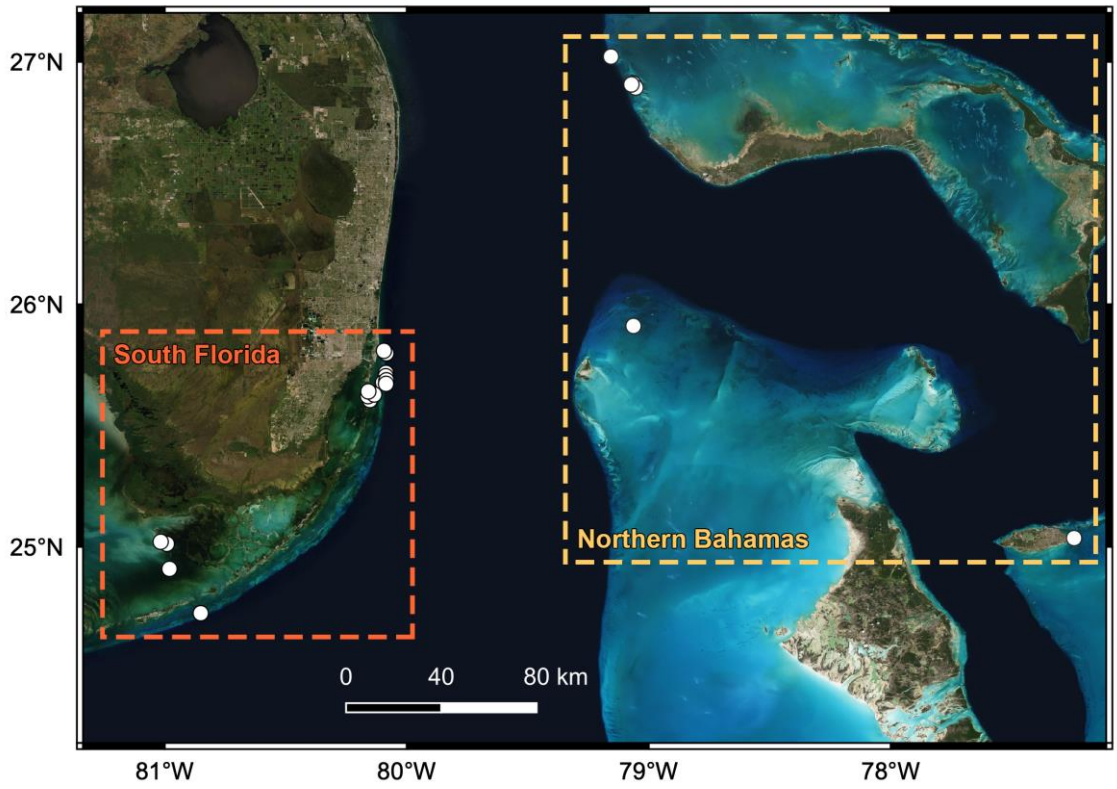

Figure 2. Map of the study area showing capture locations of tiger sharks between 2011 and 2019 by sampling locations (Bahamas/Florida). Satellite map reveals stark differences in coastal development between Florida and the Bahamas.

6.2.2. Capture and sampling

Sharks were captured using the circle-hook drumlines (details in Gallagher et al. 2014). In brief, drumlines were deployed (10 - 40 m deep) to soak for 1 h before checked for shark presence. Upon capture, sharks were brought to a partially submerged platform, where they were temporarily immobilized. A water pump was then inserted into the shark's mouth for oxygenation, and morphological measurements were taken (total length -TL, cm), sex was identified (based on the presence/absence of copulatory organs - claspers), and blood samples were obtained. After all procedures, sharks were tagged and released. Phlebotomy (~20 ml) was conducted from the caudal vein using 18-gauge needles, 10-ml heparinized syringes and immediately centrifuged (3500 rpm, 410 × g) for 2 min. Plasma was then removed, placed within a cooler on the boat and then stored frozen at −20°C for future analyses.

Procedures were approved by the University of Miami Institutional Animal Care and Use Committee (Protocol 15-238) and research permits from Florida Fish and Wildlife Conservation Commission, Biscayne National Park, the National Marine Fisheries Service, Florida Keys National Marine Sanctuary, and the Bahamas Department of Fisheries.

6.2.3. Physiological analysis

Commercially available enzyme immunoassay (microplate spectrophotometer ELISA) kits were used to quantify T_3 (K050-H1) and T_4 (K056-H1) (Arbor Assays, Ann Arbor, MI, USA) with colorimetric enzymatic reaction using a spectrophotometer ELISA (SpectraMax 250, Molecular Devices). To obtain a relative measurement of corticosteroids in the plasma, we used the corticosterone analysis kit (Cayman Chemical, Ann Arbor, MI, item # 500655). The dilution selected was 1:5 (diluted with Cayman Assays assay buffer). This corticosterone kit has been previous validated to quantify relative 1α-hydroxycorticosterone (1α-OH-B, the primary corticosteroid in elasmobranchs), by exploiting the cross-reactivity of the corticosterone antibody with 1α-OH-B concentrations (Evans et al., 2010) and excluding other corticosteroids using mass spectrometry (Lyons et al., 2019). However, as this approach is expectedly unprecise for determining concentrations of 1α-OH-B, and since we did not identify other corticosteroids (e.g. cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone) we assumed that corticosterone ELISA would be reflective of relative corticosteroid following Lyons et al. (2019). Therefore, the results are referred to as relative corticosteroid concentrations. Samples were run in duplicate. The assay kit was validated by testing different dilutions of samples. For corticosterone assay the dilution selected was 1:5 (diluted with Cayman Assays assay buffer). The mean intra-assay coefficient of variation were 11% for corticosteroids, 17% for T_3 and 16.5% for T_4.

Fatty acid profiles were analyzed in plasma (100 μL) by direct transmethylation, without lipid extraction, as described by Parrish et al. (2015a). Briefly, 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 this, 1.5 ml of Milli-Q® water and 1.8 ml of hexane and dichloromethane (4:1 v:v) were added, and then the tubes were mixed and centrifuged at 2,000 rpm for 5 min. The upper layer was removed, transferred to 2 ml injection vials and the volume reduced under a nitrogen stream. Fatty acid analysis was carried out in a gas chromatograph (Varian, Scion 436) coupled with a flame ionization detector (FID) and an auto-sampler (CP 8410). Hydrogen was used as a carrier gas at a linear velocity of 1.4 ml per minute, and the capillary column used was CP Wax, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length. The column was programmed at 170 °C for 1 min, followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The injector and FID temperatures were 250 and 260 °C, respectively. Fatty acid methyl esters were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualmix,

PUFA fish M, Menhaden Oil, Larodan). The data were presented as % of total fatty acid methyl-esters based on peak area analyses. The main specific fatty acids, sums and ratios used in the present study were based on available literature data (Table 1).

6.2.4. Statistical analysis

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; Sulikowski et al., 2016) to distinguish immature from adult females. Differences in metabolic hormone and fatty acid concentrations between sampling locations were investigated using Generalized Linear Mixed Models (GLMM) performed with the mgcv package (Wood, 2017). Models included the respective biomarker concentrations as the response variables and used Gaussian families of error distribution. Biological variation was also accounted for by including shark TL as a continuous factor, to control for individual length in the analysis. To account for the effects of sampling over multiple years the variable year was included as a random effect. Possible difference in shark TL between the two locations was tested using Student's *t*-test for independent samples and normally distributed data. All analyses were performed in the R software (version 4.0.2) and the level of statistical significance set at 0.05.

Table 1. Individual fatty acids and fatty acid sums and ratios used as biomarkers for different food resources.

Source	Fatty acid biomarkers	Reference
Seagrass and terrestrial vegetal	C18:2n6, C18:3n3, ARA	Dalsgaard et al. 2003;
Diatoms	EPA, C16:1n7, C18:1n7	Kelly and Scheibling, 2012
Dinoflagellates	DHA	Dalsgaard et al. 2003, Parrish et al. 2015b
Zooplankton	C20:1, C22:1	Parrish et al., 2000
Heterotrophic bacteria	BFA-OFA	Dalsgaard et al. 2003;
	↑ΣSFA, C16:1n7, C18:1n7	Kelly and Scheibling, 2012
Cyanobacteria	↑ C18 PUFA ↓ ΣHUFA	Muller-Navarra et al., 2004
Urban discharge	↑ C16:0 ↑C18:1n9	Jardé et al., 2005; Boëchat et al., 2014
Nutritional quality	↑ ΣHUFA, Σn3/Σn6	Tocher, 2003; Arts and Kohler, 2009

SFA: saturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids (i.e. EPA, DHA and ARA), EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, ARA: arachidonic acid, BFA: branched-chain fatty acids, OFA: odd-chained fatty acids.

6.3. Results

A total of 34 juvenile female tiger sharks were analyzed in the present study, comprising 17 sampled in the Bahamas and 17 sampled in South Florida waters. Sharks were larger in the Bahamas (258.9 ± 34.6 cm TL) than South Florida (200.7 ± 48.9 cm TL; t = 4.02, df = 32, p = <0.001).

Among all metabolic hormones compared across tiger shark size distribution and sampling locations, only the relative corticosteroids exhibited significant differences (Table 2). While the relative corticosteroid concentration did not vary significantly as a function of shark size (Table 2) the individuals sampled in South Florida had significant higher concentrations of this hormone (788.8 ± 709.3 pg ml^{-1}) than in the Bahamas (257.2 ± 204.7 pg ml^{-1}) (deviance explained = 29.0%) (Fig. 3). The thyroid hormones did not differ between sharks sampled in South Florida (T_3 = 965.7 ± 327.9 pg ml^{-1}; T_4 = 950.1 ± 442.1 pg ml^{-1}) versus the Bahamas (T_3 = 1001.7 ± 624.9 pg ml^{-1}; T_4 = 1190.9 ± 1162.2 pg ml^{-1}), and similarly with T_3/T_4 ratio in sharks sampled in South Florida (1.2 ± 0.4) versus the Bahamas (1.2 ± 1.1) (Fig. 3). Both T_3 and T_4, and T_3/T_4 ratio did vary significantly as a function of shark size (Supplemental Table S1).

Blood plasma comprised mainly SFAs, C16:0 and C18:0, for both Bahamas and South Florida sharks, followed by PUFAs, mainly DHA and ARA, in South Florida sharks, and monounsaturated fatty acids (MUFAs, mainly C18:1n9) in sharks sampled in the Bahamas

(Supplemental Table S2). The proportions of the terrestrial marker C18:2n6, n3 PUFA and the bacterial marker BFA-OFA were found to be constant in relation to TL, but to vary significantly as a function of sampling location (Table 2, Fig. 4 and 5). Tiger sharks caught in South Florida had higher C18:2n6 (deviance explained = 15.1%, Fig. 4) and n3 PUFA (deviance explained = 26.1%), and lower BFA-OFA (deviance explained = 19.5%) proportions than individuals sampled in Bahamas (Fig. 5). While proportions of C18:0 and C18:1n9 did not vary as a function of sampling location, they were found to be significantly influenced by tiger shark size (Table 2, Fig. 6). Both C18:0 (deviance explained = 22.3%) and C18:1n9 (deviance explained = 15.8%) were found in higher proportions in sharks smaller than 225 cm TL (Fig. 6).

Table 2. Generalized Linear Mixed Model of metabolic hormone concentrations and fatty acid percentages of female tiger shark (*Galeocerdo cuvier*) as a function of total length (TL) and sampling location (n= 17 -Bahamas-, n= 17 –Florida-), only for those were significant. Included are the corresponding biomarker type (Marker), response variable (biomarker), coefficient estimate (Est.), standard error (SE), t-value (t) and p-value (p) of each model. **Bold**: significant values ($p<0.05$).

Marker	Response	Variable	Est.	SE	t	p
Hormone	Corticosterone	TL	0.32	2.26	0.14	0.887
		(Location) Florida	**549.76**	**243.38**	**2.26**	**0.038**
Fatty acids	C18:0	TL	**0.01**	**0.05**	**2.19**	**0.036**
		(Location) Florida	-0.20	0.53	-0.39	0.702
	C18:1n9	TL	**0.02**	**0.01**	**2.06**	**0.048**
		(Location) Florida	0.16	0.90	0.17	0.863
	C18:2n6	TL	0.07	0.06	1.19	0.241
		(Location) Florida	**1.26**	**0.61**	**2.04**	**0.049**
	n3 PUFA	TL	-0.02	0.02	-0.90	0.373
		(Location) Florida	**3.75**	**1.81**	**2.07**	**0.047**
	BFA-OFA	TL	-0.12	0.05	-0.25	0.803
		(Location) Florida	**-1.01**	**0.47**	**-2.17**	**0.038**

n3 PUFA: omega 3 polyunsaturated fatty acid, BFA-OFA: branched chain and odd chain fatty acid.

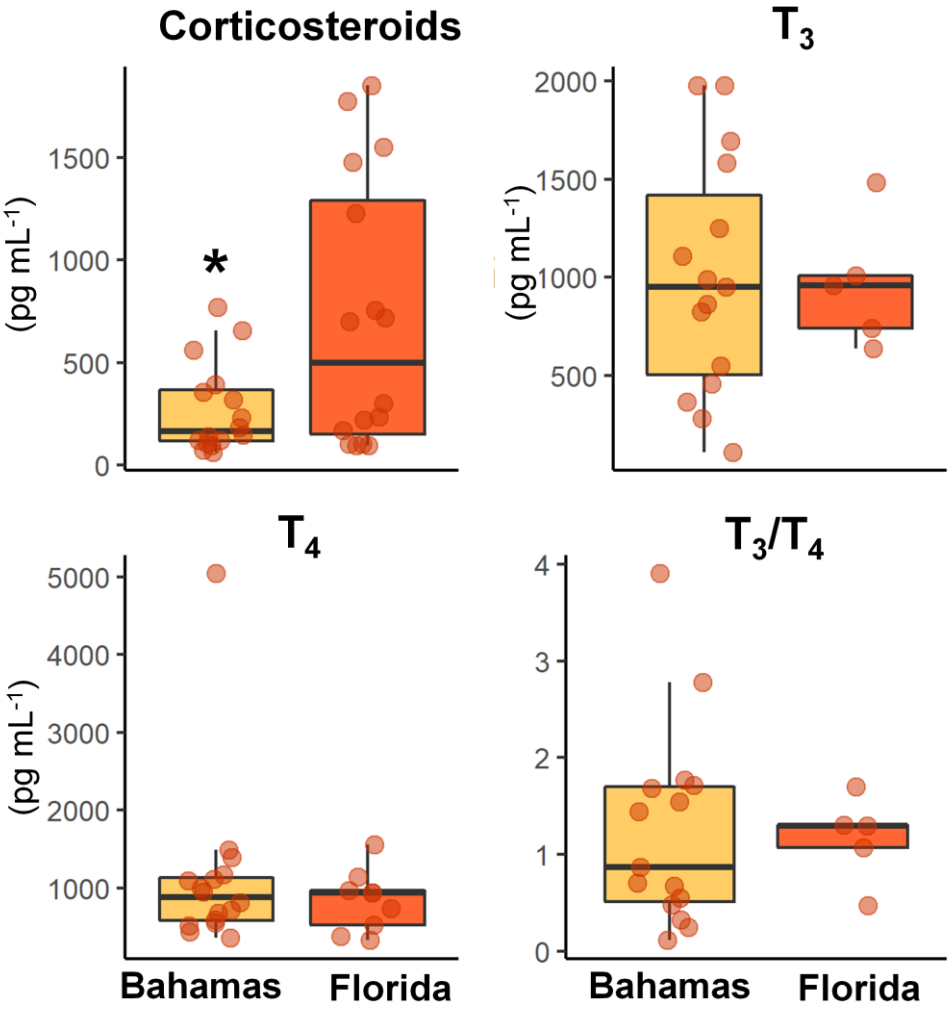

Figure 3. Differences in metabolic hormone concentrations between Bahamas and Florida sampling locations. Black line indicates the median value and black points indicate the outliers. (*) Significant differences found with the Generalized Linear Mixed Model. Legend: T_4 (thyroxine) and T_3 (triiodothyronine).

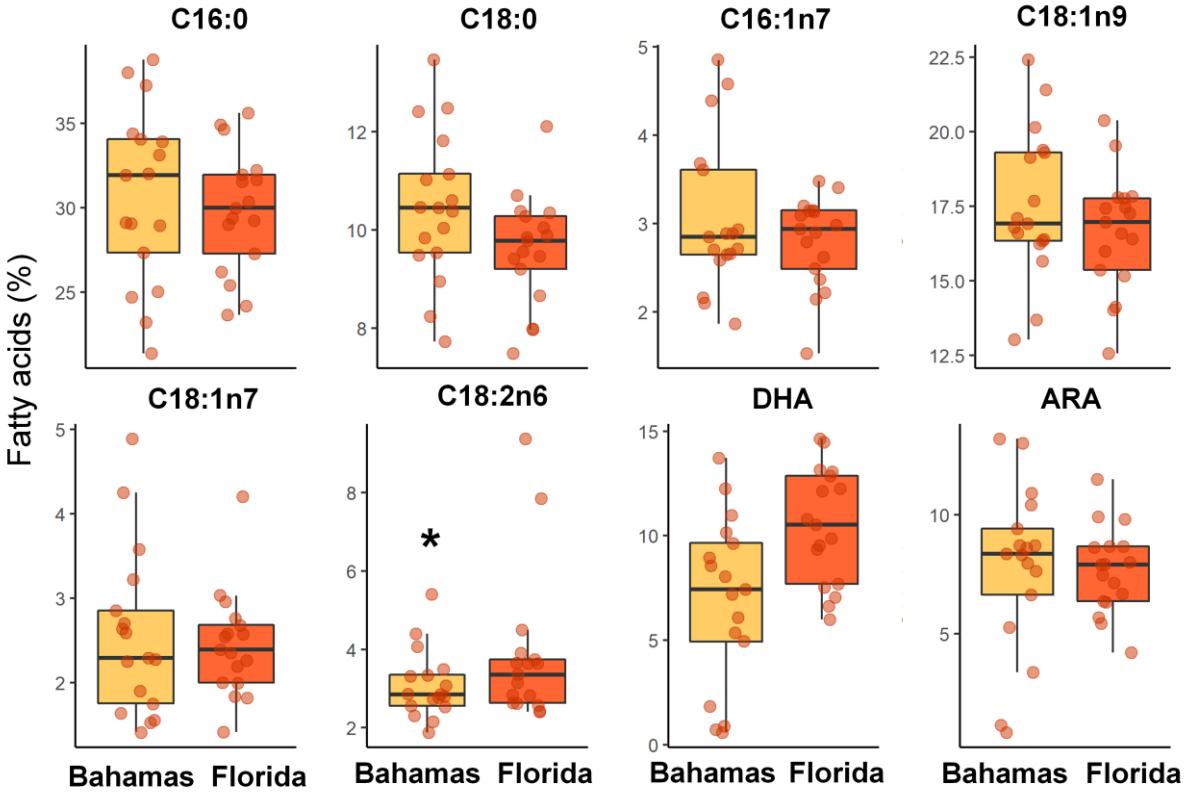

Figure 4. Differences in fatty acid percentages between Bahamas (n= 17) and Florida (n= 17) sampling locations. Black line indicates the median value and black points indicate the outliers. (*) Significant differences found with the Generalized Linear Mixed Model. ARA: arachidonic acid, DHA: docosahexaenoic acid.

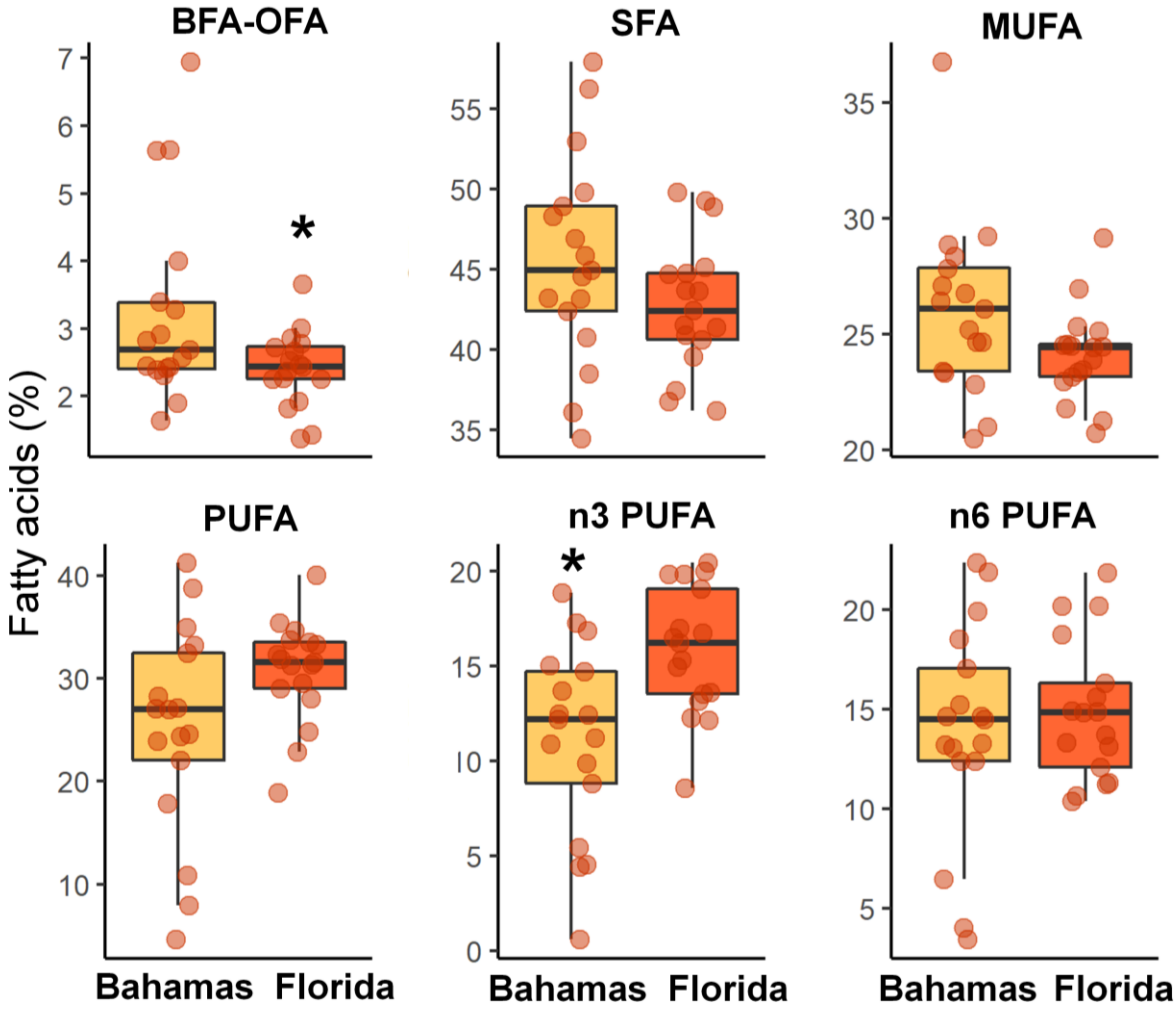

Figure 5. Differences in fatty acid percentages between Bahamas (n= 17) and Florida (n= 17) sampling locations. Black line indicates the median value and black points indicate the outliers. (*) Significant differences found with the Generalized Linear Mixed Model. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, n3 PUFA: omega 3 PUFA, n6 PUFA: omega 6 PUFA, BFA – OFA: branched chain and odd chain fatty acid.

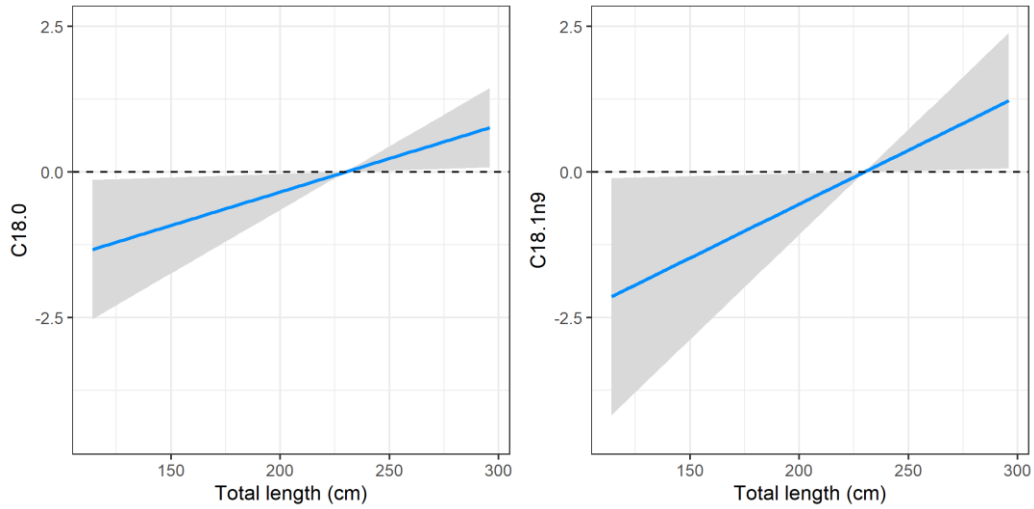

Figure 5. Generalized Linear Mixed Model of significant fatty acid variation as a function of tiger shark total length. The dashed lines and shaded areas represent null effects and 95% confidence intervals, respectively.

6.4. Discussion

Using a non-lethal approach through analyzing multiple physiological markers our results suggest that juvenile tiger sharks show limited, but significant spatial variation in both metabolic and nutritional condition between the two neighboring areas that differ significantly in the degree of coastal development and associated urbanization (South Florida versus the Bahamas). As predicted, relative corticosteroid concentrations were higher in juvenile tiger sharks sampled in closer proximity to urbanized coastal areas (i.e. South Florida), suggesting they may have higher energetic demand compared to their conspecifics foraging close to more pristine habitat (i.e. the Bahamas). However, no significant differences were observed in thyroid hormones between the two study locations. Contrary to our hypothesis about nutritional quality, we found higher proportions of n3 PUFA (indicative of high prey quality) in juvenile tiger sharks sampled off South Florida, and higher proportions of the bacterial marker BFA-OFA in sharks sampled in the Bahamas. Taken together, these findings do not suggest that the differences in nutritional quality and metabolic condition found between the two sampling locations can be fully attributed to foraging in areas exposed to differing levels of urbanization.

Physiological and ecological process may explain the elevated relative corticosteroid concentrations found in juvenile female tiger sharks sampled in South Florida waters. First, if tiger sharks sampled here are indeed foraging in areas with relatively lower prey availability, and consequently reduced foraging efficiency in waters exposed to higher levels of coastal

urbanization, increased relative corticosteroid concentrations could be associated with the need for increased foraging rates to meet dietary requirements (e.g. Romero, 2002; Landys et al., 2006). Second, human disturbance can increase allostatic load, consequently increasing glucocorticoid levels and inducing chronic stress in sharks living adjacent to high levels of coastal urbanization (Bonier, 2012). However, as chronic stress is difficult to detect in highly mobile sharks (e.g. Skomal and Mandelman, 2012), we do not speculate about this pattern in the present study. Although the effects of glucocorticoids in sharks are not well understood, elevated concentrations of both corticosterone and 1α-OH-B have been associated with energy mobilization during stressful events such as food deprivation, capture and reproduction (Rasmussen and Crow 1993; Manire et al. 2007; Iki et al. 2020). It is unlikely that relative corticosteroid concentrations found here are associated with capture stress as tiger sharks have been previously shown to exhibit minimal stress responses to fisheries capture (e.g. Gallagher et al., 2014), including no correlations between glucocorticoid concentration and capture (Newton et al., 2020). Further, it is unlikely that relative corticosteroid concentrations found here were related to sex or reproduction as all sharks analyzed were juvenile females. However, similar studies including neonates and young-of-the-year tiger shark samples would be definitive to assess possible ontogenetic variations in this parameter.

The lack of differences in thyroid hormones concentrations may be an indicative of similar metabolic activity in juvenile tiger sharks sampled in both locations. A single study reporting regional variation in the thyroid hormones of the bonnethead *Sphyrna tiburo*, suggested that higher concentrations found in maternal serum in sharks sampled in Florida Bay could be related to metabolic rate (McComb et al. 2005). An association was found between higher concentrations of thyroid hormones with high temperature and lower exposure to contaminants, compared to other bonnethead from Tampa Bay, Florida (McComb et al. 2005). Based on these findings, it is possible to infer that juvenile tiger sharks may not face major differences in environmental conditions, in terms of contaminants and temperature. However, because the effect of thyroid hormones in vertebrates is complex (e.g. Hulbert, 2000; Deal and Volkoff, 2020), further research is required to better understand the role of these metabolic hormones in sharks.

The plasma fatty acid profile suggested that juvenile tiger sharks differed in important dietary biomarkers between the two study locations. Because urbanization often leads to habitat degradation and pollution (e.g. harmful chemicals, bacteria and sediment-associated sewage), such processes can reduce food availability, and consequently alter trophic

interactions trough bottom up forcing (e.g. Faeth et al., 2005; El-Sabaawi, 2019), we hypothesized that juvenile tiger sharks sampled in South Florida would exhibit lower nutritional quality than sharks sampled in the Bahamas. Surprisingly, we found higher proportions of n3 PUFA, mainly DHA, in sharks sampled in South Florida waters, suggesting consumption of higher quality food resources compared to Bahamas. This result differ from recent research which found that nurse sharks (*Ginglymostoma cirratum*) sampled in highly urbanized areas exhibited higher levels of plasma saturated and bacterial fatty acids compared to conspecifics sampled in adjacent minimally urbanized areas (Rangel et al. 2021a). Similarly, nurse sharks from highly urbanized sites also exhibited lower proportions of essential fatty acids (i.e., highly unsaturated fatty acids, HUFAs), mainly due to low contributions of omega-6 HUFA (Rangel et al. 2021a).

One possible explanation for the results found here in juvenile tiger sharks is that n3 PUFA, DHA in particular, can be successively enriched through the trophic web (Parrish et al., 2015b; Meyer et al., 2019) and perhaps Florida sharks were consuming higher trophic level prey compared to sharks sampled in the Bahamas, rather than prey of higher nutritional quality. It is worth considering that the Bahamas is a hotspot for tiger sharks, especially the sampling site where females of mixed life-stages, including large pregnant individuals, aggregate (Sulikowski et al., 2016). Accordingly, it is plausible that smaller females have reduced foraging success due to increased competition from larger conspecifics given documented intra-specific competition found in other large sharks (e.g., Martin et al. 2009). Another possible explanation for the apparent lower nutritional food quality found in sharks sampled in the Bahamas could be related to provisioning dive tourism near the study site, where dive tourists attract and feed tiger sharks with fish carcasses. Although the provisioning doesn't appear to impact tiger shark migrations and daily habitat use (Hammerschlag et al. 2012, 2017), the use of attractants with little food reward could increase energy expenditure for low quality prey (mostly fish carcasses lacking meat) as has been found for whitetip reef sharks (*Triaenodon obesus*) provisioned at dive sites in the Red Sea (Barnett et al., 2016).

Not surprising, the C18:2n6 was found in higher proportions in South Florida sharks, indicating an influence of terrestrial and freshwater sources (mangroves and terrestrial plants) (Kelly and Scheibling, 2012; Every et al., 2016). It is common to find this marker in species using coastal areas with high influence of river systems and large outflow of freshwater (Every et al., 2016). Compared to other life-stages within the Bahamas, juvenile tiger sharks also had higher proportions of C18:2n6 than adult females, demonstrating a gradual decrease

in the dependence on coastal-associated biomarkers with increasing body size (Rangel et al., 2021b).

We expected juvenile tiger sharks sampled at urbanized coastal areas to exhibit higher proportions of bacterial markers, as increased bacterial communities are often correlated with domestic sewage effluent and organic detritus (e.g. Boëchat et al., 2014; Le Moal et al., 2019; Rangel et al., 2021a). However, higher amounts of heterotrophic bacterial marker BFA-OFA were found in sharks sampled in the Bahamas, although the percentage in both locations is low. One possible explanation is that this bacterial marker is usually found in higher concentrations in demersal prey (Käkelä et al., 2005; Kelly and Scheibling, 2012), suggesting that sharks sampled in the Bahamas may be consuming more demersal teleosts (e.g. Rangel et al., 2021a). If this were the case, it is also plausible that smaller females could be feeding more on more benthic prey types to avoid competition with larger conspecifics, as previously observed in tiger sharks in Hawaii (Lowe et al., 1996). Taken together, these findings suggest that juvenile female tiger sharks differ in their dietary patterns between the two locations, and that competitive interaction with larger conspecifics could be influencing intra-specific foraging.

Higher proportions of both C18:0 and C18:1n9 in juvenile tiger sharks smaller than 225 cm TL suggest these fatty acids vary ontogenetically. Tiger sharks exhibit high growth rates at juvenile stage (Afonso et al., 2012), thus possibly explaining higher proportions of circulating C18:0 and C18:1n9, as these fatty acids are the main constituents of fat stores catabolized for energy (Tocher 2003, Rangel et al., 2020). Moreover, it is possible that the decrease in C18:1n9 with increasing body size may reflect feeding on herbivores, such as turtles and mysticete whales (Aines et al., 2017; Dicken et al. 2017; Ferreira et al., 2017; Rangel et al., 2021b), as the C18:1n9 is indicative of piscivory/carnivory in sharks (Every et al., 2016; Bierwagen et al., 2019). However, at juvenile stages, tiger sharks primarily consume teleost fishes (~76% of total items found in stomachs; Aines et al., 2017).

Our multiple physiological markers approach allowed us to better understand the relationship between physiological condition and spatial variation in juvenile female tiger sharks. However, due to opportunistic sampling and because we used a non-lethal approach of plasma analyses, our study has several limitations. This includes the use of relative corticosteroid concentrations as a proxy for the potential effects of the main glucocorticoid in sharks, the 1α-OH-B. Further studies including 1α-OH-B and adrenocorticotropic hormone levels are required to better understand the responses related to nutritional stress. Because thyroid hormones have been closely associated with temperature in sharks (e.g. McComb et

al. 2005), the influence of abiotic variables should also be considered in future studies. Finally, although plasma fatty acid have been extensively demonstrated as a promising method to assess short-term shifts in diet in elasmobranch's trophic ecology (e.g. Semeniuk et al., 2007; Beckmann et al., 2014; McMeans et al., 2012; Bierwagen et al., 2020; Rangel et al., 2020) and in the context of urbanization (e.g. Andersson et al., 2015; Isaksson, 2015; Toledo et al., 2016, Rangel et al. 2021a), it has a limitation in terms of specifically identifying dietary and/or non-dietary origin (e.g. mobilized from storage tissues).

6.5. Conclusion

Our results suggest that juvenile tiger sharks sampled in closer proximity to an highly urbanized coastline and greater market gravity did not exhibit associated negative physiological, in terms of nutritional quality and metabolic condition, as would be expected. However, it is still possible that exposure to higher market gravity could influence the energetic demands of individuals. The lack of expected effects of coastal urbanization and greater market gravity on tiger sharks sampled off South Florida may be due to the highly migratory nature and generalist diet of tiger sharks, even at juvenile stages. It is likely that even at the juvenile stages, these sharks are moving across both study areas, and therefore, the scale of their movement and generality of their diet would not be tied to specific locations, as would be a more sedentary species and dietary specialists at a location. Importantly, while sampling was conducted along an urbanized coastline, it occurred in waters 2-5 km from shore, likely more influenced from oceanic waters than nearshore mainland waters influenced by runoff and other urban effects. Therefore, it is possible that the prey base of tiger sharks sampled off South Florida was not sufficiently impacted by urbanization. We encourage future integrated research on the ecological processes influenced by habitat quality and urbanization, and their consequences for physiological condition and movement patterns, including those related with food availability, intra- and inter-specific trophic relationships, and physiological stress.

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Supporting Information

Table S1. Generalized linear models of metabolic hormone concentrations and fatty acid percentages of female tiger shark (*Galeocerdo cuvier*) as a function of total length (TL) and sampling location (n= 17 -Bahamas-, n= 17 –Florida-), only for those were not significant. Included are the corresponding biomarker type (Marker), response variable (biomarker), coefficient estimate (Est.), standard error (SE), t-value (t) and p-value (p) of each model. **Bold**: significant values ($p<0.05$).

Marker	Response	Variable	Est.	SE	t	p
Hormones	T_3	TL	1.91	3.06	0.00	1.000
		(Location) Florida	1.51	3.49	0.43	0.672
	T_4	TL	5.87	5.89	0.99	0.334
		(Location) Florida	86.00	632.84	0.14	0.894
	T_3/T_4	TL	-0.01	0.01	-1.99	0.063
		(Location) Florida	-0.61	0.54	-1.13	0.274
Fatty acids	C16:0	TL	-0.02	0.02	-0.83	0.415
		(Location) Florida	-1.94	1.89	-1.03	0.313
	C16:1n7	TL	-0.00	0.00	-0.59	0.562
		(Location) Florida	-0.37	0.30	-1.21	0.234
	C18:1n7	TL	-5.59	3.47	-0.16	0.873
		(Location) Florida	-1.64	3.51	-0.47	0.643
	C20:5n3 (EPA)	TL	-0.01	0.00	-1.58	0.127
		(Location) Florida	-0.02	0.32	-0.07	0.947
	C22:5n3	TL	4.51	3.05	1.48	0.149
		(Location) Florida	6.37	3.13	2.04	0.051
	C22:6n3 (DHA)	TL	-0.02	0.01	-1.20	0.239
		(Location) Florida	2.53	1.46	1.73	0.093
	C20:4n6 (ARA)	TL	-0.00	0.01	-0.15	0.885
		(Location) Florida	-0.18	1.19	-0.15	0.881
	C22:4n6	TL	0.00	0.00	0.43	0.669
		(Location) Florida	-0.16	0.46	-0.36	0.722
	SFA	TL	-8.01	2.29	-0.35	0.729
		(Location) Florida	-3.43	2.31	-1.48	0.149
	MUFA	TL	0.01	0.01	1.16	0.257
		(Location) Florida	-1.11	1.26	-0.88	0.387
	PUFA	TL	-0.01	0.03	-0.18	0.857
		(Location) Florida	5.71	3.41	1.68	0.104
	n6 PUFA	TL	0.01	0.01	0.54	0.592
		(Location) Florida	1.92	1.93	0.99	0.328

Thyroid hormones: T3: triiodothyronine, T_4: thyroxine. **Fatty acids:** 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 6 PUFA.

Table S2. Fatty acid profile of plasma (% of total, mean ± standard deviation, coefficient of variation CV,%) of tiger sharks (*Galeocerdo cuvier*) sampled in Florida and the Bahamas.

Fatty acids	Bahamas	CV	Florida	CV
C15:0	2.4 ± 1.4	58.1	1.6 ± 0.4	27.1
C17:0	0.9 ± 0.3	29.7	0.9 ± 0.3	32.7
C14:0	4.0 ± 1.2	30.5	3.0 ± 0.8	26.5
C16:0	30.7 ± 5.2	17.0	29.8 ± 3.6	12.1
C18:0	10.5 ± 1.5	14.4	9.6 ± 1.1	11.7
C14:1	2.8 ± 0.9	33.1	2.0 ± 0.6	31.5
C16:1n7	3.1 ± 0.9	28.3	2.8 ± 0.5	18.3
C18:1n9	17.6 ± 2.5	14.2	16.6 ± 2.0	11.9
C18:1n7	2.5 ± 1.0	38.6	2.4 ± 0.6	25.5
C18:2n6	3.1 ± 0.9	28.3	3.8 ± 1.9	49.9
C20:5n3 (EPA)	1.6 ± 0.7	41.4	1.8 ± 0.8	42.9
C22:5n3	2.9 ± 0.8	27.8	3.2 ± 0.8	24.0
C22:6n3 (DHA)	6.9 ± 4.1	58.9	10.4 ± 2.8	26.7
C20:4n6 (ARA)	7.8 ± 3.5	45.0	7.7 ± 1.8	23.7
C22:4n6	2.6 ± 1.3	49.3	2.2 ± 0.9	40.9
BFA-OFA	3.3 ± 1.5	45.2	2.4 ± 0.6	23.5
SFA	45.6 ± 6.5	14.2	42.8 ± 4.1	9.7
MUFA	26.1 ± 3.8	14.5	24.1 ± 2.0	8.4
PUFA	25.1 ± 10.2	40.7	30.7 ± 5.0	16.3
n3 PUFA	11.1 ± 5.1	45.4	15.8 ± 3.4	21.2
n6 PUFA	14.0 ± 5.5	39.1	14.9 ± 3.6	23.9
n3/n6	0.8 ± 0.3	33.4	1.1 ± 0.3	29.4

EPA: eicosapentaenoic acid, ARA: arachidonic acid, DHA: docosahexaenoic acid, SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, n3 PUFA: omega 3 PUFA, n6 PUFA: omega 6 PUFA, BFA – OFA: branched chain and odd chain fatty acid.

Capítulo 7

Physiological state predicts space use of sharks at a provisioning tourism site

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Abstract

While a growing body of literature has shown that provisioning tourism can influence the behavior of wildlife, how physiological state might be related to the nature and magnitude of these effects remains poorly understood. Physiological state, including reproductive and nutritional status, can have profound effects on an individual's behavior and decision-making. In the present study, we used multiple physiological markers related to reproductive (testosterone, 17β-estradiol and progesterone), metabolic (corticosteroids) and nutritional ecology (stable isotopes and fatty acids), integrated with ultrasonography and passive acoustic telemetry to explore the possible relationship between physiological condition and space use of tiger sharks (*Galeocerdo cuvier*) exposed to provisioning dive tourism. Large, non-gravid female tiger sharks, with higher plasma steroid levels (i.e., testosterone, 17β-estradiol, relative corticosteroid), enriched $\delta^{15}N$, and elevated nutritional status (in terms of fatty acids), spent proportionally more time at food provisioning sites compared to conspecifics. Testosterone levels also were positively correlated with the proportion of time spent at provisioning sites. Based on these results, we speculate that physiological condition plays a role in shaping the spatial behavior of female tiger sharks within the context of food provisioning, whereby larger individuals, exhibiting higher testosterone levels and elevated nutritional status, show selective preferences for provisioning dive sites, where they outcompete conspecifics of relatively smaller size, lower testosterone levels, and depressed nutritional state. While more studies are needed to explore whether sharks are making these decisions because of their physiological state or whether spending more time at provisioning sites results in altered physiological state, our findings highlight the importance of considering animal life-stage, endocrine regulation, and nutritional condition when evaluating the biological impacts of provisioning tourism.

Keyword: ecotourism, fatty acids, *Galeocerdo cuvier*, stable isotopes, steroid hormones.

7.1. Introduction

Ecotourism can play an important role in wildlife conservation as it often replaces extractive uses of animals and their habitat (Gallagher & Hammerschlag, 2011; Cusack et al., 2021). However, feeding or attracting wildlife with food to enable better viewing opportunities by ecotourists (i.e., provisioning tourism) has the potential to alter the natural behavior and physiology of animals (reviewed by Brena, Mourier, Planes, & Clua, 2015; Trave, Brunnschweiler, Sheaves, Diedrich, & Barnett, 2017; Cox & Gaston, 2018; Patroni, Simpson, & Newsome, 2018). For instance, in the presence of tourism-based provisioning, Northern Bahamian Rock Iguanas (*Cyclura cychlura*) experienced a greater incidence of endoparasitic infection and indicators of a nutritionally unbalanced diet (Knapp et al., 2013). Similarly, southern stingrays exposed to provisioning tourism (*Hypanus americanus* at Grand Cayman) exhibited several negative physiological consequences including lower hematocrit and body condition, reduced antioxidant capacity, and altered intake of essential nutrients (Semeniuk, Speers-Roesch, & Rothley, 2007; Semeniuk, Bourgeon, Smith, & Rothley, 2009; Semeniuk, Haider, Cooper, & Rothley, 2010; Hoopes et al., 2020). Given increasing discussions framing ecotourism activities as a conservation tool, it is important to understand the physiological and behavioral impacts that provisioning has on wildlife populations, especially for threatened species (Gallagher & Huveneers 2018).

Ecotourism-related feeding of large aquatic predators, in particular, has raised concerns regarding alterations in their behavior that could have ecosystem impacts or ramifications for human safety (Gallagher et al., 2015; Macdonald et al., 2017).There is a wide discussion on the habituation of predators to human contact and dependence on the food provided, as well as the safety hazards for recreational divers and water enthusiasts during provisioning activities (Orams, 2002; Macdonald et al., 2017; Mann et al., 2021). Shark diving tourism is a global phenomenon, where sharks are usually attracted or fed (Gallagher & Hammerschlag 2011); however, studies on the potential effects of ecotourism-related feeding on sharks have revealed mixed effects on their behavior and physiology, suggesting possible population-, species-, location-, and context-dependent effects. Behavioral changes detected have included aggregation of large solitary sharks at a small spatial scale (Brunnschweiler & Baensch, 2011; Bruce & Bradford 2013), increased residency at provisioning sites (e.g., Fitzpatrick, Abrantes, Seymour, & Barnett, 2011, Heinrich et al., 2020), increased locomotor activity levels (Barnett, Payne, Semmens, & Fitzpatrick, 2016; Huveneers, Watanabe, Payne, & Semmens, 2018), and intra and interspecific aggression (Clua, Buray, Legendre, Mourier, &

Planes, 2010). Further, ecophysiological studies have indicated that provisioning can alter individual metabolic rate (Barnett, Payne, Semmens, & Fitzpatrick, 2016; Heinrich et al., 2020) and dietary patterns (Maljkovic & Coté, 2011; Brunnschweiler, Payne, & Barnett, 2018).

While a growing number of studies involving sharks have investigated the impact of provisioning tourism on an individual's behavior, studies have not examined the potential influence that a shark's physiological condition (i.e., state of the body or bodily functions) could contribute to behavioral decisions associated with provisioning (e.g., Senigaglia & Bejder, 2020). To better understand the physiological mechanisms that could mediate, or be affect by, the time spent associated with provisioning tourism, here we investigated aspects of tiger shark (*Galeocerdo cuvier*) behavior and physiology at a popular dive tourism location in The Bahamas, named "Tiger Beach," where tiger sharks are attracted and/or fed regularly by dive operations. Located within the southern core range of tiger sharks during winter months (Hammerschlag et al., 2022), Tiger Beach hosts a high number of tiger sharks dominated by females of mixed age classes (Sulikowski et al., 2016). Previous studies in the area have suggested that the provisioning dive tourism has little impact on the long-term migration and diel habitat use patterns of tiger sharks here (Hammerschlag et al., 2012, Hammerschlag, Gutowsky, Gallagher, Matich, & Cooke, 2017), but moderate effects on social behaviors (Jacoby et al., 2021). Despite being highly migratory and nomadic for much of their range, non-random associations among individual tiger sharks were detected in Tiger Beach using social network analysis on tiger shark tracking data (Jacoby et al., 2021).

In the present study, we used multiple physiological markers related to reproductive, metabolic and nutritional condition, integrated with ultrasonography and passive acoustic telemetry to explore, for the first time, the possible relationship between physiological condition and space use of tiger sharks exposed to dive provisioning tourism at Tiger Beach. We explored the gonadal steroid hormones testosterone, 17β-estradiol and progesterone, because they are thought to regulate phenotypic responses to dynamic social environments in female vertebrates, including sharks (e.g., Rosvall, Bentz, & George, 2020; Awruch, 2013). Relative corticosteroid levels were used as a proxy for energetic demand since the role of corticosteroids in regulating the acquisition and mobilization of external prey resources is highly conserved across vertebrate evolution (Crespi, Williams, Jessop, & Delehanty, 2013; Romero & Wingfield 2016). Finally, stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) and fatty acid profiles of blood plasma were used as short-term dietary biomarkers and as a proxy for nutritional quality (Beckmann, Mitchell, Stone, & Huveneers, 2014; Rangel et al., 2020; Rangel,

Hammerschlag, Sulikowski, & Moreira, 2021a). These data were used to explore if (1) steroid hormones, including reproductive hormones and corticosteroids, were correlated with spatial behavior of tiger sharks exposed to tourism-provisioning, and (2) relationships existed between nutritional condition and the proportion of time tiger sharks spend at provisioning sites.

7.2. Material and methods

7.2.1. Ethical Statement

This work was conducted under permits from the Department of Marine Resources, Ministry of Agriculture and Marine Resources, Government of The Bahamas, and the University of Miami Institutional Animal Care and Use Committee (IACUC Protocol 15-238).

All sharks were captured using standardized circle-hook drumlines, a passive, autonomous fishing technique, as described in Gallagher, Serafy, Cooke, & Hammerschlag (2014). This method permitted sharks to swim in a 23 m radius circle around the base when captured, which minimizes the capture stress. After an hour from the first deployment, each drumline was sequentially checked for shark presence. Once hooked, each shark was slowly brought to the boat and restrained on a dive platform partially submerged in the water. To facilitate respiration, a hose was immediately inserted into the shark's mouth that actively pumped water over the shark's gills. This capture and handling method was selected to promote shark vitality and reduce stress levels during sampling, as previous stress analysis showing tiger sharks do not experience physiological stress using these procedures (Gallagher et al 2014).

7.2.2. Study area and Tiger Beach

The study area is located within the northwest edge of the Little Bahama Bank (26.86° N, 79.04° W) that extends off Grand Bahama Island, Bahamas. The habitat is shallow (average 5 m deep) and mostly covered by sandy bottoms seagrass beds. As outlined in Hammerschlag et al. (2017), Tiger beach encompass a core area of 1.5 km^2, where up to four regular dive operators conduct tiger shark diving activities. These dive operations mostly occur during the day when sharks are attracted to the dive boats via chumming and are fed fish carcasses (Hammerschlag et al., 2012). While some dive tourism here occurs year-round, the majority of operations are focused during cooler months (from October-May), coinciding with the greatest presence of large female sharks. Approximately 98.5% of the diver days that

are dedicated tiger shark dives throughout The Bahamas are in Grand Bahama (Haas et al., 2017). Information from one regular operator, revealed that there were 163 entries (tourism events) between 1 Nov 2013–16 Oct 2015 (714 days; Jacoby et al., 2021).

7.2.3. Shark capture, blood sampling, and acoustic tagging

In Oct 2013, May 2014, and Nov 2014, tiger sharks were caught in the study area using sets of drumlines with baited circle hooks (described in Gallagher et al., 2014). Once caught, hooked sharks were secured alongside the boat where sex and length measurements (total length [TL] cm) were recorded, and pregnancy status was assessed through imaging the reproductive tract of each female shark, using ultrasonography (Ibex Pro portable ultrasound, EI Medical Imaging, Loveland, CO, USA), with a 60 mm curved linear array 2.5 to 5 MHz transducer (model 290470). Blood samples were taken from the caudal vasculature using 18-gauge needles and 10-mL heparinized syringes (Lawrence et al., 2020). The blood sample was stored on ice and then a subsample was centrifuged at 3500 rpm (410 × g) for 3 min to separate out plasma. Both samples of whole blood and plasma were then removed and stored frozen at −80°C for future stable isotope, hormonal and fatty acid analyses.

Tiger sharks were tagged with coded Innovasea V16-4X acoustic transmitters (Amirix Inc., Bedford, NS, Canada), with a nominal delay of 60 to 90 seconds, surgically implanted in the body cavity following Hammerschlag et al. (2017). Once the transmitter was inserted, the incision was closed promptly with one to two dissolvable sutures. All caught sharks were released in good condition at their location of capture.

7.2.4. Acoustic telemetry array

To understand the relationship between hormone levels and provisioning site use, we used passive acoustic telemetry to track tiger sharks. To measure tiger shark residencies, we established an array of 32 Innovasea VR2W receivers (Amirix Inc., Bedford, NS, Canada) as outlined in Hammerschlag et al. (2017). Receivers were arranged 750 m apart from one another to form a 12 x 3.2 km rectangle (Fig. 1). Due to receiver failure, the final array of functioning receivers to the completion of the study consisted of 23 receivers. This included receivers placed at the four primary dive sites at Tiger Beach (Fig. 1).

We performed range testing on a subset of six receiver stations in August 2017. At six different distances (100, 200, 400, 600, 800, and 1000 m) from the receiver station, an Innovasea V16-4X sentinel range testing tag with a nominal delay of 10 seconds was dropped into the water from an anchored vessel with its engine shut off. The tag was lowered

approximately one meter into the water column for one minute to allow for six transmissions. Once the detection data were downloaded, the number of detections received at each distance was divided by the number of theoretical detections (in this case, six). The detection percentages at each distance from all six stations were then plotted and fitted with a logistic regression curve to estimate the 50% detectability range of the entire receiver array.

7.2.5. Acoustic telemetry data

Before processing detection data downloaded from the acoustic receivers, they were filtered to remove any false detections – defined as a single detection that occurred alone within a one-hour period (Kessel et al., 2014) - using the R package 'GLATOS' (Holbrook et al., 2017). To monitor shark space use patterns across the acoustic array, we calculated the time spent by each individual (i.e., residency times) at both provisioning and non-provisioning sites, within the first 90 days post tagging. A 90-day period was used as it relates to the temporal scale of endocrine modulations of spatial behavior in teleost fishes (e.g., hormone/species; Birnie-Gauvin et al., 2019) (Fig. 1).

Residency was calculated for each individual at each acoustic receiver station using the *detection events* function of the R package GLATOS. This function classifies a distinct event when either an individual is detected at a different station or the time difference between subsequent detections at a given station surpasses a pre-specified time threshold (in this study, defined as one hour). Once all detection events were computed, we summed the total time spent across all detection events at each site, which resulted in two residency time measurements for each individual: (1) the total time spent at provisioning stations and (2) the total time spent at non-provisioning stations. These two values were added together to calculate the total time spent in the array. Proportion of time spent in provisioning sites was calculated by dividing the total time spent at provisioning sites by the total time spent in the array. Based on those proportions, individuals were categorized as showing 'high' (>70% of time) or 'low' use (< 30% of time) of provisioning dive sites (Fig. 1).

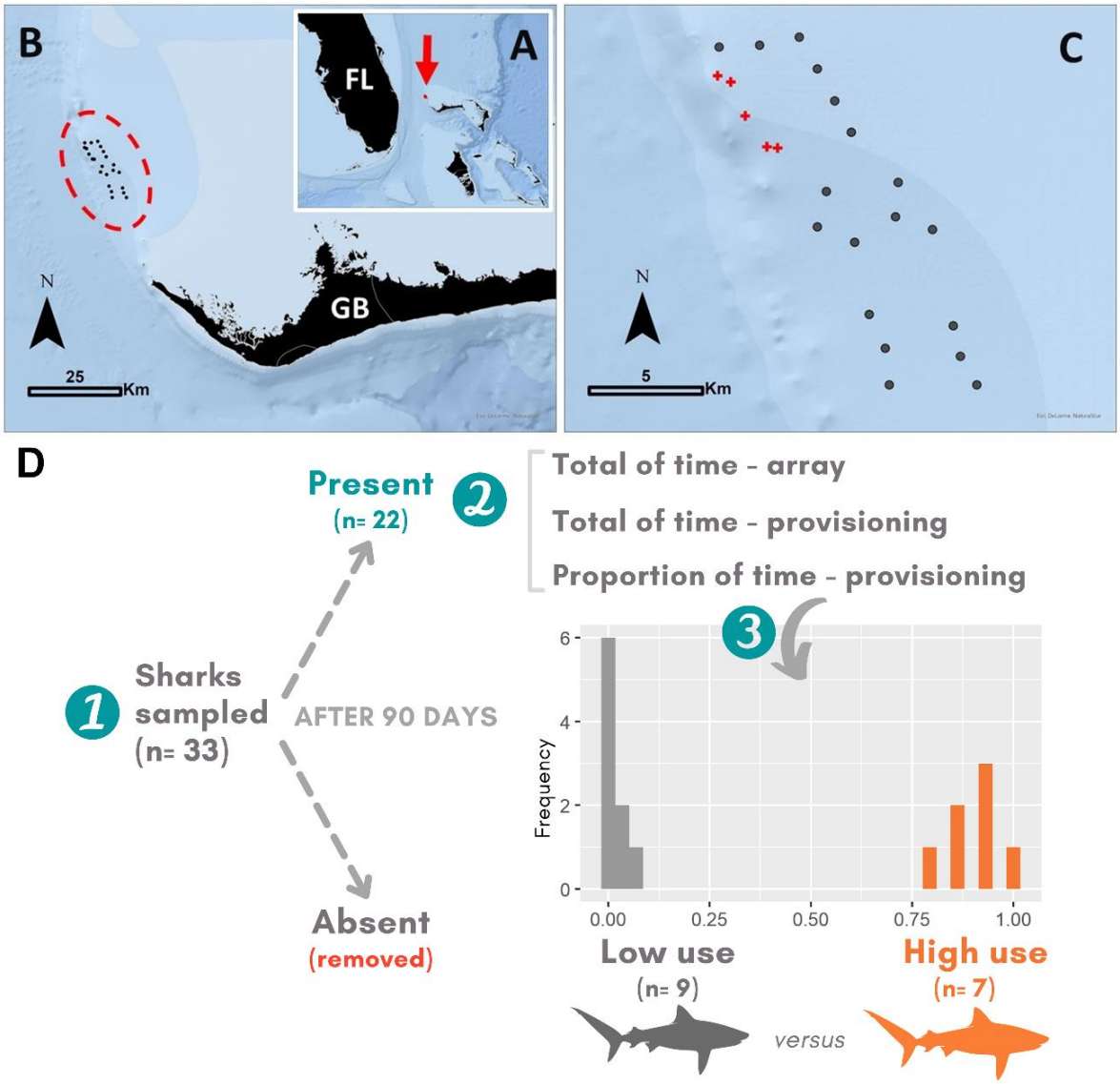

Figure 1. (A) The location of the study area in the northern Bahamas identified with a red arrow. FL = Florida, USA, as a spatial reference. **(B)** Positioning of the telemetry array on the northwestern edge of the Little Bahama Bank, off Grand Bahama [GB] Island. The 23 receivers used in this study are outlined in a red dashed oval. **(C)** Receivers were arranged in a roughly 12 km × 3 km rectangle, with the western line just inshore of the bank edge. Receivers in locations exposed to provisioning from commercial shark dive operations are identified with red crosses, referred to in the text as provisioning sites. All other receivers are identified with gray circles, referred to in the text as non-provisioning sites. **(D)** Schematic representation of the sequence of analyzes performed in the present study (from 1 to 3). Based on the proportions of time spent at provisioning sites, individuals were categorized as showing 'low (< 30% of time) or 'high (>70% of time) use of provisioning dive sites.

7.2.6. Blood hormone analysis

Animals used in this study were the same animals described in Sulikowski et al. (2016). In brief, plasma concentration values of the gonadal steroid hormones (testosterone, 17β-estradiol and progesterone) were measured using standards from Steraloids (Newport, RI) by radioimmunoassay (Sulikowski et al., 2004). A Tri-Carb 2900TR liquid scintillation analyzer (PerkinElmer, Waltham, MA) was used to measure radioactivity (see Sulikowski et al., 2016 for details). To obtain a relative measurement of corticosteroids in the plasma, we used the corticosterone ELISA kit (Cayman Chemical, Ann Arbor, MI, item # 500655). Measuring relative corticosteroid concentrations by corticosterone ELISA assay offer an alternative to studies with sharks because a commercial assay for 1α-OH-B was not available yet (Lyons Wynne-Edwards, 2019; Rangel et al., 2021a). The dilution selected was 1:5 (diluted with Cayman Assays assay buffer). Detailed description can be found in the Supplemental Material.

7.2.7. Reproductive Status

We considered length tiger sharks to be mature at >300 cm total length (Sulikowski et al., 2016). Sharks were assigned to one of three reproductive stages based on Sulikowski et al. (2016): (1) immature (<300 cm TL), (2) mature but non-gravid, and (3) mature and gravid. Reproductive states of all adult females were based on ultrasonography (see details in Sulikowski et al., 2016). Specifically, the presence of developing embryos or pups in the uterus was used to classify sharks as gravid (see details in Sulikowski et al., 2016).

7.2.8. Stable isotopes and fatty acids

Stable isotope analysis was performed on whole blood samples collected in the stable isotope laboratory at Boston University using glycine (- 34, 10.73) and peptone (- 14.73, 7.4) standards obtained from the National Bureau of Standards (see Shiffman, Kaufman, Heithaus, & Hammerschlag, 2019 for details). Plasma fatty acid profiles were generated by direct transmethylation, i.e., without lipid extraction, as described by Parrish, Nichols, Pethybridge, and Young (2015). Detailed description can be found in the Supplemental Material.

7.2.9. Data analysis

We were not able to collect the full range of physiological variables from all individuals tagged due to challenges in field blood collections. Sample sizes, therefore, were unequal across parameters measured.

To understand space use within the study area across life-stages, total times spent within range of receiver stations at provisioning sites were compared among life-stages (i.e., immature, mature, and gravid) via a one-way ANOVA with a Tukey's *post hoc* test for parametric data, or Kruskal-Wallis H-test followed by Dunn's *post hoc* for non-parametric data, using log (x+1) transformed data. We used the variable (x+1) to account for instances where an individual spent no time at provisioning sites. Binomial generalized linear models (GLM) were employed to understand the influence of hormone levels on the proportion of time sharks spent at provisioning stations, where the explanatory variables were the measured hormone levels in each individual, and the response variable was the proportion of time spent at provisioning stations during the first 90 days post release. We performed a binomial GLM for each effect of hormone level on the proportion of time sharks spent at provisioning sites. Hormone levels were transformed to help meet model assumptions (i.e., equal variance and normally distributed residuals, Table 2). A generalized linear mixed model (GLMM) was originally performed to account for the life-stage and month as random effects to control for variation in physiological variables between life stages and months; however, the random effect variance was zero and therefore these were removed from the model (Table S1).

We used GLMs with the mgcv package (Wood, 2017) to test for differences in hormone levels between sharks that frequently used the provisioning site and those that were infrequent visitors. In this test, we grouped sharks into those spending > 70% of their time at provisioning sites ("high use") and those spending < 30% of their time at provisioning sites ("low use", Fig. 1). Models included the respective biomarker values as the response variables and used Gaussian families of error distribution. We originally incorporated life-stage as a random effect to control for variation in physiological variables between life-stages and months; however, the random effect variance was zero and therefore these were removed from the model.

It is possible that high testosterone levels measured in sharks spending more time at provisioning sites could be a byproduct of the fact that non-gravid individuals, which have higher testosterone levels (Sulikowski et al., 2016), make greater use of these sites (rather than testosterone levels being a possible driver of greater use of these sites or that greater use of the sites may increase testosterone levels). To explore this potential, the correlation between hormone levels and proportion of time spent at provisioning sites were investigated using Pearson correlations on log transformed data. Additionally, we tested for potential differences in hormone levels between mature, non-gravid females that exhibited high (>

70%) versus low (<70%) use of provisioning sites. Differences between groups were evaluated with a Student's t-test, using log transformed data.

Permutational multivariate analysis of variance (PERMANOVA) with a Bonferroni correction was used to investigate potential differences in fatty acid profiles between sharks spending > 70% of their time at provisioning sites ("high use") and those spending < 30% of their time at provisioning sites ("low use"), including all life-stages (i.e., immature, mature, and gravid). Data for each fatty acid, including C16:0, C18:0, ARA, DHA, ΣSFA ΣPUFA, Σn3 PUFA, Σn6 PUFA, and BFA-OFA, was log transformed and tests were based on a Euclidean distance matrix. To identify which fatty acids contributed to average dissimilarity between groups, similarity percentage (SIMPER) tests were performed. Model analyses and Pearson correlations were performed in the R Studio software (version 4.0.2) (R Core Team, 2019). Student's t-test and multivariate analyses were performed in Past 3.20 (Hammer, Harper, & Ryan, 2001). The threshold level of statistical significance was set at 0.05.

7.3. Results

Thirty-three female tiger sharks were sampled and tagged within the study period, but only 22 individuals were detected in the acoustic array within 90 days of tagging, including six immature (264.5 ± 27.67 cm TL), seven non-gravid (343.4 ± 37.51 cm TL) and nine gravid females (355.7 ± 24.09 cm TL). Life-stage had a significant effect on total time spent at provisioning sites (Fig. 1; Kruskal-Wallis H-test, df= 21; p = 0.03). Mature, but non-gravid females spent significantly more time at provisioning sites (20.6 ± 31.4 hours) than immature females (2.3 ± 31.4 hours, Dunn's *post hoc*, z= 2.60, p= 0.009), while the proportion of time gravid females spent at the provision site (5.9 ± 9.0 hours) did not differ from the immature (z= 1.63, p= 0.10), and mature, but non-gravid females (Fig. 2; z= 0.96, p=0.34).

Females that used the provisioning site frequently (n= 9) were on average longer than those that did not use it frequently (n=7) (Fig. 3a; Table S2). Immature females showed higher use of non-provisioning sites (83% of individuals, n= 5 of 6; Fig. 3b). Four of six mature, but non-gravid females and two of four gravid females exhibited high use of provisioning sites (Fig. 3b).

Among all physiological variables measured, only testosterone concentrations (n= 21) were significantly related to the proportion of time sharks spent at provisioning sites (Fig. 4; Table 1). However, when sharks were grouped into high and low use of provisioning sites other physiological variables significantly differed across groups (Table 2 and S2). Specifically, females that frequently used provisioning sites exhibited significantly higher

testosterone (Fig. 5a), 17β-estradiol (Fig. 5b) and relative corticosteroids concentrations (Fig. 5d; Table 2 and S2). We found that testosterone levels in mature, but non-gravid females were significantly correlated with proportion of time at the provisioning sites (n= 9; Fig. S2; Table S3). Additionally, they were higher in individuals spending greater than 70% of their time at provisioning sites than low use individuals (Fig. S2; Table S4).

In terms of stable isotopes (n= 8) and fatty acids (n= 16), the high use group exhibited enriched $\delta^{15}N$ (Fig. 6; Table 2 and S2), lower percentages of ΣSFA, including C16:0, and higher percentages of ΣPUFA, including Σn3 PUFA and DHA (Fig. 7; Table 2 and S2). Multivariate analyses revealed a statistical difference in fatty acids between provisioning site low and high use groups (PERMANOVA, F= 3.93; *p*= 0.037), mainly due to the contribution of DHA (21.8%) and ARA (18.46%) (Table S5).

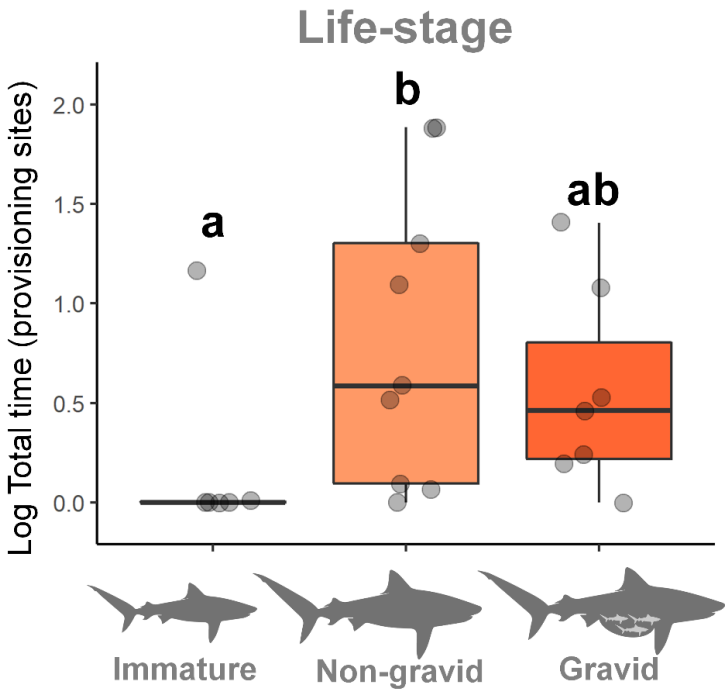

Figure 2. Comparison of total time spent at provisioning site (in hours log transformed) among life-stage of female tiger sharks (*Galeocerdo cuvier*): immature (n= 6, 2.3 ± 31.4 hours), non-gravid (n= 7, 20.6 ± 31.4 hours), and gravid females (n= 9, 5.9 ± 9.0 hours). Black line indicates the median value. Significant difference among life-stages is denoted with different superscripts above bars, which extended from minimum to maximum values (Kruskal-Wallis H-test followed by Dunn's *post hoc*).

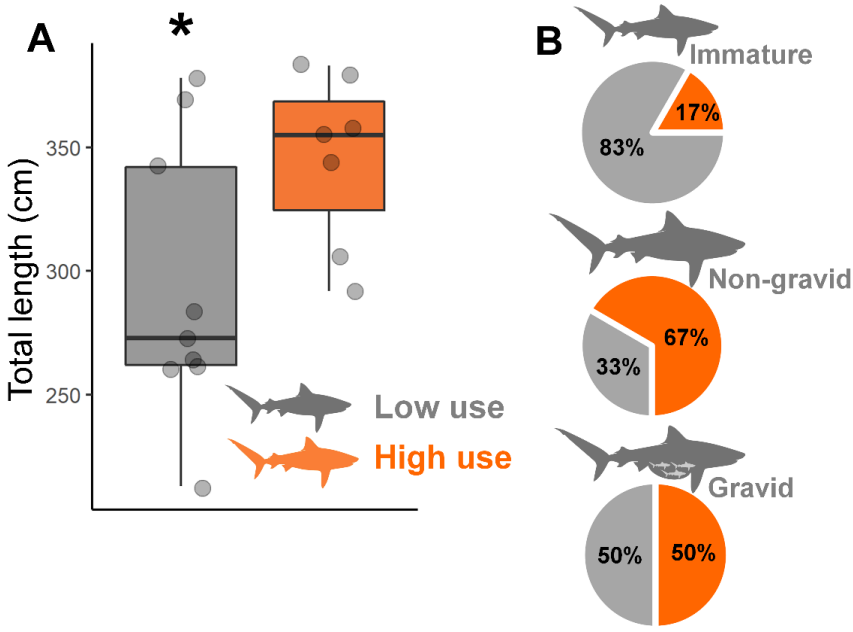

Figure 3. (a) Comparison of total length of female tiger sharks (*Galeocerdo cuvier*) between provisioning site low (n= 9) and high use (n= 7) groups. Black line indicates the median value. (b) Proportion of time females of each life-stage spent at non-provisioning (grey) and provisioning (orange) sites. Significant difference between groups is indicated by asterisks (Generalized Linear Model *p < 0.05).

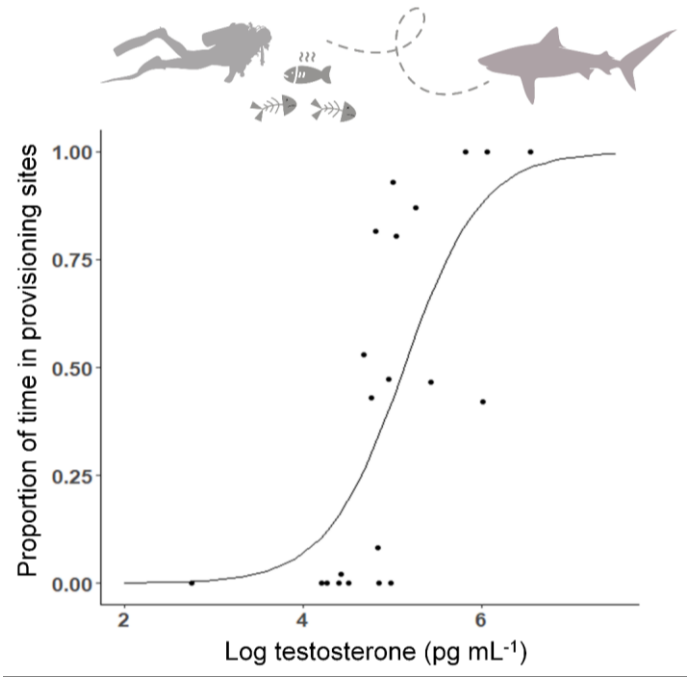

Figure 4. Scatter plot showing the relationship between the proportion of time a shark spent at provisioning sites at Tiger Beach, Bahamas, during the first 90 days of their tag life and the log transformation of testosterone levels. Points represent the actual measurements (n = 21). Line represents predictive values as derived from a binomial generalize linear model (GLM) with a logit link function.

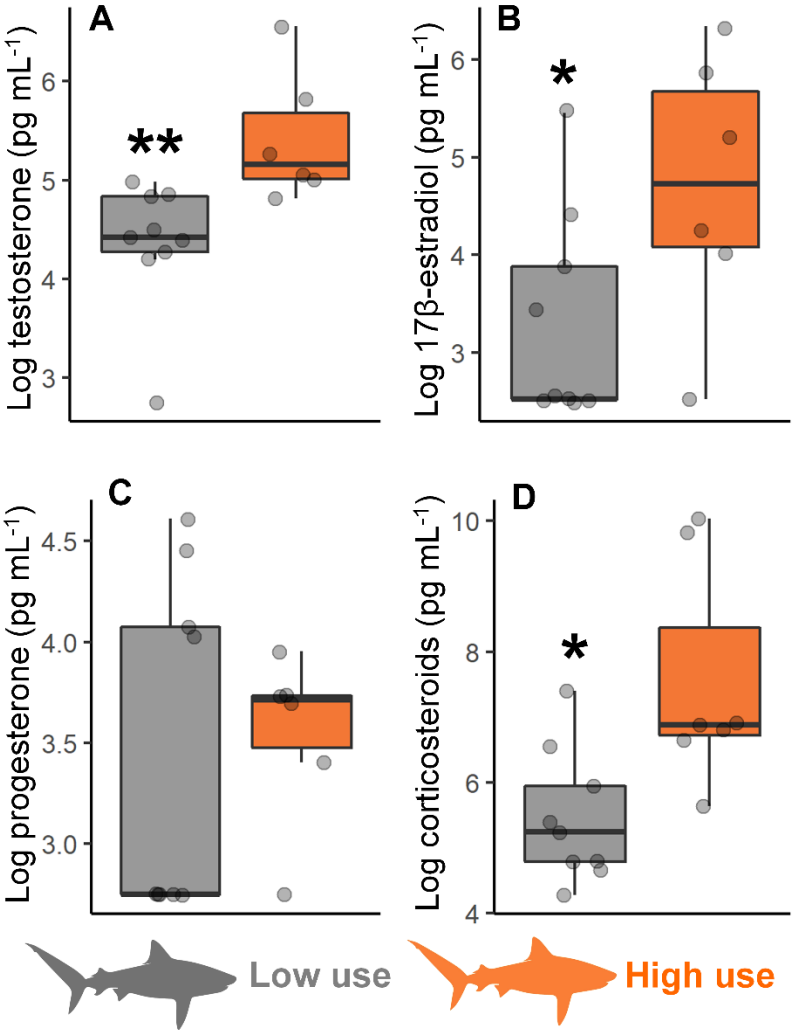

Figure 5. Comparison of steroid hormones of female tiger sharks (*Galeocerdo cuvier*) between provisioning site low (n= 9) and high (n= 7) use groups: (a) testosterone, (b) 17β-estradiol, (c) progesterone, and relative corticosteroid concentrations. Black line indicates the median value. Values are log transformed. Significant differences between groups are indicated by asterisks (Generalized Linear Model *$p < 0.05$; **$p < 0.01$).

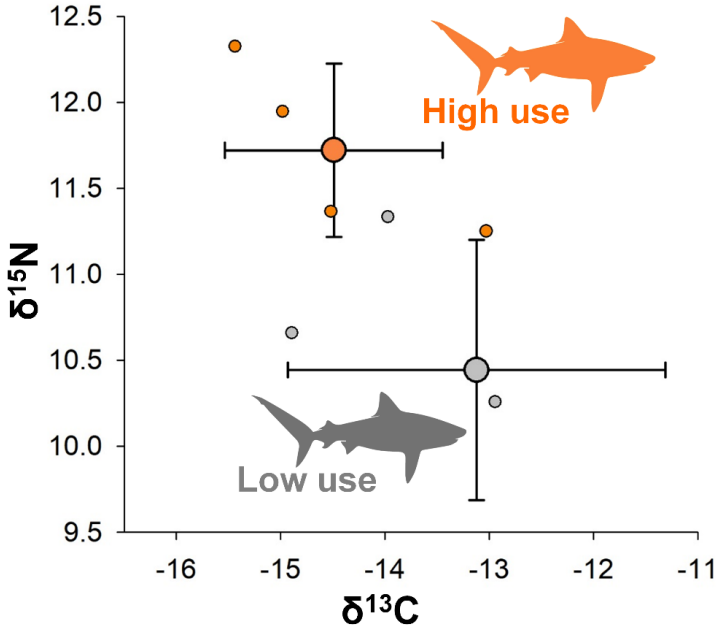

Figure 6. Stable isotope biplot of $\delta^{13}C$ and $\delta^{15}N$ values of female tiger sharks (*Galeocerdo cuvier*) for provisioning site low (n= 4) and high (n= 4) use groups.

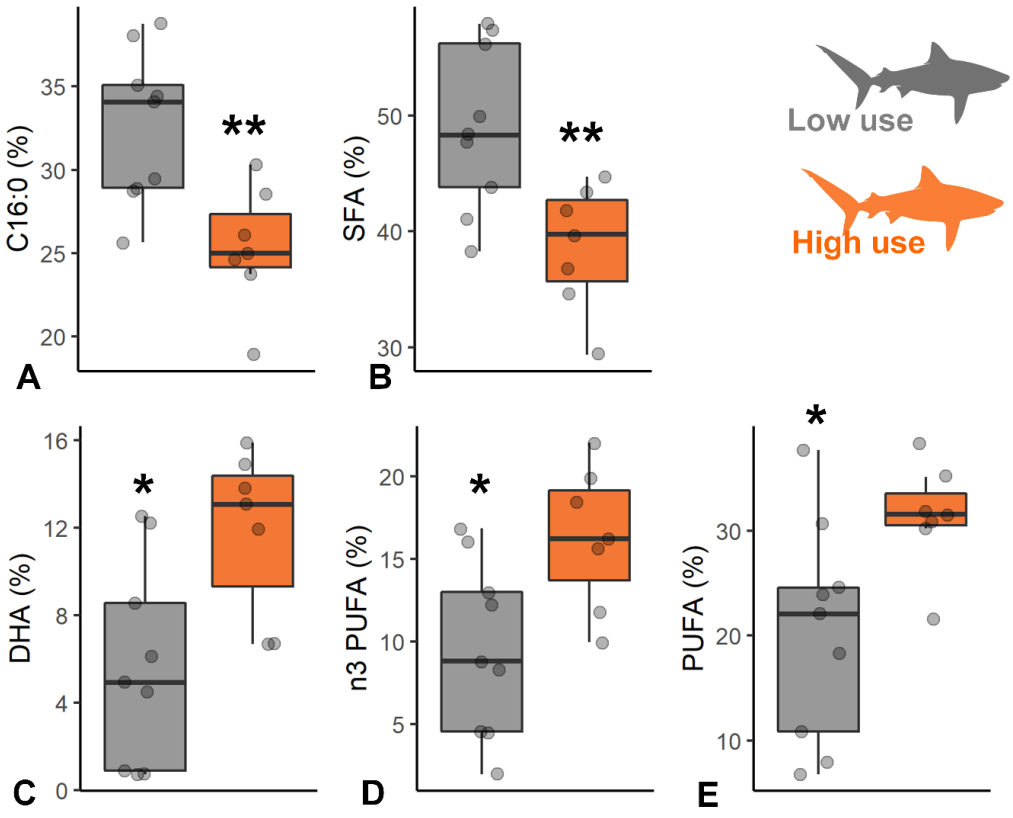

Figure 7. Comparison of plasma fatty acids of female tiger sharks (*Galeocerdo cuvier*) between provisioning site low (n= 9) and high (n= 7) use groups. (a) C18:0; (b) SFA: saturated fatty acids; (c) DHA: docosahexaenoic acid; (d) n3 PUFA: omega-3 polyunsaturated fatty acids; (e) PUFA. Black line indicates the median value. Significant differences between groups are indicated by asterisks (Generalized Linear Model *$p < 0.05$; **$p < 0.01$).

7.4. Discussion

Although physiological approaches are often used to explore decision-making in migratory animals (e.g., Lennox et al., 2016; Goossens, Wybouw, Van-Leeuwen, & Bonte, 2020), they have not been applied widely to investigations of decision-making in the context of tourism activities. The integration of multiple behavioral and physiological tools in the present study provided new insights into how physiological state and life-stage may be important factors influencing spatial use by large marine predators exposed to provisioning tourism (Fig. 8). We found that adult, but non-gravid tiger sharks spent more time at provisioning sites than immature individuals. In addition, individuals using provisioning sites more often had higher hormone levels (i.e., testosterone, 17β-estradiol, relative corticosteroid) and enriched $\delta^{15}N$ (here most likely indicating feeding at higher trophic levels) and fatty acid profiles indicative of elevated nutritional status (Fig. 8).

In a range of vertebrates, female dominance is often correlated with age or body size (e.g., Stockley & Bro-Jørgensen, 2011; Maréchal, Semple, Majolo, & MacLarnon, 2016; Francis et al., 2020). The pattern found here of higher residency of larger (>300 cm TL) tiger sharks at provisioning sites compared to the higher residency of smaller tiger sharks (<300 cm TL) at non-provisioning sites, suggests that size-based dominance interactions may be occurring, whereby larger sharks are excluding smaller individuals from the provision sites (Fig. 8). Indeed, size-based dominance interactions have previously been described in tiger sharks during temporary aggregations. Specifically, large females (>3.8m TL) exhibited dominance over smaller individuals (from 3 to 3.8m TL) while feeding from a whale carcass (Clua, Chauvet, Read, Werry, & Lee, 2013). Similar behavior has been described in white sharks (*Carcharodon carcharias*) at both seal hunting sites (Martin, Rossmo, & Hammerschlag, 2009) and whale carcasses (Dicken, 2008, Fallows, Gallagher, & Hammerschlag, 2013). In the tourism provisioning context, size-dependent dominance has also been observed in female Caribbean reef sharks *Carcharhinus perezii* (Maljkovic & Côté, 2011) and in female stingrays (e.g., *Bathytoshia brevicaudata*, Newsome, Lewis, & Moncrieff, 2004; *Hypanus americanus*, Semeniuk & Rothley, 2008).

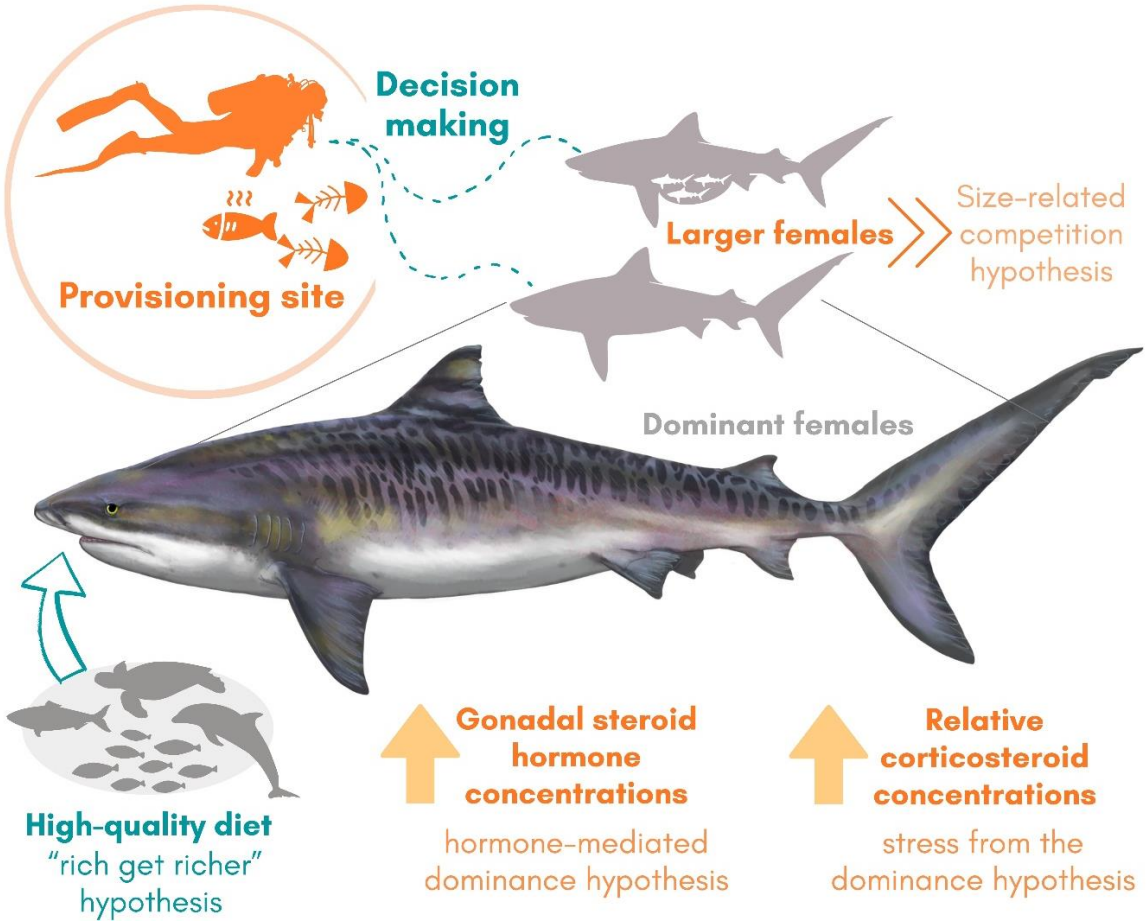

Figure 8. Conceptual figure summarizing the physiological state of female tiger sharks (*Galeocerdo cuvier*) which spent proportionally more time at food provisioning sites. The findings suggested that larger females, especially non-gravid individuals, with higher hormone levels (i.e. testosterone, 17β-estradiol, relative corticosteroid), enriched δ15N, and in better nutritional status (in terms of fatty acids), spent proportionally more time at the food provisioning sites compared to conspecifics. *A posteriori* hypotheses explaining the results are indicated and discussed for each result.

Although body size seems to play a role in observed patterns of space use, its overall importance remains unclear. Indeed, large gravid females had more variable spatial behavioral patterns compared to mature, but non-gravid females, suggesting the reproductive state and maybe the plasma testosterone levels have an influence on behavior (Fig. 8). For example, changes in shark behavior associated with their reproductive cycle have been reported for provisioned sicklefin lemon sharks, in which males increase aggression during mating (Clua, Buray, Legendre, Mourier, & Planes, 2010), probably associated with increased testosterone levels during this period (e.g., Awruch, 2013). Testosterone is naturally produced by female tiger sharks during the reproductive cycle (Sulikowski et al., 2016), however, its role in viviparous sharks remains unclear (Awruch, 2013; Sulikowski et al., 2016; Becerril-García et al., 2020). Results from previous studies suggest the role of testosterone as a

substrate for estradiol biosynthesis, which could explain the high 17β-estradiol concentrations observed in tiger sharks with increased testosterone in the present study. Testosterone is also believed to be involved in follicular development and reproductive behavior during shark mating (Awruch, 2013; Becerril-García et al., 2020). While speculated, no study has systematically investigated the androgen's role in reproductive behavior in sharks (Becerril-García et al., 2020).

Testosterone levels were significantly correlated with the proportion of time spent at provisioning sites. One possible explanation is that females with naturally elevated testosterone levels, as a response of their reproductive cycle, spent more time at provisioning sites. However, competition among females at the provisioning sites could also promote elevate testosterone levels. Indeed, during cold months, tiger shark abundance in the study area is relatively high (Hammerschlag et al., 2015, 2017), which could increase competition for concentrated food resources, including at the provisioning sites. This competition could induce physiological and behavioral responses that promote female dominance. In fact, level of aggression has been observed as an important factor of dominance in female tiger sharks during feeding events (Clua, Chauvet, Read, Werry, & Lee, 2013), and therefore, it is plausible that competitive dominance could be related to the hormonal levels observed in the present study. Like in males, testosterone can improve competitive abilities of females through mechanisms including enhanced muscle functioning and increased aggression (e.g., Stockley & Bro-Jørgensen, 2011; Cain & Ketterson, 2012; Rosvall et al., 2020).

Female tiger sharks with higher relative corticosteroid levels spent more time at provisioning sites. One possible explanation is that corticosteroids are also involved in social competition and dominance, along with the testosterone (MacDougall-Shackleton et al., 2019). Although historically considered "stress indicators", corticosteroids play diverse roles in the biology of vertebrates (MacDougall-Shackleton et al., 2019). Such a relationship could be explained by the 'stress from the dominance hypothesis' (Fig. 8), which predicts that dominant individuals will have increased glucocorticoids levels as a response of increased allostatic load (Goymann & Wingfield, 2004; Cavigelli & Caruso, 2015). Additionally, since the majority of females using provisioning sites were non-gravid females exhibiting significantly higher hormones, another possible explanation is that the high energetic demand to build gonadal tissue during the pre-mating reproductive stage (e.g., vitellogenesis, embryotrophe production; Castro, 2009, Castro, Sato, & Bodine, 2016) could be driving the high use of provisioning sites, whereby sharks have learned to restrict their movements and focus on those sites with the lowest costs of foraging. However, the most parsimonious

explanation may simply be that differences in corticosteroid levels between high and low users is just a byproduct of the fact that there were slightly more non-gravid individuals in the high user group. Future studies using a more robust sample size should be conducted to test these hypotheses. Also, experimental and comparative studies (e.g., analyzing hormonal variation throughout the reproductive cycle in sites without tourism activities) could be useful to understand this process.

The higher percentages of n3 PUFA found in female tiger sharks spending more time at provisioning sites indicated they are consuming more n3 PUFA-rich prey compared to those females using the non-provisioning sites, which exhibited higher percentages of SFAs. These results suggest that individuals spending more time at provisioning sites have a better nutritional condition (if we consider only fatty acids) (Fig. 8). Alternatively, it is possible that females achieving social dominance are able to monopolize access to a high-quality food that has a relatively short handling time (Geary, Walter, Leberg, & Karubian, 2019; Francis et al., 2020). In both scenarios, however, any potential dominance hierarchy seems to influence access to resources and possibly have fitness consequences. This because the n3 PUFA are involved in many important physiological processes, such as brain and eye development and immune and inflammatory responses (e.g., Bobe & Labbé, 2010; Lund, Steenfeldt, Suhr, & Hansen, 2008). Additionally, as discussed in detail by Rangel et al. (2021b), n3 PUFAs can directly affect reproductive success, since they act as the main component of sperm and oocyte plasma membranes (Bobe & Labbé 2010). We also found enriched $\delta^{15}N$ values in females spending more time at provisioning sites. This result could be related to the provisioning itself, since tiger sharks are often fed tuna or grouper carcasses, which have relatively high $\delta^{15}N$ values. Therefore, it is also possible that females achieving social dominance at the provisioning sites can monopolize access to these carcasses. For example, higher $\delta^{15}N$ values were found in larger, provisioned (grouper carcasses), and dominant Caribbean reef sharks in the Bahamas, compared to non-fed conspecifics (Maljkovic & Côté, 2011).

It is important to also consider that provisioning tourism activity may not impact diets and nutritional condition of sharks where prey resources are abundant, as found in the case of white sharks in South Australia (Meyer, Pethybridge, Beckmann, Bruce, & Huveneers, 2019). However, additional work is required to better understand our study system, including continuous measures of essential nutrient profiles (e.g., fatty acids, amino acids, total proteins) of tiger sharks and their prey, as well as the bait offered during tourism activities. It is also possible that, despite high nutritional quality, the composition of bait provided during

tourism activities is different from those found in natural shark prey items (Semeniuk et al., 2007). For such evaluations it would be necessary to sample and determine the physiological condition of sharks before and after feeding at the dive sites (e.g., Semeniuk et al., 2007; Meyer et al., 2019), which we could not do in this study.

One notable limitation with this study is the relatively short detection range of the passive acoustic receivers (50% detectability = ~200 m). Since the acoustic receivers are separated by 750 m on average, it is possible that sharks were not detected despite being within or just beyond the boundary of the array. Therefore, these detection data were interpreted as presence data, rather than presence and absence data. Using passive acoustic telemetry also limits our understanding of where tagged individuals go when they leave the boundary of the array. Future studies of this kind may consider both passive acoustics and satellite-linked transmitters to explore spatial movements of sharks.

Although use of multiple physiological markers allowed us to explore the relationship between physiological condition and spatial use of tiger sharks, several caveats exist. First, we used gonadal steroid hormones because of their role in regulating phenotypic responses to dynamic social environments in female vertebrates. However, the role of gonadal steroid hormones in the reproduction and behavior of female tiger sharks remains unclear (Sulikowski et al., 2016). Second, we used relative corticosteroid concentrations as a proxy for the potential effects of 1α-OH-B, the main glucocorticoid in sharks (Evans, Rimoldi, Gadepalli, & Nunez, 2010). Additionally, although plasma fatty acids have been extensively demonstrated as a promising method to assess short-term shifts in the diet and trophic ecology elasmobranchs (e.g., Semeniuk et al., 2007; Hoopes et al., 2020; Rangel et al., 2020, 2021b), this approach is limited in terms of specifically identifying dietary and/or non-dietary origin (e.g., mobilized from storage tissues) using non-lethal methods, as the stomach content was not evaluated.

Future studies combining physiological measurements and social network analysis (e.g., Jacoby et al., 2021; Pini-Fitzsimmons, Knott, & Brown, 2021) could help us better understand the involvement of hormones in the complex social interactions in wild elasmobranchs. In this case, studies could focus on the initial capture of individuals for blood sampling, and then monitoring their behavior during diving activities, through identification tags (e.g., different colored tags) and using ethograms of agonistic behaviors (Pini-Fitzsimmons et al., 2021). Furthermore, validation of hormone assays in non-invasive samples, such as skin mucus, which can be collected during the diving without the need to capture tiger sharks, could be valuable in such behavioral studies (e.g., Carbajal et al., 2019).

In summary, our study suggests that life-stage, endocrine regulation, and nutritional condition influence and/or are influenced by the time wild female tiger sharks spend exposed to provisioning tourism (Fig. 8). Through using multiple physiological markers, combined with passive acoustic telemetry tracking and ultrasonography, we found that large, non-gravid females with higher testosterone 17β-estradiol, relative corticosteroid plasma levels spent proportionally more time at food provisioning sites. Also, nutritional markers indicated that females exhibiting high use of provisioning sites are feeding on higher prey quality (in terms of essential fatty acids) with enriched $\delta^{15}N$. While more studies are needed to explore whether sharks are making these decisions because of their physiological state or whether spending more time at provisioning sites results in altered physiological state, our findings highlight the importance of considering animal life-stage, endocrine regulation, and nutritional condition when evaluating impacts of provisioning tourism.

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Supporting Information

Detailed information for blood hormone analysis

This corticosterone kit has previously been validated to quantify relative 1α-hydroxycorticosterone (1α-OH-B, the primary corticosteroid in elasmobranchs), by exploiting the cross-reactivity of the corticosterone antibody with 1α-OH-B concentrations (Evans, Rimoldi, Gadepalli, & Nunez, 2010) and by excluding other corticosteroids by mass spectrometry (Lyons & Wynne-Edwards, 2019). However, as this approach will not deliver precision for concentrations of 1α-OH-B, and we did not identify other corticosteroids (e.g. cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone) as done by (Lyons & Wynne-Edwards, 2019). Thus, we assumed that the ELISA-based corticosterone values would reflect relative corticosteroids and results herein are referred to as relative corticosteroid concentrations (Rangel et al., 2021a).

Based on the assumptions that: (1) glucocorticoids serve some physiological function including as a stress hormone (MacDougall-Shackleton, Bonier, Romero, & Moore, 2019), (2) tiger sharks exhibit very low stress responses, i.e. exhibit low lactate levels and display more subdued acceleration values during capture (Gallagher et al., 2014; 2016), and (3) that relative corticosteroid concentrations have not been associated with fight time for 12 of the tiger sharks analyzed here (Fig. S1), we assumed the corticosterone results are close to baseline values.

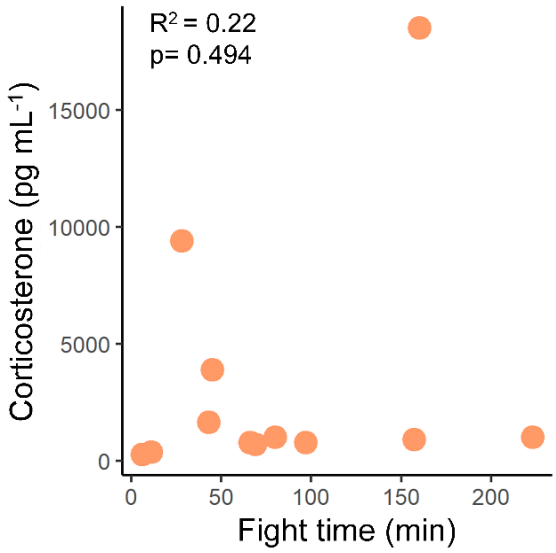

Figure S1. Plot of the interaction between hook time (minutes) and relative corticosteroid levels (n= 12).

Detailed information for stable isotope analysis

For stable isotope analysis, between 2 and 3 mg of wet weight whole blood was added to tins, which were dried overnight at 60 ºC. Samples were combusted in a EuroVector Euro EA elemental analyzer and then passed through a GV Instruments diluter before being introduced to a GV Instruments IsoPrime isotope ratio mass spectrometer. One replicate per ten samples was run, any outliers were rerun, and laboratory standards of glycine and peptone were run once per 15 samples. Lipid and urea extraction were not undertaken for plasma based on the recommendation of Kim et al. (2012) for blood. Ratios of stable isotopes were expressed as parts per thousand using standard d notation. Plasma fatty acid profiles were generated by direct transmethylation, i.e., without lipid extraction, as described by Parrish, Nichols, Pethybridge, and Young (2015).

Detailed information for fatty acid analysis

The samples (100 μL) were homogenized and directly transmethylated in 3 ml of methanol: dichloromethane: concentrated hydrochloric acid solution (10:1:1 v/v) for 2 h at 80–85 °C. Then, 1.5 ml of Milli-Q® water and 1.8 ml of hexane and dichloromethane (4:1 v:v) were added, the tubes were mixed and then centrifuged at 2,000 rpm for 5 min. The upper layer was removed, transferred to 2 ml injection vials, the volume reduced under a nitrogen stream, and then suspended in approximately 300 uL of hexane. Fatty acid analysis was carried out in a gas chromatograph (Varian, Scion 436) coupled with a flame ionization detector (FID) and an auto-sampler (CP 8410). Hydrogen was used as a carrier gas at a linear velocity of 1.4 ml/min cm/s, and the capillary column used was CP Wax, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length. The column was programmed at 170 °C for 1 min, followed by a 2.5 °C/min ramp to 240 °C and a final hold time of 5 min. The injector and FID temperatures were 250 and 260 °C, respectively. Fatty acid methyl esters (FAME) were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualmix, PUFA fish M, Menhaden Oil, Larodan). The data are presented as % of total fatty acid methyl-esters based on peak area analyses.

Fatty acids are subject to biosynthesis when transferred from prey to predator, but they remain relatively unchanged, making them appropriate dietary biomarkers if prey libraries are adequate (Darlsgaard et al., 2003; Iverson, 2009; Budge, Iverson, & Koopman, 2006). We selected physiologically important fatty acids that occur in high percentages in tiger sharks (details in Rangel, Hammerschlag, Sulikowski, & Moreira, 2021b) to test for differences between ‘low’ and ‘high’ use groups. This includes the palmitic acid (C16:0), stearic acid

(C18:0), ΣSFA, docosahexaenoic acid (DHA, C22:6n3), arachidonic acid (ARA, C20:4n6), and the total omega-3 and -6 PUFAs (Σn3 and Σn6 PUFAs), total monounsaturated fatty acids (ΣMUFA) and the bacterial markers odd chain fatty acids (OFA) and branched chain fatty acids (BFA). Specifically, we used ΣPUFA as indicators of high prey quality/nutritional quality. Because vertebrates are unable to synthesize de novo ΣPUFA, they need to obtain these essential fatty acids directly from the diet, as such, the fatty acid signature of an animal is closely linked to the quality and quantity of prey consumed (Budge et al., 2006; Iverson, 2009; Twining, Shipley, & Winkler, 2018).

Table S1. Original model incorporating life stage and month as a random effect to control for variation in testosterone among life stages.

Proportion of time spent in Provisioning			
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	0.00	0.00 – 0.44	**0.036**
log(T)	11.07	1.10 – 111.37	**0.041**
$N_{Individuals}$	21		
Random effects	Variance	Std. Deviation	
Month:Life Stage	1.505e-09	3.879e-05	
Life Stage	4.046e-26	2.011e-13	

Table S2. Mean values ± SD examined for total length and physiological markers, including reproductive hormones (testosterone, 17β-estradiol, and progesterone), metabolic hormone (relative corticosteroids), stable isotopes ($\delta^{15}N$ and $\delta^{13}C$), and fatty acids.

Variables	***N***	**Low use**	***N***	**High use**
Total length *(cm)*	9	293.8 ± 56.1	7	345.5 ± 32.4
Reproductive hormones *(pg ml^{-1})*				
Testosterone	9	89.9 ± 39.4	7	276.5 ± 221.4
17β-estradiol	9	50.8 ± 72.3	7	204.3 ± 213.2
Progesterone	9	42.2 ± 34.1	7	36.9 ± 12.6
Metabolic hormone *(pg ml^{-1})*				
Corticosteroids	9	394 ± 507.0	7	6471.5 ± 9793.9
Stable isotopes *(‰)*				
$\delta^{15}N$	4	10.4 ± 0.8	4	11.7 ± 0.5
$\delta^{13}C$	4	-13.1 ± 1.8	4	-14.5 ± 1.0
Fatty acids *(%)*				
C16:0	9	32.6 ± 4.6	7	25.3 ± 3.7
C18:0	9	11.1 ± 2.2	7	9.6 ± 1.4
C20:4n6 (ARA)	9	6.0 ± 4.0	7	9.0 ± 4.8
C22:6n3 (DHA)	9	5.7 ± 4.6	7	11.9 ± 3.7
ΣSFA	9	49.0 ± 7.2	7	38.7 ± 5.5
ΣMUFA	9	27.8 ± 3.5	7	28.3 ± 4.9
ΣPUFA	9	20.3 ± 10.4	7	31.4 ± 5.2
Σn3 PUFA	9	9.6 ± 5.3	7	16.3 ± 4.3
Σn6 PUFA	9	10.8 ± 5.4	7	15.1 ± 4.7
BFA-OFA	9	2.9 ± 1.7	7	1.6 ± 0.8

Fatty acids: SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ARA: arachidonic acid; DHA: docosahexaenoic acid; BFA: branched-chain fatty acids; OFA: odd-chained fatty acids.

Table S3. Pearson correlations between hormone levels (testosterone, 17β-estradiol, and progesterone) and metabolic hormone (relative corticosteroids) and proportion of time spent at provisioning sites in mature, but non-gravid female tiger sharks (*Galeocerdo cuvier*). Significant ($p < 0.05$) results are **bolded**.

Reproductive hormones *(pg ml^{-1})*	**r**	*t*-value	**df**	*p*-value
Testosterone	**0.77**	**3.23**	**7**	**0.014**
17β-estradiol	0.11	0.29	7	0.773
Progesterone	-0.11	-0.31	7	0.767
Metabolic hormone *(pg ml^{-1})*				
Corticosteroids	0.53	1.64	7	0.146

Table S4. Mean values ± SD examined for reproductive hormones (testosterone, 17β-estradiol, and progesterone) and metabolic hormone (relative corticosteroids) in mature, but non-gravid females tiger sharks (*Galeocerdo cuvier*) when compared between high users (n= 5) and low users (n= 4). *t* Score values for Student's *t*-test. Significant ($p < 0.05$) results are **bolded**.

Mature, but non-gravid females	**Proportion of time at provisioning sites**		**Student *t*-test**	
	< 30%	**> 70%**	*t*-value	*p*-value
Reproductive hormones *(pg ml^{-1})*				
Testosterone	**119.5 ± 29.8**	**346.7 ± 248.7**	**3.01**	**0.019**
17β-estradiol	199.4 ± 127.4	289.5 ± 215.5	0.03	0.979
Progesterone	136.5 ± 150.3	38.5 ± 5.7	0.68	0.520
Metabolic hormone *(pg ml^{-1})*				
Corticosteroids	10779.1 ± 11576.3	1427.1 ± 1674.9	1.38	0.210

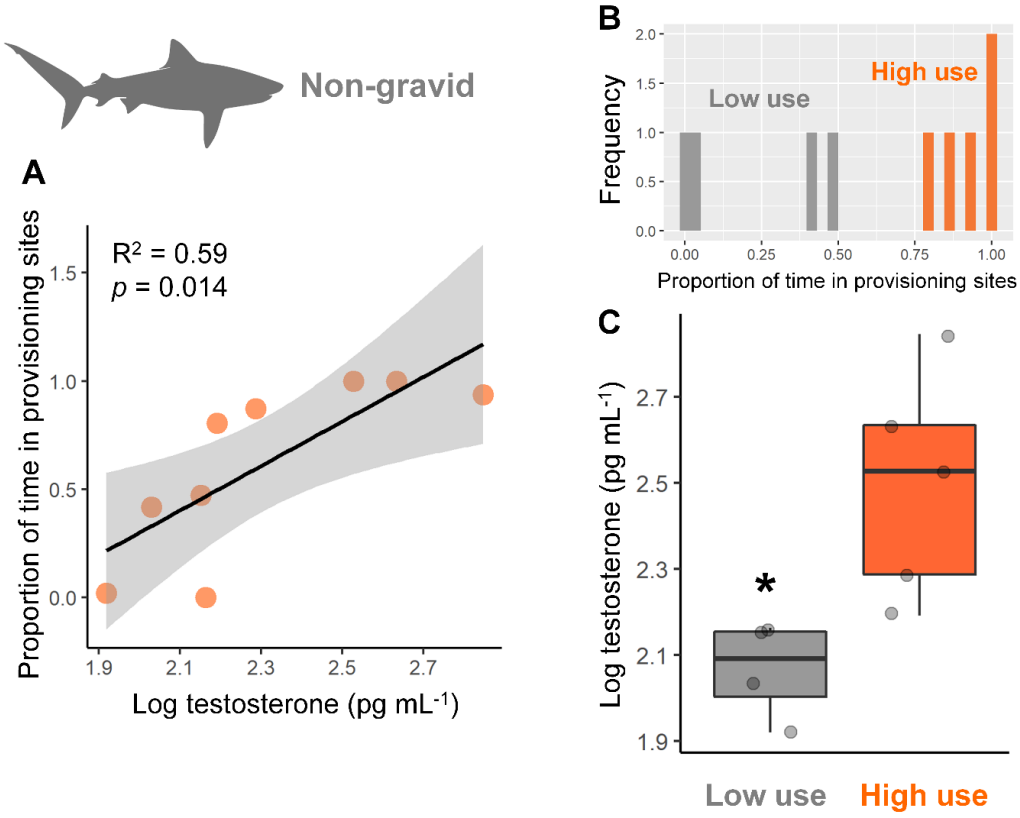

Figure S2. (A) Correlation between testosterone and proportion of time spent at provisioning sites in mature, but non-gravid female tiger sharks (*Galeocerdo cuvier*) (n= 9). Based on the proportions of time spent at provisioning sites (B), mature, but non-gravid females were categorized as showing 'low (< 70% of time, n= 4) or 'high (>70% of time, n= 5) use of provisioning dive sites. (C) Comparison of testosterone between low and high use groups. Black line indicates the median value. Significant difference between groups is indicated by asterisks (Student t-test *p < 0.05).

Table S5. Results of SIMPER analysis relative to comparative of plasma fatty acid profile of female tiger sharks (*Galeocerdo cuvier*) between provisioning site low (n= 9) and high (n= 7) use groups.

Fatty acids	Av. dissim	Contrib. %	Cumulative %	Mean Low use	Mean High use
DHA	2.628	21.37	21.37	0.551	1.05
ARA	2.191	17.82	39.18	0.654	0.824
Σn3 PUFA	1.608	13.08	52.26	0.898	1.2
BFA-OFA	1.486	12.08	64.34	0.419	0.153
ΣPUFA	1.323	10.76	75.1	1.24	1.49
Σn6 PUFA	1.226	9.969	85.07	0.974	1.16
C16:0	0.5505	4.476	89.55	1.51	1.4
ΣSFA	0.5288	4.3	93.85	1.69	1.58
C18:0	0.4447	3.616	97.46	1.04	0.978
ΣMUFA	0.3118	2.536	100	1.44	1.45

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Conclusões e Considerações Finais

Esta tese teve como objetivo investigar variações fisiológicas sazonais e espaciais associadas ao estágio de vida e comportamento de tubarões, utilizando múltiplas ferramentas não-letais para fornecer uma melhor compreensão dos padrões energéticos e reprodutivos, além de uma base fisiológica que ajude a prever os efeitos de distúrbios ambientais nos tubarões. Os resultados mostraram que os biomarcadores nutricionais, reprodutivos e metabólicos aqui usados fornecem uma poderosa ferramenta para descrever padrões ecológicos complexos como interação alimentar em um sistema insular oceânico (Capítulo 1), variações nos padrões de dieta e condição nutricional e metabólica para a reprodução (Capítulos 2 e 3). Além disso, esses biomarcadores mostraram-se especialmente úteis para entender a influência da urbanização na condição nutricional e metabólica de tubarões que vivem em locais altamente alterados (Capítulos 4, 5 e 6) e como que a condição fisiológica parece ser um importante modulador na expressão de comportamentos de tubarões expostos ao turismo de alimentação (Capítulo 7).

O estudo de espécies de diferentes histórias de vida foi especialmente importante para entender particularidades de aspectos ecofisiológicos relacionados à reprodução e efeitos da urbanização. Por exemplo, machos de tubarões-galha-preta (*Carcharhinus limbatus*) e de tubarões-lixa (*Ginglymostoma cirratum*) parecem diferir em sua estratégia energética para financiar a reprodução talvez como resultado de suas ecologias divergentes (sedentário e bentônico = tubarão-lixa versus ativo e epipelágico = tubarões- galha-preta). Enquanto tubarões-lixa mostram padrões de criadores de capital (*capital breeders*), ou seja, usando recursos endógenos armazenados (normalmente lipídios) durante o período de cópula, os tubarões-galha-preta se assemelham mais a criadores de renda (*income breeders*), que normalmente utilizam recursos exógenos (normalmente carboidratos) para financiar a reprodução. Em relação aos efeitos da urbanização, os resultados mostraram que os efeitos de viver perto de uma cidade grande parecem ser mais pronunciados em espécies de tubarões sedentários, como o tubarão-lixa (Capítulo 4), quando comparado com espécies mais ativas, como os tubarões-galha-preta (Capítulo 5) e tubarões-tigre (*Galeocerdo cuvier*, Capítulo 6) (Fig. 1). Como os tubarões-lixa não se movimentam muito, eles provavelmente são mais expostos à poluição (química, sonora e luminosa) provenientes da cidade em comparação com outras espécies que tendem a se movimentar mais.

Figura 1. Resumo dos resultados para tubarões urbanos, incluindo o tubarão-lixa (*Ginglymostoma cirratum*), ponta-preta (*Carcharhinus limbatus*) e tubarão-tigre (*Galeocerdo cuvier*). Ilustração dos tubarões-lixa e tigre cortesia de Kelly Quinn e ilustração do tubarão-galha-preta cortesia de Alexandre Huber. Outras imagens são do Canva (www.canva.com).

O capítulo 7 trouxe uma abordagem inédita na pesquisa de tubarões, combinando múltiplos marcadores fisiológicos com informações do estágio reprodutivo e de movimentação dos tubarões obtidas através de ultrassonografia e da telemetria acústica passiva, respectivamente, para entender relações entre os aspectos fisiológicos e comportamento de tubarões expostos ao turismo de alimentação. Os resultados demonstraram que o estágio de vida, a regulação endócrina e a condição nutricional influenciam e/ou são influenciadas pelo tempo que os tubarões-tigre selvagens passam expostos ao turismo de alimentação.

Este também é o primeiro trabalho que realiza uma pesquisa não-letal voltada para o estudo da ecologia nutricional de tubarões no Brasil e em uma ilha oceânica remota no Oceano Atlântico Sul. Os dados gerados poderão agora servir de base para estudos e monitoramento a longo prazo dos padrões ecofisiológicos, especialmente considerando que Fernando de Noronha é um arquipélago oceânico exposto à crescente visitação turística. O trabalho gerado com os tubarões no arquipélago foi além da importância cientifica dos dados gerados, uma vez que foi liderado por uma jovem cientista mulher e permitiu o envolvimento mais próximo com os gestores, pesquisadores comunidade local. Os resultados da presente

tese contribuiriam ainda com importantes informações para o monitoramento a longo prazo da saúde dos tubarões realizado no sul da Flórida e nas Bahamas desde 2011.