ECOLOGIA TRÓFICA DE FÊMEAS ADULTAS DA TARTARUGA-VERDE (*CHELONIA MYDAS*) QUE NIDIFICAM NO ATOL DAS ROCAS: UMA ABORDAGEM ISOTÓPICA

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UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE - UENF

CAMPOS DOS GOYTACAZES – RJ

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Orientadora: Profª. Drª. Ana Paula Madeira Di Beneditto
Coorientador: Prof. Dr. Carlos Eduardo Veiga de Carvalho

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Governo do Estado do Rio de Janeiro

Universidade Estadual do Norte Fluminense Darcy Ribeiro

Pró-Reitoria de Pesquisa e Pós-Graduação

DECLARAÇÃO

Eu, Marina Satika Suzuki, coordenadora do Programa de Pós-Graduação em Ecologia e Recursos Naturais (PPG-ERN) da Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), seguindo a Resolução CPPG nº2 de 2021, declaro validadas as assinaturas constantes da Folha de Assinaturas da Tese intitulada "**Ecologia trófica de fêmeas adultas da tartaruga verde (*Chelonia mydas*) que nidificam no Atol das Rocas: Uma abordagem isotópica"** de autoria de Karoline Fernanda Ferreira Agostinho, defendida no dia 08 de março de 2023.

Campos dos Goytacazes, 18 de maio de 2023

Marina Satika Suzuki
Coordenadora PPG-ERN / UENF
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“Quando eu penso em desistir eu subo no farol, e do alto do farol eu vejo todo o Atol e grande parte da área da Unidade e eu tenho o retrato de todo esse lugar, eu conheço cada pedacinho desse lugar, até as pedras eu conheço, até as Rocas. E eu olho e digo: você não pode desistir, olha o que você fez [...] E essa é minha maior vitória. É que existe essa parceria, esse amor e esse compromisso entre nós dois.”

Zélia Brito – Chefe da Reserva Biológica do Atol das Rocas

Fugindo do trivial, dedico esta tese as muitas versões de mim mesma que viveram esse percurso, umas mais serenas, outras mais sombrias, mas todas cientes de que, no final, essa trajetória me deixaria orgulhosa pelo que eu construí.

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"Stable isotopes as measure of niche breadth of nesting green turtles (*Chelonia mydas*) on Rocas Atoll, Brazil"

Capítulo 4:
"Trace elements and stable isotopes in egg yolk of green turtles on Rocas Atoll, Brazil"

LISTA DE ABREVIAÇÕES

ANOVA – Analysis of Variance
As – Arsênio
Ba – Bário
CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CBB – Centro de Biociências e Biotecnologia
CCL – Curved Carapace Length
Cd – Cádmio
CD – Mean Distance to Centroid
CNPQ – Conselho Nacional de Desenvolvimento Cientifico e Tecnológico
CR – Variability in Basal Resources
Cu – Cobre
CV – Coefficient of Variation
FAPERJ – Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro
Fe – Ferro
FUNBIO – Fundo Brasileiro para a Biodiversidade
GLM's – Generalized Linear Models
H_2O_2 – Peróxido de hidrogênio
HNO_3 – Ácido nítrico
IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
ICMBio – Instituto Chico Mendes para Conservação da Biodiversidade
ICP-OES – Espectrometria de Emissão Atômica por Plasma Acoplado Indutivamente
IQR – Interquartile Range
IUCN – International Union for Conservartion of Nature
LCA – Laboratório de Ciências Ambientais
LOD – Limit of Detection
LQ – Lower Quartile
MMA – Ministério do Meio Ambiente
Mn – Manganês
MNND – Mean Nearest Neighbor Distance
NR – Trophic Breadth
***p* –** Probabilidade da hipótese nula (H0) de um teste estatístico ser verdadeira
Pb – Chumbo
PDB – Pee Dee Belemnite
R_2 – Coeficiente de determinaçãomúltiplo de um modelo geral ou generalizado de regressão
SEA – Standard Ellipse Area
SIBER – Stable Isotope Bayesian Ellipses in R
SISBIO – Sistema de Autorização e Informação em Biodiversidade
SNND – Standard deviation of Nearest Neighbour Distance
U – Estatística de espaçamento de RAO
UQ – Upper Quartile.
Zn – Zinco
$\delta^{13}C$ – Resultado de ($^{13}C/^{12}C$amostra **/** $^{13}C/^{12}C$ da formação Pee Dee -1) * 1000
$\delta^{15}N$ – Resultado de ($^{15}N/^{14}N$amostra / $^{15}N/^{14}N$ do nitrogênio atmosférico -1) *1000
$2\pi/n$ – Hipótese nula de uniformidade

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RESUMO

Esta tese teve como objetivo geral compreender a ecologia trófica das tartarugas verdes que nidificam no Atol das Rocas utilizando os isótopos estáveis de carbono e nitrogênio como principal método de análise e interpretação dos resultados. O capítulo 1 apresenta o perfil isotópico das fêmeas reprodutivas, avalia a consistência temporal dos padrões tróficos e verifica se o vitelo pode ser um preditor dos valores isotópicos de carbono e nitrogênio presentes no sangue das fêmeas amostradas. O capítulo 2, através da análise de dois tecidos com diferentes taxas de *turnover*, utilizou a abordagem de nicho isotópico para compreender como esses indivíduos utilizam os recursos alimentares ao longo do tempo. Já o capítulo 3 avaliou a consistência dos nichos tróficos das tartarugas verdes por meio da análise de dois tecidos com diferentes taxas de renovação. O capítulo 4 utilizou os isótopos estáveis de carbono e nitrogênio junto a alguns elementos traço essenciais e não essenciais para avaliar se esses dois marcadores químicos são adequados para rastrear a área de alimentação desses indivíduos. Em resumo, os resultados encontrados nos capítulos supracitados sugerem que as fêmeas que utilizam o Atol das Rocas como área de nidificação possuem uma alimentação preferencialmente herbívora, destacada pelos valores de nitrogênio do vitelo, com contribuição de matéria animal a longo prazo, conforme valores observados na análise da carapaça. Além disso, conforme apresentado nos capítulos 1, 2 e 3, esses animais tendem a se alimentar em regiões costeiro-bentônicas. No capítulo 2, que apresentou uma amplitude de nicho semelhante entre os dois anos de amostragem, houve semelhança na maneira como os indivíduos utilizam o nicho trófico. Já quando os indivíduos foram analisados de forma individual, foi possível observar que a maioria apresentou variação na estratégia alimentar ao longo do tempo. Para os dados desta tese, a associação entre os elementos traço e os isótopos de carbono e nitrogênio não foi suficiente para rastrear as áreas de alimentação desses animais. Este estudo apresentou informações inéditas que contribuem para a compreensão da ecologia trófica das tartarugas verdes que usam o Atol das Rocas como área de nidificação. Entretanto, a união de múltiplas abordagens em futuro próximo pode ser interessante para consolidar a interpretação dos resultados relacionados a ecologia trófica desses animais. Por fim, além da necessidade de haver um interesse global em investigar esses animais altamente migratórios, estudos que visem compreender as relações da tartaruga-verde com o Atol das Rocas servem também como reforço para manter essa área constantemente protegida.

Palavras-chave: área de reprodução; composição isotópica; isótopos estáveis; Oceano Atlântico Sul; tartarugas marinhas.

ABSTRACT

This thesis aimed to understand the trophic ecology of green turtles that nest in Rocas Atoll using stable isotopes of carbon and nitrogen as the main method of analysis and interpretation of the results. Chapter 1 presents the isotopic profile of reproductive females, evaluates the temporal consistency of trophic patterns, and checks if the egg yolk can be a predictor of the isotopic values of carbon and nitrogen present in the blood of the sampled females. In addition, this chapter presents other studies that make up the literature on this topic for the adult green turtle. Chapter 2, through the analysis of two tissues with different turnover rates, used the isotopic niche approach to understand how these individuals use food resources over time. Chapter 3 evaluated the consistency of trophic niches in green turtles through the analysis of two tissues with different turnover rates. Chapter 4 used the stable isotopes of carbon and nitrogen together with some essential and non-essential trace elements to assess if these two chemical proxies are suitable for tracking the feeding area of these individuals. In summary, the results found in the above-mentioned chapters suggest that females that use Rocas Atoll as a nesting area have a preferentially herbivorous diet, highlighted by the values of nitrogen in the egg yolk, with a contribution of animal prey in the long-term, values observed in the carapace. Furthermore, as presented in chapters 1, 2, and 3 these animals tend to feed in coastal-benthic regions. In Chapter 2, which presented a similar niche breadth between two years of sampling, there was a similarity in the way in which individuals use the trophic niche. When the individuals were analyzed individually, it was possible to observe that the majority presented variation in the food strategy over time. For the data of this thesis, the association between trace elements and isotopes of carbon and nitrogen was not enough to track the feeding areas of these animals. This study presented new information that contributes to understanding the trophic ecology of green turtles that use Rocas Atoll as a nesting area. However, the combination of multiple approaches in the near future may be interesting to consolidate the interpretation of the results related to the trophic ecology of these animals. Finally, in addition to the need for a global interest in investigating these highly migratory animals, studies that aim to understand the relationship between the green turtle and the Rocas Atoll also serve as a reinforcement to keep this area constantly protected.

Keywords: reproduction area; isotopic composition; stable isotopes; South Atlantic Ocean; sea turtles.

ORGANIZAÇÃO DA TESE

Esta tese de doutorado está redigida em forma de capítulos e é composta de:

1. Introdução geral, que caracteriza a tartaruga-verde, incluindo sua biologia e aspectos reprodutivos; define isótopos estáveis e esclarece como eles podem ser utilizados no entendimento da ecologia trófica de indivíduos adultos de tartarugas marinhas e apresenta o Atol das Rocas como área de estudo.

2. Quatro capítulos que reproduzem os artigos científicos publicados no âmbito desta tese de doutorado, e investigam a ecologia trófica das fêmeas reprodutivas da espécie *Chelonia mydas* que utilizam o Atol das Rocas como área de nidificação. Cada capítulo possui resumo, introdução, metodologia, resultados, discussão, conclusão e referências no formato de cada revista nas quais foram publicados ou submetidos:

Capítulo 1 **–** Isotopic profile of female green turtles nesting on Rocas Atoll, northeastern Brazil. Situação: Em preparação.

Capítulo 2 **–** Stable isotopes as measure of niche breadth of nesting green turtles (*Chelonia mydas*) on Rocas Atoll, Brazil. Situação: publicado no periódico Revista Ibero-Americana de Ciências Ambientais.

Capítulo 3 **–** Individual niche trajectories in nesting green turtles on Rocas Atoll, Brazil: an isotopic tool to assess diet shifts over time. Situação: publicado no periódico Biota Neotropica.

Capítulo 4 **–** Trace elements and stable isotopes in egg yolk of green turtles on Rocas Atoll, Brazil. Situação: publicado no periódico Marine Pollution Bulletin.

3. Discussão geral, que sintetiza os principais resultados dos quatro capítulos supracitados.

1. Introdução geral

1.1. A tartaruga-verde, *Chelonia mydas*

A tartaruga-verde, *Chelonia mydas* (Linnaeus, 1758), pertence à ordem Testudines, subordem Cryptodira e família Cheloniidae, e é uma das sete espécies viventes de tartarugas marinhas. Morfologicamente, a tartaruga-verde é caracterizada pela presença de quatro escudos laterais na carapaça, um par de escamas pré-frontais e quatro pares de escamas pós-orbitais na cabeça, além de mandíbula serrilhada. As populações do Oceano Atlântico possuem carapaça de coloração marrom-esverdeada, e seus filhotes se diferenciam das demais espécies por apresentarem o plastrão branco, que se torna amarelado durante o crescimento (Márquez, 1990; Pritchard e Mortimer, 2000).

Segundo a União Internacional para Conservação da Natureza, o status de conservação da tartaruga-verde é 'Em Perigo (EN)' (IUCN, 2022). Entretanto, no Oceano Atlântico Sul, algumas subpopulações apresentaram dados recentes de recuperação (Broderick e Patrício, 2019) e no Brasil, graças ao sucesso das ações dos programas de conservação, a espécie deixou a Lista de Espécies Ameaçadas de Extinção (Portaria MMA nº 148, de 7 de junho de 2022). Apesar disso, todos os estágios do ciclo de vida das tartarugas marinhas seguem sendo protegidos por lei - Portaria IBAMA n.º 1.522 de 19 de dezembro de 1989.

A espécie se distribui em águas tropicais e subtropicais dos Oceanos Atlântico, Pacífico, Índico e Mar Mediterrâneo, e eventualmente, há registros em regiões temperadas (Márquez, 1990; Seminoff, 2004; Almeida et al., 2011). As migrações entre as áreas de forrageamento e nidificação podem incluir deslocamentos por milhares de quilômetros (Hays et al., 1999).

No Oceano Atlântico, as áreas prioritárias de desova da tartaruga-verde estão localizadas em águas tropicais. No Brasil, a reprodução ocorre preferencialmente em ilhas oceânicas: ilha da Trindade, no estado do Espírito Santo, Atol das Rocas, no estado do Rio Grande do Norte, e Fernando de Noronha, no estado de Pernambuco, com desovas ocasionais na costa dos estados do Rio Grande do Norte, Sergipe, Bahia e Espírito San(queto (Almeida et al., 2011; Marcovaldi et al., 2011).

A idade de alcance da maturidade sexual pode variar entre populações e entre indivíduos de uma mesma população. A espécie apresenta maturação sexual tardia,

estimada entre 15 e 50 anos, dependendo da população (Bjorndal e Zug, 1995; Seminoff et al., 2002; Balazs e Chaloupka, 2004; Bell et al., 2005; Watson, 2006; Goshe et al., 2010). Em geral, os indivíduos atingem a maturidade enquanto estão nas áreas de forrageamento (Miller, 1997). A tartaruga-verde pode atingir até 140 cm de comprimento da carapaça, e as taxas de crescimento estão relacionadas com a quantidade e a qualidade do alimento disponível em suas áreas de forrageamento (Bjorndal et al., 1983; Bjorndal, 1997).

A formação do vitelo dos ovos (vitelogênese) ocorre de 4 a 6 meses antes da temporada de reprodução, quando há aumento de estradiol sérico, de vitelogenina e de cálcio no sangue das fêmeas (Rostal et al., 1998). O período reprodutivo é regulado por hormônios, que no caso das fêmeas favorecem a receptividade em relação à corte do macho e a maturação dos óvulos (Owens, 1980). A cópula ocorre em locais próximos à área de nidificação, denominados "*courtship areas*" ou áreas de corte, ou durante a migração, cerca de um (1) mês antes do início das posturas (Owens, 1980; Fitzsimmons et al., 1997).

Os óvulos são liberados simultaneamente pelos dois ovários e, após a fertilização, passam pela fase de albuminação, se deslocando para o oviduto inferior. No oviduto ocorre a deposição das membranas da casca e a calcificação do ovo. Concomitantemente à calcificação dos ovos mais desenvolvidos, novos folículos ovarianos iniciam o processo de maturação. A ovulação seguinte ocorre de um a dois dias após a postura dos ovos, e o processo é então reiniciado (Owens, 1980). A mesma fêmea realiza em média cinco posturas a cada temporada de reprodução, retornando à praia a cada 12 dias, período denominado como "intervalo internidal". A migração para o mesmo local de desova é intermitente e ocorre a cada três ou quatro anos (Bellini et al., 2013).

As fêmeas retornam às praias onde nasceram para realizar a postura dos ovos, caracterizando o comportamento de filopatria (Fitzsimmons et al., 1997; Naro-Maciel et al., 2012). O ninho é escavado com as nadadeiras posteriores, acima da preamar, e, após a postura, ele é coberto por areia, camuflado e a fêmea retorna ao mar (Hailman e Elowson, 1992). Os ovos são esféricos, têm casca maleável e são recobertos por fluido transparente e espesso com propriedades antimicrobianas (Praja et al., 2021). No Atol das Rocas, o número médio de ovos em cada ninho é 120, mas os registros indicam variações de 19 a 211 ovos. O tempo médio de incubação dos ovos varia de 51 a 75 dias (Bellini et al., 1996).

O sexo das tartarugas marinhas é determinado pela temperatura da areia na área de nidificação. A temperatura ideal para o nascimento equilibrado de fêmeas e machos fica em torno de 29º (Broderick et al., 2000; Godfrey e Mrosovski, 2006). Não há cuidado parental, mas as fêmeas transferem nutrientes e elementos traço para o embrião através do vitelo, que é a sua fonte de nutrição. A qualidade do vitelo está relacionada com a alimentação da fêmea nas áreas de forrageamento, onde a vitelogênese se inicia (Miller, 1997). O tamanho e a qualidade do ovo interferirão na viabilidade e no desenvolvimento do embrião (Hewavisenthi e Parmenter, 2001).

O nascimento dos filhotes ocorre, de forma geral, no final do dia ou antes de amanhecer, quando não há incidência de luz solar ou quando ela é mínima (Miller, 1997; Lohmann et al., 1997). Os filhotes se orientam até o mar através da luminosidade no horizonte (ou da lua) e da umidade. Esse percurso é denominado '*imprinting'*, quando o filhote memoriza as características geomagnéticas e/ou químicas do local de nascimento para retorno anos depois, quando estiver apto a se reproduzir (Lohman et al., 1997; 2013).

Assim que deixam o ambiente costeiro, os filhotes iniciam a fase de migração oceânica que pode durar vários anos (Meylan e Meylan, 1999). Essa fase foi denominada como "*lost years*", anos perdidos em tradução literal, devido à carência de informações acerca desse período do ciclo de vida (Carr, 1987; Reich et al., 2007). Alguns estudos sugeriram que durante os anos iniciais de vida os filhotes permanecem em superfícies formadas por algas flutuantes à deriva, nas correntezas próximas as áreas onde nasceram, em uma associação que lhes fornece segurança contra potenciais predadores e conforto térmico (Mansfield et al., 2014; Putman e Mansfield, 2015). No entanto, atualmente há informações sobre o período, que foram obtidas através da colocação de tags satelitais nos filhotes (Mansfield et al.,2021) .

Antes de realizarem a migração para áreas de forrageamento costeiras, os filhotes de tartaruga-verde são preferencialmente carnívoros, e se alimentam de crustáceos, cnidários e ctenóforos (Bjorndal, 1997; Musick e Limpus, 1997). Na fase juvenil, quando são recrutados para águas costeiras, os animais realizam troca trófica e passam a ser preferencialmente herbívoros, apesar do registro de presa animal em sua alimentação (Di Beneditto et al., 2017; 2019). Em geral, a tartaruga-verde é preferencialmente herbívora na fase adulta, se alimentando de macroalgas e gramas marinhas (Bjorndal et al., 1997). No entanto, estudos sugerem que alguns indivíduos são onívoros nesta fase, a depender da disponibilidade de alimento nas áreas de

forrageamento (Hays et al., 1999; Hatase et al., 2006).

A alimentação das tartarugas marinhas durante o período reprodutivo ainda não é bem compreendida. Tucker e Read (2001) afirmaram que os indivíduos da tartaruga-verde reduzem a frequência alimentar, enquanto Plot et al. (2013) afirmaram que na tartaruga-de-couro não há atividade alimentar nesse período. Carr et al. (1974) justificaram a ausência de atividade alimentar na reprodução da tartaruga-verde como forma de minimizar o gasto energético decorrente da busca e captura do alimento. Em contrapartida, há autores que constataram a alimentação das tartarugas-verdes e tartarugas-oliva durante o período reprodutivo, associando-a à disponibilidade de itens alimentares no entorno das áreas de nidificação (Hays et al., 2002; Petitet e Bugoni, 2017). Independentemente dessa controvérsia, as espécies variam quanto aos seus itens alimentares preferenciais, e essa variação também pode ter caráter intraespecífico, dependendo da disponibilidade de itens alimentares ao longo das áreas de uso e dos gradientes latitudinais (Hawkes et al., 2006; Wallace et al., 2010; Ferreira et al., 2018).

Para aprimorar o entendimento do hábito alimentar e da ecologia trófica das tartarugas marinhas, nas últimas duas décadas houve o incremento de abordagens que utilizam os isótopos estáveis como método acessório a outros métodos, ou mesmo como método principal de investigação (Figgener et al., 2019).

1.2 Isótopos estáveis como ferramenta para o entendimento da ecologia trófica de indivíduos adultos de tartarugas marinhas

Isótopos são átomos de um mesmo elemento químico que possuem o mesmo número de prótons, mas diferem no número de nêutrons. Os isótopos que não se decompõem em outros elementos e que possuem combinações estáveis de prótons e nêutrons são denominados como isótopos estáveis. Os isótopos encontrados em maior abundância na natureza são os de carbono (C), nitrogênio (N), oxigênio (O), hidrogênio (H) e enxofre (S) (Fry, 2006; Martinelli et al., 2009). Dentre estes, os isótopos ^{13}C e ^{15}N são os mais utilizados em estudos sobre ecologia trófica (Martínez del Río et al., 2009).

A composição isotópica é determinada pela relação de um isótopo pesado com outro mais leve (Martinelli et al., 2009). As razões isotópicas são expressas por meio da notação delta (δ) e os valores finais são expressos em partes por mil (‰). A equação que simplifica a razão isotópica é: $\delta^{b}X = [(R_{amostra} / R_{padrão})-1]*1000$, em que

δ é a razão isotópica da amostra a ser avaliada em relação ao padrão internacional pré-estabelecido para o isótopo analisado; b representa a massa do isótopo mais pesado; e $R_{amostra}$ e $R_{padrão}$ simbolizam as frações dos isótopos mais leves e mais pesados presentes na amostra e no padrão, respectivamente (Fry, 2006; Martinelli et al., 2009).

A composição isotópica de um animal é derivada da trajetória trófica e do habitat em que está inserido (Newsome et al., 2010). Portanto, a partir dos resultados isotópicos dos tecidos corporais é possível traçar a trajetória trófica em diferentes escalas temporais e obter indicações das fontes alimentares assimiladas após os processos de digestão e excreção (Fry, 2006). Embora a composição isotópica reflita a alimentação do organismo, existe diferença entre os valores do alimento e do consumidor, que é denominado como fator de discriminação isotópica (Reich et al., 2008; Nielsen et al., 2018).

Em geral, o isótopo ^{13}C tem pouco enriquecimento ao longo dos níveis tróficos (0-1‰), mas é eficiente no reconhecimento da origem das fontes basais da cadeia trófica (e.g., costeira vs. oceânica, pelágica vs. bentônica, aquática vs. terrestre) e inferências latitudinais sobre os locais de alimentação (Peterson e Fry, 1987; Post, 2002; Petitet e Bugoni, 2017; Di Beneditto et al., 2019). O isótopo ^{15}N, por sua vez, apresenta maior enriquecimento entre níveis tróficos (3-4‰), o que possibilita a sua utilização no reconhecimento da posição trófica de um consumidor e na compreensão de interações alimentares entre os organismos (DeNiro e Epstein, 1981; Peterson e Fry, 1987; Post, 2002). Fatores como o estado nutricional, as taxas de excreção e respiração do consumidor e o estágio de vida (fase ontogenética) podem influenciar nos valores isotópicos de ^{15}N, principalmente (Caut et al., 2009; Vander Zanden et al., 2014).

A aplicabilidade da composição isotópica como ferramenta de análise da ecologia trófica vai além do reconhecimento das fontes basais de energia e da posição trófica dos consumidores. Isótopos estáveis de ^{13}C e ^{15}N são influenciados por longos períodos de estresse nutricional, tais como a ingestão de alimentos pouco nutritivos ou em pouca quantidade, e períodos de jejum ou fome. Nesse caso, durante a privação alimentar há enriquecimento de ^{15}N nos tecidos animais e, em menor proporção, depreciação dos valores de ^{13}C (McCue e Pollock, 2008; Hatch, 2012).

O tempo necessário para que a alteração na dieta do consumidor se reflita na assinatura isotópica dos seus tecidos corporais é denominado taxa de renovação (ou

turnover) (Hobson e Clark, 1992). Tecidos metabolicamente mais ativos, ou seja, com taxas de renovação mais rápidas, como plasma sanguíneo (Pfaller et al., 2020) e fígado (Di Beneditto et al., 2019), refletem a dieta mais recente. Por outro lado, tecidos inertes ou de metabolismo mais lento, como queratina da carapaça (Rodriguez et al., 2019) e ossos (Tomaszewicz et al., 2015), refletem a dieta de mais longo prazo. Dessa forma, a utilização de tecidos com diferentes taxas de renovação possibilita a interpretação temporal da dieta, que pode ir de poucas horas atrás (plasma sanguíneo, por exemplo) até toda vida do consumidor (ossos, por exemplo) (Martinez Del Rio et al., 2009; Petitet e Bugoni, 2017).

De modo geral, estudos sobre a ecologia trófica das tartarugas marinhas que utilizam os isótopos estáveis permitem a compreensão das fontes alimentares preferencialmente assimiladas, da origem dos recursos alimentares e da posição trófica dos indivíduos (e.g., Di Benedito et al., 2019; Figgener et al., 2019; Agostinho et al., 2021). Entretanto, outras abordagens utilizando esse método vêm colaborando para o conhecimento da história de vida desses animais e para ações futuras de conservação e manejo. As análises de isótopos estáveis vêm sendo utilizadas para monitorar o comportamento de forrageamento de tartarugas marinhas sobreviventes a desastres ambientais (Reich et al. 2017), para verificar estratégias alimentares durante a ontogenia (Peavey et al, 2017; Ferreira et al., 2018) e para apontar a variabilidade individual dentro da mesma espécie (Petitet e Bugoni, 2017).

As análises de isótopos estáveis também mostraram como a produtividade e a disponibilidade de recursos do local de forrageamento podem afetar o tamanho das ninhadas e os intervalos de remigração – período entre uma temporada reprodutiva e outra – das tartarugas marinhas (Ceriani et al., 2015). Além disso, estes estudos podem ser utilizados como ferramenta para idetentificar possíveis áreas de forrageamento, uma vez que os indivíduos permanecem a maior parte do ciclo de vida nessas áreas e, portanto, são prioritárias para a conservação (López-Castro et al., 2014; Rees et al., 2016; Haywood et al., 2019). Nesses estudos, a utilização de indivíduos adultos reduz vieses de interpretação, que normalmente ocorrem quando se considera filhotes e/ou juvenis, por exemplo. Nessas fases do ciclo de vida, a taxa de crescimento é elevada e a alimentação é, em geral, mais variável em comparação à fase adulta, o que limita as interpretações isotópicas (Reich et al., 2008).

Em relação a *Chelonia mydas*, a literatura que aborda a ecologia trófica a partir da análise de isótopos estáveis em indivíduos adultos é escassa (Tabela 1). Os

estudos disponíveis indicam o quão variável pode ser o hábito alimentar da espécie, apesar de destacar a preferência pela matéria vegetal. Godley et al. (1998) e Vander Zanden et al. (2013b) reforçaram a herbivoria como principal fonte de alimentação, e os últimos autores demonstraram a dinâmica alimentar da espécie pela sua composição isotópica. Eles concluíram que as fêmeas de uma área de reprodução no Oceano Atlântico (Caribe) são provenientes de várias áreas de alimentação distribuídas ao longo de ampla região geográfica, ao mesmo tempo que constataram a fidelidade de sítio alimentar de cada indivíduo e dieta consistente ao longo do tempo. A presença de matéria animal na alimentação (assimilação) foi verificada por Burkholder et al. (2011) (Austrália), e sugerida por Shimada et al. (2014) (Japão).

As relações entre os valores isotópicos e o tamanho do corpo das tartarugas verdes também já foram investigadas, se mostrando não significantes conforme dados de Burkholder et al. (2011) e Hatase et al. (2006). Por outro lado, Vander Zanden et al. (2013b) registraram diferenças sexuais nos valores de $\delta^{15}N$ de animais no Oceano Atlântico Sul, com a variância nas fêmeas maior em comparação aos machos. A distribuição das áreas de forrageamento inferida pela composição isotópica das tartarugas indica padrões interessantes, tais como fidelidade a essas áreas (Vander Zanden et al.,2013a; Shimada et al., 2014; Bradshaw et al., 2017) e variações intrapopulacionais dos habitats de forrageio (nerítico vs. oceânico) (Hatase et al., 2006). Bradshaw et al. (2017) apontaram que a fidelidade de *C. mydas* estaria condicionada a disponibilidade de recursos, com possibilidade de movimentação entre áreas de alimentação próximas.

Tabela 1. Literatura sobre a utilização de isótopos estáveis na compreensão da ecologia trófica das tartarugas verdes adultas.

					$\delta^{13}C$		$\delta^{15}N$	
Referência	**Bacia oceânica**	**Local**	**Tecido**	**N**	**Média±DP**	**Mín e Máx**	**Média±DP**	**Mín e Máx**
Vander Zanden et al., 2013a	Atlântico	Costa Rica	Epiderme	102	-9.3±0.2	-17.0 e -5.3	6.6±0.1	3.0 e 9.4
Vander Zanden et al., 2013b	Atlântico	Costa Rica	Carapaça	14	-6.5	-13.0 e -6.5	7.4	2.5 e 9.9
Chabot et al., 2019	Atlântico	Flórida	Epiderme	69	-8.9±1.4		7.3±1.2	
Chabot et al., 2019	Atlântico	Flórida	Ovo não desenvolvido	69	-10.2±1.3		6.1±1.2	
Filippos et al., 2021	Atlântico	Atol das Rocas	Células vermelhas	31	-18.7±1.3	-20.5 e -15.8	6.1±1.5	3.3 e 10.4
Ferreira et al., 2021	Atlântico	Ilha do Príncipe	Epiderme	60	-17.3±1.8	-19.4 e -8.6	13.6±1.5	7.9 e 17.3
Roche et al., 2021	Atlântico	Flórida	Pele	54	-7.9±1.3	-13.1 e -6.2	7.1±0.8	5.8 e 9.5
Roche et al., 2021	Atlântico	Flórida	Sangue total	7	-11±6.7	-9.6 e -7.2	4.5±0.8	3.4 e 5.9
Roche et al., 2021	Atlântico	Flórida	Células Vermelhas	3	-8.3±0.6	-8.9 e -7.4	5.0±1.0	3.8 e 6.2
Roche et al., 2021	Atlântico	Flórida	Plasma	12	-7.5±1.1	-9.2 e -6.4	5.6±1.0	3.6 e 6.8
Roche et al., 2021	Atlântico	Flórida	Carapaça	27	-8.8±1.2	-12.8 e -7.2	5.9±0.8	4.4 e 7.6
Agostinho et al., subm.	Atlântico	Atol das Rocas	Vitelo	55	-17.7±2.2	-21.2 e -12.6	7.0±1.3	4.7 e 9.5
Agostinho et al., subm.	Atlântico	Atol das Rocas	Sangue total	39	-18.6±1.9	-21.6 e -13.3	7.2±2.0	4.0 e 11.6
Agostinho et al., subm.	Atlântico	Atol das Rocas	Carapaça	86	-18.4±2.0	-27.0 e -12.5	7.9±1.6	4.5 e 12.4
Hatase et al., 2006	Pacífico	Japão	Vitelo	89	-17.7±1.4	-23.1 e -11.4	9.4±1.5	6.6 e 14.2
Páez-Rosas et al., 2021	Pacífico	Galápagos	Epiderme	56	-15.9±0.7		12.0±0.6	
Páez-Rosas et al., 2021	Pacífico	Galápagos	Carapaça	56	-18.2±0.7		11.4±0.8	
Godley et al., 1998	Mediterrâneo	Chipre/Turquia	Vitelo	20	-11.7±1.9	-15.3 e -8.5	5.1±1.3	3.3 e 7.5
Bradshaw et al., 2017	Mediterrâneo	Chipre	Epiderme	196	-7.6±1.8	-11.5 e -4.7	7.2±1.9	2.0 e 12.1

Ainda que a análise de isótopos estáveis traga informações importantes sobre a ecologia trófica das tartarugas marinhas, a combinação de técnicas de amostragem é capaz de fornecer resultados mais completos. Os elementos- traço, por exemplo, podem ser utilizados em estudos que buscam avaliar se os indivíduos compartilham a mesma região de forrageamento (Agostinho et al., 2021). Outras ferramentas como telemetria via satélite (Vander Zanden et al., 2013a), coleta de potenciais itens alimentares para estimar sua contribuição na dieta das tartarugas marinhas (Cardona et al., 2017), análises genéticas (Cameron et al., 2019; Medeiros et al., 2019), câmeras de vídeo (Burkholder et al., 2011; Thomson et al., 2012) e análises de conteúdo estomacal (Shimada et al., 2014) também são recomendadas para complementar e dar suporte às análises de isótopos estáveis.

1.3 O Atol das Rocas (03º51'S; 33º49'W)

Um atol é uma ilha oceânica em forma de anel com estrutura formada por corais e outros invertebrados, com uma lagoa em seu interior. O Atol das Rocas é o único atol do oceano Atlântico Sul e, apesar de ser um dos menores atóis do mundo (aproximadamente 7,5 km²), é importante na manutenção da biodiversidade marinha brasileira (Candisani, 2002; Granville, 2012). Em 1979, o local se tornou a primeira unidade de conservação marinha do Brasil, após anos de intensa atividade pesqueira no seu entorno. Atualmente, a Reserva Biológica do Atol das Rocas é administrada pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), vinculado ao Ministério do Meio Ambiente (MMA), com apoio da Fundação SOS Mata Atlântica (MMA, 2007; Pereira et al., 2010).

O Atol das Rocas está localizado no Nordeste do Brasil, 267 km a leste do município de Natal, capital do estado do Rio Grande do Norte, e 148 km a oeste do arquipélago de Fernando de Noronha, estado de Pernambuco (Figura 1). A região é um recife elíptico delimitado pela isóbata de 1.000 m, com área total de 360 km² (Almeida, 2006; Pereira et al., 2010). As marés locais são semidiurnas com amplitude de aproximadamente 2,7 m na maré de sizígia. O Atol das Rocas é banhado pela corrente Sul Equatorial, originária da costa africana e que flui para oeste (MMA, 2007; Pereira et al., 2010).

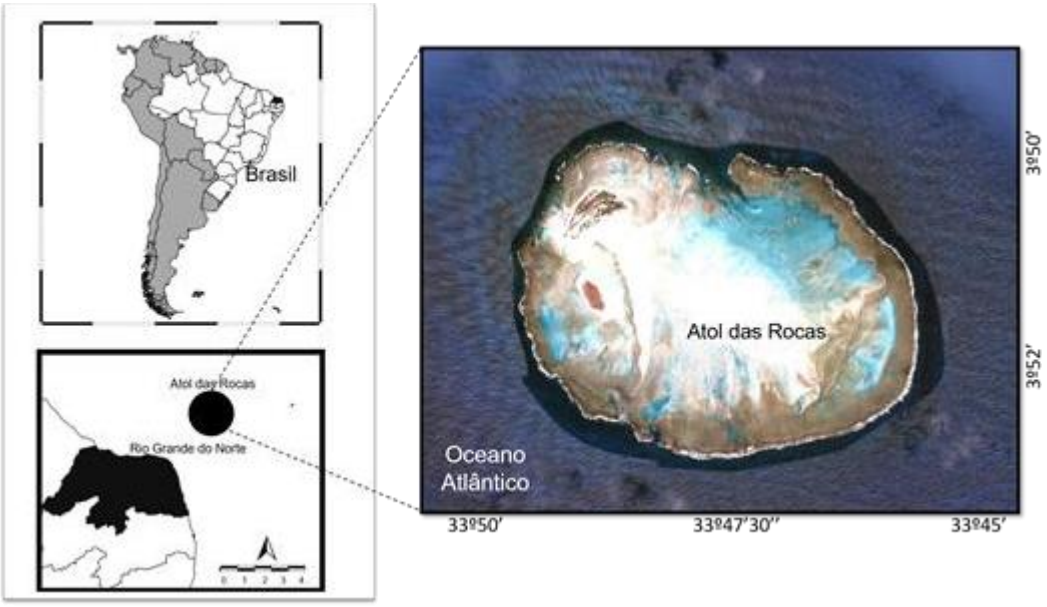

Figura 1. Mapa do Brasil com indicação da localização do estado do Rio Grande do Norte e da Reserva Biológica do Atol das Rocas.

O Atol das Rocas é o segundo maior sítio de desova da tartaruga-verde no Brasil. A temporada reprodutiva da espécie começa no final de dezembro e se estende até maio, com o nascimento dos últimos filhotes em julho. O número anual de ninhos no local é de cerca de 340 (Grossman, 2001; Bellini et al., 2013). O comprimento médio da carapaça das fêmeas que desovam na região tem diminuído ao longo dos anos (115,9 cm entre 1990 e 1992, e 112,9 cm entre 2006 e 2008), sugerindo que mais animais estão alcançando a fase reprodutiva. Isso pode ser reflexo do sucesso dos projetos de conservação da espécie ao longo de sua distribuição (Grossman, 2001; Bellini et al., 2013).

O Atol das Rocas também é área de forrageamento para juvenis de tartarugas-verdes, e de tartarugas-de-pente, *Eretmochelys imbricata* (Linnaeus, 1766) (Marcovaldi e Marcovaldi, 1999). Segundo Bjorndal et al. (2006), as agregações alimentares de juvenis do Atol das Rocas e de Fernando de Noronha são geneticamente similares, e a maior parte da contribuição genética vêm da Ilha de Ascensão, seguida do Caribe e da África ocidental. Em relação à população reprodutiva (indivíduos adultos), os animais que utilizam o Atol das Rocas e Fernando de Noronha são geneticamente iguais, diferindo da população reprodutiva da Ilha da Trindade, que se localiza ao largo da costa do estado do Espírito Santo, Sudeste do Brasil, e é o maior sítio reprodutivo da tartaruga-verde no Brasil.

Os machos adultos que chegam ao Atol das Rocas para reprodução são

geneticamente semelhantes às fêmeas reprodutivas, mas se diferenciam dos juvenis da espécie que estão no local se alimentando. Este fato pode indicar uma filopatria dos machos à área de reprodução (Naro-Maciel et al., 2012). Há indícios genéticos de que os indivíduos juvenis da tartaruga-verde que se alimentam no Atol das Rocas provêm de uma população distinta das fêmeas adultas que se reproduzem na região (Bjornal et al 2006): três haplótipos são registrados exclusivamente nos juvenis, sete haplótipos estão presentes exclusivamente em fêmeas adultas, e apenas três haplótipos são compartilhados entre ambos.

Naro-Maciel et al. (2006) sugeriram que os filhotes de tartaruga-verde se movimentam por meio de correntes oceânicas: filhotes nascidos na Ilha de Ascensão chegariam à América do Sul pela corrente Sul Equatorial. As correntes oceânicas são importantes para a movimentação, não somente de filhotes, mas de indivíduos em vários estágios de desenvolvimento. Lum et al. (1998) registraram um subadulto da espécie anilhado no estado do Ceará, Nordeste do Brasil, encalhado em uma praia de Trinidad, da região do Caribe. É possível que o animal tenha utilizado a Corrente da Guiana para deslocamento de cerca de 2.700 km. Lima et al. (2003) documentaram o registro de uma tartaruga-verde anilhada no estado do Ceará e recapturada na Nicarágua, também na região do Caribe (~5.000 km entre as áreas). Lima & Troëng (2001) relataram o movimento inverso de uma fêmea adulta, que foi anilhada na Nicarágua e recapturada no Brasil. Esses registros, mesmo que pontuais, dão pistas do padrão de deslocamento das tartarugas verdes que ocorrem no Nordeste do Brasil.

Capítulo 1:

Isotopic profile of female green turtles nesting in Rocas Atoll, northeastern Brazil

→ Em preparação

Isotopic profile of female green turtles nesting in Rocas Atoll, northeastern Brazil

Running title: Isotopic profile of female green turtles

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Abstract

This study compared the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in whole blood, carapace, and egg yolk samples of female green turtles at Rocas Atoll, Northeastern Brazil, during three consecutive nesting periods (2017 to 2019). Our analyses indicate a positive and significant relationship between total blood and egg yolk for both $\delta^{13}C$ and $\delta^{15}N$ ($p<0.01$). The median of $\delta^{13}C$ and $\delta^{15}N$ values recorded for whole blood, carapace and egg yolk were -18.9‰ and 6.6‰, -18.4‰ and 7.6‰, and -17.8‰ and 6.7‰, respectively. The isotopic composition of each analyzed tissue was similar between the nesting periods, demonstrating temporal consistency in nesting females' trophic patterns in the region. Our results suggest that these turtles are likely to prefer herbivorous food in coastal-benthic environments but with high individual variation and prey's participation in the long-term diet. This study contributes to new data on the $\delta^{13}C$ and $\delta^{15}N$ signatures for the green turtle at this life cycle stage since few studies around the world use stable isotopes to understand the trophic ecology of the adult green turtle.

Keywords: Atlantic Ocean, *Chelonia mydas*, isotopic composition, tissues

1. Introduction

The green turtle, *Chelonia mydas* (Linnaeus, 1758), is classified by the International Union for the Conservation of Nature - IUCN as Endangered (EN), even with some subpopulations showing signs of recovery, such as in the South Atlantic Ocean (Broderick and Patricio, 2019). This species is the only sea turtle presenting a preferential herbivorous feeding habit when adult (Bjorndal, 1997; Vander Zanden et

al., 2013a), but animal prey has also been observed in their diets (Amorocho and Reina, 2007; Awabdi et al., 2013; Burkholder et al., 2011; Shimada et al., 2014). The green turtle migrates between feeding and nesting areas, travelling up to thousands of kilometers (Hays et al., 1999; Baudouin et al., 2015). Migration to the same breeding site is intermittent and occurs every three or four years (Bellini et al., 2013). The species present high fidelity to feeding and reproduction areas and can feed during migratory movements (Shimada et al., 2020).

Stable isotopes are chemical markers that can be used to make inferences about the animals' trophic ecology (Newsome et al., 2010). The consumer's isotopes stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) values come from its habitat and trophic trajectory, respectively (Layman et al., 2007; Newsome et al., 2010). In general, $\delta^{13}C$ represents the habitat use or the food resource (coastal vs. oceanic, pelagic vs. benthic) and allows latitudinal inferences about foraging areas (Fry, 2006; Petitet and Bugoni, 2017; Di Beneditto et al., 2019). On the other hand, $\delta^{15}N$ indicates the consumer habit or trophic position (herbivore, carnivore, omnivore) and trophic interactions between organisms (Peterson and Fry, 1987; Post, 2002). Isotopic analysis performed on green turtles' tissues has already corroborated their fidelity to feeding areas (Vander Zanden et al., 2013b; Shimada et al., 2014; Bradshaw et al., 2017), detected intrapopulation variations in the habitat use (Hatase et al., 2006), and demonstrated trophic dynamics over time (Di Beneditto et al., 2019; Agostinho et al., 2021a).

The time required for the food ingested to reflect the isotopic composition of the consumer's body tissue is called the turnover rate (Hobson and Clark, 1992). Each tissue presents a specific renewal time varying according to its metabolism. For example, metabolically active tissues such as the liver and blood plasma show rapid turnover rates, reflecting the previous days or hours' diet, respectively (Hobson, 1999; Di Beneditto et al., 2019). Keratinous tissues, such as turtle shell scales, are metabolically inert and represent the previous months or years' diet (Hobson, 1999; Rodriguez et al., 2019). The sea turtle egg yolk is formed when the female is still in the feeding area, about six to eight months before reproductive migration (Rostal et al., 1998). Carpentier et al. (2015) indicated that the egg yolk can predict the isotopic values of other tissues, such as whole blood and epidermis, and can be used if the females cannot be directly sampled. It reinforces the use of stable isotopes to understand these animals' trophic ecology.

This study aimed to (1) validate the use of yolk instead of whole blood for green turtles' isotopic analysis, (2) to evaluate the temporal constancy in the trophic patterns of these green turtles through the study of isotopic profiles from tissues with different turnover rates (egg yolk, whole blood, and carapace) and sampled in three nesting years, and (3) present the literature available for this theme in the green turtle.

2. Materials and methods

2.1 Sampling

The Rocas Atoll (03°51'S; 33°49'W) is a biological reserve located 266 km off the Brazilian coast and is the second-largest breeding site of the green turtle in the country (Fig. 1) (Bellini et al., 2013). Nesting female green turtles (N= 128) in the Rocas Atoll were sampled for whole blood, egg yolk, and shell fragment in 2017, 2018, and 2019 (Table S1). The samplings occurred between January and May, during the species nesting period in the region. Data collection was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) through the SISBIO license number 59809.

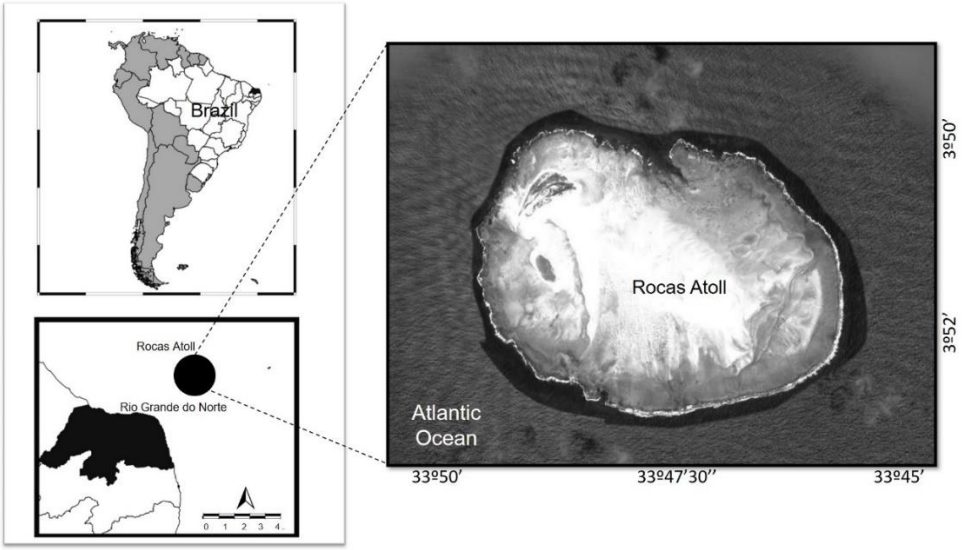

Fig. 1. Rocas Atoll Biological Reserve, Brazilian northeast, where data collection was performed between 2017 and 2019.

Females were measured for curved carapace length and microchipped for individual recognition during data collection, avoiding resampling. In total, 39 samples of whole blood (2018 and 2019), 55 of yolk, and 86 of carapace were collected. At least two tissues were sampled in 43 individuals, while only one tissue sample was collected in the other individuals (n = 85).

Blood collection (10 mL) was performed in the dorsal cervical venous sinus with previously heparinized needles (40 x 12 mm) during egg laying so as not to harm nesting. Two eggs were sampled per nest (2017 and 2018) or during laying (2019) and were washed in filtered water (N_{total} = 55). The yolk was separated from the albumen and shell and stored together, creating a composite sample for each female. Blood and yolk were stored in sterile vials, frozen (-20°C), lyophilized, and sprayed in a grate and pistil. The carapace fragment (5 cm^2) was sampled while females were laying eggs and removed with tweezers and scissors from the margin of the anterior plate, close to the nuchal notch. The fragment was immersed in pure acetone to remove possible incrustations, washed in filtered water, dried at room temperature, and transformed to powder with a mortar and pestle.

2.2 Isotopic analysis ($\delta^{13}C$ and $\delta^{15}N$)

Stable isotopic ratios ($\delta^{13}C$ and $\delta^{15}N$) were determined in 0.4 mg of each sample's dry weight in an elemental, organic analyzer (Flash 2000, Thermo Scientific) coupled to a mass spectrometer (Delta V Advantage Isotope Mass Proportion Spectrometer, Thermo Scientific) with ConFlo-VI interface (Model BR30140, Thermo Scientific). The analyses were performed at the Laboratório de Ciências Ambientais at the Universidade Estadual do Norte Fluminense Darcy Ribeiro, Rio de Janeiro, Brazil.

The reference values were Pee Dee Belemnite (PDB) and atmospheric Nitrogen, and the samples were analyzed using blank and urea analytical standards (IVA Analysentechnik-330802174). Analytical control was performed every ten samples with a certified isotopic standard (Elemental Microanalysis Protein Standard OEA). Reproducibility was based on triplicates every ten samples (± 0.2‰, $\delta^{13}C$; ±0.3‰, $\delta^{15}N$). Results are presented in parts per thousand (‰).

The yolk is a lipid-rich tissue, and it interferes with the $\delta^{13}C$ results (Post et al., 2007). Therefore, the lipid content of this tissue was extracted using a chloroform and methanol solution (2:1) (Bligh and Dyer, 1959). Then, the yolk samples were dried for 48 hours in an oven at 60°C to remove residual solvent. Since lipid extraction interferes on $\delta^{15}N$ values (Petitet and Bugoni, 2017), we analyzed the yolk samples for isotopic determination with and without lipid extraction.

2.3 Data analysis

Linear regressions were used to assess the relationships between whole blood and egg yolk samples from green turtles in 2019 since these samples are paired. Generalized linear models (GLMs) were fitted to assess possible differences in $\delta^{13}C$ and $\delta^{15}N$ across nesting periods and between tissues. Isotopes ($\delta^{13}C$ and $\delta^{15}N$) were the response variables, and nesting periods (2017, 2018, and 2019) or tissues (whole blood, egg yolk, and carapace) were the predictor variables. Due to the response variable's continuous nature, the model used a normal residual (error) structure and Gaussian family function. All analyzes were performed in the statistical environment R (R Core Team, 2021), assuming a type I error of 5% ($\alpha = 0.05$).

3. Results

In the paired samples of whole blood and egg yolk sampled in 2019, positive and significant relationships ($p<0.01$) were observed between the two tissues for carbon and nitrogen isotopes, suggesting that these tissues can predict each other (Figure 2). Regarding unpaired samples, the whole blood, egg yolk, and carapace isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) showed high individual variation in all sampling periods (Figure 3). No difference was observed between the nesting periods to the of $\delta^{13}C$ and $\delta^{15}N$ values in the three analyzed tissues ($p > 0.05$). However, comparison of raw values between tissues revealed differences in both isotopes that possibly reflect turnover rates (Fig. 3). We found a significant variation of $\delta^{13}C$ values between whole blood and egg yolk ($p = 0.04$), and between carapace and egg yolk ($p = 0.05$), with higher values in the egg yolk. For $\delta^{15}N$, differences were found between whole blood and carapace ($p = 0.04$) and between egg yolk and carapace ($p < 0.01$), with higher values in the carapace. Data from each sampled individual are shown in table S1.

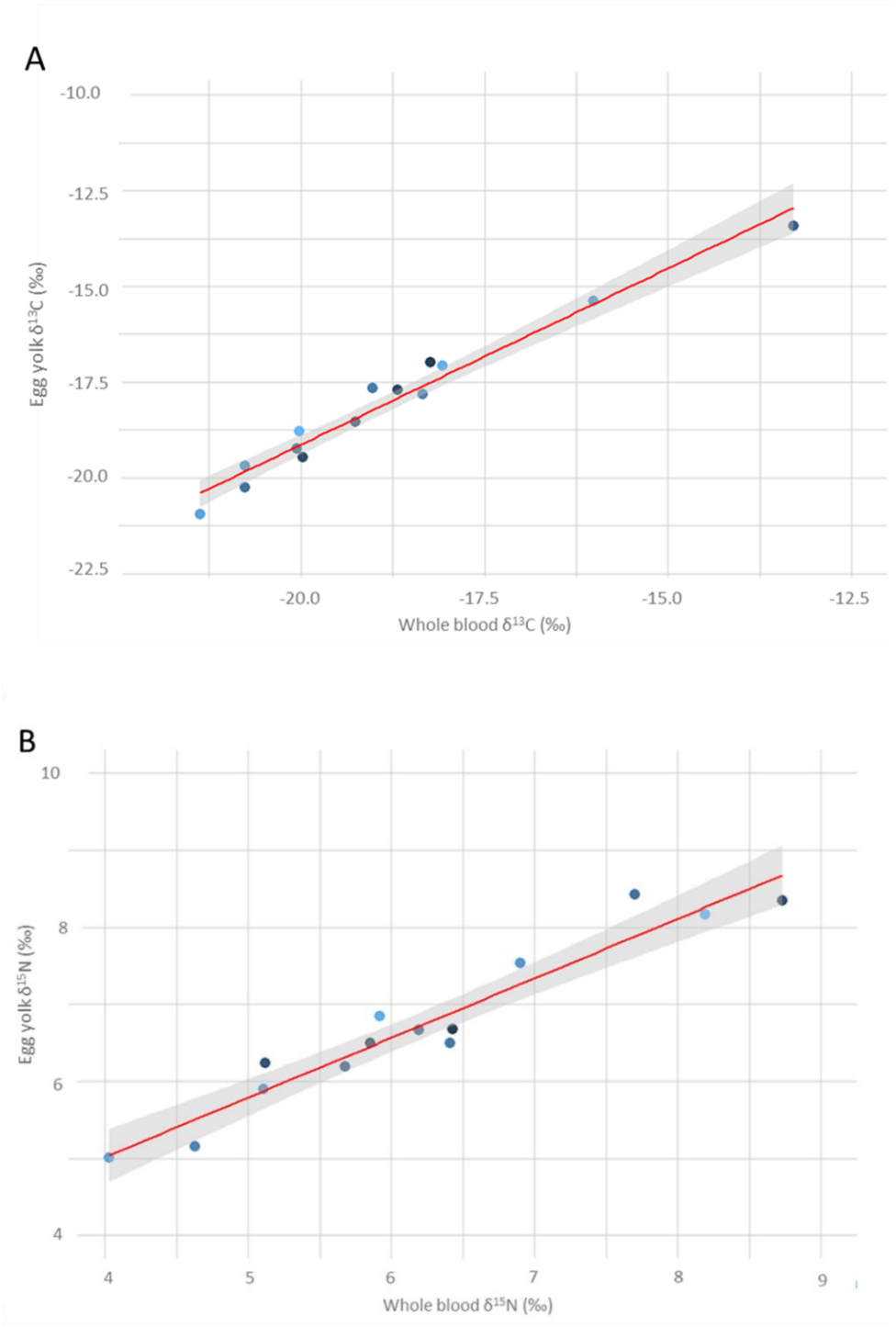

Fig. 2. Relationship between whole blood and egg yolk for (A) $\delta^{13}C$ and (B) $\delta^{15}N$ of adult female green sea turtles that nested in Rocas Atoll in 2019 (n = 14). The regression for whole blood and yolk $\delta^{13}C$ values showed a positive and significant relationship ($p<0.01$; Estimate 1.05; SE 0.06; t-value 18.31) and for values of $\delta^{15}N$, it showed a positive and significant relationship ($p<0.01$; Estimate 1.2; SE 0.1; t-value 12.43).

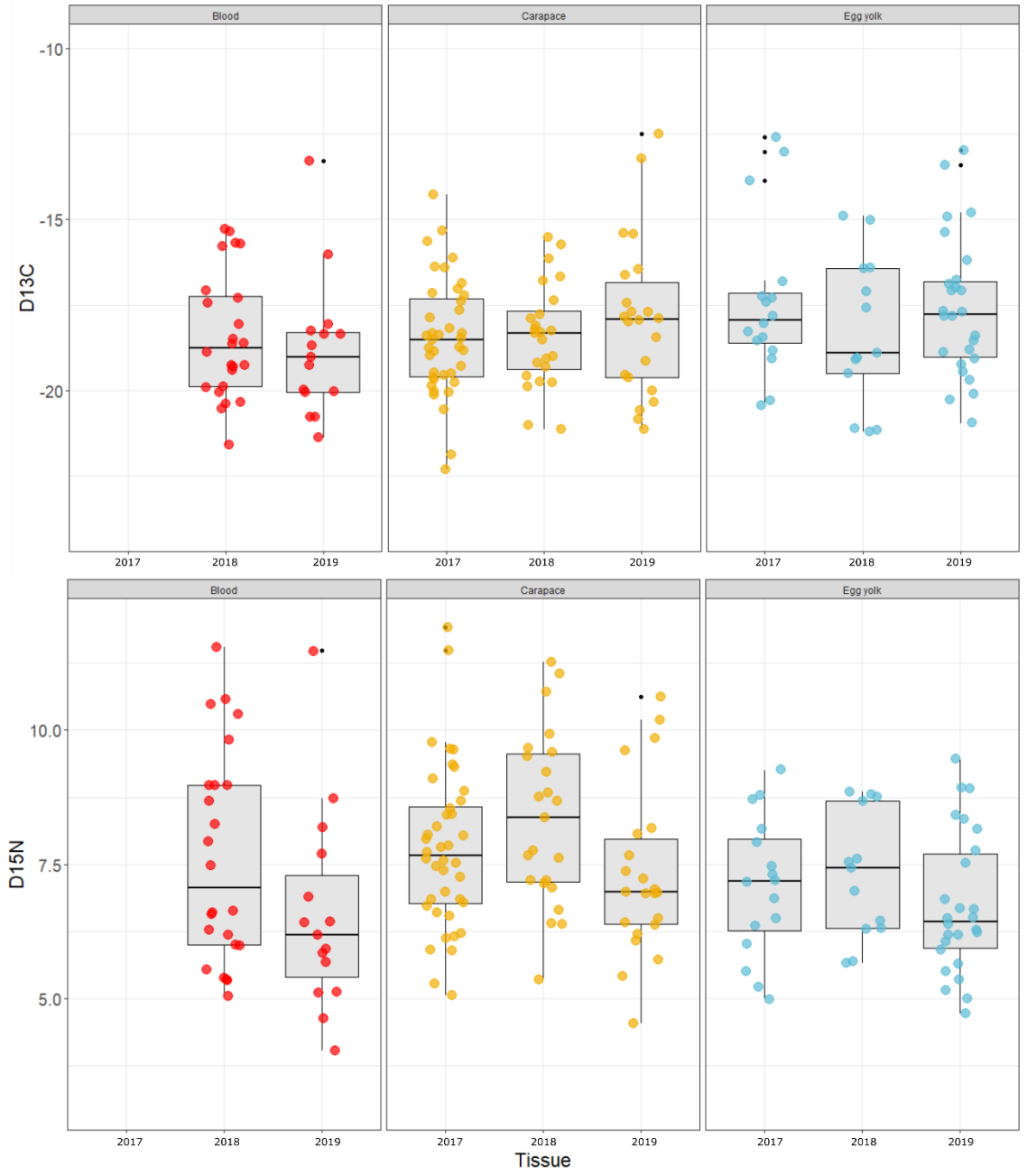

Fig. 3. Boxplots representing median (bar inside the box), interquartile range (box: first to the third quartile), minimum and maximum values of the stable isotopes ($\delta^{13}C$ e $\delta^{15}N$) in whole blood (red circles), egg yolk (yellow circles), and carapace (blue circles) samples of nesting green turtles on the Rocas Atoll in 2017, 2018 and 2019 nesting periods. Open circles are outliers (values greater than the 3rd quartile + 1.5 times the interquartile range or lower than the first quartile - 1.5 times the interquartile range). Different lowercase letters indicate significant differences ($p < 0.05$) among groups.

From the literature review on the trophic ecology of adult green turtles, we found ten studies distributed between the Atlantic and Pacific oceans and the Mediterranean sea (Table 1). In addition, previous studies have already addressed the egg yolk isotopic composition and the nesting female green turtles' carapace in the Rocas Atoll (Agostinho et al., 2020; 2021a; 2021b). However, this is the first study presenting the whole blood isotopic composition for the species. Filippos et al. (2021) analyzed red blood cells from individuals from the same study area, and the authors' results are within the isotopic range recorded here (Table 1).

Table 1: $\delta^{13}C$ and $\delta^{15}N$ (average ± standard deviation, minimum and maximum values) in different tissues of nesting female green turtles in the Atlantic and Pacific Ocean and in the Mediterranean Sea.

					$\delta^{13}C$		**$\delta^{15}N$**	
Reference	**Ocenic basin**	**Local**	**Tissue**	**N**	**Mean±SD**	**Min and Max**	**Mean±SD**	**Min and Max**
Vander Zanden et al., 2013a	Atlantic	Costa Rica	Epidermis	102	-9.3±0.2	-17.0 and -5.3	6.6±0.1	3.0 and 9.4
Vander Zanden et al., 2013b	Atlantic	Costa Rica	Scute	14	-6.5	-13.0 and -6.5	7.4	2.5 and 9.9
Chabot et al., 2019	Atlantic	Florida	Epidermis	69	-8.9±1.4		7.3±1.2	
Chabot et al., 2019	Atlantic	Florida	Unhatched egg	69	-10.2±1.3		6.1±1.2	
Filippos et al., 2021	Atlantic	Rocas Atoll	Red Blood Cell	31	-18.7±1.3	-20.5 and -15.8	6.1±1.5	3.3 and 10.4
Ferreira et al., 2021	Atlantic	Prince's Island	Epidermis	60	-17.3±1.8	-19.4 and -8.6	13.6±1.5	7.9 and 17.3
Roche et al., 2021	Atlantic	Florida	Skin	54	-7.9±1.3	-13.1 and -6.2	7.1±0.8	5.8 and 9.5
Roche et al., 2021	Atlantic	Florida	Whole blood	7	-11±6.7	-9.6 and -7.2	4.5±0.8	3.4 and 5.9
Roche et al., 2021	Atlantic	Florida	Red Blood Cell	3	-8.3±0.6	-8.9 and -7.4	5.0±1.0	3.8 and 6.2
Roche et al., 2021	Atlantic	Florida	Plasma	12	-7.5±1.1	-9.2 and -6.4	5.6±1.0	3.6 and 6.8
Roche et al., 2021	Atlantic	Florida	Scute	27	-8.8±1.2	-12.8 and -7.2	5.9±0.8	4.4 and 7.6
This study	**Atlantic**	**Rocas Atoll**	**Egg yolk**	**55**	-17.7±2.2	**-21.2 and -12.6**	7.0±1.3	**4.7 and 9.5**
This study	**Atlantic**	**Rocas Atoll**	**Whole blood**	**39**	-18.6±1.9	**-21.6 and -13.3**	7.2±2.0	**4.0 and 11.6**
This study	**Atlantic**	**Rocas Atoll**	**Scute**	**86**	-18.4±2.0	**-27.0 and -12.5**	7.9±1.6	**4.5 and 12.4**
Hatase et al., 2006	Pacific	Japan	Egg yolk	89	-17.7±1.4	-23.1 and -11.4	9.4±1.5	6.6 and 14.2
Páez-Rosas et al., 2021	Pacific	Galapagos	Epidermis	56	-15.9±0.7		12.0±0.6	
Páez-Rosas et al., 2021	Pacific	Galapagos	Scute	56	-18.2±0.7		11.4±0.8	
Godley et al., 1998	Mediterranean	Cyprus/Turkey	Egg yolk	20	-11.7±1.9	-15.3 and -8.5	5.1±1.3	3.3 and 7.5
Bradshaw et al., 2017	Mediterranean	Cyprus	Epidermis	196	-7.6±1.8	-11.5 and -4.7	7.2±1.9	2.0 and 12.1

4. Discussion

Linear regressions performed with paired samples indicated a positive and significant relationship between whole blood and egg yolk for both $\delta^{13}C$ and $\delta^{15}N$ ($p<0.01$). This result is in accordance with Carpentier et al. (2015), which indicated that the egg yolk could be used instead of whole blood in trophic inferences based on isotopic composition in adult female green turtles. The use of egg yolk, which can be sampled indirectly, minimizes the animal perturbation during egg-laying and increases the opportunities to study populations where access to females is difficult.

The $\delta^{15}N$ values determined in the individuals' carapace were higher than those determined in whole blood and egg yolk. The carapace's isotopic composition reflects a broader time window of food assimilation since the turnover rate is higher than the other analyzed tissues (Hobson, 1999; Rodriguez et al., 2019). Considering that $\delta^{15}N$ values are generally used to infer the consumers' trophic position (Fry, 2006), our results reinforce that animal prey participates in the long-term diet of nesting females in Rocas Atoll as described in Agostinho et al. (2021a; 2021b), despite their preference for a herbivorous diet.

According to the literature, the $\delta^{13}C$ and $\delta^{15}N$ values determined in nesting green turtles' whole blood, egg yolk, and carapace fragment in the Rocas Atoll reflect the preferential herbivorous feeding habits in coastal-benthic environments (Fry, 2006). This pattern was previously reported in the literature on the trophic ecology of adult green turtles (Bjorndal, 1997; Figgener et al., 2019). The temporal consistency in the trophic pattern and the individual variation in the use of food resources, revealed by the similarity between the nesting periods (2017 to 2019) and the individual variation of isotopic values, respectively, was also previously described for the species in the literature (Vander Zanden et al., 2013b; Thomson et al., 2018). In addition, the trophic pattern of nesting females in the Rocas Atoll was constant over the sampling years, regardless of the food site origin.

Studies concerning trophic ecology of adult green turtles indicate a generalized diet (Table 1). However, the preference for a herbivore diet is highlighted in some studies (e.g., Godley et al. 1998; Vander Zanden et al. 2013b), including one using isotopic composition to evaluate food dynamics (Vander Zanden et al. 2013b). This study indicated that females from a reproduction area in the Caribbean came from different feeding areas distributed in a wide geographic range, although each individual's food site fidelity and diet were consistent over time (Vander Zanden et al.

2013b).

The distribution of feeding areas inferred by isotopic composition can thus indicate interesting patterns, including food site fidelity (Vander Zanden et al.,2013a; Shimada et al., 2014; Bradshaw et al., 2017) and intrapopulation variation in foraging habitats (neritic vs oceanic) (Hatase et al., 2006). In this sense, the fidelity of *C. mydas* would be related to the available resources, allowing for movement between near feeding areas (Bradshaw et al., 2017).

In this study, the females' isotopic results sampled in the Rocas Atoll differ from those in the Caribbean Sea, where a population of adult green turtles demonstrated herbivorous preference in the short- and long-term diet (Vander Zanden et al., 2013a,2013b). On the other hand, ingestion of animal prey by adult green sea turtles was determined by isotopic analysis in Japan and Australian populations (Burkholder et al., 2011; Shimada et al., 2014). Our results and the comparison with literature data reinforce the intrapopulation variations of the species in terms of food patterns.

5. Conclusion

This study presented the egg yolk as a tissue alternative capable of inferring the isotopic values of $\delta^{13}C$ and $\delta^{15}N$ present in the female when it is not possible to collect the whole blood of the green sea turtle during laying. In addition, we present the isotopic profile of different tissues of nesting female green turtles in the Rocas Atoll, northeastern Brazil. This population of reproductive females is preferentially herbivorous, feeding in a coastal-benthic environment, but animal prey can participate in the long-term diet. The trophic pattern was homogeneous over the three years of sampling but with high individual variation in diet. The data available on the isotopic profile of adult green turtles are still minimal, considering the species' wide geographic distribution. In the case of adult females, the information available in the literature is restricted to only ten studies (Table 1). Thus, this work contributes to new data on the $\delta^{13}C$ and $\delta^{15}N$ signatures for the green turtle at this life cycle stage.

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8. Supplementary Material

Table S1. Individual stable isotopes composition in egg yolk, blood and carapace samples of nesting green turtles in the Rocas Atoll during the 2017, 2018, and 2019 nesting seasons. The stable isotopes are expressed as ‰.

			Blood		Carapace		Egg yolk	
Turtle ID	Year	ccc	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
1	2019	108	-18.24	6.43	-17.45	7.24	-16.98	6.68
2	2019	108	-18.68	5.85	-17.90	6.49	-17.71	6.50
3	2019	115	-19.97	8.73	-19.56	9.85	-19.46	8.34
4	2019	119	-19.26	5.12	-18.44	6.37	-18.54	6.24
5	2019	110	-13.29	5.68	-12.49	6.97	-13.42	6.19
6	2019	118	-20.06	6.19	-20.01	7.04	-19.24	6.67
7	2019	108	-20.76	7.70	-20.84	8.17	-20.26	8.42
8	2019	111	-19.02	6.41	-17.71	6.95	-17.67	6.50
9	2019	116	-18.34	4.63	-17.85	5.41	-17.83	5.15
10	2019	109	-20.76	5.11	-20.34	6.07	-19.69	5.90
11	2019	111	-16.01	6.90	-15.39	8.07	-15.38	7.53
12	2019	120	-21.37	4.03	-20.57	4.54	-20.95	5.01
13	2019	120	-18.07	5.92	NA	NA	-17.07	6.85
14	2019	111	-20.02	8.19	NA	NA	-18.80	8.16
15	2019	NA	-18.36	11.48	NA	NA	NA	NA
16	2019	107	NA	NA	-19.15	6.99	-18.41	6.18
17	2019	110	NA	NA	-21.14	7.67	-20.09	7.76
18	2019	120	NA	NA	-15.42	10.63	-14.92	9.46
19	2019	107	NA	NA	-16.45	6.96	-16.78	5.65
20	2019	109	NA	NA	-17.99	6.41	-17.83	5.50
21	2019	110	NA	NA	-19.63	10.19	-18.88	8.94
22	2019	112	NA	NA	-17.93	9.63	-17.07	8.91
23	2019	123	NA	NA	-17.69	5.72	-16.90	5.35
24	2019	116	NA	NA	-13.21	7.38	-12.99	6.38
25	2019	119	NA	NA	NA	NA	-19.07	6.06
26	2019	112	NA	NA	NA	NA	-16.18	4.72
27	2019	108	NA	NA	-16.63	6.19	-14.80	6.28
28	2018	112	-18.49	8.97	-17.88	9.94	NA	NA
29	2018	107	-15.78	6.57	-16.13	7.20	NA	NA

30	2018	114	-18.62	10.49	-18.33	10.72	NA	NA
31	2018	113	-20.06	6.19	-19.88	7.06	NA	NA
32	2018	117	-19.90	6.64	-19.19	7.62	NA	NA
33	2018	114	-20.40	7.93	-19.30	9.22	NA	NA
34	2018	113	-19.30	6.61	-18.11	7.75	NA	NA
35	2018	117	-19.40	8.25	-18.21	8.84	NA	NA
36	2018	115	-20.53	5.35	-19.74	6.40	NA	NA
37	2018	116	-17.30	10.31	-16.80	11.06	NA	NA
38	2018	121	-19.27	10.58	-19.00	11.27	NA	NA
39	2018	107	-18.64	5.98	-17.77	7.14	NA	NA
40	2018	107	-18.06	9.82	NA	NA	NA	NA
41	2018	115	NA	NA	-17.36	5.35	NA	NA
42	2018	108	-15.69	5.53	-15.73	7.20	NA	NA
43	2018	113	-18.87	8.98	-18.31	9.60	NA	NA
44	2018	115	-19.27	11.55	-21.02	8.37	NA	NA
45	2018	113	-17.08	5.05	-16.66	6.38	NA	NA
46	2018	117	-21.59	7.48	-19.06	12.41	NA	NA
47	2018	112	-20.34	8.98	-19.58	9.66	NA	NA
48	2018	112	-15.70	6.01	NA	NA	NA	NA
49	2018	108	-15.34	5.38	-15.53	6.64	NA	NA
50	2018	112	NA	NA	-18.25	8.68	NA	NA
51	2018	115	-17.45	8.68	NA	NA	NA	NA
52	2018	108	-15.29	5.34	NA	NA	NA	NA
53	2018	112	-19.89	6.27	NA	NA	NA	NA
54	2018	112	NA	NA	-18.52	9.52	NA	NA
55	2018	126	NA	NA	-19.77	7.66	NA	NA
56	2018	111	NA	NA	-21.12	8.76	NA	NA
57	2018	NA	NA	NA	NA	NA	-19.10	8.69
58	2018	NA	NA	NA	NA	NA	-21.16	7.44
59	2018	NA	NA	NA	NA	NA	-19.05	8.81
60	2018	NA	NA	NA	NA	NA	-16.41	6.44
61	2018	NA	NA	NA	NA	NA	-18.91	8.76
62	2018	NA	NA	NA	NA	NA	-21.12	7.60
63	2018	NA	NA	NA	NA	NA	-15.01	5.66
64	2018	NA	NA	NA	NA	NA	-21.19	7.54

65	2018	NA	NA	NA	NA	NA	-19.49	7.01
66	2018	NA	NA	NA	NA	NA	-17.58	5.69
67	2018	NA	NA	NA	NA	NA	-17.10	8.85
68	2018	NA	NA	NA	NA	NA	-14.89	6.31
69	2018	NA	NA	NA	NA	NA	-16.43	6.30
70	2017	112	NA	NA	-18.18	9.66	NA	NA
71	2017	111	NA	NA	-19.85	6.15	NA	NA
72	2017	NA	NA	NA	-17.03	8.03	NA	NA
73	2017	113.5	NA	NA	-19.66	8.43	NA	NA
74	2017	106.5	NA	NA	-16.12	11.92	NA	NA
75	2017	122.5	NA	NA	-19.76	6.73	NA	NA
76	2017	102	NA	NA	-18.72	6.12	NA	NA
77	2017	118	NA	NA	-21.88	8.87	NA	NA
78	2017	106	NA	NA	-17.65	7.82	NA	NA
79	2017	118	NA	NA	-19.48	9.36	NA	NA
80	2017	112	NA	NA	-18.33	8.68	NA	NA
81	2017	108	NA	NA	-20.55	7.72	NA	NA
82	2017	108.5	NA	NA	-14.28	7.60	NA	NA
83	2017	103	NA	NA	-19.62	8.05	NA	NA
84	2017	110	NA	NA	-20.02	9.32	NA	NA
85	2017	102	NA	NA	-18.40	8.54	NA	NA
86	2017	111	NA	NA	-18.52	6.84	NA	NA
87	2017	114	NA	NA	-19.51	8.42	NA	NA
88	2017	112	NA	NA	-19.55	6.60	NA	NA
89	2017	120	NA	NA	-18.46	7.47	NA	NA
90	2017	103	NA	NA	-20.04	5.06	NA	NA
91	2017	109.5	NA	NA	-18.83	6.79	NA	NA
92	2017	105	NA	NA	-15.63	5.90	NA	NA
93	2017	112	NA	NA	-18.32	9.77	NA	NA
94	2017	109	NA	NA	-22.31	7.27	NA	NA
95	2017	106	NA	NA	-15.32	6.21	NA	NA
96	2017	112	NA	NA	-17.14	7.97	NA	NA
97	2017	112	NA	NA	-17.87	6.54	NA	NA
98	2017	105	NA	NA	-17.23	11.48	NA	NA
99	2017	111	NA	NA	-18.85	7.57	NA	NA

100	2017	112	NA	NA	-16.87	6.99	NA	NA
101	2017	113	NA	NA	-17.39	5.28	NA	NA
102	2017	116.5	NA	NA	-18.76	8.21	NA	NA
103	2017	121	NA	NA	-18.97	7.86	NA	NA
104	2017	112	NA	NA	-16.40	6.84	NA	NA
105	2017	112	NA	NA	-19.29	7.52	NA	NA
106	2017	112	NA	NA	-20.13	9.10	NA	NA
107	2017	114.5	NA	NA	-18.38	9.63	NA	NA
108	2017	103	NA	NA	-16.39	7.38	NA	NA
109	2017	108	NA	NA	-27.01	5.91	NA	NA
110	2017	NA	NA	NA	NA	NA	-16.80	9.26
111	2017	NA	NA	NA	NA	NA	-18.82	6.86
112	2017	NA	NA	NA	NA	NA	-18.05	5.22
113	2017	NA	NA	NA	NA	NA	-17.82	6.35
114	2017	NA	NA	NA	NA	NA	-18.45	8.16
115	2017	NA	NA	NA	NA	NA	-17.41	4.98
116	2017	NA	NA	NA	NA	NA	-18.53	7.92
117	2017	NA	NA	NA	NA	NA	-13.02	5.51
118	2017	NA	NA	NA	NA	NA	-12.59	6.02
119	2017	NA	NA	NA	NA	NA	-20.42	7.20
120	2017	NA	NA	NA	NA	NA	-18.28	7.30
121	2017	NA	NA	NA	NA	NA	-17.26	8.79
122	2017	NA	NA	NA	NA	NA	-13.86	6.49
123	2017	NA	NA	NA	NA	NA	-17.30	7.17
124	2017	NA	NA	NA	NA	NA	-19.08	8.71
125	2017	NA	NA	NA	NA	NA	-20.29	7.47

Capítulo 2:

Stable isotopes as measure of niche breadth of nesting green turtles (*Chelonia mydas*) on Rocas Atoll, Brazil

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Stable isotopes as measure of niche breadth of nesting green turtles (*Chelonia mydas*) on Rocas Atoll, Brazil

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Abstract

The study analysed the niche breadth of nesting green turtles, *Chelonia mydas*, on Rocas Atoll, Brazil, through stable isotopes ($\delta^{15}N$ and $\delta^{13}C$), comparing samplings of egg yolk and carapace collected from two nesting groups (2017 and 2019). The mean $\delta^{15}N$ values in egg yolk and carapace were 7.1‰ (2017) and 6.8‰ (2019), and 7.8‰ (2017) and 7.3‰ (2019), respectively. For $\delta^{13}C$, the mean values were -17.4‰ (2017) and -17.5‰ (2019) in egg yolk, and -18.4‰ (2017) and -17.9‰ (2019) in carapace. The results suggest herbivory in coastal benthic environments as the main feeding pattern in this nesting population. The niche breadth was similar between 2017 and 2019 in both tissues. In general, the trophic diversity (NR, CR, CD and SEA) was comparable between years as well as the trophic redundancy (MNND and SDNND), which was overall high. The niche metrics pointed to a homogeneous feeding pattern in the two nesting groups (2017 and 2019). This study adds a piece to solve the puzzle of adult green turtle trophic ecology.

Keywords: Stable isotopes; Marine turtle; Trophic ecology; South Atlantic Ocean.

Isótopos estáveis como medida de amplitude de nicho de tartarugas verdes (*Chelonia mydas*) nidificantes no Atol das Rocas, Brasil

Resumo

O estudo analisou a amplitude do nicho de tartarugas verdes, *Chelonia mydas*, no Atol das Rocas, Brasil, por meio de isótopos estáveis ($\delta^{15}N$ e $\delta^{13}C$), comparando amostragens de gema de ovo e carapaça coletadas de dois grupos de nidificação (2017 e 2019). Os valores médios de $\delta^{15}N$ na gema do ovo e na carapaça foram 7,1‰ (2017) e 6,8‰ (2019), e 7,8‰ (2017) e 7,3‰ (2019), respectivamente. Para $\delta^{13}C$, os valores médios foram -17,4‰ (2017) e -17,5‰ (2019) na gema de ovo, e -18,4‰

(2017) e -17,9‰ (2019) na carapaça. Os resultados sugerem que a herbivoria em ambientes costeiros-bentônicos é o principal padrão alimentar nesta população. A amplitude do nicho foi semelhante entre 2017 e 2019 em ambos os tecidos. Em geral, a diversidade trófica (NR, CR, CD e SEA) foi comparável entre os anos, assim como a redundância trófica (MNND e SDNND), que em geral foi elevada. As métricas de nicho apontaram para um padrão de alimentação homogêneo nos dois grupos de nidificação (2017 e 2019). Este estudo adiciona uma peça para resolver o quebra-cabeça da ecologia trófica da tartaruga-verde adulta.

Palavras-chave: Isótopos estáveis; Tartaruga marinha; Ecologia trófica; Oceano Atlântico Sul.

1. Introduction

The green turtle *Chelonia mydas* (Linnaeus, 1758) occurs in all ocean basins in tropical and sub-tropical regions. The conservation efforts in Brazil and worldwide reduced intentional captures and eggs harvesting, but the species is still vulnerable to fisheries and marine pollution (BRODERICK et al., 2019). Like other marine turtles, this species does extensive migrations for thousands of kilometers between foraging and breeding sites, with high site fidelity for both sites (BELLINI et al., 2013; BAUDOUIN et al., 2015; SHIMADA et al., 2020).

The Rocas Atoll (03°51'S; 33°49'W) is a Brazilian marine reserve situated 266 km off the coastline, and it is the second largest nesting site for the green turtle in Brazil (BELLINI et al., 2013) (Figure 1). Genetic evidences highlighted the green turtles on Rocas Atoll as key individuals for maintaining the diversity of the species in the South Atlantic Ocean (BJORNDAL et al., 2006). Bellini et al. (2013) monitored the nesting females in Rocas Atoll for 19 consecutive years: the peak of nesting season occurs from February to April, the average number of females and nests per season were 73 and 335, respectively (5.2 nests per individual), internesting period ranged from 8-17 days and remigration period was 3.5 years. The authors speculate that these nesting females could come from several foraging sites, although the locations of these sites are still unknown.

Stable isotopes are chemical proxies whose utilization allow inferences on animals' trophic ecology. The isotopic composition of the animal tissues derives from its trophic pathway, corresponding to the isotopic composition of their food resources and trophic

habitat after the food assimilation and fractionation processes in the tissues (LAYMAN et al., 2007; NEWSOME et al., 2007; FRY, 2008). Each body tissue has a specific turnover time, *i.e.* time within which stable isotopes in tissue is replaced by stable isotopes derived from food (AUERSWALD et al., 2010). Keratinous tissue, like carapace scutes, is metabolically inert, representing the dietary information from years ago (HOBSON, 1999). The isotopic profile of egg yolk in marine turtles represents the food assimilation in a narrower temporal window when compared to carapace scutes. The vitellogenesis process happens 4 to 6 months before the female's migration to the breeding site, while she is still at the feeding site (ROSTAL et al., 1998).

Individuals from the same population do not necessarily have consistent foraging strategies over time, changing the consumed food resources (BEARHOP et al., 2004; RIO et al., 2009). Niche differentiation is the process by which species evolve different forms of food resource utilization (MACARTHUR, 1984). Bearhop et al. (2004) and Newsome et al. (2007) expanded the niche theory using stable isotopes as a measure of niche breadth, while Layman et al. (2007) introduced metrics from ecomorphological approaches to summarize the quantitative niche information from stable isotopes. Jackson et al. (2011) developed a Bayesian framework for comparing these metrics, allowing inferences regarding the isotopic niche of consumers. Therefore, stable isotopes are reliable tools to provide quantitative information about the consumer isotopic niche, which is associated with its ecological niche.

Based on the above assumptions, this study applied stable isotopes to analysed the niche breadth of nesting green turtles on Rocas Atoll (Figure 1), comparing samplings from two nesting groups (2017 and 2019). The egg yolk represents the food assimilation from a few months before oviposition, whereas carapace fragments represent the food assimilation from years ago, *i.e.* in a more comprehensive temporal dimension. The isotopic niche approach will provide clues on how nesting individuals use the resources over time.

2. Methodology

2.1 Sampling and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) analysis

The nesting green turtles were sampled in Rocas Atoll in 2017 and 2019 nesting seasons (January to March), herein refer as years (Figure 1). Each year represents a given nesting group, since the individuals were not resampled between years nor within the same year. The sample size in each year is in table 1. In 2017, the samplings

of egg and carapace did not necessarily belong to the same female, but in 2019 both tissues were sampled from the same individual. Fresh eggs (2019) and carapace (2017 and 2019) samplings began as soon as the female initiated egg laying. During sampling, each female was measured for curved carapace length, from the nuchal notch to the tip of the longest posterior marginal scute (cm), and microchipped for individual identification. In 2017, unhatched intact eggs were sampled directly from the nest. Since each nest had an identification tag, individual turtles were sampled only once.

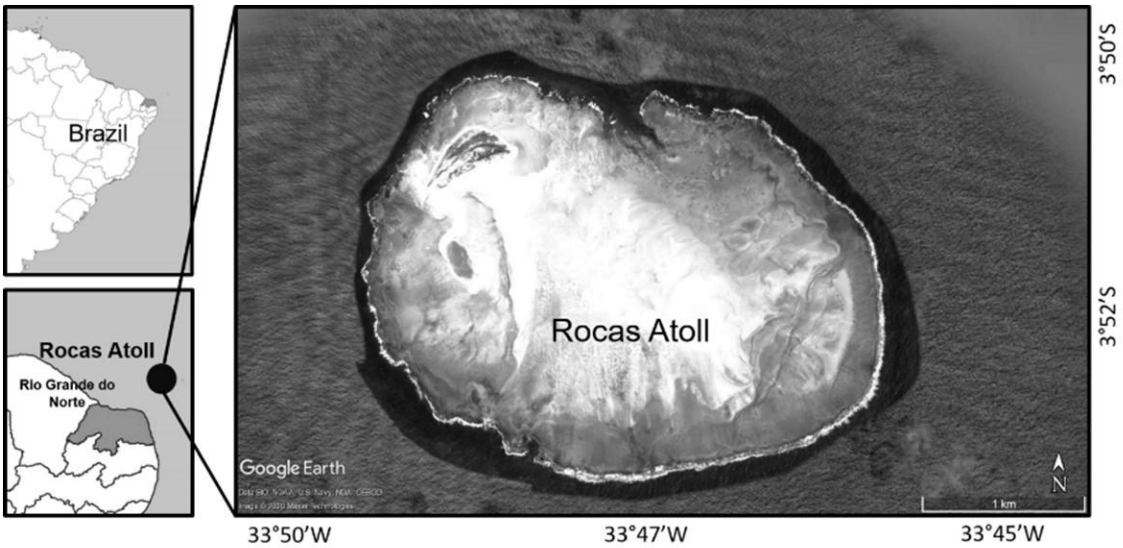

Figure 1: The location of Rocas Atoll, the nesting site for the green turtle in Brazil, southwestern Atlantic Ocean.

After sampling, the eggs were rinsed in filtered water, opened, and egg yolk separated from other fractions (albumen and shell). Then, a bulk sample with two egg yolks from the same nest/female was stored in a clean transparent plastic bag. One gram of wet weight of egg yolk (bulk sample) was freeze-dried and ground into a homogeneous powder. The egg yolk has >50% of lipid content in freeze-dried samples, and the samples were treated using a 2:1 solvent mixture of chloroform and methanol prior to lipid extraction (BLIGH et al., 1959; CARPENTIER et al., 2015). This procedure minimizes bias in $\delta^{13}C$ values interpretation (POST et al., 2007).

A carapace fragment (5 cm2) was sampled from the margin of the anterior scute, close to the nuchal notch. The sample was immersed in pure acetone to dissolve any incrustation. Then, it was rinsed in filtered water, air dried, grounded into a homogeneous powder and stored in a clean transparent plastic vial. This fragment does not represent the oldest part of the carapace (LOPÉZ-CASTRO et al., 2014), but

since all samples were collected in the same way, bias in the data interpretation is not expected.

The stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were determined in 0.3-0.4 mg of dry weight of each sample (egg yolk and carapace) in an organic elemental analyzer (Flash 2000, Thermo Scientific) coupled with a mass spectrometer (Delta V Advantage Isotope Ratio Mass Spectrometer, Thermo Scientific) through the ConFloVI interface (Model BR30140, Thermo Scientific). The stable isotope analyses were performed in the Laboratório de Ciências Ambientais at Universidade Estadual do Norte Fluminense Darcy Ribeiro. Reference values were Pee Dee Belemnite (PDB) and atmospheric nitrogen. The samples were analyzed using analytical blanks and urea analytical standards (IVA Analysentechnik-330802174). The analytical control was done for every 10 samples using a certified isotopic standard (Elemental Microanalysis Protein Standard OAS), and the reproducibility was based on triplicates for every 10 samples (±0.2‰, $\delta^{13}C$; ±0.3‰, $\delta^{15}N$). The results are presented as parts per thousand (‰).

2.2 Niche breath analysis

Quantitative metrics based on the position of each green turtle in the space $\delta^{13}C$ - $\delta^{15}N$ estimated the isotopic niche breadth for individuals sampling in each year, according to Layman et al. (2007) and Jackson et al. (2011). Since egg yolk and carapace represent different temporal windows in food assimilation (last months *vs*. years), comparisons were done only within the same tissue. The quantitative metrics were calculated by the functions for Stable Isotope Bayesian Ellipses in R (SIBER - JACKSON et al., 2011; R CORE TEAM, 2020). The Bayesian assessment for the comparison of isotopic niche metrics proposed by Jackson et al. (2011) is appropriate for small sample sizes (at least 10 samples), as in the present study (Table 1).

The first four metrics described below represent the trophic diversity in the space $\delta^{13}C$-$\delta^{15}N$, while the other two metrics represent the trophic redundancy (LAYMAN et al., 2007). The $\delta^{15}N$ range (NR metric), is the distance between maximum and minimum $\delta^{15}N$ values, which means the trophic breadth. The $\delta^{13}C$ range (CR) is the distance between maximum and minimum $\delta^{13}C$ values, indicating variability in basal resources. The standard ellipse area (SEA) is the trophic niche breadth, centred on the group centroid and scaled to encompass a 40% chance (p= 0.40) of including a subsequently sampled datum. The mean distance to centroid (CD) is the average Euclidean distance of each individual to the group centroid (mean $\delta^{15}N$ and $\delta^{13}C$

values). The mean nearest neighbor distance (MNND) is the average Euclidean distance to the individual's nearest neighbour in the space δ^{13}C-δ^{15}N, where a set of many individuals with similar trophic ecologies would have a smaller MNND than a set in which individuals have a more diverse diet. The last metric is SDNND (standard deviation of nearest neighbour distance), which is a measure of evenness in the scatterplot, with low values indicating a more even distribution of individuals in the trophic niche space.

The differences between each year regarding mean δ^{15}N and δ^{13}C values in egg yolk and carapace were assessed via t-tests. The SEAs were compared probabilistically with the posterior Bayesian distributions, calculating the proportion of ellipses for group 1 that was larger than (or equal to) ellipses for group 2 in the simulated draws (JACKSON et al., 2011). The percent of overlapping SEA between years was the measure of isotopic niche overlap. T tests were used to assess differences between years considering CD and MNND metrics because they are means. Because SDNND is a standard deviation, it was compared (after squaring) by an F-ratio test.

3. Results

Curved carapace length of the nesting green turtles showed little variation within the same year, as demonstrated by the standard deviation, and between years, as verified by the mean values (Table 1). No statistical support for mean differences between years were recorded for the isotopic values in both tissues (Table 1).

The SEAs that represent 2017 and 2019 samplings were similar in size and position in the δ^{13}C-δ^{15}N scatterplot for egg yolk samples, while for carapace samples the SEA of 2019 was greater than 2017, but with similar position in the δ^{13}C-δ^{15}N scatterplot (Figure 2 and Table 1). A probabilistic comparison between the ellipse areas based on the posterior distribution of simulated ellipses indicated that 48.6% and 91.6% of the SEAs of 2019 are larger than 2017 in egg yolk and carapace samples, respectively.

For egg yolk samples, the trophic diversity metrics (NR, CR, CD and SEA) were similar between years, and CD did not present a clear difference between them (t = 0.144, df = 30.7, p= 0.886) (Table 2). For the trophic redundancy metrics, representing how closely positioned individuals are to each other within their respective niches, MNND values were low and did not show difference between years (t = -0.54, df= 32.6,

p= 0.593), while SDNND values were low, but different between years (F= 3.94, *p*= 0.0045) (Table 2).

Table 1: Sample size (n), curved carapace length (CCL in cm) and stable isotopes values (‰) of nesting green turtles on Rocas Atoll. Values are mean ± standard deviation and *p*-values represent the t-tests results that compared the stable isotope values between years.

	n	CCL	$\delta^{13}C$	*p*-values 2017 x 2019	$\delta^{15}N$	*p*-values 2017 x 2019
Egg yolk						
2017	16	-	-17.4 ± 2.2	0.828	7.1 ± 1.2	0.499
2019	22	112.8 ± 4.9	-17.5 ± 2.1		6.8 ± 1.3	
Carapace						
2017	39	110.7 ± 5.1	-18.4 ± 2.2	0.332	7.8 ± 1.5	0.212
2019	22	112.8 ± 4.9	-17.9 ± 2.3		7.3 ± 1.5	

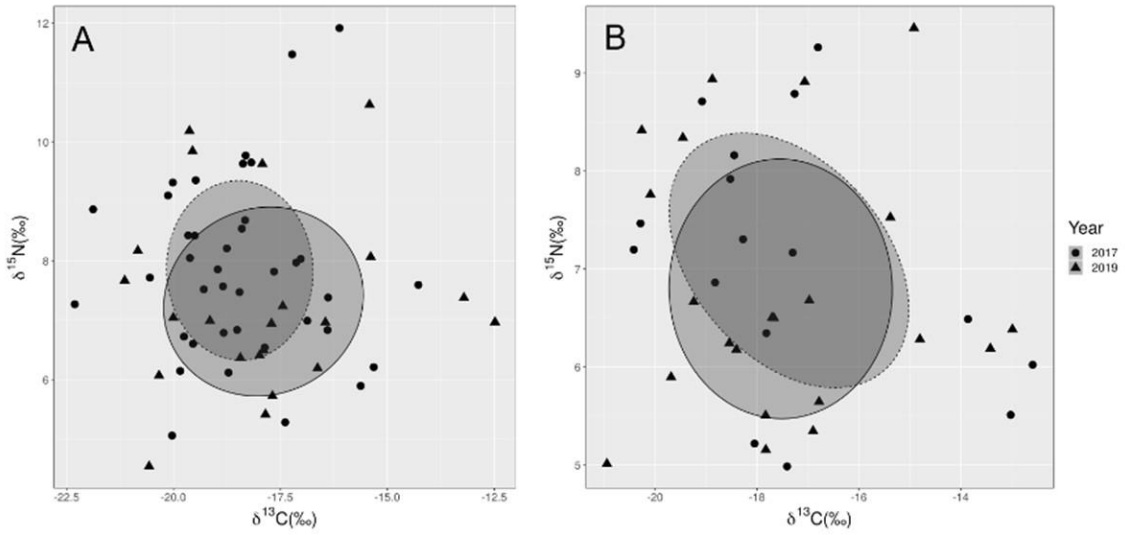

Figure 2: $\delta^{13}C$-$\delta^{15}N$ scatterplot of the nesting green turtles on Rocas Atoll (A: egg yolk and B: carapace). Lines depict the standard ellipse (the 40% confidence interval) for the isotopic niches. The dotted line is the ellipse for 2017 and the solid line is the ellipse for 2019.

For carapace samples, the trophic diversity metrics were similar between years, except for SEA as mentioned above, and the trophic redundancy metrics had similar behavior (Table 2). Neither CD nor MNND nor SDNND presented significant differences between years (CD: t= -1.27, df= 36.9, *p*= 0.209; MNND: t= - 1.63, df= 35.6, *p*= 0.112; SDNND: F= 1.64, *p*= 0.0906). Considering the percentages of SEA overlap between years, the values for egg yolk were higher than 80% in both years, and for carapace samples, they ranged from 57% to 83% (Figure 2).

Table 2: Quantitative niche metrics of nesting green turtles on Rocas Atoll for egg yolk and carapace. NR= δ^{15}N range, CR= δ^{13}C, SEA= standard ellipse area, CD= distance to centroid, MNND= mean nearest neighbour distance, SDNND= standard deviation of nearest neighbour distances, LQ = lower quartile, and UQ = upper quartile.

	NR	CR	CD	MNND	SDNND	SEA (‰^{2})		
						LQ	Median	UQ
Egg Yolk								
2017	4,3	7,8	2,2	0,6	0,3	7,9	9,1	10,6
2019	4,4	7,9	2,2	0,7	0,5	7,6	8,9	10,6
Carapace								
2017	6,8	8,0	1,9	0,5	0,5	7,2	8,0	9
2019	6,1	8,6	2,4	0,8	0,6	10,0	11,5	13,4

4. Discussion

The isotopic profile of adult female green turtles on Rocas Atoll suggests that herbivory in coastal benthic environments is the main feeding pattern for both nesting groups (2017 and 2019). Despite Thomson et al. (2018) presenting caveats in comparisons of isotopic profile at different spatial scales, even within the same species and gender-age class, comparisons between our results and current literature support this statement. The mean δ^{15}N values of our nesting green turtles (7.3‰ and 7.8‰) are within the isotopic range in carapace of nesting green turtles from the Caribbean region (2.5‰ to 9.9‰), which are known as primary herbivores (ZANDEN et al., 2013a; 2013b). Conversely, Hatase et al. (2006) determined the isotopic composition in egg yolks of omnivore green turtles in Pacific Ocean, whose mean δ^{15}N value was 11.4‰, which is 4‰ heavier than our egg yolk samples (6.8‰ and 7.1‰). This difference is large enough to distinguish a trophic level, separating herbivore from omnivore-carnivore consumers (FRY, 2008).

Heavier δ^{13}C values (less negative) are usually associated with coastal benthic environments (FRY, 2008). The mean δ^{13}C values in the egg yolk on Rocas Atoll (-17.4‰ and -17.5‰), for instance, is 2‰ heavier than egg yolk of the leatherback turtle (*Dermochelys coriacea*). The leatherback turtle is a pelagic oceanic consumer whose mean δ^{13}C values are -19.2‰ (Pacific Ocean) and -19.9‰ (Atlantic Ocean) (WALLACE et al., 2006). The difference from 2‰ in δ^{13}C values can distinguish coastal-benthic *vs*. pelagic-oceanic consumers (FRY, 2008).

The carapace δ^{15}N in nesting green turtles on Rocas Atoll is typical from an herbivore habit, but the NR metric, a measure of trophic breadth that includes the range of δ^{15}N values among individuals, indirectly suggests contribution of animal matter to

the diet over time. The NR from egg yolk, representing the diet from few months before oviposition, while females are in the foraging sites, is 1.5 times lower than NR from carapace, which tracks the diet from years ago. A larger NR implies that a consumer might overlap more than one trophic level, suggesting omnivore (LAYMAN et al., 2007). Since tissues have different metabolic and turnover rates, they were independent samples in the niche breadth analysis; however, the isotopic load of egg yolk is contained in the isotopic load of the carapace. Therefore, direct comparison between NR values in egg yolk and carapace has biological meaning, mainly for 2019 samplings when both tissues came from the same individual.

The niche breadth of nesting green turtles on Rocas Atoll was similar between years, in both tissues (egg yolk and carapace). The trophic diversity (NR, CR, CD and SEA) was comparable between years as well as the trophic redundancy (MNND and SDNND), which was overall high (values <1.0), revealing individuals with similar trophic ecologies and even distribution in the trophic niche space (LAYMAN et al., 2007). The only noteworthy difference between years was the SEA of carapace samples, larger in 2019 than in 2017; however, the SEAs overlap (57%-83%) suggest that this difference does not represent marked differences in the trophic niches.

5. Conclusions

The isotopic niche approach provided clues on how individuals from different nesting groups that nested in the same site are using the food resources over time (few months ago and years ago). The niche breadth alone does not allow inferences on foraging sites location, but this approach adds a piece to solve the puzzle of adult green turtle trophic ecology, presenting for the first time the isotopic signatures of nesting females on Rocas Atoll. Regardless of the original foraging site, the niche metrics pointed to a homogeneous feeding pattern in the two nesting groups (2017 and 2019), *i.e.* metrics are similar in both years.

Since the green turtle has a complex foraging strategy, with variations in feeding preference and trophic habitats along its wide distribution and among and within life-stages, we recommend multiple-tissue analysis to track any temporal variation in foraging habitats through stable isotopes. The utilization of trophic metrics or trophic models instead of stable isotopes values alone is interesting because some trophic details, such as suggested by δ^{15}N range (NR metric) in this study, could be lost, limiting a comprehensive understanding of the species trophic ecology.

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Capítulo 3:

Individual niche trajectories in nesting green turtles on Rocas Atoll, Brazil: an isotopic tool to assess diet shifts over time

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Individual niche trajectories in nesting green turtles on Rocas Atoll, Brazil: an isotopic tool to assess diet shifts over time

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Abstract

In this study, multi-tissue (yolk and carapace) stable isotope analysis was used to assess individual isotopic niche trajectories of nesting green turtles on Rocas Atoll, off northeastern Brazil, and to reveal a diet shift in the temporal dimension. The diet trajectories of individual green turtles were highly directional, with a stronger component towards decreasing values of $\delta^{15}N$ from carapace to yolk. When the green turtles are in their foraging sites (temporal window measured by the yolk samples), they are more herbivores. Conversely, in a broader temporal window, the green turtles demonstrate a carnivore-omnivore strategy, such as represented by heavier $\delta^{15}N$ values in the carapace. This finding confirms a temporal diet shift. This is the first study that applies trophic niche trajectories for sea turtles, adding a new isotopic tool to understand the trophic ecology of these migrant animals.

Keywords*:* *Chelonia mydas;* adult females; stable isotopes; trophic ecology; Atlantic Ocean.

Trajetórias de nicho individual em tartarugas verdes do Atol das Rocas, Brasil: uma ferramenta isotópica para verificar trocas de dieta ao longo do tempo

Resumo

Neste estudo, a análise de isótopos estáveis em múltiplos tecidos (vitelo e carapaça) foi usada para avaliar as trajetórias individuais de nicho isotópico de tartarugas verdes em nidificação no Atol das Rocas, nordeste do Brasil, e para revelar uma mudança de dieta na dimensão temporal. As trajetórias individuais da dieta de tartarugas verdes foram altamente direcionais, com um componente mais forte na direção de valores decrescentes de $\delta^{15}N$ da carapaça ao vitelo. Quando as tartarugas verdes estão em seus locais de forrageamento (janela temporal medida pelas amostras de vitelo), elas são mais herbívoras. Por outro lado, em uma janela temporal mais ampla, as tartarugas verdes demonstram uma estratégia carnívora-onívora, representada por

valores mais elevados de $\delta^{15}N$ na carapaça. Os resultados confirmam uma mudança temporal na dieta. Este é o primeiro estudo que aplica trajetórias de nicho trófico para tartarugas marinhas, adicionando uma nova ferramenta isotópica para entender a ecologia trófica desses animais migrantes.

Palavras-chave: *Chelonia mydas*; fêmeas adultas; isótopos estáveis; ecologia trófica; Oceano Atlântico.

1. Introduction

The green turtle (*Chelonia mydas* Linnaeus, 1758) is the only sea turtle species known to be herbivore after the individuals' recruitment from oceanic to coastal waters (Bjorndal 1997). However, the contribution of animal matter in its diet can be variable, as demonstrated in the last decade (e.g., Burkholder et al. 2011; Carman et al. 2012; Veléz-Rúbio et al. 2016; Di Beneditto et al. 2017; Fukuoka et al. 2019). Adult sea turtles are true migrants, moving between foraging and breeding sites thousands of kilometers apart. Comparing satellite telemetry data, Shimada et al. (2020) showed that fidelity to specific foraging sites following breeding migrations is common across several sea turtles species. For the green turtle, the authors stated high and longterm fidelity to specific foraging sites, with feeding activity happening during the migration to-and-from the breeding sites.

Stable isotopes are chemical proxies applied as a tool to analyse animals' trophic niches because they allow inferences on food resource use over many temporal scales (Newsome et al. 2007). The stable isotope of carbon ($\delta^{13}C$) represents the food resource origin, with more enriched values (less negative) in coastal than in oceanic waters and in benthic than in pelagic environments. The stable isotope of nitrogen ($\delta^{15}N$) tracks the animals' trophic position and it is more enriched at higher trophic levels, i.e. carnivore and omnivore consumers usually have more enriched $\delta^{15}N$ values in their tissues than herbivore consumers (Fry 2008).

Since each tissue has a specific metabolic rate, its turnover time, i.e. the time within which stable isotopes in tissues are replaced by stable isotopes derived from the food sources, is different (Auerswald

et al. 2010). Keratinous tissues, like hair and carapace scutes, are metabolically inert and maintain an isotopic record from the location where they were synthesized, representing the dietary information over a longer period (e.g. several months or years) (Hobson 1999). In metabolically active tissues, such as liver, this information refers to a shorter time, such as a few weeks (Hobson 1999). The vitellogenesis process of sea turtles happens 4 to 6 months before the female's migration to the breeding site, when she is still at the feeding site (Rostal et al. 1998). Thus, the egg yolk represents the dietary information in a narrower temporal window when compared to carapace scutes. By comparing the isotopic profile from different tissues, it is possible to analyse the temporal consistency of individual diet through isotopic niche trajectories (Costa-Pereira et al. 2019). The assumption is that individuals with temporally consistent diets have more similar isotope values across tissues than individuals with temporally variable diets (Martínez del Rio et al. 2009).

Individuals from the same population do not necessarily have temporally consistent foraging strategies, undergoing diet shifts over time, while others may have a more constant diet (Bearhop et al. 2004; Martínez del Rio et al. 2009). Fukuoka et al. (2019) demonstrated by stable isotope analysis and biologging experiments the seasonal diet shift in a juvenile green turtle population from Japanese Pacific waters. The temporal dimension of individual foraging strategies allows for an understanding of habitat use as a whole (Costa-Pereira et al. 2019), which is especially important for true migrant species, such as the sea turtles.

In this study, multi-tissue (yolk and carapace) stable isotopes were used to assess the consistency of trophic niches of nesting green turtles over time. This approach was adapted from Costa-Pereira et al. (2019), whose study with dozens of populations and hundreds of individuals from tropical frogs' species demonstrated the reliability of the trophic niche trajectories to measure the trophic consistency of individuals in a temporal scale. The carapace represents a comprehensive assimilation, integrating diet from several months to years ago, whereas the yolk

represents the food assimilation from a few months before oviposition, while the nesting females are still at the feeding sites. If the hypothesis of a consistent dietary pattern over time is supported, yolk and carapace isotope values will be similar, and variation in the individual trophic niche trajectories (lengths and angles) is not expected to differ from a random pattern of changes.

2. Material and Methods

2.1. Sampling

The sampling of green turtles on Rocas Atoll (03°51'S; 33°49'W) was authorized by the Brazilian Government by the license number 59809. This area is the second largest nesting site for the green turtles in Brazilian waters (Bellini et al. 2013). It is the only atoll in the South Atlantic Ocean, with 7.5 km^2 and located 266 km off the northeastern Brazilian coast. Rocas Atoll is a marine biological reserve; therefore, a pristine area. Bellini et al. (2013) conducted a comprehensive survey to monitor the nesting females in this site from 1990 to 2008. The nesting season occurs from December (beginning, with few nests) to May, with peak from February to April. The average number of nesting females per season is 73, with five nests per individual. The mean remigration period is 3.5 years. The authors estimated that 255 reproductively active females nested on the Rocas Atoll between 1990 to 2008. The authors observed a high site fidelity for the reproductive females in the Rocas Atoll nesting site, confirming its demographic independence in relation to a close reproductive site (Fernando de Noronha Island, 03°51'S; 32°25'W).

In the 2019 nesting season, 22 female individuals were sampled in Rocas Atoll, and sampling began as soon as each female initiated the first egg laying. All females had a healthy appearance, i.e. no visible tumours (Jones et al. 2016). During sampling, each female was measured for curved carapace length, from the nuchal notch to the tip of the longest posterior marginal scute (cm), and microchipped for individual identification (to avoid resampling). Two fresh eggs were sampled during the first laying, rinsed in filtered water, and the yolk separated

from other egg fractions. The bulk sample with two yolks (from the same female) was stored in a clean transparent plastic bag and kept frozen (-20°C) until analyses. A carapace fragment (5 cm^2) was sampled from the margin of the anterior scute, close to the nuchal notch. Each fragment was immersed in pure acetone to dissolve any incrustation. Then, it was rinsed in filtered water, dried at room temperature, grounded into a homogeneous powder and stored in a clean plastic vial until analyses. The fragment does not represent the oldest tissue in the carapace, as indicated in Lopéz-Castro et al. (2014), but since all samples were collected in the same way, bias is not expected.

2.2 Stable isotopes analysis

One gram of wet weight of yolk (bulk sample) was freeze-dried for 96 hours and ground into a homogeneous powder. Since yolk has a large amount of lipids (>50% in freeze-dried samples) (Carpentier et al. 2015), the samples were treated using a 2:1 solvent mixture of chloroform and methanol prior to lipid extraction (Bligh and Dyer 1959). The samples were dried at 60°C in an oven for 48 hours to remove the residual solvent. This procedure minimizes bias in $\delta^{13}C$ data interpretation (Post et al. 2007). Since the extraction of lipids can interfere with the $\delta^{15}N$ values (Petitet & Bugoni 2017); the yolk samples were analyzed twice: with and without lipid extraction. For $\delta^{13}C$, the mean values in yolk with and without lipid extraction were -17.9 ± 1.7‰ and -20.0 ± 1.7 ‰, respectively; and for $\delta^{15}N$ they were 7.2 ± 1.3‰ and 6.9 ± 1.3 ‰, respectively.

The ratios of stable isotopes were determined in 0.3-0.4 mg of dry weight of each sample (yolk and carapace) using an organic elemental analyzer (Flash 2000, Thermo Scientific) coupled with a mass spectrometer (Delta V Advantage Isotope Ratio Mass Spectrometer, Thermo Scientific) through the ConFlo-VI interface (Model BR30140, Thermo Scientific) in the Laboratório de Ciências Ambientais at Universidade Estadual do Norte Fluminense Darcy Ribeiro. Reference values were Pee Dee Belemnite (PDB) and atmospheric nitrogen. Samples were analyzed using analytical blanks

and urea analytical standards (IVA Analysentechnik-330802174). Analytical control was performed for every 10 samples using a certified isotopic standard (Elemental Microanalysis Protein Standard OAS). The reproducibility was based on triplicates for every 10 samples (±0.2‰, $\delta^{13}C$; ±0.3‰, $\delta^{15}N$).

2.3 Individual niche trajectories

Individual niche trajectories between isotope values of carapace and yolk quantified the temporal consistency of individual diets (Schmidt et al. 2007; Costa-Pereira et al. 2019). The length of trajectories in the bivariate isotopic space ($\delta^{15}N$-$\delta^{13}C$) was calculated by the Euclidean distance between starting points (carapace) and endpoints (yolk) for each individual. The trajectory direction was determined by the (counter clockwise) angle of the line connecting carapace and yolk values in relation to the x-axis ($\delta^{13}C$). The yolk and carapace are dependent measures because part of the dietary information integrated into one tissue is hierarchically integrated to another tissue. Differences between tissues regarding stable isotope values may emerge by temporal variation in environmental baselines and/or differential isotopic route, and not necessarily due to diet variation over time. Since these potential biases should be homogeneous across individuals, they are not expected to bias the results (Schmidt et al. 2007).

The vectors representing the niche trajectory between carapace and yolk for each green turtle individual in the isotopic space represent a dietary shift from the more general life pattern to the period of vitellogenesis. Differences in the angle of change indicate whether and in which direction individuals' isotopic values shift vertically ($\delta^{15}N$ or trophic level shift) and/or horizontally ($\delta^{13}C$ or base carbon sources) over time. The magnitude of the diet shift can be measured by the trajectory length. To compare the observed circular distribution of trajectories with the expected null distribution of random changes, we calculated three statistics: mean trajectory length, standard deviation of trajectory length (as a measure of variability) and Rao's spacing statistic (*U*). The spacing statistic measures the sum of differences of arc-lengths between

adjoining points (ranked by angle) and the regularly spaced arc-lengths expected for the null hypothesis of uniformity (2π/n) (Pewsey et al. 2013). The *U* values become larger as directionality increases, with less deviation between individuals' trajectories. The circular statistics were calculated in the R package *circular* (Agostinelli & Lund 2017; R Core Team 2020).

Following the procedure delineated in Costa-Pereira et al. (2019), we used a randomization process to generate stochastic individual niche trajectories in the isotopic space. For each individual, the observed isotopic starting point was kept constant (carapace isotopic values) and the isotopic endpoint was assigned by drawing randomly (without replacement) a pair of yolk isotopic values from the distribution of observed yolk isotope values in the sample of 22 individuals. For each resampling, the endpoint of random trajectories could therefore, assume isotope values observed in the conspecific individuals. The null model tested whether the mean length and standard deviation of observed niche trajectories are compatible with a random expectation, and whether the distribution of trajectory angles support a uniform circular distribution. The null distribution of trajectory statistics was based on 10,000 replicates. Probability values were calculated by the sum of permuted statistics that were equal to or more extreme than the observed, divided by 10,000. The stable isotope values used in this study, as well as the R script to calculate trajectory statistics and the randomization procedure are available as Supplementary material.

3. Results and Discussion

The multi-tissue isotopes explored the individual trophic consistency of nesting green turtles over the foraging period while individuals stay in the foraging sites (yolk samples), and in a broader temporal scale (carapace samples). Trajectory angles showed directionality towards lighter $\delta^{15}N$ values and heavier $\delta^{13}C$ values (Figure 1). The observed mean and standard deviation of niche trajectory length were smaller than expected by chance ($p < 0.0001$) (Figure 2). The observed sample was more directional than expected under a uniform circular distribution

(U = 176.568, *p* = 0.0083) (Figure 2). The curved carapace length was very homogeneous across the sample (n = 22 individuals; 112.5 ± 5.0 cm; 107 to 123 cm) and not associated with any isotopic or descriptive variable of the niche trajectory.

The results highlighted that most individuals vary in the consistency of their feeding strategies over time, following a similar pattern. Therefore, a temporal diet shift was noted for the reproductive females that nested at Rocas Atoll during the nesting season of 2019, and this shift was similar within the sample. When the green turtles are in their foraging sites (temporal window measured by the yolk samples), they are more herbivore with stronger association with coastal-benthic waters. Lighter $\delta^{15}N$ values represent lower trophic level, typical in herbivore strategy, whereas heavier $\delta^{13}C$ values are usually associated with coastal-benthic environments (Fry 2008). Conversely, in a broader temporal window, the green turtles demonstrate a carnivore-omnivore strategy, such as represented by heavier $\delta^{15}N$ values in the carapace. Heavier $\delta^{15}N$ values usually represent a greater contribution of animal matter to the consumer diet (Fry 2008), but they could represent variations in isotopic baseline across turtles' habitat. Different sources of nitrogen in turtles foraging sites influence the isotopic profile of turtles from the same trophic level (Ceriani et al. 2012; Pajuelo et al. 2012). However, if the $\delta^{15}N$ values represented the habitat baseline instead of the ingestion of animal matter, we would expect closer $\delta^{15}N$ values between yolk and carapace in the same turtle, which was noted for only three individuals (Figure 1).

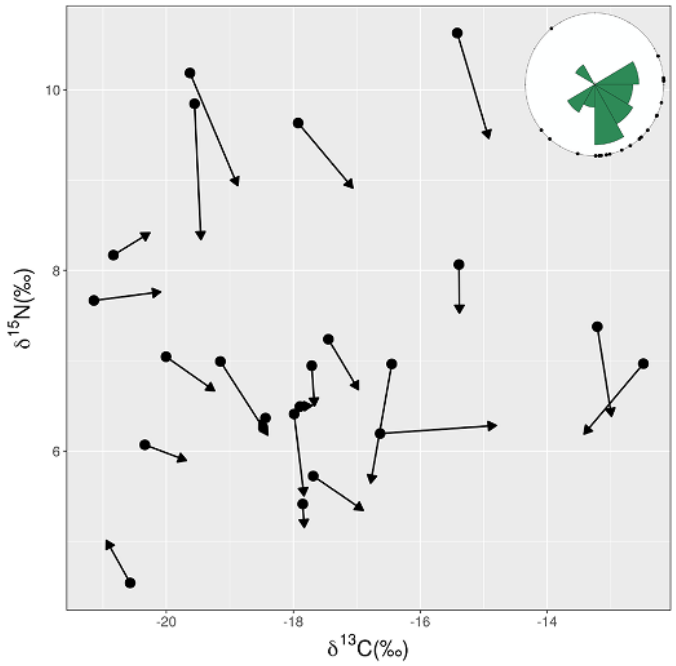

Figure 1. Individual niche trajectories represented by vectors between carapace (black circle) and yolk (black arrow) isotope values, for nesting green turtles on Rocas Atoll, Brazil. The inset represents the observed circular distribution of niche trajectory angles.

The temporal diet shift demonstrated for the green turtles that nest on Rocas Atoll had a directional pathway, albeit some individual tendencies are noted (Figure 1, Supplementary material). In the four individuals with the highest $\delta^{15}N$ values in the carapace (> 9‰), both oceanic and coastal foraging habitats are represented ($\delta^{13}C$ range), indicating isotopic enriched in different areas. In three individuals, yolk and carapace samples have similar $\delta^{15}N$ values and the temporal diet shift is not evident. Similarities between tissues regarding $\delta^{15}N$ values could represent habitat baseline. Individuals from the same population can vary in their foraging strategies over time, while others may have a more constant diet (Bearhop et al. 2004; Martínez del Rio et al. 2009). The nesting populations of sea turtles represent a mix of individuals from several foraging sites, and satellite telemetry reveals high fidelity to specific sites following breeding migrations (Meylan et al. 2011; Shimada et al. 2020). For the green turtle, Shimada et al. (2020) stated high and long-term fidelity to specific foraging sites, with feeding activity also happening during the migration to-and-from the breeding sites. These features support the individual tendencies described above.

Herbivory is recognized as the predominant feeding habit in adult green turtles (Bjorndal 1997; Burgett et al. 2018), but the adult individuals have enough feeding plasticity to take advantage of other food resources when necessary and available, allowing them to behave temporally like carnivores-omnivores (Burkholder et al. 2011). Agostinho et al. (2020) mentioned the possible foraging activity of nesting green turtles while stay at Rocas Atoll for breeding due to the high diversity of food items locally. Indeed, this area is a foraging site for juvenile green and hawksbill turtles (Bellini et al. 2013). The Rocas Atoll biodiversity includes 143 taxa of macroalgae (Villaça et al., 2010) and a range of zoobenthos and fish species (Moraes et al. 2003; Paiva et al. 2007; Batista et al. 2012; Paiva et al. 2015). The higher $\delta^{15}N$ values for oceanic habitats (more negative $\delta^{13}C$ values) noted in two nesting individuals (Figure 1) could be related to the animal matter ingested around the nesting site, that is an oceanic habitat; or even during the migration to-and-from the nesting site.

Figgener et al. (2019) organized an isotopic database for sea turtles species worldwide, demonstrating the variability of isotopic signatures among them, both inter- and intraspecifically, as well as similarities in the isotopic profile. The green turtle is the second most studied species, with 40% of the available stable isotope studies until November 2018 (Figgener et al. 2019). Meanwhile, data on the isotopic profile of nesting green turtles are still scarce, limited to five studies until the above period (Godley et al. 1998; Hatase et al. 2006; Vander Zanden et al. 2013a; 2013b; Bradshaw et al. 2017). Thus, this study also contributes to the database on $\delta^{13}C$ and $\delta^{15}N$ values in this species (Supplementary material).

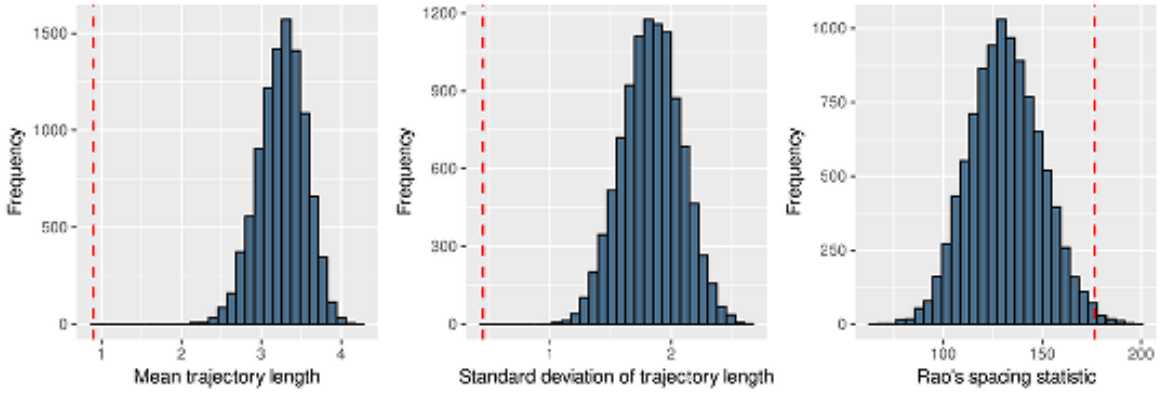

Figure 2. Null distributions of niche trajectory statistics: mean and standard deviation of trajectory length, the Euclidean distance between carapace and yolk stable isotope values from a given green turtle individual, and Rao's spacing statistic, quantifying the degree of directionality in niche trajectories. Red vertical lines indicate observed values.

Despite the small sample size (n = 22), the isotopic niche trajectories had strong statistical support to demonstrate the temporal diet shift for most green turtle individuals that nest on Rocas Atoll, off northeastern Brazil, during 2019 nesting season. To our knowledge, this is the first study that presents this approach for sea turtles, adding a new isotopic tool to understand the trophic ecology of these migrant animals. This approach has potential to be applied in other green turtle populations and/or in other sea turtle species. Since the adult green turtles can feed in the full extent of the habitat (foraging sites, breeding sites and to-and-from), changes in the food availability in these sites and over their migratory routes might compromise the health of the reproductive population. In a global scenario of rapid environmental changes, it deserves concern because sea turtles are long-lived endangered animals, and most species, like the green turtles, have high site fidelity for both feeding and breeding sites.

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6. Supplementary Material 1

Stable isotopes data of nesting green turtles on Rocas Atoll, Brazil (2019 nesting season), where CC is the curved carapace length in cm, C13C and N15C are the stable isotope values of carbon e nitrogen in carapace samples, respectively, and C13Y and N15Y are the stable isotope values of carbon e nitrogen in yolk samples, respectively (txt file format) (file ccyolk.txt in the R script).

CC	C13C	N15C	C13Y	N15Y
108	-17.448	7.238	-16.975	6.680
108	-17.896	6.493	-17.705	6.504
115	-19.557	9.846	-19.456	8.341
119	-18.439	6.366	-18.544	6.242
110	-12.485	6.967	-13.420	6.188
118	-20.006	7.044	-19.242	6.667
108	-20.836	8.169	-20.263	8.417
111	-17.711	6.945	-17.668	6.499
116	-17.852	5.414	-17.828	5.154
109	-20.340	6.069	-19.687	5.896
111	-15.391	8.065	-15.383	7.527
120	-20.572	4.540	-20.946	5.010
107	-19.150	6.992	-18.405	6.177
110	-21.143	7.667	-20.092	7.762
120	-15.418	10.628	-14.92	9.461
107	-16.450	6.964	-16.782	5.645
109	-17.988	6.410	-17.832	5.504
110	-19.629	10.18	-18.878	8.939
112	-17.925	9.632	-17.065	8.913
123	-17.688	5.724	-16.900	5.345
116	-13.211	7.377	-12.987	6.384
108	-16.633	6.194	-14.803	6.283

7. Supplementary Material 2

The R script used to calculate the individual isotopic niche trajectories for the nesting green turtles on Rocas Atoll, Brazil (text file format).

```
require(circular)
cpyk<-read.table("ccyolk.txt",header=T)
#Trajectory length
trajL<-sqrt(cpyk$C13^2 + cpyk$N15^2)
#Trajectory angles (circular object)
trajA←coord2rad(cpyk[,6:7])
#Rao's spacing statistic
rao.spacing.test(trajA)
#Arguments: x0, y0 = initial coordinates, x1,y1 = end coordinates, perm= number of permutations.
#function returns data frame with three columns (one for each statistic) and perm.n lines.
TrajPerm<-function(x0,y0,x1,y1,perm.n){require(circular)
  #define empty variable to contain permutation results
  permRao<-vector()
  permL<-vector()
  permSDL<-vector()
  #Permutation procedure
  for (i in 1:perm.n){
    np<-sample(1:length(x1))
    xp<-x1[np]-x0
    yp<-y1[np]-y0
    trajP<-coord2rad(xp,yp)
    #Calculate statistics to fill the vectors
    permL[i]<-mean(sqrt(xp^2+yp^2))
    permSDL[i]<-sd(sqrt(xp^2+yp^2))
    permRao[i]<-rao.spacing.test(trajP)$statistic}
  return(data.frame(pL=permL, pSDL=permSDL, pRao=permRao))}

#Function usage. generates data frame with 10000 rows in three columns, one for each statistic

test.perm<-TrajPerm(cpyk$C13C,cpyk$N15C,cpyk$C13Y,cpyk$N15Y,10000)
```

Capítulo 4:

Trace elements and stable isotopes in egg yolk of green turtles on Rocas Atoll, Brazil

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Trace elements and stable isotopes in egg yolk of green turtles on Rocas Atoll, Brazil

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Abstract

This study analyzed trace elements (As, Ba, Cd, Cu, Fe, Mn, Pb, Zn) and stable isotopes of carbon and nitrogen in egg yolk samples of female green turtles that nested in Rocas Atoll, Brazil, in 2017 and 2018. The trace elements concentration varied between years, with higher concentrations in 2017, suggesting that the nesting groups come from different foraging sites. The isotopic data indicated high overlap between years (73%), leading to an ambiguous interpretation on the turtles' foraging site. The Normalized Total Load presented a low association ($0.01 < R2 < 0.41$) with the stable isotopes. The Normalized Total Load that represents the trace element load in egg yolk is a holistic approach that can be applied elsewhere to predict ecotoxicology pathways in any animal species. We recommend a continuous monitoring to verify how the trace elements load behave in the nesting green turtles on Rocas Atoll.

Keywords: Chelonia mydas; Foraging site; Nesting site; Trace elements load; Isotopic composition

1. Introduction

Sea turtles are both flagship species of conservation concern and indicators of marine environment health (Figgener et al., 2019). As highly migratory species in the adult stage, the sea turtles can reflect the environmental health from many areas (Cortés-Gomez et al., 2017). Nesting populations of sea turtles represent a mix of individuals from several foraging sites, and satellite telemetry reveals high fidelity to specific foraging sites following breeding migrations (Meylan et al., 2011; Shimada et al., 2020).

Chelonia mydas (Linnaeus, 1758), known as green turtle, is widely distributed in all ocean basins and in the Mediterranean Sea in tropical and warm temperate waters. In the Atlantic Ocean, adult green turtles can migrate more than 3000 km between foraging and breeding sites (Mortimer and Carr, 1987; Baudouin et al., 2015).

Besides conservation efforts in Brazil and worldwide that reduced intentional captures and eggs harvesting (Seminoff, 2004; Broderick and Patricio, 2019), the green turtles (and other species) are vulnerable to marine pollution, both chemical and debris, assimilating or interacting with pollutants through feeding (Cort´es-Gomez et al., 2017; Bonanno and OrlandoBonaca, 2018; Muñoz and Vermeiren, 2020).

The Rocas Atoll (03º51′S; 33º49′W) is the second largest nesting site for the green turtle in Brazilian waters (Bellini et al., 2013). It is a biological marine reserve located 266 km from the northeastern Brazilian coast. The green turtles that nest in Rocas Atoll and Fernando de Noronha Island (03º51′S; 32º25′W) share haplotype frequencies, but they are genetically diverse from those on Trindade and Martin Vaz Island (20º30′S; 29º20S), which is the largest nesting site for the species in Brazil (Bjorndal et al., 2006). Thus, genetic evidence highlights green turtles from the Rocas Atoll-Fernando de Noronha Island system as key individuals for maintaining the genetic diversity of the species in the South Atlantic Ocean. Despite sharing haplotype frequencies, during 19 consecutive years only four females were observed in both nesting sites, indicating that the sites are demographically independent (Rocas Atoll and Fernando de Noronha Island), even though they are separated by only 150 km (Bellini et al., 2013).

Bellini et al. (2013) conducted a comprehensive survey (1990 to 2008) to monitor nesting females from Rocas Atoll. Briefing the authors' main results, the local nesting season occurs from December (few nests) to May, with a peak from February to April. The average number of nesting females and nests per season were 73 and 335, respectively, with 5.2 nests per individual. The inter nesting interval ranged from 8 to 17 days, and the remigration period was 3.5 years. The authors stated that the green turtles that nest in Rocas Atoll could come from several foraging sites, although the locations of these sites are still unknown.

Trace elements occur in organisms in very small quantities (<0.01%) and include essential elements with metabolic and physiological importance, such as Fe, Cu and Zn, and nonessential toxic elements, such as As, Cd, Pb and Hg (Ali and Khan, 2019). Due to its longevity, sea turtles can bioaccumulate nonessential toxic elements through the food chain and direct exposure albeit the main pathway is the trophic pathway (Cortés-Gomez et al., 2017). As an oviparous species, the maternal transference to eggs is also a pathway of trace elements in sea turtles (Sakai et al., 1995; Páez-Osuna et al., 2010a, 2010b, 2011; Ehsanpour et al., 2014; Sinaei and

Bolouki Kurandeh, 2017). The maternal transference to eggs does not include the entire trace element load of the nesting female, but a percentage of this load, which is variable among elements (*e.g.*, 0.5% for Pb: Páez-Osuna et al., 2010a; 0.2% for Cd, 7.8% for Cu, 3.4% for Zn and 21.5% for Ni: Páez-Osuna et al., 2010b; 2% for Hg: Páez-Osuna et al., 2011). Some authors stated that there are no differences in trace elements load among clutches from the same female in a given nesting season (Guirlet et al., 2008; Sinaei and Bolouki Kurandeh, 2017), *i.e.* transfer rate to their eggs is constant. However, as each female has its own trace elements load, the maternal transference to eggs may vary among clutches from different females. Zinc, for instance, can be regulated through homeostatic processes and does not exhibit significant differences among nesting sites in sea turtles populations, whereas Cd and Ni, for instance, are not actively controlled by females, with concentrations changing as a function of the degree of exposure in the environment (Sakai et al., 1995; Paez-Osuna ´ et al., 2010b).

The isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) derive from the trophic pathway, in which animals participate, *i.e.*, animals' isotopic signatures correspond to the isotopic signatures of their feeding sources and trophic habitat (Layman et al., 2007; Newsome et al., 2007). For sea turtles, stable isotopes composition can be applied to assess foraging distributions across large proportions of a nesting population, since this approach is analogous to the satellite telemetry to track individuals' movement, but with lower spatial resolution despite lower cost (Pearson et al., 2017, 2019). Carpentier et al. (2015) compared isotopic signatures of whole blood and epidermis in nesting loggerhead turtles (*Caretta caretta*) with egg yolk and hatchlings, concluding that they correlate significantly to infer information about the female foraging habits. In sea turtles, the vitellogenesis process occurs 4 to 6 months before the female's migration to the breeding site (Rostal et al., 1998). Therefore, the egg yolk represents feeding ($\delta^{13}C$, $\delta^{15}N$) when the individual is still at its foraging site.

Trace elements and stable isotopes can be used together in ecotoxicological approaches to trace the elements assimilation from a trophic habitat (Asante et al., 2008; Liu et al., 2018). Regardless the sea turtle's metabolism that regulates the trace elements load, the concentrations in eggs derive from the female's body. The meta-analysis review did by Cortés-Gomez et al. (2017) stated that the main trace elements pathway to sea turtles body is their foraging habits; therefore, due to the high fidelity

to specific foraging sites, as demonstrated in Shimada et al. (2020), we can infer that the trace elements load in egg yolk is indirectly derived from the female's foraging site. Ultimately, the egg yolk samples can be used as a proxy for the females' trace elements load and isotopic signatures.

This study analyzed essential (Cu, Fe, Mn, Zn) and nonessential (As, Ba, Cd, Pb) trace elements and stable isotopes ($\delta^{13}C$, $\delta^{15}N$) in the egg yolk of green turtles from Rocas Atoll to evaluate if these chemical proxies are suitable in order to trace the individuals' foraging site. We compare the trace elements load and isotopic signatures in egg yolks from two consecutive nesting seasons (2017 and 2018) in order to make inferences about whether the nesting green turtles come from the same foraging site or not. Since nesting sea turtles could come from mixed foraging groups (Meylan et al., 2011), we hypothesized that no difference would be anticipated between nesting seasons.

2. Material and methods

2.1. Sampling

We sampled 14 nests in 2017, and 13 nests in 2018. From each nest, two unhatched intact eggs were sampled. After sampling, the eggs were washed in ultrapure water and the yolk was separated from other egg fractions (albumen and shell). The two egg yolks formed a bulk sample that represents a given nesting female. Since each nest had an identification tag, with correspondence to each turtle (individual recognition by microchip or flipper tag), no resample occurred. Each bulk sample was kept frozen (- 20ºC) in a transparent and clean plastic bag until trace element and stable isotope analyses.

2.2. Choice and determination of the trace elements

The choice of the trace elements for this study followed Agostinho et al. (2020). From the same dataset we choose 8 from 12 trace elements, whose concentrations were above the limit of detection (LOD) in more than 30% of the sample, at least in one nesting season (2017 and/or 2018) (Table 1; details in Agostinho et al., 2020). One gram of wet weight of egg yolk was solubilized in 10 mL of 65% HNO_3 and 1.5 mL of a filtered 30% H_2O_2 solution, and the final volume was 15 mL. Since the certificate reference material recovery showed good results (Table 1), the possible trace element loss during the filtering processes did not significantly affect the results.

The trace elements were determined by inductively coupled plasmaoptical emission spectrometry (ICP-OES, 720 ES, Varian) in the Laboratorio de Ciências Ambientais at Universidade Estadual do Norte Fluminense Darcy Ribeiro. A certificate reference material (standard reference material DORM-3) was analyzed to test the precision and accuracy of the analytical method and achieved recovery values above 80% (Table 1). The coefficients of variation were lower than 15%. The concentrations of all trace elements were recorded in $\mu g.g^{-1}$ of wet weight.

The LOD for each trace element varied with the sample mass. The LODs were calculated according to Skoog and Leary (1992) as follows: LOD = ((3*SD/*a*)*v)/m, where SD is the standard deviation of 40 blank samples, *a* is the slope, m is the mean mass of each sample, and v is the final filtered volume. We performed the ICP-OES calibration for 30 samples and the quantification control with a calibration standard of 0.05 mg L- 1 for 15 samples to avoid measurement errors. The percentage of egg yolk samples with concentration values below the LOD are shown in Table 1. The concentration values for each trace element in each sample are in Supplementary material of Agostinho et al. (2020).

Table 1. Validation of the analytical procedure using DORM-3 (fish protein) certificate reference material for trace elements ($mg.kg^{-1}$) (*n* = 3) and percentage of egg yolk samples above the limit of detection (>LOD) in each nesting season. ns: not specified.

Trace Elements	Determined concentration Mean±standard deviation	Certified concentration	Recovery %	>LOD 2017	>LOD 2018
As	8.35 ± 0.30	6.88	121	93%	46%
Ba	5.57 ± 0.15	ns	-	100%	92%
Cd	0.32 ± 0.02	0.29	110	86%	8%
Cu	18.0 ± 0.4	15.5	116	93%	69%
Fe	348.0 ± 1.15	347.0	100	100%	92%
Mn	3.87 ± 0.23	4.6	84	86%	76%
Pb	0.44 ± 0.06	0.0	110	43%	23%
Zn	52.7 ± 0.3	51.3	103	100%	100%

2.3. Analysis of stable isotopes ratios (δ13C and δ^{15}N)

The $\delta^{13}C$ and $\delta^{15}N$ analyses were performed in the same samples whose trace element concentrations were previously determined. One gram of wet weight of egg yolk (bulk sample) was freeze-dried for 96h and ground into a homogeneous powder. Since egg yolk has a large amount of lipids (>50% in freeze-dried samples) (Carpentier et al., 2015), the samples were treated using a 2:1 solvent mixture of chloroform and methanol prior to lipid extraction (Bligh and Dyer, 1959). Then, the samples were dried

at 60 ºC in an oven for 24–48 h to remove the residual solvent. This procedure removes the influence of the $\delta^{13}C$ depleted values of lipids and prevents bias in $\delta^{13}C$ data interpretation (Post et al., 2007). However, the extraction of lipids can interfere with the $\delta^{15}N$ values (Petite and Bugoni, 2017); to avoid a possible second bias, the egg yolk samples were analyzed twice: with and without extraction of lipids. The ratios of stable isotopes were determined in 0.4 mg of dry weight using an organic elemental analyzer (Flash 2000, Thermo Scientific) coupled with a mass spectrometer (Delta V Advantage Isotope Ratio Mass Spectrometer, Thermo Scientific) through the ConFlo-VI interface (Model BR30140, Thermo Scientific) in the Laboratório de Ciências Ambientais at Universidade Estadual do Norte Fluminense Darcy Ribeiro. Reference values were Pee Dee Belemnite (PDB) and atmospheric nitrogen. Samples were analyzed using analytical blanks and urea analytical standards (IVA Analysentechnik-330,802,174). Analytical control and reproducibility were performed for every 10 samples using a certified isotopic standard (Elemental Microanalysis Protein Standard OAS) and were based on triplicates for every 10 samples (±0.2‰, $\delta^{13}C$; ±0.3‰, $\delta^{15}N$), respectively. The stable isotope results are presented as parts per thousand (‰).

2.4. Data analysis

All statistical analyses were performed in the R program (R Core Team, 2020) an *a priori* error of 5% was assumed ($\alpha = 0.05$) in all hypothesis tests. The descriptive statistics used in this study were the median and interquartile range (abbreviated by IQR) due to the asymmetry and presence of outlines in most of trace elements data (Table 1 - Supplementary material). If the mean (average values) had been used as a measure of centrality and the standard deviation as a measure of dispersion, the representation of the data would have been biased (mean and standard deviation are strongly affected by asymmetry and outliers, especially with lower sample sizes). The IQR and median were used for the calculation of a nonparametric coefficient of variation (CV) in place of the standard deviation and mean.

To investigate if the isotopic composition of the nesting populations from 2017 and 2018 indicates convergence or divergence regarding their isotopic profiles, the bivariate distribution of $\delta^{13}C$-$\delta^{15}N$ data was constructed (data.ellipse function, car package; Fox and Weisberg, 2019) with a 40% confidence interval, as suggested by Jackson et al. (2011). Differences between nesting seasons were tested using the maxLikOverlap function to calculate the ellipses overlap (Stable Isotope Bayesian

Ellipses in R package - SIBER; Jackson et al., 2011). The Bayesian assessment for the comparison of ellipses areas proposed by Jackson et al. (2011) is appropriate for small sample sizes (at least 10 samples), as in the present study.

To analyze how the trace element concentrations are related to the isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) in egg yolks, two assessments were carried out. First, the association of each trace element with each stable isotope was assessed using linear regression (lm function, base package, R Core Team, 2020). The trace elements and stable isotopes associations were detailed through 30 regression adjustments (eight trace element associations with each of two stable isotopes for 2017 and 2018 nesting seasons, excepted for Cd in 2018 due to only one sample > LOD) (Table 2 - Supplementary material). The equations, R2 values and *p*-values were reported (Table 2 - Supplementary material). Since the concentration of each trace element varies greatly, as indirectly demonstrated by the CV values (Table 1 - Supplementary material), we proposed a Normalized Total Load calculation of trace elements in the green turtle's egg yolk, as follows:

$$\text{Normalized Total Load } (\mu g.\, g^{-1}) = \sum_{1}^{nm8} \frac{\text{Element concentration } (\mu g.g^{-1})}{\text{N of Elements>LOD}}.$$

The equation represents the sum of trace element concentrations in each egg yolk sample (representing an individual) weighted by the number of elements detected in that individual (trace element load). Since we did not measure the carapace length of the nesting females that laid each egg sample, this variable was not included in the calculation, *i. e.* it was not normalized by turtle's size. Meanwhile, Agostinho et al. (2020) presented the curved carapace length of nesting females on Rocas Atoll from the same nesting seasons (2017 and 2018): 113.0 ± 3.7 cm. The CV is low (0.03), indicating negligible difference among sizes. Furthermore, Bellini et al. (2013) in a long-term approach with the same nesting population also report little variation in sizes (CV < 5% over 19 consecutive years). Therefore, the lack of length values to our samples will not compromises the data interpretation.

The adult green turtles feed on multiple feeding sources (Hatase et al., 2006), whose nutrients and trace elements are maternally transferred to the eggs. Since each source (or food item) has a distinct concentration of trace elements, when calculating the Normalized Total Load that brings together all the elements it is possible to reduce the noise from different sources. Thus, the Normalized Total Load represents the trace element load assimilated from maternal transference and provides a holistic view of

the ecotoxicological pathway.

The Normalized Total Load was applied in linear regressions with $\delta^{13}C$ and $\delta^{15}N$ to evaluate how they were related to each other. The regressions were presented graphically with their 95% confidence intervals, and the equations, R2 values and *p*-values were reported. The difference for each trace element and Normalized Total Load between nesting seasons was assessed using ANOVA (aov function, base package, R Core Team, 2020), and the *p*-values were reported. Data was transformed to natural logarithmic, as indicate by a maximum likelihood function (boxcox; MASS package; Venables and Ripley, 2002), to meet ANOVA requirements (linearity, normality and homoscedastic). ANOVA's assumptions were validated through the use of diagnostic plots, as suggested by Belsley et al. (1980).

3. Results

Fig. 1 represents the isotopic composition ($\delta^{13}C$-$\delta^{15}N$) of egg yolk samples from 2017 and 2018 nesting seasons, indicating high overlap between them (73% ± 27; p = 0.95), *i.e.* similar values in both years, as also showed in Table 1 - Supplementary material. The median values for each trace element concentration and Normalized Total Load indicate higher values for egg yolk samples in 2017 nesting season (Table 1 - Supplementary material). The regressions adjustments for each trace element indicate which one contributes most to the Normalized Total Load and stable isotopes associations. Since most equations (93%) were non-significant, we assumed that the contribution was similar among the trace elements (Table 2 - Supplementary material).

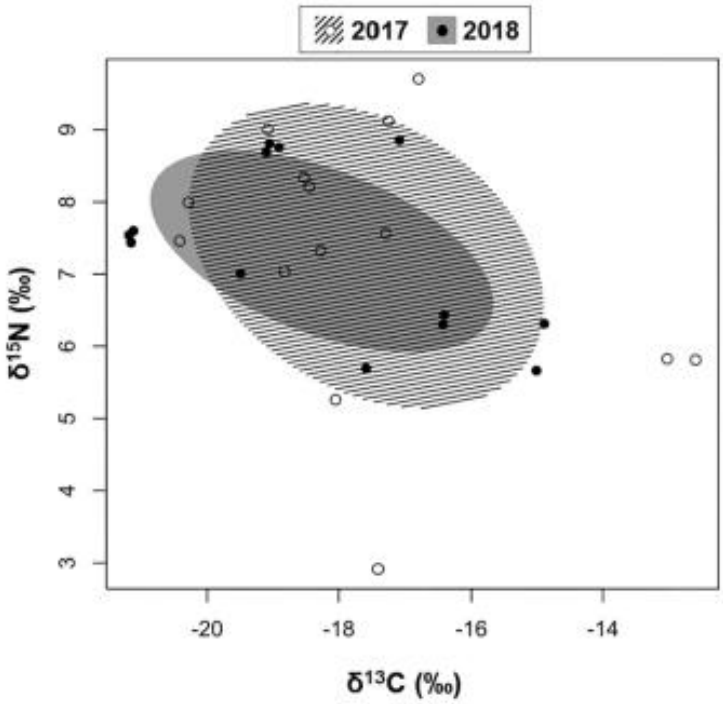

Fig. 1. Stable isotopes ($\delta^{13}C$ $\delta^{15}N$) in egg yolk samples of green turtles on Rocas Atoll in 2017 (open circles and hatched ellipse) and 2018 (closed circles and grey ellipse) nesting seasons. Hatched and grey ellipses represent the core niche area (Jackson et al., 2011) that encompasses 40% of the data for 2017 and 2018 nesting seasons, respectively.

The Normalized Total Load presented a low association (R2 < 0.08) with the stable isotopes in egg yolk samples, except for $\delta^{15}N$ in 2018 samples (R2 = 0.41; *p* = 0.03) (Fig. 2).

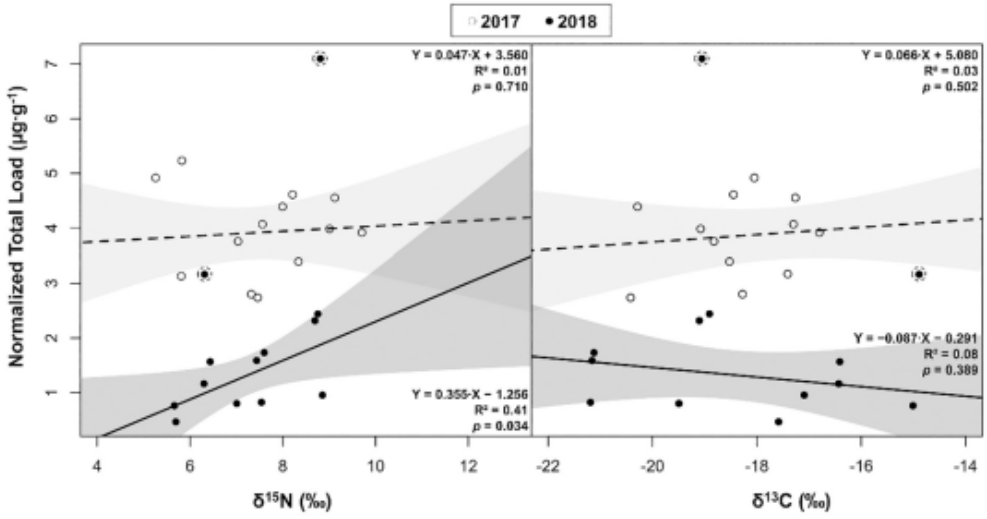

Fig. 2. Association between Normalized Total Load ($\mu g \cdot g^{-1}$) and stable isotopes in egg yolk samples of nesting green turtles in Rocas Atoll in 2017 (open circles) and 2018 (closed circles) nesting seasons. The grey shading represents the 95% confidence interval of the regressions. Regression statistics (equation, R2 values and *p*-values)

are shown inside each graph. Marked outliers were removed from the 2018 regressions analysis for both isotopes. With the outliers, the regression statistics for the $\delta^{15}N$ would be: Y = 0.686·X -3.106, R2 = 0.22, p = 0.10 and, for the $\delta^{13}C$, it would be: Y = -0.040·X + 1.168, R2 < 0.01, p = 0.86.

The trace elements concentration and the Normalized Total Load varied between the two nesting seasons, in the same way, with higher concentrations in egg yolk samples from 2017 (Table 1 – Supplementary material). For five elements (Ba, Cu, Fe, Mn, Zn) and Normalized Total Load, the difference is demonstrated by a strong statistical support (Fig. 3). For the other three elements (As, Cd, Pb), the difference is highlighted by the percentage of samples above the LOD, which were higher in 2017 than in 2018 (Table 1).

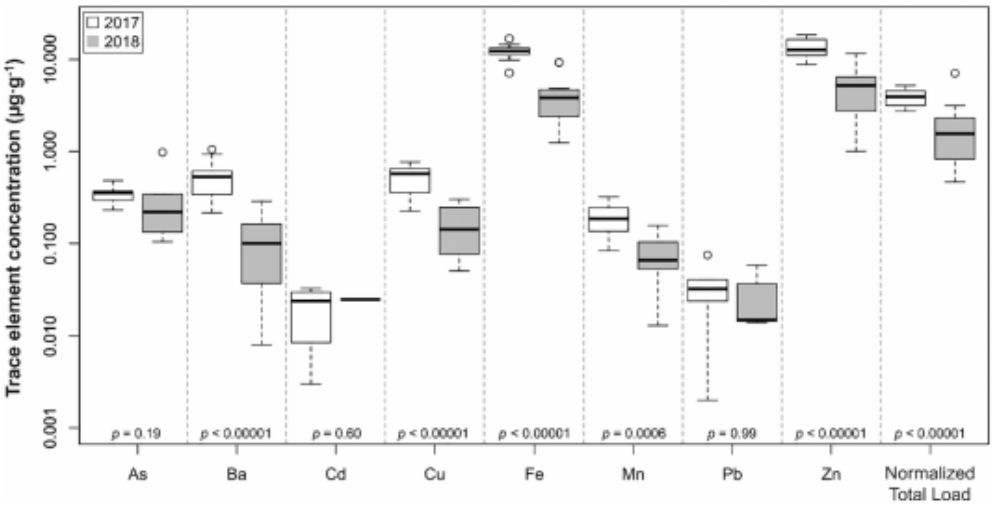

Fig. 3. Boxplots representing median (bar inside the box), interquartile range (box: 1st to 3rd quartile), minimum and maximum values of the trace elements concentrations and Normalized Total Load in egg yolk samples of nesting green turtles on Rocas Atoll in 2017 (white box) and 2018 (grey box) nesting seasons. Open circles are outliers (values greater than the 3rd quartile + 1.5 times the interquartile range or lower than the 1st quartile - 1.5 times the interquartile range) and *p*-values are the significances from ANOVA for the comparisons between nesting seasons. The y-axis distances were log-transformed.

4. Discussion

The trace elements concentration and the isotopic composition of egg yolk samples of nesting green turtles on Rocas Atoll led to different interpretations regarding the individuals' foraging site. The concentrations of As, Ba, Cd, Cu, Fe, Mn, Pb and Zn, and the Normalized Total Load suggest that the female turtles from 2017 and 2018 nesting seasons came from foraging sites with different levels of anthropic

influence and/or basal sources, which influence the trace elements concentration in the egg yolk, applied here as a proxy for the trace elements in the females' body. This finding refutes the hypothesis that no difference would be anticipated between nesting seasons because females could come from mixed foraging groups, as indicated by Meylan et al. (2011) for the sea turtles and speculated by Bellini et al. (2013) for the nesting green turtles on Rocas Atoll.

Villa et al. (2017) and Barraza et al. (2019) investigated trace elements concentration in blood of green turtles from foraging sites with different sources of pollution and natural levels of trace elements, identifying habitat-specific differences and location-specific contaminant signatures. Therefore, foraging sites with high levels of trace elements may affect the turtles in different ways. Furthermore, the maternal transference of trace elements to eggs is highly variable among elements (Paez-Osuna ´ et al., 2010b). Thus, the discriminating between foraging sites using trace elements concentrations might be more complex than it seems. The Normalized Total Load minimized somehow the biases described above, since all elements were considered to infer differences in foraging sites of green turtles that nested on Rocas Atoll during 2017 and 2018.

The isotopic composition of the egg yolks, in turn, pointed to two possible interpretations, which might lead to a misunderstanding if the stable isotopes of carbon and nitrogen were analyzed alone to track the nesting turtles' foraging site. According to the isotopic data, which are similar between 2017 and 2018, the nesting green turtles could come from the same/close foraging site, in which they feed on same resources. Conversely, the turtles could come from different foraging sites, where food resources are likely different albeit have similar isotopic profile. The latter situation was previously observed for the green turtles from Australia and Costa Rica, with the same isotopic profile even more than 15,000 km away (Thomson et al., 2018). The green turtle is mainly carnivore at early life stage, shifting to a more herbivore diet in adulthood, but the individuals can take advantage of animal matter as food resources and behave like omnivores (Bjorndal, 1997; Hatase et al., 2006; Burkholder et al., 2011; Burgett et al., 2018). Thomson et al. (2018) highlighted the complexity of green turtle trophic interactions throughout its distributional range, with different levels of specialization among geographic regions, and among and within life-stages.

Both trace elements concentration and stable isotopes composition in vertebrates represent its trophic pathway; so, both chemical proxies have the same

main route (dietary intake), reflecting the elements load and isotopic profile of the feeding resources. In general, the trophic transference of trace elements increases at higher trophic levels, and a lower load of trace elements is expected in herbivorous consumers compared to omnivorous or carnivorous (Chojnacka and Saeid, 2018). Similarly, the $\delta^{15}N$ signatures are usually more enriched at higher trophic levels, whereas for $\delta^{13}C$ signatures, a proxy for food source origin, the values are more enriched (less negative) in coastal-benthic trophic habits than in oceanic-pelagic areas (Fry, 2008). The low association between Normalized Total Load and stable isotopes in egg yolk samples, as showed in Fig. 2, reinforce that to our data these chemical proxies led to different interpretations. The only noteworthy association refers to the Normalized Total Load and $\delta^{15}N$ in 2018, with a positive and significant relationship that may represent a greater contribution of animal matter to some individuals from this nesting season. Individuals from the same population do not necessarily have consistent foraging strategies over time, changing the consumed food resources (Bearhop et al., 2004; Martínez del Rio et al., 2009).

Data on the isotopic profile of nesting green turtles are still very limited (Godley et al., 1998; Hatase et al., 2006; Vander Zanden et al., 2013a, 2013b; Bradshaw et al., 2017), and this study contributes with new data on $\delta^{13}C$ and $\delta^{15}N$ signatures for this life stage. Defining the trophic position only by isotopic composition is not so simple for sea turtles because there are large variations in isotopic baseline across its habitat. For instance, adult loggerhead turtles (*Caretta caretta* Linnaeus, 1758) from Northwest Atlantic Ocean varying in $\delta^{15}N$ values due to different sources of nitrogen in their foraging sites, and not due to differences in their trophic positions (Ceriani et al., 2012; Pajuelo et al., 2012). The leatherback turtles (*Dermochelys coriacea* Linnaeus, 1766) are strictly pelagic, foraging mainly on jellyfish, but their isotopic values are different in the Pacific and Atlantic Oceans (Wallace et al., 2006). The isotopic composition of sea turtles can assess the populations foraging distribution like in the satellite telemetry technique, such as recorded in many studies (*e.g.*, Seminoff et al., 2012; Bradshaw et al., 2017; Pearson et al., 2017, 2019). However, for the nesting green turtles on Rocas Atoll the isotopic composition ($\delta^{13}C$-$\delta^{15}N$) alone did not allow a clear interpretation of the foraging site from where they come from.

The Normalized Total Load that represents the maternal transference to eggs and, ultimately, the elements load of the nesting female assimilated from their feeding activities, is a holistic approach proposed here to predict how the trace elements load

is related to stable isotopes. This approach considered the elements load instead of each trace element separately, and it can be applied elsewhere to predict ecotoxicology pathways in any animal species. If the research interest is a particular set of trace elements (or contaminants), each relationship should be evaluated separately; however, if the aim is to evaluate the ecotoxicology pathways in a macro scenario, for instance, this methodological approach minimizes redundancies and makes data interpretation easier and consistent.

Differences in trace elements concentration regarding green turtles from 2017 and 2018 nesting seasons highlight an important conservation issue: the habitat quality, since green turtles have high site fidelity to its foraging area. In a global scenario of rapid changes that negatively affect the marine environment, these results demand concern. As a migratory species, the full extent of the green turtles habitat have to be considered for conservation purposes, including foraging sites, migratory routes and breeding sites. Therefore, we recommend a continuous monitoring to verify how the trace elements load behave in the nesting green turtles on Rocas Atoll, since this site is important to the maintenance of the species genetic diversity in the South Atlantic Ocean.

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6. Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The sampling of green turtles from Rocas Atoll was authorized by the Brazilian Government by the sampling licenses numbers 40636 and 59809.

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9. Supplementary material

Table 1. Median ± interquartile range (IQR) of the trace elements concentrations and stable isotopes composition in egg yolk samples of nesting green turtles on Rocas Atoll in 2017 and 2018 nesting seasons. The trace elements and Normalized Total Load are expressed as $\mu g \cdot g^{-1}$ wet weight and the stable isotopes as ‰.

Trace elements and stable isotopes	Egg yolk					
	2017			2018		
	Values	N	Coefficient of variation (IQR/median)	Values	N	Coefficient of variation (IQR/median)
As	0.35 ± 0.08	13	0.22	0.22 ± 0.17	6	0.77
Ba	0.53 ± 0.27	14	0.51	0.10 ± 0.12	12	1.20
Cd	0.02 ± 0.02	12	0.84	0.03 ± 0.00	1	0.00
Cu	0.57 ± 0.29	13	0.51	0.14 ± 0.15	10	1.04
Fe	12.41 ± 1.81	14	0.15	3.83 ± 2.30	11	0.60
Mn	0.19 ± 0.10	12	0.55	0.07 ± 0.05	11	0.75
Pb	0.03 ± 0.02	6	0.49	0.01 ± 0.02	3	1.50
Zn	12.77 ± 4.75	14	0.37	5.21 ± 3.71	13	0.71
Normalized Total Load	3.94 ± 1.28	14	0.32	1.56 ± 1.49	13	0.95
$\delta^{13}C$	-18.2 ± 1.5	14	0.08	-18.9 ± 3.1	13	0.16
$\delta^{15}N$	7.5 ± 2.2	14	0.29	7.4 ± 2.4	13	0.32

Table 2. Associations of the trace element concentrations with $\delta^{13}C$ and $\delta^{15}N$ in nesting green turtles on Rocas Atoll from 2017 and 2018 nesting seasons. The R^2 values and *p*-values and slope are shown. Asterisks indicate significant regressions with $R^2 > 0.30$.

Trace elements	Stable isotopes	Season	Equation	R^2	*p*-value
As	$\delta^{13}C$	2017	Y = 0.02·X 0.704*	0.36	0.03
		2018	Y = 0.043·X 1.096	0.09	0.57
	$\delta^{15}N$	2017	Y = -0.003·X 0.37	0.00	0.82
		2018	Y = 0.188·X -0.978	0.43	0.16
Ba	$\delta^{13}C$	2017	Y = -0.057·X -0.453	0.27	0.06
		2018	Y = 0.006·X 0.214	0.02	0.63
	$\delta^{15}N$	2017	Y = 0.058·X 0.123	0.18	0.13
		2018	Y = 0.033·X -0.135	0.19	0.16
Cd	$\delta^{13}C$	2017	Y = 0.001·X 0.046	0.10	0.31
		2018	------	------	------
	$\delta^{15}N$	2017	Y = -0.002·X 0.032	0.03	0.60
		2018	------	------	------
Cu	$\delta^{13}C$	2017	Y = -0.008·X 0.369	0.01	0.75
		2018	Y = 0.008·X 0.31	0.05	0.54
	$\delta^{15}N$	2017	Y = -0.021·X 0.66	0.04	0.50
		2018	Y = 0.008·X 0.104	0.01	0.80
Fe	$\delta^{13}C$	2017	Y = 0.404·X 19.385	0.17	0.15
		2018	Y = 0.252·X 8.941	0.05	0.53
	$\delta^{15}N$	2017	Y = 0.134·X 11.296	0.01	0.71
		2018	Y = 0.583·X -0.126	0.06	0.48

Mn	$\delta^{13}C$	2017	Y = -0.006·X 0.076	0.05	0.50
		2018	Y = -0.001·X 0.054	0.01	0.82
	$\delta^{15}N$	2017	Y = 0.021·X 0.043	0.27	0.08
		2018	Y = 0.006·X 0.033	0.03	0.63
Pb	$\delta^{13}C$	2017	Y = -0.007·X -0.096*	0.73	0.03
		2018	Y = 0.011·X 0.236	0.73	0.35
	$\delta^{15}N$	2017	Y = 0.004·X 0.012	0.10	0.53
		2018	Y = 0.019·X -0.128	0.33	0.61
Zn	$\delta^{13}C$	2017	Y = 0.098·X 15.116	0.01	0.81
		2018	Y = -0.111·X 2.959	0.01	0.79
	$\delta^{15}N$	2017	Y = 0.167·X 12.174	0.01	0.74
		2018	Y = 1.284·X -4.399	0.25	0.08

2. Discussão Geral

O presente estudo contribuiu para a compreensão da ecologia trófica das tartarugas verdes que utilizam o Atol das Rocas como área de nidificação, utilizando os isótopos estáveis de carbono e nitrogênio como principal método de análise e interpretação dos resultados. De modo geral, os valores de $\delta^{13}C$ e $\delta^{15}N$ determinados nos tecidos (sangue total, vitelo e carapaça) das tartarugas refletiram a alimentação preferencialmente herbívora desses animais. Entretanto, há contribuição de matéria animal na dieta a longo prazo, inferida a partir dos valores de $\delta^{15}N$ da carapaça. No capítulo 2, por exemplo, a amplitude trófica calculada indica a contribuição de matéria animal na alimentação dos animais em uma janela temporal mais ampla e de maneira uniforme. Os resultados do capítulo 4 demonstraram a associação entre a carga total de elementos traço e os valores de $\delta^{15}N$ no vitelo de alguns indivíduos da temporada reprodutiva de 2018, reforçando a contribuição de presas animais na dieta das tartarugas verdes adultas (Figura 2).

A sobreposição isotópica entre as tartarugas das temporadas reprodutivas de 2017 e 2018, conforme dados do capítulo 4, levou a duas interpretações distintas sobre sua procedência: elas são provenientes da mesma área de forrageamento, onde alimentam-se das mesmas presas, ou elas são procedentes de áreas de forrageamento diferentes, com recursos alimentares diferentes, mas que têm valores isotópicos similares. Apesar dessa dicotomia, os resultados dos capítulos 1 e 2 apontaram para um padrão trófico constante ao longo dos anos, independente das áreas de forrageamento utilizadas.

A partir dos resultados dos capítulos 1, 2 e 3 pode-se afirmar que a alimentação das tartarugas verdes adultas ocorre principalmente em ambientes costeiro-bentônicos. A amplitude de nicho apresentada no capítulo 2 foi semelhante em 2017 e 2019 (vitelo e carapaça), indicando que os individuos utilizam o nicho trófico de maneira similar. Quando os indivíduos foram analisados individualmente, conforme os resultados no capítulo 3, a maioria apresentou variação na estratégia alimentar ao longo do tempo. Quando estão em áreas de forrageamento apresentam alimentação principalmente herbívora (valores isotópicos do vitelo), enquanto no longo prazo a alimentação tende a ser onívora (valores isotópicos da carapaça). Dessa forma, nota-se a mudança temporal na dieta.

As abordagens utilizadas nesta tese de doutorado forneceram informações

inéditas sobre a ecologia trófica das tartarugas verdes que utilizam o Atol das Rocas como área de nidificação. Entretanto, a união de múltiplas abordagens em futuro próximo pode ser interessante para consolidar a interpretação dos resultados relacionados a ecologia trófica desses animais. Por exemplo, a telemetria via satélite e a coleta de potenciais itens alimentares para estimar sua contribuição na dieta podem se associar aos resultados isotópicos das tartarugas. A telemetria via satélite já foi utilizada em estudos que buscaram confirmar os locais de forrageamento das fêmeas amostradas em áreas de nidificação para verificar sua fidelidade de sítio (e.g., Zbinden et al., 2011; Ceriani et al., 2012; 2015; Vander Zanden et al., 2015). A coleta de potenciais itens alimentares para estimar a sua contribuição na dieta das tartarugas marinhas a partir de modelos de mistura isotópica também foi realizada por alguns autores (*i.e.,* Burkholder et al., 2011; Vander Zanden et al., 2013a; Shimada et al., 2014; Cardona et al., 2017), e ampliam o entendimento sobre as preferências alimentares das espécies e populações.

A utilização de isótopos estáveis para o entendimento da ecologia trófica das tartarugas marinhas tem se incrementado nos últimos 10 anos, mas ainda há lacunas a serem preenchidas e protocolos de coleta e análise a serem padronizados. O interesse em investigar esses répteis deve ser global, pois são migrantes verdadeiros, que utilizam recursos distribuídos em diversas latitudes. No caso das fêmeas de tartarugas-verdes que nidificam no Atol das Rocas, estudos que visem compreender as relações da espécie com o único atol do oceano Atlântico Sul servirão também como reforço para manter essa área constantemente protegida.

3. Referências Bibliográficas da Introdução Geral e Discussão Geral

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