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MUDANÇAS ONTOGENÉTICAS NA DIETA E NO USO DE HABITAT E ESTIMATIVA DE IDADE E CRESCIMENTO DA TARTARUGA-DE-PENTE, *Eretmochelys imbricata*

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RESUMO

Apesar de sua ampla distribuição geográfica em águas tropicais e subtropicais de todo o mundo, a história de vida da tartaruga-de-pente, *Eretmochelys imbricata*, é pouco estudada em uma escala global. Além disso, um dos maiores mistérios da história de vida das tartarugas marinhas são os seus primeiros anos de vida, que ocorrem em locais desconhecidos ou inacessíveis. Onde, como e por quanto tempo este período enigmático ocorre continua a ser um desafio especial para os herpetólogos. No presente estudo, a análise de isótopos estáveis (AIE) foi combinada com a esqueletocronologia para avaliar as fases iniciais de vida e detectar mudanças ontogenéticas de habitat e na utilização de recursos pelas tartarugas-de-pente ao longo da costa brasileira. Embora a AIE forneça informações essenciais para a ecologia animal, a interpretação dos valores isotópicos baseia-se em premissas que não estão bem estabelecidas, como a presença de lipídios nos tecidos animais e o viés que eles podem causar nos valores de δ^{13}C. Por esta razão, os efeitos de extração de lipídios nos valores de isótopos estáveis dos ossos de tartarugas marinhas também foram testados neste trabalho, a fim de estabelecer um protocolo padrão para o processamento do tecido ósseo. Neste experimento, os valores de δ^{13}C foram significativamente maiores que o grupo controle, após a extração de lipídios com clorofórmio:methanol. Em contrapartida, os valores de δ^{15}N não foram afetados por nenhum tratamento de extração. Estes resultados sugerem que a extração de lipídios é um passo importante para a determinação precisa de δ^{13}C dos ossos de tartarugas marinhas. O comprimento da carapaça e a estimativa de idade das tartarugas-de-pente variou de 26,0 a 111,0 cm e de 2 a 26 anos, respectivamente. As taxas de crescimento mantiveram-se elevadas em todas as classes de tamanho (1,12-8,26 cm) com o pico de crescimento na classe de 60-69,9 cm CCC. Com base no modelo de

crescimento, a maturação sexual para as tartarugas-de-pente do Atlântico Sul foi estimada a ocorrer em média aos 26,98 ± 10,69 anos. Os valores de δ^{13}C não diferiram entre as classes etárias e de tamanho. Além disso, os valores de δ^{13}C não apresentaram correlação com o tamanho corporal. Os valores de δ^{15}N entre 6 e 8 anos foram significativamente maiores do que no primeiro ano de vida. Os valores de δ^{15}N também foram maiores nos animais entre 40−59,9 cm CCC em comparação com as classes de tamanho menores. Observou-se uma correlação positiva entre δ^{15}N e o comprimento da carapaça, mas nenhuma correlação foi registrada entre os valores isotópicos e as taxas de crescimento. Estes dados contradizem o antigo paradigma que afirma que as tartarugas-de-pente, ao recrutarem do ambiente pelágico para o ambiente nerítico, alteram seu hábito alimentar onívoro para uma dieta preferencialmente espongívora. Ao contrário, as análises de isótopos estáveis não detectaram qualquer mudança ontogenética no habitat utilizado e na utilização de recursos alimentares. A alta variabilidade nos dados de δ^{15}N sugerem que os indivíduos de tartarugas-de-pente são especialistas dentro de uma população generalista, com um aumento da amplitude de nicho trófico a partir dos 7 anos de idade.

Palavras-chave: Extração de lipídios, Esqueletocronologia, Isótopos estáveis, Tartarugas Marinhas

ABSTRACT

Ontogenetic shifts in diet and habitat use, and estimate of age and growth of hawksbill turtle, *Eretmochelys imbricata*. Despite its worldwide tropical and subtropical distribution, the life history of hawksbill sea turtles, *Eretmochelys imbricata*, is poorly studied globally. Moreover, one of the biggest mysteries on the life history of sea turtles is their first years of life, which are spent in unknown or inaccessible locations. Where, how and for how long this cryptic lifestages occur still remain a challenge for herpetologists. In the present study, stable isotope analysis (SIA) was combined with skeletochronology to assess cryptic lifestages and detect ontogenetic shifts in habitat and resource use of hawksbill turtles. Although SIA has the potential to make important contributions to the study of animal ecology, the interpretation of these ratios relies on assumptions that are not well established, as the presence of lipids in animal tissues and the potential bias it may cause in δ^{13}C values. For this reason, the effects of lipid extraction in stable isotope values of the humerus bones of sea turtles were tested, aiming to establish a standard protocol for processing bone collagen tissue. In this experiment, the δ^{13}C values were significantly higher than the control after lipid extraction with chloroform-methanol. By contrast, the δ^{15}N values were not affected by the lipid extraction treatments with chloroform-methanol or petroleum ether. These results suggest that lipid extraction is an important procedure for accurate δ^{13}C determination in sea turtle bone collagen. The carapace length and age estimate for Brazilian hawksbill turtles ranged from 26.0–111.0 cm and 2–26 yr, respectively. Growth rates remained high for all size classes (1.12–8.26 cm) with peak growth at 60–69.9 cm size class. Based on the growth model fit, the simulated age distribution resulted in a mean age at maturation for hawksbill turtle population at 26.98

± 10.69 yr. Carbon isotope composition did not differ throughout age and size classes. Moreover, no correlation was observed between size and δ^{13}C values. δ^{15}N values at age classes 6, 7 and 8 were significantly higher than at age 1. For body size, δ^{15}N values were higher in 40−49.9 and 50−59.9 cm size classes in comparison to smaller size classes. There was a positive relationship between δ^{15}N and carapace length, but no correlation between isotope values and growth rates. These data differ from the long standing paradigm that when hawksbills recruit to neritic habitat, they switch from pelagic to benthic feeding, and from an omnivorous to a spongivorous diet. Instead, stable isotope analyses were unable to detect any ontogenetic shift in resource and habitat use. The high variability in data suggests that hawksbills are long-term specialists within a generalist population, with an increase of the dietary niche width after 7 yr old.

Key words: Lipid extraction, Skeletochronology, Stable isotopes, Sea turtles

INTRODUÇÃO

Um dos maiores mistérios da história de vida das tartarugas marinhas são seus primeiros anos de vida, que ocorrem em locais desconhecidos ou inacessíveis e, por essa razão, são chamados de "anos perdidos" (Musick & Limpus, 1997). Desvendar onde, como e por quanto tempo esta enigmática fase inicial ocorre ainda é um especial desafio para os herpetólogos. No Atlântico Norte, há indícios de que os filhotes habitam massas flutuantes de *Sargassum*, exibindo um comportamento alimentar oportunista (Witherington *et al.*, 2012). No Atlântico Sul, no entanto, esta fase é completamente desconhecida e não há registros de onde os neonatos de tartaruga permanecem, uma vez que nesta parte do Atlântico não há grandes e regulares aglomerações de algas flutuantes. Recentemente, a esqueletocronologia foi associada à análise de isótopos estáveis (AIE) para avaliar mudanças ontogenéticas em fases iniciais de vida das tartarugas-cabeçuda, *Caretta caretta* (Snover *et al*., 2010; Avens *et al*., 2013), e essa abordagem provou ser um ferramenta poderosa para revelar o complexo ciclo de vida das tartarugas marinhas.

A esqueletocronologia consiste na análise das linhas de crescimento (*Lines of Arrested Growth* - LAGs) de ossos de répteis e anfíbios (Castanet & Smirina, 1990), que permitem estimar a idade e taxas de crescimento. Tem sido amplamente aplicada na última década em estudos com tartarugas marinhas (Avens & Snover, 2013), fornecendo informações essenciais sobre as características demográficas das populações, como estrutura etária, dinâmica de crescimento, taxas de recrutamento e idade de maturidade sexual (Goshe *et al*., 2010; Petitet *et al*., 2012; Snover *et al*., 2013). Esses parâmetros são essenciais para uma adequada compreensão da história de vida das tartarugas marinhas (Heppel *et al*., 2000) e fundamentais para delinear planos de manejo

adequados para a conservação de espécies ameaçadas (Heppel & Crowder, 1996; Chaloupka & Musick, 1997; Lotze *et al*., 2011).

Nas últimas décadas, a análise de isótopos estáveis tornou-se uma ferramenta essencial na área da ecologia, particularmente para identificar fontes alimentares e relações tróficas (Mancini & Bugoni, 2014), utilização de habitat (Bjorndal & Bolten, 2010; Pajuelo *et al*., 2012), padrões migratórios (Hobson, 1999) e mudanças ontogenéticas na dieta e no habitat (Arthur *et al*., 2008; Drago *et al*., 2009). A AIE é um método adequado para tais estudos, pois os valores de isótopos estáveis de carbono (δ^{13}C) e de nitrogênio (δ^{15}N) refletem uma dieta integrada no tempo, dependendo do tecido analisado. O tecido ósseo, por exemplo, permanece relativamente inerte após sua síntese, mantendo a história isotópica do animal e fornecendo dados sobre uma escala anual, limitada apenas pela reabsorção óssea na cavidade medular (Snover *et al*., 2010). A razão entre o isótopo pesado em comparação com o leve (e comparado com um padrão conhecido) no tecido de um consumidor reflete a razão isotópica de suas presas em uma escala de tempo determinada pelas características metabólicas de cada tecido e de cada espécie (Peterson & Fry, 1987; Fry, 2006). Devido à eliminação preferencial dos isótopos mais leves, e consequentemente, a incorporação de isótopos mais pesados (Fry, 2006), a discriminação isotópica entre os organismos ocorre de forma mais ou menos previsível a cada nível trófico (~1‰ para δ^{13}C e 3–5‰ para δ^{15}N; DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Fry, 2006). Com base nesse padrão, os valores de δ^{15}N são utilizados para avaliar o nível trófico de um consumidor, enquanto que os valores de δ^{13}C permitem discriminar fontes alimentares e distinguir o uso de diferentes habitats marinhos, e.g. nerítico *vs.* oceânico e demersal *vs.* pelágico (Fry, 2006). A seleção de tecidos é um pressuposto importante para a reconstrução da dieta via AIE, pois apresentam composições e taxas de renovação (*turnover*) distintas (Fry,

2006; Perkins *et al*., 2013). O colágeno ósseo é em grande parte composto de proteína (Gannes *et al*., 1997) e apresenta uma taxa de renovação lenta, que para animais de médio e grande porte pode ser anos ou mesmo décadas (Dalerum & Angerbjörn, 2005). Por essa razão, é considerado como um tecido adequado para identificar tendências de longo prazo nos padrões alimentares ou para investigar mudanças significativas na estrutura da cadeia alimentar ao longo do tempo (Dalerum & Angerbjörn, 2005; Christensen & Richardson, 2008).

Uma questão metodológica que permeia a análise de isótopos estáveis é a de que os lipídios têm valores de $\delta^{13}C$ mais negativos em relação a outros compostos bioquímicos, tais como proteínas e carboidratos. Por outro, lado os diferentes nutrientes da dieta não são usados da mesma forma na síntese e na manutenção dos diferentes tecidos do consumidor. Consequentemente, os tecidos muitas vezes não refletem a composição isotópica total da dieta, mas sim representam a composição isotópica do componente nutricional da dieta a partir do qual o tecido foi sintetizado (Gannes *et al*., 1997). Para animais que contém dietas ricas em proteínas, essas biomoléculas são utilizadas exclusivamente para a síntese dos tecidos, enquanto que os carboidratos e os lipídios são catabolizados para suprir a demanda de energia ou armazenado em tecidos de reserva (Gannes *et al*., 1997). Na síntese lipídica, efeitos cinéticos que ocorrem durante a conversão do piruvato em acetil-CoA causam uma depleção nas razões isotópicas de carbono de aproximadamente 6–8‰ (DeNiro & Epstein, 1977). Como a análise de isótopos para estudos de ecologia trófica normalmente visa o rastreamento de proteínas, e a quantidade de lipídios varia entre os tecidos, essa carga lipídica pode potencialmente introduzir erros na interpretação dos valores de $\delta^{13}C$, particularmente em estudos utilizando modelos de mistura de isótopos (Kiljunen *et al*., 2006). Para animais aquáticos, foi proposto que razões de C:N acima de 3,5 indicam a presença de

lipídios com potencial para alterar as assinaturas de $\delta^{13}C$ e que, em tais casos, é necessária a extração de lipídios ou a normalização matemática (Post *et al*., 2007). Recentemente, a aplicabilidade da razão C:N como preditor de conteúdo lipídico foi avaliada em tecidos de peixes e demonstrou-se que a maioria dos modelos de normalização matemáticos utilizados em AIE com base neste parâmetro (por exemplo, McConnaughey & McRoy, 1979; Post *et al*., 2007; Logan *et al*., 2008) subestimam o conteúdo lipídico (Fagan *et al.*, 2011). Por outro lado, há um conjunto de evidências de que os procedimentos de extração de lipídios podem remover compostos de nitrogênio (glicolipídios ou lipoproteínas) e levam a mudanças significativas nos valores de $\delta^{15}N$ (Post *et al*., 2007; Logan *et al*., 2008; Mateo *et al*., 2008). No entanto, a padronização de protocolos de processamento de amostras para análise isotópica ainda é escassa para a maioria dos tecidos e grupos taxonômicos, incluindo as tartarugas marinhas. Por esta razão, no Capítulo 1 foram testados os efeitos da extração de lipídios nos valores de $\delta^{13}C$ e $\delta^{15}N$ do colágeno ósseo, utilizando dois tipos de solventes, com o objetivo de estabelecer um protocolo padrão para o processamento deste tecido em espécies de tartarugas marinhas.

A tartaruga-de-pente (*Eretmochelys imbricata*) apresenta uma ampla distribuição geográfica, ocorrendo em águas tropicais e subtropicais de todos os oceanos, e suas populações foram reduzidas drasticamente nas últimas três gerações devido à intensa exploração de sua carapaça para suprir um amplo comércio de bijuterias e artesanato (Meylan & Donelly, 1999). Além disso, as tartarugas-de-pente também são ameaçadas pela exploração de sua carne e ovos, pela fragmentação das áreas de desova e alimentação, pela captura incidental na pesca, pela poluição marinha, e por colisões com embarcações (Lutcavage *et al*., 1997, Meylan & Donelly, 1999). Por essas razões,

atualmente esta espécie é classificada como criticamente ameaçada de extinção pela International Union for Conservation of Nature (IUCN, 2014).

Semelhante às outras espécies de tartarugas marinhas, a tartaruga-de-pente apresenta uma complexa história de vida, caracterizada por extensas migrações, mudanças ontogenéticas de habitat e alimentação, e fidelidade às áreas de nidificação (Mortimer & Donnelly, 2008; Meylan *et al*., 2011). No Oceano Atlântico, estima-se que os "anos perdidos" durem de 1 a 3 anos, quando as tartarugas então recrutam para o ambiente nerítico, com cerca de 20–25 cm de comprimento da carapaça (Boulon, 1994). Após o recrutamento, as tartarugas-de-pente passam a ocupar recifes de corais, pois apresentam uma dieta baseada predominantemente em esponjas (principalmente *Chondrilla nucula*; Meylan, 1988; Berube *et al*., 2012) e zoantídeos (Proietti *et al*., 2012), embora populações da Grande Barreira de Corais, na Austrália, alimentem-se preferencialmente de algas marinhas (Bell, 2012). Além disso, esta espécie também é associada a outros habitats bentônicos, como pradarias de macrófitas (Bjorndal & Bolten, 2010), costões rochosos (Proietti *et al*., 2012), e baías com manguezais (Gaos *et al*., 2012).

O conhecimento sobre taxas de crescimento individuais e a idade em que ocorre a maturidade sexual são características importantes para compreender a dinâmica populacional das espécies. No caso das tartarugas-de-pente, sabe-se que as populações do Oceano Pacífico tendem a apresentar padrões de crescimento menores do que as populações do Caribe e do Atlântico Norte Ocidental (Hawkes *et al*., 2014). Além disso, as tartarugas-de-pente do Atlântico Norte mostram um pico de crescimento em uma classe de tamanho menor (30-40 cm; Boulon, 1994; León & Diez, 1999; Diez & van Dam, 2002; Bjorndal & Bolten, 2010) em comparação com as populações do Pacífico, cujo pico de crescimento é comumente observado entre 55 e 70 cm (Chaloupka & Limpus, 1997; Bell & Pike, 2012). Recentemente, um estudo realizado com a população

do Havaí estimou que indivíduos de *E. imbricata* de 20 a 80 cm de Comprimento Curvilíneo da Carapaça (CCC) crescem em média 2,24-4,77 cm/ano, enquanto que indivíduos acima de 80 cm apresentam crescimento de 0,3 cm/ano e atingem a maturidade sexual entre 17 e 22 anos de idade (78,6 cm de CCC) (Snover *et al.*, 2013). No Atlântico Sul, no entanto, informações sobre esses parâmetros são escassas para a tartaruga-de-pente, o que dificulta um manejo adequado para a conservação desta espécie.

O litoral brasileiro abriga a maior população reprodutiva remanescente de tartarugas-de-pente do oceano Atlântico Sul (Marcovaldi *et al*., 2007) e serve, principalmente, como habitat para o desenvolvimento de juvenis (Proietti *et al.*, 2012). Estudos genéticos mostraram que as áreas reprodutivas brasileiras e as agregações nas áreas de alimentação constituem unidades demográficas distintas (Vilaça *et al*., 2013; Proietti *et al*., 2014). Análises de estoques mistos revelaram que as áreas de alimentação no Brasil são compostas principalmente de tartarugas provenientes das áreas reprodutivas da Bahia e Rio Grande do Norte, no nordeste do Brasil, com contribuições menores de colônias da África e do Caribe (Proietti *et al*., 2014). No entanto, apesar da importância que o Brasil apresenta para o status de conservação das populações de tartaruga-de-pente, informações sobre ecologia e demografia ainda são escassos. Diante deste cenário, a presente dissertação utilizou a esqueletocronologia para descrever padrões de crescimento (Capítulo 2) e aliou esta técnica à análise de isótpos estáveis para detectar mudanças ontogenéticas de habitat e alimentação (Capítulo 3) das tartarugas-de-pente do Atlântico Sul Ocidental.

OBJETIVOS

Os objetivos deste trabalho são: (1) analisar os efeitos da extração de lipídios nas assinaturas isotópicas de carbono e nitrogênio do tecido ósseo; (2) estimar a idade, as taxas de crescimento e a maturidade sexual das tartarugas-de-pente a partir das marcas de crescimento no úmero e suas relações com o tamanho corporal; e (3) avaliar mudanças de habitat e hábitos alimentares através de valores isotópicos de $\delta^{13}C$ e $\delta^{15}N$ em úmeros.

MATERIAL E MÉTODOS

a. Experimento de extração de lipídios

As amostras utilizadas no Capítulo 1 foram coletadas em monitoramentos de praia ao longo da costa sul do Rio Grande do Sul, entre a Lagoa do Peixe e o Arroio Chuí, no período de outubro de 2009 a março de 2010. Foram coletados úmeros das nadadeiras anteriores de espécimes de tartaruga-cabeçuda (*C. caretta*) que encalharam mortos na região. Os úmeros direito e esquerdo foram removidos, armazenados em sacos permeáveis e macerados. A maceração consiste em manter os ossos imersos na água por aproximadamente duas semanas, até a remoção total dos tecidos moles. Após essa etapa, os úmeros secaram em temperatura ambiente por mais duas semanas. De cada úmero foram extraídas três secções transversais de 1 mm de espessura cada. Os cortes foram realizados na porção média da diáfise, próximo à crista deltapeitoral, com o auxílio de uma serra metalográfica de baixa rotação (Buehler®). Os cortes foram separados aleatoriamente em três grupos. No primeiro grupo, os lipídios foram removidos com uma solução de éter de petróleo e etil-éter (1:1); no segundo grupo, os

lipídios foram extraídos com solução de clorofórmio:metanol (2:1); e o terceiro grupo, o grupo controle, não passou por nenhum processo de extração. As extrações foram realizadas com aparelho Soxhlet por um período de 4 h para cada grupo. Após esta etapa, as amostras de todos os grupos foram submetidas a um processo de acidificação com HCl 10% para eliminar o carbonato de cálcio presente no tecido ósseo e reter apenas o colágeno ósseo. Por fim, as amostras foram liofilizadas durante 6 h e reduzidas a pó com o auxílio de graal e pistilo. Aproximadamente 1 mg de amostra pulverizada foi armazenado em cápsula de estanho para a análise isotópica. A vidraria utilizada durante este procedimento permaneceu em banho ácido de HCl 10% por no mínimo 12 h para evitar contaminação das amostras.

b. Coleta e processamento de amostras

Para os Capítulos 2 e 3 foram coletados os úmeros de natimortos (filhotes que eclodiram dos ovos, mas que não conseguiram emergir dos ninhos) e de espécimes de tartaruga-de-pente que encalharam mortos ao longo da costa brasileira, no período de dezembro de 2012 a fevereiro de 2014. As coletas provenientes dos encalhes ocorreram na praia de Pipa, no litoral do Rio Grande do Norte; em Maceió, Alagoas; entre a Costa do Sauípe e Arembepe, no litoral norte da Bahia; de Itaúnas, no Estado do Espírito Santo, a Araruama, no Rio de Janeiro; em Pontal do Sul, no Paraná; e entre a Lagoa do Peixe e o Arroio Chuí, no Rio Grande do Sul. Os exemplares de natimortos são provenientes de ninhos de Regência (ES) e de Maceió (AL) (Vide mapa no Capítulo 2). De cada espécime encalhado foi obtido o comprimento curvo da carapaça (CCC), conforme Bolten (1999), com o auxílio de fita métrica flexível com 0,1 cm de precisão. Para os natimortos, foi medido o comprimento retilíneo da carapaça (CRC) com o auxílio de um paquímetro (± 0,1 mm). O CRC dos filhotes foi convertido em CCC com

base na equação proposta por Hawkes *et al.* (2014). Os úmeros foram macerados e o diâmetro de cada osso foi medido na altura da cicatriz de inserção do músculo delto-peitoral, com o auxílio de um paquímetro (Zug *et al*., 1986).

c. Estimativa de idade e crescimento

Os úmeros foram cortados com uma serra metalográfica de baixa rotação em duas partes com diferentes espessuras, a partir da extremidade distal da cicatriz de inserção do músculo delto-peitoral. Primeiro, foi realizado um corte transversal de 1 mm para análise de isótopos estáveis. Em seguida, para a análise das marcas de crescimento, uma secção transversal de 2–3 mm foi cortada adjacente ao local de seccionamento. Para a análise de esqueletocronologia os úmeros foram preparados conforme Avens & Goshe (2007). Os cortes para histologia foram fixados em formalina 10% por 2 a 3 h e, em seguida, enxaguados com água destilada. Posteriormente, as secções foram descalcificadas por 6 a 12 h com RDO, uma solução descalcificadora comercial cujo ingrediente ativo é o ácido hidroclorídrico. Após este processo, os ossos ficaram imersos em água destilada por mais 12 h para remover qualquer resíduo de RDO. Cada corte foi novamente seccionado em porções de 25 µm com micrótomo de congelamento (LEICA SM2000 R). Estas fatias foram coradas com solução 1:1 de hematoxilina de Ehrlich e água destilada, e montadas em lâminas de microscopia com glicerina. Como a lâmina histológica descora rapidamente, fotos com ampliação de 4x foram tiradas de porções sequenciais de cada secção com uma câmera digital de 17,28 megapixels acoplada a um microscópio binocular invertido (Olympus DP73). As imagens parciais de cada secção foram montadas usando Adobe Photoshop Elements 8.0 para compor uma imagem de alta resolução, o que permite a visualização e análise das linhas de crescimento. Cada secção foi interpretada por apenas um leitor, que

conduziu três leituras independentes com intervalos mínimos de 5 dias. Quando o número de marcas de crescimento variou entre as leituras, uma quarta leitura foi realizada.

Em anfíbios e répteis, a primeira linha de crescimento (*annulus*) é observada próxima ao centro do úmero como uma linha difusa. As linhas depositadas posteriormente ao longo do crescimento das tartarugas marinhas são depositadas subsequencialmente, de dentro para fora, e cada uma equivale a um ano de idade (Zug *et al*., 1986). As LAGs visíveis foram contadas e seus diâmetros medidos com o software ImageJ. A idade foi definida como sendo o número de LAGs visíveis para aqueles indivíduos que ainda possuíam *annulus.* Durante o crescimento das tartarugas marinhas, as primeiras linhas de crescimento são reabsorvidas, impossibilitando a determinação da idade por contagem direta (Zug *et al*., 1986). Com base no número e nas medidas dos diâmetros das LAGs dos animais com *annulus*, foram ajustados modelos de regressão linear e não-linear com duas estruturas de erro: Näive e hierárquico (Faraway, 2006). Detalhes sobre as estruturas dos modelos estão descritos no Capítulo 2.

Para calcular as taxas de crescimento, foi aplicada a técnica do "retro-cálculo", método aplicado e validado para *C. caretta* por Snover *et al.* (2007). Esta técnica baseia-se na premissa de que há uma taxa constante de deposição de marcas de crescimento e que há uma proporcionalidade previsível entre o diâmetro do úmero (*D*) e o tamanho corporal. Para encontrar a melhor relação entre o diâmetro do úmero e o tamanho corporal, foram ajustados os quatro modelos propostos por Snover *et al.* (2007) e cujas estruturas estão detalhadas no Capítulo 2. Para avaliar o padrão de crescimento das tartarugas-de-pente do Atlântico Sul Ocidental, foi ajustado o modelo de crescimento de von Bertallanfy e a partir dos parâmetros estimados, foi calculada a

idade média de maturação sexual para o tamanho médio das tartarugas que desovam no estado da Bahia (97,4 cm CCC; Marcovaldi *et al*., 1999).

d. Análise de isótopos estáveis

Para a análise de isótopos estáveis, foram extraídos lipídios de cada corte de 1 mm, conforme protocolo estabelecido no Capítulo 1. Para coletar amostras das linhas de crescimento, as imagens de alta resolução dos cortes histológicos e as medidas de diâmetro das LAGs foram usadas como guia. Nos cortes histológicos é possível observar LAGs finas e escuras que se alternam com áreas mais claras e largas, que representam a zona de crescimento ativo. Esta zona de crescimento ativo representa o crescimento anterior à deposição da LAG (Snover & Hohn, 2004; Avens & Snover, 2013). As amostras das linhas de crescimento foram coletadas nessas áreas de incremento com uma broca elétrica manual (Dremel®) com 300 µm de espessura e imediatamente acondicionados em cápsulas de prata esterilizadas de 4 x 6 mm. Em alguns casos, as linhas individuais estavam muito próximas umas das outras (a uma distância menor do que 300 µm), resultando em baixa quantidade de material (<0,3 mg). Assim, nem todas as LAGs visíveis nos cortes histológicos foram passíveis de coleta e algumas amostras podem representar mais de uma linha de crescimento. Como a broca pulveriza as linhas de crescimento no momento da coleta, cada secção transversal foi lavada com água destilada e seca em estufa entre cada amostragem, a fim de evitar a contaminação. Todas as amostras foram acidificadas com HCl a 10%.

Os valores de isótopos estáveis foram determinados por um espectrômetro de massa de razão isotópica de fluxo contínuo no Laboratório de Química Analítica da Universidade da Geórgia (EUA). Os valores de isótopos estáveis são expressos com a notação δ, em partes por mil (‰), em relação aos padrões internacionais (PDB - *Peedee*

Belemnite limestone para C, e N_2 atmosférico para N), de acordo com a seguinte equação (Bond & Hobson, 2012):

$$\delta X = (R_{amostra}/R_{padrão})-1 \quad (1)$$

onde X é o valor de $\delta^{13}C$ ou $\delta^{15}N$, e R é a razão correspondente de $^{13}C/^{12}C$ ou $^{15}N/^{14}N$ (Peterson & Fry, 1987). Dois padrões laboratoriais foram analisados a cada 12 amostras desconhecidas. A precisão de medida de ambos, carbono e nitrogênio, foi de 0,2‰.

e. Análises estatísticas

Todas as análises estatísticas foram realizadas utilizando o software R versão 3.0.3 (R Core Team, 2014). No Capítulo 1 e 3, a normalidade e a homoscedasticidade dos valores isotópicos foram testadas por meio do teste de Shapiro-Wilk e o teste de Bartlett, respectivamente. As variações nos valores de $\delta^{13}C$ e $\delta^{15}N$ entre os tratamentos apresentados no Capítulo 1, e entre as classes etárias e de tamanho no Capítulo 3 foram testadas separadamente aplicando ANOVA de uma via ou o teste não-paramétrico de Kruskal-Wallis, quando os requisitos de normalidade e homocedasticidade não foram atendidos. Regressões lineares simples foram calculadas no Capítulo 1 a fim de avaliar a viabilidade de uma normalização matemática a partir do conjunto de dados. A precisão da normalização matemática foi testada a partir do modelo de eficiência (EF) proposto por Mayer & Butler (1993). No Capítulo 3, correlações de Spearman foram aplicadas para verificar a relação dos valores de $\delta^{13}C$ e $\delta^{15}N$ com o tamanho corporal e as taxas de crescimento.

No Capítulo 2, as estimativas de idade e crescimento das tartarugas-de-pente abordaram a análise bayesiana. Estimativas de parâmetros desconhecidos são dadas

como distribuições de probabilidade, denominadas de posteriores (Gelman *et al.*, 2003). Essas distribuições posteriores são obtidas através da integração da função de verossimilhança dos dados com informações prévias, sintetizadas em uma distribuição de probabilidade denominada *priori*, tendo como base o Teorema de Bayes (Gelman *et al.*, 2003). Em todos os modelos foram realizadas simulações de Monte Carlo por Cadeias de Markov (MCMC) para obter as distribuições posteriores. A escolha entre os modelos foi baseada no critério de informação de deviância (DIC; Spiegelhalther *et al*., 2002).

RESULTADOS

No Capítulo 1 foram utilizados os úmeros de 11 tartarugas-cabeçuda, cujo tamanho da carapaça variou de 50,0 a 93,0 cm (média ± DP, 73,8 ± 13,0 cm). Em ambos os tratamentos de extração de lipídios, os valores de $\delta^{13}C$ foram superiores ao controle (Fig. 1A). Os valores variaram de -16,96 a -13,36‰ (-15,00‰ ± 0,89) e de -15,37 a -13,10‰ (-14,38‰ ± 0,73), para o tratamento com éter de petróleo:éter etílico e o com clorofórmio:metanol, respectivamente. A ANOVA mostrou diferenças significativas nos valores de $\delta^{13}C$ ($F_{(2,30)} = 7,31$, $p < 0,05$). O teste *post hoc* de Tukey indicou que os valores de $\delta^{13}C$ foram significativamente maiores após a extração de lipídios com clorofórmio:metanol em relação ao grupo controle ($p < 0,05$). Por outro lado, os valores de $\delta^{15}N$ não foram afetados pelos protocolos de extração de lípidos ($F_{(2,30)} = 0,04$, $p = 0,95$; Fig. 1B). Com base nesses dados, a viabilidade de normalização matemática foi testada utilizando-se apenas o grupo controle ($\delta^{13}C_{bulk}$) e o grupo cuja extração foi feita com clorofórmio:metanol ($\delta^{13}C_{le}$). Foi observada uma relação significativa entre os valores do grupo controle e os valores de $\Delta^{13}C$ (resultantes da

diferença entre $\delta^{13}C_{le}$ e $\delta^{13}C_{bulk}$). Com base nos coeficientes estimados na regressão linear, uma equação de correção foi estruturada. No entanto, apesar da evidência de correlação entre $\Delta^{13}C$ e $\delta^{13}C_{bulk}$, a eficiência do modelo foi negativa (EF = -19.676), indicando que os parâmetros do modelo não devem ser utilizados como um fator de correção de lipídios.

No Capítulo 2 e 3 foram utilizados úmeros de 59 tartarugas-de-pente, dos quais 10 eram filhotes que não conseguiram emergir dos ninhos e 49 consistiram de tartarugas que encalharam mortas ao longo da costa brasileira. O tamanho das tartarugas encalhadas variou de 26,0 a 111,0 cm de CCC (média ± DP = 42,93 ± 20,34 cm). Para filhotes, o CRC variou de 3,55 a 4,79 cm (4,16 ± 0,49) e o diâmetro do úmero de 1,5 a 1,9 mm (1,73 ± 0,12 cm).

A primeira linha de crescimento (*annulus*) foi detectada nas secções transversais de 35 tartarugas-de-pente, que apresentaram de 2 a 7 LAGs visíveis e variavam em tamanho de 26,0 a 53,9 cm. Para as tartarugas sem *annulus*, a idade foi estimada a partir do modelo que melhor se ajustou aos dados, o modelo hierárquico não-linear (DIC= -365,6). O CCC destas tartarugas variou de 49,5 a 111,0 cm (71,94 ± 17,23 cm) e a idade estimada foi de 6 a 26 anos de idade. Para o "retro-cálculo", o modelo alométrico não-linear, que incorpora os interceptos biológicos (l_{op} e d_{op}) e o coeficiente alométrico c mostrou o melhor ajuste (DIC = -79,9). As taxas de crescimento mostraram-se elevadas em todas as classes etárias (1,12–8,26 cm/ano), com um o pico de crescimento na classe de idade 60–69,9 cm. Com base no ajuste do modelo, observou-se uma correlação positiva entre a taxa de crescimento e o CCC ($r = 0,44$; $p < 0,01$), mas a relação entre a idade estimada e a taxa de crescimento não foi significativa ($r = 0,16$; $p = 0,26$). O modelo de crescimento de von Bertallanfy teve um bom ajuste para a relação entre a idade estimada e os dados de comprimento da carapaça. Embora o conjunto de dados

contemple um amplo intervalo de idades, a amostra é constituída basicamente de juvenis (n = 36, CCL <40 cm), o que pode ter influenciado no comportamento da assíntota do modelo, que apresentou um comportamento quase linear. A estimativa de idade de maturação sexual resultou em uma idade média de maturação de 26,98 ± 10,69 anos (95% intervalo de credibilidade: de 12,72 a 53,61 anos).

Os valores de $\delta^{13}C$ das linhas de crescimento variaram de -21,08 a -12,95‰ (-16,26‰ ± 1,14). Esses valores não variaram entre as classes etárias (H_{11} = 5.12, p = 0,92) e as classes de tamanho (H_8 = 15,89; p = 0,04). Embora o valor de p indique que há diferença significativa nos valores de $\delta^{13}C$ entre as classes de tamanho, o teste de Wilcoxon não detectou diferenças entre os grupos, provavelmente devido ao ajuste de Bonferroni utilizado para comparações múltiplas. Além disso, não foi observada correlação entre o tamanho corporal e os valores de $\delta^{13}C$ (r_s = 0,15; p = 0,08). Os valores de $\delta^{15}N$ variaram de 5,42 a 18,30‰ (10,06‰ ± 2,06). O teste de Kruskal-Wallis mostrou diferenças dos valores de $\delta^{15}N$ entre as idades (H_{11} = 35,12; p < 0,01) e entre as classes de tamanho (H_8 = 35,40; p < 0,01). Os valores de isótopos de nitrogênio nas classes 6 a 8 anos foram significativamente maiores em comparação ao primeiro ano de vida das tartrugas. Em relação ao tamanho corporal, os valores de $\delta^{15}N$ com CCC entre 40 e 59.9 cm foram superiores à classe de 20 a 29,9 cm. Além disso, observou-se uma relação positiva entre $\delta^{15}N$ e comprimento da carapaça (r_s = 0,39; p < 0,01). Por fim, constatou-se que não há correlação entre os valores de isótopos e as taxas de crescimento ($\delta^{13}C$: r_s = 0,07; p = 0,48; $\delta^{15}N$: r_s = 0,01; p = 0,90).

CONCLUSÕES

- O aumento significativo nos valores de δ^{13}C observado após a extração de lipídios com solução de clorofórmio:metanol sugere que a extração de lipídios é uma etapa importante para a determinação precisa de δ^{13}C do colágeno ósseo de tartarugas marinhas. Portanto, a extração de lipídios deve ser levada em conta em estudos que utilizam este tecido para avaliar a ecologia trófica e mudanças ontogenéticas.

- As tartarugas-de-pente do Atlântico Sul Ocidental apresentaram elevadas taxas de crescimento em todas as classes etárias (1,12–8,26 cm/ano), com pico de crescimento na classe de idade 60–69,9 cm, o que poderia indicar que o tamanho da população está abaixo da capacidade de suporte do ambiente e corrobora com a hipótese de que, historicamente, o tamanho das populações era maior.

- No caso das tartarugas-de-pente do Atlântico Sul Ocidental, estimou-se que demora quase 30 anos para a substituição das gerações. O tempo de maturação é reconhecidamente um parâmetro essencial para determinar por quanto tempo planos de gestão devem ser implementados para identificar respostas populacionais.

- Os valores de δ^{13}C sugerem que a espécie não passa por nenhuma alteração significativa de habitat, ou, alternativamente, transitam entre diferentes habitat com assinaturas isotópicas semelhantes.

- Os valores de δ^{15}N apresentaram alta variabilidade ao das idades e entre as classes de tamanho, com um aumento significativo entre 6 e 8 anos de idade, quando atingem 40 a 50 cm de CCC. Estes resultados podem ser um indicativo de uma mudança ontogenética, quando as tartarugas-de-pente passam a ter um comportamento alimentar mais seletivo, beneficiando-se do aumento no tamanho corporal para capturar presas maiores.

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CAPÍTULO 1

Effects of lipid extraction on the isotopic values of sea turtle bone collagen: is extraction really necessary?

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Effects of lipid extraction on the isotopic values of sea turtle bone collagen: is extraction really necessary?

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ABSTRACT: Many stable isotope analysis (SIA) studies aim to track protein, which is assimilated in animal tissues from their food sources, to assess feeding ecology, movements and ontogenetic shifts of marine animals. Lipids are known to be a potential source of bias because they are depleted in ^{13}C compared to ^{12}C. Although lipids are usually removed before SIA, there is a lack of standardized analytical protocols for this procedure. We tested the effects of lipid extraction with two chemical solvents in the humeri of the loggerhead sea turtle *Caretta caretta* to establish a standard protocol for processing the bone collagen of marine animals and to develop a mathematical correction. In both lipid extraction treatments, $\delta^{13}C$ values were higher than the control, but only lipid extraction with chloroform-methanol showed significant differences. By

contrast, the $\delta^{15}N$ values were not affected by the lipid extraction treatments with chloroform-methanol or petroleum ether. The linear regression between $C:N_{bulk}$ and $\Delta^{13}C$ was not significant, which does not support the assumption that there is a predictable relationship between C:N and lipid content. Nevertheless, a significant positive relationship between $\Delta^{13}C$ and $\delta^{13}C_{bulk}$ was observed, but such a model is not recommended as a mathematical lipid correction method because the model efficiency had a negative value, which indicates that the mean value of $\delta^{13}C_{bulk}$ is a better predictor than the model itself. These results suggest that lipid extraction should be taken into account in SIA of bone collagen tissues for accurate $\delta^{13}C$ determination.

KEY WORDS: collagen, lipid extraction, lipid normalization, sea turtle, reptiles

RUNNING HEAD: Effects of lipid extraction on stable isotopes

INTRODUCTION

Over the past several decades, stable isotope analysis (SIA) has become a useful tool in ecological research, particularly for identifying dietary sources and trophic relationships (Dodge et al. 2011), habitat use (Bjorndal & Bolten 2010, Pajuelo et al. 2012), migration patterns (Hobson 1999) and ontogenetic shifts (Arthur et al. 2008, Drago et al. 2009). SIA is a well-suited method for such studies because the stable isotope values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) reflect a time-integrated diet, *i.e.* the stable isotope ratio of consumer tissues reflects the similar ratio of its prey items within a timescale determined by the metabolic traits of each tissue and the species analyzed (Peterson & Fry 1987, Fry 2006). Due to preferential excretion of the lighter isotopes,

and therefore the selection of heavier isotopes (Fry 2006), δ^{15}N measurements can be used to assess the trophic level of a consumer with a 3–5‰ increase in δ^{15}N values with each trophic level (DeNiro & Epstein 1981, Minagawa & Wada 1984). δ^{13}C is often used to discriminate food sources by using the large variation in the isotopic carbon value in food webs due to photosynthetic baselines (Fry 2006).

An important methodological issue in SIA is that lipids have more negative δ^{13}C values relative to other biochemical compounds, such as proteins and carbohydrates. The different nutrients in the diet are not used in the same way in the synthesis and maintenance of the different tissues of the consumer. Consequently, tissues often do not reflect the isotopic composition of the bulk diet but instead represent the isotopic composition of the nutrient component of the diet from which the tissue was synthesized (Gannes et al. 1997). For animals that have high-protein diets, dietary proteins are exclusively used for the synthesis of tissues, whereas carbohydrates and lipids are catabolized to supply energy demands or stored in reserve tissues (Gannes et al. 1997). In lipid synthesis, kinetic effects that occur during the conversion of pyruvate to acetyl CoA cause a depletion in carbon stable isotope ratios of approximately 6–8‰ (DeNiro & Epstein 1977). Thus, the amount of lipid in bulk tissues could introduce a potential bias in δ^{13}C values and lead to misinterpretation of trophic relationships, particularly in studies using isotope mixing models (Kiljunen et al. 2006).

According to Post et al. (2007), C:N ratios higher than 3.5 in aquatic animals indicate the presence of lipids with the potential to alter δ^{13}C signatures; in such cases, lipid extraction or mathematical normalization is required. Questions have arisen regarding the general applicability of these thresholds and techniques (Fagan et al. 2011), and there is a growing concern on the need to describe the effects on isotopic

signatures caused by pre-analytical procedures, as sample acidification, distilled water rinsing and lipid extraction (Mateo et al. 2008, Mintenbeck et al. 2008, Fagan et al. 2011). Recently, the usefulness of the C:N ratio as a predictor of lipid content was evaluated in fish tissues and demonstrated that most mathematical normalization models utilized in SIA based on this parameter (e.g., McConnaughey & McRoy 1979, Post et al. 2007, Logan et al. 2008) underestimated the lipid content (Fagan et al. 2011). On the other hand, there is a body of evidence that lipid extraction procedures can remove nitrogen compounds (glycolipids or lipoproteins) and lead to significant changes in δ^{15}N values (Post et al. 2007, Logan et al. 2008, Mateo et al. 2008). However, the standardization of sample processing protocols for isotopic analysis is still scarce for most tissues and taxa.

Given the differences in tissue composition, tissue selection is an important requirement for dietary reconstruction (Perkins et al. 2013). Bone collagen is largely composed of protein (Gannes et al. 1997) that turns over very slowly and has a long half-life, which for medium- and large-sized animals can reflect years or even decades (Dalerum & Angerbjörn 2005). It is therefore the most suitable tissue to identify long-term trends in animal dietary patterns or to investigate fundamental changes in food web structure over time (Dalerum & Angerbjörn 2005, Christensen & Richardson 2008).

Recently, skeletochronology has been coupled with SIA to assess the ontogenetic shifts and early life stages of loggerhead sea turtles, *Caretta caretta* (Snover et al. 2010, Avens et al. 2013). Skeletochronology is the study of growth marks in bones of reptiles and amphibians (Castanet & Smirina 1990), which allow researchers to estimate age and growth rate. This methodology has been extensively applied over the last decade in studies with sea turtles (Avens & Snover 2013). Because bone tissues remain relatively inert after synthesis, the tissue retains the isotopic history of the

animal, providing data on an annual scale, limited only by bone resorption at the core (Snover et al. 2010). Although the combination of these two techniques is still in its infancy, it appears that this approach will greatly expand our knowledge on the complex life cycle of sea turtles.

Although SIA has the potential to make important contributions to animal ecology, the interpretation of these ratios relies on assumptions that are not well established. For this reason, we tested the effects of lipid extraction with two common chemical solvents in the humerus bones of loggerhead sea turtles with the aim to establish a standard protocol for processing bone collagen tissue from sea turtle species and to develop a mathematical normalization for SIA.

MATERIALS AND METHODS

Sample collection and processing

Samples consisted of 11 humeri from loggerhead turtles that stranded dead on the south coast of Rio Grande do Sul, southern Brazil, between Lagoa do Peixe (31°20'S, 51°05'W) and Arroio Chuí (33°45'S, 53°22'W); samples were collected from October to December 2009. For each turtle, curved carapace length (CCL) was measured from nuchal notch to posterior-most tip of carapace with a flexible metric tape (± 0.1 cm) (Bolten 1999). Humeri samples were macerated by immersing bones in freshwater for 2 to 3 weeks until the total removal of soft tissue and were then air-dried for 2 weeks. Three cross-sections (1-mm thick) were cut off from each humerus with a low speed isomet saw (Buehler®) with a diamond-embedded blade.

In order to assess the effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values, the humerus sections of each individual turtle had the lipids extracted by two solutions:

petroleum ether:ethyl ether (1:1) and chloroform:methanol (2:1); the samples were compared with a control that had no lipid extraction. The removal of lipids for each group was performed with a Soxhlet apparatus for 4 h. All samples had carbonates removed by demineralization with 10% HCl using the "drop-by-drop" technique (Jacob et al. 2005) until no gas bubbles were produced and were then washed with distilled water. The resultant collagen from the humerus subset samples was frozen in an ultrafreezer at -20ºC and then freeze-dried for at least 6 h.

The bone collagen of each section was powdered using a mortar and pestle, and ~1 mg of sample was placed in individual 4 x 6 mm tin cups and analyzed using a continuous-flow isotope-ratio mass spectrometer in the Laboratory of Analytical Chemistry, University of Georgia (USA). Stable isotope values are expressed in δ-notation as parts per thousand (‰) difference from the international standard material according to the following equation (as in Bond & Hobson 2012):

$$\delta X=(R_{sample}/R_{standard})-1 \quad (1)$$

where X is the ^{13}C or ^{15}N value, and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson & Fry 1987). Vienna Pee Dee Belemnite limestone and atmospheric nitrogen (Air) were used as the carbon and nitrogen standards, respectively. Two laboratory standards were analyzed for every 12 unknown samples. The measurement precision of both $\delta^{13}C$ and $\delta^{15}N$ analysis was 0.2‰.

Statistical analysis

Data were analyzed using a Shapiro-Wilk test to assess normality, and Bartlett's test was used to verify the homogeneity of variance between groups. One-way ANOVA was conducted separately for $\delta^{13}C$ and $\delta^{15}N$, followed by Tukey's pairwise *post hoc* comparisons when significant differences were found.

In order to evaluate the feasibility of mathematical normalization from our data set, simple linear regression models were performed to determine the relationship between (a) $\delta^{13}C$ values of lipid extracted ($\delta^{13}C_{le}$) and untreated samples ($\delta^{13}C_{bulk}$); (b) $\Delta^{13}C$ ($\delta^{13}C_{le}$ - $\delta^{13}C_{bulk}$) and C:N_{bulk}; (c) $\Delta^{13}C$ and carbon content (%C); and (d) $\Delta^{13}C$ and $\delta^{13}C_{bulk}$. Model predictability was tested by performing model efficiency (EF) as follows:

$$EF = 1 - \frac{\sum(y_i - x_i)^2}{\sum(y_i - \bar{y})^2} \quad (2)$$

where y_i is the $\delta^{13}C_{le}$ value, $\bar{y}$ is the mean of $\delta^{13}C_{le}$ values, and x_i is the model predicted $\delta^{13}C$ value. EF values range between negative infinity and 1; values close to 1 indicating a "near-perfect" model, whereas values close to 0 indicate a poor fit model (Mayer & Butler 1993). Any model with negative values indicates that the mean value of $\delta^{13}C_{le}$ is a better predictor than the model itself. In our case, such a model would not be recommended as a mathematical lipid correction method. The significance level for all tests was α = 0.05. All statistical analyses were carried out using R software version 3.0.3 (R Core Team 2014).

RESULTS

The carapace sizes of the 11 sampled turtles ranged from 50.0 to 93.0 cm CCL (mean ± SD, 73.8 ± 13.0 cm), representing juveniles and adults based on the size range of mature loggerhead sea turtles from Brazilian nesting areas in Espírito Santo state (83.0 to 120.0 cm CCL; Baptistotte et al. 2003) and Rio de Janeiro (86.5 to 114.5 cm; Lima et al. 2012). In both lipid extraction treatments, the $\delta^{13}C$ values were higher than the control (Fig. 1a). The values ranged from -16.96 to -13.36‰ (-15.00 ± 0.89‰) and from -15.37 to -13.10‰ (-14.38 ± 0.73‰) for the petroleum ether:ethyl ether group and the chloroform:methanol group, respectively. One-way ANOVA comparing treatment groups showed differences in the $\delta^{13}C$ values ($F_{(2,30)} = 7.31$, $p < 0.05$), whereas Tukey's *post hoc* comparisons indicated that the $\delta^{13}C$ values were significantly higher after lipid extraction with chloroform-methanol relative to the non-extracted samples ($p < 0.05$). By contrast, the $\delta^{15}N$ values were not affected by the lipid extraction protocols ($F_{(2,30)} = 0.04$, $p = 0.95$; Fig. 1b). The mean $\Delta^{13}C$ was 0.86 ± 0.68‰ (range from 0.16 to 1.93‰) for the petroleum ether lipid extraction and 1.49 ± 1.07‰ (range from 0.03 to 2.99‰) for the chloroform-methanol treatment. The mean bulk C:N ratio was 3.1 ± 0.6, with lower values after lipid extraction with the chloroform-methanol solvent and higher, but non-significant, values following the petroleum ether lipid extraction ($F_{(2,30)} = 2.10$, $p = 0.13$; Table 1).

Because the control and chloroform-methanol lipid extraction groups differed in the $\delta^{13}C$ values in bone collagen, the feasibility of mathematical normalization was tested using regression analyses. However, neither the relationship between $\delta^{13}C_{le}$ and the untreated samples nor between $C:N_{bulk}$ and $\Delta^{13}C$ were significant (Table 2). Hence, these regression results do not support the idea that there is a predictable relationship between bulk C:N and lipid content. The linear regression was also not significant for the $\Delta^{13}C$ and carbon content (%C) relationships. Nevertheless, a significant relationship

was observed between $\Delta^{13}C$ and $\delta^{13}C_{bulk}$ (r^2 = 0.55, p < 0.01). Based on the linear regression coefficients of the latter model, we could potentially provide a lipid correction equation (Fig. 2). Nevertheless, despite the evidence of the relationship among $\Delta^{13}C$ and $\delta^{13}C_{bulk,}$ the model efficiency was negative (EF = -19,676), indicating that the model parameters should not be used as a lipid correction factor.

DISCUSSION

To the best of our knowledge, this is the first study to measure the effects of lipid extraction methods on the isotopic values of bone collagen from loggerhead humeri. Because lipids have low $\delta^{13}C$ values compared with other molecules (DeNiro & Epstein 1977), it may confound the results of SIA and lead to erroneous ecological interpretations. The sea turtle humerus is a long bone that grows by collagen and calcium carbonate deposition, which is driven by physiological cycles synchronized with local environmental factors (Avens & Snover 2013). Although the use of bone tissue in SIA is still scarce, its recent application as a method of linking SIA with skeletochronology highlighted the usefulness of the analysis of this tissue to unravel the complex life cycle of sea turtles (Snover et al. 2010, Avens et al. 2013). The significant increase in $\delta^{13}C$ values observed after lipid removal using the chloroform-methanol solution suggests that lipid extraction is an important step for accurate $\delta^{13}C$ determination in sea turtle bone collagen. Therefore, lipid extraction should be taken into account in studies using the bone collagen tissue of marine animals to assess their trophic ecology and ontogenetic shifts.

Overall, the lipids in the bone tissue of loggerhead sea turtles exhibited decreased $\delta^{13}C$ values by approximately 1.5‰ after lipid extraction. The tissues of other

species with high lipid contents showed similar changes in $\delta^{13}C$ values, such as in the muscle tissues of eel *Anguilla anguilla* and Baltic herring *Clupea harengus membras* (Kiljunen et al. 2006), the muscle and liver tissues of cormorants (*Phalacrocorax carbo* and *P. auritus*; Doucette et al. 2010), and the skin of bottlenose dolphin *Tursiops truncatus* (Wilson et al. 2012). Although changes in $\delta^{13}C$ at this magnitude may appear biologically meaningless, it may influence the quantitative interpretation of food source partitioning when using mixing models or even in qualitative assumptions about movements and cryptic life stages. Kiljunen et al. (2006) demonstrated that the output of mixing models are largely influenced by whether the prey or consumer is lipid-normalized and recommended caution in the interpretation of results from these models pending further experimental evidence.

Lipid extraction did not affect the $\delta^{15}N$ values of bone collagen for both treatments. Similar to our results, a previous study showed no difference in the $\delta^{15}N$ values of skin of *T. truncatus* following lipid extraction with chloroform-methanol. Another experiment observed no lipid extraction effects in the nitrogen isotopic signatures of the exoskeleton and soft tissues of grain aphids *Sitobion avenae* (Perkins et al. 2013). By contrast, the effects of lipid extraction on $\delta^{15}N$ values have also been documented. Skin samples from 6 species of 4 families of cetaceans exhibited smaller, but statistically significant changes in $\delta^{15}N$ values after lipid extraction with a mean depletion of 0.14‰ in Balaenopteridae, and mean enrichment values of 0.30‰ and 0.22‰ in porpoises *Phocoena phocoena* and beluga *Delphinapterus leucas*, respectively (Lesage et al. 2010). For seabirds, the effects on stable nitrogen isotope values were observed in the muscle of white-tailed tropicbird (*Phaethon lepturus*) in which the lipid-extracted $\delta^{15}N$ values were, on average, 0.20‰ higher than bulk tissue (Kojadinovic et al. 2008). The large variability in nitrogen isotope ratios indicates the

need to evaluate the effects of the solvents on several tissues and species (Sotiropoulos et al. 2004). We tested the chemical solvents most commonly used in lipid extraction protocols and observed no significant change in the δ^{15}N values for both chloroform:methanol and petroleum ether. Generally, the analysis of carbon and nitrogen isotopes in separate subsamples is recommended to ensure accuracy in δ^{15}N values (Sotiropoulos et al. 2004, Post et al. 2007, Kojadinovic et al. 2008). However our data support that it is not necessary to separate subsamples of sea turtle bone collagen for SIA. This is a welcomed result because it represents a decrease in the cost of analysis and optimizes the processing work in laboratory.

As an alternative to lipid extraction, models have been proposed for the mathematical normalization of δ^{13}C values (e.g., McConnaughey & McRoy 1979, Post et al. 2007, Logan et al. 2008). However, their applicability has been questioned because generalized corrections may be inaccurate for specific datasets (Fagan et al. 2011), and taxon-specific models have been proposed in some cases (Kiljunen et al. 2006, Sweeting et al. 2006, Bodin et al. 2007, Mintenbeck et al. 2008, Logan et al. 2008). Due to the high carbon content of lipids, mathematical normalization models are typically based on the correlation between the C:N ratios of the bulk tissue and the percent lipid content in the tissue (Post et al. 2007). This assumption is based on the premise that the lipid content contains negligible nitrogenous components and therefore the removal of any lipid amount is reflected in a decrease in the C:N ratio. Post et al. (2007) postulated that the bulk C:N ratio in tissue accurately predicts the Δ^{13}C among the lipid extracted and untreated samples for a range of consumer species. For aquatic animals, C:N ratios >3.5 was established as a suitable indicator of the potential presence of a large fraction of lipids that could affect δ^{13}C analyses, and this predictor has been widely applied since then (McClellan et al. 2010, Dodge et al. 2011, Pajuelo et al. 2012,

Rosenblatt & Heithaus 2012). Although the utility of the C:N ratio as a predictor of lipid content was statistically validated (McConnaughey & McRoy 1979, Post et al. 2007, Logan et al. 2008), several studies noted that due to species-specific variation in lipid composition it could be difficult to apply a generic correction factor and recognized that any lipid-normalization method should be tested before application to a particular tissue (Kiljunen et al. 2006, Fagan et al. 2011, Ryan et al. 2012). For our dataset, it was not possible to employ the lipid normalization equation proposed by Post et al. (2007) to develop a new mathematical correction because the C:N ratio was not a suitable predictor of lipid content, and there was a weak correlation between $\Delta^{13}C$ and $\delta^{13}C$. We recognize that the limited sample size in our study could preclude the development of efficient models for lipid normalization, and more extensive studies are required to understand lipid content variation in humerus bones and the relationships associated with biochemical and physiological processes.

As expected, a decrease in the C:N ratio following lipid extraction with chloroform-methanol was observed. However, petroleum ether lipid extraction increased the C:N ratio. Chloroform-methanol is considered one of the most suitable chemicals for removing total lipid content, as it removes both polar and nonpolar lipids (Sotiropoulos et al. 2004). On the other hand, petroleum ether removes only polar lipids (Dobush et al. 1985). Thus, petroleum ether appears to be less effective, probably because it is unable to remove all the lipids in bone samples, which cause biases in the carbon and nitrogen stable isotope ratios.

Another issue that has arisen in the methodological protocols of SIA regards the preservation methods. A study performed with tissues of the spectacled petrel *Procellaria conspicillata* reported that the $\delta^{13}C$ values in ethanol showed high variation according to brand and batch and could account for the differences found in the $\delta^{13}C$

ratios in ethanol-preserved samples (Bugoni et al. 2008). Humeri do not require any chemical for preservation, but lipid extraction was performed with substances that contain carbon, such as chloroform, methanol and ether. It is possible that these chemicals may have influenced the carbon isotope values, similar to what was suggested for the spectacled petrel, and contributed to the variation in the C:N ratio in current the study.

Zug et al. (1986) validated that the humerus is the most suitable bone for age estimates of hard-shelled sea turtles. Because it is a long bone, its morphology is characterized by the presence of a medullary cavity filled by bone marrow (Castanet & Smirina 1990). Bone marrow varies considerably in composition, both within and between individuals due to functional demand and age (Dietz 1946). Rabbit bone marrow in the center of the humerus is composed of water (54.8%), lipids (32.6%), lipid free solids (12.6%), total nitrogen (1.94%) and lipid nitrogen (0.189%; Dietz 1946). The variation in bone marrow composition is a plausible explanation for the variability in stable isotope values between treatments, as well as the lack of a relationship between the C:N ratio and the $\Delta^{13}C$ or $\delta^{13}C$ values. It is also notable that bone maceration before processing for SIA could cause high variability in lipid content because maceration does not degrade marrow homogeneously, potentially causing an inherent lipid variation in bone tissue. Studies addressing the influence of maceration methods on the lipid content in bones are required and could improve our understanding on the observed variation in the stable isotopes of bone tissues.

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Table 1. Loggerhead sea turtle *Caretta caretta*. Mean ± SD (min–max) stable carbon and nitrogen values and C:N ratios of bone collagen with the different lipid extraction treatments vs. control group

Treatment groups	$\delta^{13}C$	$\delta^{15}N$	C:N
Petroleum-ether	-15.00 ± 0.89	12.87 ± 1.83	3.6 ± 0.8
	(-16.96 – -13.36)	(10.4 – 15.41)	(2.9 – 5.4)
Chloroform-methanol	-14.38 ± 0.73	12.99 ± 2.26	2.9 ± 0.8
	(-15.37 – -13.10)	(9.92 – 16.35)	(1.0 – 4.7)
Control	-15.86 ± 1.08	12.74 ± 2.06	3.1 ± 0.6
	(-17.96 – -14.60)	(10.17 – 16.56)	(2.2 – 4.0)

Table 2. Loggerhead sea turtle *Caretta caretta*. Parameter estimates of linear regressions ($y = \beta x + \alpha$) for lipid normalization based on $\delta^{13}C_{bulk}$ or $C{:}N_{bulk}$ from a subset of samples of bone collagen treated with chloroform-methanol vs. control group

Models	Coefficient	r^2	p
$\delta^{13}C_{le} = \beta.\delta^{13}C_{bulk} + \alpha$	$\alpha = -10.54$		
	$\beta = 0.24$	0.03	0.27
$\Delta^{13}C = \beta.\ C{:}N_{bulk} + \alpha$	$\alpha = 0.002$		
	$\beta = 0.47$	-0.03	0.45
$\Delta^{13}C = \beta.\%C_{bulk} + \alpha$	$\alpha = 0.76$		
	$\beta = 0.01$	-0.07	0.58
$\Delta^{13}C = \beta.\delta^{13}C_{bulk} + \alpha$	$\alpha = -10.54$		
	$\beta = -0.75$	0.55	<0.01

FIGURE CAPTIONS

Fig. 1. Loggerhead sea turtle *Caretta caretta*. Mean ± SD (‰) of $\delta^{13}C$ (A) and $\delta^{15}N$ (B) values of bone collagen in the lipid extraction treatment vs. control group. The horizontal lines represent the mean values, the gray box indicates standard deviation, and the vertical lines are the maximum and minimum values

Fig. 2. Loggerhead sea turtle *Caretta caretta*. Relationship between the change in carbon isotopic values ($\Delta^{13}C$) and (a) $\delta^{13}C_{bulk}$ and (b) the C:N ratio of bulk bone collagen before lipid extraction with the chloroform-methanol (2:1) solvent

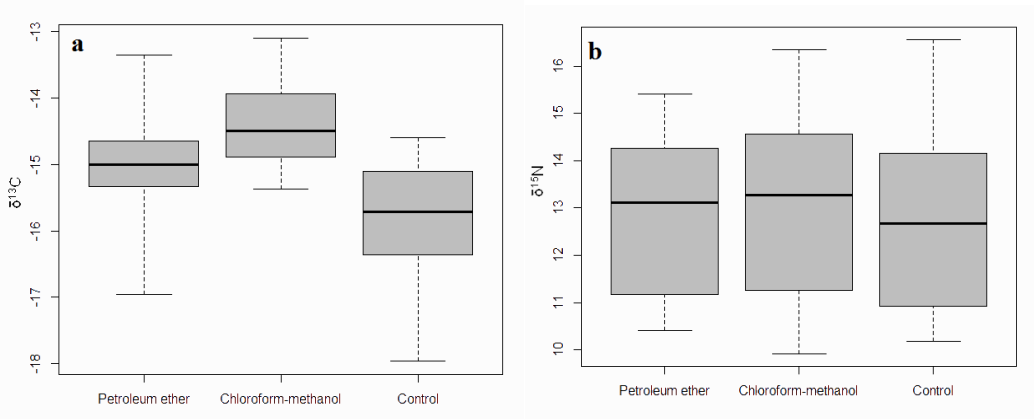
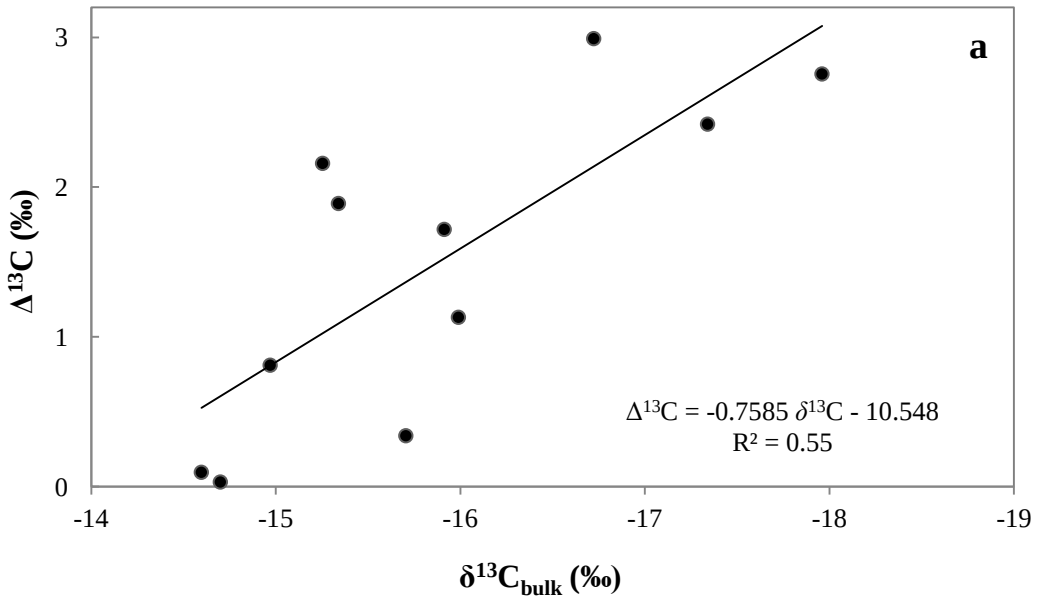

a
b
δ13C
δ15N
-13
-14
-15
-16
-17
-18
16
15
14
13
12
11
10
Petroleum ether
Chloroform-methanol
Control
Petroleum ether
Chloroform-methanol
Control

Fig. 1.


a
3
2
1
0
-14
-15
-16
-17
-18
-19
Δ13C (‰)
δ13Cbulk (‰)
Δ13C = -0.7585 δ13C - 10.548
R² = 0.55

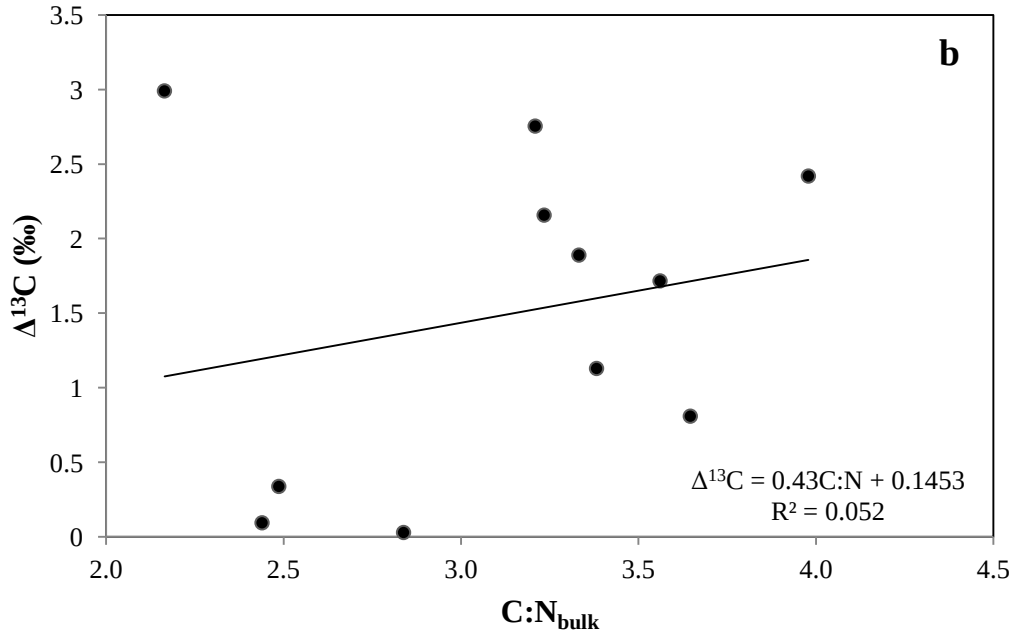

b
3.5
3
2.5
2
1.5
1
0.5
0
2.0
2.5
3.0
3.5
4.0
4.5
Δ13C (‰)
C:Nbulk
Δ13C = 0.43C:N + 0.1453
R² = 0.052

Fig. 2.

CAPÍTULO 2

Age and growth of hawksbill turtles (*Eretmochelys imbricata*) in the southwestern Atlantic Ocean

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Age and growth of hawksbill turtles (*Eretmochelys imbricata*) in the southwestern Atlantic Ocean

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ABSTRACT: The growth dynamics and age structure of hawksbill sea turtles *Eretmochelys imbricata* are poorly studied in Brazil. In the present study, we estimated age and growth rate of hawksbill turtles in the southwestern Atlantic Ocean by skeletochronological analysis. The carapace length and estimated ages for Brazilian hawksbill turtles ranged from 26.0–111.0 cm and 2–26 yr, respectively. Growth rates remained high for all size classes (1.12–8.26 cm) with peak growth at 60–69.9 cm size class. Higher growth rates in large juveniles may be a result of better quality foraging habitats or a size that allows better exploitation of available resources. The growth dynamics of hawksbill turtles from southwestern Atlantic is similar to that observed in Caribbean and Hawaiian populations, although the peak of growth is similar to that observed at the Great Barrier Reef, Australia. The von Bertalanffy growth model was fit to age-at-length data, with age at sexual maturity estimated using mean size of nesting females from the Brazilian coast (97.4 cm). Based on the growth model fit, the simulated age distribution resulted in a mean age at maturation for hawksbill turtle population at 26.98 ± 10.69 yr (95% probability interval: 12.72–53.61 yr), which is higher than that reported for Caribbean and Hawaiian populations. This difference could be related to distinct recovery strategies among population after historical depletion, intrinsic different patterns of each population, or effects related to foraging resources availability.

KEY WORDS: Skeletochronology, Bayesian inference, von Bertalanffy, growth model, sea turtle

RUNNING HEAD: Age and growth of hawksbill turtles

INTRODUCTION

Growth dynamics and age structure are important elements for a proper understanding of demography and life history of sea turtles (Heppel et al. 2000). Demography parameters, such as age-at-size maturity, sex-specific growth, rates of recruitment and growth rate variability are essential for adequate conservation and management of threatened species (Heppel & Crowder 1996, Chaloupka & Musick 1997, Lotze et al. 2011).

The hawksbill sea turtle (*Eretmochelys imbricata*) has a worldwide tropical and subtropical distribution and global numbers have been greatly reduced, as much as 80% over the last three generations, due to extensive exploitation to supply an intensive trade of tortoise shell jewelry and other crafts (Meylan & Donelly 1999). In addition, hawksbills are also threatened by exploitation for meat and eggs, fragmentation of nesting and foraging habitats, incidental capture in fisheries, marine pollution, and boat collisions (Lutcavage et al. 1997, Meylan & Donelly 1999). Consequently, hawksbill turtles are currently classified as Critically Endangered on the IUCN Red List (IUCN 2014).

Similar to other sea turtle species, hawksbills have complex life histories that encompass migratory behaviour, ontogenetic shifts and nesting site fidelity (Mortimer & Donnelly 2008, Meylan et al. 2011). As soon as they born, hawksbill hatchlings enter the sea and spend their first years in oceanic habitats. This initial life stage is called "lost years" because it is a poorly-known phase, estimated to last 1 to 3 years, when turtles then recruit to neritic habitats at a size of 20–35 cm carapace length (Limpus 1992, Boulon 1994). Once in neritic habitats, hawksbills occupy coral reefs due to their preferentially spongivorous diet (León & Bjorndal 2002, Meylan et al. 2011), although

they also inhabit other benthic habitats such as seagrass beds (Bjorndal & Bolten 2010), rocky shores (Proietti et al. 2012), and mangrove bays (Gaos et al. 2012). These traits make it difficult to access demographic parameters of this species. Although analyses of mark-recapture data have provided essential information about such parameters, this kind of study requires intensive long-term fieldwork and generally is restricted to a restricted area and includes a few size classes (Bjorndal & Bolten 2010, Bell & Pike 2012, Hawkes et al. 2014).

On the other hand, the skeletochronological method provides age-related data more rapidly and has the potential to yield information for a broad age class. Skeletochronology is the study of growth marks in bones of reptiles and amphibians (Castanet & Smirina 1990), which allows researchers to estimate age and growth rates. This methodology has been extensively applied over the last decade in studies with sea turtles (Avens & Snover 2013) and has provided key information on demographic settings, such as age structure, growth dynamics, rates of recruitment and age at sexual maturity (Goshe et al. 2010, Avens et al. 2012, Petitet et al. 2012, Avens et al. 2013, Snover et al. 2013).

Growth data on hawksbill turtles have been reported in Pacific Ocean – Australia (Limpus 1992, Chaloupka & Limpus 1997, Bell & Pike 2012), Hawaiian Islands (Snover et al. 2013), Western Samoa (Witzell 1980) – in western North Atlantic (Wood et al. 2013, Bjorndal & Bolten 2010), and Caribbean (Boulon 1994, Leon & Diez 1999, Diez & van Dam 2002, Beggs et al. 2007, Blumenthal et al. 2009, Krueger et al. 2011, Hawkes et al. 2014). However, no data on growth is available for hawksbill turtles in the Atlantic, south of the Equator.

The Brazilian coastline is an important habitat for sea turtle populations (Marcovaldi & Marcovaldi 1999). Besides hosting the largest remaining population of

nesting hawksbill turtles in the South Atlantic Ocean (Marcovaldi et al. 2007), Brazilian waters serve primarily as development habitat for early juveniles from a range of Atlantic populations (Proietti et al. 2012). Most studies in this area had focused on nesting females (Marcovaldi et al. 1999, Marcovaldi et al. 2007, Camillo et al. 2009, Santos et al. 2010, Santos et al. 2013) and addressed genetic issues (Lara-Ruiz et al. 2006, Vilaça et al. 2012, Vilaça et al. 2013, Proietti et al. 2014). Some of these studies showed that Brazilian rookeries and feeding aggregations are distinct demographic units (Vilaça et al. 2013, Proietti et al. 2014). Mixed stock analysis indicated that Brazilian feeding aggregations are mostly composed of animals originating from the domestic rookeries at Bahia and Pipa, northeastern Brazil, with minor contributions from African and Caribbean rookeries (Proietti et al. 2014). Nonetheless, a limited number of studies had been carried out in foraging aggregations (Sanches & Bellini 1999, Proietti et al. 2012, Leite et al. 2013) and thus demographic information of hawksbill turtles is still scarce. In this study we applied skeletochronological analysis to describe growth patterns of hawksbill turtles in western South Atlantic Ocean, based on samples collected along a wide area of the Brazilian coastline, and including nesting as well as developmental grounds.

MATERIAL AND METHODS

Sample collection and processing

Humeri samples were collected from stranded dead hawksbill turtles in nesting and foraging grounds along the Brazilian coast (Fig. 1). Hatchlings were recovered dead from nesting areas in Espírito Santo and Alagoas states, juveniles at all sampling sites, while subadults and adults at nesting grounds in Rio Grande do Norte, Bahia, Alagoas

and Espírito Santo states. For each stranded turtle, Curved Carapace Length (CCL) was measured with a flexible metric tape (± 0.1 cm) from nuchal notch-to-the-posterior end to the posterior marginal scute (Bolten 1999). Size of hatchlings was measured as the Straight Carapace Length (SCL) taken with calipers (± 0.1 mm). SCL of hatchlings were converted to CCL based on the linear regression equation provided by Hawkes et al. 2014:

$$\mathrm{CCL} = 1.1\mathrm{SCL} + 0.1 \quad (1)$$

Because sex could not be determined in most individuals by gonad examination due to decomposition, data analysis was based on both sexes pooled. Each humerus was dissected and immersed in water for 2–3 weeks for complete removal of soft tissues, and then air-dried for 2 weeks. After cleaning, the medial width was measured prior to removing cross-sections for histological processing (Zug et al. 1986).

The methodology of Avens & Goshe (2007) was used to prepare humerus for skeletochronological analysis. A low-speed isomet saw (Buehler®) with a diamond-embedded blade was used to cut 2−3 mm thick cross-sections of each humerus at a location just distal to the delto-pectoral crest, using the distal end of the insertion scar for the delto-pectoral muscle as a reference point. Sections were fixed in 10% formalin, rinsed with tap water and decalcified with the commercial decalcifying agent RDO. Decalcification time varied with size of the bone, between 4 and 16 h. After decalcification, cross-sections 25 μm thick were cut using a freezing-stage microtome (LEICA SM2000 R). Thin sections were then stained with 1:1 solution of Erlich's hematoxylin and distilled water, and mounted on slides in 100% glycerin. As the stain quickly bleached from the cross-sections (within ~4−5 weeks), multiple digital images

were taken of sequential portions of humeri sections at 4x magnification using a 17.28 megapixels digital camera Olympus DP73 coupled to a binocular inverted microscope. These partial images were combined using Adobe Photoshop Elements 8.0, resulting in a high resolution digital image used for growth mark analysis. In amphibians and reptiles, including marine turtles, a growth mark consists of a broad lightly stained area followed by a thin, dark line, denoted as Line of Arrested Growth (LAG), which could appears as a defined or a diffuse mark (Zug et al. 1986, Snover & Hohn 2004, Snover & Rhodin 2007). Other two growth mark morphologies were also typically observed: the double and split lines (Snover & Hohn 2004, Goshe et al. 2010, Snover et al. 2013). The first consists of two closely spaced dark lines that follow the same direction along the humerus circumference and represent 1 yr old. The second appears as a dark line that splits in multiple lines in some areas along the bone circumference. Each multiple line must be counted as one LAG. Each section was interpreted by one reader who conducted 3 independent LAG counts at a minimum of 5 days interval. If the number of growth marks varied between readings, a further reading was carried out.

Age estimation

Annual marks have been validated for 4 out of the 7 sea turtle species: *Lepidochelys kempii* (Snover & Hohn 2004), *Caretta caretta* (Klinger & Musick 1992, Coles et al. 2001, Snover & Hohn 2004), *Chelonia mydas* (Goshe et al. 2010), and *Dermochelys coriacea* (Avens et al. 2009). Due to the lack of known-age individuals of hawksbill turtles up to date, no direct validation exists for this species[1]. Three sources of indirect validation were presented by Snover et al. (2013) that encompass morphology of the growth marks, marginal increment analysis and growth data from a tagged and

[1] For the next version of this manuscript, we aim to process and include in the analysis two known-age hawksbill turtles raised in captivity for 2 and 14 yrs at Praia do Forte (Bahia State) and Guriri (Espírito Santo State), respectively. This will provide the first validation for the species.

later recaptured hawksbill turtle in Hawaii. This hawksbill turtle was tagged as a small juvenile (32.9 cm SCL) on October 1989 and recaptured twice: on January 1990, measuring 36.2 cm SCL, and again on January 1992, measuring 46.4 cm SCL (annual growth rate = 5.2 cm year^{-1}). From validation based on bones, the first growth mark observed was a diffuse mark, similar to that one observed in Kemp's ridley turtles (Snover & Hohn 2004). The first growth mark (*annulus*) settles closest to the centre of the bone and later growth marks are deposited sequentially throughout the outer circumference (Zug et al. 1986). Assuming that bones grow cyclically (Snover & Rhodin 2007, Avens & Snover 2013) and that each growth mark indicates 1 yr old (Coles et al. 2001, Snover & Hohn 2004, Goshe et al. 2010), the age for turtles retaining the *annulus* could be estimated as the number of growth layers observed in the outermost region of bone sections. However, due to body growth, there is bone resorption in the center of humeri, leading to the disappearance of the *annulus* and earlier lines, denoted as "lost LAGs" (Zug et al. 1986). In order to estimate the age for such turtles, the number of LAGs lost to resorption was estimated using a correction factor (Parham & Zug 1997). This correction factor derives from a relationship between the number of growth layers (x) and the corresponding growth layer diameters (y).

First, LAG diameters were measured in all cross-sections and LAGS numbered from the inner to the outer edge of the bone from turtles retaining an *annulus*, resulting in pairs (x,y). Regression models were assessed to determine the relationship which best described our data set. Model parameters were estimated based on two error structures (Faraway 2006). The structure called 'naïve' has three parameters (a or A, b and σ) and take on that:

$$y_i = A + Bx_i + v_i \quad (2)$$

$$y_i = ax_i^b e^{v_i} \quad (3)$$

where v_i are independent normal random variables with mean 0 and variance σ^2 for a total of n pairs (y_i, x_j), i = 1,..., n and a = ln$A$. The other error structure, denoted 'hierarquical', has five parameters (μ_a, σ_a, μ_b, σ_b, σ) and takes into account the inter and intra-individual variability. This error structure assumes that:

$$y_{ij} = A_i + B_i x_{ij} + v_{ij} \quad (4)$$

$$y_{ij} = a_i x_{ij}^{b_i} e^{v_{ij}} \quad (5)$$

where within individual i = 1,..., m, the variables v_{ij} are independent normal random variables with mean 0 and variance σ^2 for a total of n_i pairs (x_{ij}, y_{ij}), j = 1, ..., n_i and $n = \sum_{i=j}^{m} n_i$. Moreover, the pairs of parameters (a_i, b_i) represent individual-specific variation and were modelled as independent normal random variables with mean and variance (μ_a,σ^2_a) and (μ_b,σ^2_b), respectively.

To estimate the number of missing LAGs for turtles without an *annulus*, the resorption core diameters were measured (y_{core}) and substituted for LAG diameter in the correction factor. Hence, the corresponding number of lost lines was inferred by reverse prediction (x_{core}). As a result, the turtle's estimated age was calculated by the number of growth layers observed in the cross-section (x_{obs}) plus the number of missing LAGs ($x = x_{core} + x_{obs}$).

Back calculation and growth rates

Back-calculation is a technique developed in ichthyology to estimate growth rates by using dimensions of growth mark of otoliths or scales to back-calculate body

length at an earlier time (Francis 1990). This method has also been applied and validated for loggerhead and green turtles (Snover et al. 2007, Goshe et al. 2010) and is based on the premise that there is a constant rate of deposition of growth marks (e.g. daily or annual) and there is a predictable proportionality between some measurements of the hard structures being used and the animal body size.

To find a model that describes the best relationship between humerus diameter and carapace length over all size classes from our data set, we fitted the 4 models applied by Snover et al. (2007), who used least-squares regression to determine whether the relationship is allometric (nonlinear) or isometric (linear):

$$L = l_{op} + b(D - d_{op})^c \quad (6)$$

$$L = l_{op} + b(D - d_{op}) \quad (7)$$

$$L = a + bD^c \quad (8)$$

$$L = a + bD \quad (9)$$

where L is carapace length (cm), D is humerus section diameter (mm), L_{op} is average hatchling carapace length (cm), D_{op} is average hatchling humerus diameter (mm), and c is the allometric coefficent, which is equal to 1 for linear models (eqs. 7 and 9). For L_{op} and D_{op}, we used the mean CCL (L_{op} = 4.68 cm) and humerus diameter (D = 1.73) from 10 hatchlings collected in this study.

After fitting all four models, we applied the Body Proportional Hypothesis (BPH) to enable estimation of carapace length from diameters of early growth marks. This hypothesis assumes that the ratio of the real size of the animal (L) and the estimated size by retro-calculation model associated with the humerus diameter

$\hat{L} = f(D)$ is the same for all values of *D*. Hence, the BPH generic equation takes the form:

$$L_b = [f(D_b)][L_{final}][f(D_{final})]^{-1} \quad (10)$$

where L_b is the back-calculated length for a given diameter D_b, L_{final} is the CCL of a turtle at death, and $f(D_{final})$ is the back-calculated CCL, based on humerus diameter.

In order to estimate annual growth rates, we used all back-calculated size-at-age data. Back-calculated CCLs at the innermost LAG was subtracted from the outermost LAG for each LAG pair and growth rate data were then binned into 10 cm size categories based on the average of each size class.

Growth model

A von Bertalanffy growth curve was fitted to our data set. The von Bertalanffy function is:

$$L = L_\infty(1 - e^{-k(t-t_0)}) \quad (11)$$

where L_∞ is the asymptotic length, k is the allometric coefficient, and t_0 is the hypothetical age when length is equal to zero. The parameter t_0 was replaced by L_0 which represents the length-at-age-zero. Ultimately, the reparametrized von Bertalanffy growth model takes the form:

$$L = L_\infty - (L_\infty - L_0)e^{-kt} \quad (12)$$

Once the growth model had been adjusted, the estimated parameters were used to estimate age-at-sexual-maturity, based on the eq. 13. The estimated age distribution

was based on the mean size of hawksbill nesting females in northern Bahia (97.4 cm CCL, Marcovaldi et al. 1999).

$$t = -\frac{1}{k}\ ln\frac{(L - L_{\infty})}{(L_{\infty} - L_0)} \quad (13)$$

Statistical analysis

Statistical analyses were performed based on Bayesian inference. Markov Chain Monte Carlo (MCMC) was used to obtain the posterior distributions of parameters by integrating the data likelihood and non-informative prior distributions (Gelman et al. 2003). Non-informative priors were used in all models, except for the von Bertalanffy growth model, for which the prior for the parameters L_0 and L_{∞} were a normal distribution with 6 degrees of freedom, centred at the mean of the log likelihood and with a precision equal to the inverse of the logarithm of the variance. Model selection was based on the Deviance Information Criterion (DIC; Spiegelhalter et al. 2002). All analyses were performed with software R (R Core Team 2014) and OpenBugs (Thomas et al. 2006) through R libraries R2WinBUGS (Sturtz et al. 2005) and BRugs (Thomas et al. 2006). Simulated posterior samples were checked for convergence using library CODA (Plummer et al. 2006). Adjustments in burn-in length and thinning were performed until good convergence diagnostics had been achieved.

RESULTS

Our sample contained 59 hawksbill turtles[2], of which 49 consisted of turtles washed ashore in nesting and foraging grounds along the Brazilian coast and 10

[2] For the next version of this manuscript, we aim to process and include in the analysis other 13 humeri samples: five early juveniles, two large juveniles, one subadult and five adults.

hatchlings recovered dead from nests. Sizes of stranded turtles ranged from 26.0 to 111.0 cm CCL (mean ± SD = 42.93 ± 20.34 cm; Fig. 2). For hatchlings, SCL ranged from 3.55 to 4.79 cm (4.16 ± 0.49) and humerus diameter varied between 1.5 and 1.9 mm (1.73 ± 0.12 cm).

Age estimation

Although there is no direct validation of the annual nature of the growth marks of hawksbill turtles, indirect methods support this assumption (Snover et al. 2013). In this study, the growth marks observed in humeri cross-sections were morphologically similar to validated marks in other species (Fig. 3). The diffuse *annulus* was detected in 35 cross-sections and these turtles retained between 2 and 7 LAGs, with age being equal to the number of LAGs, and ranging in size from 26.0 to 53.9 cm. Based on DIC, the hierarquical power function model provided the best fit to our data set (Table 1). The following power function was fitted to the relationship between LAG diameter and LAG number for turtles that retained an *annulus*, with posteriors as parameter point estimates, and the correction factor equation took the form:

$$LAG\ diameter\ (mm) = 2.01\ (LAG\ number)^{0.29} \quad (14)$$

For the 14 turtles lacking the first-year mark, the LAG diameter (y) was replaced by the resorption core diameter (y_{core}), and the solved equation provided the number of lost LAGS. The CCL of these turtles ranged from 49.5 to 111.0 cm (71.94 ± 17.23 cm) and the estimated ages were between 6 and 26 yr old.

Back calculation and growth rates

The nonlinear allometric model in Eq. (6) provided the best fit, which incorporated the biological intercepts (l_{op} and d_{op}) and the constant c, despite other 3 models also had good fit to the data, but with higher DIC values (Table 2, Fig. 4). Based on the model fit, we observed a significant positive relationship between growth rates and CCLs ($r = 0.44$, $p < 0.01$), suggesting that turtle growth rates are higher as turtles increase in size. The relationship between estimated age and growth rate was not significant ($r = 0.16$, $p = 0.26$).

Growth model

The von Bertallanfy growth model had a good fit for the relationship between estimated age and carapace length data (Table 4, Fig. 5). Such model exhibit an asymptote, which is highly sensitive to missing data points, making necessary that the sampling had included all age classes (Siegfried & Sansó 2006). Although data includes a wide age interval, our sample consists mostly of juveniles (n = 36, CCL <40 cm), which could had influenced the behaviour of the asymptote, shaping it almost linear. The simulated age distribution resulted in a mean age at maturation for hawksbill turtle population at 26.98 ± 10.69 yr (95% probability interval: 12.72–53.61; Fig. 6).

DISCUSSION

To the best of our knowledge, this study provides the first description of age structure and growth dynamics of hawksbill turtles from the southwestern Atlantic Ocean. Growth data on hawksbill turtles have been reported in Australia (Limpus 1992, Chaloupka & Limpus 1997, Bell & Pike 2012), Hawaiian Islands (Snover et al. 2013), Western Samoa (Witzell 1980), western North Atlantic (Wood et al. 2013, Bjorndal &

Bolten 2010), and Caribbean (Boulon 1994, Leon & Diez 1999, Diez & van Dam 2002, Beggs et al. 2007, Blumenthal et al. 2009, Krueger et al. 2011, Hawkes et al. 2014).

Age estimation

The hierarquical power model showed greater stability compared to the näive model. The best fit occurred due to the lower residual variance, which incorporate the parameters σ_a and $\sigma_{b,}$ and takes into account the individuals and interindividual variation (Petitet et al. 2012). This variation is possibly due to the stochastic nature of environmental traits which depends on the spatial and temporal variability of resources and, hence, reflects on variation of LAG deposition and compensatory growth (Bjorndal et al. 2003).

Although hawksbill turtles nesting in Brazil represents the largest remaining population in the South Atlantic (Marcovaldi et al. 2007), Brazilian waters serve primarily as development habitat for early juveniles (Proietti et al. 2012). The main feeding areas for hawksbills in Brazil are the Archipelago of Fernando de Noronha (Sanches & Bellini 1999), the Saint Peter and Saint Paul Archipelago (SPSPA), the Abrolhos Marine National Park, and the Arvoredo Biological Reserve (Proietti et al. 2012). The size of captured turtles at these sites ranged from 30.5–75.5 cm at Fernando de Noronha, 24.5–63.0 cm CCL at Abrolhos (mean = 37.9 cm), 30–75 cm at SPSPA (mean = 53.7 cm), and 30.0–59.5 cm (mean = 41.3 cm) at Arvoredo. An occasional report of hawksbill foraging aggregation was also made for Anchieta Island State Park, ranging in size from 30 to 79.5 cm CCL (mean = 46.0 cm, Leite et al. 2013). In this study, the CCL and age estimates of hawksbill turtles ranged from 26 to 111 cm and 2 to 26 yr, respectively. However, our sample consists mainly of early juveniles (n = 36 CCL <40 cm), ranging in age from 2 to 6 yr. It has been suggested that hawksbills in the

western Atlantic Ocean recruit to neritic habitats when they reach 20–25 cm CCL, after living in pelagic environment for 1–3 years (Boulon 1994, Meylan et al. 2011). Our data corroborate this hypothesis and, hence, the large number of stranded early juveniles supports the assumption that southwestern Atlantic Ocean is an important feeding ground and a development habitat for recent recruits.

Growth rates

An issue that has arisen in the age estimation from growth data is the inherent uncertainty involved in the inference of age from size, mainly due to individual variation in growth (Congdon et al. 2001, Armstrong & Brooks 2014). Sea turtle species show high growth rate variability over time and among turtles (Goshe et al. 2010, Petitet et al. 2012, Wood et al. 2013). For this reason, finding the best relationship between carapace length and LAG deposition is essential to assess growth parameters (Snover et al. 2007). Recently, Bayesian inference proved to be a workable statistical framework to deal with such variability (Petitet et al. 2012, Armstrong & Brooks 2014) because it is based on predictive probabilities over all possible ages and sizes from data sets, which provide more accuracy to estimated parameters (Ellison 2004). Based on this premise, it is not surprising that the nonlinear allometric model provided the best fit, which incorporates the biological intercepts (l_{op} and d_{op}) and the allometric coefficient *c*.

Demographic parameters can vary within and among populations, even within the same geographic scales, and thus may not necessarily agree with theoretical life history patterns (Johnson et al. 2010). Our results showed a positive relationship between growth rate and CCL, but this relationship was weak and it is not appropriate to assume that hawksbill growth rates are higher as turtles increase in size because this

approach contradicts the growth pattern from other regions of the Atlantic and Pacific Oceans, where it had been indicated a negative relationship between size and growth rate of hawksbill turtles (Bjorndal & Bolten 2010, Bell & Pike 2012, Wood et al. 2013, Snover et al. 2013, Hawkes et al. 2014, and references therein). As our data set is mostly composed by juveniles, and considering the high variability observed in growth rates between and within size classes, this contradictory result may be a consequence of the low representation of subadult and adult individuals in our sample. Therefore, the high variability between these few individuals does not reflect the true pattern of growth of their respective size classes. As shown in Table 3, growth rates increase to a peak in the 60-69.9 cm size class, followed by a decline in later classes, represented by adults and sub-adults, with the exception of 90-99.9 size class cm (n = 1), which shows a growth rate of 5.39 $cm.yr^{-1}$ and that could influence the observed result.

The growth rate patterns of hawksbill aggregations from the Pacific Ocean tend to be smaller than those in the Caribbean and western North Atlantic (Hawkes et al. 2014). Furthermore, Atlantic hawksbills show peak growth in a smaller size class (30–40 cm; Boulon 1994, León & Diez 1999, Diez & van Dam 2002, Bjorndal & Bolten 2010) in comparison to Pacific populations, whose peak growth is commonly observed in the 55–70 cm size classes (Chaloupka & Limpus 1997, Bell & Pike 2012). The growth rates reported here (Table 3) are similar to those reported in age classes of Caribbean (Boulon 1994, León & Diez 1999, Diez & van Dam) and Hawaiian hawksbill's populations (Snover et al. 2013). However, we observed a peak in mean growth rate in the 60–69.9 cm CCL age class (8.26 $cm.yr^{-1}$), the highest growth rate recorded for hawksbill turtles for this size class. Similar peak growth was also observed in a mark-recapture study in southern Great Barrier Reef, Australia (Chaloupka & Limpus 1997, Bell & Pike 2012), although growth rates recorded have been much lower

than in our study (2.2 $cm.yr^{-1}$ for females and 1.7 $cm.yr^{-1}$ for males). Higher growth rates in large juveniles may be a result of better quality foraging habitats or a size that allows better exploitation of available resources, such as sponges and zoanthids in crevices of reefs or rocky shores (Bjorndal & Bolten 2010).

It had been assumed that the slower growth rates observed in early juveniles is a response to stochastic environments and could lead to a compensatory growth, i.e. after a period of suboptimal environmental conditions with a reduced growth, turtles could undergone an accelerated growth when exposed to better conditions (Bjorndal et al. 2003). Based on two small juveniles of hawksbill turtles which had high growth rates (14.3 and 15.6 $cm.yr^{-1}$), Bjorndal & Bolten (2010) suggested that delayed growth is a result of ontogenetic shifts from oceanic to neritic habitats. High growth rates were recorded in all size classes of our sample (ranging from 10.03 to 23.68 cm^{-1}, Table 3). These individual differences in growth rates could be related to a variety of factors, such as water temperature, food resources and differential fitness consequences. While differences in water temperature can alter metabolic rates or cause changes in food availability (Carrillo et al. 1999), foraging aggregations may drive a pressure on limited resources (Bjorndal & Bolten 2010), thereby influencing somatic growth. Detailed studies on foraging habitats are needed to verify how differences in foraging behaviour and habitat use affects growth dynamics of sea turtles and how these traits shape the complex life history and the ecological role of hawksbill turtles within an evolutionary scale.

Growth model

The von Bertalanffy growth model is commonly applied in ecology to assess the growth pattern of species. This growth model exhibit an asymptote, which is highly

sensitive to missing data points, making necessary that sampling includes all age classes (Siegfried & Sansó 2006). Hence, fitting the von Bertalanffy model in a Bayesian framework increases robustness and provides a much larger asymptote than classical regression methods (Siegrified & Sansó 2006, Armstrong & Brooks 2014).

Time to maturation is recognized as important for determining how long management measures must be implemented to increase turtle numbers on nesting beaches and identify population recovery (Boulon 1994). Based on the von Bertalanffy growth model, the estimated age at maturation was about 27 yr. A skeletochronological analysis of Hawaiian hawksbill turtles suggests that hawksbills reach 78.6 cm SCL (mean size of nesting females) on average between 17 and 22 yr (Snover et al. 2013). For Caribbean turtles, it was estimated that females from Buck Island, in St. Thomas United States Virgin Islands, require approximately 16.5 yr and 19.3 yr to reach the minimum (78.8 cm) and mean (88.7 cm) size at maturity, respectively (Boulon 1994). The growth curve presented by Witzell (1980) indicates that captive hawksbill turtles from Western Samoa mature at about 50 cm and 3.5 yrs old. However, the minimum nesting size reported for Samoan hawksbill is 60 cm, which indicates that the Samoan turtles either grow faster in their natural habitat or mature at considerably older ages than estimated. Differences in the mean and minimum sizes of nesting individuals may be related to recovery strategies intrinsic to each population after a severe historical reduction in population size, such as early sexual maturity of individuals with smaller sizes. Additionally, foraging success is intimately linked to reproductive success and hence population viability (Lescroël et al. 2010). Therefore, processes that drive foraging success may strongly shape the conservation status of hawksbill populations.

CONCLUSIONS

Management of exploited populations requires knowledge of demographic settings, such as age structure, growth dynamics, rates of recruitment and age at sex maturity (Heppel & Crowder 1996, Lotze et al. 2011). Our data indicates that the southwestern Atlantic Ocean is an important feeding ground and a development habitat for recent recruits, ranging in age from 2−6 yr. Growth rates can be used as indicators of the health of a population or ecosystem, and provide a baseline for assessing habitat quality and environmental changes (Bell & Pike 2012). Brazilian hawksbill population show high growth rates in all size classes, which could indicates population size well under the carrying capacity of the environment, which agrees with suggested much larger historical population sizes (Marcovaldi et al. 2011). Ultimately, the estimated age at sexual maturity was higher than that reported in Caribbean and Hawaiian populations, lasting almost 3 decades.

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Table 1. Hawksbill sea turtle *Eretmochelys imbricata*. Bayesian fits of the 4 correction factor models. *a*, *b* and *σ* represent the posterior mean; values within brackets are the 95% credibility intervals. DIC is the Deviance Information Criteria; lower deviance indicates the best model fit

	Naïve Model		Hierarquical model	
Parameter	$y = a + bx$	$y = ax^b$	$y = a + bx$	$y = ax^b$
A and *a*	-6.08 (-21.54−9.29)	2.27 (2.14−2.40)	6.73 (6.23−7.22) σ_a = 1.28 (0.95−1.70)	2.01 (1.95−2.07) σ_a = 0.17 (0.13−0.22)
B and *b*	14.75 (7.48−22.09)	-0.01 (-0.05−0.02)	1.14 (1.01−1.29) σ_b = 0.27 (0.15−0.43)	0.29 (0.26−0.32) σ_b = 0.06 (0.03−0.09)
σ	9.48 (8.37−9.98)	0.22 (0.20−0.25)	0.57 (0.48−0.67)	0.05 (0.04−0.06)
DIC	83.5	-8.7	293.5	-365.6

Table 2. Hawksbill sea turtle *Eretmochelys imbricata*. Bayesian fit of the allometric and isometric models. In each model *L* is the Curved Carapace Length and *D* is the humerus diameter; l_{op} is the carapace length at hatching, d_{op} is the humerus diameter at hatching. Estimated parameters are posterior means; values within brackets are the 95% credibility intervals. DIC is the deviance information criteria; lower deviance indicates the best model fit. --- Not applicable

Model	Parameters				
	a	b	c	σ	DIC
$L = l_{op} + b(D - d_{op})^c$	---	3.89 (3.26−4.57)	0.89 (0.83−0.96)	0.10 (0.08−0.12)	-79.9
$L = l_{op} + b(D - d_{op})$	---	2.88 (2.81−2.95)	---	4.02 (3.31−4.92)	278.8
$L = a + bD^c$	0.83 (0.00−7.20)	3.36 (1.97−4.02)	0.94 (0.88−1.07)	3.73 (3.0−4.6)	274.4
$L = a + bD$	3.72 (1.14−6.37)	2.67 (2.52−2.82)	---	3.69 (3.02−4.56)	275.3

Table 3. Hawksbill sea turtle *Eretmochelys imbricata*. Size-specific growth rates from back-calculated Curved Carapace Lengths (CCLs) of all measurable LAG diameters (n = 204 growth intervals) in the humeri of 49 turtles. Growth rates were divided into size classes based on the mean CCL of the back-calculated CCL pairs. SD is standard deviation. --- Not applicable

Size class (cm CCL)	Mean growth rate ($cm.year^{-1}$)	SD	Growth rate range ($cm.year^{-1}$)	n
15.5−19.9	5.29	3.30	1.69−10.03	7
20.0−29.9	3.88	2.26	1.17−10.53	62
30.0−39.9	2.81	2.83	0.81−15.44	66
40.0−49.9	4.84	4.96	0.62−23.68	21
50.0−59.9	5.76	4.86	0.37−15.91	10
60.0−69.9	8.26	8.14	2.28−22.00	5
70.0−79.9	2.81	4.34	0.27−17.49	15
80.0−89.9	1.11	0.96	0.39−3.42	10
90.0−99.9	5.39	---	---	1
> 100	1.12	1.36	0.07−4.18	8

Table 4. Hawksbill sea turtle *Eretmochelys imbricata.* Bayesian fit of von Bertalanffy growth model for curved carapace length (CCL) and estimated age data. Estimated parameters are posterior means; values in parenthesis are 95% credibility intervals. DIC is deviance information criterion to select among models

von Bertalanffy model	
L_{∞}	95.64
	(84.79−107.74)
L_0	3.93 (3.07−4.98)
k	0.11 (0.09−0.14)
σ	0.20 (0.17−0.25)

FIGURE CAPTIONS

Fig. 1. Approximate location of sampling sites along the Brazilian coast: RS − Rio Grande do Sul State (n = 7), PR − Paraná State (n = 4), RJ − Rio de Janeiro State (n = 3), ES − Espírito Santo State (n = 20), BA − Bahia State (n = 11), AL − Alagoas State (n = 3), RN − Rio Grande do Norte State (n = 2). Dashed lines represent the stretch of beach monitored searching for stranded turtles. Numbers represents known developmental areas of early juveniles of hawksbill turtles: (1) Saint Peter and Saint Paul Archipelago (SPSPA), (2) Archipelago of Fernando de Noronha, (3) Abrolhos Marine National Park, (4) Arvoredo Biological Reserve

Fig. 2. Hawksbill sea turtle *Eretmochelys imbricata*. Frequency distribution of Curved Carapace Lengths (CCLs) from dead stranded turtles in this study and hatchlings, measured from notch-to-tip (n = 59)

Fig. 3. Hawksbill sea turtle *Eretmochelys imbricata*. Stained humerus cross-section of a Brazilian turtle. The black arrows indicate (a) the morphology of a growth mark consisted of a lightly stained area followed by a dark Line of Arrested Growth (LAG); and (b) *Annulus*, the first year mark

Fig. 4. Hawksbill sea turtle *Eretmochelys imbricata*. Relationship between Curved Carapace Length (CCL) and humerus diameter (n = 49). Black solid line: model [$L = l_{op} + b(D - d_{op})^c$] fitted to the stranded turtle data set; l_{op} is CCL at hatching, d_{op} is humerus diameter at hatching, *b i*s the slope of line and *c* is a allometric coefficient

Fig. 5. Hawksbill sea turtle *Eretmochelys imbricata*. Bayesian fit of von Bertallanfy growth model. Solid line represents posterior median, dashed red lines are the 80% probability intervals, and dotted blue lines are the 50% probability intervals. Solid black horizontal line shows approximate size at sexual maturity in Bahia state, Brazil

Fig. 6. Hawksbill sea turtle *Eretmochelys imbricata.* Estimated age at maturation for the southwestern Atlantic Ocean population

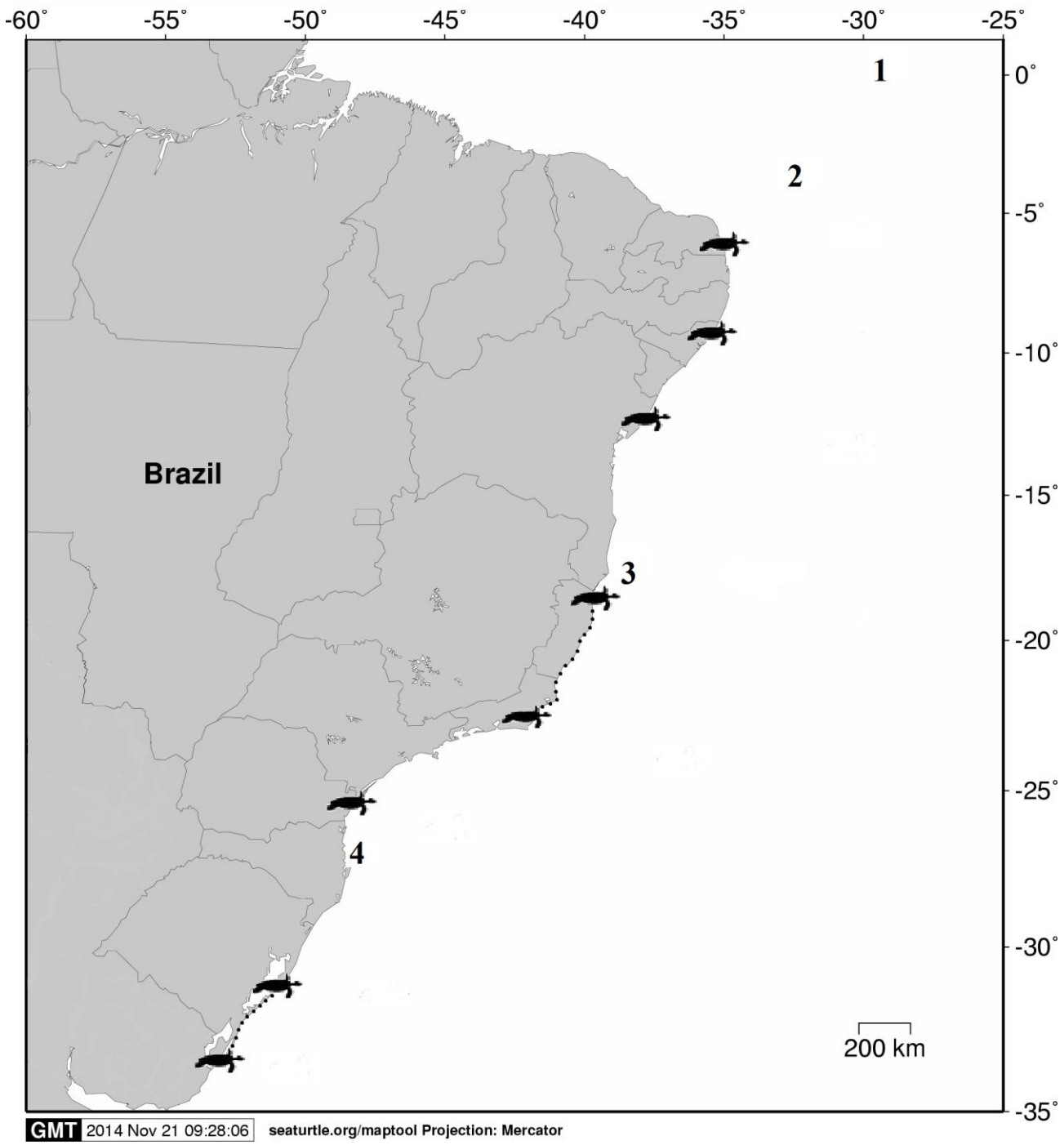
-60˚
-55˚
-50˚
-45˚
-40˚
-35˚
-30˚
-25˚
0˚
-5˚
-10˚
-15˚
-20˚
-25˚
-30˚
-35˚
1
2
3
4
Brazil
200 km
GMT 2014 Nov 21 09:28:06
seaturtle.org/maptool Projection: Mercator

Fig. 1.

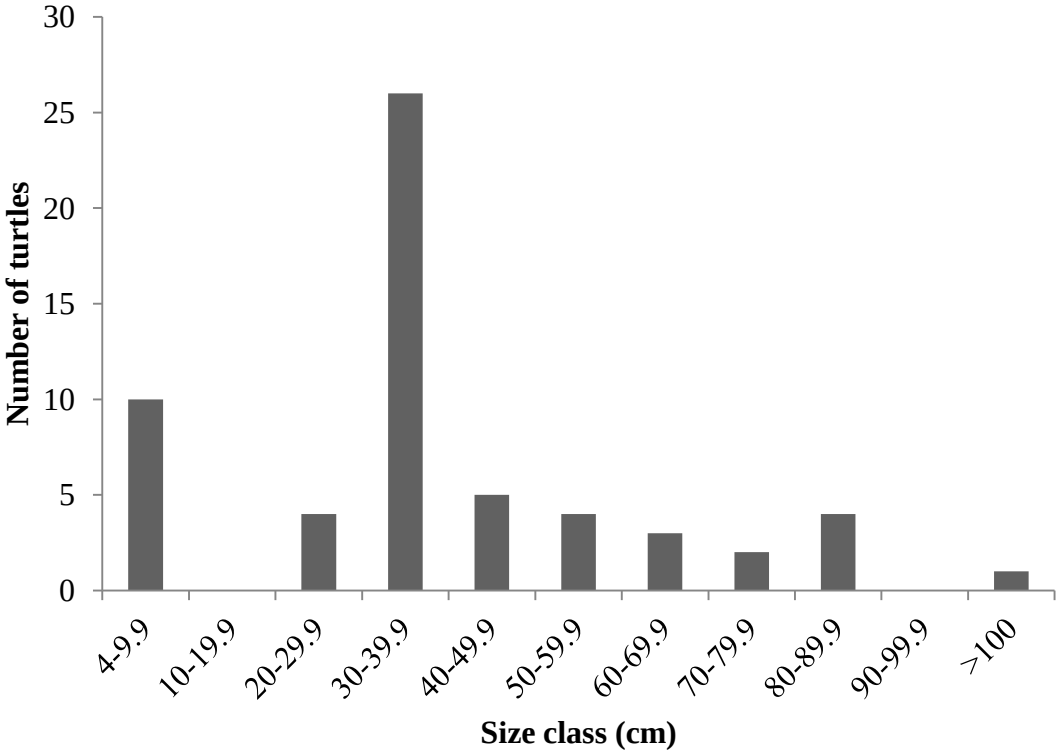
30
25
20
15
10
5
0
Number of turtles
4-9.9
10-19.9
20-29.9
30-39.9
40-49.9
50-59.9
60-69.9
70-79.9
80-89.9
90-99.9
>100
Size class (cm)

Fig. 2.

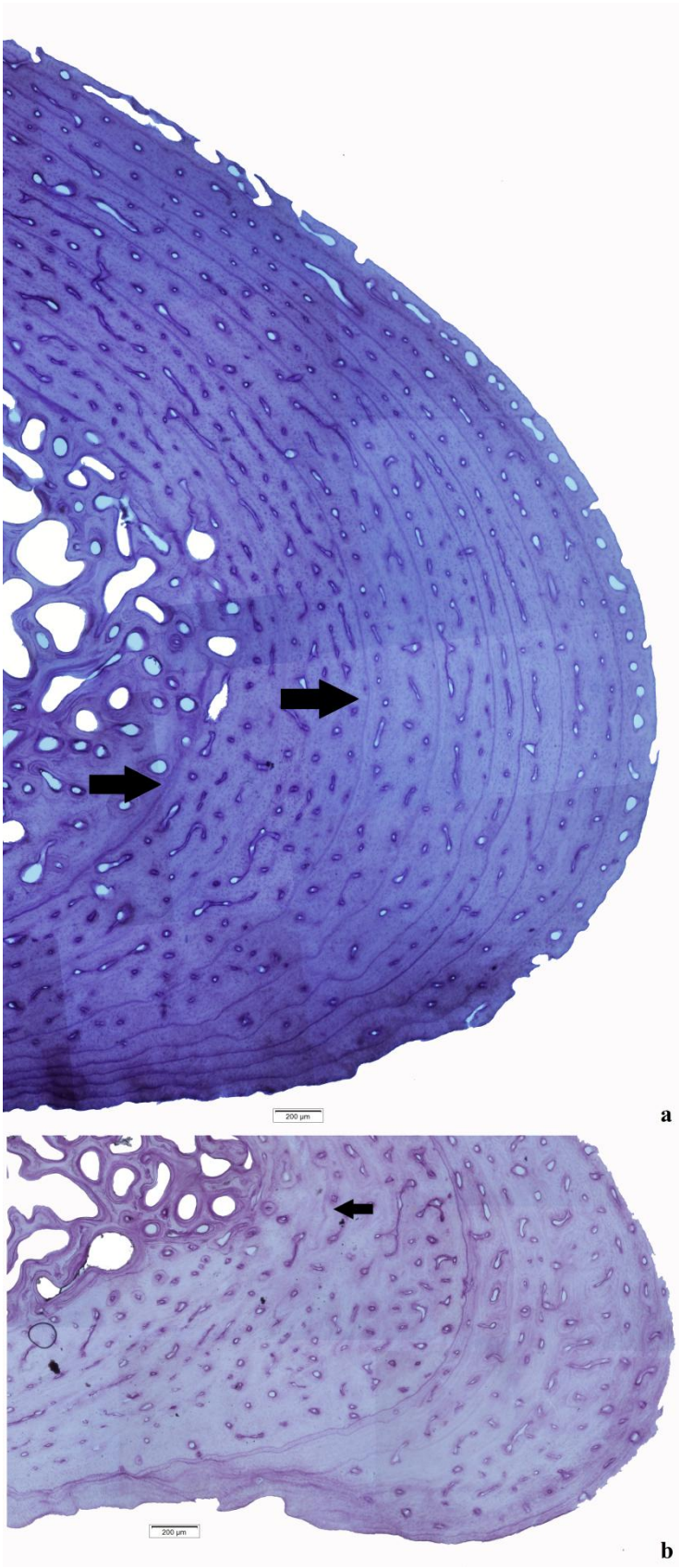

Fig. 3.

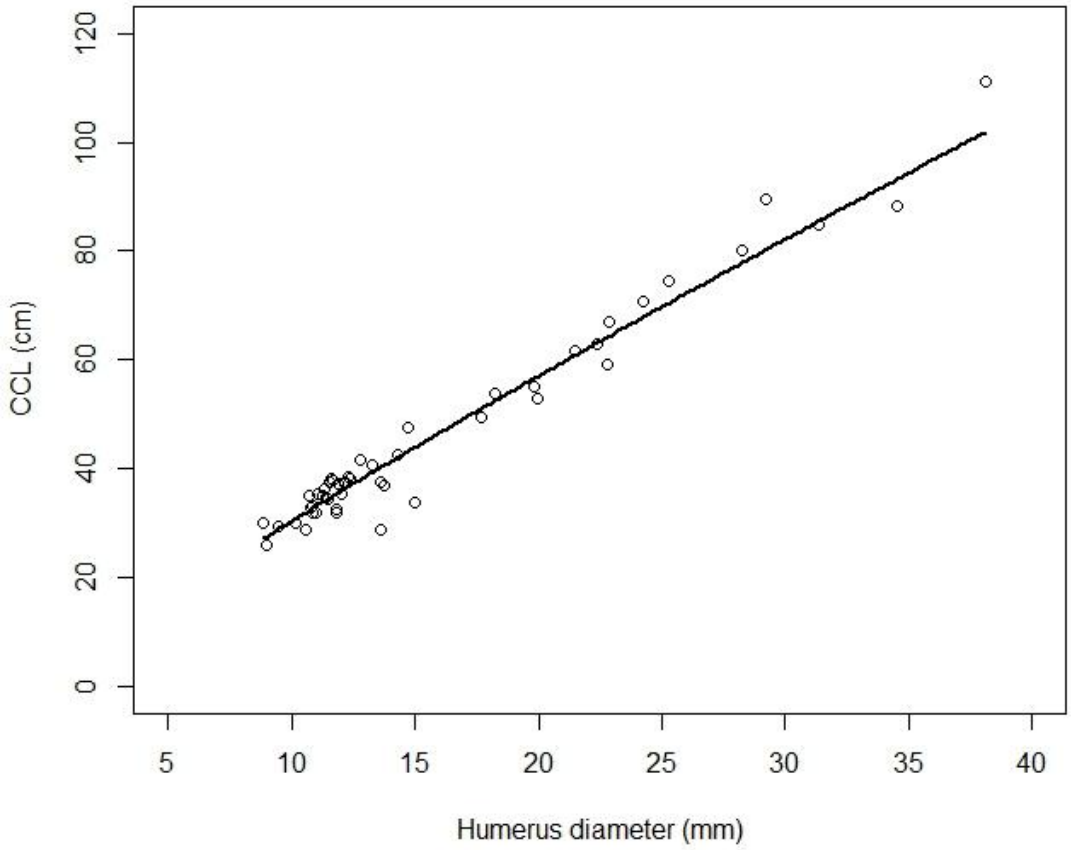
CCL (cm)
0
20
40
60
80
100
120
5
10
15
20
25
30
35
40
Humerus diameter (mm)

Fig. 4.

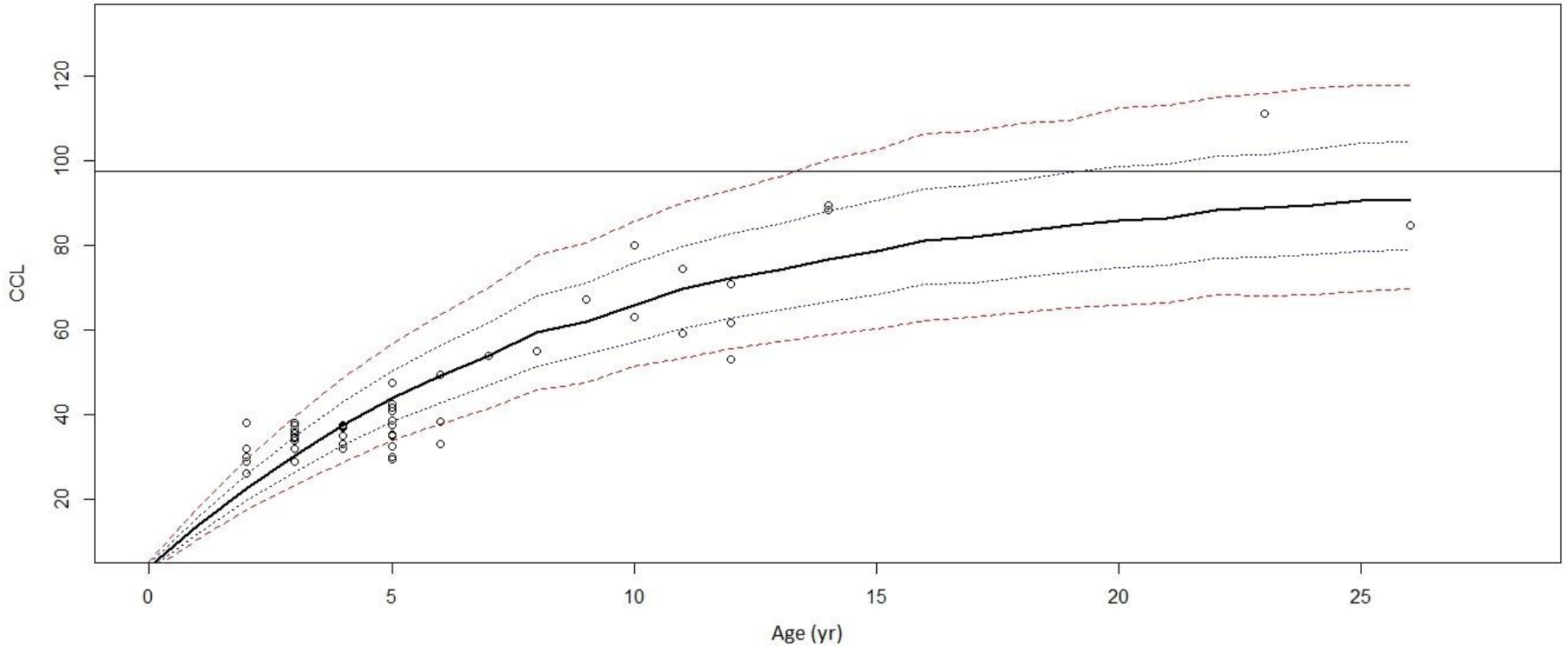

Fig. 5.

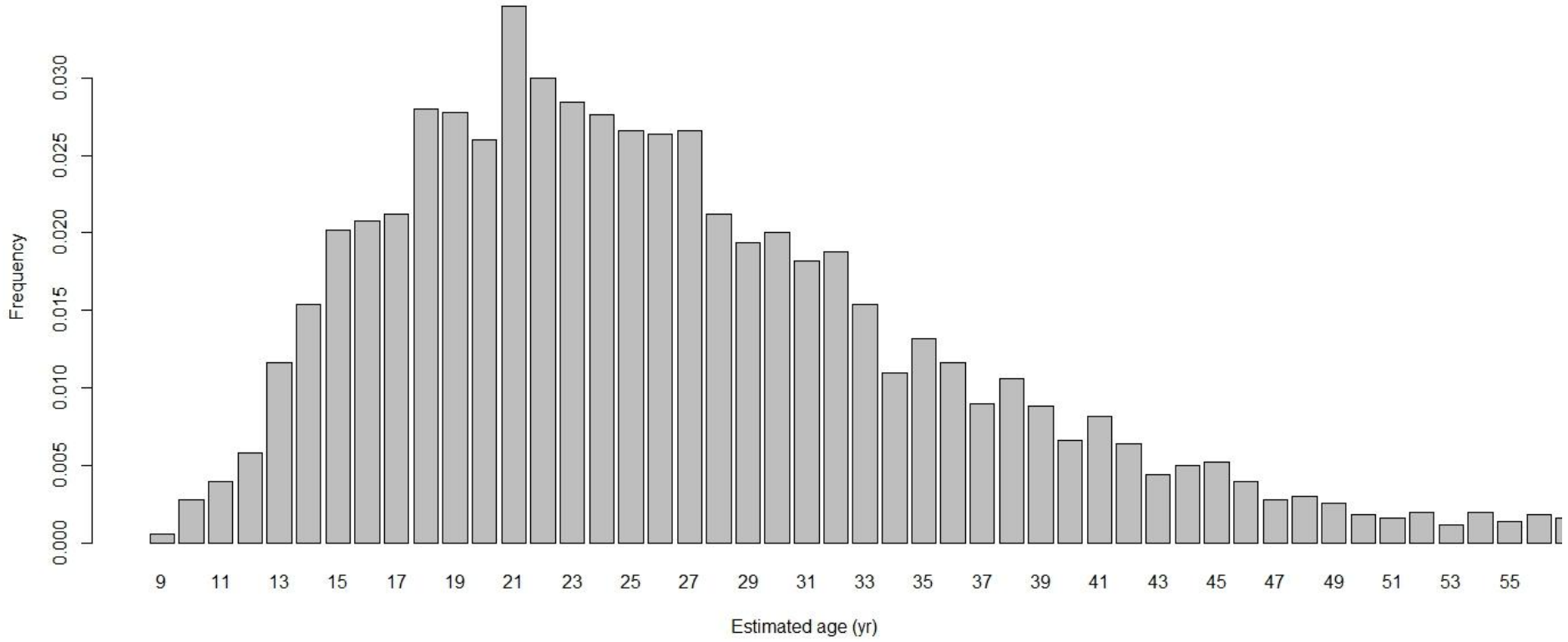
Frequency
0.000
0.005
0.010
0.015
0.020
0.025
0.030
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
51
53
55
Estimated age (yr)

Fig. 6.

CAPÍTULO 3

Life history of hawksbill sea turtles: Do they really use different habitats during ontogenetic development?

Luciana Medeiros & Leandro Bugoni

Artigo em construção. Redigido conforme as normas utilizadas nos capítulos anteriores para fins de padronização

Life history of hawksbill sea turtles: Do they really use different habitats during ontogenetic development?

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ABSTRACT: Despite its worldwide tropical and subtropical distribution, the life history of hawksbill sea turtles, *Eretmochelys imbricata*, is poorly studied in a global scale. In the present study, we combined stable isotope analysis with skeletochronology to assess cryptic lifestages and detect ontogenetic shifts in habitat and resource use of hawksbill turtles. Carbon isotope values did not differ throughout age and size classes. Moreover, no correlation was observed between size and δ^{13}C values. δ^{15}N values ranged from 5.42 to 18.30‰ (10.06‰ ± 2.06), and values at age classes 6, 7 and 8 were significantly higher than age 1. For body size, δ^{15}N values were higher in the 40−49.9 and 50−59.9 cm size classes in comparison to smaller size classes. There was a positive relationship between δ^{15}N and curved carapace length, but no correlation was found between nitrogen isotope values and growth rates. These data differ from the long standing

paradigm that when hawksbills recruit to neritic habitats, they switch from pelagic to benthic feeding, and from an omnivorous to a spongivorous diet. Instead, stable isotope analyses were unable to detect any ontogenetic shift in resource and habitat use. The high variability in data suggests that hawksbills are long-term specialists within a generalist population, with an increase in trophic niche width after 7 years old.

KEY WORDS: Stable isotopes, Skeletochronology, Cryptic lifestages, Intraspecific variability, Brazil, *Eretmochelys imbricata*

RUNNING HEAD: Ontogenetic shifts in hawksbill turtles

INTRODUCTION

One of the biggest mysteries on the life history of sea turtles is their first years of life, which occur in unknown or inaccessible locations. Where, how and for how long this cryptic lifestages occur still remain a special challenge for herpetologists. In the North Atlantic, post-hatchlings of loggerhead, *Caretta caretta*, green, *Chelonia mydas*, hawksbill, *Eretmochelys imbricata*, and kemp's ridley, *Lepidochelys kempii*, sea turtles are known to inhabit floating rafts of *Sargassum*, displaying an opportunistic feeding behavior (Witherington et al. 2012). In the South Atlantic Ocean, however, this phase is completely unknown and there is no record on where turtle's neonates remain in the first stages of their lives, as in this part of the Atlantic there are no massive and regular patches of floating algae. Recently, skeletochronology was coupled with stable isotope analysis (SIA) to access the ontogenetic shifts and early life stages of loggerhead sea

turtles (Snover et al. 2010, Avens et al. 2013), and this multiple methods approach proved to be a powerful tool to increase our understanding on the complex life cycle of sea turtles.

Over the past several decades, SIA has become a useful tool in ecological research, particularly for identifying dietary sources and trophic relationships (Mancini & Bugoni 2014), habitat use (Bjorndal & Bolten 2010, Pajuelo et al. 2012), migration patterns (Hobson 1999) and ontogenetic shifts (Arthur et al. 2008, Drago et al. 2009). SIA is a well suited method for such studies because the stable isotope values of carbon (δ^{13}C) and nitrogen (δ^{15}N) reflect a time-integrated diet, i.e. the stable isotope ratio of consumer tissues reflects the similar ratio of its prey items within a timescale determined by the metabolic traits of each tissue and the species analyzed (Peterson & Fry 1987, Fry 2006). Due to preferential elimination of the lighter isotopes, and therefore the selection of heavier isotopes (Fry 2006), measurements of δ^{15}N can be used to assess the trophic level of a consumer (DeNiro & Epstein 1981, Minagawa & Wada 1984) with a 3−5‰ increase in δ^{15}N values at each trophic level. δ^{13}C is often used to discriminate food sources by using the large variation in the isotopic carbon signatures in food webs due to photosynthetic baselines (Fry 2006). Given the differences in tissue composition, tissue selection is an important assumption for dietary reconstruction (Perkins et al. 2013). Bone collagen is largely composed of protein (Gannes et al. 1997) that turns over very slowly and has a long half-life, which, for medium- and large-sized animals, can reflect yrs or even decades (Dalerum & Angerbjörn 2005). Therefore, it is the most suitable tissue to identify long-term trends in animal dietary patterns or to investigate fundamental changes in food web structure over time (Dalerum & Angerbjörn 2005, Christensen & Richardson 2008).

On their turn, skeletochronology is the study of growth marks in bones of reptiles and amphibians (Castanet & Smirina 1990), which allow researchers to estimate age and growth rate and has the potential to provide information for a broad age class. This methodology has been extensively applied over the last decade in studies with sea turtles (Avens & Snover 2013), providing essential information on demographic settings, such as age structure, growth dynamics, rates of recruitment and age-at-sex maturity (Goshe et al. 2010, Avens et al. 2012, Petitet et al. 2012, Avens et al. 2013, Snover et al. 2013). Because bone tissues remain relatively inert after synthesis, the tissue retains the isotopic history of the animal, providing data on an annual scale, limited only by bone resorption at the bone core (Snover et al. 2010).

The hawksbill sea turtle (*Eretmochelys imbricata*) has a worldwide tropical and subtropical distribution and populations have been greatly reduced over the last three generations, due to a variety of human activities (Lutcavage et al. 1997, Meylan & Donelly 1999). Consequently, hawksbill turtles are currently classified as Critically Endangered in the IUCN Red List (IUCN 2014). Similar to other sea turtle species, hawksbill has complex life histories that encompass migratory behaviour, ontogenetic shifts and nesting site fidelity (Mortimer & Donnelly 2008, Meylan et al. 2011). As soon as they born, hawksbill hatchlings enter the sea and spend their first years in oceanic habitats. This initial life stage is called "lost years" because it is a poorly-known phase, estimated to last 1 to 3 yrs, when turtles are then supposed to recruit into neritic habitats at a size of 20–35 cm carapace length (Limpus 1992, Boulon 1994). After reaching at neritic habitats, hawksbills occupy coral reefs due to their preferentially spongivorous diet (León & Bjorndal 2002, Meylan et al. 2011), although also inhabit other benthic habitats such as seagrass beds (Bjorndal & Bolten 2010), rocky shores (Proietti et al. 2012), and mangrove bays (Gaos et al. 2012).

The Brazilian coastline is an important habitat for sea turtle populations (Marcovaldi & Marcovaldi 1999). Besides hosting the largest remaining population of nesting hawksbill turtles in the South Atlantic Ocean (Marcovaldi et al. 2007), Brazilian waters serve primarily as development habitats for early juveniles (Proietti et al. 2012). Most studies with hawksbill and other sea turtle species in this area used to focus on nesting females (Marcovaldi et al. 1999, 2007, Camillo et al. 2009, Santos et al. 2010, 2013), or addressed genetic issues (Lara-Ruiz et al. 2006, Vilaça et al. 2012, 2013, Proietti et al. 2014). Some of these studies showed that Brazilian rookeries and feeding aggregations are distinct demographic units (Vilaça et al. 2013, Proietti et al. 2014). Mixed stock analysis indicated that Brazilian feeding aggregations are mostly composed of animals originating from the domestic rookeries at Bahia and Pipa, in northeastern Brazil, with minor contributions from African and Caribbean rookeries (Proietti et al. 2014). Nonetheless, a limited number of studies had been carried out in foraging aggregations (Sanches & Bellini 1999, Proietti et al. 2012, Leite et al. 2013) and thus demographic information of hawksbill turtles is still scarce. In this study, SIA was linked to skeletochronological analysis to assess the temporal consistency in resource and habitat use, to detect ontogenetic shifts and to provide the first insights into the "lost years" of hawksbill turtles in the western South Atlantic Ocean.

MATERIALS AND METHODS

Sample collection and processing

Humeri samples were obtained from stranded dead hawksbill turtles in nesting and foraging grounds along the Brazilian coast (Fig. 1). Juveniles were collected at all sampling sites, while subadults and adults at nesting grounds in Rio Grande do Norte,

Bahia, Alagoas and Espírito Santo states. For each stranded turtle, Curved Carapace Length (CCL) was measured with a flexible metric tape (± 0.1 cm), from nuchal notch-to-the-posterior end to the posterior marginal scute (Bolten 1999). Because sex could not be determined in most individuals by gonad examination due to decomposition, data analysis was based on both sexes pooled.

Each humerus was dissected and immersed in water for 2–3 weeks for complete removal of soft tissues, and then air-dried for 2 wk. After cleaning, the medial width was measured prior to removing cross-sections for histological processing (Zug et al. 1986). Humeri were cut in two portions with different thicknesses, from the distal end of the insertion scar of delto-pectoral muscle. First, a 1 mm cross section was taken and saved unpreserved for stable isotope analysis. Then, for skeletochronological analysis, a 2−3 mm cross section was cut adjacent to the sectioning location. The methodology of Avens & Goshe (2007) was used to prepare humeri for skeletochronological analysis. Bayesian models, as in Petitet et al. (2012) were fitted to estimate age and growth rates. The model structures and results from those estimates are detailed in Chapter 2.

Stable isotope analysis

Because lipid content of a tissue is a potential confounding factor in SIA (Post et al. 2007, Chapter 1), humerus sections collected for SIA had lipids extracted by a 2:1 chloroform:methanol solution with a Soxhlet apparatus for 4 h. After that, bone sections were rinsed with distilled water and oven-dried. In order to find the growth marks on humerus sections collected for SIA, the stained thin sections and growth layer diameters were used as a guide. Growth marks were collected with a manual electric drill (Dremel®) with 300 µm thick and immediately packed into 4 x 6 mm sterilized silver capsules (Fig. 2). In some cases, individual lines were hard to remove because they

were too close to each other (at a distance smaller than 300 μm), resulting in low amount of material (<0.3 mg). Thus, some samples could be a result of more than one line. As the drill pulverizes growth lines at the time of collecting, bone section was washed with distilled water and dried at every sampling of a line or group of lines to avoid contamination. Sea turtle humeri contain carbonates that could potentially alter $\delta^{13}C$ values (Biasatti 2004) and it is a common procedure to acidify samples before analysis (Jacob et al. 2005). Because tin capsules degrade rapidly in the presence of acid, silver capsules were used instead, given their larger acid resistance (Bosley & Wainright 1999). Finally, all samples of potential food sources had carbonates removed by demineralization with 10% HCl using the "drop-by-drop" technique (Jacob et al. 2005) until no gas bubbles were produced and then oven-dried.

All samples were analyzed using a continuous-flow isotope-ratio mass spectrometer in the Laboratory of Analytical Chemistry, University of Georgia (USA). Stable isotope values are expressed in δ-notation as parts per thousand (‰) differences from the international standard material, Vienna Pee Dee Belemnite limestone and atmospheric nitrogen (Air) for carbon and nitrogen, respectively, according to the following equation (as in Bond & Hobson 2012):

$$\delta X = (R_{sample}/R_{standard})-1 \quad (1)$$

where X is the ^{13}C or ^{15}N value, and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson & Fry 1987). Two laboratory standards were analyzed for every 12 unknown samples. The measurement precision of both $\delta^{13}C$ and $\delta^{15}N$ analysis was 0.2‰.

Statistical analysis

For stable isotope data, Shapiro-Wilk's test was applied to assess normality, and Bartlett's test was used to verify the homogeneity of variance between groups. Because these requirements were not fulfilled, Kruskal-Wallis non-parametric test was conducted, separately for $\delta^{13}C$ and $\delta^{15}N$ values, followed by pairwise comparisons using Wilcoxon rank sum test with Bonferroni adjustment when significant differences were found. It was tested for distinction in isotopic values between ages and between size classes. Spearman correlation was also calculated to assess the relationship between size classes and growth rates with $\delta^{13}C$ and $\delta^{15}N$ values.

RESULTS

Our sample contained 49 hawksbill turtles washed ashore in nesting and foraging grounds along the Brazilian coast. The carapace length and age estimates for Brazilian hawksbill turtles ranged from 26.0 to 111.0 cm and 2–26 yr, respectively (Chapter 2), and mean carapace length back-calculated for the first yr old was 23.41 ± 3.88 cm of CCL (range 15.85–32.11 cm).

$\delta^{13}C$ values in hawksbill sea turtle bones ranged from -21.08 to -12.95‰ (-16.26‰ ± 1.14, Table 1, Fig. 3). Carbon isotope values did not differ throughout age (H_{11} = 5.12, p = 0.92) and size (H_8 = 15.89, p = 0.04) classes. Although the p value indicates significant difference between $\delta^{13}C$ and size classes, the Wilcoxon test indicates no difference between groups, probably due to the Bonferroni adjustment for multiple comparisons. Moreover, no correlation was observed between size and $\delta^{13}C$ values (r_s = 0.15, p = 0.08). $\delta^{15}N$ values ranged from 5.42 to 18.30‰ (10.06‰ ± 2.06, Table 1, Fig. 4). By contrast, Kruskal-Wallis test showed differences in $\delta^{15}N$ values between age (H_{11} = 35.12, p < 0.01) and size classes (H_8 = 35.40, p < 0.01). The nitrogen isotope values of age 6, 7 and 8 were significantly higher than age 1. In

relation to body size, δ^{15}N values of 40–49.9 and 50–59.9 cm size classes were higher than 20–29.9 cm size class. Additionally, the 40–49.9 cm size class was significantly higher than 30–39.9 cm size class. Furthermore, there was a positive relationship between δ^{15}N and CCL ($r_s = 0.39$, $p < 0.01$). No correlation was found between isotope values and growth rates (δ^{13}C: $r_s = 0.07$, $p = 0.48$; δ^{15}N: $r_s = 0.01$, $p = 0.90$; Fig. 5). High variability in stable isotopes of individual growth marks were found (Fig. 6)

DISCUSSION

This seems to be the first study to assess the temporal consistency of foraging ecology and habitat use in successive life stages of hawksbill turtles. Moreover, results presented here provide the first insights on the "lost years" of hawksbill sea turtles from southwestern Atlantic Ocean.

Because individuals acquire the isotopic composition of their foraging environments, the isotopic composition of consumer's tissue can be used to verify their movements through isotopically distinct habitats (Graham et al. 2010). In our study, carbon isotope values in humeri growth marks of turtles showed no significant difference between age and size classes. Moreover, δ^{13}C values and carapace length were not correlated, suggesting that the species does not undergo marked change in habitat, or alternatively, commute between habitats with similar isotopic signatures. Isoscapes are geographical distribution patterns of isotopic values in the environment, on temporal and spatial scales, that are an ecological proxy for animal movements (Graham et al. 2010). Based on this concept, a marine consumer is regarded as resident when the individual has similar isotopic values compared to local isotopic baseline, whereas distinct values indicate an immigrant consumer. Isoscape profiles were proposed for Atlantic Ocean based on meta-analysis of published isotopic values of

zooplankton (McMahon et al. 2013). Nevertheless, current marine isoscapes were not useful in this study to infer sea turtle movements, apparently because baseline signatures are coarse, while foraging areas of hawksbill turtles in the area (Bjorndal & Bolten 2010, Berube et al. 2012, Proietti et al. 2012) seems to be isotopically similar (Graham et al. 2010, McMahon et al. 2013).

In addition, similar to other sea turtle species, hawksbills have complex life histories that encompass migratory behaviour, ontogenetic shifts and nesting site fidelity (Mortimer & Donnelly 2008, Meylan et al. 2011). It has been suggested that hawksbills in the western Atlantic Ocean recruit to neritic habitats when they reach 20–25 cm CCL, after living in pelagic environment for 1–3 years (Boulon 1994, Meylan et al. 2011). In this study, the data set consists mainly of early juveniles (n = 36 with CCL <40 cm), ranging in age from 2 to 6 yr. Apparently, based on δ^{13}C values, there is no ontogenetic change in habitat of hawksbill turtles from southwestern Atlantic Ocean during the first years, despite an increase in mean and in the variance of δ^{15}N values, which could be due to ingestion of larger prey as turtles get bigger.

Mangroves and seagrass beds play a key function as nursery of marine vertebrates (Nagelkerken et al. 2002, Dorenbosch et al. 2004). The densities of fishes from reefs in islands in the Caribbean with, as well as without, mangroves and seagrass beds were compared and it was concluded that in reefs of islands lacking these habitats, there is a complete absence or low densities of 11 out of the 17 species studied (Nagelkerken et al. 2002). The main known feeding areas for hawksbills in Brazil are the Archipelago of Fernando de Noronha (Sanches & Bellini 1999), the Saint Peter and Saint Paul Archipelago (SPSPA), the Abrolhos Marine National Park, and the Arvoredo Biological Reserve (Proietti et al. 2012). The size of captured turtles at these sites ranged from 30.5–75.5 cm at Fernando de Noronha, 24.5–63.0 cm CCL at Abrolhos

(mean = 37.9 cm), 30–75 cm at SPSPA (mean = 53.7 cm), and 30.0–59.5 cm (mean = 41.3 cm) at Arvoredo. An occasional report of hawksbill foraging aggregation was also made in Anchieta Island State Park, ranging in size from 30 to 79.5 cm CCL (mean = 46.0 cm, Leite et al. 2013). It is known that in some areas of the Abrolhos Archipelago there are patches of macroalgae associated with seagrass beds (*Halodule wrightii*) (Creed & Amado-Filho 1999, de Paula et al. 2003). The macroalgal community in the seagrass beds at Abrolhos is diverse and contains a large number of Phaeophyceae taxa. Sanches & Bellini (1999) demonstrated that the Baía do Sueste is the main foraging areas of hawksbill and green turtles in Fernando de Noronha Archipelago, possibly due to protected waters and the high abundance of macroalgae and seagrass meadows. Although there is no record to date, it is possible that hawksbill post-hatchlings are using seagrass beds and algae banks near the Brazilian islands as nursery and shelter sites, reducing mortality and benefiting from the availability of food resources. The carapace length back-calculated for the first yr old on (Chapter 2) also support this idea, because they are within the size range observed in Brazilian islands (Sanches & Bellini 1999, Proietti et al. 2012). Additionally, mixed stock analysis indicated that Brazilian feeding aggregations are mostly composed of animals originated from the domestic rookeries at Bahia and Rio Grande do Norte states, with minor contributions from African and Caribbean rookeries (Proietti et al. 2014).

In this study, the δ^{15}N values in growth marks exhibited high variability throughout time and among size classes, with a significant increase on δ^{15}N values between 6 and 8 yr old, when they reach 40 to 50 cm. These results could be an indicative of an ontogenetic shift, when hawksbills change to a more selective feeding behavior. However, caution is needed in interpreting these data. Given the high variability of δ^{15}N values, it seems that there is an increase in niche width with body

size. Intraspecific variability is an important ecological feature for the maintenance of species coexistence and the dynamics of communities because reduces intra and interspecific competition or predation (Bolnick et al. 2011). Co-occurring individuals have a more specialized behavior in shared habitats, selecting different food sources even among individuals of a given size and sex (Araújo et al. 2011). This 'individual specialization', i.e. when individuals use a small fraction of the population's resource base, have been recorded for several vertebrate and invertebrate taxa (Bolnick et al. 2003, Araújo et al. 2011), including sea turtles. It has been demonstrated that individual loggerhead sea turtles are long-term specialists within a generalist population (Vander-Zanden et al. 2010, 2013). Data presented here suggest that hawksbill turtles are also displaying individual specialization throughout their lives, but more detailed analyses are needed to properly address this subject (Fig. 6).

The nitrogen availability is regarded as an important factor promoting or constraining growth rate of animals (White 1978). However, no correlation was found between δ^{15}N values and growth rates, possibly due to the stochastic nature of environmental traits which depends on the spatial and temporal variability of resources, and hence reflects on variation of LAG deposition and compensatory growth (Bjorndal et al. 2003). It has been assumed that the slower growth rates observed in early juveniles is a response to stochastic environments and could lead to a compensatory growth, i.e. after a period of suboptimal environmental conditions with a reduced growth, turtles could undergone an accelerated growth when exposed to better conditions (Bjorndal et al. 2003). Additionally, differences in water temperature can alter metabolic rates (Bjorndal 1980) or cause changes in food availability (Carrillo et al. 1999), driving a pressure on limited resources (Bjorndal & Bolten 2010), and thereby influencing somatic growth.

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Table 1. Hawksbill sea turtle *Eretmochelys imbricata*. Stable isotope values of size classes. Values are reported as mean ± standard deviation (min-max). Growth rates were divided into size classes based on the mean CCL of the back-calculated CCL pairs

Size class (cm)	$\delta^{13}C$	$\delta^{15}N$	n
<20.0	-15.89 ± 0.71 (-16.44 – -14.69)	8.48 ± 1.18 (6.77 – 9.83)	5
20.0−29.9	-16.50 ± 1.20 (-21.18 – -15.08)	9.19 ± 1.10 (6.95 – 11.82)	46
30.0−39.9	-16.13 ± 0.82 (-18.07 – -14.46)	10.12 ± 2.18 (7.48 – 18.21)	45
40.0−49.9	-16.68 ± 1.16 (-19.37 – -15.35)	11.68 ± 1.23 (9.44 – 14.78)	15
50.0−59.9	-15.71 ± 1.33 (-17.82 – -12.98)	11.18 ± 1.20 (9.35 – 13.23)	8
60.0−69.9	-15.90 ± 1.90 (-18.04 – -12.95)	9.20 ± 2.65 (2.65 – 5.42)	5
70.0−79.9	-15.26 ± 1.30 (-16.43 – -13.11)	10.52 ± 2.35 (7.02 – 13.48)	5
80.0−89.9	-16.87 ± 1.41 (-19 – -15.35)	12.50 ± 4.34 (8.17 – 18.30)	5
>90.0	-15.16 ± 0.06 (-15.20 – -15.10)	9.25 ± 1.01 (8.35 – 10.34)	3

FIGURE CAPTIONS

Fig. 1. Approximate location of sampling sites along Brazilian coast: RS − Rio Grande do Sul State (n = 7), PR − Paraná State (n = 4), RJ − Rio de Janeiro State (n = 3), ES − Espírito Santo State (n = 20), BA − Bahia State (n = 11), AL − Alagoas State (n = 3), RN − Rio Grande do Norte State (n = 2). Dashed lines represent the stretch of beach monitored searching for stranded turtles. Numbers represent known developmental areas of early juveniles of hawksbill turtles: (1) Saint Peter and Saint Paul Archipelago (SPSPA), (2) Fernando de Noronha Archipelago, (3) Abrolhos Marine National Park, (4) Arvoredo Biological Reserve

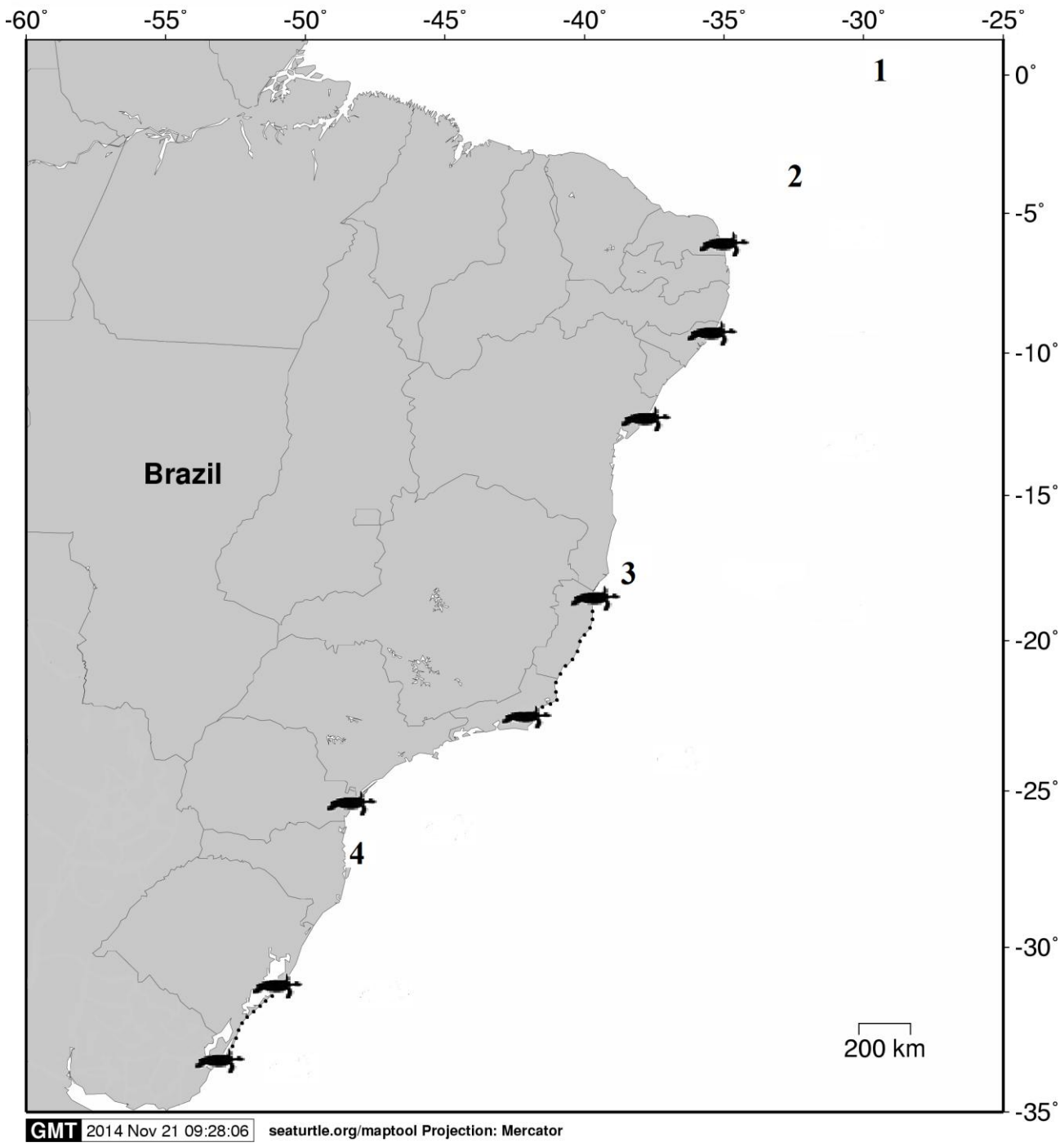
Fig. 2. Hawksbill turtle *Eretmochelys imbricata*. Humerus cross section with the LAGs marked with yellow dotted lines depicting their locations. The black dots indicate the active growth zone where LAG samples were taken with drill.

Fig. 3. Hawksbill turtle *Eretmochelys imbricata*. Boxplots showing δ^{13}C values of (A) age classes and (B) size classes. Gray box shows interquartile range, solid black line shows median value, and the vertical lines are the maximum and minimum values

Fig. 4. Hawksbill turtle *Eretmochelys imbricata*. Boxplots showing δ^{15}N values of (A) age classes and (B) size classes. Gray box shows interquartile range, solid black line shows median value, and the vertical lines are the maximum and minimum values

Fig. 5. Hawksbill sea turtle *Eretmochelys imbricata*. Correlation between growth rates (cm/yr) and (A) δ^{13}C values and (B) δ^{15}N values (n = 101)

Fig. 6. Hawksbill sea turtle *Eretmochelys imbricata*. Isotopic profiles of selected individuals of our sample (n = 15)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-35˚
-30˚
-25˚
0˚
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Fig. 1.

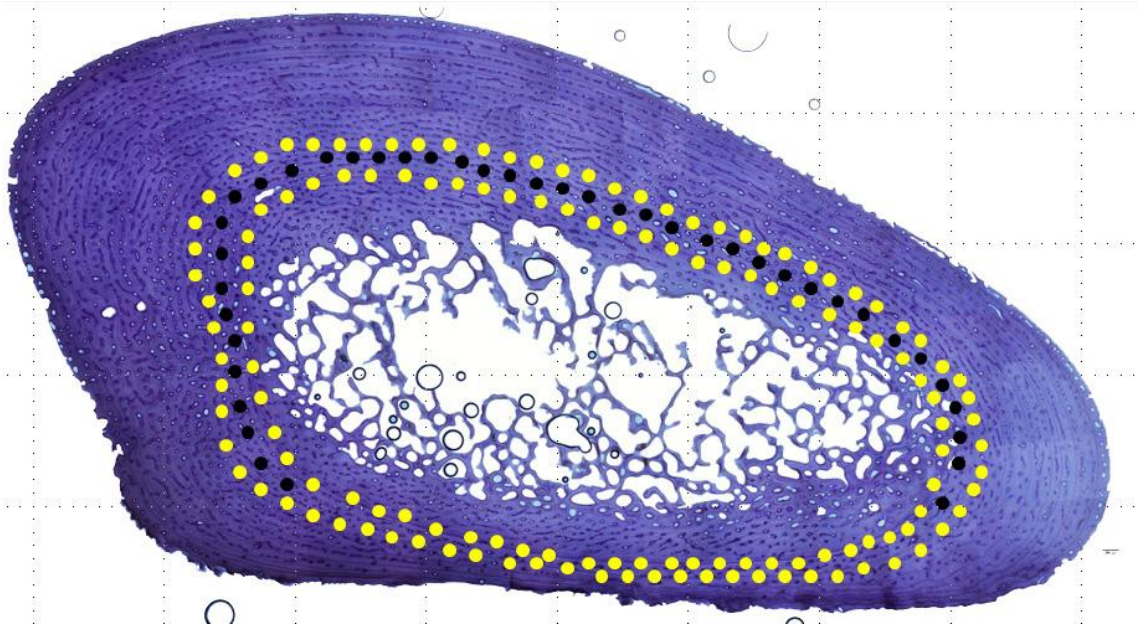
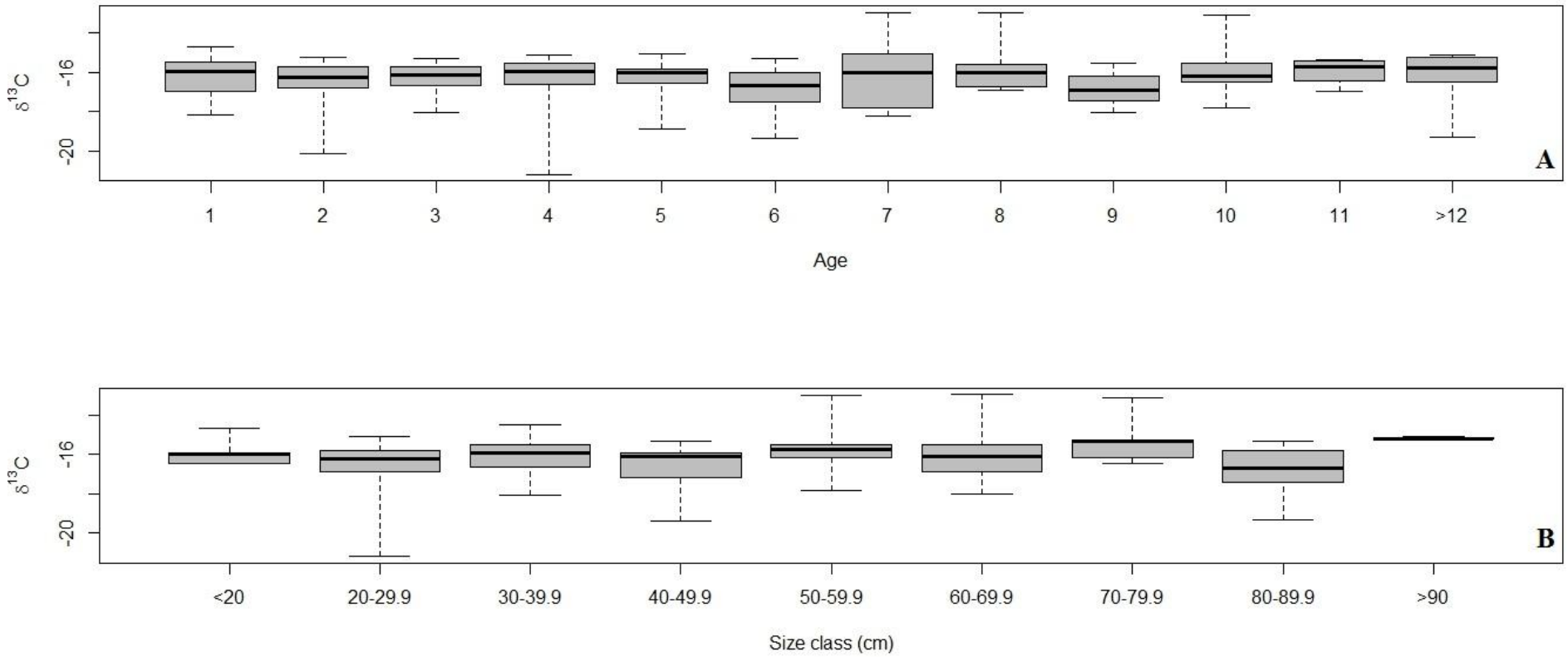


Fig. 2.


δ13C
-16
-20
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10
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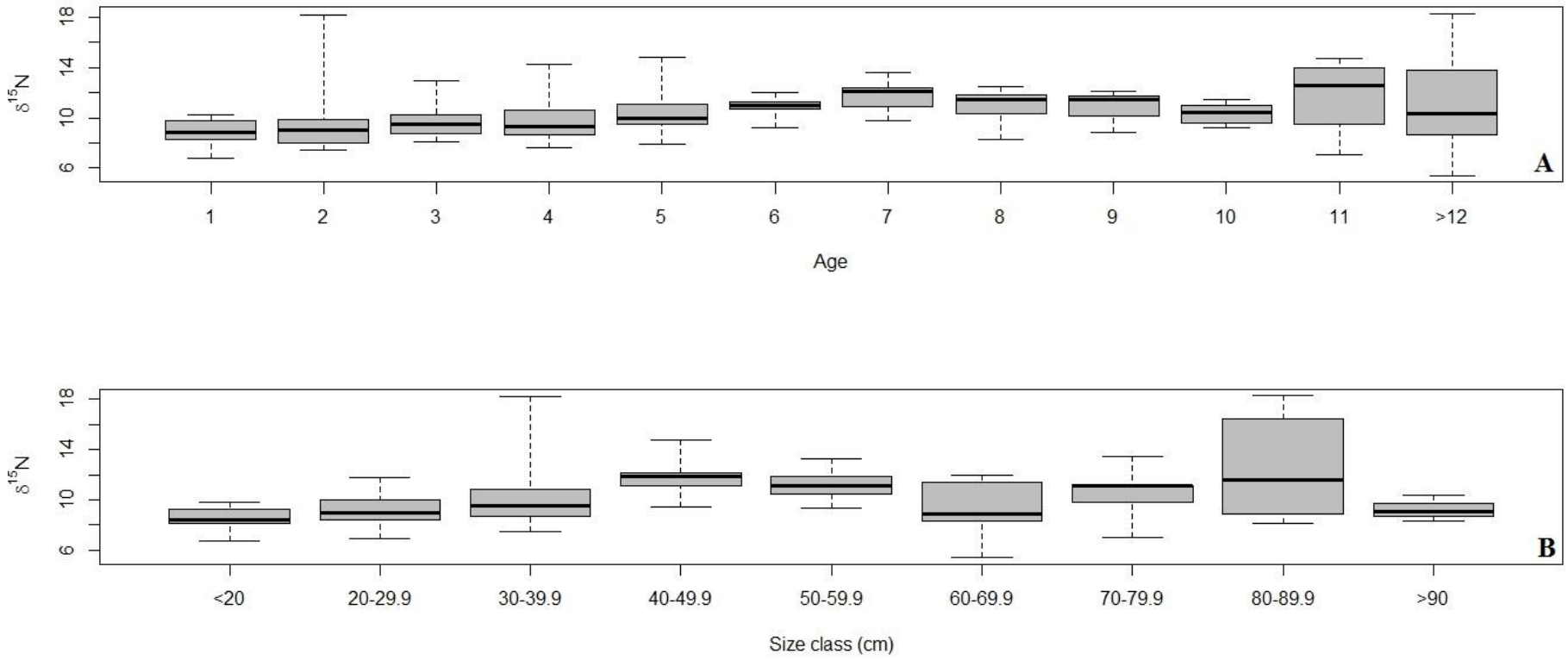
Age
δ13C
-16
-20
B
<20
20-29.9
30-39.9
40-49.9
50-59.9
60-69.9
70-79.9
80-89.9
>90
Size class (cm)

Fig. 3.


δ15N
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Age
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<20
20-29.9
30-39.9
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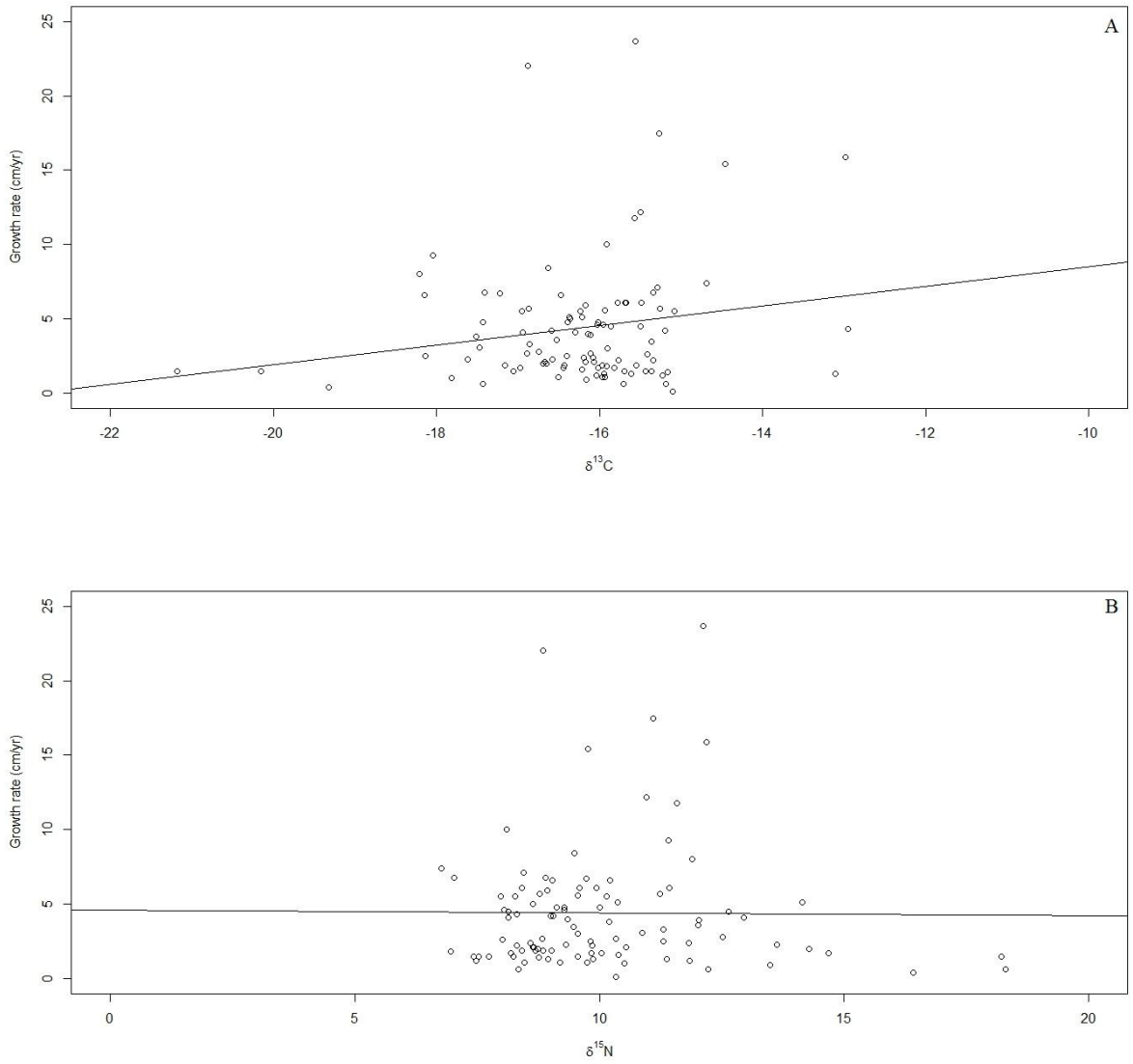
50-59.9
60-69.9
70-79.9
80-89.9
>90
Size class (cm)

Fig. 4.


A
Growth rate (cm/yr)
0
5
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B
Growth rate (cm/yr)
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δ15N

Fig. 5.

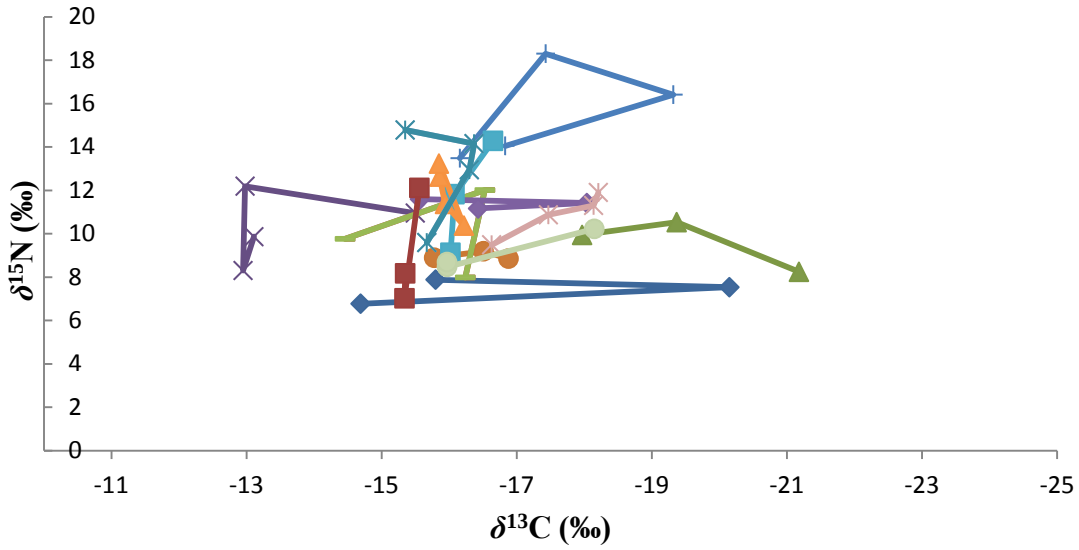

Fig. 6.