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**VARIAÇÃO ONTOGENÉTICA E TEMPORAL NA
ECOLOGIA TRÓFICA DA FOCA-CARANGUEJEIRA,
Lobodon carcinophaga, NO LESTE DA PENÍNSULA
ANTÁRTICA**

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*“Loco es aquel que haciendo siempre lo mismo,
espera resultados distintos”
–Albert Einstein–*

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RESUMO

Como predador especializado em krill-antártico (*Euphausia superba*), a foca-caranguejeira (*Lobodon carcinophaga*) é potencialmente vulnerável aos efeitos das alterações climáticas de longo prazo e à sobrepesca da sua principal presa. Assim, o objetivo deste trabalho foi caracterizar a ecologia trófica das focas-caranguejeiras e sua potencial variação temporal no leste da Península Antártica (LPA). Dentes e bigodes de focas mumificadas encontradas na região da Ilha Marambio/Seymour no LPA foram utilizados para estimar as idades (dentes) e para análises de isótopos estáveis de carbono e nitrogênio (dentes e bigodes). Os dentes caninos e pós-caninos foram processados utilizando técnicas convencionais para estimativa de idades de pinípedes, com o objetivo de comparar as estruturas e determinar qual é a mais indicada para a foca-caranguejeira. A estimativa da idade dos exemplares contando os grupos de camadas de crescimento no cemento dos dentes pós-caninos foi considerada a mais adequada. Subsequentemente, foi analisada a composição isotópica individual ($\delta^{13}\text{C}$ e $\delta^{15}\text{N}$) das GLG dos caninos das focas mumificadas (i.e. focas históricas) e dos bigodes de 5 destes exemplares. Adicionalmente os bigodes de 6 focas amostradas vivas (i.e. focas atuais) na região foram analisados para avaliar diferenças temporais. Os valores de $\delta^{13}\text{C}$ não mostraram diferença estatística entre as focas históricas e atuais, porém, os valores de $\delta^{15}\text{N}$ foram mais altos nestas últimas. A maior área de nicho nas focas atuais em comparação com as mumificadas poderia estar relacionada à incorporação de presas de maior nível trófico em indivíduos atuais, assim como variações potenciais na linha de base das cadeias alimentares modernas. Elevados valores de $\delta^{15}\text{N}$ e mais baixos de $\delta^{13}\text{C}$ encontrados nos filhotes (GLG1) estariam associados ao período de lactação assim como ao catabolismo de tecidos durante o período de jejum pós-desmame. Após este primeiro ano, os valores isotópicos de carbono e de nitrogênio seguem trajetórias de aumento e diminuição, respectivamente, até as GLGs 5-6, consideradas como a idade de maturidade sexual nesta espécie. Estas tendências podem ser explicadas tanto por diferenças no padrão de forrageio destes indivíduos mais jovens como pelo efeito da taxa de crescimento elevada sobre os valores de discriminação isotópica entre as presas e os tecidos do predador, esta última afetando principalmente os valores de $\delta^{15}\text{N}$. Os resultados deste trabalho mostraram que, apesar da região do LPA ser considerada menos afetada pelas mudanças climáticas globais, alterações no padrão de forrageio de

espécies residentes podem estar indicando possíveis câmbios nos ecossistemas marinhos adjacentes que merecem ser avaliados com mais detalhamento em estudos futuros. Adicionalmente, foram evidenciadas mudanças ontogenéticas nos perfis isotópicos destes predadores possivelmente associados a fatores tróficos e/ou fisiológicos.

Palavras-chave: Dentes, estimativa de idade, focídeos, isótopos estáveis, Mar de Weddell, vibrissas.

ABSTRACT

Temporal and ontogenetic variation in the trophic ecology of crabeater seals (*Lobodon carcinophaga*) from the east Antarctic Peninsula

As a specialized predator in Antarctic krill (*Euphausia superba*), the crabeater seal (*Lobodon carcinophaga*) is potentially vulnerable to the effects of long-term climate change and overfishing of its main prey. Thus, the objective of this work was to characterize the trophic ecology of crabeater seals and their potential temporal variation in the eastern Antarctic Peninsula (LPA). Teeth and whiskers of mummified seals found in the Marambio / Seymour Island region in the LPA were used to estimate ages (teeth) and for stable carbon and nitrogen isotope analyses (teeth and whiskers). Canine and post-canine teeth were processed using conventional pinniped age estimation techniques, with the purpose of comparing the structures and determining which is the most suitable for the crabeater seal. The estimation of the age of the specimens counting the growth layer groups in the cementum of the post-canine teeth was considered the most appropriate. Subsequently, the individual isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of the canine GLGs of the mummified seals (i.e. historical seals) and the whiskers of 5 of these specimens was analysed. Additionally, whisker samples from 6 alive seals (i.e. recent seals) in the region were analysed to assess temporal differences. The values of $\delta^{13}\text{C}$ showed no statistical differences between historical and recent seals, however, the values of $\delta^{15}\text{N}$ were higher in the latter. The larger niche area in recent seals compared to historic seals could be related to the incorporation of higher trophic prey in recent individuals, as well as potential variations in the baseline of modern food chains. High $\delta^{15}\text{N}$ and lower $\delta^{13}\text{C}$ values found in yearlings (GLG1) could be associated with lactation as well as tissue catabolism during the post-weaning fast period. After this first year, isotopic carbon and nitrogen values follow trajectories of increase and decrease, respectively, up to GLGs 5-6, considered as the age of sexual maturity in this species. These trends can be explained either by differences in the foraging pattern of these younger individuals or by the effect of high growth rate on isotopic discrimination values between prey and predator tissues, the latter mainly affecting $\delta^{15}\text{N}$ values. The results of this study showed that, although the LPA region is considered less affected by global climate change, differences in the foraging pattern of resident species may be indicating possible changes in adjacent marine ecosystems that deserve further

evaluation in future studies. Additionally, ontogenetic changes were observed in the isotopic profiles of these predators possibly associated with trophic and / or physiological factors.

Keywords: age estimation, phocids, stable isotope analysis, teeth, Weddell Sea, whiskers.

1. INTRODUÇÃO GERAL

O continente Antártico está rodeado pela Corrente Circumpolar Antártica (CCA) e isolado pelas massas de água que formam o oceano Austral (Moline 2008). Esta região caracteriza-se pela elevada concentração de nutrientes, assim como por uma alta biomassa de fitoplâncton, que tornam esta região uma área altamente produtiva (Mendes et al. 2012). Neste ambiente os crustáceos e as salpas são um componente importante do macrozooplâncton, sendo os crustáceos o principal item da dieta de alguns predadores de topo desta teia trófica. Nesta área concentra-se a maior densidade do krill-antártico (*Euphausia superba*), sendo uma espécie chave dentro das, relativamente curtas, cadeias tróficas antárticas (Laws 1985). Esta espécie está associada aos bancos de gelo (banquisas) os quais servem de habitat de refúgio e desova assim como substrato no qual se aderem as microalgas consumidas pelo krill (Flores et al. 2012, Moline 2008) que, por sua vez, sustenta as espécies de níveis tróficos superiores como pinguins, skuas, focas e baleias (Atkinson et al. 2004).

A Península Antártica tem experimentado oscilações climáticas desde 1950 (Oliva et al. 2017). Embora, a Península Antártica Ocidental (PAO) apresente um rápido aquecimento, redução do gelo marinho e dos glaciais (Ducklow et al. 2007, 2012, Moline 2008), a costa leste da Península Antártica (LPE) apresenta-se mais estável, com menos oscilações de temperatura. Dentre as possíveis causas, hipotetizou-se que as montanhas da Península Antártica poderiam funcionar como uma barreira às diferenças climáticas entre as costas oeste e leste (Skvarka et al. 1998, Siegert et al. 2019). Assim, estas oscilações ambientais no gelo podem também ter consequências para a disponibilidade de krill, e afetar principalmente aqueles predadores que são especialistas no consumo deste recurso (Bengtson 2009, Polito et al. 2011, Hückstädt et al. 2012, Botta et al. 2018).

Além das oscilações ambientais, a disponibilidade do krill-antártico tem sido condicionada por atividades antrópicas como a caça de baleias que começou no século 19. Embora ainda hoje não seja bem compreendido o efeito da remoção de milhões de toneladas deste cetáceo (Estes et al. 2006), hipotetizou-se que esta redução nas populações de baleias teria levado a um aumento na disponibilidade do krill, permitindo que outras espécies consumidoras deste recurso incrementem suas populações (Estes et al. 2007, Southwell et al. 2008). Por outro lado, a pescaria de krill vem aumentando de

forma gradual desde os anos 1960s podendo ser também um fator que afete o recrutamento deste importante recurso e, em consequência, a fauna que depende dele (Nicol and Foster 2003). Como consequência das mudanças climáticas e efeitos antrópicos, os predadores de topo poderiam reagir de formas diferentes: modificar a sua distribuição, comportamento de forrageio, taxas de crescimento, sucesso reprodutivo e migração. Além disso, a sobrevivência dos indivíduos poderia ver-se afetada em decorrência destas mudanças (Walther et al. 2002, Polito et al. 2011). Particularmente espécies dependentes do gelo (e.g. foca-de-Weddell, *Leptonichotes weddelli*; foca-caranguejeira, *Lobodon carcinophaga*), poderiam sofrer efeitos negativos se as mudanças na extensão do gelo marinho persistirem (Siniff et al. 2008).

A foca-caranguejeira (Fig. 1) é o pinípede mais abundante do continente antártico, são endêmicas dessas águas e distribuem-se ao longo do continente todo. Sua população é estimada em 10-15 milhões de indivíduos (Southwell et al. 2008, Bengston 2009, Bengston et al. 2011). Esta espécie é considerada uma espécie dependente dos bancos-de-gelo (Southwell et al. 2003) pois habita áreas com altas concentrações de gelo da plataforma continental e águas profundas (McMahon et al. 2002, Wall et al. 2007). Porém, Wall et al. (2007) e Nachtsheim et al. (2017) mostraram que não apenas utilizam os bancos de gelo, mas também áreas mais oceânicas, até as águas da quebra de plataforma, e podem frequentar as costas principalmente na época pós-reprodutiva (Laws et al. 2003).

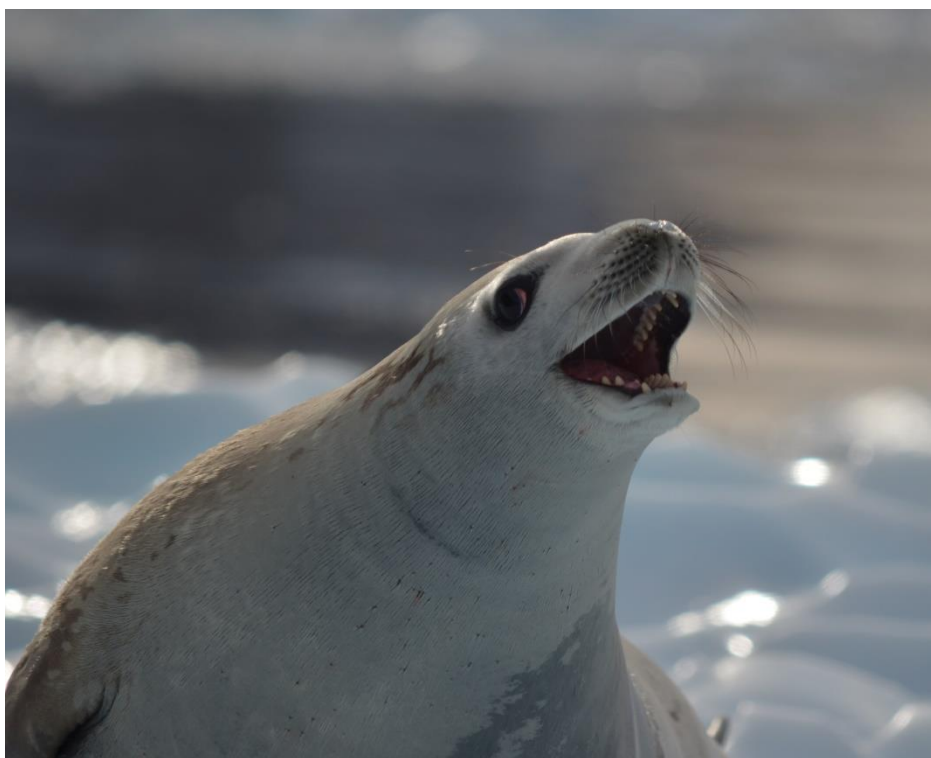


Figura 1. Exemplar de foca-caranguejeira (*Lobodon carcinophaga*) encontrada na Costa Danco, Antártida. Foto: Pablo Moscoso-IAA

O período reprodutivo começa no final da primavera (setembro) e continua até novembro (Laws 1984) e, como espécie dependente de gelo, estas focas reproduzem-se no gelo flutuante. No entanto, na etapa pós-reprodutiva, esta espécie pode passar um tempo considerável em águas abertas (Wall et al. 2007). O período de lactação das focas-caranguejeiras é variável, mas estima-se que pode durar entre 3 e 6 semanas (Siniff et al. 1979, Laws 1985).

Através de diferentes análises (por exemplo, o conteúdo de fezes e estômago) diversos autores confirmaram que a dieta das focas-caranguejeiras é dominada principalmente pelo krill-antártico em áreas pelágicas e do talude, e pelo krill-de-cristal (*E. crystallorophias*) em áreas neríticas (Laws 1981, Southwell et al. 2003). O consumo anual é estimado em aproximadamente 60 a 70 milhões de toneladas de krill (Wall et al. 2007). Lulas e peixes complementam a sua dieta, sendo consumidos em menor proporção (Zhao et al. 2004, Bengston 2009, Huckstadt et al. 2012, Botta et al. 2018). Por ter uma dieta especialista, as focas-caranguejeiras são vulneráveis às variações na disponibilidade de krill (Hückstädt et al. 2012) podendo resultar em modificações nas proporções do krill consumido respeito de outros itens alternativos. Análises de isótopos

estáveis (AIE) em focas-caranguejeiras subfósseis do Mar de Ross e PAO, por exemplo, mostraram que houve uma mudança na alimentação das focas nos últimos 700 anos. Estas focas, além de apresentar um nível trófico maior, consumiriam maiores proporções de presas de níveis tróficos mais altos do que as focas atuais, sendo que esta mudança poderia ter acontecido aproximadamente 200 anos atrás (Brault 2017). Assim, as mudanças nas proporções de krill na dieta desta espécie podem ser usadas como um indicador da disponibilidade deste recurso no meio ambiente.

O habitat preferencial da espécie está estreitamente associado à distribuição do krill, que é abundante nos primeiros 2 m embaixo do gelo e nas águas superficiais do oceano aberto principalmente durante o verão (Flores et al. 2012). As focas mergulham na coluna d'água geralmente até os primeiros 50 m de profundidade coincidindo com a maior abundância do krill (Croxall et al. 1985, Nachtsheim et al. 2017). Os mergulhos são mais frequentes à noite, coincidindo com as migrações verticais desta presa (Nachtsheim et al. 2017). Indivíduos juvenis/sub-adultos, por outro lado, possuem uma capacidade de mergulho menor que os adultos (Burns 1999), o que poderia levar a uma diferenciação no habitat de forrageio, sendo mais restrito a regiões costeiras nos primeiros e mais variáveis nos segundos (Aubail et al. 2011, Brault 2017).

AIE tem sido um complemento às técnicas tradicionalmente utilizadas para estudos de dieta tais como as análises de conteúdo estomacal e fecal e de regurgito (Green e Williams 1986, Lowry et al. 1988, Bowen e Iverson 2013). Estas técnicas tradicionais possuem como desvantagens a sub ou sobre- representação de presas de rápida ou lenta digestão, respectivamente, além de geralmente oferecerem uma visão de curto prazo do espectro trófico (Pierce et al. 2004), sobre tudo quando se tem pouca representatividade temporal de amostras. Assim, a AIE tem sido aplicada como ferramenta para avaliar a dieta dos animais, permitindo também investigar o uso do habitat, movimentos e fisiologia dos consumidores (Newsome et al. 2010). Estas análises estão baseadas principalmente na medição dos isótopos estáveis de alguns elementos, tipicamente carbono e nitrogênio, pois as composições isotópicas dos consumidores estão relacionadas com aquelas das suas presas (Vander Zanden e Rasmussen 2001). Os valores de $\delta^{15}\text{N}$ permitem estimar o nível trófico dos consumidores, pois existe uma discriminação do isótopo mais leve (^{14}N) durante a assimilação das presas, sendo preferencialmente excretados, ficando o predador com

valores de $\delta^{15}\text{N}$ enriquecidos no isótopo mais pesado (^{15}N). Os isótopos mais leves do carbono (^{12}C) também sofrem discriminação através de níveis tróficos, porém esta é menos acentuada. Desta forma, os valores de $\delta^{13}\text{C}$ são geralmente utilizados para inferir as fontes de carbono da base das redes tróficas onde ocorreu o forrageio (Peterson e Fry 1987). Fontes nutritivas, composição dos produtores primários (C3 e C4) e características oceanográficas regionais resultam em diferentes padrões isotópicos entre regiões, permitindo investigar e compreender o uso do habitat e padrões migratórios (Graham et al. 2010, McMahon et al. 2013). A possibilidade de obter informação sobre o ambiente de forrageio e sobre os recursos utilizados permite, ainda, utilizar estes valores como eixos que delimitam o “nicho isotópico”, o qual é utilizado como um *proxy* para o nicho ecológico (Bearhop et al. 2004, Newsome et al. 2007).

Devido aos tecidos de um consumidor serem compostos por diferentes macromoléculas, e possuírem taxas de renovação distintas, a medição da composição de isótopos estáveis nestes pode refletir informações da dieta em diferentes escalas temporais: curto prazo (por exemplo, sangue), intermediário (por exemplo, bigodes) e longo prazo (por exemplo, ossos) (Hobson e Clark 1992, Hobson 1993, Newsome et al. 2010). Por outro lado, tecidos inertes, como dentes e bigodes, são comumente usados em estudos históricos (dentes e bigodes) e paleontológicos (dentes), pois oferecem a vantagem de reconstruir eventos de alimentação de longo prazo (Newsome et al. 2010, Bocherens 2015, Brault et al. 2017, Hückstädt et al. 2017), podendo evidenciar mudanças históricas na dieta das espécies (Bocherens 2015, Hückstädt et al. 2017).

Vários trabalhos já foram realizados utilizando bigodes de pinípedes para avaliar mudanças de longo prazo na ecologia trófica dos indivíduos (Cherel et al. 2009, Kernaléguen et al. 2012). No entanto, o tempo integrado pelos bigodes cobre apenas alguns anos ou menos (Cherel et al. 2009). Por outro lado, os dentes dos pinípedes, compostos por dentina, esmalte e cemento, (McCann 1993), podem incorporar informações da dieta e do uso do habitat dos animais que refletem toda sua vida (Newsome et al. 2006, 2009, Martin et al. 2011, Albernaz et al. 2017), devido à característica monofiodôncia do grupo. A deposição sequencial de dentina e cemento nos dentes de pinípedes, é refletida em evidentes grupos de camadas de crescimento (*Growth Layer Groups*, GLGs, Perrin e Myrick 1980 cuja contagem permite estimar a idade dos indivíduos. Esta deposição em grupos discretos permite ainda, a obtenção de a

amostras cronologicamente ordenadas que possibilitam a investigação de variações ontogenéticas nas composições isotópicas que permitam investigar mudanças de longo prazo (i.e. ontogenéticas) na dieta dos pinípedes (Newsome et al. 2006, Albernaz et al. 2017).

A utilização deste tipo de metodologia depende da correta evidencia e contagem das GLGs, assim como a calibração destas estruturas. No caso das focas-caranguejeiras, diversas metodologias têm sido utilizadas para estimar as idades, sendo aplicadas tanto a dentes caninos e pós-caninos (Bengtson e Sniff 1981, Laws 1985, Laws et al. 2002, Loza et al. 2016). Estes trabalhos, reportaram diferentes dificuldades e limitações para a contagem de GLGs dependendo do tecido (i.e. cemento vs. dentina) e do tipo de dente (caninos vs. pós-caninos) (Laws et al. 2002, Loza et al. 2016). Estes trabalhos, porém, carecem de imagens e descrições detalhadas que permitam a replicação da técnica e a correta identificação das GLGs nestas estruturas. Este tipo de exercício torna-se essencial para a adequada utilização desta metodologia de obtenção deste importante parâmetro.

A vantagem de poder acessar dados sobre o uso de recursos cronologicamente ordenados, somado a que, devido à natureza resistente dos dentes faz com que quase sempre sejam encontrados bem preservados, mantendo a composição isotópica original (Bocherens 2015), permitem que sejam preferencialmente utilizados para a reconstrução histórica da dieta nestes organismos (Hobson e Sease 1998, Laws et al. 2006, Hückstädt et al. 2017). A disponibilidade de material biológico de focas-caranguejeiras mumificadas, datadas em aproximadamente 100 anos (Negrete et al. 2015), e de focas recentes amostradas na Ilha Marambio/Seymour (LPA), permitiu investigar a variação temporal na ecologia trófica das focas-caranguejeiras nesta região, assim como verificar possíveis mudanças ontogenéticas no nicho ecológico, por médio da AIE.

2. HIPÓTESES

2.1. O nicho isotópico das focas mumificadas (amostras históricas) é menor do que nas focas atuais;

2.2. Os filhotes/sub-adultos apresentam um nicho isotópico menor do que os adultos.

3. OBJETIVOS

Caracterizar as variações temporais e ontogenéticas na ecologia trófica e, uso do habitat de focas-caranguejeiras do leste da Península Antártica.

3.1 Objetivos específicos:

1. Estimar as idades de focas-caranguejeiras na Ilha Seymour, leste da Península Antártica, utilizando duas metodologias conhecidas;
2. Determinar a técnica de estimação da idade mais apropriada para a foca-caranguejeira;
3. Investigar a existência de mudanças temporais na dieta e uso do hábitat da espécie no leste da Península Antártica;
4. Verificar se existe variação ontogenética na ecologia trófica e/ou uso do habitat de forrageio na espécie.

4. MATERIAIS E MÉTODOS

4.1 Área de estudo, coleta de material

Caninos, pós-caninos e bigodes de focas históricas (mumificadas) foram coletados entre 2012 e 2016 na Ilha de Marambio (64°15'S, 56°48'W, Fig. 1- Anexo II), Mar de Weddell ocidental, na Antártica. A análise radiocarbônica dessas focas concluiu que o evento da morte ocorreu aproximadamente nos últimos 100 anos (Negrete et al. 2011). Essas carcaças de focas mumificadas apresentaram diferentes estados de decomposição que variaram de ossos desarticulados a corpos totalmente dessecados, bem conservados e com perfeita conservação dos tecidos moles (Negrete et al. 2015). O sexo destes espécimes, não pôde ser verificado na maioria dos casos. Assim, de um total de 48 dentes de foca mumificados examinados, uma subamostra de 32 indivíduos apresentava tanto caninos como pós-caninos e os 16 restantes apresentavam caninos ou pós-caninos. Bigodes de 5 focas foram extraídos conservando a raiz dos mesmos.

Paralelamente, durante o ano 2016, foram extraídos desde a raiz bigodes de 6 focas vivas que foram imobilizadas e sedadas usando uma combinação de tiletamina/zolazepam 250 mg (Higgins et al. 2002).

Os caninos e pós-caninos foram removidos do osso sempre que necessário, limpos com uma escova de dentes e água destilada para remover qualquer carne remanescente, e secos à temperatura ambiente. Uma subamostra de 26 dentes foram fervidas em solução 100% de peróxido de hidrogênio (H_2O_2) durante 1 h para facilitar a extração dos dentes e remover a carne remanescente (Loza et al. 2016). Devido às suas diferentes dimensões, os caninos e os pós-caninos foram processados para determinação da idade, utilizando dois métodos convencionais, conforme especificado nas próximas seções.

4.2 Estimativa de idade

Para determinar a idade dos exemplares, foi realizada a contagem das GLGs (Laws 1993) presentes na dentina ou no cimento dos caninos e pós-caninos, sendo cada GLG considerada como correspondente a um ano de vida do exemplar (Laws et al. 2002).

4.2.1 Metodologia 1: Estimativa da idade nos caninos

Os dentes caninos foram processados seguindo metodologias previamente descritas (Rosas et al. 1993; Rust et al., 2019). Cada dente foi bisseccionado longitudinalmente com uma serra metalográfica diamantada Buehler IsoMet® de baixa velocidade (3" de diâmetro x 0,006"). A superfície exposta foi polida com lixas d'água com diferentes granulações. Cada metade foi descalcificada com ácido fórmico 25% durante 1 h e enxaguada durante 12 h.

Embora a leitura dos GLGs seja normalmente melhorada esfregando a superfície gravada com pó de grafite (Evans & Robertson, 2001; Molina-Schiller & Pinedo, 2004; Loza et al., 2016), neste trabalho esta etapa foi evitada, devido à interferência potencial do pó de grafite com as análises de isótopos estáveis da dentina.

4.2.2 Método 2: Estimativa de idade em cimento e dentina de pós-caninos

Um dente pós-canino de cada exemplar foi cortado com uma serra metalográfica em seções longitudinais. A parte central do dente foi fixada numa solução de formaldeído a 10 %, durante 24 h. Após lavagem com água, foram então descalcificados usando uma mistura comercial de ácidos (RDO®) por 48-96 h, dependendo do tamanho, densidade e espessura da seção do dente. Quando prontos, os dentes foram bem lavados em água corrente e lâminas de 25 µm foram obtidas usando um micrótomo gelado a -20 °C. As lâminas foram coloridas usando hematoxilina de Mayer e fixadas em 100% de glicerina. As duas melhores lâminas foram montadas para ser examinadas sob estereomicroscópio.

As idades (i.e. contagem de GLGs) foram estimadas independentemente por dois autores, nos casos de discordância cada dente era analisado e discutido em conjunto.

4.3 Análises de isótopos estáveis

4.3.1 Bigodes

Os bigodes foram lavados com água destilada, limpos com uma solução 1:1 de etanol metanol para remoção de lipídios e secos ao ar. Uma vez secos, os bigodes foram seccionados a cada 5 mm com corta-unhas. Cada seção foi cortada e aproximadamente 0,7 -1 mg de amostra foram colocados em cápsulas de estanho para AIE. As subamostras próximas à ponta foram pesadas juntas para se obter o peso necessário.

4.3.2 Dentina de caninos

Após ter realizado a estimativa da idade nos caninos, pó de cada GLG foi obtido utilizando um Micromill com uma broca de 300 micras. As GLGs foram amostradas até a última camada visível. Devido à baixa resolução, GLGs correspondentes às idades maiores foram agrupadas e os valores isotópicos médios foram calculados para estes grupos.

4.4 Análise de isótopos estáveis

As amostras foram analisadas usando um analisador elementar Costech (4010) acoplado a um espectrômetro de massa de razão isotópica Thermo Scientific Delta V no Centro de Isótopos Estáveis da Universidade do Novo México (UNM - CSI). As razões isotópicas ($^{13}\text{C}/^{12}\text{C}$ e $^{15}\text{N}/^{14}\text{N}$) serão expressas na notação delta (δ) em partes por mil (‰) dos padrões VPDB (Vienna Pee Dee Belemnite limestone) para carbono e ar atmosférico para nitrogênio, através da seguinte equação:

$$\delta(\text{‰}) = (R_{\text{sample}}/R_{\text{standard}}) - 1$$

Os padrões internos de laboratório utilizados foram: proteína de soja, proteína de soro de leite, caseína, atum, IAEA-N1, IAEA-N2, USGS-4 e USGS-43. A precisão analítica (DP) desses padrões internos foi medida como <0,2 ‰ para valores de $\delta^{15}\text{N}$ e <0,04 ‰ no caso de valores de $\delta^{13}\text{C}$.

4.5 Análises estatísticas

Testes de normalidade Shapiro Wilks e teste de Bartlett foram aplicados para verificar normalidades e homocedasticidade dos dados.

Com o objetivo de visar diferenças entre as estruturas dentárias (dentina-canino, dentina e cemento-pós-canino) e entre os procedimentos de tratamento de limpeza foram realizados Modelos Mistos Lineares com distribuição Gaussiana e funções de ligação identidade. Como efeito aleatório foi utilizado a identidade individual para explicar medidas repetidas da variável resposta (diferentes estimativas de idade do mesmo indivíduo).

Utilizando dados de indivíduos que possuíam ambas as estruturas dentárias, regressões lineares foram usadas para comparar as contagens de GLG em dentina de caninos, dentina e cemento de pós-caninos. Essas regressões também foram realizadas para comparar as contagens entre dentes tratados e não tratados com H_2O_2 , separadamente.

Para avaliar as diferenças em $\delta^{13}C$ e $\delta^{15}N$ entre focas históricas e atuais e entre as diferentes classes etárias, foram utilizados os testes t de Student e ANOVA / Kruskal-Wallis (para dados não normais), respectivamente, com um nível significativo de $p < 0,05$. Também foram realizadas comparações post-hoc aos pares para os testes paramétricos e não paramétricos.

Os GLGs foram agrupados em três categorias de idade, de acordo com Laws (1953) e Bengston e Sniff (1981): filhote - incluindo o 1º GLG, subadulto - do 2º GLG à idade na maturidade sexual (5º/6º GLG). Os GLGs correspondentes às idades após a maturação sexual (ou seja, categoria de idade adulta) foram considerados aqueles depositados após a mudança na espessura média dos GLGs (ver resultados do Anexo 1).

Calcularam-se os nichos isotópicos para as focas históricas e atuais, usando os valores de $\delta^{13}C$ e $\delta^{15}N$ dos bigodes, e para as classes etárias usando valores isotópicos das GLGs. Foi calculada a sobreposição do nicho entre as focas históricas e atuais e entre as diferentes classes etárias. As áreas de nicho isotópico e as sobreposições foram calculadas usando o pacote SIBER (Jackson et al. 2011) em R v. 3.6.0.

Adicionalmente se aplicaram modelos mistos aditivos generalizados (GAMM) para avaliar a variação ontogenética em dos valores isotópicos entre os animais. Identificação individual foi incluída como efeito aleatório. O modelo foi estruturado com uma distribuição gaussiana, com função de ligação de Identity e curvas suavizadas.

5. SINTESE DOS RESULTADOS

5.1 Estimativa das idades

- A idade mínima obtida para dentina (caninos e pós-caninos) e cemento (pós-caninos) foi 0 anos e a máxima 31 anos, esta última registrada no cemento dos pós-caninos corados com Hematoxilina nos dentes que não receberam tratamento com H_2O_2 ;

- Os modelos lineares mistos mostraram uma interação significativa entre os termos (dente * tratamento), onde os dentes tratados apresentaram idades menores no cimento do que dentes não tratados. Nestes últimos, a estimativa de idade não foi significativamente diferente entre as estruturas utilizadas;
- Uma alta similaridade nas estimativas de idades utilizando as diferentes estruturas foi encontrada para idades menores a 13 anos. Conforme a cavidade pulpar fecha-se, porém, maiores inconsistências na estimativa da idade foram encontradas entre as estruturas analisadas;
- As leituras realizadas na dentina de caninos e pós-caninos, apresentaram maiores dificuldades devido principalmente à presença de linhas acessórias. Por isso, considera-se a leitura no cimento dos pós-caninos mais simples para estimar a idades de focas-caranguejeiras;
- O tratamento prévio com H_2O_2 afeta consideravelmente a estrutura do cimento causando a perda das GLGs mais externas (mais recentemente depositadas);
- Uma área de transição (diferença de largura nas GLGs) foi identificada na dentina e no cimento de caninos e post-caninos, usualmente associada à maturidade sexual, estando em média na idade 5,8 e 5,1 anos para a dentina dos caninos e pós-caninos, respectivamente, e 5,9 anos para o cimento dos pós-caninos.

5.2 Isótopos estáveis

5.2.1 Valores isotópicos das focas-caranguejeiras históricas vs. atuais

- Os valores médios de $\delta^{13}C$ foram similares para os grupos de focas analisados: $-22,3 \pm 1,3 \text{ ‰}$ e $-22,0 \pm 1,18 \text{ ‰}$ para históricas e atuais, respectivamente. Os valores isotópicos de nitrogênio foram estatisticamente diferentes; as focas atuais apresentaram valores maiores de $\delta^{15}N$;
- O nicho isotópico das focas atuais é levemente mais amplo que o nicho das focas históricas, mostrando uma elevada sobreposição (55,3 % e 70,2 %, respectivamente);

5.2.2 Variação ontogenética nos valores de $\delta^{13}C$ e $\delta^{15}N$ nas focas históricas

- Os valores isotópicos na dentina de caninos das focas-caranguejeiras históricas mostraram uma variação de -23,3 a -18,6 ‰ para o $\delta^{13}\text{C}$ e 6,7 a 12,0 ‰ para o $\delta^{15}\text{N}$. Diferenças significativas encontraram-se entre as classes etárias. Juvenis apresentaram valores de $\delta^{15}\text{N}$ mais elevados e os menores valores de $\delta^{13}\text{C}$ em comparação com os adultos e subadultos. Adultos e subadultos não apresentaram diferenças significativas para os valores de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$;
- Filhotes apresentaram áreas de nicho isotópico maiores que os subadultos e adultos, enquanto estes apresentaram áreas de nicho isotópico similares. A sobreposição de nichos entre subadultos e adultos foi elevada (84,7 % e 83,4 %, respectivamente). Por sua vez, juvenis apresentaram sobreposição parcial com o nicho dos subadultos e adultos (33,1 % e 34,7 %, respectivamente);
- O GAMM mostrou que existem diferenças significativas nos efeitos das GLG nos valores isotópicos de carbono e nitrogênio. Com as curvas suavizadas foi possível registrar um aumento do $\delta^{13}\text{C}$ com o aumento na idade, atingindo o máximo aos 6 anos, depois dessa idade os valores começam a diminuir. Para o caso dos valores do $\delta^{15}\text{N}$, a curva mostra uma descida abrupta logo após primeiro ano, seguidas de um leve incremento e estabilização dos valores de $\delta^{15}\text{N}$ a partir do 5º ano de vida;
- Nos casos onde um mesmo indivíduo apresentava amostras de bigode e dente – apenas para as focas históricas-, os valores isotópicos de carbono e nitrogênio da dentina foram 1,6 ‰ e 2,5 ‰ maiores do que nos bigodes, respectivamente.

6. CONCLUSÕES

Ao avaliar diferentes estruturas dentarias através de diferentes metodologias podemos concluir que a estimação da idade pode ser feita em nas dentinas como no cimento dos pós-caninos e utilizando qualquer das duas metodologias analisadas nesse trabalho enquanto a idade das focas seja menor que 13 anos. Porém, as leituras feitas no cimento dos pós-caninos são mais precisas e apresentam menor dificuldade de execução. Além disso, recomenda-se evitar o uso de tratamento químico como a finalidade de remover restos de tecido mole nos dentes que forem utilizados para a contagem de GLGs.

Os nichos isotópicos das focas-caranguejeiras históricas e atuais do leste da Península Antártica permaneceram sem grandes mudanças durante o século XX e começos do XXI. As focas atuais apresentaram $\delta^{15}\text{N}$ o levemente maiores, podendo estar relacionados a uma diminuição da contribuição do krill na dieta dessas focas. No entanto, mudanças nos níveis basais de nitrogênio não devem ser desconsideradas, recomendando-se futuras avaliações por meio da análise de compostos específicos em aminoácidos (CSI-AA) para distinguir os efeitos da linha de base dos hábitos de forrageio.

Perfis ontogenéticos nos valores isotópicos das amostras históricas, mostraram padrões opostos nas mudanças dos valores de $\delta^{15}\text{N}$ e $\delta^{13}\text{C}$, possivelmente pelo consumo de leite, desmame rápido e a elevada taxa de crescimento dos animais. Após atingir a maturidade sexual, a tendência é a estabilizar os valores, refletindo uma similaridade no consumo de recursos nestes indivíduos ao longo da vida adulta.

Para finalizar, este trabalho permitiu um aumento do conhecimento e um melhor entendimento desta espécie no ecossistema do Mar de Weddell, o qual está menos estudado em comparação com os ecossistemas marinhos do oeste da PA, devido, principalmente, à cobertura de gelo permanente que impossibilita a logística para realizar trabalhos de campo. Levando em consideração os efeitos contrastantes das mudanças climáticas globais entre ambas as regiões da Península Antártica, aconselham-se futuras pesquisas direcionadas a avaliar outros componentes da rede trófica destes ecossistemas, assim como a aplicação de técnicas complementares (CSI-AA, rastreamento satelital) que ajudem a compreender melhor a estruturação trófica e

os padrões de uso de hábitat e forrageio das focas-caranguejeiras no leste da Península Antártica.

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ANEXO I

Age estimation and growth layer patterns in teeth of crabeater seals: a comparison of methods

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Age estimation and growth layer patterns in teeth of crabeater seals: a comparison of methods

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Abstract

Age estimation of marine mammals provides important information about ecological and life history parameters. Growth Layer Groups (GLGs) count in dentine and cement of teeth is the main method for age estimation used in pinnipeds. In this work, we used acid-etched canines and decalcified stained post-canine sections to calibrate readings in the crabeater seal (*Lobodon carcinophaga*). A subsample of this group received a prior cleaning treatment of boiling teeth with H₂O₂. Cement from post-canines showed higher estimated ages than dentine from canine and post-canines, mainly in those teeth that did not receive prior treatment. Boiling with H₂O₂ has shown to affect cement structure and thus results in underestimated ages, but dentine did not seem to be affected. The evaluation of the structures and methods used to age crabeater seals showed that accurate age estimations can be obtained with either structure and method, at least for seals <13 yr. However, easier and more accurate estimations are obtained from stained thin sections of post-canine teeth.

KEYWORDS

Aging, Etched-canines, Growth Layer Groups, *Lobodon carcinophaga*, Stained post-canines.

1. INTRODUCTION

Age estimation of marine mammals provides indispensable information used for calculating life history parameters, such as age-at-sexual maturity, mortality and longevity (Amano, Miyazaki, and Petrov, 2000; Evans, Kemper, McKenzie, & McIntosh, 2007; Hewer, 1963). Furthermore, age estimation can be useful to detect changes in growth rates due to climate changes (Boyd & Roberts, 1993; Dellabianca et al., 2012; Knox, Stuart-Williams, Warneke, Hoskinsv, & Arnould, 2013; Wittmann et al., 2016) or pollutants exposure through tooth mineralization anomalies (Luque et al., 2013). The most widely used method consists in the examination of incremental layers in dentine and/or cementum of teeth and bones usually referred to as *Growth Layer Groups* or GLGs (Perrin & Myrick, 1980).

Among marine mammals, teeth are preferred for age estimation because cetaceans and pinnipeds are monophyodont, thus growth layers are deposited throughout life without remodelling and GLGs can be directly related to the modern age of the individual (Bowen 1983; Hohn, 2018). Teeth are composed of three sections; the crown, the neck and the root. The crown is formed of enamel exposed above the gum, covering the dentine. The dentine is present as pre-natal (deposited before birth) and secondary dentine (deposited after birth). The cement covers the root and is usually below the gum line, allowing it to anchor tooth to its alveolus (Hohn, 2018; Lockyer, Mackey, Read, Härkönen, & Hasselmeier, 2010; Mc Cann, 1993). The pulp cavity, surrounded by dentine, becomes partially or fully filled in older animals. However, in some species with large teeth (e.g. elephant seals), the pulp cavity remains open throughout life (Laws 1953). Contrary to dentine, cement is deposited on the outer surface, where the outermost layers represent the most modern ones. Cement deposition continues regularly throughout life, filling the alveolar cavity.

Growth Layer Groups can be recognized by a predictably cycling repetition with constant spacing (Hohn, 2018). In stained thin-sections, such repetition is usually composed by alternating dark and light layers, which are orientated parallel to the tooth within the dentine or cement (Evans et al., 2007; Lockyer et al., 2010). In the case of pinnipeds, each GLG usually represents one year of life (Bowen, Sergeant, & Oritsland, 1983; Childerhouse, Dickie, & Hessel, 2014; Frie et al. 2011; Hewer, 1963; McCann,

1993; Scheffer, 1950). In many cases, sub annual layers known as accessory lines can be present, being recognizable by the thickness and irregular occurrence (Hohn, 2018). Some authors suggest that these lines are related to different or abnormal feeding patterns or life history events such as moult, reproduction or lactation (Knox et al., 2013; McCann, 1993; Rust, Danil, Melin, & Wilkerson, 2019).

Although age estimation through GLG counting in dentine and cement of teeth is similar among marine mammal groups, some particularities exist. Cetaceans, for example, are homodont, and all teeth present the same growth layer pattern, thus simplifying the choice of what structure to use for age estimation. Pinnipeds, however, present an heterodont dentition, which means that growth layer patterns are not equal between structures (Hohn, 2018). Therefore, various methods were developed for estimating ages using different tooth structures (Laws, 1953, 1958; Lockyer et al., 2010; Murphy et al., 2012; Rust et al., 2019). Indeed, age can be estimated by counting external rings in the canines (Laws, 1953; Molina-Schiller & Pinedo, 2004), or by counting GLGs in the dentine or/and cement of canines, post-canines and incisive teeth (Bowen et al., 1983; Evans et al., 2007; Frie et al., 2011, Hewer, 1963; Laws, 1953; Mansfield & Fisher, 1960). Moreover, GLGs in canines and post-canines can be identified in thick unstained and undecalcified sections (Lockyer et al., 2010; Molina-Schiller & Pinedo, 2004; Schiavini, 1992) or after decalcification, sectioning and staining (Bengtson & Siniff, 1981; Evans et al., 2007; Molina-Schiller & Pinedo, 2004; Schiavini, 1992; Wittmann et al., 2016). Nevertheless, since each tooth processing technique can introduce biases during age counting, Murphy et al. (2012) suggested developing species-specific age methods. However, in most cases, age estimation depends on the availability of equipment and time. It will also depend on which teeth are available for age determination, and their sizes will determinate how each tooth should be processed.

This work focus on the age estimation in crabeater seals (*Lobodon carcinophaga*), the most abundant of all Antarctic seals (Forcada et al., 2012; Laws, 1981). It is a krill-feeding specialist and its tri-lobbed post-canine teeth allow them to be distinguished from the other seals (Adam, 2005; Bengtson, 2009). The age of sexual maturity for this species has been estimated between 2 and 6 years old (Laws, 1984). Its reproduction

occurs during the austral spring (Oct-Nov), when they form groups that consist in a female with her pup and male guarding female until lactation is over (Bengston, 2009). The lactation period of this species is quite short, from days to 6 weeks (Laws, 1958).

In this species, age determination has been performed by means of different methodologies and structures (e.g. Bengtson & Siniff, 1981; Laws, 1957; Laws, Baird, & Bryden, 2002). In an early work, Laws (1957) used transversal sectioned canines to age crabeater seals by counting GLGs in the dentine. However, due to the non-linear axis of the canine that hampers its extraction from the mandible and the relatively narrow cement deposited, Laws et al. (2002) recommended the use of the post-canine for ageing this species. Therefore, counting GLGs in the cement of longitudinal thin sections of post-canines mounted in glass slides was proved to be the most reliable method for age estimation, particularly in the case of old specimens (Laws et al., 2002). However, these studies did not directly compare the structures as different sample sets were used. Counting GLGs in stained thin sections of teeth is usually the preferred method for estimating ages in pinnipeds and odontocets (Hohn & Fernandez, 1999; Hohn, Scott, Wells, Sweeney, & Blair Irvine, 1989; Murphy et al., 2012; Schiller & Pinedo 2004). Nevertheless, because this technique requires more steps and specific equipment, ageing seals by counting GLGs in dentine of acid-etched canines is sometimes the only available alternative. In other cases, the selected structure for ageing will depend on which tooth is available. In such cases, the calibration of GLG counting among different structures is desirable in order to make them comparable.

In this study, paired canines and post-canines of crabeater seals were processed for age estimation using two different and frequently used methodologies for age determination in marine mammals. We then compared these methods in order to calibrate readings between structures and recommend the most appropriate technique for crabeater seals.

2. METHODS

Study area, collecting and sampling

Canines and post-canines from mummified crabeater seals were collected between 2012 and 2016 at Marambio Island, (64°15'S, 56°48'W), western Weddell Sea, Antarctica. Radiocarbon analysis of those seals concluded that death event occurred approximately

in the last 100 yr (Negrete et al., 2011). These mummified seal carcasses presented different states of decay that varied from disarticulated bones to entirely desiccated, or well-preserved bodies with perfect soft tissue conservation (Negrete et al., 2015). Thus, from a total of 48 mummified crabeater seal teeth examined, a subsample of 32 individuals presented both canines and post-canines and the remaining 16 seals presented either canine or post-canine (Fig. 1, Table 1). Even though Laws et al. (2002) recommended using the third post-canine from the left mandible for age determination, in most cases we could not specify which post-canine was available for age estimation and in one case an incisive was used. For whole-preserved seals with mandibles attached, these were removed from the skull using bolt cutters and cleaned from remaining flesh using scalpel. The canines and post-canines taken from disarticulated carcasses (mandibles and teeth from 22 specimens) were removed from the bone using bolt cutters whenever necessary, cleaned with a toothbrush and distilled water to remove any remaining flesh, and dried at room temperature. A subsample of 26 teeth were obtained from seal mandibles that were boiled in 100% hydrogen peroxide (H_2O_2) solution for 1 h to facilitate the extraction of teeth and remove the remaining flesh (Loza et al., 2016). Due to their different sizes, canines and post-canines were processed for age determination using two conventional methods as specified in the next sections.

Method 1: GLGs counts in the dentine of canines

Canines were processed following conventional procedures described elsewhere (e.g. Rosas, Haimovici, & Pinedo, 1993; Rust et al., 2019). Each tooth was mounted onto wooden blocks with thermoplastic glue and sectioned in the anterior-posterior cutting plane with a Buehler IsoMet® diamond metallographic low-speed saw (3" diameter x 0.006"). The most on-centered section (i.e., with the largest percentage of opened pulp cavity) was chosen and the dentine surface was grounded with a series of sandpapers (320, 500, 600, 1200, 12000 grit). Although teeth can be cut either in a transverse or longitudinal plane, there is no agreement on which is better to count GLGs (Laws, 1958; Loza et al., 2016). Nevertheless, in this work, we used longitudinal sections in order to provide a larger area of examination.

Each half-tooth obtained was etched with 25% formic acid during 1 h, washed for 12 h with tap water and air-dried. Although GLGs reading is usually enhanced by rubbing

the etched surface with graphite powder (Evans & Robertson, 2001; Loza et al., 2016; Molina-Schiller & Pinedo, 2004), in this work this step was skipped, because of the potential interference of the graphite powder with future dentine chemical analysis (e.g. stable isotopes or trace elements).

Since canines show relatively little cement deposition (Laws, 1958), only dentinal layers were counted. Tooth surfaces were photographed using a digital camera attached to a stereomicroscope at 4x.

Method 2: GLGs counts in cement and dentine of post-canines

Each post-canine was mounted onto wooden blocks to cut thick longitudinal sections, that contain the most central part of the tooth. Each section was fixed in a 10% formaldehyde solution for 24 h, and then washed out overnight in tap water. Tooth sections were then decalcified using a commercial mixture of acids (RDO®) for 48-96 h, depending on the size, density and thickness of the tooth section. Generally, older teeth have smaller pulp cavity and require longer decalcification times (Molina-Schiller & Pinedo, 2004). Sections were considered ready when they were entirely flexible (Evans & Robertson, 2001). Teeth were then well washed in tap water and 25 µm slides were obtained using a freezing microtome at -20 °C.

Slides were then colored using Mayer's haematoxylin during 40 min, blued in weak ammonia and washed under tap water. Slides were submerged in 50% glycerine for 30 min and fixed in 100% glycerine, in which were stored until mounted. Before mounting, all sections were examined in order to determine the 2 best slides -the ones with the largest pulp cavity and clearest GLGs (Molina-Schiller & Pinedo, 2004). These sections were mounted in 100% glycerine sealed with Entellan® and examined under a transmitted light microscope with 4x and 10x magnifications, coupled to a digital camera.

Age estimation

Age estimation of each individual was performed by counting GLGs, without reference to biological data of the specimen (i.e. sex, size). The patterns of GLGs deposition in the dentine of canines described in Laws (1958) and in Loza et al. (2016) were used as a

reference for the identification of the annual layering. In the case of the post-canines, cement and dentine layering were analyzed following Laws et al. (2002).

Ages were estimated in each tooth section by two independent researchers (J.C. and S.B.). In case of disagreement, the readers analyzed and discussed images together in order to decide the best age estimation.

A transitional zone in seal teeth was previously described by Laws (1953) for the southern elephant seal (*Mirounga leonina*) and Hewer (1963) for the grey seal (*Halichoerus grypus*) and considered as the age-at-sexual maturation. In the crabeater seal, Bengtson and Siniff (1981) and Laws et al. (2002) reported a transition zone in the cement of the post-canines, usually placed at 5 years of age, defined as a change in the average thickness of the GLGs (i.e. from larger ones in the younger GLGs to narrower layers in the older ones). Therefore, the GLG at the transition zone was identified in each of the structures used for age estimation in order to investigate the average age at transition and the consistency of this transition zone among the structures of each individual.

Statistical analysis

Linear Mixed Models with Gaussian distribution and identity link functions were used to examine differences among tooth structures (canine dentine, post-canine dentine and cement) used for age estimation, and between clean-up treatment procedures (boiled in hydrogen peroxide or not) and their interaction on the estimated ages. Residuals plots were also performed in order to validate the model. The model was run using individual identity as a random effect to account for repeated measures of the response variable (i.e. different age estimations of the same individual).

Using data from individuals that had both dental structures, linear regressions were used to compare GLG counts in dentine of canines, dentine and cement of post-canines. These regressions were also performed for H₂O₂ treated and untreated teeth separately.

RESULTS

Considering all samples, the minimum age obtained for dentine (canines and post-canines) and cement (post-canines) was 0 yr and maximum age was 31 yr, registered in

the cement of a stained post-canine without H₂O₂ treatment. In the dentine, the maximum number of GLGs counted was 24 yr in the post-canines and 25 yr in a canine without H₂O₂ treatment. Among treated teeth, post-canine sections show a maximum age of 22 yr and 19 yr for cement and dentine, respectively, while 25 yr was the maximum count among canines.

Linear Mixed Models results showed no influence of the structure used for age estimation (dentine in canines and post-canines, and cement in post-canines) or treatment in the estimated ages. However, the interaction term (tooth*treatment) was significant ($p < 0.05$).

A good agreement in the estimated ages was obtained between different methods, although GLG counts in acid-etched post-canines (Fig. 2) were more difficult to identify than layers in stained post-canine dentine and cement (Fig. 3). However, the paired-GLG counts in the dentine of the canines and the dentine of the post-canines showed a slightly better agreement ($R^2 = 0.85$; $p < 0.001$) than with the cement of post-canines ($R^2 = 0.81$; $p < 0.001$) (Fig. 4). Furthermore, the estimated ages in cement and dentine of the post-canines were also consistent ($R^2 = 0.82$, $p < 0.001$). In general, a close agreement between estimated ages was found in teeth with less than 13 yr, while larger inconsistencies appeared in older animals in which the pulp cavity were almost closed (Fig. 2.B). Moreover, GLG counting in the dentine of both canines and post-canines was, in general, more difficult due to the presence of accessory layers that confound the identification of the annual layering. Thus, the estimation of ages in the cement of post-canines was proved to be more straightforward than in the dentine.

When comparing the ages estimated for the H₂O₂ treated and non-treated teeth, a closer agreement was found between paired-counts for the non-treated teeth (Figs. 4, 5 and 6). The correlation between the ages estimated in the dentine of canines and those in the dentine of post-canines was similar in treated and non-treated teeth ($R^2 = 0.85$, $p < 0.001$ and $R^2 = 0.86$, $p < 0.001$, respectively). Similarly, a high agreement was found in non-treated teeth when comparing the counts in the cement of post-canines and those in the dentine of canines ($R^2 = 0.95$, $p < 0.001$) or post-canines ($R^2 = 0.91$, $p < 0.001$). However, the H₂O₂ treatment considerably affects the structure of the cement causing the outermost loss of growth layers (Fig. 7); this was evident in the lower maximum

estimated ages. As a consequence, a low agreement in the estimated ages between the cement of post-canines and those in the dentine of canines ($R^2 = 0.53$, $p = 0.001$) or post-canines ($R^2 = 0.65$, $p < 0.001$) was found in these treated teeth.

The transition zone was identified in both dentine (canines and post-canines) and cement (post-canines) of most individuals. In the case of the canine dentine, it was identified in 69% of the teeth and the mean age at transition was 5.8 yr (4 - 7 yr). The transition zone was observed in 73% and 54% of the post-canine dentine and cement, respectively, with the average age-at-transition of 5.1 yr (2 - 7 yr) in the former and 5.9 yr in the latter (Figs. 2 and 3).

3. DISCUSSION

The sample set analyzed in this work provided advice on the best structure for age estimation in crabeater seals. Our findings support the accuracy of estimating ages by counting GLGs in the dentine of canines, and in the dentine and cement of post-canines. Nevertheless, GLGs in stained thin sections of the post-canines are more easily identified, especially in the wide cement of the crabeater seal tooth. Furthermore, it was demonstrated that the cleaning method could potentially influence age estimation, by affecting the structure of the cement. Indeed, Loza et al. (2016) had previously estimated the ages of a subset of the same H_2O_2 -treated teeth used in the present work, which were consistently lower especially in the case of older animals (Table 1). Boiling the teeth in hydrogen peroxide solution caused the loss of the outermost cement layers, thus causing an underestimation of the ages of these animals. In consideration of these results, we strongly recommend to avoid chemical treatment teeth before processing in order to prevent potential damage to the most modernly deposited GLGs in cement.

Currently, no calibration of GLG counting was performed in crabeater seals due to the absence of known-age specimens. Nevertheless, considering the estimated ages in the post-canine cement, where GLGs are more clear and easier to identify (Laws et al., 2002; this work), the age accuracy of the estimated ages could be addressed by comparing counts in these stained-GLGs in the post-canines with those in the other tooth/ structures. Although GLGs in the stained post-canine dentine are also clearly visible, at old ages the pulp cavity begins to close thus interfering with the

individualization of GLGs. Furthermore, the dentine tends to have more irregularities in the GLG width, as well as more accessory lines within GLGs, both having the potential to cause errors in ageing (Lockyer, 1993; Rust et al., 2019).

In the case of teeth that did not receive prior H₂O₂ treatment, age determination coincided up to about 13 yrs when comparing etched canines and cement from post-canines. Generally, age estimation was higher in cement than in dentine for older individuals. For this reason, we consider that cement gives more accurate age estimation than dentine, at least for older ages, as Laws et al. (2002) stated. Therefore, at earlier ages either of the two methodologies can be applied, whereas for animals 15 yr-old or older the pulp cavity begins to close and age determination in the dentine of canines and post-canines can be inaccurate.

Although we also expected to find differences between counts in the dentine and cement, as GLGs become narrower in the dentine of older ages and may lead to undercounting. In fact, correlation graphics showed clearly that estimated ages in cement were higher than in dentine.

In this work, the transitional zone was evident in both dentine of canines and post-canines and cement of post-canines. In general, the first 5-6 yrs GLGs are wider in all structures observed, becoming considerably narrower at older ages. These changes in deposition rate may be related to additional energetic cost influenced by sexual maturation. Furthermore, any event that delays or accelerates juvenile growth (i.e. variation in krill availability) may also be reflected in a change of age at sexual maturity (Eberhardt, 1977). Based on the analysis of reproductive tracts and transitional zone in teeth of crabeater seals collected from 1950s to 1970s, Bengtson and Laws (1983) reported that the age-at-sexual maturation dropped during the whaling period (from 4.4 to 2.5 yrs) and started to rise again after the cessation of major whaling in 1963. The authors postulated that these fluctuations in age-at-maturity would be related to the fluctuation in krill availability due to reduction in whale numbers during and after the major whaling period. Nevertheless, the age-at-transition found in this study (5 - 6 yr) is older than that calculated for the pre-whaling period. Considering that the present samples were obtained from mummified seals that probably died during the last 100 yr (Negrete et al., 2011) it is not possible to state whether this older age-at-maturity is due

to the lower krill availability during the pre-whaling period or because of the more modern increase in krill catches that began in the 1970s (Nicol & Foster, 2003). A more precise dating of the mummified seals is needed in order to hypothesize about the probable causes of the higher age-at-maturity found in this group of seals. Additionally, future work must include the measurement of growth layers and development of change-point-models in order to more accurately identify the age at transition.

CONCLUSION

The evaluation of the structures and methods used to age crabeater seals showed that accurate age estimations can be obtained with either structure and method, at least for seals <13 yr-old. However, easier and more accurate estimations are obtained from stained thin sections of post-canine teeth. Nevertheless, when choosing the most appropriate method for ageing this and other species, it is important to consider the costs of the experimental approach and the availability of equipment. Therefore, results presented here are important for making decisions about which structure and method to use for age estimation, considering the time, costs and sample availability. Furthermore, we advise against the use of chemical cleaning procedures in teeth that will be used for age estimation in order to preserve tissues used for counting GLGs.

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Table1. Summary of estimated ages (yr) of crabeater seals (*Lobodon carcinophaga*) counting GLGs in acid-etched canines (C, dentine) and stained post-canines (PC, dentine and cement). Whether the teeth received a cleaning treatment with H₂O₂ is indicated. Ages previously reported by Loza et al. (2016), specifying the structure use for age estimation (C, PC or NA – information not available), are also shown.

Individual	DENTIN E-C	CEMENT-PC	DENTINE-PC	H₂O₂ TREATMENT	AGE (Loza et al.2016)
1	3	3	3.5	NO	--
2	4	4	4	NO	--
3	2	2	3	NO	--
4	20	20	19	NO	--
5	31	31	18	NO	--
6	7	7	7	NO	--
7	18	21	18	NO	--
8	6	6	6	NO	--
9	5	5	5	NO	--
10	22	28	24	NO	--
11	22	26	17	NO	--
12	14	14	14	NO	--
13	10	10	10	NO	--
14	14	19	16	NO	--
15	21	21	21	NO	--
16	9	11	11	NO	--
17	NA	1	0	NO	--
18	23	27	22	NO	--
19	25	NA	NA	NO	--
20	7	7	7	NO	--
21	NA	19	18	YES	4-PC
22	7	NA	NA	YES	4-PC
23	20	15	13	YES	14-PC
24	22	14	18	YES	13-PC
25	NA	17	19	YES	10-PC
26	12	0	11	YES	5-NA
27	8	5	8	YES	10-NA
28	12	12	12	YES	6-PC
29	10	10	10	YES	5-PC
30	NA	5	6	YES	3-PC
31	13	NA	NA	YES	7-NA
32	13	13	11	YES	4-NA
33	19	NA	NA	YES	10-PC
34	12	12	10	YES	4-PC

35	NA	15	11	YES	7-PC
36	16	10	12	YES	6-PC
37	NA	10	10	YES	4-PC
38	6	NA	NA	YES	6-NA
39	18	19	16	YES	8-NA
40	9	NA	NA	YES	7-PC
41	NA	5	8	YES	6-PC
42	NA	22	NA	YES	12-PC
43	6	1	5	YES	4-PC/C
44	6	3	6	YES	5-NA
45	NA	5	9	YES	7-PC
46	25	NA	NA	YES	9-NA
47	0	0	1	NO	--
48	16	23	16	NO	--



Figure 1. Canine (left) and post-canine (right) from an adult specimen of crabeater seal, *Lobodon carcinophaga*.



Figure 2. Longitudinal sections of etched canines of crabeater seal, *Lobodon carcinophaga*. GLGs are represented by horizontal black lines. A) Dentine with 3 GLGs, B) and 19 GLGs. Pulp cavity (PC), Dentine (D), Cement (C) and Pre-natal dentine (PN) are indicated.

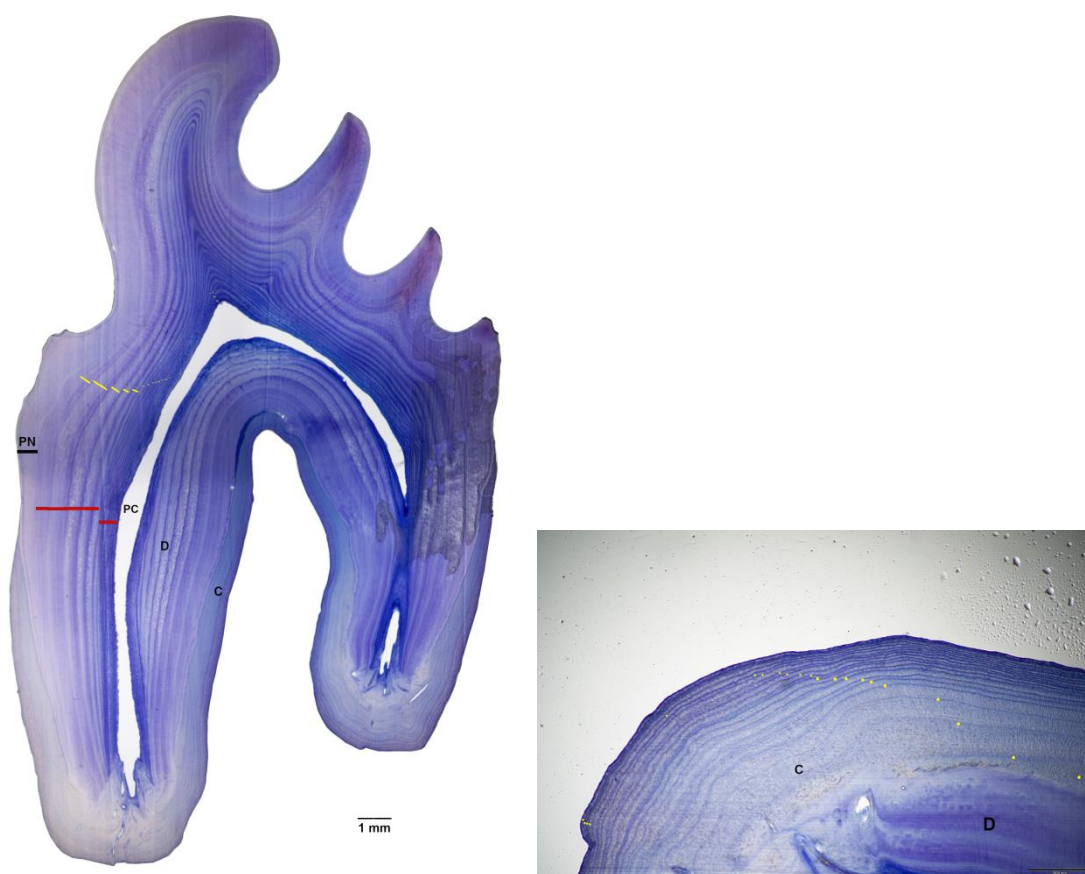


Figure 3. Stained section from a post-canine of crabeater seal, *Lobodon carcinophaga* without treatment. Dentine with 18 GLGs indicated as yellow bars (A) and cement with 21 GLGs represented by yellow dots (B). The Red horizontal lines show the two sections with differing GLG width (transition zone), in this case around the 5th GLG. Pulp cavity (PC), Dentine (D), Cement (C) and Pre-natal dentine (PN) are indicated.

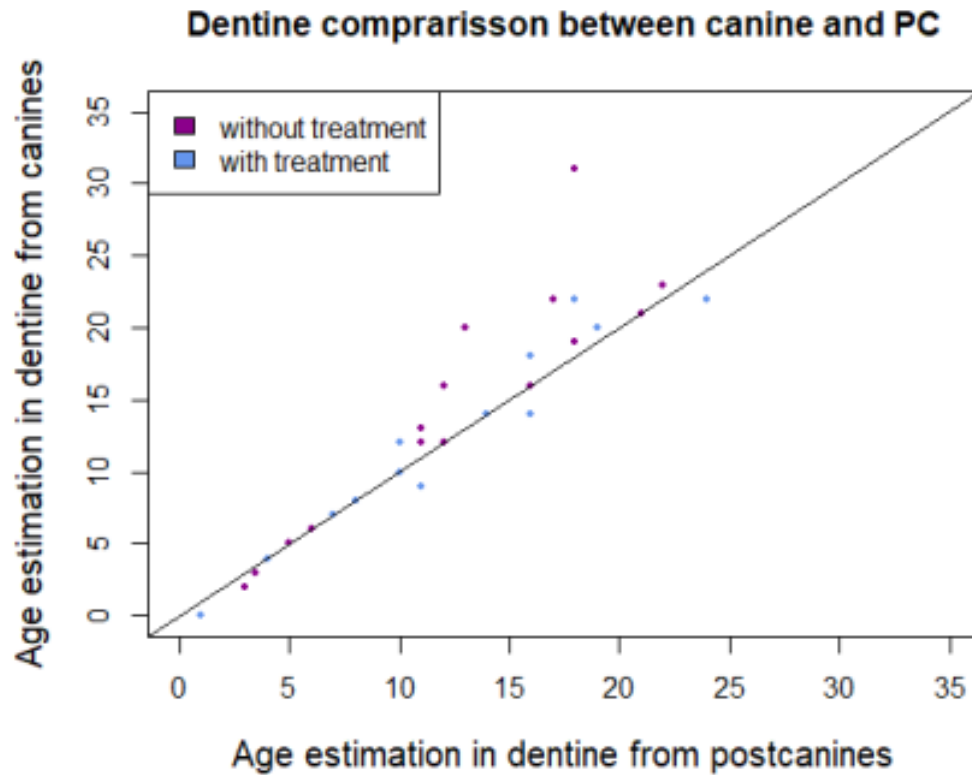


Figure 4. Relationship between ages estimated in the dentine of canines and post-canines of crabeater seals, *Lobodon carcinophaga*. Teeth that were treated with hydrogen peroxide and those not treated are indicated with different colors.

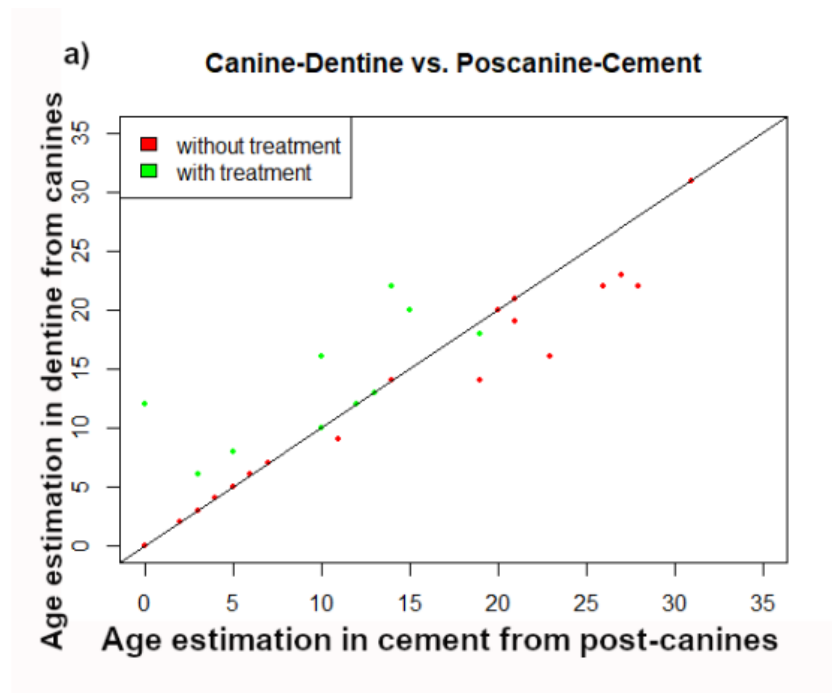


Figure 5. Relationship between ages estimated in the dentine of canines and cement of post-canines of crabeater seals, *Lobodon carcinophaga*. Teeth that were treated with hydrogen peroxide and those not treated are indicated with different colors.

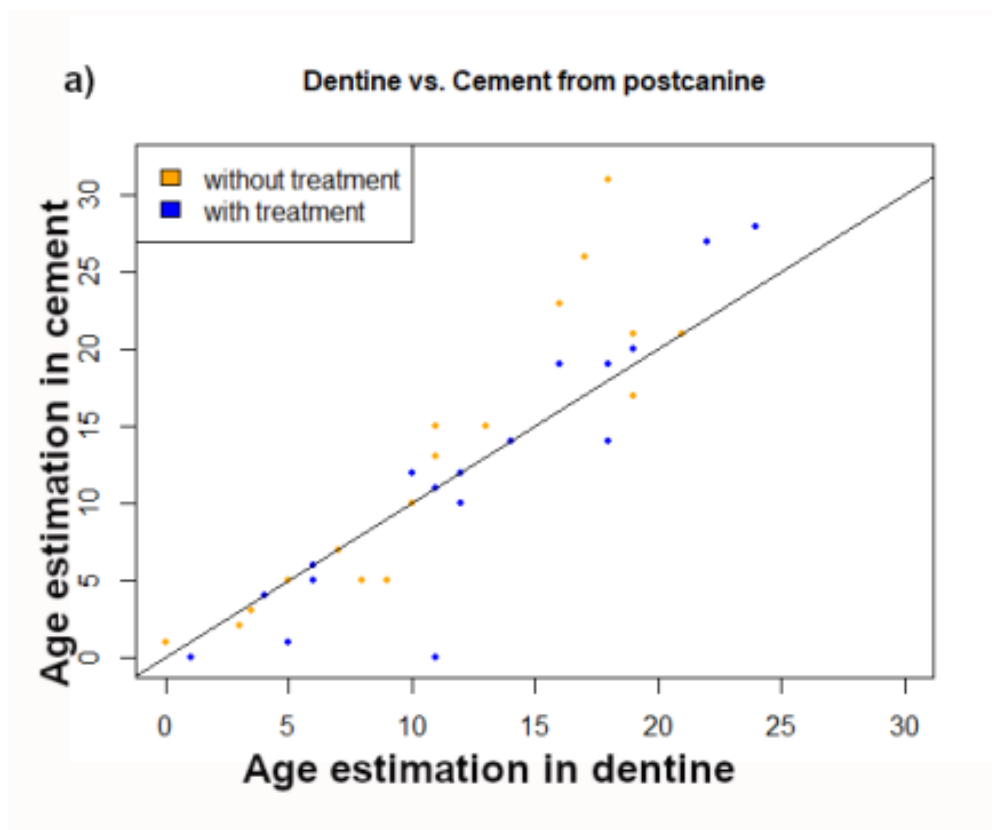


Figure 6. Relationship between ages estimated in the dentine and cement of postcanines of crabeater seals, *Lobodon carcinophaga*. Teeth that were treated with hydrogen peroxide and those not treated are indicated with different colors.



Figure 7. Stained section from a post-canine of crabeater seal, *Lobodon carcinophaga* that was previously treated with hydrogen peroxide. This individual was estimated to be 6 yr of age at the time of its death. GLGs are shown as yellow lines. Pulp cavity (PC), Dentine (D), Cement(C) and Pre-natal dentine (PN) are indicated.

ANEXO II

Temporal and ontogenetic variation in trophic ecology of crabeater seal (*Lobodon carcinophaga*) from the east Antarctic Peninsula

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Temporal and ontogenetic variation in trophic ecology of crabeater seal (*Lobodon carcinophaga*) from the east Antarctic Peninsula

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ABSTRACT

The crabeater seal is a krill specialist feeder and is vulnerable to possible negative effects of climate change or overfishing on its main prey. In this work, we aim to assess temporal and ontogenetic variation in the trophic ecology of the crabeater seals from the East Antarctic Peninsula. For such, we analysed stable isotopic composition in serial whiskers of historical and recent crabeater seals and dentine GLGs of canines. It was possible to infer temporal changes in prey availability, as well as ontogenetic dietary shifts. Recent seals have slightly higher $\delta^{15}\text{N}$ values and a larger isotopic niche area than historic seals, that might be interpreted as the probable inclusion of other higher trophic level prey in the former. Despite of crabeater seals being krill specialists, squids and fishes may be included in their diet. No significant differences were found for $\delta^{13}\text{C}$ in whiskers through time. Isotopic niche of yearlings were significantly wider when compared with subadults and adults. Yearlings showed higher $\delta^{15}\text{N}$ and lower $\delta^{13}\text{C}$ than subadult and adult seals. isotopic values in this age class may be influenced by the lactation period and by tissue catabolism after weaning. GAMMs for historical crabeater seals showed significant effects of the GLG on isotopic values with an increasing trend in $\delta^{13}\text{C}$ and decreasing trajectory of nitrogen isotopic values from the GLG1 up to GLGs 5/6 which represent the age at sexual maturity. These trends were interpreted as a mixture of changing foraging habitats and/or a change in trophic discrimination factors after sexual maturation. This work contributes to increase knowledge of this endemic Antarctic species in the Weddell Sea ecosystem, which is less studied in comparison to the west AP, mainly due to its permanent ice cover that imposes logistic constrains to field work.

Key-words: Global climate change, isotopic niche, mummified seals, phocids, stable isotope analysis, Weddell Sea.

1. Introduction

The Southern Ocean ecosystems are highly productive and their relatively short trophic chains are supported by the Antarctic krill (*Euphausia superba*) a keystone species (Laws 1985). This keystone species serves as food for several trophic level organisms, including high trophic level predators such as penguins, skuas, seals and whales (Atkinson et al. 2005). Krill is associated with sea ice, which provides both forage and refuge habitat for larval and juvenile stages (Flores et al. 2012, Moline 2008), thus changes in the availability of this habitat would likely affect its survival (Loeb et al. 1997, Flores et al. 2012, Hill et al. 2013).

The Antarctic Peninsula has been subjected to temperature oscillations since 1950, whereas some regions are warming, others are cooling (Oliva et al. 2017). Indeed, the western Antarctic Peninsula (WAP) is experiencing a rapid warming, with a reduction of winter sea ice extension and glaciers (Ducklow et al. 2007, 2012, Moline 2008). On the other hand, the Eastern Antarctic Peninsula (EAP) is not suffering similar temperature oscillations as the WAP; probably because the mountains of the Antarctic Peninsula act as a barrier leading to climate differences between the west and east coasts (Skvarka et al. 1998, Siegert et al. 2019). Therefore, less intense or imperceptible changes in the EAP food webs are expected in comparison to those recorded in the WAP (Hill et al. 2013, Etourneau et al. 2019, Siegert et al. 2019).

As a consequence of habitat changes, top predators may alter their distribution, foraging behaviour, growth rates, reproductive success and migration; potentially affecting their survival and eventually becoming extinct (Walther et al. 2002, Polito et al. 2011). In the case of ice-dependent species, such as Weddell seals (*Leptonichotes weddelli*) and crabeater seals (*Lobodon carcinophaga*), negative effects are expected due to the long-term reduction of the sea ice extent (i.e population size and distribution may be affected) (Siniff et al. 2008).

The crabeater seal is the most abundant Antarctic pinniped and its population is estimated in 10-15 million individuals (Southwell et al. 2008, Bengston 2009, Bengston et al. 2011). This species is considered a pack-ice seal (Southwell et al. 2003). Its breeding period begins in September and lasts until November (Laws 1984), and depends on the floating pack ice, where pregnant females give birth and nurse for a period of 3 to 6 weeks (Laws 1985, Siniff et al. 1979).

The diet of crabeater seals is dominated mainly by the Antarctic krill in pelagic waters and near continental slope areas, and cristal krill (*E. crystallorophias*) in neritic areas (Southwell et al. 2003); although squids and fishes can be also consumed in lower proportions (Zhao et al. 2004, Bengston 2009, Huckstadt et al. 2012, Botta et al. 2018). As a krill specialist (Adam 2005, Bengston 2009), the crabeater seal perform dives of varying length and depth according to the daily vertical migration and seasonal movements of its main prey (Burns 2004). It has been estimated that crabeater seals consume around 60 to 70 million tonnes of krill per year (Wall et al. 2007) and thus are vulnerable to changes in the krill availability (Hückstädt et al. 2012). Therefore, changes in krill proportions in the crabeater seal diet can be used as a proxy of the availability of this resource in the environment.

Stable isotope analysis (SIA) has been used as a complement to traditional and direct dietary techniques such as scat, stomach content and regurgitation analysis (Green and Williams 1986, Lowry et al. 1988, Bowen and Iverson 2013). Traditional dietary techniques tend to under or over-estimate the contribution of certain prey items because its different digestibility, and offer a short-term view of the trophic spectrum (DeNiro and Pierce et al. 2004). Thus, SIA has been applied to study predators' diet, habitat use, movements and physiology (Newsome et al. 2010). Additionally, the trophic, scenopoetic and temporal dimensions of the ecological niche can also be investigated through its proxy, the isotopic niche (Bearhop et al. 2004, Aubail et al. 2011, Brault et al. 2019).

Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) are the most commonly used isotopes, as they can offer insights into the predator's trophic position and habitat use patterns. This is because the $\delta^{15}\text{N}$ in a predator is typically enriched in the heavy isotope (^{15}N) in comparison with its prey (DeNiro and Epstein 1981). On the other hand, changes in $\delta^{13}\text{C}$ are minimal between prey and predator, and thus, it is mostly used as a proxy for baseline carbon of their foraging area (Deniro and Epstein 1979, Post 2002). The composition of primary producers (C3 and C4) as well as regional oceanographic characteristics result in different isotopic patterns among regions (Graham et al. 2010, Mc Mahon et al. 2013).

Since tissues typically differ in their macromolecules, elemental composition and turnover, SIA of different tissues from the same individual can provide dietary

information in the short (e.g. blood), intermediate (e.g. whiskers) and long (e.g. bones) time intervals (Hobson 1993). Inert tissues, such as teeth and whiskers, are commonly used in historical and paleontological studies, because they offer long-term resource use information (Newsome et al. 2010, Bocherens 2015, Brault 2017, Hückstädt et al. 2017).

Therefore, several studies used whiskers and teeth of pinnipeds as recording tissues for assessing seasonal and/or temporal shifts in the trophic ecology of these predators (Cherel et al. 2009, Kernaléguen et al. 2012). In this way, ontogenetic changes in resource use can be studied by analysing stable isotopic values chronologically archived along the whisker and/or along annual growth layer groups (GLGs, Perrin and Myrick 1980) in teeth (Newsome et al. 2006, 2009, Martin et al. 2011, Albernaz et al. 2017).

The main aim of this work is to investigate the temporal and ontogenetic variation in the trophic ecology of crabeater seals from the EAP. Analysing the stable isotopic composition of serial whisker segments of historic and recent crabeater seals and dentine GLGs of canines we try to understand temporal changes in prey availability, as well as dietary shifts during ontogeny.

2. Methods

2.1 Study area and sampling

Samples of mummified (i.e. historic) and alive (i.e. recent) seals were collected between 2012 and 2016 at Marambio Island (64°15'S, 56°48'W), western Weddell Sea, Antarctica (Figure 1). Mummified carcasses presented different states of decay that varied from disarticulated bones to completely well-preserved bodies (reported by Negrete et al. 2015). Through radiocarbon techniques applied to these seals, it was concluded that the death event of these bodies occurred within the last 100 yrs (Negrete et al. 2011). In this work, canines (n=38) and whiskers from historic (n=5) and recent seals (n=6) were collected (Table S1).

One canine from each seal carcass was extracted from the superior mandible whenever possible. Otherwise, an inferior canine was sampled. Whiskers were collected by plucking from its root. In the case of the recent samples, whiskers were collected from seals that were darted and sedated using a combination of tiletamine/zolazepam

250 mg (Higgins et al. 2002). Captured seals were measured in total length (nose to tail) and sex was determined by visual inspection of the external genitalia. Individuals were classified as yearling/subadults/adults according to Laws (1957) and Bengtson e Sniff (1981), Walker e Macko (1999). For two individuals it was not possible to record the length measurements; however, they were considered as subadults or adults according to their size, based on the experience of the research team.

All historic seals had their age estimated by counting cement GLGs in post-canines and were also identified in the dentine of sectioned canines (see Cebuhar et al. in review). The latter were chosen for stable isotope analysis due to their larger size that allowed a more precise individual sampling. Canine GLGs were exposed after mounting the tooth in a wood block and sectioned in the anterior-posterior cutting plane with a Buehler IsoMet® diamond metallographic low-speed saw (3" diameter x 0.006"). The section with the largest percentage of opened pulp cavity was chosen and the dentine surface was grounded with sandpaper of different grids.

2.2 Stable Isotope Analysis (SIA)

2.2.1 *Whisker sampling*

Whiskers were washed with distilled water, cleaned with a 1:1 ethanol-methanol solution for lipid removal and air dried. Once dried, whiskers were sectioned every 5 mm with nail clippers. Each section was chopped and approximately 0.7 -1 mg of sample was placed in tin capsules for SIA. Subsamples close to the tip were merged in order to get the required mass.

2.2.2 *Dentine sampling*

About 0.7 -1 mg of bulk canine dentine powder from each GLGs identified during the age estimation procedure was collected using a high-resolution micromill system (Merchantek) with a 300 microns drill. Dentine was drilled to a depth of 200 microns to avoid contamination with other layers. Samples of each GLG were collected to the last visible layer; GLGs corresponding to older ages were grouped during drilling and for these groups isotopic mean values were calculated. Dentine powder was not acidified since it was demonstrated that acidification did not modify the $\delta^{13}\text{C}$ values (Martin et al. 2011, Carrasco et al. 2018). A total of 377 powder samples were collected from the dentine of 38 drilled teeth.

2.2.3 Laboratory Analysis

Stable isotopes were analysed using a Costech (4010) elemental analyser coupled to a Thermo Scientific Delta V isotope ratio mass spectrometer at the Centre for Stable Isotopes of the University of New Mexico (UNM - CSI). Isotopic data was expressed in delta (δ) notation as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (‰), through the following equation:

$$\delta^{13}\text{C} \text{ or } \delta^{15}\text{N} (\text{‰}) = (R_{\text{sample}} / R_{\text{standard}}) - 1$$

Internationally accepted standards were Vienna Pee Dee Belemnite (V-PDB) and atmospheric N_2 for carbon and nitrogen, respectively. The delta values were calculated using multiple-point normalization. The internal laboratory standards used were soy protein, whey protein, casein, tuna, IAEA-N1, IAEA-N2, USGS-4 and USGS-43. The analytical precision (SD) of these internal standards was measured to be $<0.2\text{‰}$ for $\delta^{15}\text{N}$ values and $<0.04\text{‰}$ in the case of $\delta^{13}\text{C}$ values.

2.3 Data analysis

Shapiro Wilk and Bartlett tests were applied to verify the normality and homocedasticity of the isotopic data. To assess differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between historic and recent samples and age classes, Student's t tests and ANOVA or their equivalent non-parametric (Mann Whitney and Kruskal Wallis tests followed by post-hoc tests were used, respectively, with a significant level of $p < 0.05$.

Paired isotopic values of whisker and canine dentine (last deposited GLGs) ($n=5$) were compared using paired- t tests. The isotopic values from teeth used for this comparison represented a variable number of GLGs, due to the narrowing of the GLGs they were impossible to be individually sampled. As these seals were considered adults (14 to 26 yrs), GLGs averaged values were considered as representative of the adult resource use. In the case of whisker mean isotopic values, no models of whisker growth exist for crabeater seals. However, based on available whisker growth models for other Phocidae species, it was assumed that the whole whisker integrates the isotopic ratios for several months (up to one year) preceding the date of death (Hirons et al. 2001, Rogers et al. 2016).

In order to assess the individual variation in isotopic composition among individuals, linear plots of whisker $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values against the distance from the tip (oldest deposited) of the whisker were produced for each individual seal (Fig. S1).

GLGs were grouped into three age categories according to Laws (1953) and Bengston and Sniff (1981): yearling including just the 1st GLG isotopic value, subadult –from the 2nd GLG to age at sexual maturity (5th/6th GLG). GLGs corresponding to ages after sexual maturation (i.e. adult age category) were considered as those deposited after the change in average thickness of the GLGs, following Cebuhar et al. (in review).

Isotopic niches were calculated for historic and recent seals, using individual whisker $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, and for age classes using GLGs isotopic values. Standard Ellipse Areas (SEAc) corrected for small sample sizes were generated. The niche overlap between historic and recent seals, and among age classes were calculated as the percentage of a given SEAc that is overlapped with another SEAc. Niche ellipse areas and overlaps were calculated using SIBER package (Jackson et al. 2011) in R 3.6.0 (R Core Team 2019).

Generalized additive mixed models (GAMM) were applied to evaluate ontogenetic variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ among individuals. GLGs isotopic values from the first GLG up to the 13th GLG were used, as older GLGs were not individually sampled due to the narrow width of them. Individual identification was included as random effect. Models were structured using a Gaussian distribution, identity link function and smoothed curves (thin plate regression splines) as smoothing functions. Predicted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and their standard errors were calculated using the final models for nitrogen and isotopes and plotted against the explanatory variable (i.e. GLG). All models considered were subject to the customary residual analyses and were found to have a satisfactory fit (results not shown). The models were fitted under the statistical environment R 3.6.0 (R Development Core Team 2019) using the mgcv package (Wood, 2006).

3. Results

3.1 Historical vs. Recent isotopic values in whiskers

A total 28 fragments were obtained from 5 whiskers of the historic group, and 77 fragments from the 6 recent seals. Fragments that did not reach the minimum required

weight were grouped together. Mean $\delta^{13}\text{C}$ values in whiskers were similar for historic (-22.3 ± 1.3 ‰) and recent (-22.0 ± 1.18 ‰) seals (Student's t test, $p > 0.05$). On the other hand, recent seals (6.6 ± 0.69 ‰) had significantly higher nitrogen isotopic values in whiskers than historical seals (6.2 ± 0.58 ‰) (t-test, $p < 0.01$) (Table 1).

Paired isotopic values of dentine and whiskers of historic seals (Table 2) were significantly different (paired-t test, $p < 0.001$). Dentine $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were 1.6 ‰ and 2.5 ‰ higher, in average, respectively, than values in whisker.

According to whisker sections, the isotopic niche area of recent crabeater seals was slightly larger than the historic seals (Table 3, Figure 2). Ellipse areas of historic and recent seals showed a high overlap 55.3% and 70.2% of historic and recent seals SEAc, respectively).

Individual isotopic values along the length of historical whiskers showed no change, except for 2 individuals that showed a slightly increase towards the base of the whisker (i.e. most recently deposited). Whisker profiles of recent crabeater seals showed more variability, especially in the case of the carbon isotopic values (Fig. S1).

3.2 Ontogenetic variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in dentine canine of mummified seals

Stable isotope values in dentine GLGs varied from -23.3 to -18.6 ‰ for $\delta^{13}\text{C}$, and from 6.7 to 12.0 ‰ for $\delta^{15}\text{N}$ (Table 4). There were significant differences for both isotopes among age classes ($\delta^{13}\text{C}$: $F = 6.15$, $p = 0.002$; $\delta^{15}\text{N}$: $H = 11.43$, $p = 0.003$). Yearlings presented higher $\delta^{15}\text{N}$ values than subadults and adults (Wilcoxon tests, $p < 0.01$ and $p < 0.05$, respectively). Carbon isotopic values, on the other hand, were lower $\delta^{13}\text{C}$ in yearlings with respect to subadults and adults (Tuckey's post-hoc test, $p < 0.01$ and $p < 0.01$, respectively). No significant differences were found between adults and subadults for both isotopes.

Isotopic niche areas were larger in yearlings than in subadults and adults (Table 5, Figure 3) while areas of subadults and adults were similar in size. A partial overlap in the isotopic niches of yearlings with subadults and adults was observed, representing between 33.1 % and 34.7 % of the yearling's niche and 74.7 % and 79.5 % of the subadult and adult niches, respectively. On the other hand, a high overlap between adults and subadults was observed (84.7 % and 83.4 % of their niche area, respectively).

The GAMMs for historical crabeater seals showed significant effects of the GLG on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Table 6). Smooth splines showed an increase in $\delta^{13}\text{C}$ values with age, reaching a maximum at 6 yr and decreasing after this age. On the other hand, nitrogen isotope values showed an abrupt decrease after the first year and up to the 3rd GLG, where an inflection point is detected. GLG $\delta^{15}\text{N}$ values increase after this point, being stabilized from the fifth GLG onwards (Figure 4).

4. Discussion

In this work, isotopic carbon and nitrogen values for historical and recent samples of crabeater seals were reported for the first time for the eastern Antarctic Peninsula. No long-term (i.e. historical vs. recent) differences in carbon isotopic values in whiskers could be detected, thus revealing similar habitat use patterns of crabeater seals during the last 100 years. On the other hand, nitrogen isotopic values were significantly higher in modern seals, probably reflecting either a change in resource use or baseline isotopic values. Ontogenetic profiles obtained by sampling GLGs from canine dentine revealed changes in isotopic values derived from the contrasting values of milk and solid food (i.e. mainly krill) and, probably, the effect of growth rates on the discrimination of ^{15}N and ^{13}C during metabolism.

Whiskers from recent seals had moderately higher nitrogen isotopic values and a wider niche area than historic samples. These differences can be interpreted as changes in the diet composition of seals through time and/or a reflection of modified basal nitrogen values. Although the crabeater seal is highly specialized in the consumption of Antarctic krill (Croxall et al. 1985), it may include other prey species such as squids and fishes (Adam 2005). In this sense, a higher consumption of these higher trophic level prey may have increased the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of modern seals. The commercial krill fishery in Antarctica began in 1934, and since 1962 it has experienced a progressive development. It involves ships from different nationalities that had caught hundreds of tonnes until 1984, when overfishing started to impact krill predators' reproduction (Nicol and Foster 2003, CCAMLR 2018). In order to regulate this activity in the Southern Ocean, the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) has established the delimitation of marine management areas, among them, area 48.1 comprises the Antarctic Peninsula including Marambio/Seymour

Island adjacencies in the western Weddell Sea. Although high krill catches are reported for some of the subareas within the 48.1 area, catch reports for the eastern Antarctic Peninsula are considered low (CCAMLR 2018), either because of the lower abundance of krill (Atkinson et al. 2014), or due to the permanent sea ice cover. Nevertheless, although the most recent CCAMLR fishery report showed that in 2018, krill fishing Area 48.1 at Marambio region has increased from 25 tons in 1996 to 1092 tons in 2018, this amount is still minimal probably not potentially affecting the availability of this important resource for crabeater seals. Even though fisheries are unlikely a source for a lower availability of krill and, consequently, the incorporation of higher trophic level prey in crabeater seals diet, it was well documented that in 1900s whaling drastically reduced Southern Ocean baleen whale populations, leading to an increase in krill biomass (Southwell et al. 2008). Therefore, it was hypothesized that this increased availability of krill resulted in an advantage to other krill consumers (e.g. penguins, seals, whales) allowing them to increase population levels (Estes et al. 2007, Southwell et al. 2008) and possibly to consume a high proportion of this prey. Therefore, a higher specialization in krill could not be discarded as the cause for the reduced niche area, and the lower $\delta^{15}\text{N}$ values found in historical samples of crabeater seals from the eastern AP.

In cases of low availability of krill, crabeater seals may increase the proportion of complementary prey in their diet (e.g. fish, cephalopods). In this sense, Huckstadt et al. (2012) reported variable percentage of krill contribution among individuals for crabeater seals from the Western AP. The western AP is a region that is being affected by increasing temperatures, which is altering the structure of the food web and thus, affecting the availability of krill to predators (Polito et al. 2011, Flores et al. 2012, Cavan et al. 2018, Seyboth et al. 2018). On the other hand, Brault (2017) showed that subfossil crabeater seals from the Ross Sea had higher bulk $\delta^{15}\text{N}$ values than modern ones. However, by analysing compound specific isotopes of amino acid, the author concluded that this difference was not due to a shift in baseline isotopic values, but a change from a diet of subfossil crabeater seals including prey of higher trophic level to a more specialized krill-based diet in the modern ones (Brault 2017). Baseline information for the Weddell Sea is virtually inexistent. In this way, it is difficult to interpret changes in either the baseline vs trophic shifts for these groups of seals

Nevertheless, a change in basal nitrogen values in this region could not be discarded, and should be better investigated through compound-specific isotope analysis of amino acids (CSI-AA) in order to distinguish potential isotopic baseline shifts *versus* trophic habits on the foraging ecology of these seals. Considering the contrasting effects of the global climate change between these two regions of the AP, we recommend to conduct more research focusing on assessing the role of baseline isotopic values in the isotopic patterns found in the crabeater seals from the eastern AP

Furthermore, ontogenetic profiles of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in dentine of mummified crabeater seals showed more detailed differences which may suggest differences in foraging habits among age classes. Yearlings had higher $\delta^{15}\text{N}$ values than adults and subadults, probably influenced by the lactation period, as well as the consumption of solid food after weaning. This high $\delta^{15}\text{N}$ values in the first GLG showed a marked decreasing trend until the 3rd GLG. This change in the trajectory of nitrogen isotopic values in ontogenetic series is usually interpreted as the signal of weaning in pinnipeds (e.g. southern elephant seals, *Mirounga leonina*, Martin et al. 2011, fur seals, *Arctocephalus* sp., Albernaz et al. 2017, sea lions, *Eumetopias jubatus*, fur seals, *Callorhinus ursinus* and northern elephant seal, *Mirounga angustirostris*, Hobson and Sease 1998) as well as other marine mammals (e.g. killer whales, *Orcinus orca*, Newsome et al. 2007, *Tursiops tuncatus*, Fruet et al. 2015). Milk is synthesized from the catabolism of mother's tissues, so during the lactation period pups occupy a higher trophic level than its mothers (Newsome et al. 2006). Crabeater seals usually lactate for a short period of about 3 to 6 weeks after birth (Bengston 2009) and the milk composition is extremely rich in nutrients and polyunsaturated fatty acids (Green et al. 1993). This lactating period is followed by an abrupt weaning after which the pup needs to rely on this energy budget during the post-weaning fast (Laws 1957, Green et al. 1993), until they start feeding on solid food around 10 days after weaning (Shaugnessy and Kerry 1989). Therefore, the initial high $\delta^{15}\text{N}$ values, showed for the GLG 1 can be attributed both to the milk ingestion and to the catabolism of the pup on tissues during the post-weaning fast.

After this initial high $\delta^{15}\text{N}$ values, a decreasing trend was observed until GLGs 2-3. Lower nitrogen isotopic values for these GLGs were expected due to the absence of the

lactation signal and are probably different from the other GLGs attributed to the sub-adult period (GLGs 4 and 5/6) due to differences in forage habitats. Since younger seals forage less efficiently and present less diving ability than seals for older ages, they might be exploring inshore foraging habitats (Brault 2017, Aubail et al. 2011). The decreasing trend towards the age at sexual maturity (Bengtson & Laws 1983, Harding & Härkönen 1995, Cebuhar et al. in review) marked by an inflection point after which nitrogen isotopic values rise again, might be related to higher growth rates of immature seals (Laws 1957). High growth rates of sub-adult seals can affect the trophic discrimination factor, thus decreasing the nitrogen isotopic values in the predator tissues, due to lower $\delta^{15}\text{N}$ discrimination during tissue synthesis (Newsome et al. 2009, Albernaz et al. 2017). After sexual maturation, a greater investment in reproduction and lowered growth rates towards the physical maturation may result in higher trophic discrimination factors, thus probably explaining the increasing trend in the older GLG $\delta^{15}\text{N}$ values.

Carbon isotopic values also showed changes in the ontogenetic profiles with values showing an increasing trend from the first GLG until around the 6th where a change in the trajectory towards a smoothed decreasing trend in values was detected. Therefore, the same factors affecting nitrogen isotopic values seemed to influence $\delta^{13}\text{C}$ values as well. The high lipid content of milk is usually associated to lower $\delta^{13}\text{C}$ values found in pup tissues from several species (*Orcinus orca*, Newsome et al. 2009, *Mirounga leonina* Martin et al. 2011, *Arctocephalus sp.*, Albernaz et al. 2017). In addition, differences in $\delta^{13}\text{C}$ values exhibited by yearlings and adults and subadults can indicate some differences in feeding habitats among these groups (Zhao et al. 2004). Since it is common to find greater $\delta^{13}\text{C}$ values in benthic/nearshore habitats compared to more offshore/pelagic habitats, these lower values of $\delta^{13}\text{C}$ suggest that yearlings forage mainly in the water column (Aubail et al. 2011), while adults could take advantage of their diving capacity to forage at deeper depths (Burns et al. 2004). In agreement with our results, lower $\delta^{13}\text{C}$ in young seals were also found by other authors (Zhao et al. 2004, Aubail et al. 2011). Therefore, differences in foraging behaviour such as diving capacity could explain these isotopic values (Burns 1999). Several authors have reported differences in diving abilities and foraging behaviour between adults and juvenile seals resulting in ontogenetic shifts in diet (Burns 1999, Holst et al. 2001, Born

et al. 2004, Aubail et al. 2011, Brault et al. 2019). Host et al. (2001) reported that yearlings forage less efficiently in comparison with older seals which can be the cause for the ontogenetic differences shown in isotopic values.

5. Conclusion

The isotopic niche of historic and recent crabeater seals from the eastern Antarctic Peninsula seem to have remained relatively unaltered during the XX and beginning of the XXI centuries. However, slightly higher nitrogen values a larger isotopic niche could be detected in the recent samples, that was attributed to moderately decrease in the contribution of krill to the diet of these seals. Ontogenetic profiles of isotope values in historic samples showed opposite changes in the trajectories of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, probably resulting from the consumption of milk, the post-weaning fast and also due to the high growth rates of the immature animals. After sexual maturity, trends in the isotopic values seemed to stabilize, reflecting similar trophic resource use in the long term of adult seals.

This work contributes to the knowledge and better understanding of this Antarctic species in the Weddell Sea ecosystem, which is less studied in comparison to the west AP, mainly due to its permanent ice cover that imposes logistic constraints to fieldwork.

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Table 1. Stable isotope values of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) (mean $\pm$ standard deviation) in whiskers of historic (i.e. mummified) and recent crabeater seals (*Lobodon carcinophaga*) from Seymour Island, eastern Antarctic Peninsula.

Time period	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
	Mean $\pm$ SD	Mean $\pm$ SD
Historic	-22.3 $\pm$ 1.3	6.2 $\pm$ 0.6
Recent	-22.0 $\pm$ 1.2	6.6 $\pm$ 0.7

Table 2. Comparison of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (in ‰) in whiskers (mean of values for subsamples) and dentine from the last GLGs (most recently deposited) from the same individual

Id - sample	Whisker		Dentine		Offset	
	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
Lc11	-21.4	6.4	-20.7	9.3	0.7	2.9
Lc12	-21.7	6.4	-20.1	8.7	1.6	2.3
Lc14	-24.6	5.3	-22.0	7.7	2.6	2.4
Lc15	-22.4	6.7	-20.5	9.2	1.9	2.5
Lc19	-22.0	6.4	-20.7	8.6	1.3	2.2

Table 3. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic niche metrics (in ‰²) for historical and recent crabeater seals (*Lobodon carcinophaga*) from Seymour Island, eastern Antarctic Peninsula. Standard ellipse areas (SEA), standard ellipse areas corrected (SEAc) for small samples and total area of the convex hull (TA) are indicated for each group.

	Historical	Recent
TA	5.51	8.67
SEA	1.80	2.35
SEAc	1.88	2.38

Table 4. Stable isotope values of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) (mean $\pm$ standard deviation) in bulk dentine of canines from crabeater seals (*Lobodon carcinophaga*) from Seymour Island, eastern Antarctic Peninsula.

Age Class	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
	Mean $\pm$ SD	Mean $\pm$ SD
Yearlings	-21.6 $\pm$ 0.9	8.9 $\pm$ 1.2
Subadults	-21.1 $\pm$ 0.8	8.2 $\pm$ 0.6
Adults	-21.1 $\pm$ 0.8	8.3 $\pm$ 0.6

Table 5. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic niche metrics (in ‰²) for yearlings, subadult and adult of crabeater seal (*Lobodon carcinophaga*) from Seymour Island, eastern Antarctic Peninsula. Standard ellipse areas (SEA), standard ellipse areas corrected (SEAc) for small samples, total area of the convex hull (TA) are presented for each age class.

	Yearlings	Subadults	Adults
TA	10.9	8.3	9.0
SEA	3.1	1.4	1.4
SEAc	3.2	1.4	1.4

Table 6. Summary of the output from the Generalized Additive Mixed Models (GAMM) applied to analyse the effect of GLG on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in dentine of historic crabeater seals (*Lobodon carcinophaga*) from Seymour Island, eastern Antarctic Peninsula.

Model	Smooth terms			
	R^2	Edf	F	Prob
GAMM $\delta^{13}\text{C}$	0.05	3.2	5.3	0.001
GAMM $\delta^{15}\text{N}$	0.08	5.8	7.9	<0.001

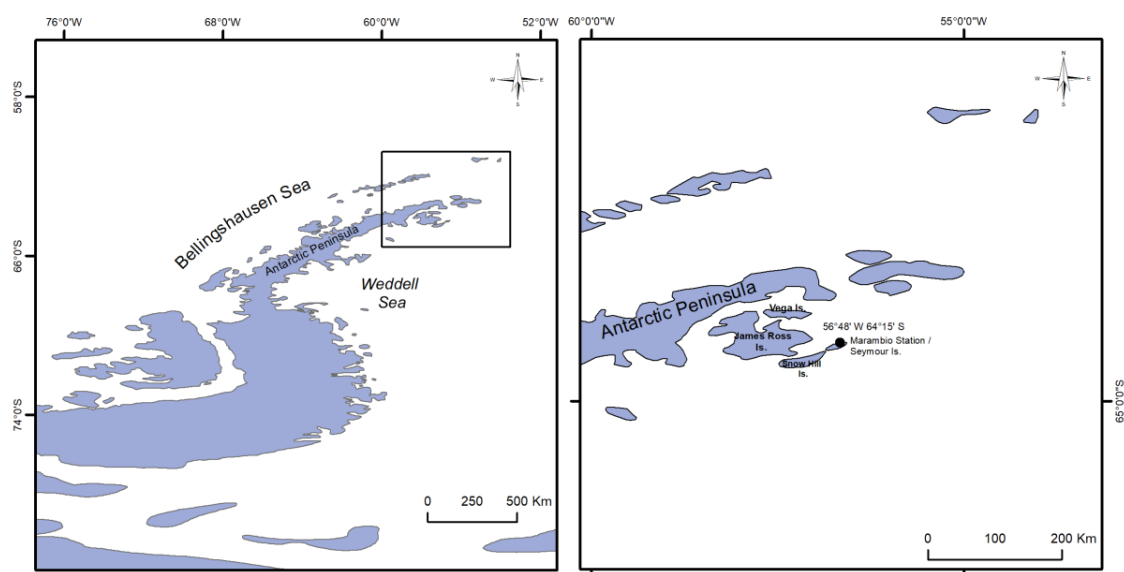


Fig. 1 Map of the sampling area of crabeater seal (*Lobodon carcinophaga*) in East Antarctic Peninsula on Weddell Sea

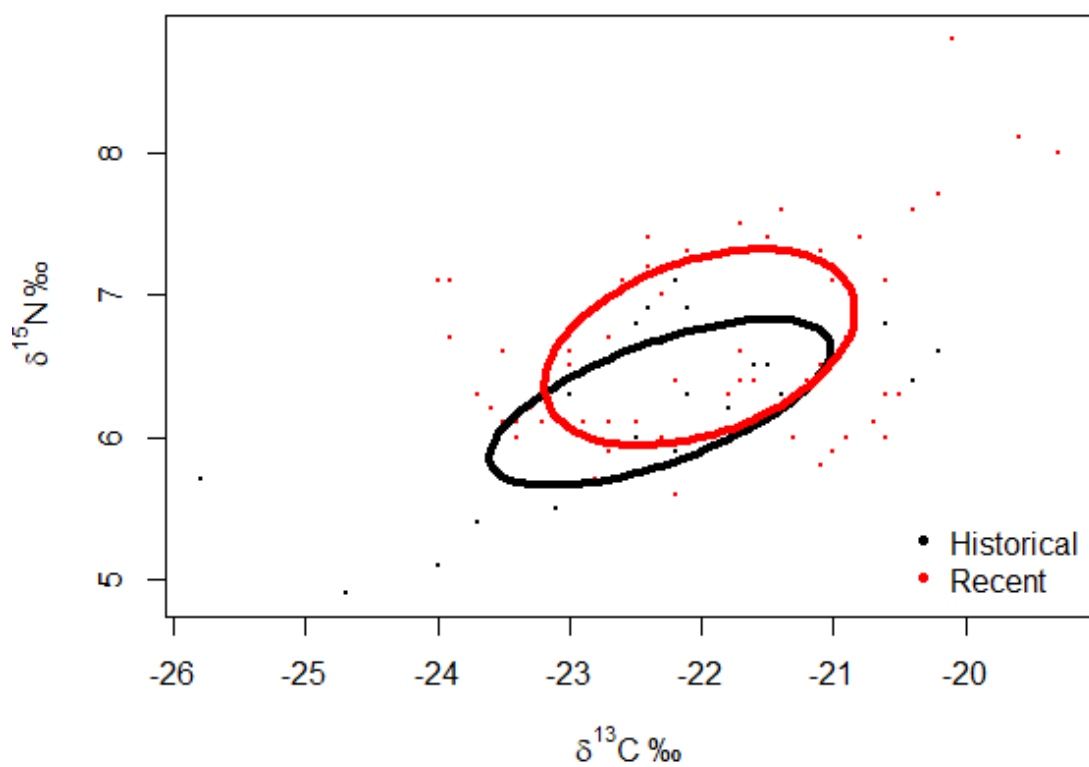


Fig. 2 Isotopic standard ellipses representing variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for historical and recent samples of whiskers of crabeater seal (*Lobodon carcinophaga*).

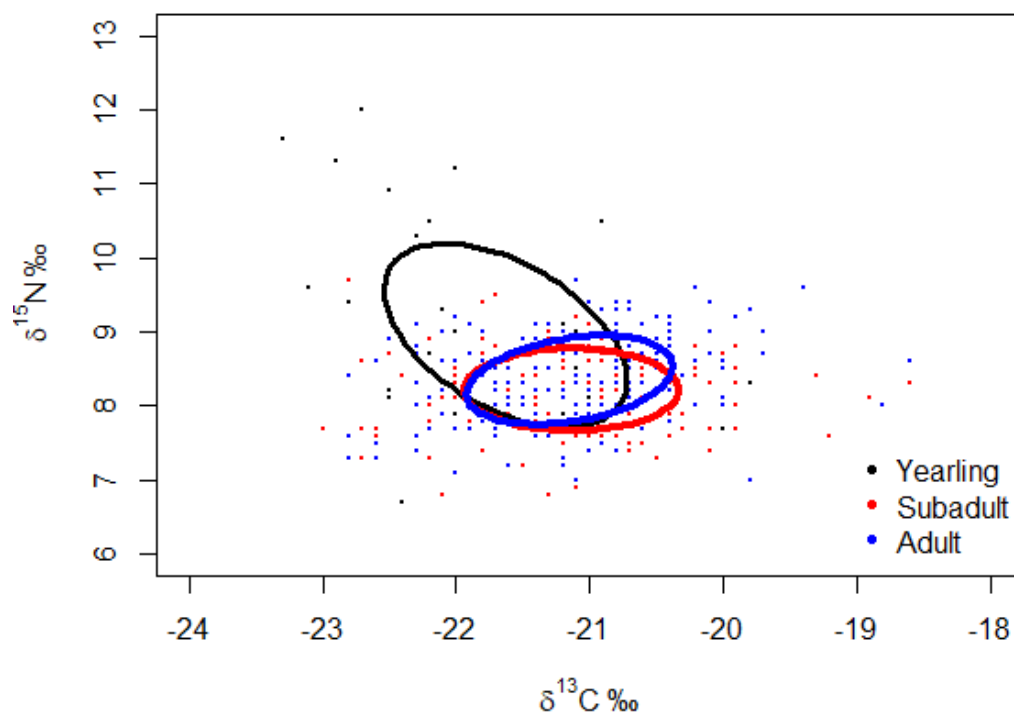


Fig. 3 Isotopic standard ellipses representing the variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in canine dentine from yearling, subadult and adult historical crabeater seals (*Lobodon carcinophaga*).

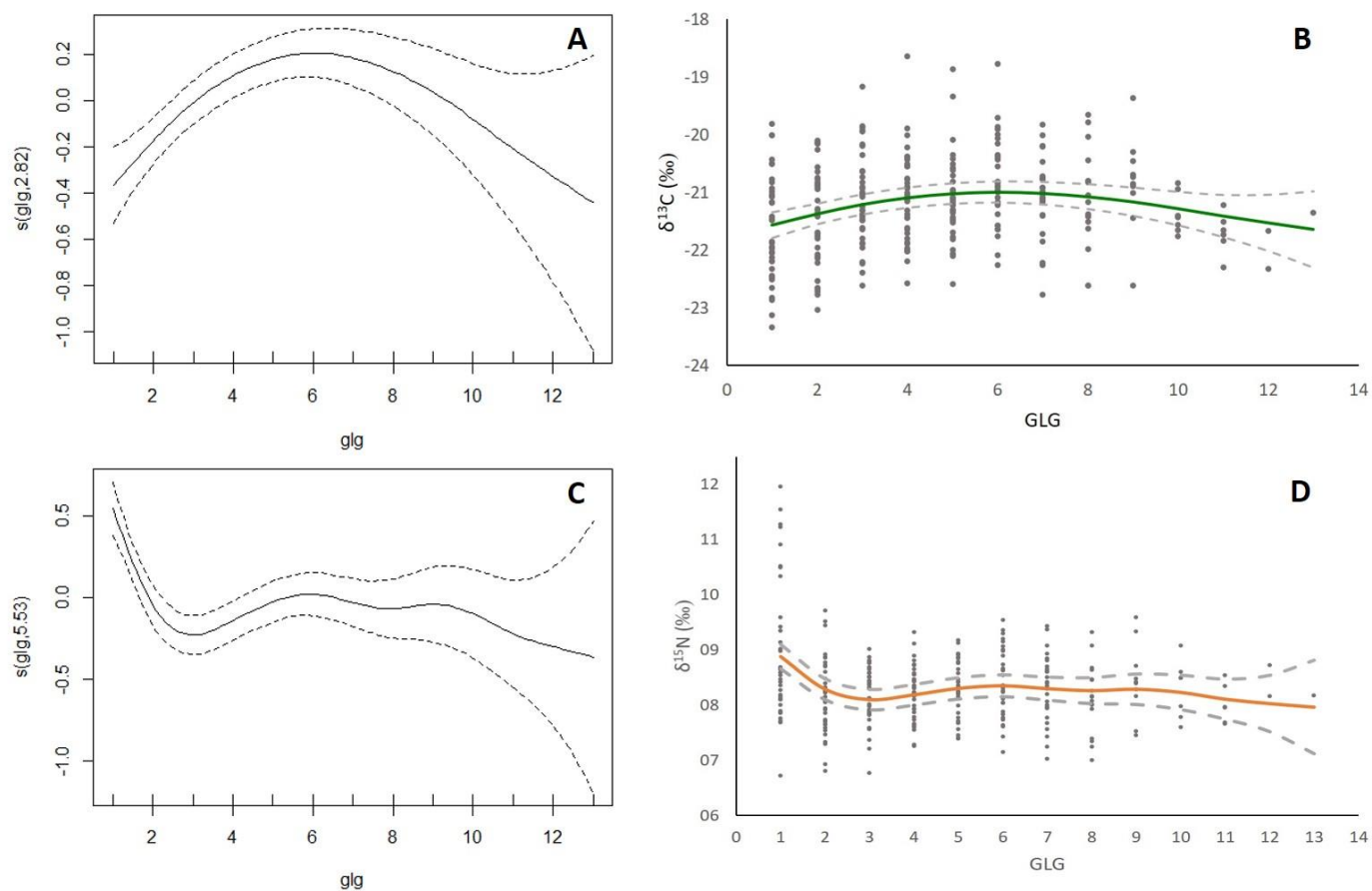


Fig. 4 Generalized Additive Mixed Model (GAMM) smoothing splines and 95% confidence interval (dotted lines) fit to $\delta^{13}\text{C}$ (A) and $\delta^{15}\text{N}$ (C) values and GLGs of crabeater seals (*Lobodon carcinophaga*). Estimated smoothers (thick green and orange lines) and $\delta^{13}\text{C}$ (B) and $\delta^{15}\text{N}$ (D) data (dots) are shown.

SUPPLEMENTARY MATERIAL

Table S1. List of samples of crabeater seals (*Lobodon carcinophaga*).

ID	Date	Tissue-Tooth/whisker	Sampled GLG	Age	Whisker size (mm)	Number of samples
1	Historical	Tooth	3	3	-	-
2	Historical	Tooth	4	4	-	-
3	Historical	Tooth	2	2	-	-
4	Historical	Tooth	11	20	-	-
5	Historical	Tooth	11	31	-	-
6	Historical	Tooth	7	7	-	-
7	Historical	Tooth	14	18	-	-
8	Historical	Tooth	6	6	-	-
9	Historical	Tooth	5	5	-	-
10	Historical	Tooth	7	22	-	-
11	Historical	Tooth/ whisker	7	22	26.5	5
12	Historical	Tooth/ whisker	8	14	15	3
13	Historical	Tooth	8	10	-	-
14	Historical	Tooth/ whisker	8	14	20	4
15	Historical	Tooth/ whisker	10	21	24	5
16	Historical	Tooth	9	9	-	-
18	Historical	Tooth	10	23	-	-
19	Historical	Tooth/ whisker	11	25	56	7
20	Historical	Tooth	7	7	-	-
22	Historical	Tooth	6	7	-	-
23	Historical	Tooth	10	20	-	-

24	Historical	Tooth	12	22	-	-
26	Historical	Tooth	9	12	-	-
27	Historical	Tooth	8	8	-	-
28	Historical	Tooth	7	12	-	-
29	Historical	Tooth	8	10	-	-
31	Historical	Tooth	9	13	-	-
32	Historical	Tooth	9	13	-	-
33	Historical	Tooth	12	19	-	-
34	Historical	Tooth	8	12	-	-
36	Historical	Tooth	8	16	-	-
38	Historical	Tooth	6	6	-	-
39	Historical	Tooth	8	18	-	-
40	Historical	Tooth	6	9	-	-
43	Historical	Tooth	6	6	-	-
44	Historical	Tooth	4	6	-	-
46	Historical	Tooth	5	25	-	-
48	Historical	Tooth	9	16	-	-
Lc1	Recent	Whisker		Adult	52	11
Lc2	Recent	Whisker		Subadult	87	17
Lc3	Recent	Whisker		Subadult	56	11
Lc4	Recent	Whisker		Subadult	90	17
Lc5	Recent	Whisker		Adult	52	10
Lc6	Recent	Whisker		Adult	55	11

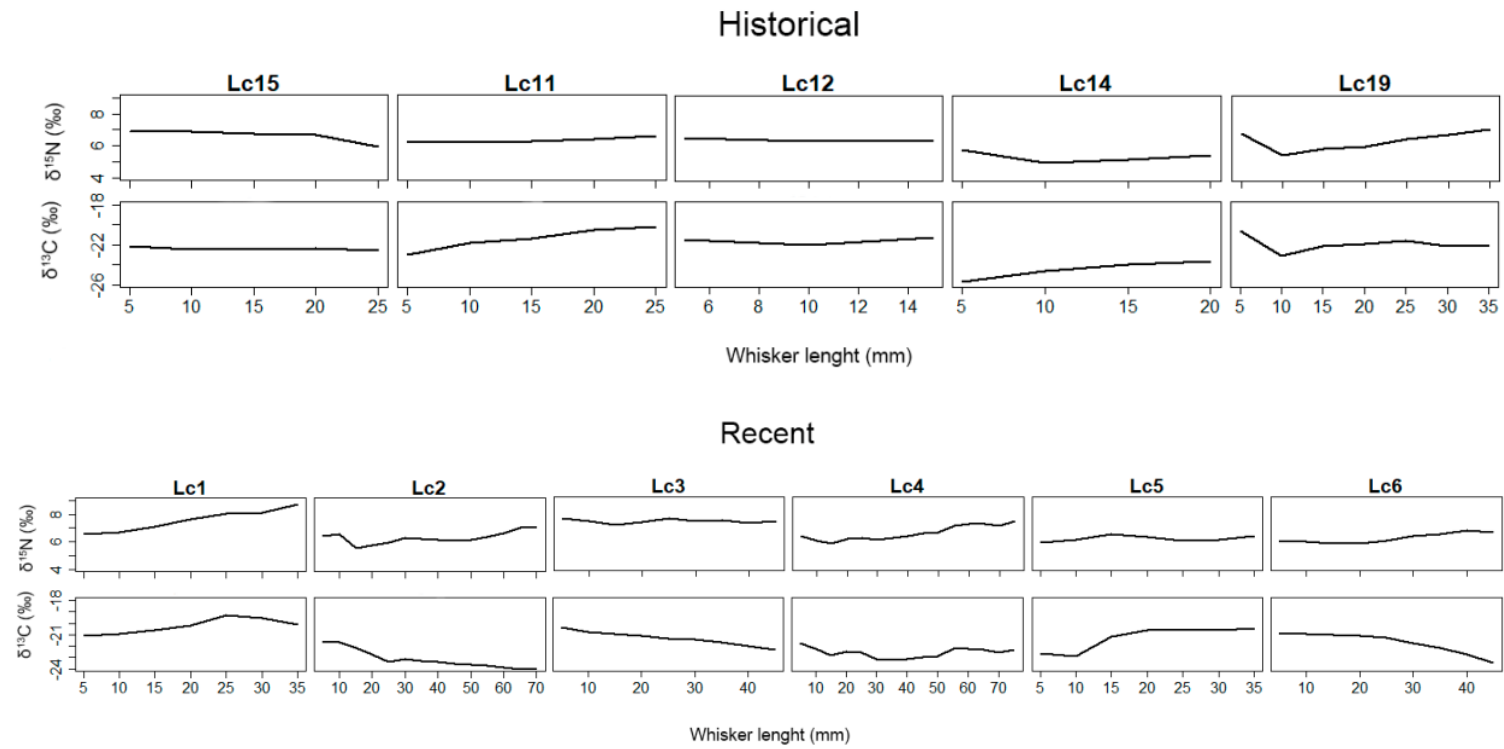


Figure S1. Whisker $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ individual variability for historical and recent crabeater seals (*Lobodon carcinophaga*).