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ORIGINAL PAPER

Variability in tissue-specific trophic discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) between Antarctic krill *Euphausia superba* and free-ranging *Pygoscelis* penguins

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Abstract

For top consumers in marine environments, trophic discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) between food and consumers' tissues are expected to be similar among related species. However, few studies conducted in the laboratory indicate a large variability among species, which should be potentially higher in free-ranging animals. Here, we test for differences in tissue-specific $\Delta^{13}C$ and $\Delta^{15}N$ values of two wild penguin species (Chinstrap *Pygoscelis antarctica* and Gentoo *P. papua*) breeding in sympatry at Livingston Island, Antarctica. A total of 41 adults and 28 chicks, and food items comprised exclusively by Antarctic krill (*Euphausia superba*, $n = 22$) in Chinstraps and almost exclusively in Gentoos, were sampled for stable isotope analyses. Overall, $\Delta^{13}C$ values varied between -1.8 and 4.0 ‰ and $\Delta^{15}N$ values ranged from 1.2 to 6.1 ‰, and these differed between species, tissues and age-classes. $\Delta^{13}C$ in adult penguins differed between species for feather and blood. Species-specific differences in $\Delta^{13}C$ and $\Delta^{15}N$ were seen in chick nail and muscle, while only $\Delta^{13}C$ values differed between species in feathers. Our results show that trophic discrimination factors can differ substantially between closely related species consuming similar prey, especially in $\Delta^{13}C$ value. Variation in $\Delta^{13}C$ was driven by species, tissue and age-class, while variation in $\Delta^{15}N$ was mostly driven by tissue type. Trophic discrimination factors may be associated to physiological and/or stress factors which may fluctuate in the wild, and this was particularly evident on chicks. This study highlights the use of diet-specialised species for the determination of trophic discrimination factors in the wild.

Keywords Carbon · Nitrogen · Stable isotopes · Chinstrap · Gentoo · Isotopic variability

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Introduction

Assessment of trophic ecology, through stable isotope analyses (SIA) relies on the principle that individual consumers acquire the isotopic composition of their prey (see Newsome et al. 2007 for a review). Thus, important ecological and trophic information on marine environments can be gathered with the use of stable isotopes of carbon ($^{13}C/^{12}C$, $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$, $\delta^{15}N$), the most assessed stable isotopes in marine organisms (Newsome et al. 2007). The use stable isotope data is now routinely applied for tracing food-web structure and function, e.g. species trophic positions can be estimated, and the relative dietary importance of food sources can be quantified (Phillips et al. 2014). However, basal information on trophic relationships between consumers and prey need to be assessed to investigate different aspects of trophic ecology and food-web structure (Hoeinghaus and Zeug 2008). Consequently, information on trophic discrimination factors between food and consumers' tissues

(also termed fractionation factors or enrichment factors, hereafter ΔX) are essential to reconstruct diets, assess variability in isotopic values, estimate trophic level, investigate food-web structures and understand how stable isotopes flow through food webs (Caut et al. 2009; Auerswald et al. 2010; Boecklen et al. 2011; Barton et al. 2019).

For practical reasons, ΔX values are often assumed to be constant (typically around + 1‰ for $\delta^{13}C$ and + 3.5‰ for $\delta^{15}N$, but see Hussey et al. 2014) but, in reality, many variables affect trophic discrimination factors (Barnes et al. 2007; Newsome et al. 2007), which may vary considerably (i.e. from − 3 to + 3‰ in $\delta^{13}C$, from − 1 to + 10‰ in $\delta^{15}N$, Peterson and Fry 1987). It is known that trophic discrimination factors can vary depending on the taxon. Taxonomically similar organisms generally have similar physiological processes, and thus it is expected to show ΔX values related to taxonomic identity (Vanderklift and Ponsard 2003). However, inherent variation (i.e. isotopic deviations that arise from individual differences in physiology despite consuming the same diet) is not limited to taxon, and intra-specific variability in ΔX values might be affected by physiological stresses such as lack of proteins (Vanderklift and Ponsard 2003; Barnes et al. 2008; Vander Zanden et al. 2012). This should affect individuals in different forms and be particularly relevant considering for instance different age-classes due to differentiated physiological characteristics and individual mass-adjusted metabolic rate (Vanderklift and Ponsard 2003; Costantini 2008). Thus, the effect of growth (and respective metabolism) may induce variability in ΔX values in chicks versus adults. This may result into overestimated populations' isotopic niches, which usually assumes the isotopic variation observed is purely due to differences in habitat and diet (Vander Zanden et al. 2012) due to movement between geographic locations with different baseline isotopic values (and their temporal variability) or shifts in dietary composition (Hinke et al. 2015). However, it is important to note that consumer stable isotope values can change over time due to shifts in dietary composition or movement between geographic location with differing isotopic baselines (Cherel and Hobson 2007; McMahon et al. 2013). There is also evidence the ΔX values can be dependent at the diet scale (Vanderklift and Ponsard 2003; Caut et al. 2009), for instance, the type of food (e.g. animal matter, plant matter, detritus), the diet protein quantity and quality, and the diet isotopic ratios (Vander Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; Robbins et al. 2005). Therefore, studies using SIA should adopt species-diet-specific values available in literature, when possible/appropriate (Cherel et al. 2005a). In the lack of such studies, average values from related species consuming similar prey should be adopted, acknowledging that a sensitivity analysis should be used in order to estimate how much error there might be (Cherel et al. 2005a). Additionally, trophic discrimination factors are tissue-specific due to differences in biochemical composition in lipid content and amino acids composition, also taking into account the variation in protein turnover leading to differences in isotopic incorporation rates among tissues (Wolf et al. 2009). In birds, for instance, feathers show typically higher $\Delta^{13}C$ and $\Delta^{15}N$ values than blood (Cherel et al. 2005a, 2014). As feathers are mainly composed of keratin and metabolically inert after synthesis, they reflect the diet during periods of growth, whereas blood retains information on diet up to the previous 3–4 weeks (Hobson and Clark 1992). Moreover, the catastrophic moult of adult penguins that renew their whole plumage while fasting ashore during a 2–4 weeks period leads to identical isotopic values in all the body feathers, thus contrasting with large inter-feathers $\delta^{13}C$ and $\delta^{15}N$ values induced by the protracted moult of other avian species (Carravieri et al. 2014). Thus, feather stable isotope values in penguins should reflect the diet prior to moult, since feather growth requires endogenous reserves to be synthetized, and this might result in enriched nitrogen values (Cherel et al. 2005b)(but see Polito et al. 2011c).

Currently, Bayesian models are widely used in ecological studies, including diet reconstruction through stable isotope mixing models (e.g. MixSIAR, Parnell et al. 2010). These models allow users to incorporate variability in trophic discrimination factors, but they are highly sensitive to variation in ΔX values (Bond and Diamond 2011; Healy et al. 2018). Therefore, the use of proxy trophic discrimination factors may not be appropriate for species or tissues where the specific value is unknown (Bond and Diamond 2011), leading to misused and misinterpreted results (Phillips et al. 2014). This assumption gains further relevance considering wild population studies where physiological and environmental factors may considerably influence variation in ΔX values among individuals (McCutchan et al. 2003; Barton et al. 2019).

Trophic discrimination factors are species-, tissue- and diet-specific, and although several experiments have been carried out in top predators (e.g. Lesage et al. 2002; Caut et al. 2011; Borrell et al. 2012; Giménez et al. 2016), these remain largely unknown for the great majority of upper-trophic-level marine organisms. Evidently, trophic discrimination factors cannot be predicted for all species in all environments (Barnes et al. 2007). The recently developed SIDER package for R (Healy et al. 2018) allows a greater degree of confidence on the choice of trophic discrimination factors, and it is especially recommended in cases where study-specific data from feeding trials is unavailable. However, the uncertainty of adopting precise ΔX values in food-web modelling is extremely high for most species and environments (Phillips et al. 2014), especially when there are no specific available studies (Barton et al. 2019). For instance, in the Southern Ocean, it is crucial to determine

species-specific trophic discrimination factors between consumers and the Antarctic krill *Euphausia superba* for a better understanding of the food-web structure (Polito et al. 2013). Antarctic krill is a key species that constitutes the link between lower- and upper-trophic levels, and thus relevant in food-web modelling (Polito et al. 2013; Reiss et al. 2017). Moreover, and from the taxon point of view, there are very few studies publishing trophic discrimination factors for consumers from different taxa in general (Caut et al. 2009), and seabirds in particular (Bond and Jones 2009). Most studies are conducted on captive animals on controlled diets and are not often measured experimentally in field because a varied diet increases potential errors when estimating trophic discrimination factors (Wolf et al. 2009). However, trophic discrimination factors derived from captive studies might not be realistic in wild-caught animals (e.g. due to different prey ingestion), and there is a lack of experimental studies conducted in wild animals testing and comparing how much ΔX values vary within and among species (Wolf et al. 2009).

In this study, we estimated trophic discrimination factors (Δ^{13}C and Δ^{15}N values) in two closely related seabird species breeding in sympatry at Livingston Island, South Shetland Islands (Antarctica), the Chinstrap *Pygoscelis antarctica* and Gentoo *P. papua* penguins. We focussed on potential variability in three factors: species, tissue and age-class. To our knowledge, no information on Δ^{13}C and Δ^{15}N values from any tissue of Chinstrap penguins exists, but Polito et al. (2011b) estimated 1.3‰ for Δ^{13}C and 3.5 ‰ for Δ^{15}N between herring *Clupea harengus* and Gentoo penguins breast feathers measured in laboratory. However, such values may vary considerably between penguin species, e.g. in the blood and feathers of King penguins *Aptenodytes patagonicus* and Rockhopper penguins *Eudyptes chrysocome* (Cherel et al. 2005a), or between tissues (blood and claws) in African penguins *Spheniscus demersus* (Barquete et al. 2013). Thus, while reviewing available studies on trophic discrimination factors in penguins' species, we foresee a large overall variation in Δ^{13}C and Δ^{15}N values in this study conducted in the wild. For each penguin species, we analysed different tissues in both adults (whole blood and feathers) and chicks (down feathers, nails and muscle) to determine species- and age-class-specific isotopic discrimination for each penguin's tissue and Antarctic krill. Euphausiids (mainly Antarctic krill) represented almost the entirety of items found in stomachs of Chinstrap penguins (i.e. 99.9%) and Gentoo penguins (i.e. 99.4% krill; 0.5% fish) on King George Island, South Shetlands Islands, during the breeding season (Panasiuk et al. 2020). Likewise, both penguin species are known to feed predominantly on Antarctic krill at Livingston Island (Miller et al. 2010; Polito et al. 2015; Dimitrijević et al. 2018), although fish may be also a component of the Gentoo Penguin's diet (Miller et al. 2010). This constitutes an excellent opportunity to study and determine for the first time precise trophic discrimination factors between the consumers and specific keystone prey (i.e. Antarctic krill) in two wild penguins' populations, under similar environmental conditions. Specifically, the goals of this study were to: (1) evaluate any differences in Δ^{13}C and Δ^{15}N values between two related and sympatric penguin species consuming a similar diet in a natural environment, (2) test for differences in tissues with different biochemical composition, and (3) test the potential effect of growth (i.e. between age-classes) in the two penguin's species by evaluating age-class-specific trophic discrimination factors in feathers of adults and chicks.

Materials and methods

Fieldwork was carried out simultaneously on Chinstrap and Gentoo penguins during the chick-rearing period at Livingston Island, South Shetland Islands, between December 2011 and January 2012. Specifically, tissue samples of Chinstrap penguins were collected at the breeding colony of Miers Bluff point on Hurd Peninsula (62° 43′ S, 60° 25′ W; January 9th) and the shore close to the Bulgarian Base (62° 38′ S, 60° 21′ W; December 17th) and those of Gentoo penguins at the breeding colony of Hannah Point (62° 39′ S, 60° 36′ W; December 20th) and Bulgarian Base (January 13th and 14th). All these areas are located on the south part of the island distanced by c.a. 11 km from each other (Fig. 1).

Blood samples (c.a. 1 mL blood from the brachial vein using 25G hypodermic needles) and 6–8 breast feathers were collected from randomly captured adults (Chinstrap: $n = 12$, Gentoo: $n = 29$). Tissue samples of penguin chicks (i.e. down feathers, toenails and muscle tissue) were collected from dead birds (Chinstrap: $n = 13$, Gentoo: $n = 15$), which had recently died from unknown causes (carcasses were in a very good condition, with very well-preserved stomach contents), and found randomly in their colonies. Six to eight down feathers were collected from chicks; toenails from the middle toe of the left leg, and muscle tissue (i.e. from thigh muscle) of the left leg. Blood samples, toenails and muscle tissue were stored frozen until isotopic analyses and feathers were stored in dry plastic bags. In chicks, toenails and muscle tissues grow slowly but continuously, and they reflect the diet from the moment they were formed (i.e. in the egg, the diet of the mother in the pre-laying period) and throughout the growth of the chick after the egg hatching (i.e. the onset of the breeding season prior to their death) (Vasil et al. 2012). On the other hand, as down feathers are metabolically inert after synthesis (Hobson and Clark 1992), their isotope signatures reflect mother's diet (during the pre-laying). Vasil et al. (2012) demonstrated that down feather and toenail isotope values from dead and living *Pygoscelis* penguin chicks displayed similar isotopic values (although muscle has not

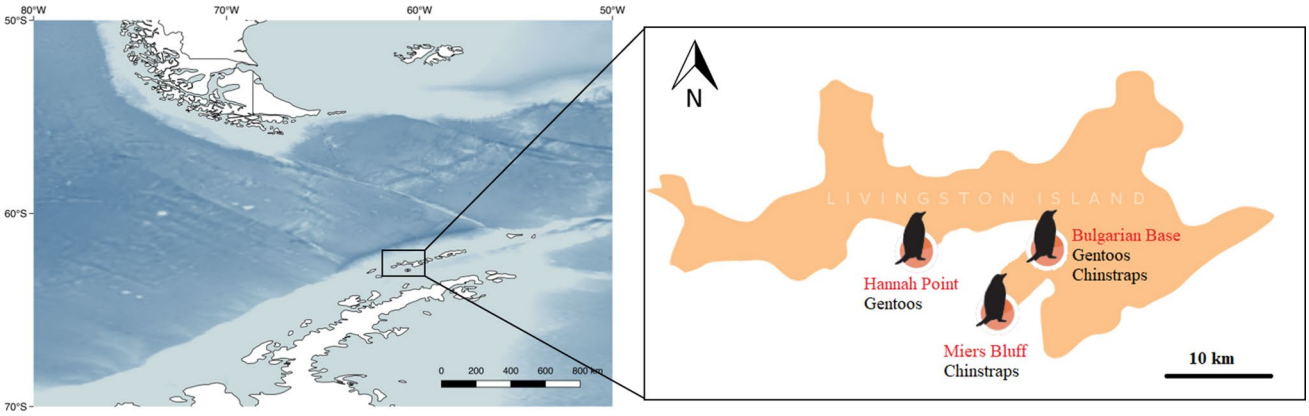

Fig. 1 Location of the study sites at Livingston Island, more specifically at Miers Bluff (62° 43′ S, 60° 25′ W), Bulgarian Base (62° 38′ S, 60° 21′ W) and Hannah Point (62° 39′ S, 60° 36′ W)

been tested), despite the cause of death, supporting the use of opportunistic sampling in stable isotope analyses.

Collection of faecal and stomach content samples to determine diet

Fresh feces (i.e. recently produced, still viscous, not dried) from adults (Chinstrap: $n = 59$, Gentoo: $n = 73$) were collected randomly from Miers Bluff (in December 28th, January 9th and 16th), Hannah Point (in December 20th, 29th and January 3rd) and the shore close to the Bulgarian Base (in December 15th, 24th and January 13th). Stomach contents were collected from 3 Chinstrap penguin chicks and 15 Gentoo penguin chicks (all dead chicks tissue samples were obtained from). All diet samples were stored frozen and analysed within 24 h in the laboratory of the Bulgarian base "St. Kliment Ohridski". Stomach contents of recently dead chicks and feces of adults were then examined, and the frequency of occurrence (FO; %), number (N) and mass (M; %, for stomach contents only) were determined for all prey following Xavier et al. (2015) and Petta et al. (2020). Crustacean (through their carapaces) and fish (through their otoliths) species were identified using reference collections and identification guides (Reid 1996; Xavier et al. 2020).

Antarctic krill exclusively dominated stomach contents/faeces in Chinstrap penguins and nearly exclusively in Gentoo penguins (Table 1). Antarctic krill was highly abundant (i.e. a total of 1526 individuals) and present in 100% of samples collected from individuals of both species and age-classes. Other prey was extremely rare, and only found in the diet of Gentoo penguins. These included 11 small crustaceans (i.e. amphipods and Mysidacea) from three adults and four chicks, and one individual fish (i.e. one otolith of mackerel icefish *Champsocephalus gunnari*) from one adult.

Antarctic krill sampling for stable isotope analyses

A total of 327 Antarctic krill specimens (87 from Chinstrap chicks and 240 from Gentoo chicks) were measured (carapace and total length) to test for any differences in the size of specimens consumed between species. Intact specimens of Antarctic krill (i.e. complete items, still fresh) were

Table 1 Number (N) and frequency of occurrence (FO; %) of prey species collected from feces of adults and from stomach contents of dead chicks of Gentoo penguins (*Pygoscelis papua*) and Chinstrap penguins (*Pygoscelis antarctica*) at Livingston Island, South Shetland Islands, Antarctica

Species	Antarctic krill *Euphausia superba*			Mackerel icefish *Champsocephalus gunnari*			Other crustaceans		
	N	FO (%)	M (%)	N	FO (%)	M (%)	N	FO (%)	M (%)
Chinstrap adult (feces, $n = 59$)	474	100	–	0	0.0	–	0	0.0	–
Gentoo adult (feces, $n = 73$)	725	100	–	1	1.4	–	3	4.1	–
Chinstrap chick (stomach contents, $n = 3$)	87	100	100.00	0	0.0	0.00	0	0.0	0.00
Gentoo chick (stomach contents, $n = 15$)	240	100	99.66	0	0.0	0.00	8	26.6	0.34

Mass (M; %) of prey species collected from stomach contents of dead chicks is also indicated. Other crustaceans include amphipods and Mysidacea

collected from Chinstrap chick stomachs ($n = 10$ Antarctic krill individuals) and Gentoo chick stomachs ($n = 12$ Antarctic krill individuals) for stable isotope analyses (Cherel 2008), although a potential initial degradation due to bacterial activity might have occurred.

Stable isotope analyses (SIA)

$\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) values were measured in penguin tissues and in Antarctic krill obtained from stomach contents of both species. Prior to SIA, feathers and toenails were cleaned of surface contaminants using successive rinses in a 2:1 chloroform–methanol solution. All tissues were dried in an oven at 60 °C for 24 h and then homogenised. Feathers were cut into small fragments (removing the base) and toenails and thigh muscle from penguins and Antarctic krill (removing the exoskeleton) were milled with a mortar and pestle. Lipids were extracted from muscle and Antarctic krill, using a 2:1 chloroform–methanol solution. The low lipid content of whole blood does not typically require lipid extraction (Cherel et al. 2005a).

Carbon and nitrogen isotope ratios were determined by a continuous-flow isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific, Bremen, Germany) coupled to an elemental analyser (Flash EA1112, Thermo Scientific) at Marine and Environmental Sciences Centre, University of Coimbra. Approximately 0.3 mg of each sample was combusted in a tin cup for simultaneous determination of carbon and nitrogen isotope values. Stable isotope values are present in the usual δ notation based on the Vienna Pee-Dee Belemnite (V-PDB) for carbon and atmospheric N_2 (Air) for nitrogen and expressed as: $\delta^{13}C$ (‰) or $\delta^{15}N$ (‰) $= [(R_{sample}/R_{standard}) - 1] * 1000$, where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$, respectively. Replicate measurements of secondary isotopic reference material (acetanilide STD, Thermo scientific-PN 338 36700) in every batch, indicate precision < 0.2 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

Calculation of diet-tissue trophic discrimination

The Antarctic krill sampled from the stomachs of Chinstrap and Gentoo penguin's chicks did not differ significantly in either stable isotopes (i.e. $\delta^{13}C$ and $\delta^{15}N$) and biometry measurements (i.e. carapace and total length) (see Table A1, Online Resource 1). These results show that the Antarctic krill eaten at the time of sampling and consumed by both penguin species is isotopically identical and have similar sizes, suggesting no Antarctic krill partitioning during the chick-rearing period of Chinstrap and Gentoo penguins. Thus, Antarctic krill obtained from Gentoo and Chinstrap chicks were pooled, and the resulting mean isotopic values were used to estimate trophic discrimination factors. Diet-tissue trophic discrimination factors between tissues of consumers (i.e. penguin species and age-classes) and their diet (i.e. Antarctic krill) was calculated as:

$$\Delta X_{\text{consumer - diet}} = \delta X_{\text{tissue}} - \delta X_{\text{krill}}$$

where X is ^{13}C or ^{15}N. The notation $\Delta X_{\text{consumer-diet}}$ was calculated for each individual based on its specific δX_{tissue} value and the overall mean δX_{krill} value of Antarctic krill (i.e. − 25.53‰ for $\delta^{13}C$ and 5.61‰ for $\delta^{15}N$, $n = 22$), and was abbreviated to ΔX in this study.

Data analyses

All data were tested for normality (Kolmogorov–Smirnov test) and homogenous variances (Levene's test). Groups (i.e. Antarctic krill sampled from the stomach of Chinstrap and Gentoo penguin chicks, and stable isotope values of adults and chicks) were compared using Mann–Whitney *U* tests (for non-parametric data, i.e. biometric measurements of Antarctic krill) and *t*-tests (for parametric data, i.e. stable isotope values). Table A2 (Online Resource 1) shows stable isotope values for the different tissues in adult and chicks of Chinstrap and Gentoo penguins, as well as species comparisons in $\delta^{13}C$ and $\delta^{15}N$ values (and respective $\Delta^{13}C$ and $\Delta^{15}N$ values), and Table A3 (Online Resource 1) details the individual isotopic data for both Antarctic krill and consumers. We tested for differences in $\Delta^{13}C$ and $\Delta^{15}N$ values between the two penguin species and among the different tissues with different biochemical composition in the adults and chicks separately using linear mixed-effects models. The potential effect of growth (i.e. between age-classes) was tested in the two penguin's species using feathers as it was the only tissue sampled for both adults and chicks. In these analyses the individual was treated as a random effect. Values are presented as mean ± SD. All statistical tests were performed with Statistica 10.0.

Results

Species-, tissue- and age-class comparisons in $\Delta^{13}C$ and $\Delta^{15}N$ values *of Pygoscelis* penguins

In adults, $\delta^{13}C$ values measured in the tissues (i.e. blood and feathers) of Gentoo penguins were higher than in Chinstrap penguins. However, similar $\delta^{15}N$ values between adults of the two species were detected (Fig. A1 and Table A2, Online Resource 1). We found statistically significant differences in adults' $\Delta^{13}C$ values between species (Chinstraps vs. Gentoos) and tissues (blood vs. feathers), with no tissue*species interaction (Table 2). There were also statistically significant differences in the $\Delta^{15}N$ values of adults between tissues, but not between species and tissue*species interaction (Table 2).

Table 2 Statistical results from mixed-effect ANOVA showing the effect of species (Gent—Gentoo penguins *Pygoscelis papua* and Chin—Chinstrap penguins *Pygoscelis antarctica*), and tissue type (blood, feathers, nails and muscle) and their interaction in adults and chicks

	Species			Tissue			Species*tissue			Individual	
	F	*p*	Main effect	*F*	*p*	Main effect	*F*	*p*	Main effect	*F*	***p***
Adults											
Δ^{13}C (‰)	18.86	**< 0.0001**	Gent > Chin	267.56	**< 0.0001**	Feathers > blood	0.91	0.3465	–	0.50	0.9829
Δ^{15}N (‰)	0.27	0.6080	–	28.81	**< 0.0001**	Feathers > blood	0.42	0.5210	–	1.07	0.4203
Chicks											
Δ^{13}C (‰)	43.73	**< 0.0001**	Gent > Chin	127.66	**< 0.0001**	Feathers = nails > muscle	2.09	0.1351	–	3.61	**< 0.0001**
Δ^{15}N (‰)	4.91	**0.0357**	Gent > Chin	38.11	**< 0.0001**	Feathers > mails = muscle	6.65	**0.0028**	Gent, Chin feathers > all others	2.81	**0.0009**

The individual was used as a random effect. Statistically significant results in bold

In chicks, statistically significant differences were found in Δ^{13}C values between species (Chinstraps vs. Gentoos) and tissues (feathers vs. nails vs. muscle), with no tissue*species interaction (Table 2). Also, statistically significant differences were found in chicks' Δ^{15}N values between species (Chinstraps vs. Gentoos), tissues (feathers vs. nails vs. muscle), and in tissue*species interaction (Table 2).

Considering age-classes, statistically significant differences in Δ^{13}C values of feathers were found either in Chinstraps (adults vs. chicks: $F_{1,23} = 35.04$, $p < 0.0001$) or Gentoos (adults vs. chicks: $F_{1,42} = 25.52$, $p < 0.0001$). Adults of both species showed higher Δ^{13}C values in feathers than chicks (Table 3). However, no differences were found in Δ^{15}N values between adults and chicks of both Chinstrap ($F_{1,23} = 1.27$, $p = 0.2715$) and Gentoo penguins ($F_{1,42} = 1.44$, $p = 0.2367$).

Trophic discrimination factors between penguins' tissues and Antarctic krill

A variation in trophic discrimination factors was detected among and within groups (Fig. 2). Overall, Δ^{13}C values varied between − 1.8 and 4.0‰ (i.e. 5.8‰) and Δ^{15}N values ranged from 1.2 to 6.1‰ (i.e. 4.9‰) and, in general, feathers showed the highest enrichment in both isotopes, and blood and muscle the lowest. We found negative Δ^{13}C values between Antarctic krill and the blood of both adult Chinstrap (− 0.4‰) and Gentoo penguins (− 0.1‰), and an increment of 2.0 and 2.7‰ in feathers, respectively (Table 3, Fig. 2). The two adult penguin species were similar in Δ^{15}N values for both blood (Chinstrap: 2.51 ± 0.37; Gentoo: 2.34 ± 0.30) and feathers (Chinstrap: 3.10 ± 0.78; Gentoo: 3.11 ± 0.81) (Fig. 2 and Table 2, and Fig. A1 and Table A2, Online Resource 1). However, in relation to penguins' chicks, our results show very distinct Δ^{13}C and Δ^{15}N values between both species (in general higher ΔX values in Gentoo chicks), with the only exception of Δ^{15}N values in feathers (Table 3, Fig. 2).

Discussion

This study highlights that trophic discrimination factors can differ substantially from typical values often assumed for free-ranging species, and especially in Δ^{13}C (overall Δ^{13}C values varied between − 1.8 and 4.0 ‰ and Δ^{15}N values between 1.2 to 6.1‰). This variation was species-, tissue-, and age-class-specific in free-ranging *Pygoscelis* penguins, possibly associated to biochemical and/or metabolic processes during tissue synthesis and physiological and/or stress factors which can fluctuate in the wild.

We assumed that sampled Chinstrap and Gentoo penguins fed exclusively upon Antarctic krill to estimate ΔX values, as previous studies demonstrated that both species are specialised on this item at South Shetland Islands (Miller et al. 2010; Panasiuk et al. 2020), and particularly at our study site (i.e. Livingston Island) (this study; Miller et al. 2010; Polito et al. 2015; Dimitrijević et al. 2018). Since this study was conducted in wild animals, our results on potential consumption of fish and other items could be underestimated, especially in Gentoo penguins, and we cannot be 100% assured that individuals did not eventually prey on other items. Indeed, an underestimated fish contribution to the diet might have occurred when evaluating diet of both penguin species (Polito et al. 2015). Our results based on representative faecal and stomach content samples showed that Chinstrap penguins were extremely specialised in Antarctic krill (at least during the short term no other prey was found), while some Gentoo penguins exhibited slightly broader dietary niche consuming a few other items. In fact, despite the high predominance of Antarctic krill, there is evidence of fish consumption by Gentoo penguins (up to 29% of diet composition by mass) at South Shetland Islands

Table 3 Estimates of trophic discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) between food (lipid-free diet, otherwise stated) and penguin tissues in this study (in bold) and from other available studies

Species, Age-class	Tissue	Diet[a], condition[b]	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)	Reference
King penguin *Aptenodytes patagonicus*, Adult	Whole blood	Herring, captive	−0.81	2.07	Cherel et al. (2005a)
	Feather	Herring, captive	0.07	3.49	Cherel et al. (2005a)
Humboldt penguin *Spheniscus humboldti*, Adult	Feather	Anchovy (lipids not extracted), captive	2.9 ± 0.2	4.8 ± 0.5	Mizutani et al. (1992)
African penguin *Spheniscus demersus*, Adult	Whole blood	Sardine / Hake (lipids not extracted, but samples were normalised in C), captive	−0.12 ± 0.42	2.45 ± 0.22/1.83 ± 0.30	Barquete et al. (2013)
	Plasma			2.49 ± 0.27/2.01 ± 0.30	Barquete et al. (2013)
	Erythrocyte			2.51 ± 0.21/1.86 ± 0.33	Barquete et al. (2013)
	Nail			1.53 ± 0.66	Barquete et al. (2013)
Rockhopper penguin *Eudyptes chrysocome*, Adult	Whole blood	Capelin, captive	0.02	2.72	Cherel et al. (2005a)
	Feather	Capelin, captive	0.11	4.4	Cherel et al. (2005a)
Magellanic penguin *Spheniscus magellanicus*, Adult	Whole blood	Anchovy, captive	0.41 ± 0.12	2.31 ± 0.17	Ciancio et al. (2016)
Little penguin *Eudyptula minor*, Adult	Whole blood	Sprat, captive	0.2	3.9	McKenzie (2011)
	Feather	Sprat, captive	1.1	3.5	McKenzie (2011)
Gentoo penguin *Pygoscelis papua*, Adult	**Whole blood**	**Antarctic krill, wild**	**−0.05 ± 0.44**	**2.34 ± 0.30**	**This study**
	Feather	**Antarctic krill, wild**	**2.73 ± 0.77**	**3.11 ± 0.81**	**This study**
	Feather	Herring, captive	1.3 ± 0.5	3.5 ± 0.4	Polito et al. (2011a)
	Eggshell organics	Herring, captive	1.4	1.8	Polito et al. (2009)
	Eggshell carbonate	Herring, captive	7.2		Polito et al. (2009)
	Eggshell membrane	Herring, captive	2.9	4.4	Polito et al. (2009)
	Egg Albumen	Herring, captive	0.8	4.7	Polito et al. (2009)
	Egg Yolk (lipid-free)	Herring, captive	0.0	3.5	Polito et al. (2009)
Gentoo penguin *Pygoscelis papua*, Chick	**Feather**	**Antarctic krill, wild**	**1.65 ± 0.42**	**3.42 ± 0.77**	**This study**
	Nail	**Antarctic krill, wild**	**1.18 ± 0.39**	**2.76 ± 0.55**	**This study**
	Muscle	**Antarctic krill, wild**	**0.15 ± 0.40**	**2.63 ± 0.53**	**This study**
Chinstrap penguin *Pygoscelis antarctica*, Adult	**Whole blood**	**Antarctic krill, wild**	**−0.43 ± 0.67**	**2.51 ± 0.37**	**This study**
	Feather	**Antarctic krill, wild**	**2.01 ± 0.68**	**3.10 ± 0.78**	**This study**
Chinstrap penguin *Pygoscelis antarctica*, Chick	**Feather**	**Antarctic krill, wild**	**0.54 ± 0.56**	**3.55 ± 1.19**	**This study**
	Nail	**Antarctic krill, wild**	**0.40 ± 0.51**	**1.89 ± 0.40**	**This study**
	Muscle	**Antarctic krill, wild**	**−0.63 ± 0.34**	**1.96 ± 0.34**	**This study**

Trophic discriminations factors are provided as mean ± SD when available

[a]Herring *Clupea harengus*, anchovy *Engraulis japonica*, sardine *Sardinops sagax*, hake *Merluccius paradoxus/capensis*, capelin *Mallotus villosus*, sprat *Sprattus sprattus* and Antarctic krill *Euphausia superba*

[b]Diet and study condition (i.e. captive or wild) are taken from references listed

in the past (i.e. between 1997 and 2004) (Miller et al. 2010). Polito et al. (2015), found that niche partitioning between Chinstrap and Gentoo penguins is a function of the higher krill consumption and a greater use of pelagic and mesopelagic foraging habitats by the former. It is known that Gentoo penguins feed more in benthic, inshore habitats than Chinstrap penguins (Polito et al. 2011c, 2015), leading to a more diversified diet which might influence isotopic values observed. However, other items in the diet of Gentoo penguins only comprised 1.2% of total food items consumed in 2011/2012, from which the majority (i.e. 1.1%, see Table 1) were small crustaceans (amphipods and Mysidacea) related to Antarctic krill. Additionally, Antarctic krill collected from both penguin species were isotopically similar. Although we acknowledge there is a small temporal mismatch between temporal integration of feces/stomach contents (snapshot) and the stable isotope method (tissue-specific isotopic turnover), this should not substantially drive the variation (and differences) in isotopic values between species. This is particularly true considering the time of sampling (i.e. in

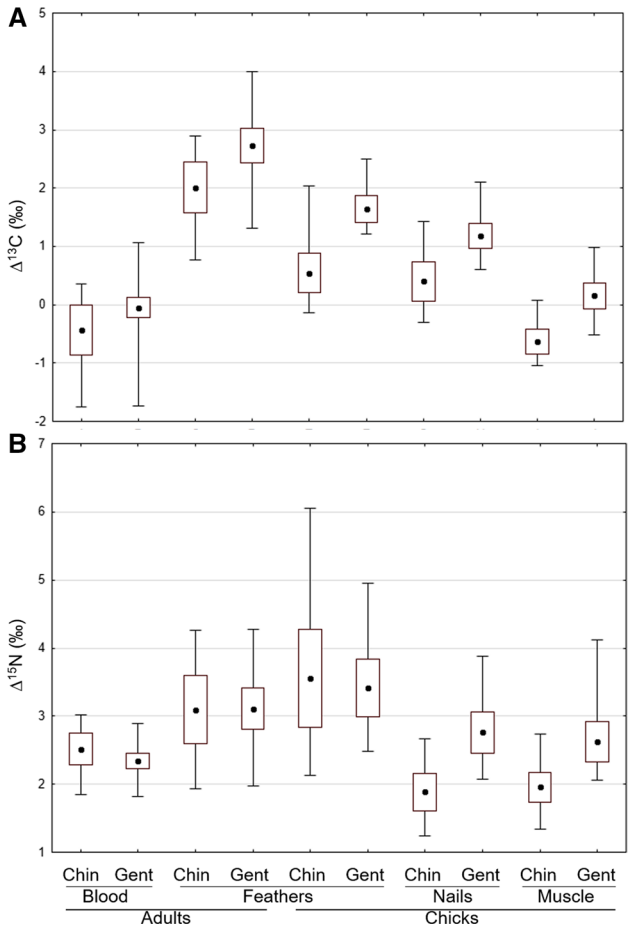

Fig. 2 Variability in trophic discrimination factors **a** $\Delta^{13}C$ (‰) and **b** $\Delta^{15}N$ (‰) between Antarctic krill *Euphausia superba* and tissues of Chinstrap (*Pygoscelis antarctica*) and Gentoo (*Pygoscelis papua*) penguins. Circles are mean values for each group, boxplots show 95% confidence intervals and lines are minimum and maximum values

the short term), reflected by tissues with high turnover rates such as blood, and the recently synthesised tissues of chicks (Hobson and Clark 1992; Vasil et al. 2012; Vander Zanden et al. 2015). However, since the isotopic composition of feathers reflects the diet of Gentoo and Chinstrap adult penguins during moult (Cherel et al. 2000), these are indicators of diet at the end of the previous breeding season (i.e. from late March 2011). The growth of new feathers occurs once during this period, near their breeding colonies (Trivelpiece et al. 1987), and we assumed that both species fed on Antarctic krill during such period with no isotopic changes over different time-scales (Polito et al. 2015; Dimitrijević et al. 2018). Still, just as for blood, $\delta^{15}N$ values of feathers did not differ between adults of the two species, suggesting that similar prey was consumed during both short- and long-term timescale. However, we acknowledge some variability in feathers could potentially derive from different prey and/or isotopic values of Antarctic krill during the previous breeding season and drive potential differences in feathers between adults and chicks.

To our knowledge, this is the first study determining trophic discrimination factors in Chinstrap penguins and in penguins' chicks, and the first aimed at free-ranging penguins in the wild (Table 3). There is only one study addressing trophic discrimination factors in feathers of adult Gentoo penguins, based on herring in captivity, which estimated 1.3‰ for $\Delta^{13}C$ and 3.5‰ for $\Delta^{15}N$ (Polito et al. 2011a). While $\Delta^{15}N$ values were similar, the $\Delta^{13}C$ values contrasted considerably with our results for the same tissue (i.e. 2.7‰ for $\Delta^{13}C$ and 3.1‰ for $\Delta^{15}N$). Following a similar pattern, our results showed more pronounced differences between the two studied species in $\Delta^{13}C$ (in both adults and chicks) than in $\Delta^{15}N$ (only on chicks). In a broad sense, such differences may thus arise from different environmental conditions (i.e. captivity vs. wild) and/or specific diet (i.e. herring vs. Antarctic krill), although we must highlight that small differences in the diet and/or foraging habitat may have driven differences between the two sympatric studied species. Interestingly, we found negative $\Delta^{13}C$ values in the whole blood of both Chinstrap and Gentoo adult penguins (and in muscle of Chinstrap chicks), indicating a depletion in ^{13}C regarding food consumed (i.e. Antarctic krill). Similarly, Cherel et al. (2005b) also found negative $\Delta^{13}C$ values (− 0.81‰) between blood of King penguins and food (i.e. herring), but in captivity. This is somewhat unexpected because normally there are both a ^{13}C and ^{15}N enrichment in consumers' tissues relative to foods, but these can vary depending on physiological and environmental factors (Newsome et al. 2007), especially in the wild where environmental conditions are not stable and stress responses of birds may differ considerably (Costantini 2008).

Differences were found in trophic discrimination factors among tissues. Information on tissue-specific trophic discrimination factors in *Pygoscelis* penguins is only available for captive Gentoo penguins. Polito et al. (2009) found a large variation in trophic discrimination factors across different egg components of Gentoo penguins, especially in $\Delta^{13}C$ values, suggesting that such variation likely reflects differences in biochemical and metabolic processes during tissue synthesis. Although we did not perform analyses on egg components, we agree with Polito et al. (2009), as both $\Delta^{13}C$ (in adults and chicks) and $\Delta^{15}N$ (in chicks) differed significantly between the tissues analysed in our study. In general, and independently on the species, $\Delta^{13}C$ and $\Delta^{15}N$ values are higher in feathers than in blood of adults (Table 3, see also Cherel et al. 2014), and our results also corroborate higher values of down feathers in comparison to muscle and nails (from chicks). Such differences in diet-tissue trophic discrimination factors are supposedly explained by tissue-specific biochemical composition (Wolf et al. 2009); accordingly, lipid content and amino acid composition in different

tissues are the main sources of variation in stable isotopes, and particularly in $\delta^{13}C$ (Cherel et al. 2014). However, the low C:N mass ratios in our samples (up to 3.5, Tables A1 and A2), which is a good proxy of lipid content in animal tissues (Post et al. 2007), suggest that amino acid composition was the main source of tissue's variation during this study. Still, the slightly higher C:N mass ratios (and respective lipid content) in blood of both adult penguins' species, especially in relation to feathers, may explain some of the variation detected between tissues (Cherel et al. 2014). A similar approach to ours was performed by Borrell et al. (2012) who estimate diet-tissue trophic discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) between a wild population of fin whales *Balaenoptera physalus* feeding on the euphausiid krill *Meganyctiphanes norvegica*. They found tissue-specific ΔX values (in bone and brain) ranging from 1.3 to 3.1‰ in carbon and from 2.0 to 4.3‰ in nitrogen. This variation was not as large as in penguins, but they attributed variation to tissue composition, which is consistent with our results.

To the best of our knowledge, no studies addressed trophic discrimination factors on penguins' chicks. Our results point to age-class-specific trophic discrimination factors, at least in feathers. However, potential underlying physiological or biochemical differences between adult feathers and chick down feathers that could contribute to differences in the stable isotope values and resulting discriminant factors might influence these outputs. Adults and chicks of both species presented distinct $\Delta^{13}C$ values (lower in chicks), but similar $\Delta^{15}N$ values, resulting thus into an apparent effect of growth (i.e. between chicks and adults) in the variability of $\Delta^{13}C$ values. The stress in wild birds associated with age-class-specific traits that are continually exposed to changing environments might affect metabolic rates of individuals (Costantini 2008). Moreover, the effect of anabolism/catabolism influencing in different ways organisms in a steady state or in those that decrease in body mass/fasting, may affect individual variation in trophic discrimination factors, especially between different age-classes (Vanderklift and Ponsard 2003; Aguilar et al. 2014).

While reviewing published studies on trophic discrimination factors estimated from penguins (Table 3), we found that, overall, mean ΔX values ranged from − 0.81 to 2.9‰ (7.2‰ if considering eggshell carbonate) in carbon and from 1.8 to 4.8‰ in nitrogen. These are mean values that do not take into consideration individual variability; still, it is evident that trophic discrimination values may vary considerable among species, tissues and age-classes (i.e. between adults and chicks, this study), and diet also should affect Δ*X* values (Vanderklift and Ponsard 2003). Potential differences between wild-caught and captive animals should be also taken into consideration, as our results displayed considerable differences in comparison with the bulk of $\Delta^{13}C$ values estimates from penguins in captivity. We draw attention to the potential high variability in trophic discrimination factors in birds, even among related species, because the current widely used mixing models are highly sensitive to variation in Δ*X* values (Bond and Diamond 2011). Limiting the potential bias to a minimum is currently required, and highly specialised species/populations are of great advantage to determine species-specific trophic discrimination factors in the wild, acknowledging the impossibility of conducting studies in every species and environments.

Conclusions

Contrary to the general assumption that trophic discrimination factors are relatively constant between taxonomically related species, we found statistically significant differences in $\Delta^{13}C$ and $\Delta^{15}N$ values between two sympatric *Pygoscelis* penguin species preying on the same food source (i.e. Antarctic krill). Differences in $\Delta^{13}C$ and $\Delta^{15}N$ values were particularly pronounced between Gentoo and Chinstrap penguins' chicks. In adults, however, differences in $\Delta^{13}C$ (but not in $\Delta^{15}N$) were meaningful across tissues analysed. While differences in $\Delta^{13}C$ were detected overall between species, tissues and age-classes (i.e. between adults and chicks), differences in $\Delta^{15}N$ were detected among tissues in both adults and chicks and between species only in chicks. This highlights that particular attention on the selection of $\Delta^{13}C$ values in trophic ecology models should be adopted.

This study provides the first data from penguins in the wild and also for chinstrap penguins and for penguins' chicks. Overall, our results show that Δ*X* values may differ substantially from typical ones often assumed (i.e. around + 1‰ for $\delta^{13}C$ value and + 3.5‰ for $\delta^{15}N$ value) in free-ranging individuals. Among other potential factors (e.g. prey, sex and population), this variation can be tissue-, species- and age-class-specific. Moreover, this study highlights inherent variation possibly associated to physiological and/or stress factors. While expanding substantially the range of species for which trophic discrimination factors are known, we strongly contribute to minimising potential bias in trophic ecology models by providing specific Δ*X* values in wild bird species. This gains further relevance considering that many species are dependent of Antarctic krill as a primary resource.

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Author contribution Study design: FRC, YC and JCX. Fieldwork and collection of the samples: JS, AB, NC and JCX. Data analysis and processing: FRC, YC and JCX. FRC led the writing of the manuscript. All authors edited and revised the manuscript, contributed critically to revisions and gave final approval for publication.

Declarations

Conflict of interest There are no conflicts of interest to declare.

Research involving animal rights The fieldwork was conducted under a research permit from the project PENGUIN of the Portuguese Polar Programme PROPOLAR. The sampling methods used for this research were in accordance with recommendations from the Scientific Committee for Antarctic Research (SCAR).

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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