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Blood-specific isotopic discrimination factors in the Magellanic penguin (*Spheniscus magellanicus*)

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RATIONALE: The use of stable isotopes for ecological studies has increased exponentially in recent years. Isotopic trophic studies are based on the assumption that animals are what they eat plus a discrimination factor. The discrimination factor is affected by many variables and can be determined empirically. The Magellanic penguin is a highly abundant marine bird that plays a key role in the southern oceans. This study provides the first estimation of the Magellanic penguin blood discrimination factor for ^{13}C and ^{15}N.

METHODS: A two and a half month feeding experiment was performed, in which ten captive penguins were fed their main natural prey (anchovy *Engraulis anchoita*). The discrimination factors were estimated by comparing anchovy δ^{13}C and δ^{15}N values (obtained with isotope ratio mass spectrometry using lipid-extracted and bulk anchovy muscle) with penguin blood δ^{13}C and δ^{15}N values.

RESULTS: Penguin blood was shown to be enriched, compared with anchovies, for ^{13}C and ^{15}N. No changes were observed in the stable isotope ratios of anchovies and discrimination factors during the experiment. The overall discrimination factors were 0.93 ± 0.12 (bulk) and 0.41 ± 0.12 (lipid-free) for ^{13}C; and 2.81 ± 0.17 (bulk) and 2.31 ± 0.17 (lipid-free) for ^{15}N.

CONCLUSIONS: Having an accurate discrimination factor for the studied species is key in any trophic or food web isotopic study. Comparisons of estimated diet-to-blood discrimination factors with published values of aquatic piscivore birds showed that the ^{13}C discrimination factor is particularly variable, and therefore ecologists should be cautious when using a surrogate value from other species. In this study, the Magellanic penguin discrimination factor of a tissue that does not require euthanasia was obtained, a fundamental input for trophic isotopic modeling of the species.

In recent years, stable isotope analysis (SIA) has become part of the ecologist's toolbox for the study of trophic ecology,[1] animal migrations,[2] ecosystem nutrient pathways, and other important topics.[3] Based on the assumption that animals are what they eat plus a discrimination factor (usually represented with a Δ symbol), diverse mixing models are used in trophic studies to estimate the trophic level or diet of predators. The Δ value varies as a result of many factors such as species, tissues within a single species (for which a 'tissue-to-diet discrimination' is estimated), diet composition, diet and trophic level of the organism,[4–6] and it can be determined empirically by comparing the isotope ratios between a predator and its food in controlled feeding experiments (e.g. [7–10]) or in natural experiments of free-living animals whose diet is precisely known.[11] Because these experiments are not easy to implement, there is a gap of information for this parameter in the literature and many diet or trophic ecology studies use a surrogate value of Δ,[5] either by using an average value for related grouped species (e.g. for aquatic organisms[12,13]) or by borrowing it from a closely related species (e.g. [14–16]). This practice incorporates a large fraction of the uncertainty into stable isotope mixing models.[17,18] In protected or endangered species non-lethal sampling for SIA is mandatory. For this reason, feathers and blood are commonly used for bird isotopic studies.[19,20]

The Magellanic penguin breeds along the Patagonian coast[21] and is mainly a schooling fish predator (anchovy *Engraulis anchoita* and sprats *Sprattus fuegensis* are its dominant prey[15,22–24]). Due to the large body size and population, this species consumes large amounts of prey in Patagonian coastal ecosystems during its breeding season.[25–27] This study attempts to provide the first estimate of blood Δ values for ^{13}C and ^{15}N of the Magellanic penguin, a dominant marine top predator,[25] which has a key role in the highly productive South West Atlantic Ocean, and for which no blood Δ values are available.

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EXPERIMENTAL

Experiment design and stable isotope analysis

A ten-week feeding experiment was performed where ten Magellanic penguins (7 males and 3 females) held in the Oceanarium Aquarium Mar del Plata were fed exclusively with anchovy (birds are regularly fed with anchovy and horse mackerel *Trachurus lathami* in captivity all year round) from the same batch. The experimental diet included fish specimens of similar size to the ones consumed by free-living birds.[28] During this period, five anchovy samples were randomly collected every week (length and weight of fish were recorded) and blood samples of 0.5 mL were obtained from the metatarsal veins of penguins during the second, fifth, and tenth week from the beginning of the experiment. Blood samples were preserved in 70% ethanol prior to C and N stable isotope analysis. Blood preservation in ethanol is suggested when no freezing is available, for which no significant effect has been shown.[29] A considerable effect has, however, been noticed in some cases, for example, in the ethanol-preserved blood C stable isotope ratios of the spectacled petrel *Procellaria conspicillata*.[30] Subsequently, anchovy muscle from the five fish collected on weeks 2, 4, 6, 8, and 9 and penguin blood samples were dried at 60°C for 48 h and then ground to a fine powder. Lipids from a subset of anchovies (weeks 2, 6, and 9) were extracted using a chloroform/methanol solution (2:1).[31] SIA was carried out at the Stable Isotope Facility, University of California, Davis (Davis, CA, USA). The stable isotope ratios were measured against the reference standards Vienna PeeDee Belemnite for ^{13}C and atmospheric air for ^{15}N expressed as δ values ‰:

$$X = [(\text{Rsample}/\text{Rstandard}) - 1],$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio, ^{13}C/^{12}C or ^{15}N/^{14}N.

The secondary isotopic reference materials used were Nylon (standard deviations (SDs) were 0.056 and 0.05 for δ^{13}C and δ^{15}N values, respectively), Bovine Liver (SDs 0.007 and 0.07 for δ^{13}C and δ^{15}N values, respectively), USGS-41 Glutamic Acid (SDs 0.17 and 0.16 for δ^{13}C and δ^{15}N values, respectively), and Glutamic Acid (SDs 0.06 and 0.26 for δ^{13}C and δ^{15}N values, respectively). Discrimination factors for ^{13}C and ^{15}N (Δ^{13}C and Δ^{15}N) were calculated by subtracting the mean isotopic ratio of anchovies from the penguin isotopic ratio.

Data treatment

Residuals were inspected for heterogeneity of variance and for normality, the latter by using visual Q–Q plots. To check if there was a change in size or stable isotope ratios in anchovies and penguins during the experiment, we ran a one-way analysis of variance (ANOVA) with week of sample as a categorical explanatory variable and δ^{13}C value, δ^{15}N value, length, and weight of anchovies as dependent variables. To investigate if there was an effect on the Δ value of the week of blood sampling and penguin sex, linear mixed-effect models (LMEs[32]) with normal distribution were used. Individual was used as a random effect to control for lack of independence between repeated measures over the same penguin. Sex and week were treated as fixed effects in the model. All statistical analyses were performed in R version 3.3.0[33] (NMLE package for the mixed-effects model[34]).

RESULTS

The stable isotope ratios ranged between –17.95 and –17.45 ‰ for δ^{13}C values, and between 17.86 and 18.43 ‰ for δ^{15}N values for Magellanic penguin whole blood, and between –19.52 and –17.18 ‰ for δ^{13}C values, and between 14.31 and 16.93 ‰ for δ^{15}N values for anchovy muscle (bulk and lipid-free samples, Fig. 1, Table 1). During the experiment, no

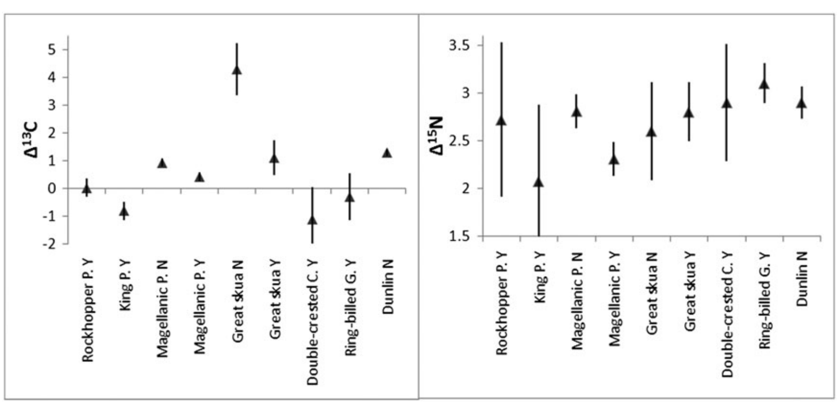

Figure 1. Comparison of ^{13}C and ^{15}N diet-to-blood discrimination factors of blood estimated for penguins and other aquatic bird species fed with fish. Triangles represent mean values ± SD. In the case where the SD of the discrimination factor was not mentioned in the study, the SDs of the bird's or prey's stable isotope ratios were used. The letter 'Y' denotes that lipids were extracted from prey items, and the 'N' denotes that they were not. Note the higher inter-species variability in Δ^{13}C and high intra-variability in Δ^{15}N. Data were taken from: Rockhopper Penguin (*Eudyptes chrysocome*) and King Penguin (*Aptenodytes patagonicus*),[8] Great Skua (*Catharacta skua*),[39] Double-crested Cormorant (*Phalacrocorax auritus*),[40] Ring-billed gulls (*Larus delawarensis*),[41] and Dunlin (*Calidris alpina pacifica*).[42]

Table 1. Length, size, and stable isotope ratios for anchovy muscle and penguin blood

	Anchovies						Magellanic penguins					
Week	weight (g)	length (cm)	δ^{13}C (‰)	δ^{15}N (‰)	δ^{13}Clf (‰)	δ^{15}Nlf (‰)	δ^{13}C (‰)	δ^{15}N (‰)	Δ^{13}C	Δ^{13}Clf	Δ^{15}N	Δ^{15}Nlf
1	25.31 ± 5.85	13.98 ± 0.54										
2	26.22 ± 5.05	13.7 ± 0.62	−18.72 ± 0.36	14.89 ± 0.61	−18.25 ± 0.10	15.40 ± 0.57	−17.68 ± 0.13	18.20 ± 0.19	0.90 ± 0.13	0.38 ± 0.13	2.85 ± 0.19	2.35 ± 0.19
3	27.21 ± 4.42	13.8 ± 0.45										
4	28.71 ± 4.49	14.06 ± 0.55	−18.66 ± 0.34	15.48 ± 0.35								
5	27.73 ± 4.76	13.96 ± 0.90					−17.70 ± 0.12	18.14 ± 0.16	0.88 ± 0.12	0.36 ± 0.12	2.79 ± 0.16	2.29 ± 0.16
6	24.45 ± 3.58	13.52 ± 0.62	−18.71 ± 0.58	15.58 ± 0.70	−18.02 ± 0.22	16.18 ± 0.75						
7	24.48 ± 2.72	13.6 ± 0.69										
8	27.56 ± 4.44	14.22 ± 0.67	−18.50 ± 0.23	15.26 ± 0.48								
9	30.21 ± 9.72	14.32 ± 1.33	−18.31 ± 1.01	15.51 ± 0.68	−17.91 ± 0.73	15.96 ± 0.69						
10	21.10 ± 3.21	12.72 ± 0.52					−17.57 ± 0.08	18.15 ± 0.15	1.01 ± 0.08	0.49 ± 0.08	2.79 ± 0.15	2.29 ± 0.15
Total	26.26 ± 5.25	13.78 ± 0.79	−18.58 ± 0.55	15.35 ± 0.59	−18.06 ± 0.43	15.85 ± 0.71	−17.65 ± 0.12	18.16 ± 0.17	0.93 ± 0.12	0.41 ± 0.12	2.81 ± 0.17	2.31 ± 0.17

Columns 4 to 9 refer to isotope ratios and 10 to 13 refer to discrimination factor (Δ) (mean SD). lf refers to lipid-free values, either of stable isotope ratios or discrimination factors.

changes were observed in either the length ($F_{(49)} = 1.12$, $p = 0.29$), weight ($F_{(49)} = 0.34$, $p = 0.56$), δ^{13}C values ($F_{(23)} = 1.43$, $p = 0.24$), or δ^{15}N values ($F_{(23)} = 1.54$, $p = 0.22$) of anchovies fed to penguins, or in the δ^{15}N values ($F_{(27)} = 0.32$, $p = 0.72$) and δ^{13}C values ($F_{(27)} = 0.39$, $p = 0.045$) of penguin blood.

The overall diet-to-blood discrimination factors were 0.93 ± 0.12 (bulk) and 0.41 ± 0.12 (lipid-free) for Δ^{13}C values; and 2.81 ± 0.17 (bulk) and 2.31 ± 0.17 (lipid-free) for the Δ^{15}N values. The Δ values estimated with lipid-free anchovy samples were different from those estimated with bulk samples in both isotopes (both t-test $t_{58} = 15.92$ for Δ^{13}C and 11.35 for Δ^{15}N; both $p < 0.001$) (Table 1).

The LMEs indicated no effect of sex or week sampled on the discrimination factors (estimated using both bulk and lipid-free anchovies) for Δ^{13}C (sex $F_{(1,18)} = 0.11$, $p = 0.74$, week $F_{(2,18)} = 0.11$, $p = 0.74$) and Δ^{15}N (sex $F_{(1,18)} = 0$, $p = 0.99$, week $F_{(2,18)} = 0.87$, $p = 0.43$).

DISCUSSION

Blood and feathers are routinely used as non-lethal tissue samples for stable isotope studies in bird species.[35] Magellanic penguins molt all their feathers at once, during periods of inshore fasting.[36] For this reason, the stable isotope ratios of feather samples contain little information about the trophic ecology of this species, which is restricted to a short time frame. This study provides the first estimation of diet-to-blood discrimination factors for the Magellanic penguin, one of the top marine consumers in terms of biomass of the Patagonian coastal ecosystems, and this constitutes the base for dietary isotopic modeling of this key species. Although the birds were fed with their main natural prey (schooling fish) during the experiment, Magellanic penguins breed along the Patagonian coast[37] and the diet from penguins in some colonies includes large fractions of other prey such as squid or crustaceans.[15,38] The Δ values presented here could differ in the penguins of these colonies that feed on other natural prey. The results underscore the importance of utilizing species-specific Δ values, as noted by other authors.[17] In addition, the Δ values seem to be particularly species-sensitive for the Δ^{13}C value (Δ^{13}C of birds fed with fish ranged from –1.1 to 4.3 – about 4 trophic levels, while the Δ^{15}N value varied by 1/3 of a trophic level between species, Fig. 1). It was also observed that lipid-extracted prey strongly affects the Δ estimation for both isotopes,[8,39] another consideration to be taken into account when choosing the discrimination factor for stable isotope modeling.

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