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Title

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Permalink

<https://escholarship.org/uc/item/8b0461kp>

Journal

Ornithology, 140(3)

ISSN

0004-8038

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Publication Date

2023-07-13

DOI

10.1093/ornithology/ukad013

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Peer reviewed

1 **Sulfur isotopic discrimination factors differ among avian tissues and diets: Insights**
2 **from a case study in Gentoo Penguins (*Pygoscelis papua*)**

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4

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13 **ACKNOWLEDGMENTS**

14 We thank S. Abel, K. Vires, J. Beck, K. McGrath, T. Solberg and D. Rivard and
15 the staff of the Scott Kingdoms of the Seas Aquarium at Omaha's Henry Doorly Zoo for
16 their invaluable assistance with this study. This research was funded by a Scientific
17 Committee on Antarctic Research (SCAR) Fellowship to N. Rosciano. This research as
18 completed in accordance with an approved IACUC protocol at Omaha's Henry Doorly
19 Zoo (HDZ#07-800). Authors' contribution: Conceived idea, design research (MP, NR),
20 collected data (MP), conducted laboratory work (MP, AS), analyzed data (MP, NR),
21 wrote the paper (NR, MP, AS), obtained funding (MP, NR).

22

23 **Data depository:** Data obtained for this study is available on Dryad:

24 <https://doi.org/10.5061/dryad.3j9kd51pk>

25 ABSTRACT

26 The use of stable isotopes of sulfur ($\delta^{34}\text{S}$) to infer avian diets, foraging habitats, and
27 movements is relatively uncommon, resulting in a lack of information on patterns of
28 $\delta^{34}\text{S}$ incorporation in avian tissue. In a controlled study of Gentoo Penguins (*Pygoscelis*
29 *papua*), we found that diet-tissue isotopic discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) differed
30 among egg components and feathers synthesized from a common diet, ranging from –
31 0.4 to –1.7 ‰. We also found that methodical choices such as lipid extraction and prey
32 tissue selection influenced calculated $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values. Specifically, $\Delta^{34}\text{S}_{\text{diet-tissue}}$
33 values were lower (i.e., more negative) when calculated using whole fish relative to fish
34 muscle and lipid-extraction biased egg yolk, but not fish tissue, $\delta^{34}\text{S}$ values. $\Delta^{34}\text{S}_{\text{diet-tissue}}$
35 values obtained for Gentoo Penguins fed a marine fish diet were generally lower than
36 those reported for freshwater fish consuming Double-crested Cormorants
37 (*Phalacrocorax auritus*), the only other bird species in which $\Delta^{34}\text{S}_{\text{diet-tissue}}$ has been
38 quantified. We found support for the hypothesis that tissue $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values are
39 inversely related to dietary $\delta^{34}\text{S}$ values in birds, similar to what has been observed in
40 mammals. Given this relationship, the discrimination factors reported here for Gentoo
41 Penguins may be broadly applicable to other avian species with a similar marine diet.
42 Finally, we provide recommendations for future studies seeking to quantify $\Delta^{34}\text{S}_{\text{diet-tissue}}$
43 in avian tissues and guidance to allow for greater application of sulfur stable isotope
44 analysis in ornithological research.

45 **Keywords:** Stable isotopes, Sulfur, Discrimination factors, Diet, Eggs, Feathers

46 LAY SUMMARY

- 47 • Stable isotope analysis of sulfur can provide new discoveries on birds' diets and
48 migration patterns if we gain a better understanding of how isotope values in
49 bird tissues are related to those in their diets.
- 50 • We measured the difference in sulfur stable isotope values between Gentoo
51 Penguins diets and their eggs and feathers in a controlled study.
- 52 • We found that the offset in sulfur isotope values from diets differed among
53 tissues and is influenced by the choices researchers make when selecting and
54 analyzing their samples.
- 55 • The difference in sulfur stable isotope values between diets and bird tissues is
56 related to the isotope values of the diet itself, which can help researchers
57 studying other birds in the absence of similar controlled studies.
- 58 • We provide recommendations for future research and guidance to promote the
59 increased use of sulfur stable isotope analysis in bird studies.

60 INTRODUCTION

61 Stable isotope analysis is a commonly used method in studies of avian foraging
62 ecology (Inger and Bearhop 2008, Hoenig et al. 2022). This is because the stable
63 isotope values of avian tissues act as a time integrated measure of these same isotopes in
64 their prey, allowing researchers to infer avian diets and/or foraging habitats (Hobson
65 and Clark 1992a, b). Stable isotope values of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) are
66 commonly applied to ornithological research (Figure 1), as they provide information on
67 avian foraging habitat use and trophic position, respectively (Hobson 1999, Kelly
68 2000). In contrast, the use of sulfur stable isotope values ($\delta^{34}\text{S}$) in ornithological
69 research has been less common (Figure 1), even though sulfur isotopes ($^{34}\text{S}/^{32}\text{S}$) have a
70 broad range of biological applications (Krouse 1989).

71 Past studies have used sulfur isotopes to reconstruct Earth's biogeochemical
72 history, study microbial activity, quantify nutrient inputs into estuaries, and identify the
73 sources and bioaccumulation patterns of pollutants (Peterson and Howarth 1987, Fry et
74 al. 1988, Canfield 2001, Fry and Chumchal 2012, Lavoie et al. 2015, Penland et al.
75 2018, Elliott et al. 2021, Jouanneau et al. 2022). Sulfur containing amino acids
76 (methionine, cystine, cysteine and taurine) and sulfolipids are important dietary sources
77 of organic sulfur to animals (Farooqui and Horrocks 1985, Brosnan and Brosnan 2006).
78 While limited, avian studies have exploited the ability of $\delta^{34}\text{S}$ values to discriminate
79 between marine and terrestrial resource and habitat use (Hobson et al. 1997, 2015), due
80 to the generally higher $\delta^{34}\text{S}$ values observed in primary producers from marine relative
81 to terrestrial/aquatic ecosystems (Connolly et al. 2004). Prior studies have also used
82 $\delta^{34}\text{S}$ values in birds to discriminate between benthic vs. pelagic prey resources
83 (Morkune et al. 2018), identify the salinity zones of foraging locations (Yohannes et al.
84 2014), infer the relative importance of capital vs. income nutrient source to egg

production (Jones et al. 2022), trace the dietary sources of environmental contaminants (Ramos et al. 2013), and quantify the use of anthropogenic derived resources (Abdennadher et al. 2014). However, $\delta^{34}\text{S}$ measurements on biological material such as avian tissues are analytically complex and expensive relative to other light elements. This is due to the generally low elemental concentration of sulfur in organic matter, the challenge of efficiently converting organic matter to SO_2 prior to isotopic analysis, and the tendency of sulfur to “stick” to stainless steel surfaces within instrumentation, and hazards associated with using sulfur dioxide (SO_2) as a reference gas (Kaczmarek and Gondko 1986, Krouse 1989, Griffiths 1991).

This legacy of analytical and financial constraints have contributed to a general lack of information on patterns of $\delta^{34}\text{S}$ values in birds. This includes data on diet-tissue isotopic discrimination factors, which are key requirements in many methods of dietary analyses using stable isotopes, such as mixing models (Phillips et al. 2014). Diet-tissue discrimination factors are calculated as the differences in stable isotope values between diet and consumer tissues. DeNiro and Epstein (1976) colloquialized this concept as “you are what you eat, plus a few per mil”. Multiple factors including tissue-type, diet quality, diet isotopic composition, and taxa-specific physiology can affect discrimination factors (Hobson and Clark 1992a, Vanderklift and Ponsard 2003, Robbins et al. 2010). For example, Florin et al. (2011) found that dietary $\delta^{34}\text{S}$ values accounted for 89% of the variation in sulfur isotopic discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) for mammalian blood obtained from controlled feeding studies. However, to date there has been only one controlled feeding study that has experimentally calculated $\Delta^{34}\text{S}_{\text{diet-tissue}}$ in avian tissues (Craig et al. 2015).

The scarcity of research on $\Delta^{34}\text{S}_{\text{diet-tissue}}$ in avian tissues is likely a limiting factor in the use of $\delta^{34}\text{S}$ in ornithological research, especially for those seeking to quantify

avian diets. In the absence of species and tissue-specific $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values studies using sulfur isotopes in dietary mixing models have applied the discrimination factors reported by Craig et al. (2015) for Double-crested Cormorants (*Phalacrocorax auritus*) fed on freshwater catfish to other bird species (e.g., Peterson et al. 2017). Other studies have used discrimination factors derived from invertebrates, fish, and/or mammals (e.g., Winiarski et al. 2012) or assumed no differences in $\delta^{34}\text{S}$ values between diets and avian tissues (e.g., Morkune et al. 2018). This is of potential concern as mixing model results are highly sensitive to variation in discrimination factors and the use of inappropriate values can bias estimates of avian diets (Bond and Diamond 2011).

However, recent methodological advances make $\delta^{34}\text{S}$ analysis in biological material more accessible and affordable. Specifically, commercially available elemental analyzer isotope ratio mass spectrometer systems are now able to regularly obtain simultaneous, high-precision measurements of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values from a single sample in a manner that avoids many of the historic issues with $\delta^{34}\text{S}$ analyses (Hansen et al. 2009, Fourel et al. 2014, Mambelli et al. 2016). Moreover, the price of simultaneous $\delta^{13}\text{C}$ / $\delta^{15}\text{N}$ / $\delta^{34}\text{S}$ analyses is commonly between \$20 to \$30 USD per sample at university-based laboratories in North America. This provides ornithologists the opportunity to quantify $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values in avian tissues more easily and facilitates the expanded use of sulfur stable isotopes in mixing model analyses of avian diets. This is a worthwhile goal, as incorporating $\delta^{34}\text{S}$ values as a third isotope value, along with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, can greatly improve mixing models by providing better discrimination among potential food source (Connolly et al. 2004, Phillips et al. 2014) and more constrained estimated of avian diets (e.g., Moreno et al. 2010).

Our study focuses on a captive breeding population of Gentoo Penguins (*Pygoscelis papua*) to provide new data on sulfur stable isotope discrimination between

avian diets and tissues. This research builds on prior studies quantifying carbon and nitrogen isotopic discrimination factors in the tissues of this species (Polito et al. 2009, 2011). Our specific goals were to: 1) Quantify isotopic discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) in egg and feather tissues, 2) explore the effects that methodical choices such as prey tissue selection and lipid extraction may have when calculating $\Delta^{34}\text{S}_{\text{diet-tissue}}$ in avian tissues, 3) compare $\Delta^{34}\text{S}_{\text{diet-tissue}}$ among penguin tissues and with a prior study of Double-crested Cormorants (Craig et al. 2015), and 4) test the applicability of the predictive relationship between dietary $\delta^{34}\text{S}$ values and isotopic discrimination developed Florin et al. (2011) in mammals to $\Delta^{34}\text{S}_{\text{diet-tissue}}$ in avian tissues.

METHODS

Penguin diet and tissue collection

Egg and feather collection methods are fully detailed in Polito et al. (2009) and Polito et al. (2011), respectively. In summary, we focused this research on a captive breeding population of Gentoo Penguins (*Pygoscelis papua*) at the Henry Doorly Zoo in Omaha, Nebraska. Penguins were hand-fed ad libitum a consistent diet of Atlantic herring (*Clupea harengus*; hereafter “herring”) for eight and ten months prior to the start of egg tissue collection and feather molt, respectively. We sampled five individual herring randomly per month during the two months prior to the mean date of egg-laying (11 November 2007) and during each of the three months leading up to and including the molt period (January to March 2008), measured (i.e., standard length in mm), then stored frozen prior to analysis. We marked eggs as they were laid using a black permanent marker to track laying order. Approximately seven days after laying, we candled eggs to determine if they were fertile. We collected and froze a sub-sample of five infertile eggs from different nests for later isotopic analysis of egg albumen and yolk. Following Hobson (1995), we assume that infertile eggs would be similar to

fertilized eggs in isotopic value, as fertilization is independent of egg formation. The remaining eggs were allowed to be incubated by their parents until they either hatched or were determined to be addled, at which time eggshell and membrane samples were collected. This provided us with eggshell membrane samples from nine two-egg clutches and two three-egg clutches for a total of 20 eggs. Finally, we collected three newly grown breast feathers from 20 adult Gentoo Penguins (8 males, 12 females) following their annual molt in late March 2008.

Sample preparation and analyses

We homogenized whole fish and fish muscle samples and then dried them for 48 h in an oven at 60 °C. We separated eggshell, shell membrane, albumen and yolk by hand and sub-samples of yolk and albumen were collected and freeze-dried. Dried fish, egg albumen and yolk were ground to a powder using an analytical mill. We removed lipids from whole fish, fish muscle, and yolk samples using a Soxhlet apparatus with a 1:1 Petroleum–Ether: Ethyl–Ether solvent mixture for 6–8h (Seminoff et al. 2007). We cleaned feathers of surface contaminants using a 2:1 chloroform: methanol rinse and cut into small fragments with stainless steel scissors. Prior studies suggest little to no effect of lipid extraction on the $\delta^{34}\text{S}$ values of teleost fish tissues (Moradi et al. 2011, Larocque et al. 2021), but that lipid extraction may increase $\delta^{34}\text{S}$ values in avian egg yolks (Oppel et al. 2010). As such, we tested for an effect of lipid extraction by analyzing whole herring collected during the pre-egg laying period and all penguin yolk $\delta^{34}\text{S}$ values before and after lipid-extraction.

We loaded approximately 0.5 mg of each of the above materials into tin cups for $\delta^{34}\text{S}$ analysis. These samples were then flash-combusted using a Thermo Scientific™ EA IsoLink™ Elemental Analyzer System coupled to a Thermo-Fisher Delta V Advantage continuous-flow stable isotope ratio mass spectrometer for sulfur stable

isotope ($\delta^{34}\text{S}$) analysis. We normalized stable isotope values using a two-point system with silver sulfide reference materials (IAEA-S2 and IAES-S3; International Atomic Energy Agency). A certified reference material of known isotopic composition (Gelatin: EA Consumables, LLC.) was measured after every seven samples to allow for drift corrections using a linear model (Werner and Brand 2001). Our sample precision was $\pm 0.3\text{‰}$ based on the standard deviation of repeated reference materials and a Red Drum (*Sciaenops ocellatus*) muscle internal laboratory standard. Sulfur stable isotope values are expressed in standard delta (δ) notation referenced to Vienna-Canyon Diablo Troilite (VCDT) and reported in per mil units (‰).

Data analysis

We used paired t-tests to compare $\delta^{34}\text{S}$ values between lipid extracted and non-lipid extracted whole herring and penguin egg yolk samples. We also used a t-test to examine if there were differences in the herring body size between the egg-laying and pre-molt periods. We also tested for differences in $\delta^{34}\text{S}$ values between fish tissue (whole fish vs. fish muscle) and the period sampled (pre-laying vs. pre-molt), using a generalized least square model (GLS) with the VarIdent function (nlme package; Pinheiro et al. 2015) to allow different variances per period sampled. If significant differences were found, we examined them with pairwise comparisons (lsmeans package; Lenth and Herve 2015).

For penguin tissue samples, we first tested for an effect of laying order (egg one vs. egg two) and sex (female vs. male) on the $\delta^{34}\text{S}$ values of eggshell membrane and breast feathers, using paired and two samples t-tests, respectively. We then tested for differences in $\delta^{34}\text{S}$ values between tissues using a GLS with the VarIdent function to allow different variances per tissue. Pairwise comparisons were performed afterwards.

209 Following the approach of Polito et al. (2009) and Polito et al.(2011) used to
 210 estimate carbon ($\Delta^{13}\text{C}_{\text{diet-tissue}}$) and nitrogen ($\Delta^{15}\text{N}_{\text{diet-tissue}}$) discrimination factors in
 211 penguin tissues, we calculated $\Delta^{34}\text{S}_{\text{diet-tissue}}$ by subtracting the $\delta^{34}\text{S}$ values of each
 212 individual egg tissue and feather sample from the mean isotopic values of the diets
 213 (herring) consumed prior to tissue synthesis. Herring samples from the pre-lay and pre-
 214 molt periods were used to calculate $\Delta^{34}\text{S}_{\text{diet-tissue}}$ for egg tissues and feathers,
 215 respectively. We also separately calculated $\Delta^{34}\text{S}_{\text{diet-tissue}}$ using herring muscle and whole
 216 fish $\delta^{34}\text{S}$ values. We used the GLS with the pairwise comparison method described
 217 above to test for differences in $\Delta^{34}\text{S}_{\text{diet-tissue}}$ among penguin and fish tissues.

218 Last, we incorporated the mean $\delta^{34}\text{S}$ values of whole fish and fish muscle fed to
 219 penguins into the equation developed by Florin et al. (2011) to predict $\Delta^{34}\text{S}_{\text{diet-tissue}}$ in
 220 mammals:

$$221 \quad \Delta^{34}\text{S}_{\text{diet-tissue}} = 1.16 - 0.26 \times \delta^{34}\text{S}_{\text{diet}}$$

222 This allowed us to assess the transferability, as previous investigators using sulfur
 223 isotopes in dietary mixing models have applied Florin et al.'s (2011) equation to
 224 estimate $\Delta^{34}\text{S}_{\text{diet-tissue}}$ avian tissues (e.g. Winiarski et al. 2012, Yohannes et al. 2014).

225 All statistical analyses were performed in R software version 4.2.1 (R Core
 226 Team 2015). Assumptions for each statistical analysis (e.g., normality and homogeneity
 227 of variances) were tested beforehand. Statistical significance was assumed at the $\alpha =$
 228 0.05 level and all means are presented $\pm$ standard deviation (SD).

229 RESULTS

230 There were no differences in mean $\delta^{34}\text{S}$ values between lipid extracted
 231 ($18.9 \pm 0.2\text{‰}$) and non-lipid extracted ($18.8 \pm 0.3\text{‰}$) whole fish collected during the pre-
 232 egg laying period ($t_9 = 0.89$, $P = 0.40$; Figure 2). Given their similar $\delta^{34}\text{S}$ values and the
 233 greater availability of archived lipid-extracted herring samples, we used lipid extracted

234 whole fish and fish muscle $\delta^{34}\text{S}$ values for all subsequent analyses. However, the mean
235 $\delta^{34}\text{S}$ values of lipid extracted yolk ($18.0 \pm 0.1\text{‰}$) were significantly higher than those of
236 non-lipid extracted yolk ($17.2 \pm 0.1\text{‰}$; $t_4 = 10.85$, $P < 0.001$; Figure 2). Therefore, we
237 used non-lipid extracted yolk $\delta^{34}\text{S}$ values for all subsequent analyses.

238 Herring fed to penguins prior to egg lay were longer (250 ± 9 mm) than those
239 during the pre-molt period (187 ± 10 mm; $t_{20} = 16.22$, $P < 0.001$). $\delta^{34}\text{S}$ values differed
240 between fish tissues (muscle vs. whole fish; $F_{1,46} = 20.3$, $P < 0.001$) and between
241 feeding periods (pre-egg laying vs pre-molt; $F_{1,46} = 16.7$, $P < 0.001$), and there was a
242 significant interaction between tissue type and feeding period ($F_{1,46} = 15.5$, $P < 0.001$).
243 The $\delta^{34}\text{S}$ values of fish muscle during the pre-molt period was the only significant
244 pairwise comparisons among groups (all $P < 0.001$), with lower mean $\delta^{34}\text{S}$ values
245 relative to other tissue types and/or feeding periods (Table 1).

246 The $\delta^{34}\text{S}$ values of egg membrane did not differ between first ($17.2 \pm 0.6\text{‰}$) and
247 second ($17.6 \pm 0.6\text{‰}$) eggs laid in the same clutch ($t_7 = -1.78$, $P = 0.12$). The mean $\delta^{34}\text{S}$
248 values of egg membrane from the two females that laid a third eggs was $17.4 \pm 0.1\text{‰}$.
249 Breast feather $\delta^{34}\text{S}$ values did not differ between males ($17.8 \pm 0.5\text{‰}$) and females
250 ($17.8 \pm 0.3\text{‰}$; $t_{10} = -0.50$, $P = 0.65$). Therefore, egg membrane and feather $\delta^{34}\text{S}$ values
251 were combined across egg order and sex, respectively for all subsequent analyses. $\delta^{34}\text{S}$
252 values differed among tissues ($F_{3,46} = 15.4$, $P < 0.001$). Pairwise comparisons indicated
253 that yolk $\delta^{34}\text{S}$ values were significantly lower than albumen ($t_8 = 5.1$, $P = 0.005$; Table
254 1) and feathers ($t_{23} = 5.98$, $P < 0.001$; Table 1). Feather $\delta^{34}\text{S}$ values were significantly
255 higher than albumen ($t_{23} = -3.25$, $P = 0.02$, Table 1) and egg membrane $\delta^{34}\text{S}$ values did
256 not differ from other tissues (all $P > 0.10$, Table 1).

257 Calculated discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) ranged from -0.4 to -1.7‰
258 (Table 2). $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values significantly differed when calculated using whole fish vs.

fish muscle ($F_{1,92} = 53.09$, $P < 0.001$) and differed among penguin tissues ($F_{3,92} = 54.16$,
 $P < 0.0001$). In addition, there was a significant interaction between these two terms
($F_{3,92} = 5.71$, $P = 0.001$). Specifically, when averaged across all tissues $\Delta^{34}\text{S}_{\text{diet-tissue}}$
values were lower when calculated using whole fish ($-1.3 \pm 0.5\text{‰}$) relative to fish muscle
($-0.9 \pm 0.6\text{‰}$; $t_{74} = 5.63$, $P < 0.001$). In general, feathers had the highest $\Delta_{\text{diet-tissue}}^{34}\text{S}$
values, yolk had the lowest $\Delta_{\text{diet-tissue}}^{34}\text{S}$ values, and egg membrane and albumen were
intermediate of other tissues (Table 2). Pairwise comparisons indicated that only feather
($t_{38} = 6.05$, $P < 0.001$) and albumen ($t_8 = 4.47$, $P = 0.003$) $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values differed
when calculated using whole fish or fish muscle (Table 2). All other within-tissue,
pairwise comparisons were non-significant ($P > 0.50$; Table 2).

Using the mean $\delta^{34}\text{S}$ values of whole fish (18.9‰) and fish muscle (18.2‰) fed
to penguins in our study (Table 1), the predictive relationship developed by Florin et al.
(2011) between dietary $\delta^{34}\text{S}$ values and isotopic discrimination estimated $\Delta^{34}\text{S}_{\text{diet-tissue}}$
values of -3.6‰ and -3.8‰ , respectively.

DISCUSSION

We quantified sulfur isotopic discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) between diet
and egg and feather tissues in a controlled dietary study of Gentoo Penguins. To our
knowledge, this is the second study to report $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values in avian tissues (e.g.,
Craig et al. 2015), and the first to report $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values in egg components. Our
results indicate that estimates of sulfur isotope discrimination in avian tissues can be
influenced by methodological choices such as lipid-extraction and the use of whole prey
vs. prey muscle. In addition, we found that $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values varied among avian egg
components and feathers even when synthesized from a common diet. Moreover, the
range of $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values across tissues we obtained for Gentoo Penguins generally
differed from those reported for Double-crested Cormorants by Craig et al. (2015).

284 These sources of variation highlight the need to better quantify sulfur isotopic
285 discrimination in avian tissues so that appropriate values can be selected and applied in
286 trophic studies.

287 Variation in lipid content among tissues reflects a common methodological
288 challenge in stable isotope studies. This is most significant for carbon ($\delta^{13}\text{C}$) and
289 hydrogen stable isotope ($\delta^2\text{H}$) values as lipids are generally depleted in ^{13}C and ^2H
290 relative to proteins (Hobson et al. 1999, Post et al 2007). Studies seek to remove and/or
291 account for lipid effects on $\delta^{13}\text{C}$ values by using chemical extraction or mathematical
292 normalization (Post et al. 2007, Logan et al. 2008, Yurkowski et al. 2015). Little is
293 known about the potential effects of chemical lipid extraction in $\delta^{34}\text{S}$ values, though
294 there is concern that the incidental loss of sulfur-bearing amino acids and/or sulfolipids
295 could bias $\delta^{34}\text{S}$ values (Oppel et al. 2010). However, we found no differences between
296 the $\delta^{34}\text{S}$ values of lipid extracted and not lipid extracted whole herring. One prior study
297 also examined the influence of lipid extraction on $\delta^{34}\text{S}$ values in teleost fish tissues.
298 Larocque et al. (2021) found little to no effect of chemical lipid extraction on the $\delta^{34}\text{S}$
299 values of salmonids muscle and fin tissues. Moreover, Riverón et al. (2022) examined
300 white muscle and liver samples from two species of sharks and skin samples from two
301 species of pinnipeds and found that tissues with higher lipid content (e.g, shark liver)
302 showed the greatest differences in $\delta^{34}\text{S}$ values due to lipid extraction. The similar
303 findings of our study and others suggest that the $\delta^{34}\text{S}$ values of fish tissues may be
304 broadly robust to chemical lipid extraction when they contain low amount of sulfolipids
305 (Moradi et al. 2011). When validated *a priori*, this will allow researchers to use lipid-
306 extracted and not extracted teleost fish tissue $\delta^{34}\text{S}$ values interchangeably in trophic
307 studies.

308 In contrast, there is growing evidence that the lipid-extracted and not extracted
309 avian egg yolk tissue $\delta^{34}\text{S}$ values may not be used interchangeably. Oppel et al. (2010)
310 found that lipid extraction of wild King Eider (*Somateria spectabilis*) yolk increased
311 $\delta^{34}\text{S}$ values by $2.3 \pm 1.1\text{‰}$ on average likely due to the loss of sulfolipids. We also found
312 higher $\delta^{34}\text{S}$ values in lipid-extracted Gentoo Penguin egg yolks compared to non-lipid
313 extracted ones, though at a smaller magnitude ($0.8 \pm 0.2\text{‰}$). This difference could be an
314 effect of our differing lipid-extraction methods. We used a Soxhlet apparatus with 1:1
315 Petroleum–Ether:Ethyl–Ether solvent mixture while Oppel et al (2010) used a 2:1
316 chloroform:methanol solvent following the method of Bligh and Dyer (1959). Larocque
317 et al. (2021) study of salmonid tissues also used the lipid extract of Bligh and Dyer
318 (1959) method, though Riverón et al. (2022) study applied the methods of Folch et al.
319 (1957) with an additional deionized water rinse to remove urea from elasmobranch
320 tissues (Kim and Koch 2012).

321 Elliott and Elliott (2016) found that polar solvents (i.e., chloroform–methanol)
322 more thoroughly removes lipids and likely a greater degree of hydrophobic proteins
323 compared with nonpolar solvents (i.e., petroleum ether). Our results indicate a need for
324 future studies that quantify the effect of differing lipid-extraction methods on tissue $\delta^{34}\text{S}$
325 values, like those that have examined $\delta^{13}\text{C}$ values (Elliott and Elliott 2016). When lipid
326 extraction is not advisable due to biased $\delta^{34}\text{S}$ values and/or logistically feasible (e.g.
327 simultaneous measurements of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values) the use of mathematical
328 models to lipid-normalize $\delta^{13}\text{C}$ values represents an alternative option (e.g., Larocque et
329 al. 2021). However, care should be taken when applying mathematical approaches to
330 avian yolks, as they assume that all egg macronutrients are derived from the same
331 dietary sources (Oppel et al. 2010).

332 The results of our study also underscore the importance of the prey tissue
333 selection when quantifying discrimination factors. We found that the $\delta^{34}\text{S}$ values of
334 whole herring were generally higher than those of herring muscle, especially for the fish
335 sampled prior to penguin molt. Stable isotope values can vary across fish tissues
336 (Hesslein et al. 1993, Jardine et al. 2005, Matley et al. 2016). While the $\delta^{34}\text{S}$ values of
337 homogenized whole fish reflect an integration of muscle, organs, and bone tissues,
338 published ratios indicates that muscle likely represented > 90% of the total body mass
339 of herring consumed by gentoo penguin in our study (Braune 1987). Moreover, muscle
340 protein can account for approximately 50% of the body protein in fish (Kaushik and
341 Seiliez 2010), and prior studies indicate that amino acid concentration, and the sulfur
342 containing amino acid methionine, can be the same or higher in fish muscle relative to
343 whole bodies (Arzel et al., 1995, Borges Tesser et al. 2014). Given this information, we
344 might have expected more similar $\delta^{34}\text{S}$ values between whole herring and herring
345 muscle.

346 One possible explanation is that tissues differing in their proportional
347 contribution to body mass also had differing rates of isotopic turnover (Hesslein et al.
348 1993, Donovan and Miles 2010). For example, the isotopic values of internal organs
349 turnover more rapidly compared to muscle tissue (Vander Zanden et al. 2015). In
350 addition, herring fed to penguins during egg-laying were slightly larger than those
351 consumed by penguins during the pre-molt period, and small rapidly growing fish can
352 have faster tissue turnover rates compared to larger fish (Hesslein et al. 1993, Donovan
353 and Miles 2010). Moreover, inorganic sulfur and/or the synthesis of sulfur containing
354 amino acids by bacteria in fish guts could also lead to differing $\delta^{34}\text{S}$ values between
355 whole herring and herring muscle (Mekhtiyeva et al. 1976).

356 In the context of our focal species, $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values derived from whole prey
357 are likely the most physiologically and ecologically relevant as Gentoo Penguins
358 consume their prey whole. In contrast, $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values derived from prey muscle are
359 more applicable to birds that scavenge and/or selectively consume prey tissues (e.g.
360 raptors, skuas, gulls, etc.). However, it is not always logistically possible for researchers
361 to collect or homogenize whole prey, and in many cases muscle may reflect the
362 dominate source of dietary protein even when prey are consumed whole (e.g., Bruane
363 1987, Kaushik and Seiliez 2010). Therefore, we calculated $\Delta^{34}\text{S}_{\text{diet-tissue}}$ relative to both
364 whole fish and fish muscle in an effort to complement previous avian studies that also
365 compared whole prey and prey muscle $\Delta^{13}\text{C}_{\text{diet-tissue}}$ and $\Delta^{15}\text{N}_{\text{diet-tissue}}$ values (Cherel et al.
366 2005, Polito et al. 2009).

367 Excluding differences due to prey-tissue choice, mean discrimination factors
368 ($\Delta_{\text{diet-tissue}}^{34}\text{S}$) varied among tissues in our study, ranging from -1.7 to -1.1 ‰ when
369 calculated using whole herring. In addition, tissue $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values were lower than
370 the corresponding carbon ($\Delta_{\text{diet-tissue}}^{13}\text{C}$) and nitrogen ($\Delta_{\text{diet-tissue}}^{15}\text{N}$) discrimination
371 factors reported by Polito et al. (2009) and Polito et al. (2011) in their analyses of these
372 same samples (Table 2). Our $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values for Gentoo Penguin tissues were also
373 lower than those reported by Craig et al. (2015) for Double-crested Cormorant tissues (-
374 1.1 to 0.5 ‰) fed a diet of channel catfish (*Ictalurus punctatus*). For example, mean
375 $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values in feathers, the only tissue examined in both studies, were 1.0 ‰
376 lower in Gentoo Penguins relative to Double-crested Cormorants (Craig et al. 2015).
377 While both studies calculated avian tissue $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values using whole fish $\delta^{34}\text{S}$
378 values, there was a large difference in the $\delta^{34}\text{S}$ values of the prey fish in these studies.
379 The whole catfish fed to cormorants had an average $\delta^{34}\text{S}$ value of 2.0 ± 2.2 ‰ (Craig et
380 al. 2015), while whole herring fed to penguins had an average $\delta^{34}\text{S}$ value of 18.9 ± 0.2 ‰

381 (Table 1). This is a reflection of freshwater prey fish generally having lower $\delta^{34}\text{S}$ values
382 (e.g., Hesslein et al. 1991, Connolly et al. 2004) compared to marine species (e.g.,
383 Moreno et al. 2010, Morkune et al. 2018).

384 Florin et al. (2011) obtained a very strong inverse relationship between dietary
385 $\delta^{34}\text{S}$ values and diet-tissue discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) in mammalian blood,
386 plasma and serum. They proposed that this correlation was due to the sulfur containing
387 amino acid methionine acting as a limiting, essential amino acid in diets. Moreover,
388 Florin et al. (2011) found that reducing dietary protein quality and quantity did not alter
389 in $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values, unlike $\Delta^{15}\text{N}_{\text{diet-tissue}}$ values which has been found to vary with both
390 of these dietary parameters (Podlesak & McWilliams 2006, Martínez del Rio 2009,
391 Robbins et al. 2010). Unfortunately our study and Craig et al. (2015) did not directly
392 measure dietary protein content or quality (e.g., methionine content as a percentage of
393 total dietary protein; Florin et al. 2011) or explore the potential for sulfur containing
394 macromolecules to undergo different metabolic routing from diet to incorporation into
395 consumer tissues (e.g., Martinez del Rio et al. 2009).

396 Even so, in agreement with Florin et al. (2011) cormorants feeding on whole fish
397 with lower $\delta^{34}\text{S}$ value (Craig et al. 2015) had higher $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values relative to the
398 Gentoo Penguins that were fed whole fish with higher $\delta^{34}\text{S}$ value. To explore these
399 similarities further, we applied the equation developed by Florin et al. (2011) to predict
400 cormorant and penguin $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values based on their dietary $\delta^{34}\text{S}$ values. The Florin
401 et al. (2011) equation predicted a $\Delta^{34}\text{S}_{\text{diet-tissue}}$ value of 0.6 ‰ for Double-crested
402 Cormorants feeding on catfish with a $\delta^{34}\text{S}$ value of 2.3 ‰. This predicted $\Delta^{34}\text{S}_{\text{diet-tissue}}$
403 value was generally higher than the $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values empirically measured by Craig et
404 al. (2015) for whole blood (0.5 ‰), red blood cell (0.1 ‰), and blood plasma (-0.5 ‰).
405 In contrast, the $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values predicted by the Florin et al. (2011) equation for

406 Gentoo Penguins feeding on herring (-3.8 to -3.6 ‰) were much lower than the range of
407 $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values were measured in our study (-1.7 to -0.4 ‰; Table 1), though we did
408 not examine blood tissues.

409 These results suggest that, like mammals (Florin et al. 2011), $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values
410 are inversely related to dietary $\delta^{34}\text{S}$ values in birds. However, based on our results we
411 urge caution to those who might apply Florin et al.'s (2011) predictive equation to
412 estimate $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values for birds due to a lack of more appropriate discrimination
413 factors (e.g., Winiarski et al. 2012, Yohannes et al. 2014). In addition, the range of
414 $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values observed among the different tissues in our study and Craig et al.
415 (2015) indicate that sulfur isotopic discrimination is tissue-specific. Therefore, we
416 recommend that future studies of avian tissues over a more continuous range of dietary
417 $\delta^{34}\text{S}$ values is needed to obtain predictive equations for diet-tissue sulfur isotopic
418 discrimination in birds.

419 In conclusion, our study provides sulfur isotopic discrimination factors ($\Delta^{34}\text{S}_{\text{diet-tissue}}$)
420 between the diets and tissues of Gentoo Penguins. Given the observed relationship
421 between tissue $\Delta^{34}\text{S}_{\text{diet-tissue}}$ and dietary $\delta^{34}\text{S}$ values, it is likely that the discrimination
422 factors reported here may also be applicable to tissues from other bird species who
423 consume a similar marine diet. However, methodological choices such as lipid-
424 extraction and prey-tissue selection should be considered as we found that they can
425 introduce tissue-specific variation in avian $\Delta^{34}\text{S}_{\text{diet-tissue}}$ values. In addition, we
426 encourage more studies of $\Delta^{34}\text{S}_{\text{diet-tissue}}$ across a broader set of avian species and tissues
427 in the future. Studies that include terrestrial birds and/or those that examine a wide
428 range of dietary $\delta^{34}\text{S}$ values are especially needed to improve our understanding of diet-
429 tissue sulphur isotopic discrimination in avian tissues and allow for greater application
430 of sulfur stable isotope analysis in ornithological research.

431

432 **LITERATURE CITED**

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FIGURES & TABLES

Figure 1. The total number of published papers in which sulfur ($\delta^{34}\text{S}$), nitrogen ($\delta^{15}\text{N}$), or carbon ($\delta^{13}\text{C}$) stable isotope analysis was applied to avian research. We conducted our literature search on 13 July, 2022 using Web of Science and the terms “avian/bird” and “stable isotope” and either “sulphur/sulfur/34S/delta S-34”, “nitrogen/15N/delta N-15”, or “carbon/13C/delta C-13”, respectively.

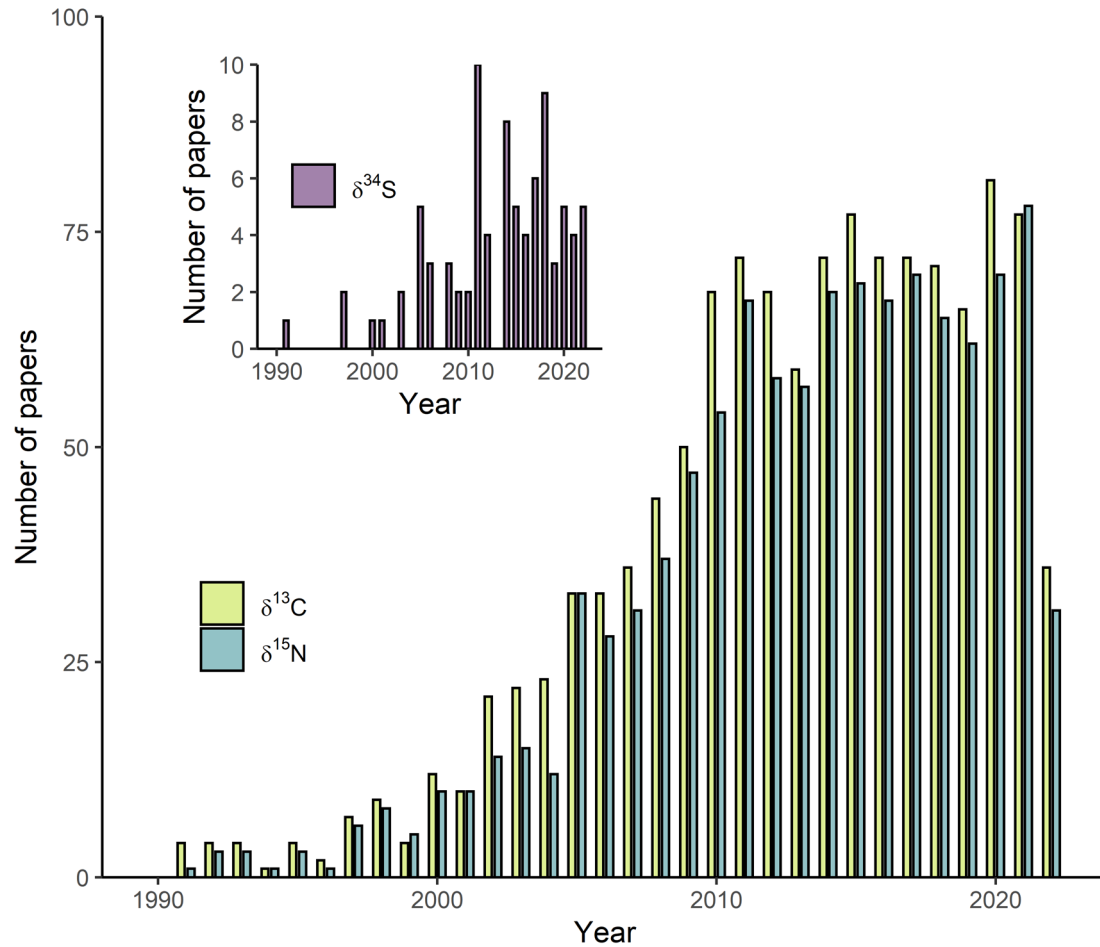


Figure 2. The sulfur stable isotope values ($\delta^{34}\text{S}$) of lipid extracted and not lipid extracted whole herring fish and yolks of captive Gentoo Penguins. Boxplots include median, interquartile range, minimum and maximum values. Large points represent mean values while jittered points represent individual values for each tissue and treatment. Treatment with differing superscript letters reflects significant differences identified from paired t-tests between lipid extracted and not lipid extracted tissues.

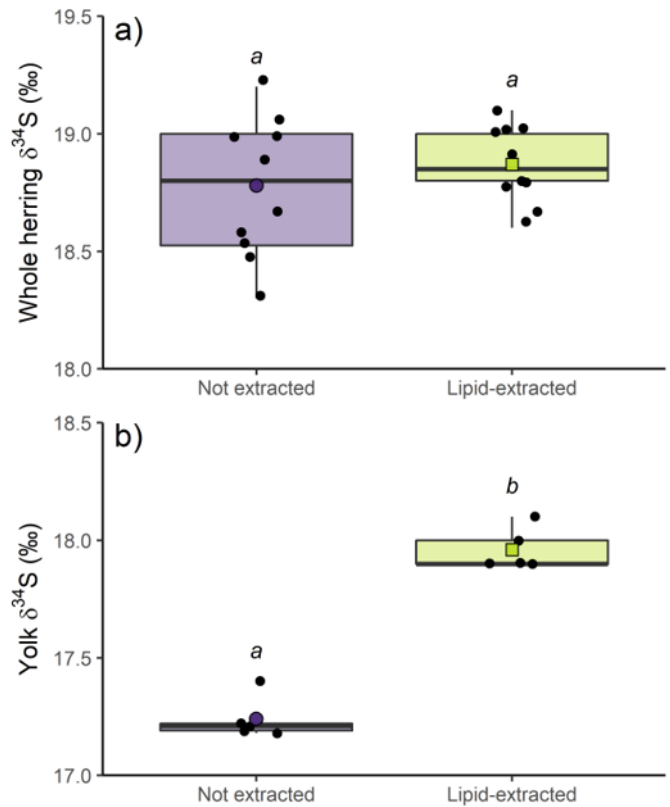


Table 1. The sulfur concentration (%S) and stable isotope values ($\delta^{34}\text{S}$) of food (herring), egg tissues, and feathers of captive Gentoo Penguins. Values are reported as mean $\pm$ SD. Test statistics reflect paired t-test between whole fish and fish muscle $\delta^{34}\text{S}$ values within the laying and molt periods, a paired t-test between egg one and two for shell membrane $\delta^{34}\text{S}$ values, paired t-test between lipid extracted and non-lipid extracted egg yolk, and a two-sample t-test between male and female breast feathers $\delta^{34}\text{S}$ values.

Sample	<i>n</i>	% S	$\delta^{34}\text{S}$ (‰)
Herring (laying)			
Whole fish	10	1.33 $\pm$ 0.03	18.9 $\pm$ 0.2
Fish muscle	10	1.25 $\pm$ 0.01	18.7 $\pm$ 0.1
Herring (molt)			
Whole fish	15	1.31 $\pm$ 0.01	18.9 $\pm$ 0.3
Fish muscle	15	1.29 $\pm$ 0.02	18.2 $\pm$ 0.4
Shell membrane	20	4.20 $\pm$ 0.35	17.4 $\pm$ 0.6
Egg albumen	5	1.35 $\pm$ 0.01	17.5 $\pm$ 0.1
Egg yolk	5	0.47 $\pm$ 0.03	17.2 $\pm$ 0.1
Breast feathers	20	3.31 $\pm$ 0.15	17.8 $\pm$ 0.4

Table 2. Carbon ($\Delta^{13}\text{C}_{\text{diet-tissue}}$), nitrogen ($\Delta^{15}\text{N}_{\text{diet-tissue}}$), and sulfur ($\Delta^{34}\text{S}_{\text{diet-tissue}}$) isotopic discrimination factors between food (whole herring and herring muscle), egg tissues, and feathers of captive Gentoo Penguins. Values are reported as mean $\pm$ SD. $\Delta^{13}\text{C}_{\text{diet-tissue}}$ and $\Delta^{15}\text{N}_{\text{diet-tissue}}$ values are obtained from Polito et al. (2009) and Polito et al. (2011). $\Delta^{34}\text{S}_{\text{diet-tissue}}$ are obtained from this study.

Tissue, diet	Discrimination Factor (‰)		
	$\Delta^{13}\text{C}_{\text{diet-tissue}}$	$\Delta^{15}\text{N}_{\text{diet-tissue}}$	$\Delta^{34}\text{S}_{\text{diet-tissue}}$
Shell membrane			
Whole fish	2.8 $\pm$ 0.5	4.4 $\pm$ 0.5	-1.5 $\pm$ 0.6
Fish muscle	2.9 $\pm$ 0.5	4.2 $\pm$ 0.5	-1.3 $\pm$ 0.6
Egg albumen			
Whole fish	0.8 $\pm$ 0.7	4.7 $\pm$ 0.5	-1.4 $\pm$ 0.1
Fish muscle	0.9 $\pm$ 0.7	4.5 $\pm$ 0.5	-1.2 $\pm$ 0.1
Egg yolk			
Whole fish	0.1 $\pm$ 0.5	3.7 $\pm$ 0.5	-1.7 $\pm$ 0.1
Fish muscle	0.0 $\pm$ 0.5	3.5 $\pm$ 0.5	-1.5 $\pm$ 0.1
Breast feathers			
Whole fish	1.3 $\pm$ 0.5	3.5 $\pm$ 0.4	-1.1 $\pm$ 0.4
Fish muscle	-	-	-0.4 $\pm$ 0.4