Carbon, Nitrogen and Sulphur isotopic fractionation in captive juvenile hooded seal (*Cystophora cristata*): application for diet analysis

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Abstract

Rationale: Intrinsic biogeochemical markers, such as stable isotope ratios of carbon, nitrogen and sulphur are increasingly used to trace the trophic ecology of marine top predators. However, insufficient knowledge of fractionation processes in tissues continues to hamper the use of these markers.

Methods: We performed a controlled feeding experiment with eight juvenile hooded seals (*Cystophora cristata*) that were held on a herring-based diet (*Clupea harengus*) for two years. Stable isotope ratios were measured via isotope ratio mass spectrometry in three of their tissues and related to values of these markers in their diet.

Results: Diet-tissue isotope enrichment (trophic enrichment factor, TEF) values between dietary herring and seal tissues for carbon ($\Delta^{13}C$) were + 0.7 ‰ for red blood cells, + 1.9 ‰ for hair and + 1.1 ‰ for muscle. The TEFs for nitrogen trophic ($\Delta^{15}N$) were + 3.3 ‰ for red blood cells, + 3.6 ‰ for hair and + 4.3 ‰ for muscle. For sulphur, the $\Delta^{34}S$ values were +1.1 ‰ for red blood cells, + 1.0 ‰ for hair and + 0.9 ‰ for muscle.

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Conclusions: These enrichment values were greater than those previously measured in adult seals. This increase may be related to the higher rate of protein synthesis and catabolism in growing animals. This study is the first report on sulphur isotope enrichment values for a marine mammal species.

Keywords: isotope fractionation, hooded seal, discrimination model, growth.

1. Introduction

Stable isotope ratios of carbon (C), nitrogen (N) and sulphur (S) allow quantitative and time-integrated estimation of diet composition [1–3]. They have been successfully used to identify diet structure and trace nutrients sources in top predators, whose life history are often difficult to deduce [4]. The $\delta^{15}N$ value shows a stepwise increase from one trophic level to the other (1 - 4‰) [5]. For this reason the $\delta^{15}N$ value is usually used to infer information about food web interactions and to estimate trophic position of species [2]. Variation of $\delta^{13}C$ and $\delta^{34}S$ values along food webs is minor (~1‰ and 0.5‰, respectively), and these values vary instead among the different sources at the base of the food web [6], permitting discrimination between the different origins of predator dietary sources (benthic *vs* pelagic, offshore *vs* nearshore). Although the utility of S in ecological studies was already known, its use was limited until recently, due to the laborious analytical techniques required for $\delta^{34}S$ measurements [6].

The isotope fractionation (IF) observed between a consumer and its prey results from the numerous metabolic processes linked with prey digestion and assimilation. Such processes are commonly represented by the calculation of the trophic enrichment factor (TEF, commonly noted as Δ) which depicts the net isotopic difference between a consumer and its food source [7]. Diet-tissue trophic enrichment factors can be estimated through controlled experiments in which organisms are fed a constant diet of known isotopic composition [8]. Since the turnover of stable isotopes within tissues varies accordingly to metabolic rates, measurement of such values in several tissues from the same individual can provide short-, intermediate- and long-term dietary information [9]. Tissues that are metabolically very active (i.e. muscle, blood, kidney) can integrate a period of days to months; while hairs, skin, vibrissae, nails, which are considered as inert, reflect the diet and behaviour during a year or even the entire life of the individual [10]. Moreover, knowledge of differences and relationships among tissues allows the alternative use of non-destructive sampling for the analysis of free-ranging endangered species [11–13].

Even if the use of several tissue types has the power to increase the efficiency of the stable isotope approach in food web investigations, the way in which isotopes fractionate between diet and the various tissues still remain poorly understood [7,9,14]. This greatly affects the correct use of recently developed approaches such as the application of isotopic mixing models (e.g. SIAR, MixSIAR), which strongly depend on TEF information. Indeed, several studies have shown how a moderate variation in fractionation estimates can lead to important differences in the model outputs and therefore to misleading conclusions [15]. This becomes especially important when TEF average values are used, or when there is not a good isotopic separation between the sources used for the model [16].

The main objective of this study was to estimate C, N and, for the first time in marine mammals, sulphur-IF in growing individuals of hooded seals *Cystophora cristata* (Erxleben, 1777) kept in human care for two years under a diet exclusively based on herring *Clupea harengus* (Linnaeus, 1758). The hooded seal is one of the most physiologically specialised *Phocidae* hunting and breeding in the cold Arctic and sub-Arctic waters [17]. In the last decade, a strong decrease in population numbers in combination with higher rates of juveniles stranding out of the normal distribution range was observed [18]. Several studies have proposed that such phenomena may be caused by climate change [19–21]; but the reason why this seems to affect hooded seals more than other species it is not yet clear. However, only fragmented information about the physiology and ecology of this species is present in the literature.

Conversely to previous captive feeding reported in the literature, our animals were completely healthy individuals, which avoided eventual effects of body condition on IF results. Moreover, the 2-year long experiment allowed a complete diet-to-tissue equilibrium to be reached and thus exclude the potential influence given by the shifts in diet or distribution that normally occur in the wild, and obtain a baseline of C, N and S IF exclusively linked with the physiology of these organisms. This has enhanced our knowledge on the basal metabolic fractionation rates of growing hooded seals, which may be lead to a better interpretation of future ecological studies.

We also compared our measured results with the fractionation factors resulting from the application of two models developed by Caut *et al*[7] and Healy *et al*[22] with the aim of assessing their use in marine mammal ecology. The urgency for the development of models, which can accurately calculate TEFs, comes from the substantial lack of experimental data on

physiologically complex species, severely denounced by modern scientific literature, and the difficulties encountered during the sampling and analysis of wild animals.

2. Materials and Methods

2.1. Sampling and captivity of hooded seals

Eight hooded seals (4 females and 4 males) were captured after the reproduction period in March 2012 (Table 1), immediately after weaning, on the whelping grounds along the pack-ice of the Greenland Sea, North-West of Jan Mayen Island (off the North-eastern coast of Greenland). Sampling was conducted during a research cruise with R/V *Helmer Hanssen*. Seals were caught using hoop nets while they were on the ice floes (Norwegian Directorate of Fisheries, capture permit n.12/1352). They were brought on-board, where they were kept in 1.5- by 2.5-m pens during the transit back to Tromsø (Norway). A captivity experiment was approved by the Norwegian Food Safety Authority (permit n°2012/030044), and initiated in the framework of several physiological studies at the Arctic University of Tromsø (UiT). The animal facility approved for such captivity experiment (Norwegian Animal Research Authority, approval #089) was situated at the Department of Arctic and Marine Biology (DAMB), UiT. The experiment lasted in total 22 months (until February 2014), during which all seals were fed on a constant diet of freshly frozen, thawed Norwegian Spring Spawning herring (*Clupea harengus*) in quantities appropriate for the sustainment of a correct development and a natural growth curve based on the animals' mass[23]. In this regard, food was also supplemented with a vitamin complex (Seatabs MA III, Pacific Research Labs, Inc., Vashon Island, WA, USA). Herring were purchased from the same company each year: they were thus sampled in the same area following the same protocol for both fishing and storing conditions. All fish used for the experiment belonged to the same length range (25 – 30 cm). The indoor light regime simulated that of outdoor natural light-darkness cycles at 70˚N latitude. Seals were maintained in two indoor 40,000-L (1.5-m deep) seawater pools with a wooden ledge along one side on which the animals could haul out at will. After two years, seals were euthanized and different organs were collected for analysis. Two seals (K5 and K6) were euthanized after one year (March 2013) for other physiological studies at the UiT; and only blood was available for our analysis. After death, the age of each seal was estimated using the two equations developed especially for hooded seals by Wiig [24]:

$$\text{for females:} \quad L_x = 200\,(\,1 - e^{-0.202\,(x + 0.61)}\,)^{\,0.336}$$

$$\text{for males:} \quad L_x = 221.1\,(\,1 - e^{-0.129\,(w + 0.61)}\,)^{\,0.309}$$

2.2. Stable isotope analysis

Fifteen herring were selected to measure the isotopic composition of the diet. In particular, one muscle sample was collected from each herring. Stable isotope ratios of N, C and S of muscle, hair and red blood cells (RBCs) of captive hooded seals, as well as muscle of herring, were measured using an isotope ratio mass spectrometer (IsoPrime100, Isoprime, Cheadle, UK) coupled in continuous flow to an elemental analyser (vario MICRO cube, Elementar Analysensysteme GmbH, Hanau, Germany). Isotopic ratios were conventionally expressed in delta (δ) notation in ‰[25], according to the following equation:

$$\delta^{i/j}E_{S,Std} = \frac{(R(\,^{i}E/\,^{j}E)s - R(\,^{i}E/\,^{j}E)Std)}{R(\,^{i}E/\,^{j}E)Std}$$

where R is the isotopic ratio of the heavier isotope ^{i}E and lighter isotope ^{j}E of element E (i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample S and international standard Std. The isotopic ratios were estimated relative to international standards such as Vienna Pee Dee Belemnite (VPBD) for carbon, Atmospheric Air for nitrogen and Vienna Canyon Diablo Troilite (VCDT) for sulphur.

We used International Atomic Energy Agency (IAEA, Vienna, Austria) certified reference materials calibrated against the international isotopic references sucrose (IAEA-C_6, $\delta^{13}C$ = -10.8 ± 0.5 ‰; mean ± SD), ammonium sulphate (IAEA-N_2, $\delta^{15}N$ = 20.3 ± 0.2 ‰; mean ± SD) and silver sulphide (IAEA-S_1, $\delta^{34}S$ = - 0.3 ‰; mean ± SD) as primary standards, and sulphanilic acid ($\delta^{13}C$= -25.9 ± 0.3 ‰ ; $\delta^{15}N$ = -0.1 ± 0.4 ‰ ; $\delta^{34}S$= 5.9 ± 0.6 ‰ ; mean ± SD in each case) as secondary analytical standard. The isotopic ratios of samples were normalized using primary analytical standards. The standard deviations on multi-batch replicate measurements (one every 12 analyses) of sulphanilic acid and a randomly selected seal muscle were 0.2 ‰ for $\delta^{13}C$ and $\delta^{15}N$ values and 0.5 ‰ for $\delta^{34}S$ values.

Lipids extraction in herring muscle was not possible due to the small quantity of tissue material available for the analysis; however, the large C/N variability found in these organisms forced us to mathematically correct for the C signature. Thus, the lipid normalization equation of McConnaughey and McRoy[26] adapted by Post[27] for aquatic animals ($\delta^{13}C_{normalised}$= $\delta^{13}C_{untreated}$ - 3.32 + 0.99 x (C:N)) was used to correct for the lipid depletion effect.

2.3. Isotope fractionation calculation

The TEFs of N, C and S were calculated using the following equation:

$$\Delta^{n}X = \delta^{n}X_{p} - \overline{\delta}^{n}X_{f}$$

where Δ represents the TEF, ^{n}X the isotopic ratio of a particular element for a predator p, $\overline{\delta}$ is the average of stable isotope ratios X of all food items *f* used during captivity.

The TEFs measured on our captive hooded seals were compared with those resulting from the application of the regression models of Caut *et al* [7] and the Bayesian models of Healy *et al* [22] in order to check for their validity for species for which no measurement is available (Figure 2). Following Caut *et al* [7] we calculated $\Delta^{13}C$ and $\Delta^{15}N$ for muscle, hair and red blood cells through the application of their regression equations, which either considered all tissues together ($\Delta^{13}C = -0.417\delta^{13}C - 7.878$ and $\Delta^{15}N = -0.141\delta^{15}N + 3.975$) or each tissue separately ($\Delta^{13}C_{hair} = -0.474\delta^{13}C - 9.064$, $\Delta^{13}C_{muscle} = -0.366\delta^{13}C - 7.030$, $\Delta^{13}C_{RBC} = 1.16 \pm 0.19‰$; $\Delta^{15}N_{muscle} = -0.214\delta^{15}N + 3.938$, $\Delta^{15}N_{hair} = 2.59 \pm 0.41‰$ and $\Delta^{15}N_{RBC} = 2.06 \pm 0.23‰$). The Bayesian model was instead used to calculate all TEFs for the different tissues using the newly developed DEsIR (Stable Isotope Discrimination Estimation In R) package (now renamed as SIDER) in the R v3.4.0 statistical environment (R Core Team, 2016) [28]. For the application of the Healy *et al* method [22], several parameters needed to be specified as input information: such as *Cystophora cristata* habitat ("marine"), taxonomic class ("mammalia"), tissue ("blood", "hair" or "muscle") and diet type ("carnivore"). Since no specific values for red blood cells were available in the literature, we used TEFs measured on whole blood to run the model.

2.4. Statistical analyses

The Shapiro-Wilk test was used to check for normality, while the Levene's test was used to assess the homogeneity of all sets of data. A one-way analysis of variance (ANOVA) followed by Dunnett's pairwise multiple comparisons test ($p < 0.05$) was conducted to examine the variability of IF between the three tissue of hooded seals; while parametric Student t tests ($p < 0.05$) were performed to compare delta values between each seal tissue and the muscle of herring. All statistical analyses were performed using GraphPad Prism version 6 for Mac (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Biometric analysis

At the time of death, the 8 captive seals (4 males and 4 females) weighed between 75 and 103 kg, with lengths ranging from 138 to 148 cm (Table 1). We applied the equation proposed by Wiig [24] for hooded seals specifically, to estimate the age of all juveniles except K2, K7 and K8, whose measurement of standard length was not available. These animals were estimated to be between 19 and 22 months old.

3.2. Stable isotope ratios

No difference was found in all the considered parameters between the two seals euthanized in 2013 and those sacrificed in 2014 ($p >= 0.05$); therefore, the 8 animals will be considered in our analysis as the same group. The stable isotope ratios of C, N and S and the C/N ratio of each tissue of hooded seals are presented in Table 2, together with the results of the ANOVA. The lipid-normalized $\delta^{13}C$ values of the herring ($n = 15$) used to feed the seals during the experiment were -20.5 ± 1.2 ‰, the $\delta^{15}N$ values were 9.5 ± 0.5 ‰, and the $\delta^{34}S$ values were 17.2 ± 0.5 ‰. Their C/N ratios ranged from 5.2 to 11 (mean + SD: 7.0 ± 1.6). In captive seals the $\delta^{13}C$ values significantly differed among all tissues with hair being the most ^{13}C-enriched tissue, followed by muscle and RBCs. Muscle showed the highest $\delta^{15}N$ values, while hair and RBCs had similar ranges. No difference was observed for $\delta^{34}S$ values among all three tissues. A significant difference between captive seals and herring was found in hair $\delta^{13}C$ values ($p = 0.006$, $t = 3.042$, $df = 17$). For muscle, such a difference was weaker ($p = 0.033$, $t = 2.300$, $df = 19$), while it did not exist for RBCs ($p = 0.067$, $t = 1.932$, $df = 21$). With regard to $\delta^{15}N$ values, a significant difference between seals and herring was found for all muscle ($p < 0.0001$, $t = 19.94$, $df = 19$), hair ($p < 0.0001$, $t = 14.14$, $df = 17$) and RBCs ($p < 0.0001$, $t = 18.97$, $df = 21$).

The C/N ratio varied very little within tissues, with hair having a significantly lower mean (3.0 – 3.1, $p < 0.0001$) than RBCs (3.3 – 3.3) and muscle (3.3 – 3.4). The seals-herring comparison of $\delta^{34}S$ values showed a significant difference in muscle ($p = 0.007$, $t = 3.043$, $df = 19$) and RBCs ($p = 0.008$, $t = 2.906$, $df = 21$) but not in hair ($p = 0.175$, $t = 1.416$, $df = 17$).

3.3. Experimentally-estimated TEFs

The $\Delta^{13}C$ significantly varied among all tissues (ANOVA, $p < 0.0001$, $F = 184.5$) with hair showing the largest fractionation ($\Delta^{13}C = 1.9 \pm 0.1$ ‰), followed by muscle ($\Delta^{13}C = 1.1 \pm 0.1$ ‰) and RBCs ($\Delta^{13}C = 0.8 \pm 0.1$ ‰) (Figure 1). The $\Delta^{15}N$ differed between muscle ($\Delta^{15}N$

= 4.3 ± 0.4 ‰), which showed the largest fractionation, and the other two tissues ($\Delta^{15}N$ of hair = 3.6 ± 0.3 ‰; $\Delta^{15}N$ of RBCs = 3.3 ± 0.1 ‰) (ANOVA, $p < 0.00001$, $F = 26.15$). Hair and RBCs showed a less pronounced difference ($p = 0.049$). The $\Delta^{34}S$ of muscle was 0.9 ± 0.8 ‰, that of hair 0.4 ± 0.1 ‰ and that of RBCs 1.0 ± 0.8 ‰, and no difference was observed among any tissues for $\Delta^{34}S$ (ANOVA, $p = 0.208$, $F = 1.766$).

3.4. Comparison with model-estimated TEFs

High variation was obtained between the TEFs measured in captive hooded seals and those resulting from the application of models (Figure 2). For muscle our ^{15}N-measured TEFs resulted in the largest values, followed by those calculated by the Bayesian method of Healy *et al* [22] ($\Delta^{15}N = 4.0 \pm 1.1$ ‰), the Caut *et al* [7] all-tissue regressions ($\Delta^{15}N = 2.0 \pm 0.1$ ‰) and the Caut *et al* [7] tissue-specific regressions ($\Delta^{15}N = 0.8 \pm 0.1$ ‰). For hair, our ^{15}N-TEFs were the largest, followed by those of Healy *et al* [22] ($\Delta^{15}N_{hair} = 3.1 \pm 0.0$ ‰), the Caut *et al* [7] tissue-specific regressions ($\Delta^{15}N_{hair} = 2.6 \pm 0.3$ ‰) and the Caut *et al* [7] all-tissue regressions ($\Delta^{15}N_{hair} = 2.0 \pm 0.02$ ‰). For RBCs, the Healy *et al* [22] ^{15}N-TEFs were the largest ($\Delta^{15}N_{RBC} = 4.1 \pm 1.0$ ‰), followed by our measured values, the Caut *et al* [7] all-tissue regressions ($\Delta^{15}N_{RBC} = 2.2 \pm 0.02$ ‰) and finally the Caut *et al* [7] tissue-specific regressions ($\Delta^{15}N_{RBC} = 2.1 \pm 0.1$ ‰).

For muscle the ^{13}C-TEFs calculated by the model of Healy *et al* [22] were the largest ($\Delta^{13}C = 1.5 \pm 1.4$ ‰), followed by our measured values, the Caut *et al* [7] all-tissue model ($\Delta^{13}C = 0.1 \pm 0.04$ ‰) and the Caut *et al* [7] tissue-specific model ($\Delta^{13}C = -0.05 \pm 0.04$ ‰). For hair the ^{13}C-TEFs calculated by the model of Healy *et al* [22] were the largest ($\Delta^{13}C = 3.1 \pm 0.0$ ‰), followed by our measured values, the Caut *et al* [7] all-tissue model ($\Delta^{13}C = -0.1 \pm 0.04$ ‰) and the Caut *et al* [7] tissue-specific model ($\Delta^{13}C = -0.2 \pm 0.04$ ‰). Finally, the ^{13}C-TEFs in RBCs were largest in the Healy *et al* [22] model ($\Delta^{13}C = 1.4 \pm 1.3$ ‰), followed by the Caut *et al* [7] tissue-specific model ($\Delta^{13}C = 1.2 \pm 0.2$ ‰), our measurements and the Caut *et al* [7] all-tissue model ($\Delta^{13}C = 0.3 \pm 0.03$ ‰).

Overall, the Bayesian model developed by Healy *et al* [22] resulted in TEFs included in the same range as the experimentally-calculated ones, while the equations of Caut *et al* [7] gave more different values, which in some cases also gave negative estimates.

4. Discussion

4.1. Comparison with literature

Prey-predator isotope fractionation occurs during the several biochemical pathways that a particular food item undergoes during digestion and assimilation by a predator[29]. Within an organism N-IF occurs primarily during processes of deamination and transamination of amino acids from the assimilated food, for the synthesis of new body proteins [30,31]. During such processes, different nitrogenous waste products (e.g. NH_4^+) are formed and excreted as urea, uric acid or ammonia, causing consumer-diet ^{15}N enrichment [30]. This is in accordance with the significant difference found in $\delta^{15}N$ values between our hooded seals and their given diet. However, when comparing our results with those in the the literature, the $\Delta^{15}N$ in our hooded seals was larger than that of other seal species or animals [32]. Taking RBCs as an example, the $\Delta^{15}N$ values found in previous captivity experiments ranged from 1.5 to 2.2‰ for captive harp and harbour seals, to 2.5‰ in foxes, while our values are higher than 3.0‰ [8,33].

The main process determining C IF is respiration, in which ^{12}C is lost as $^{12}CO_2$, during the oxidation of acetyl groups derived from the catabolism of lipids, proteins, and carbohydrates [30]. Our $\Delta^{13}C$ values of RBCs and muscle are comparable with those measured in other species of phocids [9,14,32,34,35] or other terrestrial mammals [33,36]. However, hooded seal hair was significantly enriched in ^{13}C with respect to diet; in contrast to previous studies that manifest a generally less pronounced fractionation [37]. Very little is known on S metabolic pathways within the organisms; to our knowledge this is the first study to assess S fractionation values in animals with complex physiology such as *Phocidae*. The main sources of S to a consumer are the organic S (contained in amino acids from the diet) and the inorganic S from the environment [36]. As for C, little or no fractionation is commonly associated with S assimilation into animals tissues and this is confirmed by the absence of significant increase in ^{34}S between hooded seal hair and herring [38]. Nevertheless, we found a significant enrichment in ^{34}S in muscle and RBCs compared with diet, which is in the same range of S fractionation as that measured in the muscle of different aquatic vertebrates (1.9 ± 1.4 ‰) [36].

Several factors may influence organisms IF, such as what taxonomic group they belong to [39,40]. The higher fractionation of ^{15}N in our hooded seals with respect to terrestrial animals, as for example the red foxes analysed by Roth and Hobson [33], may be due to the particular physiological adaptations linked with the diving behaviour of marine mammals [9]. Indeed, seals' blood has far higher haemoglobin and haematocrit levels than that of other non-diving homeotherms. It is possible that such differences in blood composition influence IF values [9]. However, a difference in N fractionation is observed not only between our seals and other organisms, but also when comparing our results with the values calculated by Beltran *et al* [34] for other phocids. One possibility is that the higher $\Delta^{15}N$ values shown by hooded seals is a consequence of the difference between diets [33]. Indeed while our captive seals were fed with a diet mainly composed of proteins (enriched in ^{13}C by about 4‰ over carbohydrates and by 6‰ over lipids), Beltran and colleagues [34] used another nutrition strategy, consisting of a mix of commercial pellets, herring and capelin that possibly include higher proportions of carbohydrates [34]. Such variation may also be determining the seal-herring S enrichment observed in our study, since McCutchan and colleagues [36] demonstrated that a diet high in proteins can increase the S fractionation rate of consumers with respect to diet.

Isotopic routing is considered as the dependency of consumers' isotopic composition on dietary proteins, carbohydrates and lipids [41]. This process determines a particular isotope incorporation during the formation of a tissue, based on the isotopic nature of the nutrients assimilated through the diet [10,42]. Even if the precise mechanisms are still poorly known, a few studies have demonstrated that age can influence N and, to a lesser degree, C routing during assimilation in consumers' tissues [43]. In fact in the early life-history stages, marine mammals such as seals undergo important physiological modifications regarding routing and rates of utilisation of elements, in order to sustainably maintain the organism [44,45]. The incorporation of the isotope composition of the diet in growing predators depends more on the addition of new material to the tissue than on the replacement of materials exported from the tissue [46]. This means that almost the totality of protein C and N assimilated by a young growing predator in the muscle derives from its prey [10,42]. Several studies have shown how growing birds and fish have higher rates of protein synthesis and catabolism that cause faster isotopic turnover and magnify the trophic effect, causing an even greater loss of the isotopically light N in urine [10,47]. These mechanisms could probably explain the greater $\Delta^{15}N$ values of our juvenile hooded seals.

Finally, independently of diet the variability of N stable isotope composition between our hooded seals and the other seals may be directly associated with the health status of these animals. In the case of a particular disease, a metabolic shift occurs toward utilizing more N for protein synthesis rather than urea production; as a consequence IF processes are directly affected [48]. In humans for example, liver $\delta^{15}N$ values in cirrhotic patients result in a ^{15}N depletion of 3.2‰ with respect to healthy persons, without significant differences in $\delta^{13}C$ values [49]. While we conducted our captivity experiment on healthy captured seals, Hobson *et al* [9], Germain *et al* [31,32] and Beltran *et al* [34] measured their fractionation on juvenile seals and sea lions in rehabilitation, and therefore with a poorer health status.

4.2. Comparison between tissues

For $\Delta^{13}C$, hair exhibited the greatest fractionation, followed by muscle and RBCs. In contrast, $\Delta^{15}N$ was much higher in muscle than in RBCs or hair. Finally, no differences were found in $\Delta^{34}S$ between tissues.

The turnover rate of a tissue influences elements incorporation rates and consequently IF processes. Indeed, Hobson and Clark [50] found that the C turnover rate correlated linearly with the metabolic rate of tissues. For example, they found that in quails and American crows the half-life of C ranged from 2.6 days in liver, and 20 days in muscle, to circa 50 days in hair. Moreover, Pearson and colleagues [51] showed that in warblers, the N half-life ranged from 0.5 to 1.7 days for plasma and from 7.5 to 28 days for whole blood. The higher tissue activity leads to quicker C or N incorporation with less metabolic changes of these elements, and results in a smaller IF [51]. Therefore, the higher $\Delta^{13}C$ values found in the inert hair of our hooded seals than in muscle and RBCs may be a direct consequence of tissue turnover rates [50,51].

In addition, the quantity of a certain element determines the effects of fractionation processes. The higher the abundance of a specific compound available for biosynthesis the smaller the fractionation of its isotopic composition [52,53]. The C/N ratios of our three tissues muscle and RBCs were very similar to each other, while hair presented much lower C/N value. The C/N ratios represent the quantity of C-containing molecules with respect to N in one sample [54]. Therefore, the lower C/N value results from the fact that hair is mostly composed of keratin, while blood and muscle present several C sources. The presence of a single C source forces the multiple usage of the same C pool by the hair tissue during its catabolism, leading to larger fractionation processes.

The pattern of variation for $\Delta^{13}C$ seen in across tissues was not observed for $\Delta^{15}N$, with larger fractionation for N observed in muscle tissue than in hair. One possible explanation may be linked to the fact that the different metabolic components (i.e. lipids, proteins, carbohydrates, etc.) of cells, and at larger scale of tissues, do not have the same isotopic composition as a consequence of their varied histories [55]. For example, the C used for the production of fatty acids via the Acetyl-CoA route (oxidation of pyruvate to acetyl coenzyme A) is ^{13}C-depleted with respect to glucose; and this phenomenon explains the well-known ^{13}C depletion of lipids [55]. In addition, amino acids present a large variability in both their ^{13}C and ^{15}N isotopic signature, as shown in the collagen of pigs [56,57]. Therefore, the selection of one specific C, N or S "pool" for the metabolic renewal of a tissue will influence isotope incorporation rates, fractionation processes and ultimately tissue isotopic composition [29].

The number of metabolic steps that an element undergoes in a tissue may also determine variation of IF; therefore, the more elevated rates of protein metabolism for energy expenditure in muscle may determine a significant larger discrimination in this tissue. In our hooded seals this is shown by the larger prey-predator ^{15}N enrichment found in muscle than in RBCs or hair [58]. The same process can lie behind the fact that even if no statistical difference is observed in S fractionation among hooded seals tissues, ^{34}S presented greater consumer-tissue enrichment in RBCs and muscle, than in hair. Sulphur is present in the mammalian body in four main S-containing amino acids: methionine, cysteine, homocysteine, and taurine. Only the first two are incorporated in proteins [59]. Cysteine can be synthesized by the body starting from methionine, which is an essential amino acid assimilated by the diet [60]. These two amino acids are integrated in numerous protein compounds in the different tissues based on metabolic requirements [60]. In hair, for example, both cysteine and methionine are part of the filamentary complex of keratin [61]. Once incorporated into keratin they are no longer available for other metabolic processes [61]. This could result in the very low consumer-diet S fractionation in hooded seal hair. In RBCs and muscle instead, such amino acids are subjected to several changes during the formation of a vast set of proteins (vitamins, glutathione, etc.) that may play an important role, for example in the case of seals, in antioxidant response during diving hypoxia, and therefore result in larger fractionation rates [62,63]. This is further confirmed in Figure 1 where RBCs and muscle present TEF values distributed along a range of 2.5 and 1.7‰, respectively, whilst hair is quite homogenous.

4.3. Assessment of model efficiency in calculating isotopic fractionation

The large number of potential factors which seem to be influencing IF processes in juvenile hooded seals really calls for further measurements, especially for growing individuals. However, in the case of marine mammals, captivity studies are limited by ethical and practical problems. In this direction Caut *et al* [7] and Healy *et al* [22] developed two models that allow TEFs to be calculated (called by Caut *et al* [7]: "*Discrimination factors*"; by Healy *et al* [7]: "*TDF Trophic Discrimination Factor*") for animals for which no direct measurement exists. Two different statistical approaches are considered by these authors: the first model proposes the application of a multiple-regression Diet-Dependent Discrimination Factor method (DDDF) in which diet isotopic ratio is considered as the main factor controlling IF variation [7]. Healy *et al* [7] instead use a Bayesian model (SIDER) to calculate IFs for birds and mammals through the incorporation of multiple sources of variation, among which are the phylogenetic structure and the error associated with measurements within a species [22]. The application of the Caut *et al* [7] model to calculate hooded seal IFs of muscle, hair and blood gives very different results from those measured during our experiment (Figure 2). The $\Delta^{15}N$ estimates were smaller than our values using either equations for all tissues pooled together, or equations specific to each tissue. The $\Delta^{13}C$ values were also smaller than those obtained in our experiment with the one exception of C-TEF calculated in RBCs by tissue-specific equations. The output of the Healy *et al* [7] model, instead, resulted in TEFs within the same range as those measured in this study. This shows that the hypothesis of Caut *et al* [7] that fractionation factors are mostly driven by the isotopic composition of preys does not seem to be appropriate for calculating the IF of seals, especially during the growing period. On the other hand, a comparatively more complex model taking into account multiple factors as drivers for fractionation does a rather good predictive job.

5. Conclusions

This study shows how the "growth effect" on C and N IF, already demonstrated in birds, fish and terrestrial small mammals, is exacerbated in physiologically-complex animals such as Arctic hooded seals. For the first time, it also demonstrates that S can present a significant isotopic enrichment between the consumer and the diet, as a result of diet composition or tissue metabolic needs.

The great N fractionation found in muscle raises concern about the study of juvenile seals ecology and distribution based on stable isotopes analysis. Indeed, we suggest that the

application of the classic 3.5 ‰ N consumer-diet enrichment to calculate predator trophic level or prey's potential dietary proportion in growing marine mammals, leads to a misleading interpretation; therefore, a larger IF should be considered.

Since a small change in diet seems to greatly influence IF between two seal species, a standardisation of feeding protocols may be needed for IF-related captive experiment with phocids. This could prevent misinterpretation of IF variation data between different species during comparison with the literature.

Moreover, the large difference observed especially for N or C isotopic fractionation rates between the different tissues again confirms that: (1) between-tissue fractionation variability cannot be neglected when using "non-intrusive", external tissues (i.e. hair) as a proxy of internal organs (i.e. muscle); and (2) in order to avoid data analysis errors, captive feeding experiments on Pinnipeds must be long enough to ensure that all the tissues with different metabolic rates have reached a complete diet-to-tissue isotopic equilibrium.

Finally, the results of our comparison among different TEF estimation models with experimentally-calculated values negate the oversimplification in the development of such models, which cannot consider only a single factor at the time but has to keep in mind the great complexity of the natural world.

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7. References

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Table 1. List of the 8 hooded seals sampled in the Greenland Sea in March 2012 and included in the captivity experiment with all biometric information and the tissues on which the analysis was conducted. Standard length is expressed in cm, weight in kg.

Seal Id code	Sex	Weight (Kg)	Standard Length (cm)	Tissues sampling day	Sampled Tissue		
					Muscle	Hair	RBCs
K1-12	F	77	144	14.02.14	✓	✓	✓
K2-12	F	103	NA[a]	28.02.14	✓	✓	✓
K3-12	F	88	138	27.02.14	✓	✓	✓
K4-12	M	81.5	144	19.02.14	✓	✓	✓
K5-12	M	85	148	02.12.13[b]			✓
K6-12	F	93	148	04.12.13[b]			✓
K7-12	M	75	NA[a]	21.02.14	✓		✓
K8-12	M	86	NA[a]	26.02.14	✓		✓

[a] NA = Not available
[b] Euthanized before in the framework of physiological studies at UiT.

Table 2. Carbon, Nitrogen and Sulphur isotope ratios and C/N ratios of muscle, hair and red blood cells (RBCs) of captive hooded seals. Stable isotope ratios are represented in δ notation (‰) as Mean ± SD (Min – Max). N represents the number of samples in which the analysis was conducted for each tissue.

Values	Tissues			Inter-tissue ANOVA*
	Muscle	Hair	RBCs	
N	6	4	8	–
δ^{13}C value	-19.4 ± 0.103 (-19.5 – -19.1)	-18.7 ± 0.1 (-18.8 – -18.6)	-19.6 ± 0.1 (-19.7 – -19.5)	**F = 182, p < 0.0001, df = 15**
δ^{15}N value	13.8 ± 0.3 (13.4 – 14.5)	13.1 ± 0.2 (12.9 – 13.3)	12.8 ± 0.1 (12.5 – 13.0)	**F = 26.15, p < 0.0001, df = 15**
δ^{34}S value	18.2 ±0.8 (17.1 – 19.3)	17.5 ±0.1 (17.4 – 17.6)	18.0 ± 0.8 (16.7 – 19.4)	F = 0.818, p = 0.458, df = 15
C/N ratio	3.2(3.3)±0.05 (3.3 – 3.4)	3.1(3.1)±0.04 (3.0 – 3.1)	3.3(3.2)±0.01 (3.3 – 3.3)	**F = 106, p < 0.0001, df = 15**

* Bold values represent a signific

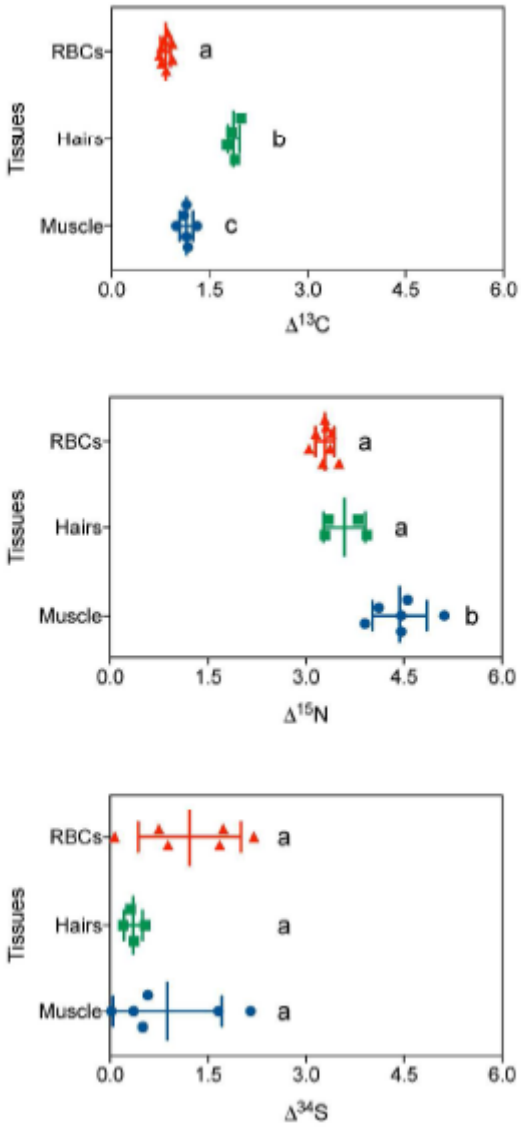

Figure 1. Calculation of isotopic fractionation (TEF: Δ) factors of Carbon ($\Delta^{13}C$), Nitrogen ($\Delta^{15}N$) and Sulphur ($\Delta^{34}S$) in muscle (N = 6), hair (N = 8) and red blood cells (RBCs) (N = 8) of juvenile hooded seals subjected to controlled feeding experiment. Values are presented as Mean (the middle bar ± SD lines) in per mill (‰). Statistical groups are represented by the letters a,b and c.

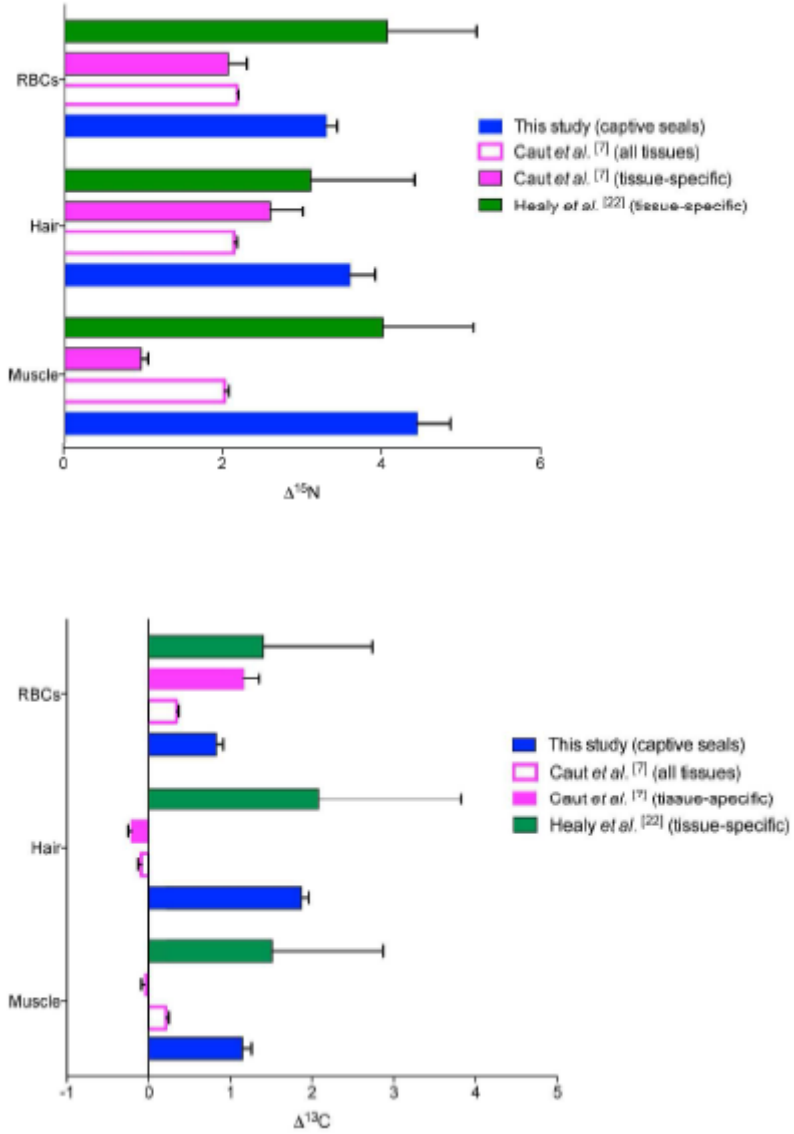

Figure 2. Comparison of the experimentally-estimated TEFs in this study for captive juvenile hooded seals (blue) and the model-estimated TEFs resulting from the application of the Diet-based regression model of Caut *et al.* [7] (filled and empty pink) and the Bayesian model of Healy *et al.* [22] (green).