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RESEARCH ARTICLE

Carbon isotopic fractionation in eider adipose tissue varies with fatty acid structure: implications for trophic studies

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SUMMARY

Carbon isotopic fractionation was investigated in fatty acids (FA) of adipose tissue and blood serum of threatened Steller's eiders (*Polysticta stelleri*) and spectacled eiders (*Somateria fischeri*) relative to the FA in their diets. Captive eiders were fed a known diet for 180 days with serum sampled at 60, 120 and 180 days immediately after a 12 h fast; adipose was collected at 180 days. Essential FA (EFA) in the adipose showed varying degrees of isotope fractionation (0–4‰), depending on FA structure. The $\delta^{13}C$ values of long-chain FA 20:5n-3 and 22:6n-3 did not differ from those in the diet, while those of 18:2n-6 and 18:3n-3 were ~2‰ greater than in the diet. The $\delta^{13}C$ values of free FA (FFA) in serum were not consistent within individuals or sampling dates; fractionation varied randomly, suggesting that FFA were arising from diet, rather than mobilization from adipose tissue. Discrimination factors were used in combination with a mixing model incorporating FA and lipid concentrations to estimate the diet of eiders fed a binary mixture with contrasting isotopic signatures. Diet estimates varied with FA but mean values closely approximated the actual proportions consumed. By tracking EFA, this study avoided the complications in interpretation arising from isotopic routing of carbon in bulk isotope analyses and serves as a basis for the development of compound-specific isotopic methods to trace dietary input in wild eiders. However, our understanding of the processes contributing to the variation in isotopic signatures of FA in nature is currently limited, and we recommend that future research directions focus on elucidating these mechanisms.

Key words: diet estimate, discrimination, lipid, compound-specific isotope analysis, stable isotope, Steller's eider, spectacled eider.

INTRODUCTION

The spectacled eider, *Somateria fischeri* (Brandt 1847), and the Alaskan breeding population of the Steller's eider, *Polysticta stelleri* (Pallas 1769), have experienced precipitous population declines of breeding birds in Alaska and are currently listed as threatened under the provisions of the US Endangered Species Act (Federal Register, 1993; Federal Register, 1997). Reasons for the declines remain largely unknown, but changes in the marine ecosystem leading to variation in the availability of food resources have been listed as potential causes and threats to the recovery of both species (US Fish and Wildlife Service, 1996; US Fish and Wildlife Service, 2002). Both Steller's and spectacled eiders use a range of marine habitats in the Bering Sea for staging, molting and wintering, and are thought to forage on a variety of prey items, including bivalves, crustaceans and snails (Petersen, 1980; Petersen, 1981; Petersen et al., 1998). Both eider species nest in tundra habitats along the coastal Bering and Beaufort Seas, and feed on insects, insect larvae, seeds and plant materials in tundra ponds (Petersen et al., 2000; Fredrickson, 2001). Reproductive problems and low productivity have been documented for both species (Grand et al., 2002; Rojek, 2008). In Steller's and spectacled eiders, the relative importance of marine and terrestrial nutrients for reproduction is unknown, but there is evidence to suggest that some sea duck species acquire nutrients for successful breeding from both the terrestrial nesting and marine staging grounds (Bond et al., 2007; Sénéchal et al., 2011). A better understanding of diet sources and timing of nutrient acquisition for these eiders will provide information to help identify important pre-breeding habitats of threatened eider populations in Alaska.

There are several well-known challenges and biases associated with traditional methods of estimating marine bird diets (reviewed in Barrett et al., 2007). New techniques using minimally invasive sampling methods and biochemical signatures in stored fat (Iverson et al., 2007; Wang et al., 2010), blood (Käkelä et al., 2009; Federer et al., 2010), stomach oil (Connan et al., 2005; Wang et al., 2007), feathers (Hobson and Clark, 1992) or eggs (Hobson, 1995; DeVink et al., 2011) provide promising alternatives for diet assessments in avian populations. These biomarker techniques typically involve either stable isotope or fatty acid (FA) analysis. For instance, stable isotopic analyses of carbon have been used to determine the relative contributions of sources of diet items (i.e. marine *versus* terrestrial, benthic *versus* pelagic), while stable nitrogen isotope analyses can indicate an animal's trophic level (Hobson and Welch, 1992; Hobson et al., 2002). However, there are limitations to the bulk isotope (i.e. measurement of the isotopic composition of total organic carbon or nitrogen in a sample) approaches, including isotopic routing and fractionations from diet to tissue that can complicate interpretation (Federer et al., 2010). As an alternative, FA signature analysis has been used to investigate diets

of higher predators (e.g. Thiemann et al., 2007; Williams et al., 2008; Cooper et al., 2009), including captive eiders (Wang et al., 2010). FA are the primary components of most lipids and have a variety of structures. Because of biochemical restrictions, many dietary polyunsaturated FA (PUFA) are incorporated into marine animals with little modification to structure, making these FA valuable as indicators, or biomarkers, of their source. However, these biomarker techniques can only offer general information on temporal and spatial variation in consumer diets; much more complex models (i.e. quantitative fatty acid signature analysis, QFASA) (Iverson et al., 2004) are necessary to estimate proportions and types of prey species in predator diets using FA.

Recently, we demonstrated the use of stable carbon isotopes of individual FA to trace carbon flow from prey to predator across multiple trophic levels in the Arctic (Budge et al., 2008). This approach tracks the stable carbon isotopes of specific FA structures that can only arise from diet and has two clear advantages over the bulk isotope technique. First, this technique avoids the problem of isotopic routing encountered with bulk isotope analyses. Isotopic routing occurs when the carbon associated with a dietary component (protein, carbohydrate or lipid) is routed to a specific tissue component. For instance, dietary protein carbon is likely to be routed to body muscle tissue. Difficulties arise, however, when a dietary component, such as carbohydrate or lipid, is catabolized and used as a carbon source to synthesize another component, such as protein. The extent of routing can be influenced by nutrient content and digestibility of diets (Focken, 2004; Codron et al., 2011) and environmental factors (Bloomfield et al., 2011), and techniques such as lipid removal before isotopic analysis attempt to minimize its impact; however, it remains a complication in the interpretation of bulk carbon isotope analysis (Gannes et al., 1997; Wolf et al., 2009). Analysis of essential dietary FA that cannot be synthesized by animals thus avoids some of the difficulties associated with isotopic routing. Second, this technique acquires $\delta^{13}C$ data of several FA within each sample near-simultaneously; when combined with mixing models to estimate source contributions, this allows several independent assessments of diet to be made, each based on an individual FA, thereby increasing the accuracy of the results. Thus, the combination of these features in the analysis of stable carbon isotopes of essential FA (EFA) offers clear benefits compared with the analysis of isotopes of bulk carbon.

While several studies have reported the stable carbon isotope composition (expressed as $\delta^{13}C$ values) of FA in members of various food webs (Hammer et al., 1998; Ruess et al., 2005; Budge et al., 2008) and provided evidence to suggest that essential dietary FA $\delta^{13}C$ values are preserved in the predator's tissues (Stott et al., 1997; Howland et al., 2003), none of these have adequately addressed the issue of molecular fractionation, or change in $\delta^{13}C$ values during metabolism of dietary FA. The basic processes involved in avian lipid digestion, transport and deposition are fairly well understood (Klasing, 1998). In the simplest case, dietary lipids are predominantly triacylglycerols (TAG) consisting of three FA esterified to a glycerol backbone. FA in TAG molecules undergo a number of enzymatic processes (e.g. lipolysis and re-esterification) before being deposited in the adipose for storage. During periods of fasting, FA in the adipose are mobilized for energy and transported in the blood, bound to proteins, as free FA (FFA). FA may also be catabolized for energy in the liver or, in some situations, elongated or desaturated. These processes all involve enzyme-catalyzed reactions and introduce the potential for isotopic fractionation of the FA. Such fractionation may have a substantial impact on the interpretation of the FA $\delta^{13}C$ values; thus, the extent of fractionation must be known before attempting to relate consumers' stable carbon isotope compositions to their diets.

Our objective was to determine the extent of molecular carbon isotopic fractionation that dietary FA experience during fat deposition and mobilization through controlled feeding studies with captive Steller's and spectacled eiders. Specifically, we measured the $\delta^{13}C$ values of several essential and non-essential FA in diet, adipose and serum to create a mixing model incorporating information from molecular fractionation in the eider tissues, and proportions and $\delta^{13}C$ values of specific FA in dietary end-members. We then tested this model on samples preserved from a previous captive feeding experiment (Wang et al., 2010) in which eiders were switched from a diet based on terrestrial material to experimental diets spiked with a different marine food item.

MATERIALS AND METHODS

Controlled feeding studies

Feeding trials were conducted during the non-breeding season with eight male Steller's eiders and eight male spectacled eiders housed at the Alaska SeaLife Center in Seward, Alaska. Research was conducted under Institutional Animal Care and Use protocol number 09-008. For 2 years, these birds had been consistently fed a diet based on Mazuri© sea duck formula (Purina Mills, St Louis, MO, USA), which consisted of approximately 6.5% lipid, 21.6% protein, 8.4% fiber, 10.9% ash and 46.6% nitrogen-free extract along with vitamins (http://www.mazuri.com; accessed 15 March 2006). This was supplemented with ≤5% of other diet items, including Antarctic krill (*Euphausia superba*), Atlantic silverside (*Menidia menidia*), Atlantic surf clam (*Spisula solidissima*) and blue mussel (*Mytilus edulis*) (diet 1). This diet was also supplied throughout the 180 days of this experiment, with the intake of diet items recorded daily by species so that the actual diet could be calculated. Blood serum was sampled between 09:00 h and 12:00 h at 60, 120 and 180 days immediately after a 12 h fast. Up to 3 ml of whole blood was collected from the jugular vein using a 23 gauge needle and a 5 ml syringe, transferred to collection tubes (BD Vacutainer SST Plus Blood Collection Tubes, Franklin Lakes, NJ, USA) and allowed to clot at 4°C. Adipose tissues were collected from all birds at the end of the 180 days. A biopsy technique was used to obtain approximately 0.1 g of synsacral adipose tissue samples from individuals *via* a 1 cm skin incision (Iverson et al., 2007; Wang et al., 2010). The area was disinfected prior to biopsy with a betadine swab and lidocaine spray. Lidocaine was also injected subcutaneously. After surgery, the biopsy site was sealed with Vet-bond© (3M, St Paul, MN, USA). The adipose tissue sample was placed in a vial of chloroform with 0.01% butylated hydroxytoluene added as an antioxidant.

For comparison, samples from a previous controlled feeding experiment (Wang et al., 2010) were also analyzed. In that study, eiders were fed a consistent diet of predominantly Mazuri© (88%), supplemented with the same diet items as in diet 1 described above. After 69 days, the diets of the Steller's and spectacled eiders were switched to diet 2 which consisted of only Mazuri© and krill, at proportions of 66% and 34%, and 56% and 44%, respectively. These diets were maintained for 21 days and synsacral adipose tissue was collected as described above. For both experiments, samples of the Mazuri© and diet supplements were collected each month to monitor the consistency of FA level and isotopic composition (N=25; 5 samplings × 5 diet types consisting of Mazuri© and supplements of krill, silverside, clam and mussel). Samples were frozen intact and stored at –20°C until extracted.

Isolation of blood lipids, lipid extraction and FA analyses

Whole-blood samples were centrifuged to isolate serum at 1500 ***g*** for 10 min in a Clay Adams® TRIAC® centrifuge (Becton Dickinson Company, Franklin Lakes, NJ, USA) within 1–2 h of the blood draw. Lipids were extracted from all sample types (serum, adipose, diet samples) using a modified Folch extraction (Folch et al., 1957; Budge et al., 2006) with 2:1 chloroform:methanol. Thin-layer chromatography using a developing solvent of 85:15:1 hexane:diethyl ether:acetic acid was used to isolate FFA in fasting blood samples. FA methyl esters (FAME) were prepared directly from ≤100 mg of the extracted and isolated lipid using H_2SO_4 in methanol. FAME were then extracted into hexane and concentrated to 50 mg ml^{-1}.

FAME were quantified using temperature-programmed gas chromatography (GC) as described previously (Budge et al., 2006), using a Perkin Elmer Autosystem II (Perkin Elmer, Boston, MA, USA) flame ionization detector (FID) gas chromatograph fitted with a 30 m×0.25 mm i.d. column coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness; J&W DB-23; Folsom, CA, USA) and operating in split injection mode. For all sample types, 1.0 μl of a 50 mg ml^{-1} solution of FAME in hexane was injected. FA were identified using known standard mixtures (Nu Check Prep., Elysian, MN, USA) and GC-mass spectrometry (Thermo Finnigan Polaris Q; Thermo Finnigan, Austin, TX, USA). All sample chromatograms and FA identifications were individually checked, corrected, and reintegrated as necessary. Each FA was described using the shorthand nomenclature of *A*:*B*n-*X*, where *A* represents the number of carbon atoms, *B* the number of double bonds and *X* the position of the double bond closest to the terminal methyl group.

The $\delta^{13}C$ values of FAME sub-samples were determined by routing the effluent from a GC through a combustion interface (IsoLink; www.isolink.com) to an isotope ratio mass spectrometer (IRMS) (Thermo Finnigan Delta V). FAME were separated using the same GC column and temperature program as described above. The GC was operated in splitless injection mode and for adipose and serum samples, 1.0 μl of a 1.0 mg ml^{-1} FAME solution in hexane was injected. For diet samples, the concentration was reduced to 0.5 mg ml^{-1}. Monounsaturated FAME isomers were adequately resolved by GC-FID but co-eluted with GC-IRMS so 16:1 and 18:1 represent the sum of those monounsaturated FAME species. FAME analyzed here differed from the FA present in the source material because of the addition of a methyl group derived from the methanol used in transesterification. To correct for the contribution from this extra carbon, we transesterified individual FFA standards 16:0 and 18:0 with the same reagents described above. The $\delta^{13}C$ values for FFA and FAME derived from those were determined using an elemental analyzer (Costech ECS4010; Valencia, CA, USA) routed to the IRMS. The difference between the $\delta^{13}C$ values for FFA and FAME with the same chain length was due to the added methyl group. The proportional contribution of this methyl group to a given FA depends on the chain length; an average $\delta^{13}C$ value for this added methyl group was therefore calculated using the following equation:

$$\delta^{13}C = (n + 1)\,[\delta^{13}C_{FAME}] - n[\delta^{13}C_{FFA}]\,, \quad (1)$$

where n is the number of C atoms in the FFA (Abrajano et al., 1994). The methyl C had an average value of –49.0±0.3‰. All FAME data were corrected for the contribution of this methyl group by rearranging the above equation. Analytical precision throughout the runs was tracked using an 18:0 standard and was ≤0.3‰. All $\delta^{13}C$ values are reported relative to Vienna Pee Dee Belemnite using delta notation. These values were calibrated using a standard mixture consisting of ethyl and methyl esters of 14:0, 16:0, 18:0 and 20:0 supplied by Indiana University Stable Isotope Reference Materials, where r^2 of the known *versus* expected relationship was >0.98.

Data analyses

A three-level one-way analysis of variance (ANOVA) was used to test for differences in $\delta^{13}C$ values of each FA between species and diet 1. The $\delta^{13}C$ values of FA of diet 1 were identical for the two species so were not compared. *Post hoc* testing was carried out using Tukey's test with an adjusted α-level of 0.017 (0.05/3). The $\delta^{13}C$ values of FA of diet 2 were not identical so a four-level one-way ANOVA was conducted using an adjusted α-level of 0.013 (0.05/4) for *post hoc* testing. A two-level ANOVA was used to compare $\delta^{13}C$ of FA of diet and the third serum sample, while repeated-measures ANOVA was used to test for differences between adipose and serum. All analyses were performed using SPSS 11. Stable isotopic discrimination factors (Δ) between FA in diet (D) and adipose (A) were calculated by subtracting the isotopic value of diet for each FA and individual eider from the corresponding value in the adipose tissue, so that Δ_{A-D}=A–D. Similarly, discrimination factors were calculated between adipose (A) and serum (S) following the same equation (Δ_{S-A}=S–A).

To test the efficacy of FA-specific isotope analysis in estimating dietary contributions, a two-end member mixing model was used to determine the relative contributions of Mazuri© and krill (diet 2) (Wang et al., 2010) to carbon in the FA examined:

$$\delta^{13}C_{eider,j} - \Delta j = x_j\delta^{13}C_M + (1 - x_j)\,\delta^{13}C_k\,, \quad (2)$$

where $\delta^{13}C_{eider,j}$ is the $\delta^{13}C$ of the eider adipose for j, the FA of interest, Δ_j is the discrimination factor for diet to adipose transfer for j, x_j is the proportion of Mazuri© carbon contribution to j, $\delta^{13}C_M$ is the $\delta^{13}C$ of the Mazuri© FA and $\delta^{13}C_k$ is the $\delta^{13}C$ of the krill FA. To determine the contribution of Mazuri© carbon to all FA in general in the eider adipose tissue, the relative amounts of lipid and FA in the diet items must be taken into account:

$$x_{FA} = \frac{\left(\dfrac{x_j}{FA_{j,M}L_M}\right)}{\left(\dfrac{x_j}{FA_{j,M}L_M}\right) + \left(\dfrac{1 - x_j}{FA_{j,k}L_k}\right)}\,, \quad (3)$$

where x_{FA} is the proportional contribution of Mazuri© to all FA in the eider adipose, $FA_{j,M}$ is the average proportion of FA_j in the Mazuri©, $FA_{j,k}$ is the average proportion of FA_j in the krill, L_M is the average lipid content of the Mazuri© and L_k is the average lipid content of the krill.

RESULTS

The major FA in all diet items were similar, with 16:0, 18:0 and 18:1 generally dominating the profiles (Table 1). The Mazuri© contained 18:2n-6 at 38% by mass of total FA and was distinct from the other diet items, all of which had 18:2n-6 levels <2%. Except for the mussel, levels of 18:3n-3 were also much greater in Mazuri© (4% *versus* <1%) than in the diet supplements. Conversely, clam, krill, mussel and silverside contained the marine FA 20:5n-3 and 22:6n-3 at levels 3–10 times greater than Mazuri©. The FA compositions in adipose and serum were similar for both Steller's and spectacled eiders. The adipose FA of both eider species were broadly similar to that of Mazuri©, the major component in diet 1, with 16:0, 18:1 and 18:2n-6 dominating both profiles; however, relative proportions in adipose and Mazuri© differed (Tables 1 and 2). For example, the ratio of 18:2n-

Table 1. Mass percent and $\delta^{13}C$ values of FA in diet items for diet 1 (this study) and diet 2 (Wang et al., 2010)

	Diet 1					Diet 2	
	Mazuri© (*N*=5)	Krill (*N*=5)	Clam (*N*=5)	Mussel (*N*=4)	Silverside (*N*=5)	Mazuri© (*N*=3)	Krill (*N*=3)
FA composition (mass%)							
16:0	15.87±0.25	21.38±0.45	15.29±0.34	14.66±0.64	16.87±0.20	15.81±0.10	
22.35±0.19							
16:1	2.59±0.17	8.69±0.71	5.29±0.21	11.35±0.24	11.40±0.12	2.28±0.01	9.32±0.37
18:0	6.30±0.04	1.24±0.15	6.46±0.28	2.42±0.15	3.29±0.15	5.29±0.06	1.12±0.04
18:1	20.68±0.14	18.37±0.44	8.51±0.64	5.18±0.33	16.85±0.70	19.26±0.35	
17.91±0.34							
18:2n-6	37.94±0.39	2.22±0.08	0.41±0.02	2.03±0.21	0.84±0.01	38.20±0.47	1.36±0.08
18:3n-3	4.15±0.06	0.95±0.14	0.30±0.02	1.47±0.14	0.70±0.04	4.15±0.04	0.65±0.06
20:5n-3	2.11±0.10	13.59±0.48	10.29±0.74	21.30±2.63	7.68±0.35	1.99±0.04	
13.16±0.81							
22:6n-3	2.37±0.17	6.62±0.58	17.83±0.79	10.63±1.24	18.70±0.49	2.82±0.11	5.93±0.12
FA $\delta^{13}C$ (‰)							
16:0	–26.0±0.1	–33.8±0.3	–24.7±0.2	–25.6±0.4	–23.8±0.2	–23.6±0.9	–29.3±1.9
16:1	–24.9±0.1	–36.2±0.4	–24.3±0.4	–25.6±0.2	–25.1±0.1	–23.5±1.4	–28.7±1.1
18:0	–27.1±0.1	–31.9±0.3	–24.1±0.2	–26.7±0.2	–24.4±0.2	–24.6±1.3	–26.2±0.8
18:1	–25.8±0.1	–35.1±0.3	–25.5±0.2	–26.4±0.3	–25.7±0.1	–22.9±0.3	–28.5±0.9
18:2n-6	–28.6±0.1	–39.2±0.2	NA	–29.2±0.7	–29.2±1.0	–27.1±0.6	–33.6±2.7
18:3n-3	–32.0±0.0	–38.5±0.2	NA	–30.9±0.3	–32.0±0.2	–29.9±0.5	–40.6±2.8
20:5n-3	–26.5±0.2	–36.8±0.2	–25.1±0.2	–25.9±0.1	–25.7±0.2	–24.5±2.5	–33.1±0.8
22:6n-3	–25.8±0.3	–34.0±0.2	–24.3±0.2	–25.3±0.3	–26.5±0.1	–22.8±0.8	–29.9±0.3
Actual proportion consumed (%)							
Steller's eider	92.5	1.7	0.9	1.4	3.5	34	66
Spectacled eider	93.4	1.5	0.9	1.2	3.0	44	56

Mass% and $\delta^{13}C$ data are means ± s.e. FA, fatty acids.

Table 2. FA composition in adipose tissue and blood serum of Steller's and spectacled eiders fed diet 1

	Adipose 180 days	Serum 60 days	Serum 120 days	Serum 180 days
Steller's eider	(*N*=8)	(*N*=8)	(*N*=7)	(*N*=8)
16:0	18.92±0.41	27.06±1.17	28.03±0.79	27.08±0.90
16:1	2.52±0.07	3.18±0.20	2.71±0.20	2.96±0.10
18:0	8.70±0.34	13.00±0.77	16.91±2.26	15.39±0.91
18:1	41.32±0.79	34.00±1.47	28.66±2.91	30.92±1.20
18:2n-6	21.91±0.39	13.72±0.53	12.58±1.16	13.35±0.74
18:3n-3	1.37±0.03	0.88±0.04	1.12±0.06	0.79±0.07
20:5n-3	0.24±0.02	0.25±0.05	0.38±0.07	0.27±0.06
22:6n-3	0.46±0.05	0.35±0.03	0.46±0.05	0.38±0.04
Spectacled eider	(*N*=8)	(*N*=8)	(*N*=8)	(*N*=8)
16:0	17.18±0.40	26.86±0.90	25.81±1.16	23.79±1.00
16:1	1.98±0.04	2.49±0.07	2.37±0.12	2.98±0.20
18:0	8.35±0.52	16.11±0.83	16.70±1.71	13.05±0.87
18:1	44.76±1.37	30.90±1.38	34.06±2.67	36.98±1.67
18:2n-6	21.56±0.51	13.21±0.96	11.46±0.96	14.00±1.08
18:3n-3	1.38±0.05	0.83±0.08	1.02±0.07	0.80±0.10
20:5n-3	0.17±0.02	0.24±0.04	0.35±0.04	0.17±0.02
22:6n-3	0.36±0.06	0.47±0.07	0.51±0.05	0.29±0.05

FA (fatty acid) composition is given as mass% total FA (means ± s.e.).

6 to 18:1 in Mazuri© was approximately 2:1 but only 1:2 in the adipose. Serum FFA also showed variation in proportions relative to the adipose from which they were mobilized. For instance, serum consistently contained greater amounts of 16:0 and 18:0, and lower levels of 18:1 and 18:2n-6 than adipose.

The $\delta^{13}C$ values of individual FA were generally similar in the Mazuri©, clam, mussel and silverside in diet 1 (Table 1). For instance, the $\delta^{13}C$ values of 18:2n-6 ranged from –29.2‰ to –28.6‰ in the four diet items, while 22:6n-3 ranged from –26.5‰ to –24.3‰. Krill FA were all much more isotopically depleted, with $\delta^{13}C$ values in the six FA analyzed varying from –39.2‰ to –31.9‰. Similar patterns were evident in the Mazuri© and krill of diet 2. Based on the known lipid contents (data not shown), concentrations and $\delta^{13}C$ values of FA in individual diet items (Table 1), and proportions of each diet item consumed (Table 1), a $\delta^{13}C$ value of each FA in the total diet was calculated (Fig. 1). For all FA except 20:5n-3 and 22:6n-3, a consistent and significant ($P<0.017$) isotopic enrichment in carbon of 1–4‰ was evident in the adipose of both eider species relative to diet 1 (Fig. 1). In general, this enrichment was less for EFA (18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3) than for non-EFA,

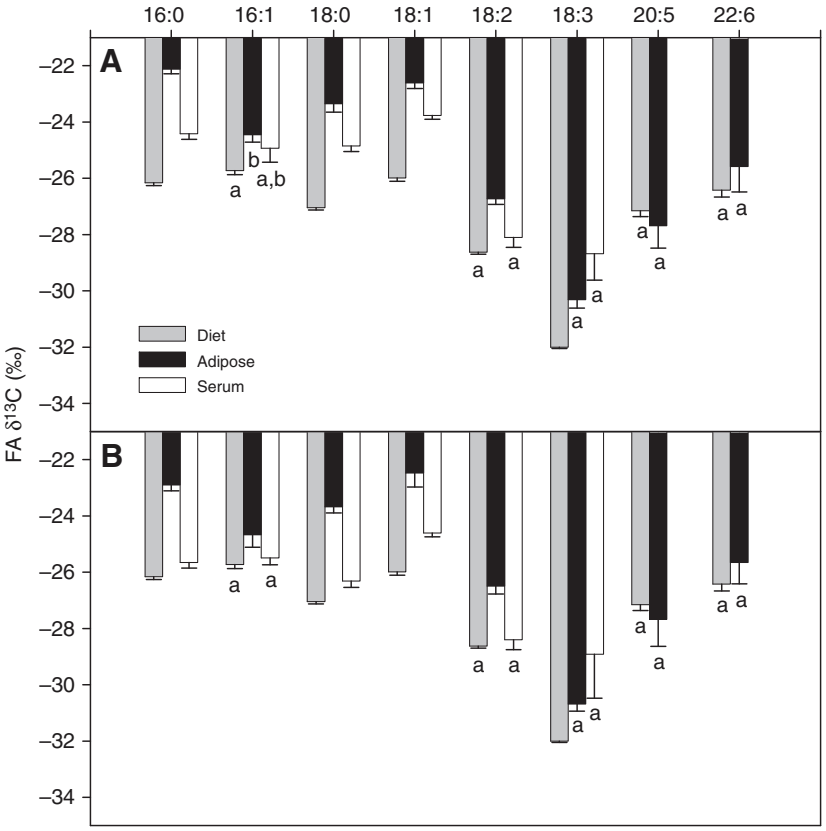

Fig. 1. $\delta^{13}C$ values (means and s.e.) of diet, adipose and serum fatty acids (FA) for (A) Steller's eider and (B) spectacled eider fed diet 1 for 180 days. The same letters indicate statistically similar values. All other comparisons are significantly different (P<0.017).

with the exception of 16:1 (Fig. 1); this FA had a Δ_{A-D} of ~1, similar to that of the EFA. Values for $\delta^{13}C$ of 20:5n-3 and 22:6n-3 in the adipose of both species were not significantly different from diet 1. There was also a very limited species effect for eiders fed diet 1; only $\delta^{13}C$ of 16:0 showed a subtle but significant difference (P<0.017) between the two species (–23.7±0.2‰ and –24.4±0.2‰, for Steller's and spectacled eiders, respectively). Isotopic enrichment of the FA in eider adipose was less consistent between species for eiders fed diet 2 (Fig. 2); however, no significant differences in $\delta^{13}C$ values of each FA in adipose between species were found, due to the large variance (up to ~2‰) associated with the measurements. Isotopic values were more similar between diet 2 and adipose, with 16:0, 18:0 and 18:3n-3, in addition to 20:5n-3 and 22:6n-3, showing similarity (P>0.013) in $\delta^{13}C$ values in both species.

Mean stable isotopic discrimination factors of FA in adipose and diet varied from –0.5‰ to +4‰ within a species (Table 3); within a FA, there was not a significant difference between species (Table 3). The estimated proportions of Mazuri© consumed in diet 2 were calculated based on the discrimination factors of strictly dietary FA (18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3; Table 3), the measured FA $\delta^{13}C$ value in the adipose (Fig. 2), and the lipid content (not shown), FA proportion and FA $\delta^{13}C$ value of each of the two diet items (Table 1), following Eqns 2 and 3. Estimated proportions varied widely among the FA but estimates based on 20:5n-3 were closest to actual

Table 3. Discrimination factors calculated from comparison of $\delta^{13}C$ values in adipose and diet 1

	Discrimination factors (Δ_{A-D})		Estimated proportion Mazuri© consumed	
Calculated	Steller's eider	Spectacled eider	Steller's eider	Spectacled eider
16:0	4.04±0.20	3.26±0.24	NA	NA
16:1	1.28±0.30	1.06±0.46	NA	NA
18:0	3.69±0.31	3.36±0.23	NA	NA
18:1	3.38±0.23	3.51±0.50	NA	NA
18:2n-6	1.90±0.21	2.13±0.29	69±5	55±4
18:3n-3	1.69±0.30	1.32±0.26	14±8	27±9
20:5n-3	–0.53±0.82	–0.52±0.98	30±79	46±116
22:6n-3	0.84±0.93	0.77±0.80	2±51	36±33
Mean	NA	NA	29±95	41±121
Mean 18:2 and 18:3 only	NA	NA	42±9	41±10
Assumed				
20:5n-3	0	0	–33±21	11±22
22:6n-3	0	0	34±362	54±21

Discrimination factor data are means ± s.e.

Proportions of Mazuri© consumed in diet 2 (means ± s.e.) were estimated based on derived discrimination factors, FA (fatty acid) proportions, lipid levels and $\delta^{13}C$ of FA in Mazuri© and krill. Actual proportions of Mazuri© fed were 34% and 44% for Steller's and spectacled eiders, respectively.

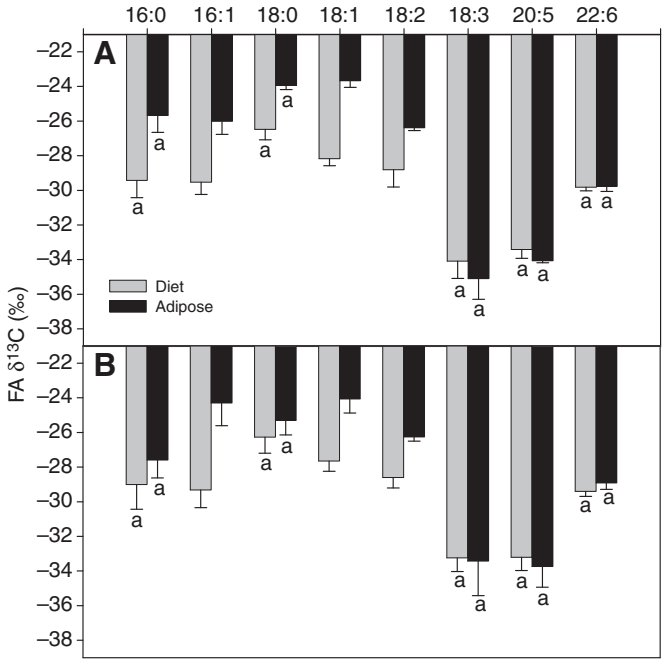

Fig. 2. $\delta^{13}C$ values (means and s.e.) of diet and adipose FA for (A) Steller's eider and (B) spectacled eider fed diet 2 for 21 days. The same letters indicate statistically similar values. All other comparisons are significantly different (P<0.013).

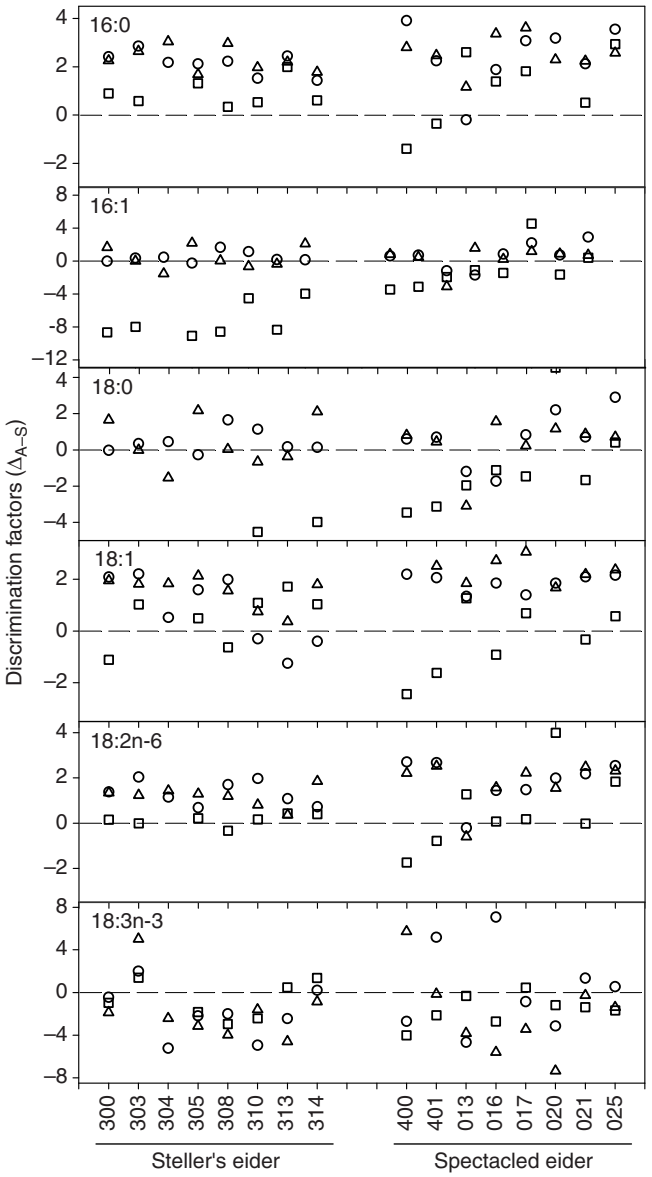

Fig. 3. Discrimination factors for the mobilization of free FA (FFA) from adipose to serum. Data are displayed for each individual at each of three collection periods (circle, 60 days; square, 120 days; triangle, 180 days).

in both species (Table 3). However, the errors associated with estimates that included contributions from 20:5n-3 and 22:6n-3 were large because of the large uncertainty associated with the discrimination factors for those two FA. Accurate estimates with much lower errors were obtained when means were calculated using only 18:2n-6 and 18:3n-3 (estimates of 42±9% and 41±10% *versus* actual of 34% and 44% in Steller's and spectacled eiders, respectively).

With the exception of 16:1 in the Steller's eiders and 18:3n-3 in both species, $\delta^{13}C$ values of serum FA were all significantly depleted (P<0.05) relative to adipose, and were much more similar to dietary isotopic values (Fig. 1). Discrimination factors for the mobilization of FA from adipose to serum varied widely and showed little consistency within individuals and FA (Fig. 3). The most extreme example of variability among serum collections in one individual occurred for 18:3n-3 in spectacled eider 016, where discrimination factors ranged from a low of –7.1 to a high of +5.6. In many individuals, the isotopic values of two of the three serum samples agreed quite well (Fig. 3); however, there was no regularity in the pattern and the dissimilar sample was not associated with a particular blood collection.

DISCUSSION

Diet to adipose isotopic discrimination factors

Potential explanations for the variation in discrimination factors with FA structure require consideration of the metabolic pathways that FA can follow after consumption (see Stevens, 1996; Klasing, 1998; Price, 2010). Dietary lipids, after crossing through the epithelial cells in the intestine, are first transported in the blood as portomicrons, the avian equivalent of mammalian chylomicrons, and routed directly to the liver. There they can be reassembled into very low-density lipoproteins (VLDL) for transport to other tissues (e.g. muscle and adipose), elongated/desaturated, stored or catabolized for energy (Stevens, 1996). If dietary lipids are in excess, some proportion is transported as VLDL to adipose tissue for storage. In a fasting state, FFA (or non-esterified FA) are mobilized from adipose for transport to tissues where they are catabolized as fuel. Within this context, a number of mechanisms could explain our results.

De novo synthesis of the saturated and monounsaturated FA could be occurring and would explain some of the observed fractionation. Similarly, elongation and desaturation of precursors could also occur to form saturates and monounsaturates, likely resulting in fractionation (Monson and Hayes, 1982). However, the birds were fed nutritionally sound diets with an excess of lipids and in that situation, enzymes associated with these processes are commonly down-regulated so it is unlikely that either biosynthesis or modification of FA was occurring (Stevens, 1996; Gurr, 1997). Synthesis of the n-3 and n-6 PUFA is impossible, as vertebrates do not possess the necessary enzymes to do so.

When FA are packaged into portomicrons for transport in the blood or deposited in the liver for storage, they must first be hydrolyzed, or cleaved, from a glycerol backbone to cross the cell membrane of epithelial and endothelial cells and then re-esterified into an intact TAG. The hydrolysis and re-esterification reactions are mediated by enzymes and therefore are subject to kinetic fractionation. However, both hydrolysis and re-esterification involve breaking or forming a bond between a carbon atom in the glycerol backbone and an oxygen atom in the fatty acyl chain; there is no reasonable mechanism for a FA with an isotopically lighter carbon atom to be selected, as only the oxygen atom on the FA chain takes part in the reaction. Thus, portomicron formation and FA deposition in the liver can be eliminated as causes of the observed fractionation. In fact, all lipid-transport processes supplying TAG to tissues involve these processes of hydrolysis and re-esterification and can be eliminated as sources of fractionation. Similarly, selective mobilization of isotopically lighter FA from the adipose tissue during fasting proceeds through hydrolysis of TAG and is unlikely to result in fractionation for the same reason.

The only probable explanation that remains for fractionation is catabolism of FA for energy. Catabolism of FA in the liver before deposition in adipose could lead to these results if enzymes associated with β-oxidation discriminate FA substrates according to their masses and favor those with lighter masses. This selection would result in more rapid oxidation of the isotopically lighter FA, leaving a greater proportion of isotopically heavier FA to be routed to the adipose for deposition, and offers a reasonable explanation for the enrichment in ^{13}C found in most FA in the adipose relative to the diet. Such a process also agrees with the original data of Deniro and Epstein where respired CO_2 was found to be isotopically lighter than that of either diet or tissue, likely the result of catabolism (Deniro and Epstein, 1978).

McMahon and colleagues found variable carbon isotopic fractionation in non-essential amino acids between diet and muscle tissue in a fish species, but practically no fractionation of essential amino acids (McMahon et al., 2010); similar results have also been reported for amino acids in insects (O'Brien et al., 2005). Here, we present data for four FA that would normally be considered essential, based on an inability of the eiders as vertebrates to synthesize these FA *de novo*: 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 (Stevens, 1996; Klasing, 1998; Cunnane, 2003). We found no discrimination of 20:5n-3 and 22:6n-3 between diet and adipose (Figs 1 and 2); however, we found substantial variation in the discrimination factors of 18:2n-6 and 18:3n-3 (~2‰ and 1.5‰, respectively). This seemingly differential fractionation of EFA may be tied to the criteria used to define essentiality. Vertebrates lack the enzymes necessary to form 18:2n-6 and 18:3n-3 from precursors so these FA are normally considered essential because they cannot be synthesized *de novo*. However, it is the longer chain PUFA, 20:4n-6, 20:5n-3 and 22:6n-3, rather than 18:2n-6 and 18:3n-3, that have important physiological functions, such as nervous system development and hormone signaling (Gurr, 1997; Ackman and Cunnane, 1992). Although 18:2n-6 and 18:3n-3 themselves have no known essential functions, they can serve as precursors for the synthesis of the longer chain PUFA through elongation and desaturation, and eliminate symptoms of EFA deficiency (Ackman and Cunnane, 1992; Cunnane, 2003), so they are therefore also considered EFA [see Cunnane (Cunnane, 2003) for full discussion of criteria for essentiality]. There is evidence from fish (Tocher, 2003) that the enzymes responsible for elongation and desaturation of precursors are much less active in marine environments where long chain essential PUFA are plentiful. Similar arguments have been proposed to explain the inability of most humans in Western society to form long chain PUFA from precursors when consuming diets high in PUFA (Siguel and Maclure, 1987; Ackman and Cunnane, 1992). If the 'essential' function of 18:2n-6 and 18:3n-3 is to serve as precursors for synthesis of 20:5n-3, 20:4n-6 and 22:6n-3 but those long chain PUFA are not being formed by elongation and desaturation, then 18:2n-6 and 18:3n-3 would not have a direct essential function. In that situation, there would be no need to spare them from oxidation and we might then expect discrimination of these FA. However, this line of reasoning fails for 18:3n-3 when diet 2 is considered. For diet 2, 18:3n-3 showed no net fractionation from diet to adipose, despite the presence of even larger amounts of 20:5n-3 and 22:6n-3 in diet 2 than in diet 1 (Table 4). If 18:3n-3 were being catabolized because it was not essential as a result of sufficient levels of long chain PUFA in the diet, one would expect catabolism to continue in eiders fed diet 2, which contains even higher levels of those PUFA.

Catabolism of specific FA may also be driven by dietary excess of those FA. Essentiality implies that there is some absolute dietary intake of a FA that must be met. Amounts consumed in excess of that minimum amount could simply be used as a substrate for oxidation, like a 'non-essential' FA. The major FA in diet 1 was 18:2n-6 (Table 4), at approximately 39% of total FA. Free-ranging eiders would not normally consume such high levels of 18:2n-6, with that FA typically present in proportions <12% in most marine bivalve species (Table 1) (Joseph, 1982), as well as aquatic insects (Goedkoop et al., 2000; Sushchik et al., 2003) and plants (e.g. Copeman et al., 2009). Known diet items for free-ranging eiders would also be expected to have low lipid contents, so that 18:2n-6 would make very little contribution to total FA consumed. In captive eiders fed high quality diet *ad libitum*, it is possible that the 18:2n-6 in the diets is in excess of their dietary needs. Such an excess is likely to be catabolized for energy, as with any other FA. In fact, it might be that 18:2n-6 is being preferentially catabolized simply because of this potential excess in the diet. Ackman and Cunnane proposed the same explanation for the preferential oxidation of 18:2n-6 in Western human diets (Ackman and Cunnane, 1992); the effect is also well known in aquaculture fish species (Turchini et al., 2009). A similar scenario could also be true with 18:3n-3. The

Table 4. Approximate concentrations of FA consumed with each diet

FA composition	Diet 1	Diet 2
Steller's eider		
16:0	814±40	1627±29
16:1	146±7	605±17
18:0	305±16	158±2
18:1	1017±51	1417±26
18:2n-6	1775±94	733±15
18:3n-3	195±10	111±3
20:5n-3	142±6	834±33
22:6n-3	166±7	409±9
Spectacled eider		
16:0	812±40	1500±25
16:1	143±7	531±14
18:0	307±16	175±3
18:1	1018±51	1348±24
18:2n-6	1792±95	912±18
18:3n-3	197±10	125±2
20:5n-3	138±6	723±28
22:6n-3	160±7	368±7

Data are mg 100 g^{-1} (total), means ± s.e. FA, fatty acids.

amount of that FA consumed by the eiders is far less than that of 18:2n-6 but it might remain in excess of some unknown minimum, thus resulting in selective catabolism and the observed fractionation when diet 1 is consumed. For diet 2, the levels of 18:3n-3 consumed dropped substantially so that amounts of 18:3n-3 were much less than those of 20:5n-3 and 22:6n-3 and there was no net fractionation between diet and adipose. It may be that amounts of 18:3n-3 had fallen below some minimum dietary threshold so there was no excess to oxidize, while 20:5n-3 and 22:6n-3 remained below their minimum necessary levels, suppressing catabolism of those FA. A similar explanation may apply to the discrimination factor observed for 16:1. This FA was present at amounts <150 mg 100 g^{-1} in diet 1, levels similar to 18:3n-3, 20:5n-3 and 22:6n-3; these relatively low levels may make it a poor substrate for oxidation. In diet 2, the concentration of 16:1 increased almost 4-fold, and showed fractionation more similar to the other abundant FA.

Finally, our results may also be influenced by turnover times of FA in the adipose tissue. If one accepts the idea that fractionation is due to oxidation of excess FA, one might expect that less fractionation would be observed with a drop in levels of dietary 18:2n-6 and 18:3n-3. Certainly, the result for 18:3n-3 in diet 2 agrees with this; however, very similar isotopic enrichment was observed for 18:2n-6 in the two diets, despite the drop in intake. Eiders were only fed diet 2 for 21 days; that period of time may not have been sufficient to fully replace the large amount of 18:2n-6 originally deposited in the adipose tissue. Indeed, the proportions of 18:2n-6 did not fall to match dietary levels, unlike most other FA including 18:3n-3, 20:5n-3 and 22:6n-3 [data in Wang et al. (Wang et al., 2010)]. Thus, it is likely that the $\delta^{13}C$ value for adipose after 21 days on diet 2 was still heavily influenced by the $\delta^{13}C$ of the previously fed diet, rather than the new diet.

All of this discussion raises important questions about fractionation of FA that should be addressed before FA-specific isotope analysis is used to estimate diets of consumers. Based on the above arguments, only PUFA that cannot be synthesized *de novo* would be useful in tracking diet and it seems reasonable to conclude that eiders foraging in their natural environment would experience little fractionation in 18:3n-3, 20:5n-3 and 22:6n-3. However, from the present data, it is difficult to assign a reliable discrimination factor to 18:2n-6. More experimentation, using diets that are more similar to those consumed by free-ranging eiders, is needed to address these questions.

Adipose to serum isotopic discrimination factors

During fasting, FA are mobilized from adipose and transported in the blood to other tissues where they can be catabolized for energy (Stevens, 1996; Price, 2010). Thus, it is reasonable to expect that the $\delta^{13}C$ values of serum FFA would resemble the adipose lipids that they were derived from. Analysis of serum FFA therefore represented a much less invasive manner to indirectly measure adipose FA $\delta^{13}C$ values. The process of mobilization involves hydrolysis of FA from TAG molecules in the adipose and, as described above, does not involve the cleavage of a bond involving a carbon atom in the FA chain. There is also no opportunity during this mobilization for synthesis or modification of FA so all six FA measured in serum, including saturated and monounsaturated FA, could be compared between the tissues without concern for biosynthetic modification of $\delta^{13}C$ of FA. Because of these restrictions, we did not expect to observe fractionation arising from the mobilization of FA from adipose to serum. Instead, we found fractionation for that transfer and it appeared to be random (Fig. 3), which suggests some challenges associated with the methodology.

Fasting, as well as strenuous exercise, results in increased mobilization of FFA from adipose stores into the bloodstream in both humans (Cortright et al., 1997) and birds (Lien et al., 1999; Price et al., 2008) but there are few data available to indicate the minimum time period necessary to promote this mobilization. Lien and colleagues (Lien et al., 1999; Lien and Jan, 2003) used a fasting period of 3 days with Tsaiya ducks (*Anas platyrhynchos* var. *domestica*), a larger species, while Käkelä and colleagues (Käkelä et al., 2006; Käkelä et al., 2007; Käkelä et al., 2009) used a period of 24 h to ensure that portomicrons derived directly from diet were eliminated from circulation in several seabird species. Eiders in this study were fasted for a shorter period of 12 h prior to blood sampling. We used thin-layer chromatography to isolate FFA from other lipid classes in the serum samples, so that the $\delta^{13}C$ data would only reflect those of FFA mobilized from adipose. However, FFA can also arise through natural hydrolysis of lipids, such as TAG and phospholipids, during sample collection and analysis (Christie, 2003), so the FFA that were isolated in eider serum may have represented degradation products of either dietary lipids in the form of portomicrons or lipids present in blood lipoproteins. Typical fasting levels of FFA seem to vary by species and likely by body mass but are generally of the order of 20–50% that of TAG. For instance, Lien and collegues found FFA at ~25% of TAG after a 3 day fast in ducks (Lien et al., 1999). With much smaller body masses of <30 g, white-throated sparrows (*Zonotrichia albicollis*) also had FFA levels ranging from 25% to 50% that of TAG after an overnight fast of unspecified duration (Smith et al., 2007). In contrast, analysis of lipid class data showed FFA at trace levels (<5% of TAG) in all eider serum samples, suggesting that the FFA were derived from the breakdown of circulating lipids, rather than mobilized from TAG in adipose. The $\delta^{13}C$ values of serum FFA were intermediate between those of diet and adipose (Fig. 1) and, for 16:1 and 18:2n-6, were not significantly different from diet in both eider species. This suggests a strong dietary influence; if portomicrons were still circulating after a 12 h fast, hydrolysis of the dietary TAG carried within them would likely result in the similarity in $\delta^{13}C$ values of the two FA. It is likely that a longer fasting period is necessary to reduce levels of circulating TAG and to promote mobilization of FFA from adipose.

Because it was not possible to sample adipose at all three time points when blood was collected, it was necessary to make the assumption that the $\delta^{13}C$ of FA in adipose did not vary from day 60 to day 180 so that discrimination factors calculated at 60 and 120 days incorporated $\delta^{13}C$ from adipose at 180 days. The diets fed for the duration of the experiment were very similar to the maintenance diets fed prior to and after the study, consisting of ~95% Mazuri© with supplementation with other diet items. Thus, it is likely that the $\delta^{13}C$ values of FA in adipose were quite similar at all time points.

Regardless of the cause of the variance in discrimination factors for mobilization of FFA from adipose to blood, there is clearly a need for further research. A first essential step is to determine the minimum time necessary to promote mobilization of FFA from adipose. This could be accomplished by simple tracer experiments where a dietary pulse of FA that is not present in the regular diet is provided. For instance, 23:0 could be fed in a large dose to the eider and the time required for its clearance from the blood determined by periodic analysis of serum FFA. With that time frame established, it would be relatively simple to repeat the serum and adipose collections to initially establish consistency of discrimination factors for FA within individuals and likely within species, assuming a constant diet was maintained.

Diet estimation

The development of an additional technique to estimate diet in eiders was the ultimate goal of this work. A necessary criterion for this application is the presence of distinct isotopic signatures of FA in different sources. Marine FA would be expected to follow the same trend as observed with bulk carbon analysis and be enriched in ^{13}C relative to terrestrial plant material produced *via* C_3 photosynthesis. However, Mazuri© is based primarily on corn, a C_4 plant that is typically enriched in ^{13}C relative to C_3 plants, and such enrichment in C_4 plants can lead to isotopic signatures that are very similar to marine values. We clearly saw this effect in our data with little consistent variation in the $\delta^{13}C$ values of FA among Mazuri©, clam, mussels and silverside. Conversely, the $\delta^{13}C$ values of Antarctic krill analyzed here were more depleted by at least 6‰ compared with the Mazuri© in the four PUFA used in the diet estimates (Table 1); such depletion is typical of Antarctic krill (Schmidt et al., 2003) and provided a convenient opportunity to test our mixing model. Estimates using all four PUFA allowed an accurate mean to be calculated. In the wild, our interest in eider diets is to differentiate between terrestrial/aquatic and marine sources but it is unlikely in that application that 20:5n-3 and 22:6n-3 will provide useful estimates. Terrestrial plants will contain very little, if any, of those two FA (Gunstone et al., 1986), and it will be necessary to rely on FA with limited synthesis pathways common to both systems, such as 18:2n-6 and 18:3n-3. In the present study, if only those two FA are used, estimates for diet 2 for both species would be ~41±10% Mazuri© (Table 3), despite very different estimates for individual birds, suggesting that the results would remain accurate with fewer FA analyzed.

No significant difference was found between the $\delta^{13}C$ values of diet 1 and adipose for 20:5n-3 and 22:6n-3 because of their high variance, implying that $\Delta_{A-D}=0$. However, when such a value was used in the diet calculation as the discrimination factor for 20:5n-3, estimates of proportions of Mazuri© consumed for Steller's eiders had a meaningless, negative value (Table 3). Thus, non-zero discrimination factors were calculated and applied. Earlier discussion has offered explanations to support the similarity in $\delta^{13}C$ values in adipose and diet for those FA, and highlighted the implications; the need to use non-zero Δ_{A-D} does not necessarily contradict this and arises because of the high variance in the measurements rather than a clear dissimilarity in values.

While the mean of the individual estimates did provide an accurate assessment of proportions, the error associated with 20:5n-3 and 22:6n-3 was large. Although only three individuals of each species were available to test diet estimates, the large errors were not a consequence of variation due to low sample number; rather, they were derived from the discrimination factors calculated in the initial experiment where eight individuals were analyzed. Both 20:5n-3 and 22:6n-3 were present at low concentrations (<0.5% of total FA), resulting in lower sample precision. These errors were then propagated through the calculation, generating the high variation in the individual estimates for those FA. Conversely, 18:2n-6 and 18:3n-3 were present at higher concentrations (~22% and 1.5% for 18:2n-6 and 18:3n-3, respectively) and variations in the resulting estimates were small. When only those two FA were used to estimate diet, the associated errors were much more reasonable at 9% and 10% for Steller's and spectacled eiders, respectively (Table 3), demonstrating that it is possible to achieve diet estimates with low variation. However, none of the estimates based solely on individual FA were accurate. Clearly, in this application, the accuracy of an estimate is not necessarily linked to its reproducibility.

Implications and recommendations

This study establishes a starting point for estimating predator diets using stable isotopes of FA, and highlights several key considerations. Perhaps the most important issue in the application of this technique is the research question to be addressed. Like analyses of bulk isotopes, this technique can yield information about the origin of material in predator diets but only sources with distinct isotopic values can be differentiated. Thus, we would expect this method to be useful in situations similar to those in which bulk isotope values are commonly used. For instance, we have previously demonstrated the potential utility of the compound-specific approach in differentiating between ice algal and phytoplankton carbon (Budge et al., 2008), and it is likely to also be useful in addressing questions that differentiate between benthic and pelagic sources, and marine and terrestrial material. In more complicated situations, with more than two distinct sources, simple mixing models will not suffice and much more complex statistics will be required to assign sources (e.g. Ward et al., 2010). It may also be possible to combine information from proportional FA composition (e.g. Iverson et al., 2004; Wang et al., 2010) with $\delta^{13}C$ values of FA so that more reliable diet estimates can be made.

Several issues remain to be addressed before this technique can be used to reliably trace carbon sources in free-ranging birds. For instance, nutritional quality and digestibility of food is known to influence discrimination factors at the bulk level (Codron et al., 2011) and the subjects in our captive study showed differential catabolism, depending upon the amounts of EFA in their diets. In free-ranging birds, it would be impossible to know the levels of dietary FA consumed and therefore difficult to assign appropriate discrimination factors. More controlled feeding studies, with diets of varying FA concentrations similar to wild diets, would help resolve this issue. Specifically, we must understand the variation in discrimination factors with changing dietary FA levels. This also relates to a second issue concerning the period of feeding represented by dietary FA in adipose tissues, as well as their turnover times. Both are poorly understood in birds and will also influence the accuracy of diet estimates. In fact, we suspect that the variation in our diet estimates was linked to a failure in equilibration of adipose tissue FA with the new diet. Turnover time will depend on the concentrations of FA in the diet and would also best be established by feeding ecologically relevant diets. Thus, we recommend that carefully designed feeding studies be used to address both issues of differential catabolism and turnover time.

Another hurdle is simply demonstrating the utility of this compound-specific approach compared with bulk stable carbon isotope analyses. We believe the advantages associated with isotopic routing of EFA and the ability to obtain multiple diet estimates from several FA in a single sample make the FA-specific approach superior to analyses of bulk stable carbon isotopes. However, this technique requires more involved sample preparation and expensive instrumentation; it also relies on information from lipids, a dietary component that is usually removed before bulk analysis is conducted (Post et al., 2007). A clear advantage, either in accuracy or reproducibility of diet estimates generated with FA stable carbon isotopes, is required. We therefore recommend that future studies also determine bulk stable carbon isotopes of the same lipid-extracted material as analyzed with the FA-specific technique, so that diet estimates derived from the two methods based on different dietary components can be compared.

While some questions do remain concerning the application of this FA-specific stable carbon isotope technique, we believe the study of EFA will allow us to ultimately derive accurate

discrimination factors because we will be able to focus on the specific pathways involved, rather than relying only on empirical factors. Analyses of bulk $\delta^{13}C$ values have been used since the 1970s in ecological studies but many uncertainties remain concerning fractionation of isotopes (Hussey et al., 2010; Wessels and Hahn, 2010). We hope that the limited metabolic pathways that EFA can follow will allow faster progress to be made in the compound-specific area. Because our understanding of the processes contributing to the variation in isotopic signatures of FA in nature is currently limited, we recommend that future research directions focus on elucidating these mechanisms.

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