

A PRACTICAL INTRODUCTION TO STABLE-ISOTOPE ANALYSIS FOR SEABIRD BIOLOGISTS: APPROACHES, CAUTIONS AND CAVEATS

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Received 21 May 2008, accepted 15 March 2009

SUMMARY

BOND, A.L. & JONES, I.L. 2009. A practical introduction to stable-isotope analysis for seabird biologists: approaches, cautions and caveats. *Marine Ornithology* 37: 183–188.

Stable isotopes of carbon and nitrogen can provide valuable insight into seabird diet, but when interpreting results, seabird biologists need to recognize the many assumptions and caveats inherent in such analyses. Here, we summarize the most common limitations of stable-isotope analysis as applied to ecology (species-specific discrimination factors, within-system comparisons, prey sampling, changes in isotopic ratios over time and biological or physiological influences) in the context of seabird biology. Discrimination factors are species specific for both the consumer and the prey species, and yet these remain largely unquantified for seabirds. Absolute comparisons across systems are confounded by differences in the isotopic composition at the base of each food web, which ultimately determine consumer isotopic values. This understanding also applies to applications of stable isotopes to historical seabird diet reconstruction for which historical prey isotopic values are not available. Finally, species biology (e.g. foraging behaviour) and physiologic condition (e.g. level of nutritional stress) must be considered if isotopic values are to be interpreted accurately. Stable-isotope ecology is a powerful tool in seabird biology, but its usefulness is determined by the ability of scientists to interpret its results properly.

Key words: $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, assumptions, diet reconstruction, mixing model, seabird, stable isotopes

INTRODUCTION

Stable-isotope ratio analysis is now commonly used by seabird biologists to infer diet and trophic relationships, to gain insight into the foraging ecology of species, and to inform population management (Inger & Bearhop 2008). First recognized in the mid-1980s (Peterson & Fry 1987), the use of stable-isotope analysis in avian ecology became widespread only after a series of experiments and field studies in the early 1990s (Hobson & Clark 1992a, 1992b, 1993; Hobson *et al.* 1994). However, as early as 1997, concerns were raised about untested assumptions of the properties of stable isotopes and a lack of controlled laboratory experiments (Gannes *et al.* 1997). Since then, considerable advances have been made (Wolf *et al.* 2009), although in a recent thorough review of seabird diet studies and methods (Barrett *et al.* 2007), stable-isotope analysis was the sole common method for which biases and drawbacks were not discussed thoroughly. As a result, seabird biologists who wish to use stable-isotope analysis face a daunting and often massive task to navigate the conflicting papers and knowledge gaps in the scientific literature. Considerable gaps remain in our knowledge of how elemental isotopes behave in biological systems, and little controlled experimentation has been conducted. Here, we present an introduction to stable-isotope analysis for seabird biologists new to this emerging, yet widespread, tool. For brevity, we discuss only the isotopes commonly used in seabird studies: carbon and nitrogen.

Isotopic ratios are expressed as a parts-per-thousand difference in the ratio of the heavier (more rare) to the lighter (more common) isotope (i.e. ^{13}C to ^{12}C), compared with the ratio found in an international standard (Pee Dee Belemnite for carbon, atmospheric air for nitrogen) such that

$$\delta X = \left(\frac{R_{\text{sample}}}{R_{\text{std}}} - 1 \right) \times 1000 \quad [1]$$

where δX is either $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$, and R is either the ratio $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$.

The value of $\delta^{15}\text{N}$ increases predictably with increasing trophic level, because ^{14}N is excreted preferentially in nitrogenous waste (Steele & Daniel 1978, Minagawa & Wada 1984, Kelly 2000). The carbon ratio also changes, but in smaller amounts, and only at lower trophic levels (DeNiro & Epstein 1978, Rau *et al.* 1983, Hobson & Welch 1992). Moreover, carbon exhibits a gradient, with inshore food sources being enriched in ^{13}C as compared with offshore sources in the marine environment (Peterson & Fry 1987, Kelly 2000). Carbon can therefore potentially act as a geographic identifier (Quillfeldt *et al.* 2005).

Isotopic ratios are determined at the time of tissue synthesis in the consumer (Hobson & Clark 1992a) and therefore offer themselves to non-destructive sampling in live animals (i.e. blood, feathers,

claws). These ratios can provide insight into seabird biology away from the breeding colony if the proper tissue (e.g. moulted feathers) is sampled.

DISCUSSION

Lipids

Compared with carbohydrates, lipids have less ^{13}C because of fractionation caused by the oxidation of pyruvate to acetyl coenzyme A during lipid synthesis (DeNiro & Epstein 1977). Nevertheless, some researchers have found significant effects of lipid content on $\delta^{13}\text{C}$; others have not (McConnaughey & McRoy 1979, Hobson & Clark 1992b, Pinnegar & Polunin 1999).

Traditionally, lipids were removed from lipid-heavy tissues (C:N > 4.0) chemically (e.g. Bligh & Dyer 1959) to reduce variation in the isotopic ratio, but chemical extraction can also affect $\delta^{15}\text{N}$ values (Murry *et al.* 2006). Two recent reviews (Post *et al.* 2007, Logan *et al.* 2008) compared mathematical modelling methods and chemical extraction techniques, and concluded that analysing a subset of samples before and after chemical lipid extraction will allow researchers to develop unique mathematical lipid models that can be applied to the remainder of the data in a given study.

Seabird tissues such as feathers and egg albumen do not require lipid extraction (Kojadinovic *et al.* 2008), and blood typically does not. However, some Procellariiformes may have lipid-rich blood that would require lipid correction (Bond *et al.* 2010). Tissues

such as muscle, liver and egg yolks almost certainly require lipid correction (Kojadinovic *et al.* 2008).

Tissue preservation

For many field studies, especially those involving seabirds on remote islands, the issue of tissue-preservation effects is of paramount importance. Formalin and genetic buffers can alter stable-isotope ratios drastically (Hobson *et al.* 1997, Gloutney & Hobson 1998), and results were mixed when tissues were preserved in ethanol (Kaehler & Pakhomov 2001, Barrow *et al.* 2008). For avian tissue, freezing is the preferred method, but freezing may not always be practical in the field, and so air drying (especially for blood samples) using an oven or similar smokeless heat source is also feasible (Bugoni *et al.* 2008). For a comprehensive review of preservation techniques for stable-isotope samples, we direct the reader to Barrow *et al.* (2008).

Discrimination factors

As prey nutrients are incorporated into the consumer, the isotopic ratio changes by a “discrimination factor” (also called a “fractionation factor”). In general, this factor falls between 0‰ and 2‰ for $\delta^{13}\text{C}$, and between 2‰ and 5‰ for $\delta^{15}\text{N}$ (Peterson & Fry 1987, Kelly 2000), and evidence is increasing that these ratios are unique to each tissue–consumer–prey combination (Bearhop *et al.* 2002, Cherel *et al.* 2005b, Caut *et al.* 2009). In addition, discrimination factors have long been regarded as an important aspect of stable-isotope ecology (Mizutani *et al.* 1992) and are often applied poorly

TABLE 1
Published mean discrimination factors for carbon and nitrogen stable isotopic ratios in seabird tissues based on a lipid-free fish diet^a

Species	Consumer tissue	Discrimination factor (‰)		Reference
		C	N	
King Penguin <i>Aptenodytes patagonicus</i>	Whole blood	−0.81	+2.07	Cherel <i>et al.</i> 2005b
	Feathers	+0.07	+3.49	Cherel <i>et al.</i> 2005b
Humboldt Penguin <i>Spheniscus humboldti</i> ^b	Feathers	+2.9	+4.8	Mizutani <i>et al.</i> 1992
Rockhopper Penguin <i>Eudyptes chrysocome</i>	Whole blood	+0.02	+2.72	Cherel <i>et al.</i> 2005b
	Feathers	+0.11	+4.4	Cherel <i>et al.</i> 2005b
Great Cormorant <i>Phalacrocorax carbo</i> ^b	Feathers	+3.8	+3.7	Mizutani <i>et al.</i> 1992
Great Skua <i>Stercorarius skua</i>	Whole blood	+1.1	+2.8	Bearhop <i>et al.</i> 2002
	Feathers	+2.1	+4.6	Bearhop <i>et al.</i> 2002
Ring-billed Gull <i>Larus delawarensis</i>	Whole blood	−0.3	+3.1	Hobson & Clark 1992b
	Liver	−0.4	+2.7	Hobson & Clark 1992b
	Muscle	+0.3	+1.4	Hobson & Clark 1992b
	Bone collagen	+2.6	+3.1	Hobson & Clark 1992b
	Feathers	+0.2	+3.0	Hobson & Clark 1992b
Black-tailed Gull <i>L. crassirostris</i> ^b	Feathers	+5.3	+3.6	Mizutani <i>et al.</i> 1992
Common Murre <i>Uria aalge</i>	Feather	+1.2	+3.6	Becker <i>et al.</i> 2007
Rhinoceros Auklet <i>Cerorhinca monocerata</i>	Whole blood	—	+3.49	Sears <i>et al.</i> 2009

^a No discrimination factors have been published for members of the Diomedidae, Procellariidae, Pelecanoididae, Hydrobatidae, Phaethontidae, Pelecanidae, Fregatidae, Sulidae or Rhyncopidae, or for other diets.

^b Lipids not extracted from prey items. Lipids result in a lower $\delta^{13}\text{C}$ value, and therefore can change discrimination factors significantly.

(Caut *et al.* 2009). A recent review by Caut *et al.* (2009) provided a decision tree for approximating discrimination factors for avian tissues, but we urge caution when applying these generalizations to marine birds, because the estimates were generated using non-marine birds. Indeed, Caut *et al.* (2009) caution that other factors, such as physiology, may play an important role in determining discrimination factors and should not be ignored by researchers.

To quantify discrimination factors accurately, consumers must be held on a controlled, isotopically constant diet covering the length of time required for complete turnover of the tissue of interest (Hobson & Clark 1992b). Most commonly, blood or feathers are sampled from seabirds. Whole-blood isotopic values are typically representative of diet for the previous 12–15 days (Hobson & Clark 1993); feathers indicate the isotopic ratios at the time of growth (Hobson & Clark 1992a). Even when feathers and blood are synthesized over the same time period, consistent differences in stable-isotope ratios are detectable, with feathers being enriched in both ^{15}N and ^{13}C as compared with blood (Quillfeldt *et al.* 2008). Proper assessment of discrimination factors therefore requires individuals to be held for lengthy periods of time—in some cases, for months or years (Hobson & Clark 1992a, Becker *et al.* 2007).

Maintaining seabirds in captivity for lengthy periods can be difficult (e.g. Oehler *et al.* 2001). Consequently, few discrimination factors have been published (summarized in Table 1). Discrimination factors are cited consistently as the weakest link in stable-isotope ecology (Phillips & Koch 2002, Post 2002, Cherel *et al.* 2005b), but they are essential for inferences about diet composition (see “Isotope mixing models,” later in this paper). When controlled laboratory studies are not possible, it may be possible to estimate discrimination factors from field studies (e.g. Bearhop *et al.* 2002). It should also be noted that captive and wild individuals may differ physiologically, which could alone alter stable-isotope ratios. Should researchers wish to make use of captive individuals, we encourage collaboration with zoos and research centres having existing captive birds.

Comparisons among and within food webs

The major assumption in stable-isotope ecology is that the signatures of consumers reflect those of their prey species, which is largely true (Post 2002). Seabirds are multi-taxa predators, consuming a wide variety of prey species in an almost infinite number of combinations. This variation presents a problem when attempting to estimate the proportion of each prey item in the consumer’s diet (see “Isotope mixing models,” later in this paper), because different combinations of prey species and proportions can result in the same isotope signature in the consumer. It is therefore possible for two seabirds exploiting two different food webs in the same location to have identical stable-isotope signatures. Although that example is extreme, many seabird diets overlap during the breeding season (Ashmole 1963, Diamond 1978, Bearhop *et al.* 2004), and so making accurate estimates of diet composition is often desired. Knowledge about the isotopic composition in the food web of interest is therefore required (Post 2002). Such knowledge can be obtained relatively easily at seabird breeding colonies by collecting food samples (Barrett *et al.* 2007). Often a combination of traditional gut-content analysis and stable-isotope analysis can provide valuable insight (Cherel *et al.* 2007).

For migratory species, problems also arise when comparing isotopic values of tissues grown in different locations or at different times of year. For example, feathers are inert once fully grown, and

their isotope signatures reflect the diet during the period of growth (Hobson & Clark 1992a). Therefore, a diet comparison across species using isotopic ratios from feathers grown away from the breeding colony is invalid because there is no certainty concerning the similarity of the isotopic composition of the food web, especially when the species of interest show geographic segregation. For many seabird species, winter diet and distribution are poorly known or completely unknown (Gaston & Jones 1998, Brooke 2004, Gaston 2004, Barrett *et al.* 2007), and so a valid assessment or comparison of isotopic ratios is challenging. Comparison of similar tissue types would alleviate some of the potentially confounding factors.

Isotope mixing models

If discrimination factors are known or can be approximated, prey and consumer isotopic ratios can be used in a mathematical model to estimate the proportion of each prey group in the consumer’s diet (Phillips & Gregg 2001, 2003). These models function on the assumption that a plot of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the prey species will create a polygon (mixing space) within which the consumer’s isotopic ratios [corrected for discrimination, (see “Discrimination factors,” earlier)] will fall (Phillips & Gregg 2001, 2003). Depending on the specific question, models such as Isoerror, Isosource or MixSIR are appropriate (Phillips & Gregg 2001, 2003; Phillips *et al.* 2005; Moore & Semmens 2008). In these models, ranges are given for the possible contribution of each prey source to the consumer’s isotope signature, but these can be very wide (e.g. Urton & Hobson 2005, Major *et al.* 2007) and meaningful biological interpretation can be challenging, although not impossible (Cherel *et al.* 2005b).

Models are as useful as the data that go into them, and thus when approximations are used in applying discrimination factors, the resulting model inherits and magnifies the uncertainty. Small changes in discrimination factors can not only change the estimates of the proportions of each prey species, but also may dictate whether the consumer’s isotope signature actually falls into the mixing space (the polygon bounded by source isotopic ratios on a $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$ plot). Critical to these models are proper discrimination factors (Caut *et al.* 2008, 2009). Recently, Bayesian models have been able to incorporate the uncertainty in discrimination factors (Moore & Semmens 2008).

Tissue heterogeneity

Within-tissue heterogeneity has received some attention in non-marine birds, with δD (ratio of hydrogen–deuterium) being assessed within feathers (Wassenaar & Hobson 2006; Smith *et al.* 2008, 2009), but interest is also emerging in how within- and between-individual isotope heterogeneity both affect the conclusions drawn from stable-isotope ratios (Jardine & Cunjak 2005). Only recently have mixing models accounted for this uncertainty (Moore & Semmens 2008). In studies on captive fish, the inherent variability in captive and wild individuals ranged from about 2% to 10% for $\delta^{15}\text{N}$ and up to 19% for $\delta^{13}\text{C}$ (Barnes *et al.* 2008). To date, tissue heterogeneity in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from avian tissue has not yet been examined, and such heterogeneity remains a significant gap in advances of laboratory methodology.

Reconstructing historical diet

Barrett *et al.* (2007) suggested that stable-isotope analysis is an ideal method for the reconstruction of historical diet, but those authors did

not review the inherent biases of the approach. Recently, two studies (Becker & Beissinger 2006, Norris *et al.* 2007) attempted to quantify the historical diet of Marbled Murrelets *Brachyramphus marmoratus*, a threatened alcid from the west coast of North America (Nelson 1997), by sampling feathers from museum specimens for stable carbon and nitrogen isotopes. Based on changes in murrelet $\delta^{15}\text{N}$, it was estimated that Marbled Murrelets experienced a significant decrease in trophic position and proportion of fish over the preceding 100 years (Becker & Beissinger 2006, Norris *et al.* 2007).

These studies lead us to a useful re-examination of some of the fundamental principles of stable-isotope ecology. Present-day isotopic ratios of prey items were used to infer historical diet, but it is impossible to know if the prey isotopic composition remained constant over time. Isotope signatures of Marbled Murrelets can change as drastically as 62% in trophic position (Norris *et al.* 2007), but prey diets (and consequently isotope signatures) may have changed as well (Quay *et al.* 2003). It may be that the proportion of prey species changed, that the isotope signatures of prey changed or that a combination of the two occurred. This situation is not testable, and it limits the valid inferences that can be made from historical data without detailed quantitative historical data from low trophic levels.

Even when historical prey samples are available, isotopic ratios are prone to artificial changes caused by preservation techniques (Hobson *et al.* 1997, Kaehler & Pakhomov 2001, Sarakinos *et al.* 2002, Feuchtmayr & Grey 2003, Rau *et al.* 2003). In addition, some “baseline” $\delta^{13}\text{C}$ values—those that ultimately determine the ratios in consumers (Post 2002)—may not remain constant over time, because burning of fossil fuel emits gases depleted in $\delta^{13}\text{C}$ as compared with background levels [dubbed the “Suess effect” (Keeling 1979, Quay *et al.* 2003)]. Changes in CO_2 , which is increasing in seawater over time (Louanchi & Hoppema 2000), may also affect $\delta^{13}\text{C}$ values. When examined in southern waters, the change in $\delta^{13}\text{C}$ was on the order of a decrease of between 0.009‰ and 0.018‰ per year (Hilton *et al.* 2006).

Confounding biologic factors

Finally, a consideration of the biology of the focal species is crucial to interpreting lab-generated data (Cherel & Hobson 2007). Factors other than diet may influence isotopic composition in seabird tissues, including foraging area (Quillfeldt *et al.* 2005, Cherel & Hobson 2007), body condition (Hobson *et al.* 1993) and metabolic rate (Kitaysky 1999). Under nutritive stress, nitrogen is metabolised when proteins replace lipids as an energy source, resulting in changes to $\delta^{15}\text{N}$ in some tissues, such as blood (Hobson *et al.* 1993, Cherel *et al.* 2005a, Williams *et al.* 2007). Currently, the level of nutritive stress required to affect $\delta^{15}\text{N}$ is not known, but as with discrimination factors, it is likely to be species-specific. Stress level may be of special interest to seabird biologists who use stable isotopes to document diet shifts over time: When the proportion of a high-quality prey source decreases over time, at what point is $\delta^{15}\text{N}$ affected?

Because of metabolic differences, it is difficult to use stable isotopes to compare adult and chick diets (Williams *et al.* 2007, Harding *et al.* 2008). Chicks are almost certainly metabolizing nutrients at rate different from that of adults, resulting in different integration periods for the same tissue (Sears *et al.* 2009). In addition, there may be potential carryover effects from maternal nutrients in eggs; more study is therefore required.

Nutritive stress and fasting are of particular concern, because many seabird species fast either during incubation or because of spatial segregation between foraging and breeding grounds. A clear understanding of the physiology of the species of interest is critical to proper biologic interpretation of stable-isotope results.

Foraging area may also change the isotopic composition in the tissues of consumers. For example, the Southern Ocean shows a latitudinal gradient in $\delta^{13}\text{C}$ that is reflective of the isotopic composition of the base of the food web there (Quillfeldt *et al.* 2005). Other areas remain untested; however, a gradient is likely present in all oceans (Goericke & Fry 1994). There is a great need to better understand the marine “isoscares” of the world’s oceans (Cherel *et al.* 2008).

RECENT DEVELOPMENTS

Despite the heterogeneity and differences in stable-isotope ratios mentioned earlier, some researchers have been able to take advantage of these differences to examine where species forage (e.g. Cherel *et al.* 2008) and consequently to better understand at-sea mortality of seabirds through fisheries bycatch (Gómez-Díaz & González-Solís 2007). With continued research, and a growing community of researchers using stable-isotope analysis, many of the potential pitfalls mentioned above will likely be overcome.

ACKNOWLEDGEMENTS

We thank R. Cunjak, K. Hobson and T. Jardine for valuable discussions on isotope ecology, and Y. Cherel and two anonymous reviewers for providing valuable comments on previous drafts of this manuscript.

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