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Stable isotopes and elasmobranchs: tissue types, methods, applications and assumptions

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Stable-isotope analysis (SIA) can act as a powerful ecological tracer with which to examine diet, trophic position and movement, as well as more complex questions pertaining to community dynamics and feeding strategies or behaviour among aquatic organisms. With major advances in the understanding of the methodological approaches and assumptions of SIA through dedicated experimental work in the broader literature coupled with the inherent difficulty of studying typically large, highly mobile marine predators, SIA is increasingly being used to investigate the ecology of elasmobranchs (sharks, skates and rays). Here, the current state of SIA in elasmobranchs is reviewed, focusing on available tissues for analysis, methodological issues relating to the effects of lipid extraction and urea, the experimental dynamics of isotopic incorporation, diet-tissue discrimination factors, estimating trophic position, diet and mixing models and individual specialization and niche-width analyses. These areas are discussed in terms of assumptions made when applying SIA to the study of elasmobranch ecology and the requirement that investigators standardize analytical approaches. Recommendations are made for future SIA experimental work that would improve understanding of stable-isotope dynamics and advance their application in the study of sharks, skates and rays.
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Key words: $\delta^{13}C$; $\delta^{15}N$; lipid extraction; mixing models; niche width; specialization; trophic position; turnover.

INTRODUCTION

Knowledge of diet, trophic position and movement patterns of species have long been recognized as critical factors, the tenets of ecology, required for the successful conservation and management of species and the environment they inhabit. New technologies offer a rapidly expanding toolbox for ecologists to examine these factors, ranging from advanced telemetry methods to the use of chemical tracers. These tools have led to breakthrough discoveries in animal ecology, physiology and behaviour, and have greatly resolved understanding of species' roles within ecosystems.

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There is mounting evidence of a global decline of large marine predators since the advent of industrial fishing (Baum *et al*., 2003, 2005), which is likely to have had significant effects on many marine ecosystems (Baum & Worm, 2009; Ferretti *et al*., 2010). Recent insights into predator top-down control effects in marine ecosystems (Heithaus *et al*., 2008; Baum & Worm, 2009), coupled with the scale of reported population declines (Baum *et al*., 2003, 2005) have increased both the scientific community and general public's awareness of the issue of large predator removal. This has led to repeated calls for global elasmobranch conservation and management plans, and a realization of high levels of data deficiency for many species (Stevens *et al*., 2000; Dulvy *et al*., 2008), motivating a new wave of directed research into shark, skate and ray behaviour, biology and ecology.

Since the development of bulk stable-isotope analyses (SIA) and reduced analysis costs, the application of stable isotopes, in particular those of carbon and nitrogen, has rapidly grown as a standard tool for ecologists to examine and link processes at multiple scales, from individual cells to ecosystems (Martínez del Rio *et al*., 2009). Specifically for animal ecology, the marked fractionation of nitrogen isotopes (^{15}N:^{14}N) between prey and consumer have been used to examine diet, foraging ecology, trophic position and food-web structure, while the conservative fractionation of carbon (^{13}C:^{12}C) between primary consumers and predators provides a tool to examine animal habitat use and movement–migration patterns. For large, highly mobile animals that inhabit environments where they are difficult to observe, SIA provided a significant tool to unveil previously unknown behaviours and interactions (Fisk *et al*., 2002; Estrada *et al*., 2006; Newsome *et al*., 2009; Matich *et al*., 2010*a*; Olson *et al*., 2010; Hussey *et al*., 2011*a*). The successful application of SIA to address ecological questions, however, is dependent on several assumptions, an understanding of which is critical for accurately interpreting data. Since an early review by Gannes *et al*. (1997) and two subsequent reviews by Martínez del Rio *et al*. (2009) and Wolf *et al*. (2009), there have been major advances in the understanding of the methodological approaches and assumptions of SIA in ecological applications. The recognition of SIA as a powerful ecological tracer is dependent on experimental data to support the numerous assumptions made during the interpretation of often complex field data (Gannes *et al*., 1997). Furthermore, the continued success of SIA is based on the accumulation of large field and experimental data sets that have inspired new theoretical SIA questions and the development of quantitative models that are able to amalgamate large field data sets and expand the utility of SIA data (Martínez del Rio *et al*., 2009). This has enabled animal ecologists to refine their approaches to SIA ecology and to address more complex questions, including specialist and generalist-feeding behaviour, modelling of multiple diet items to infer seasonal and size-based diet switches and species or community niche width and overlap. It is through these advances in SIA dynamics and mathematical modelling that SIA is increasingly being used in the study of elasmobranch biology and ecology.

Gannes *et al*. (1997), Martínez del Rio *et al*. (2009) and Wolf *et al*. (2009) have previously reviewed much of the experimental advances in SIA for general animal ecology applications. Considering the unique physiology of elasmobranchs compared to other marine vertebrates, notably the retention of urea for osmoregulation, this review focuses on seven priority areas in the current application of SIA: (1) potential tissue types available for analysis, (2) sample preparation, (3) the dynamics of isotopic incorporation, (4) diet-tissue discrimination factors and trophic position, (5) diet

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and mixing models, (6) the potential of SIA for investigating movement and (7) individual specialist–generalist and niche-width SIA. The review is structured around these main topics, with examples from the current literature, and makes recommendations for improving the application of SIA in elasmobranch studies and identifying potential avenues for future research. Finally, the use of other isotope tracers is touched upon: oxygen, hydrogen, sulphur and compound-specific analysis of amino acids.

NOMENCLATURE

Stable-isotope ratios are expressed in delta (δ) values as the ratio of an unknown sample to a recognized standard and are expressed in parts per thousand (or per mille, ‰) using the following equation: $\delta^b X = 1000\ [(R_{\text{sample}}\ R^{-1}_{\text{standard}}) - 1] \times 1000$, where X is the element, b is the mass of the heavy isotope (less abundant) and R_{sample} and R_{standard} are the heavy to light isotope ratio (*e.g.* nitrogen ^{15}N:^{14}N, carbon ^{13}C:^{12}C, hydrogen H^2:H^1, oxygen ^{18}O:^{16}O and sulphur ^{34}S:^{32}S) of the sample and standard, respectively (Peterson & Fry, 1987). The internationally accepted standard reference materials are Pee Dee Belemnite carbonate (V-PDB) for carbon, atmospheric N_2 for nitrogen, Vienna Standard Mean Ocean Water (V-SMOW) for hydrogen and oxygen and Canyon Diable Troilite for sulphur. By definition the values of these standards is 0‰.

POTENTIAL SAMPLE MATERIAL FOR ELASMOBRANCH SIA

The type of tissue analysed is recognized as a fundamental aspect of experimental design when applying SIA in ecological research. Dependent on the question, the isotopic incorporation time dictates the period when an animal is in equilibrium with its diet and environment determining the time frame over which the investigator can reliably examine diet, trophic position and movement. Tieszen *et al.* (1983) first stated that isotopic incorporation varies between tissues and suggested that more metabolically active tissues would have a faster turnover rate than less metabolically active tissues. More recently, experimental evidence suggests that the rate of protein turnover directly affects isotopic incorporation into different tissues (Carleton & Martínez del Rio, 2005). Isotopic incorporation and protein turnover are most likely linked by two key components of protein turnover: synthesis, the incorporation of dietary nutrients to build or manufacture proteins, and catabolism, the breakdown and loss of tissue materials. Therefore, structural components with low rates of protein turnover, such as collagen, striated muscle tissue and red blood cells, will have lower rates of isotopic incorporation than splanchnic organs (*i.e.* liver) and plasma proteins (Waterlow, 2006; Martínez del Rio *et al.*, 2009). For elasmobranchs, soft tissues used for SIA include muscle, whole blood, red blood cells, plasma and liver.

Considering the slow growth rates of most elasmobranchs compared to teleosts (Garcia *et al.*, 2008), white-muscle tissue is viewed as a long-term integrated measure of elasmobranch feeding habits and environment and is one of the most commonly sampled tissues. In certain instances, the long-term integrated stable-isotope values ($\delta^{15}N$ and $\delta^{13}C$) of muscle tissue can provide an overview of: trophic position

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(Estrada *et al*., 2003; Andrews & For, 2009; Revill *et al*., 2009; Sampson *et al*., 2010; Borrell *et al*., 2011*a*; Dale *et al*., 2011; Drymon *et al*., 2011; Escobar-Sánchez *et al*., 2011; Hussey *et al*., 2011*a*, 2012), interspecies variation in diet and resource use (Domi *et al*., 2005; Dale *et al*., 2011; Hussey *et al*., 2011*a*; Kim *et al*., 2011; Vaudo & Heithaus, 2011), the role of elasmobranchs within an overall food web (McMeans *et al*., 2010; Abrantes & Barnett, 2011; Botto *et al*., 2011; Vaudo & Heithaus, 2011), the effects of human provisioning of sharks in ecotourism activities (Maljkovic & Coté, 2011) and can identify coarse-scale movement patterns (Abrantes & Barnett, 2011; Dale *et al*., 2011; Hussey *et al*., 2011*a*). In addition, muscle tissue can be sampled from multiple individuals of different size, sex or maturity to provide an integrated view of a species over ontogeny (Papastamatiou *et al*., 2010; Abrantes & Barnett, 2011; Borrell *et al*., 2011*b*; Hussey *et al*., 2011*a*, 2012). Undertaking SIA of a single tissue, however, is limited to the isotopic incorporation time frame of that tissue.

MacNeil *et al*. (2005) advanced the use of SIA in elasmobranchs by analysing several tissue types sampled from individual blue shark *Prionace glauca* (L. 1758), shortfin mako *Isurus oxyrinchus* Rafinesque 1810 and thresher shark *Alopias vulpinus* (Bonnaterre 1788). First proposed by Bearhop *et al*. (2004), multiple tissues with different isotopic incorporation rates sampled from an individual provide a measure of dietary use over varying temporal scales. SIA of these different tissues can then be used to determine seasonal diet switches and resource partitioning and enable dietary breadth or niche calculations to elucidate intra and inter-variation in resource use at the population level. By comparing the relative δ^{15}N and δ^{13}C values in muscle (slow incorporation rate) and liver (high incorporation rate), MacNeil *et al*. (2005) observed a known diet switch in *I. oxyrinchus* associated with changes in the availability of a migratory prey species. No such diet switches were observed in *P. glauca* or *A. vulpinus*. More recently, Matich *et al*. (2010*a*) modified this approach and undertook SIA of muscle tissue and fin (slow incorporation rate) *v*. plasma (high incorporation rate) to determine specialist and generalist feeding behaviours in tiger shark *Galeocerdo cuvier* (Péron & LeSueur 1822) and bull shark *Carcharhinus leucas* (Müller & Henle 1839), while Kinney *et al*. (2011) used SIA of muscle tissue and red blood cells (slow incorporation rate) *v*. plasma (high incorporation rate) to provide the first evidence of resource partitioning within a communal shark nursery. Tissues with high isotopic incorporation rates such as liver and blood plasma have successfully been used to examine short-term diet profiles of several elasmobranch species (Fisk *et al*., 2002; MacNeil *et al*., 2005; McMeans *et al*., 2009; Matich *et al*., 2010*a*; Kinney *et al*., 2011; Olin *et al*., 2011). Elasmobranch liver, however, is a dynamic and highly complex tissue used for buoyancy and lipid storage, and as yet only three SIA studies have investigated its potential use (Fisk *et al*., 2002; MacNeil *et al*., 2005; Olin *et al*., 2011); liver has, however, a high lipid content that may lead to potential SIA bias (Hussey *et al*., 2010*a*). There has also been a move to conduct SIA of elasmobranch soft tissues in conjunction with stomach-content data (McMeans *et al*., 2010; Abrantes & Barnett, 2011; Dale *et al*., 2011; Hussey *et al*., 2011*a*, *b*), acoustic tracking data (Papastamatiou *et al*., 2010; Abrantes & Barnett, 2011; Maljkovic & Cote, 2011) and other chemical tracers such as fatty acids (Wai *et al*., 2011), mercury (McMeans *et al*., 2010) and organic contaminants (Fisk *et al*., 2002).

When considering the location of soft tissue sampling from an animal, the δ^{15}N and δ^{13}C values of multiple muscle tissue and liver samples taken from along the

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length of the body and length of both lobes, respectively, of individual large sharks were similar for each tissue (Hussey *et al*., 2010*a*). These findings are in agreement with data for marine mammals (Todd *et al*. 2010) and indicate that sampling location has minimal effect on the stable-isotope values of single soft tissues.

Tissues that continually accrete and are deposited at discrete intervals in which the material retains the stable-isotope value at the point of incorporation can provide a unique insight into the ecology of an individual animal over long time periods. For sharks, the vertebral centra, a biomineralized structure, provide such a structural component. Campana *et al*. (2002) first determined that the organic component of shark vertebral centra was stable based on ^{14}C profiles, providing the validation required for the confident interpretation of $\delta^{15}N$ and $\delta^{13}C$ data. Estrada *et al*. (2006) then examined the trophic ecology of white sharks *Carcharodon carcharias* (L. 1758) by drilling incremental points every 5 mm across the vertebral centra of 27 individuals, finding evidence for an increase in trophic position with size, corroborating previous stomach content studies. Although several studies have presented vertebral centra stable-isotope data as part of bomb carbon ageing studies (Campana *et al*., 2002; Kerr *et al*., 2006), Estrada *et al*.'s (2006) study remains the only one to date to determine ontogenetic stable-isotope profiles in an extant elasmobranch using this type of structure. Thorns embedded in the caudal tissue of skate and ray species or the rostral teeth of Pristidae and Pristiophoridae may also be accretionary and, if so, could be used for incremental SIA, but their potential has yet to be investigated.

With documented declines in elasmobranch populations and concern over lethal sampling (Heupel & Simpfendorfer, 2010; Hammerschlag & Sulikowski, 2011), non-invasive or minor invasive tissue sampling methods of endangered species are required for SIA. For example, recent studies have obtained muscle tissue samples using a biopsy punch similar to those used for marine mammal sampling (Matich *et al*., 2010*a*, *b*; Kim *et al*., 2011; Kinney *et al*., 2011). Red blood cells and plasma can also be sampled easily in the field through the minor invasive puncture of the caudal vein and spinning down and separation of blood components (Matich *et al*., 2010*a*, *b*; Kinney *et al*., 2011). Similar to endangered teleosts (Sanderson *et al*., 2009), investigators have recently started sampling elasmobranch fin clips for SIA (Matich *et al*., 2010*a*, *b*; Hussey *et al*., 2011*b*; Vaudo & Heithaus, 2011). Fin clips provide a valuable source material, but are not a single tissue, instead composed of multiple structural components including cartilage, skin and to a lesser extent muscle and connective tissue. This can complicate the interpretation of stable-isotope data as the different structural components may have different isotopic incorporation rates and variable diet–tissue discrimination factors; combined they provide a confused representation of the diet and environment of a study animal. Given that the proportions of these components vary by sampling location, Hussey *et al*. (2011*b*) recommended standardizing to a specific area (*e.g.* trailing edge of the fin) and sampling a single fin type for intra and interspecies comparisons. Ideally investigators would sample a single structural tissue of fin material (Hussey *et al*., 2011*b*). Further SIA experimental work on fin is required to determine isotopic incorporation rates and how this varies among structural components.

Of all the tissues investigated for SIA in elasmobranchs, teeth have received the least attention. Most work on elasmobranch teeth has analysed oxygen isotopes ($\delta^{18}O$) in phosphate ($\delta^{18}O_p$) and carbonate within the phosphate [$\delta^{18}O_c$; Vennemann *et al*. (2001)], focusing on palaeoenvironmental reconstructions using fossilized teeth

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of extinct species (Pellegrini & Longinelli, 2008). Teeth are biomineralized structures composed of fluor-apatite ($Ca_5(PO_4)F$) and carbonate apatite ($Ca_5(PO_4)OH$), fluor-apatite being the least soluble form of apatite and therefore the most resistant to subsequent alteration (Posner *et al*., 1984). Vennemann *et al*. (2001) undertook $\delta^{13}C$ analysis of tooth carbonate and suggested that whole tooth or dentine (the base of the tooth) $\delta^{13}C$ values were reflective of a dietary carbon source, while $\delta^{13}C$ values in enameloid indicated a different source, probably dissolved inorganic carbon. Dentine in shark teeth contains *c*. 25–30% organic material in contrast to low organic content of enameloid (Carlson, 1990), suggesting this material will be the most useful for SIA to reconstruct diet, trophic position and habitat or movement patterns. Teeth can be sampled minor invasively or acquired from museum collections as a unique archival source material. Furthermore, rows of elasmobranch teeth grow incrementally in the jaw (several rows are present at one time), providing a time series of samples for SIA from an individual. For teeth SIA to be effective, additional work will be required to understand tooth turnover rates amongst species and variation with increasing animal size (Moss, 1967; Reif *et al*., 1978; Luer *et al*.,1990). Experimental work will also be required to determine teeth stable-isotope diet-tissue discrimination factors, turnover rates and preparation steps required to deal with the biomineralized inorganic component.

SAMPLE PREPARATION FOR SIA

Critical to the successful application of SIA in elasmobranchs is confidence in species and tissue-specific sample preparation and storage techniques prior to analytical determination. Several sample preparation approaches have been suggested for specific tissue components (Hussey *et al*., 2010*a*, *b*, 2011*b*; Logan & Lutcavage, 2010*a*; Kim & Koch, 2011; N. E. Hussey, J. A. Olin, M. J. Kinney, B. C. McMeans & A. T. Fisk, unpubl. data). Here, lipid extraction, urea, decalcification of vertebrae and sample storage methods are examined.

LIPID EXTRACTION

An important consideration when preparing samples for SIA is the effect of lipids and the lipid extraction process, particularly for $\delta^{13}C$ data. Lipids are depleted in ^{13}C relative to carbohydrates and proteins (DeNiro & Epstein, 1977) with $\delta^{13}C$ values up to 5‰ lower than associated proteins (Newsome *et al*., 2010). Lipid extraction prior to SIA is therefore required to standardize data among individuals, across tissues and across species within a food web. In addition, recent work has highlighted unexpected changes in $\delta^{15}N$ values of various marine and freshwater fishes and invertebrates as a result of lipid extraction (Murry *et al*., 2006; Logan *et al*., 2008). Although extensive work has tested the effects of lipid extraction techniques on stable-isotope values in teleosts (Sotiropoulos *et al*., 2004; Sweeting *et al*., 2006; Logan *et al*., 2008), questions remain over the influence of lipid extraction on SIA in elasmobranch tissues. Moreover, lipid levels in certain elasmobranch tissues such as muscle can be relatively low, raising questions about the necessity of lipid extraction at all (Matich *et al*., 2010*a*).

Lipid extraction of elasmobranch tissues is typically undertaken using a chloroform–methanol (2:1) extraction following a modified Bligh & Dyer (1959)

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technique (Estrada *et al*., 2003; MacNeil *et al*., 2005; McMeans *et al*., 2010; Borrell *et al*., 2011*a*; Kinney *et al*., 2011; Hussey *et al*., 2011*a*, 2012), however, Kim & Koch (2011) used a more non-polar alternative, petroleum ether, that potentially minimizes the loss of amino acids and more efficiently extracts lipids (Dobush *et al*., 1985). Although other methods can be more accurate than that of Bligh & Dyer (1959) when quantitative estimates of total lipid content are of interest (Iverson *et al*., 2001), previous experimental work has found chloroform–methanol to be the most efficient solvent for lipid extraction of teleost tissues prior to SIA when compared to more non-polar solvent alternatives (Logan *et al*., 2008). No work to date has compared the effects of different lipid extraction methods on stable-isotope values across elasmobranch tissue types and among species. Future work is needed to experimentally compare the effectiveness of polar and more non-polar alternatives for removing lipids while retaining proteins in elasmobranch tissues.

In a semi-controlled experiment on large sandtiger shark *Carcharias taurus* Rafinesque 1810 and lemon shark *Negaprion brevirostris* (Poey 1868), Hussey *et al*. (2010*a*) reported that there was an increase in δ^{13}C values of *c*. 0·4‰ in muscle tissue, as expected following chloroform-methanol lipid extraction. In turn, the authors also found that δ^{15}N values increased following lipid extraction by *c*. 0·5‰, with an associated decline in total per cent nitrogen. Similarly, an examination of lipid extraction effects on muscle tissue of 21 elasmobranch species found an overall mean ± S.D. δ^{13}C increase of 0·6 ± 1·2‰, but there was a marked increase for some species, specifically Greenland shark *Somniosus microcephalus* (Bloch & Schneider 1801): 5·2 ± 0·5‰ and whale shark *Rhincodon typus* Smith 1828: 3·3‰ (N. E. Hussey, J. A. Olin, M. J. Kinney, B. C. McMeans & A. T. Fisk, unpubl. data). Both Kim & Koch (2011) and Logan & Lutcavage (2010*a*) also reported an increase in δ^{13}C following lipid extraction in leopard shark *Triakis semifasciata* Girard 1855 and spiny dogfish *Squalus acanthias* L. 1758 muscle tissue, respectively. Although the overall reported increase in δ^{13}C values are minimal, and lipid content is reportedly low in elasmobranch muscle tissue (Bone & Roberts, 1969; Devadoss, 1984), the variable lipid content between species, indicates that extraction is required; this could apply to high lipid content in *S. microcephalus* muscle tissue and inter and intraspecies variation in δ^{13}C values following lipid extraction (Logan & Lutcavage, 2010*a*; N. E. Hussey, J. A. Olin, M. J. Kinney, B. C. McMeans & A. T. Fisk, unpubl. data). This is particularly important for standardizing δ^{13}C values for food web reconstructions, determining carbon sources, examining movement and in determining tissue-specific diet-tissue discrimination factors. For elasmobranch liver tissue, which has known high lipid content (Remme *et al*., 2006), lipid extraction is critical to standardize data among individuals and across species (Fisk *et al*., 2002; MacNeil *et al*., 2005; McMeans *et al*., 2009; Olin *et al*., 2011). For blood components, Kim & Koch (2011) reported that petroleum ether extraction did not alter either red blood cell or plasma δ^{13}C values of *T. semifasciata*. Red blood cells generally have very low lipid content and probably do not require lipid extraction. Plasma, however, is the vehicle for gut-tissue and inter-tissue transport of lipids in elasmobranchs {predominantly *via* chylomicrons [Ballantyne (1997)]}. The observation that non-esterified fatty acid concentrations can vary in elasmobranch plasma depending on the timing of the last meal (Wood *et al*., 2010) suggests that lipid extraction should be performed. Kerr *et al*. (2006) undertook lipid extraction of vertebral centra material prior to SIA analysis. Cartilaginous material would not be expected to

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contain lipids, but further work is required to determine if this methodological step is required.

Matich *et al*. (2010*a*) reported C:N ratios (reported in mass%) of non-lipid extracted muscle tissue, red blood cells and plasma of *C. leucas* of 3·1, 2·7 and 2·0, respectively. Citing Post *et al*. (2007), these authors suggested that their values were below the recommended C:N ratio of <3·5 and therefore lipid extraction was not required. Reported C:N ratios of non-lipid extracted elasmobranch muscle tissue of <3·0 (Hussey *et al*., 2010*a*; Logan & Lutcavage, 2010*a*; Kim & Koch, 2011; N. E. Hussey, J. A. Olin, M. J. Kinney, B. C. McMeans & A. T. Fisk, unpubl. data), however, are not indicative of pure protein with an expected C:N ratio of 3·0 (DeNiro, 1987; Kilijunen *et al*., 2006; Logan *et al*., 2008). Furthermore, the increase in C:N ratio following lipid extraction (as a result of a decrease in total per cent nitrogen) reported by Hussey *et al*. (2010*a*) and Kim & Koch (2011) may suggest that urea content may influence stable-isotope values in elasmobranchs in addition to lipid content. There is the potential for arithmetic correction of δ^{13}C values in shark tissues similar to those developed for teleosts (Sweeting *et al*., 2006; Post *et al*., 2007; Logan *et al*., 2008) which would negate the requirement for chemical extraction methods. Recent work, however, has highlighted that these mathematical corrections may not always be appropriate (Fagan *et al*., 2011) and sample-specific corrections are advised (Lesage *et al*., 2010; Fagan *et al*., 2011). Whether these approaches are applicable to elasmobranchs requires rigorous testing. In addition, the likely effect of lipid extraction on δ^{15}N suggests that lipid extraction is required to standardize both δ^{13}C and δ^{15}N values.

UREA

Elasmobranchs are unique in that they maintain high levels of urea and trimethylamine oxide (TMAO) in their tissues for the purposes of maintaining osmotic balance (Olson, 1999). Fisk *et al*. (2002) suggested that the mismatch between δ^{15}N values and contaminant and stomach-content estimated trophic position of *S. microcephalus* was due to the presence of urea and potentially trimethylamine N-oxide, which is synthesized to counteract the destabilizing effect of urea on protein (Ballantyne, 1997). Urea is a waste product of metabolism and consequently is expected to be ^{15}N depleted, resulting in an artificially lower δ^{15}N value and a lower trophic position estimate than expected (Fisk *et al*., 2002). In addition, urea and TMAO tissue concentrations vary among species and animals of different size probably due to: (1) rescaling of urea–TMAO concentrations according to the environment the animal inhabits (*i.e.* fresh water, estuarine, marine, tropical or polar; Goldstein *et al*., 1968; Pillans *et al*., 2005), (2) the timing since last feeding (Wood *et al*., 2007) and (3) potential species-specific physiologies. Tissue urea and TMAO concentrations also vary with tissue type (Ballantyne, 1997). Variable urea content among species and tissues may greatly complicate accommodation of urea effects on SIA when examining elasmobranchs in conjunction with other components of the food web.

Logan & Lutcavage (2010*a*) undertook a controlled experiment to examine the effect of urea on δ^{15}N values in blood and muscle tissue of coastal skates (*Leucoraja* spp.) and *S. acanthias*. The authors followed standard water-rinsing methods to remove urea (Mathew *et al*., 2002) and concluded that urea content did not have an adverse effect on δ^{15}N values. In contrast, Kim & Koch (2011) reported that δ^{15}N

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values increased significantly in multiple muscle tissue samples of an individual *T. semifasciata*, when treated using a similar water-rinsing technique. As solvents such as chloroform–methanol can extract urea (Christie, 1993), Hussey *et al*. (2010*a*) suggested that the observed increase in δ^{15}N values and decrease in total per cent nitrogen of shark muscle tissue following lipid extraction was probably a result of the removal of soluble urea. Kim & Koch (2011) reported that, given the low dissolution constant of urea, more non-polar solvents like petroleum ether may also be effective at removing soluble urea fractions from elasmobranch muscle tissue. These authors found, however, that both water-rinsing and petroleum ether treatment had the greatest effect on muscle δ^{15}N values (*i.e.* were the most effective for urea removal) and subsequently recommended both techniques be performed to decrease variation associated with both lipid and urea.

Reported adverse effects of water rinsing on amino acid composition of blood are less well understood, and further experimental work is required (Kim & Koch, 2011). Such work is warranted because elasmobranch plasma is a promising tissue for diet reconstruction, but contains high concentrations of urea and TMAO (Wood *et al*., 2010) that could bias δ^{13}C and δ^{15}N values. In the first comprehensive paper examining bulk stable isotopes of carbon and nitrogen and nitrogen isotopes of individual amino acids in an elasmobranch, Dale *et al*. (2011) estimated that the trophic enrichment factor (TEF) for brown stingrays *Dasyatis lata* (Garman 1880) and scalloped hammerheads *Sphyrna lewini* (Griffith & Smith 1834) was lower (5·0‰) than previously reported for plankton [7·6‰ (Chikaraishi *et al*., 2009)]. Dale *et al*. (2011) suggested that because elasmobranchs use a unique carbamoyl phosphate synthetase (CPSase III), ^{15}N enrichment should be lower in glutamic acid leaving the liver to form muscle protein in species retaining high urea concentrations as a result of oxidative deamination of glutamic acid during urea formation. This would suggest that, although there may be a soluble fraction of urea that can be removed either by lipid extraction or water rinsing, ^{15}N depleted glutamic acid is incorporated during the tissue formation process and simply removing soluble urea from tissues will not remove all urea influence on SIA. It is critical that further work is undertaken to clarify the effect of urea on SIA in elasmobranchs and among species with known variable urea concentrations. Advancing the experimental work of Logan & Lutcavage (2010*a*), captive animals could be maintained in variable salinity levels and actual urea concentrations measured. Both bulk SIA and SIA of individual amino acids of non-treated and treated (lipid-extracted, water-rinsed and lipid-extracted and water-rinsed) tissues could then be undertaken to determine the direct effects of urea retention on stable-isotope values. This is of particular importance for determining appropriate diet-tissue discrimination factors.

DECALCIFICATION OF VERTEBRAL CENTRA

The vertebral centra of elasmobranchs are cartilaginous structures composed of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and collagen. Given that hydroxyapatite typically contains inorganic carbon that differs in isotopic composition to protein (Newsome *et al*., 2010), it is suggested that the organic collagen component must be isolated prior to SIA. Typically demineralization of bone is undertaken using HCl, however, Kim & Koch (2011) and H. M. McCann, N. E. Hussey & A. T. Fisk (unpubl. data) found that HCl rapidly dissolved the finely powdered vertebral centra (*i.e.* potentially

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both the collagen and inorganic mineral matrix). Kim & Koch (2011) therefore used EDTA following a technique devised by Tuross *et al.* (1988) which yields more collagen from small, finely powdered samples than HCl treatment. Kim and Koch (2011) found that the average percentage dry mass of collagen from EDTA-treated vertebrae was *c.*19% in agreement with the range of 17–27% reported by Porter *et al.* (2006). This indicates that EDTA is effective at removing the mineralized component of small finely powdered vertebral samples while retaining the collagen component. To date, no experimental data have been presented to demonstrate the effect of decalcification on elasmobranch vertebral centra stable-isotope values and whether or not this step is required. On close examination of stable-isotope values derived from *C. carcharias* vertebral centra which were decalcified (Kerr *et al.*, 2006) and non-treated (Estrada *et al.*, 2006), there would appear to be little effect on either δ^{13}C or δ^{15}N values. Future experimental work is required to clarify the required processing steps for elasmobranch cartilaginous material. This work should undertake SIA on non-treated, EDTA- and HCl-treated samples of vertebral material of different species (considering known variation in calcification of vertebral structures) and from animals of different sizes.

SAMPLE STORAGE METHOD

In contrast to other marine and terrestrial taxa (Arrington & Winemiller, 2002; Sweeting *et al.*, 2006; Barrow *et al.*, 2008; Fanelli *et al.*, 2010; Krab *et al.*, 2011), limited experimental work has been undertaken to examine the effects of storage methods, for example freezing or preservation in ethanol or dimethyl sulphoxide (DMSO) on elasmobranch tissues and its subsequent effect on SIA. Variable effects of ethanol and salt solution storage on δ^{13}C values have been reported across taxa (Lesage *et al.*, 2010; Krab *et al.*, 2011) and could affect the interpretation of results, for example those of Borrell *et al.* (2011*a*, *b*), where elasmobranch muscle samples were preserved in salt. Kim & Koch (2011) found that ethanol preserved muscle tissue of longnose skate *Raja rhina* Jordan & Gilbert 1880 had a lower δ^{13}C value when compared to frozen samples, with no observed effect on δ^{15}N values. The authors concluded that the δ^{13}C alteration was variable amongst individuals and thus no single correction could be applied. In addition, Kim & Koch (2011) stated that caution should be exerted when interpreting diet or habitat differences of ethanol preserved muscle tissues that have δ^{13}C differences of $<1‰$. Considering the potential to analyse large archival genetic sample sets constituting both muscle and fin tissue (Chapman *et al.*, 2009), understanding preservation effects on elasmobranch tissue is of importance for future SIA.

EXPERIMENTAL DYNAMICS OF ISOTOPIC INCORPORATION

Rarely will the diet of elasmobranchs remain constant and most species experience seasonal and ontogenetic shifts in prey selection (Wetherbee & Cortés, 2004). As the stable-isotope values of prey are likely to be different, such changes in feeding patterns will result in a shift in isotope values in the consumer. For example, the δ^{15}N values of many fish species increase with size, which is often attributed to the consumption of larger, higher trophic level prey (Papastamatiou *et al.*, 2010; Hussey

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et al., 2011*a*). The isotope values of prey can also vary temporally and spatially (MacAvoy *et al*., 2001; Bergamino *et al*., 2011), particularly in lower trophic level organisms (Syvaranta & Rautio, 2010) as a result of changes in carbon and nutrient sources to a system or between systems. Movement of elasmobranchs between ecosystems can result in marked changes in both carbon and nitrogen stable-isotope value exposure for an organism (Abrantes & Barnett, 2011; Dale *et al*., 2011; Hussey *et al*., 2011*a*). Thus, the stable-isotope values of most organisms are dynamic and in a constant state of change.

Quantifying the rate of isotopic incorporation, or turnover, is important for interpreting isotope values measured in the field and is dependent on controlled laboratory studies (Gannes *et al*., 1997; Martínez del Rio *et al*., 2009; Wolf *et al*., 2009). Such experimental studies are challenging as creating isotopically distinct diets that have similar chemical characteristics, such as protein content, are difficult to achieve, but are important as the characteristics of food can alter isotope incorporation (Arneson & MacAvoy, 2005). A number of recent studies have attempted to incorporate heavy isotopes (^{15}N and ^{13}C) in a food source by growing worms or maggots with enriched inorganic chemicals, with the objective of creating identical diets with varying isotopic values (MacNeil *et al*., 2006; Overmyer *et al*., 2008; Fisk *et al*., 2009; Dennis *et al*., 2010). Creating sufficient amounts of such food for long-term studies of large organisms, however, such as elasmobranchs is difficult, and there are concerns over whether the heavy isotopes have been incorporated in a natural way into the food.

The isotope value of an organism is influenced by the metabolic exchange of elements, the addition of tissue, or growth and protein turnover. Growth has been found to be a major determinate of isotope turnover in organisms, particularly fishes (Hesslein *et al*., 1993), and accounts for the majority of turnover rate in smaller fast growing fishes (Herzka & Holt, 2000). During growth, the amount of tissue replacement due to catabolic turnover is also a key determinant of tissue turnover rates. Large, long-lived fishes such as elasmobranchs undergo determinate growth and therefore the effects of catabolism turnover and growth are more pronounced for younger animals. For larger, mature animals, growth slows and metabolic turnover of isotopes becomes more important. Studies that aim to quantify metabolic turnover of isotopes need to control for growth, either through modelling and statistical analysis (MacNeil *et al*., 2006) or by providing a maintenance diet that does not allow for growth (Fisk *et al*., 2009). Limiting growth, however, may result in changes to the metabolism in the organism that could influence turnover. Starvation, for example, has been found to result in enrichment of heavy isotopes in animals (Hobson & Clark, 1992) including whole blood in sharks (Logan & Lutcavage, 2010*a*). In general, the metabolic turnover of isotopes is complicated and can also be influenced by environmental factors, such as temperature (Power *et al*., 2003), and diet, such as the isotope values of food (Caut *et al*., 2008*a*, *b*; Overmyer *et al*., 2008; Dennis *et al*.,2010). Furthermore, isotopic turnover rates vary among tissue types, most likely as a result of variable rates of protein turnover (Carleton & Martínez del Rio, 2005).

To date, there have been two studies that have measured isotope incorporation or turnover in elasmobranchs under controlled experimental conditions. The first, quantified δ^{15}N turnover in a freshwater stingray *Potamotrygon motoro* (Müller & Henle 1841) fed worms with varying δ^{15}N values that had been created by raising

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the worms in a ^{15}N enriched soil (MacNeil *et al*., 2006). Metabolic turnover rates (day^{-1}), after correcting for growth, were greatest in liver, followed by blood, muscle and cartilage, which is consistent with the metabolic activity of these tissues and has been observed in other fish species (Buchheister & Latour, 2010). The authors noted that the turnover rate was greater when the fish had been switched to a diet that had a lower $\delta^{15}N$ value than a diet with a higher $\delta^{15}N$ value. Based on the minimum (liver tissue) and maximum (cartilage) turnover rates, *P. motoro* would need 166–576 days following a diet shift to achieve an isotope value that was within 95% of a steady-state value, with liver achieving such a value *c*. 1·5, 2·5 and 3·5 times faster than blood, muscle and cartilage, respectively.

Logan & Lutcavage (2010*a*) quantified $\delta^{15}N$ and $\delta^{13}C$ isotope turnover in wild-caught sandbar sharks *Carcharhinus plumbeus* (Nardo 1827) by switching the sharks to a diet with lower $\delta^{15}N$ and $\delta^{13}C$ values. Metabolic turnover rates for $\delta^{15}N$ were similar to MacNeil *et al*. (2006), being greater in blood than muscle and of the same magnitude. Turnover rates for $\delta^{13}C$ were greater than those measured for $\delta^{15}N$, but had a time to 95% turnover in the 220–457 day range, depending on the diet-tissue discrimination factor used. Faster turnover of carbon isotopes, as compared to nitrogen isotopes, has been observed in other teleosts (Buchheister & Latour, 2010) and other animals (Overmyer *et al*., 2008; Fisk *et al*., 2009). The reason for this is unknown, but could relate to carbon being routed through different metabolic pathways and carbon content being significant in non-protein compounds such as carbohydrates that have faster turnover than proteins; however, this has not been explicatively researched and warrants additional study.

Based on two published studies, the $\delta^{15}N$ and $\delta^{13}C$ incorporation or turnover rate in sharks for most tissues is slow, complicating the quantification of seasonal diet shifts, although liver and blood plasma have potential (MacNeil *et al*., 2006; Matich *et al*., 2010*a*). These turnover rates, however, were determined in relatively small and growing elasmobranchs, consequently the rate of incorporation could be much lower in large species, and dominated by metabolic turnover (Nelson *et al*., 2011). If an elasmobranch were growing during the diet shift, however, the time to achieve a near steady-state isotope value could be much quicker.

There is a need for additional research into the dynamics of isotope turnover in elasmobranchs, as published studies have used small animals, and one a freshwater species. In addition, recent work has suggested that multiple isotope pools may exist within an organism, and that each of these pools may have different turnover rates (Ayliffe *et al*., 2004; Cerling *et al*., 2007). Dependent on the tissue type, isotopic turnover rates may therefore be better expressed by using either a single exponential function or multiple linear functions (Cerling *et al*., 2007; Carleton *et al*., 2008; Martínez del Rio & Anderson-Sprecher, 2008; Kurle, 2009). As the husbandry of most sharks, particularly large species is difficult, experimental work determining isotopic incorporation rates presents a challenge. Future diet-switching experiments should address: (1) the allometric relationship between body size and isotopic turnover rates in multiple tissue types of elasmobranchs and (2) the relevance of single or multi-compartment models. Long-term controlled studies in commercial aquaria (Hussey *et al*., 2010*a*) provide some of the best opportunities to undertake this type of experiment. There is also a need to try and move from a qualitative use of turnover rates to one that is more quantitative, but this remains a challenge for SIA ecology.

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DIET-TISSUE DISCRIMINATION FACTORS

A key assumption for applying stable-isotope data to most ecological studies, particularly diet studies and trophic position estimates, is knowledge of stable-isotope fractionation. Fractionation denotes the difference in stable-isotope composition between a consumer and its diet (*i.e.* prey items consumed) and is often referred to as a diet-tissue discrimination factor (DTDF) or a trophic discrimination factor (Tieszen *et al*., 1983; Hobson & Clark, 1992). Diet-tissue discrimination factors, typically reported as Δ^{15}N and Δ^{13}C, are calculated using the following equation: $\Delta^b X_{\text{consumer-food}} = \delta^b X_{\text{consumer}} - \delta^b X_{\text{food}}$, where $\Delta^b X_{\text{consumer-food}}$ is the diet-tissue discrimination factor or trophic discrimination factor for a particular isotope, $\delta^b X_{\text{consumer}}$ and $\delta^b X_{\text{food}}$ are the isotopic composition of the consumer and diet, respectively, and b is the mass of the heavy isotope. Consumers are typically enriched in the heavy isotope relative to diet, so derived $\Delta^b X_{\text{consumer-food}}$ values are positive [Minagawa & Wada (1984) provide negative values for certain species and conditions].

For nitrogen, isotopic fractionation is thought to be a result of the excretion of urea and other nitrogenous wastes that are ^{15}N depleted relative to total body nitrogen pools during deamination and transamination of amino acids (Macko *et al*., 1987). The magnitude of the fractionation or discrimination probably relates to the amount of nitrogenous waste products excreted. For carbon, respiration of isotopically light CO_2 is one probable mechanism for higher δ^{13}C in an animal *v*. its diet (DeNiro & Epstein, 1978). The magnitude of Δ^{13}C in fishes is thought to be influenced by the balance between isotope routing of dietary protein to tissues, *de novo* biosynthesis of non-essential amino acids and protein catabolism, all of which probably depend on the abundance of amino acids in the diet (McMahon *et al*., 2010). The most commonly applied DTDFs in SIA studies is a Δ^{15}N value of 3·4‰ and Δ^{13}C of 1·0‰, which is based on a paper by Post (2002) that summarized a series of early isotope studies. As this paper acknowledged and reported, however, there was significant variation around these estimates, and experimental work has since demonstrated that DTDFs can vary among species (Vanderklift & Ponsard, 2003; Caut *et al*., 2009) and tissues (Pinnegar & Polunin, 1999; Caut *et al*., 2009), including sharks (Hussey *et al*., 2010*a*).

Similar to isotope incorporation or turnover rates, DTDFs can also be influenced by several factors, primarily diet protein quantity and quality (Robbins *et al*., 2005; Martínez del Rio *et al*., 2009; Dennis *et al*., 2010; Robbins *et al*., 2010; Florin *et al*., 2011), but also temperature (Power *et al*., 2003) amongst other factors (Caut *et al*., 2009). It is generally accepted that the use of DTDFs with a new species, and particularly in the case of a distinctive physiological group such as elasmobranchs, requires experimental validation under controlled laboratory conditions (Caut *et al*., 2009; Wolf *et al*., 2009). Similar to tissue incorporation rate experiments, such validation studies for elasmobranchs present challenges, especially for large species that are difficult to maintain in captivity.

A majority of the elasmobranch SIA studies that have employed Δ^{15}N and Δ^{13}C values have used Post's (2002) values (3·4 and 1·0‰, respectively; Estrada *et al*., 2003, 2006; Kerr *et al*., 2006; Borrell *et al*., 2011*a*). A study of diet in *S. microcephalus* found that a DTDF of 3·8‰ for δ^{15}N, commonly used for Arctic organisms (Hobson *et al*., 1995), underestimated the trophic position of this species when

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compared to stomach contents and organochlorine contaminant concentrations (Fisk *et al.*, 2002); Fisk *et al.* (2002) suggested this may have been influenced by urea in the tissue. A recent review of DTDFs based on 66 published studies, suggested DTDFs for δ^{15}N and δ^{13}C of 2·75 and 0·75‰, respectively (Caut *et al.*, 2009), which are lower than those previously reported by Post (2002). Moreover, there is substantial evidence to suggest that Δ^{15}N values decrease with increasing protein quality (Robbins *et al.*, 2005, 2010; Dennis *et al.*, 2010) and that mixed diets (typical of wild feeding animals rather than controlled experimental studies which typically use a single diet prey item) may provide complementary amino acid profiles potentially lowering nitrogen isotope discrimination (Robbins *et al.*, 2005, 2010).

To date, there are only two experimentally derived DTDFs reported for elasmobranchs and these studies report conflicting results. The first study was based on four individual sharks (three *C. taurus* and one *N. brevirsotris*) that were held in commercial aquaria and fed mixed diets (Hussey *et al.*, 2010*a*). In this semi-controlled experiment, estimated Δ^{15}N and Δ^{13}C of lipid extracted muscle and prey data were 2·3 ± 0·2‰ and 0·9 ± 0·3‰ (mean ± s.d.), lower than average DTDFs reported for teleosts. Hussey *et al.* (2010*a*) reported that DTDFs varied widely by tissue type, for example, liver DTDF values for δ^{15}N and δ^{13}C were 0·5 ± 0·5‰ and 0·2 ± 1·2‰ (mean ± s.d.) and for cartilage were 1·5 ± 0·61‰ and 3·8 ± 0·44‰, respectively. The authors cautioned about the influence of lipid on DTDFs, particularly in high lipid liver tissue. It is likely that lower DTDFs for elasmobranch relate to the formation, retention and cycling of nitrogen waste in elasmobranchs; Hobson & Clark (1992) related lower DTDFs in birds to their unique nitrogen waste cycle. The study of Hussey *et al.* (2010*a*) calculated DTDFs on a reconstructed diet because the sharks were fed multiple prey items similar to experimental work on captive seals by Hobson *et al.* (1996), consequently the sharks were not in equilibrium with a single prey item. Although the authors accounted for the proximate contribution of prey (the fractional contribution of total per cent nitrogen and carbon within each prey item and prey biomass), it is possible that the varied and mixed diets of each shark may have been routed differentially to muscle tissue (*i.e.* isotopic routing; Tieszen & Fagre, 1993). Given that the calculated Δ^{15}N for the three sharks were similar (*C. taurus*: 2·3, 2·2 and 2·1‰ and *N. brevirostris*: 2·6‰), however, would suggest these values provide a reasonable DTDF estimate for large sharks.

The second experimental study, by Kim *et al.* (2011), was based on three *T. semifasciata*, held under controlled conditions and fed a constant diet of a single prey item, squid *Luligo opalescens*, for >1000 days. Kim *et al.* (2011) reported much higher Δ^{15}N and Δ^{13}C values for elasmobranch muscle tissue of 3·7 ± 0·2‰ and 1·7 ± 0·5‰ (mean ± s.d.), but in agreement with Hussey *et al.* (2010*a*), the Δ^{15}N and Δ^{13}C values varied between tissues [plasma and red blood cell Δ^{15}N and Δ^{13}C values were 2·2 ± 0·7, 2·8 ± 0·6 and 2·4 ± 0·5‰, 2·3 ± 0·5‰ (mean ± s.d.)]. In their introduction, Kim *et al.* (2011) suggested that carnivores, including elasmobranchs, should have higher Δ^{15}N than the commonly applied value of 3·4‰. Many of the values reported for carnivores of the above study, however, were for different tissue types, which would be expected to have different DTDFs (Pinnegar & Polunin, 1999; Hussey *et al.*, 2010*a*). Because experimental work has demonstrated that animals feeding on higher δ^{15}N prey generally have lower Δ^{15}N values (Felicetti *et al.*, 2003; Overmyer *et al.*, 2008; Caut *et al.*, 2008*a*, *b*, 2009; Dennis *et al.*, 2010;

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Robbins *et al*., 2010), it might be expected that Δ^{15}N values for large predatory elasmobranchs would be lower than for herbivores. When considering only muscle tissue, Δ^{15}N values of large predators, such as Atlantic bluefin tuna *Thunnus thynnus* (L. 1758) 1·6‰ (Varela *et al*., 2011), harp seal *Phoca groenlandica* 2·4‰ (Hobson *et al*., 1996) and killer whale *Orcinus orca* 1·2‰ (Caut *et al*., 2011), were lower than 3·4‰ and for values reported for herbivores [4·5–5·7‰; (Mill *et al*., 2007)], being more similar to the value suggested by Hussey *et al*. (2010*a*) for large sharks. The current experimental studies on isotope fractionation between an elasmobranch consumer and its prey by Hussey *et al*. (2010*a*) and Kim *et al*. (2011) would indicate that DTDFs are variable for elasmobranchs and that this variation is probably related to the type of diet and the δ^{15}N value of the diet that the study animal is consuming.

Abrantes & Barnett (2011) compared the δ^{15}N values of sevengill sharks *Notorynchus cepedianus* (Péron 1807) with main prey items by correcting sharks using a Δ^{15}N value of 2·4‰ according to Hussey *et al*. (2010*a*). The authors reported that the DTDF corrected δ^{15}N values of these fish were still lower than expected and suggested that either the DTDF is lower than 2·4‰ for this species owing to it feeding on high δ^{15}N value prey and that the sharks are only temporally resident in coastal areas and therefore slow tissue turnover affected the corrected shark δ^{15}N data relative to coastal prey isotope values. In addition, Abrantes & Barnett (2011) did not extract the lipid from the muscle tissue samples they used. Given the high urea content and large changes in δ^{15}N values observed in *S. microcephalus* following lipid extraction, soluble urea may be affecting these data (N. E. Hussey, J. A. Olin, M. J. Kinney, B. C. McMeans & A. T. Fisk, unpubl. data). *In lieu* of using values from the literature, McMeans *et al*. (2010) used a combination of prey stable-isotope and stomach-content data to estimate Δ^{15}N and Δ^{13}C values for lipid extracted *S. microcephalus* muscle of 4·0 and 0·4%, respectively. The authors stated that this was within the range of accepted Δ^{15}N values, but also indicated that the small stomach-content data set ($n = 22$ individuals) that did not include known marine mammal prey of *S. microcephalus* may have biased the Δ^{15}N value.

Given the wide variation in DTDFs reported in the literature, even for species within a genus (Caut *et al*., 2009), and common use of different review DTDF estimates in current published elasmobranch studies, further experimental work is imperative. As suggested by Hussey *et al*. (2010*b*) and Logan & Lutcavage (2010*b*), experimental work should examine DTDFs for indicator species that inhabit different environments and have specific feeding habits or diets such as large zooplanktivores [manta ray *Manta birostris* (Walbaum 1792)], large mobile generalists (*C. plumbeus* and *C. leucas*) and epibenthic generalist species (*T. semifasciata* and southern stingray *Dasyatis americana* Hildebrandt & Schroeder 1928). Controlled experimental studies should also aim to investigate the effects of high *v*. low diet quantity and quality, mixed and single prey item diets and growth rate (juvenile *v*. adult) on derived DTDFs. In addition, for elasmobranch species with well characterized diets (from extensive stomach-content data) and restricted home-ranges (*i.e.* coastal movements to limit mixed pelagic and coastal isotope diet values), extensive SIA of prey items should be undertaken to reconstruct DTDFs from stomach-content data. The Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Richardson 1836) may be a good model species for this type of study (Drymon *et al*., 2011).

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ECOLOGICAL APPLICATIONS

DIET AND MIXING MODELS

Potentially the most powerful application for SIA is for the reconstruction of diet. To use SIA for diet reconstruction requires an accurate DTDF, variable stable-isotope values in potential prey items, and stable-isotope values of consumer tissues that are close to equilibrium with the diet. The importance of DTDFs is highlighted above, and was recently demonstrated for elasmobranchs (Hussey *et al*., 2010*b*; Logan & Lutcavage, 2010*b*). If prey items have similar stable-isotope values, it is not possible to distinguish their relative importance to a consumer. This is particularly problematic for more generalist-feeding species or species that reside in a single habitat with minimal carbon and nutrient sources and small ranges of isotope values (*e.g.* open ocean). If the isotope values of an animal are not in equilibrium with its current diet, then diet reconstruction will be compromised, providing unreliable prey contributions. For example, newborn sharks are provisioned with maternal reserves in the form of an enlarged liver (Hussey *et al*., 2010*c*) that confounds the stable-isotope values of offspring because muscle and liver tissue δ^{15}N and δ^{13}C values reflect varying contributions of the diet of the mother and the young shark for up to 1 year (Matich *et al*., 2010*b*; Olin *et al*., 2011). As a result, diet reconstruction of newborn and juvenile sharks using stable isotopes in these tissues is only possible when the maternal influence has been replaced by the shark's own diet.

It is recommended that diet reconstruction in elasmobranchs be coupled with stomach-content data and other chemical tracers when possible (such as fatty acids or other stable isotopes such as δ^{34}S). Stomach-content data can also inform an investigator about which prey items should be collected and analysed for SIA. In addition, undertaking a pilot study to determine stable-isotope variation in potential prey samples is recommended prior to commencing a SIA diet reconstruction research programme. Analysing multiple tissues will provide a temporal component to the analysis (MacNeil *et al*., 2005), and can help resolve whether an animal's stable-isotope values are near equilibrium with diet. It should be noted, however, that an animal that has stable-isotope values near equilibrium with its diet usually has different isotope values in different tissues (Pinnegar & Polunin, 1999; Hussey *et al*., 2010*a*; Kim & Koch, 2011).

When a diet study is limited to only two stable isotopes, usually δ^{13}C and δ^{15}N, distinguishing the relative importance of more than three prey items is difficult and exact solutions are not possible (Phillips & Gregg, 2003; Boecklen *et al*., 2011). This complication has led to the development of mixing models for estimating diet, which use DTDFs and isotope values of prey items to generate a range of potential solutions for the diet composition of an animal based on a mass-balance approach (must sum to 100%). The first stable-isotope mixing model, ISOSOURCE, considered all possible combinations of each diet item (0–100%) in small increments (*e.g.* 1%) to generate feasible solutions (Phillips & Gregg, 2003). Recently, more advanced Bayesian models to account for the uncertainty associated with the multiple prey sources have been developed allowing estimation of the DTDF value and consumer stable-isotope values [MixSIR; (Moore & Semmens, 2008) and SIAR (Parnell *et al*., 2010)]. Concerns have been raised, however, about these models (Jackson *et al*., 2009; Semmens *et al*., 2009) and they have been found to be highly sensitive to the assumed DTDF values (Bond & Diamond, 2011).

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To date, two studies have estimated the diet proportions of elasmobranch species using stable-isotope mixing models. Kim *et al*. (2011) used MixSIR to estimate the stable-isotope diet contributions of principal prey items identified from stomach-content analysis in muscle tissue samples of blue *P. glauca* and smooth hammerhead *Sphyrna zygaena* (L. 1758) sharks. For *P. glauca and S. zygaena*, stable-isotope values of identified stomach-content data did not characterize known diets, estimating a lower proportion of red crab *Pleuroncodes planipes* and a larger contribution of sardine *Sardinops sagax* (Jenyns 1842) and *Etrumeus teres* (Dekay 1842) in the diets of each species, respectively. Kim *et al*. (2011) suggested that discrepancies in the results between the two methods highlighted the limitations of stomach-content analysis; that it only provides a snapshot of recent feeding and can be biased by materials that are more resistant to digestion (*i.e.* cephalopod beaks). In a study of *R. terraenovae* in the Gulf of Mexico, Drymon *et al*. (2011) found that SIAR analysis using muscle tissue was confounded by the fact that the most abundant fish prey items sampled had similar isotope values. In addition, the authors found that the stable-isotope values of important diet items identified from stomach contents fell well beyond the observed range of isotope muscle tissue values of the sharks. Both of these studies demonstrate the challenges of using stable-isotope mixing models to reconstruct elasmobranch diet: the requirement for comprehensive prey sampling, slow tissue turnover rates, highly mobile species that move between isotopically distinct systems and knowledge of species-specific parameters such as DTDFs. These studies also highlight two important points that investigators should consider for SIA of elasmobranchs: (1) the need for standardized tissue processing (*i.e.* the complication of combined lipid extraction and arithmetic lipid correction in Drymon *et al*., 2011) and (2) potential issues of using stable-isotope derived prey data from the literature rather than co-ordinated predator-prey sampling (Kim *et al*., 2011).

Two further studies have applied mixing models of stable isotopes in elasmobranchs, but these were focused on assessing carbon sources. Wai *et al*. (2011) used fatty acid and SIA results in a Bayesian mixing model to demonstrate that juvenile white-spotted bamboo shark *Chiloscyllium plagiosum* [Anonymous (Bennett) 1830] used a greater proportion of terrestrial carbon than adults. In a similar approach, Vaudo & Heithaus (2011) estimated that most elasmobranchs in a nearshore community were highly dependent on a seagrass-based food web, with the contribution of the seagrass food web increasing with animal size for certain species.

The application of mixing models to assess elasmobranch diet has great potential, but similar to other taxa, is primarily limited by the lack of validated DTDFs and complicated by the effects of dietary protein composition and mixed *v*. single diet effects on DTDFs (Robbins *et al*., 2005, 2010; Florin *et al*., 2011). With experimentally derived tissue-specific DTDFs, mixing models could potentially be used to quantify seasonal diet switches by assessing prey contributions to different tissues with different incorporation rates. This would advance the approach used by MacNeil *et al*. (2005) to assess diet switching in sharks by using tissue-specific DTDFs rather than the invalid assumption that DTDFs are equal across tissues. This approach could be validated by selecting a species with a known seasonal diet switch, or that feeds seasonally on a specific prey resource, for example large carcharhinids feeding on the annual sardine *S. sagax and E. teres* run in South Africa (Dudley & Cliff, 2010). Combining other chemical tracers, such as fatty acids, mercury or contaminants, with stomach-content data in mixing models holds potential for improving diet

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reconstruction of elasmobranchs and increasing the confidence in SIA mixing model outputs. It is also recommended that investigators undertake extensive sampling and SIA of prey items to increase confidence in model predictions.

ESTIMATING TROPHIC POSITION

Elasmobranchs, in particular the large predatory sharks, are apex predators in marine ecosystems and understanding their role or trophic position (T_P) in terms of top-down control on community structure is of ecological and conservation importance (Baum & Worm, 2009). Traditional studies have used stomach-content data to estimate T_P for sharks (Cortés, 1999) and skates (Ebert & Bizzarro, 2007), but this approach can be limited by the snapshot sampling of stomach-content data and the broad functional prey categories used to calculate T_P (Hussey *et al*., 2011*a*). The simplest and most commonly applied method to calculate T_P is to use only $\delta^{15}N$ values and the following equation:

$$T_{\text{Pelasmobranch}} = (\delta^{15}N_{\text{elasmobranch}} - \delta^{15}N_{\text{baseline organism}})(\Delta^{15}N)^{-1} + T_{\text{Pbaseline organism}}, \quad (1)$$

where $\delta^{15}N_{\text{baseline organism}}$ and $T_{\text{Pbaseline organism}}$ are the $\delta^{15}N$ value and predicted T_P for a low trophic level or baseline organism in the ecosystem, normally a sessile organism such as a clam or mussel and $\Delta^{15}N$ is the DTDF (Post, 2002). A more complex measure of T_P involves a two-source food web that accounts for diet contributions from different components of the ecosystem (*e.g.* coastal *v*. offshore) having different stable-isotope values (Post, 2002). This model has appeal for elasmobranchs, particularly larger more mobile species and is calculated using the following equations:

$$T_{\text{Pelasmobranch}} = \lambda + \{\delta^{15}N_{\text{elasmobranch}} - [\delta^{15}N_{\text{base1}} \times \alpha + \delta^{15}N_{\text{base2}} \times (1 - \alpha)]\} (\Delta^{15}N)^{-1}, \quad (2)$$

where λ is the T_P of the selected baseline consumers (often low trophic level organisms similar to those for equation 1), $\delta^{15}N_{\text{base1}}$ is the value of one base consumer (*e.g.* coastal), $\delta^{15}N_{\text{base2}}$ is the value of the second base consumer (*e.g.* offshore) and α is the calculated proportion of the nitrogen in the consumer derived from the base of the coastal food web ($\delta^{15}N_{\text{base1}}$):

$$\alpha = (\delta^{13}C_{\text{elasmobranch}} - \delta^{13}C_{\text{base2}})(\delta^{13}C_{\text{base1}} - \delta^{13}C_{\text{base2}})^{-1}. \quad (3)$$

Trophic position estimates are highly dependent on the DTDF value used, which can vary between species and has not been validated for most elasmobranchs. In one of the first applications of stable isotopes towards calculating a T_P of an elasmobranch, Fisk *et al*. (2002) raised concern because the liver tissue of *S. microcephalus* in Cumberland Sound, Baffin Island, was not enriched in ^{15}N relative to known prey. Specifically, *S. microcephalus* had similar $\delta^{15}N$-based trophic positions to Greenland halibut *Reinhardtius hippoglossoides* (Walbaum 1792) and ringed seal *Pusa*

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hispida, although both of these species were identified in stomach contents (Fisk *et al*., 2002). Furthermore, *S. microcephalus* had higher concentrations of biomagnifying organochlorine contaminants than either *R. hippoglossoides* or *P. hispida*, suggesting that the sharks fed at a higher trophic position than these species, and that δ^{15}N values had underestimated *S. microcephalus* T_P (Fisk *et al*., 2002). In contrast to results from Cumberland Sound, *S. microcephalus* from Icelandic waters had higher δ^{15}N values relative to several known teleost prey species by *c*.2–5‰ (McMeans *et al*., 2010). Thus, the authors found no reason to doubt the trophic position of 4·3 for *S. microcephalus* calculated using a Δ^{15}N of 4·0‰ (McMeans *et al*., 2010). This trophic position estimate was also in agreement with stomach-content estimated trophic position for *S. microcephalus* (Cortés, 1999). If, however, the trophic position of *S. microcephalus* reported in McMeans *et al*. (2010) is recalculated using a Δ^{15}N of 2·3‰ (Hussey *et al*., 2009) an average trophic position of 5·2 is obtained. Considering that stomach-content data often underestimates T_P due to simplified diet categories (Hussey *et al*., 2011*a*) and that *S. microcephalus* frequently consume piscivorous fishes and marine mammals (Fisk *et al*., 2002; McMeans *et al*., 2010; LeClerc *et al*., 2011), 5·2 is a reasonable estimate of trophic position for this species.

Dale *et al*. (2011) used a Δ^{15}N value of 2·7‰ based on the review by Vanderklift & Ponsard (2003), to estimate the trophic position of *D. lata*. The resulting agreement between estimated SIA T_P and stomach-content calculated T_P suggests the Δ^{15}N value was appropriate for a ray species. Similarly, stable isotope estimated T_P of *P. glauca*, *I. oxyrinchus*, *A. vulpinus* and the basking shark *Cetorhinus maximus* (Gunnerus 1765) using a Δ^{15}N value of 3·4‰ were in agreement with stomach-content data T_P (Cortés, 1999). In the only study to date, Hussey *et al*. (2011*a*) used the two-source model (equations 2 and 3) and δ^{15}N values for inshore and offshore fish species as baseline organisms to calculate ontogenetic trophic profiles of *S. lewini* and *Carcharhinus obscurus* (LeSueur 1818). There were discrepancies, however, between stable-isotope and stomach-content calculated T_P estimates that the authors suggested were a result of the two-source model failing to account for the highly variable movements of sharks between isotopically distinct environments and the limitations of using broad prey categories to calculate stomach-content T_P, leading to possible T_P underestimation.

An additional complication when calculating the T_P of elasmobranchs, or any higher trophic level species, is that the DTDF can vary with each step in the food web owing to the significant relationship between Δ^{15}N and diet δ^{15}N value (Robbins *et al*., 2005, 2010; Caut *et al*., 2009; Dennis *et al*., 2010). Thus, if a lower trophic level animal is used as the reference baseline organism in equations (1) and (2) and a single DTDF applied, the estimate of trophic position for top predators may be underestimated. This is a particular concern for elasmobranchs given their apparently lower DTDF. For example, most sharks T_P estimates use a reference baseline organism feeding at trophic level three (Estrada *et al*., 2003, 2006; Borrell *et al*., 2011*a*; Hussey *et al*., 2011*a*) on the premise that sharks feed around trophic level four (Cortés, 1999). Trophic level four animals are piscivores that, by definition, feed on zooplanktivorous fishes. Considering that most sharks feed on piscivorous teleosts (*i.e.* fishes that eat zooplanktivorous fishes), a single DTDF applied to a trophic level three baseline organism may lead to a downward bias in T_P. Therefore, staggered DTDFs may be required at each trophic level, similar to ideas proposed by

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Hobson *et al.* (1995). Alternatively, defining trophic guilds by sampling known indicator trophic level organisms would increase confidence in derived T_P estimates of related elasmobranchs. As for mixing models, a blended approach that incorporates multiple lines of evidence will lead to the most accurate picture of food web trophic structure.

Despite the uncertainty surrounding T_P calculations, stable-isotope estimated T_P is an important metric as it provides a quantitative estimate of trophic structure that has not been otherwise possible. It also allows for comparison of spatial and temporal feeding ecology of a species or population by examining the variability in stable-isotope values within and between ecosystems. Importantly, T_P provides a measure of the relative position and role of a species in a food web (Pauly *et al.*, 1998; Navia *et al.*, 2010) and a unique measure of human pressure on ecosystems (Pinnegar *et al.*, 2002; Branch *et al.*, 2010).

MOVEMENT AND MIGRATION

The application of SIA to examine animal origin and movements has grown in recent years, revealing detailed residency, movement and migration patterns that have proved valuable for investigating cryptic and highly threatened species (Hobson, 1999; Rubenstein & Hobson, 2004; Graham *et al.*, 2010; Hobson *et al.*, 2010). The use of SIA to track animal movements is based on the retention of stable-isotope signatures in an animal's tissues that reflect the local food web where the animal resides for an extended period of time (Peterson & Fry, 1987; Michener & Schell, 1994). Because the stable-isotope values of food webs vary spatially depending on localized biogeochemical processes, the stable-isotope values of an animal's tissues from within a given food web will reflect those particular habitats or environments (DeNiro & Epstein, 1978, 1981). Importantly, the retention time of the stable-isotope value of a given food web within an animal's tissue depends on the rate of isotopic incorporation, whereby an animal could move between isotopically distinct food webs but retain the isotopic value of the previous feeding location for some time. The rate of tissue isotope incorporation, in conjunction with *a priori* knowledge of movement patterns, will determine the tissue type to analyse for SIA of residency. Structures that grow incrementally (*e.g.* vertebral centra) are metabolically inert following formation and therefore maintain a chronological isotopic record of foraging location over time.

Hussey *et al.* (2011*a*) examined the potential of SIA of muscle tissue for determining coarse scale ontogenetic movement patterns of *C. obscurus* and *S. lewini* off the south-east coast of Africa. With known spatial latitudinal and inshore *v.* offshore gradients in $\delta^{13}C$ values (Hill *et al.*, 2006, 2008), the authors identified a degree of sexual separation in sub-adult *S. lewini* and a movement to shelf-edge foraging in large *C. obscurus*. These isotopic reconstructed movements were supported by previous tag-recapture and observation data for these species (Klimley, 1987; Hussey *et al.*, 2009; Diemer *et al.*, 2011). Juvenile *C. obscurus* also showed variable $\delta^{13}C$ values compared to small *S. lewini*, reflecting a known migration along the South African coastline (Hussey *et al.*, 2009).

Using both bulk SIA and SIA of individual amino acids, Dale *et al.* (2011) noted ontogenetic separation in feeding areas of *D. lata* in Kaneohe Bay, Hawaii, U.S.A. Because of a lack of spatial variation in the diet of *D. lata*, the authors concluded that

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both bulk SIA and SIA of individual amino acids identified variability in baseline values between bay zones attributable to different environmental conditions. Dale *et al*. (2011) concluded that a movement to foraging in offshore waters was related to the onset of maturity.

Abrantes & Barnett (2011) used SIA to examine intra-population variations in habitat use of *N. cepedianus*. Overall, δ^{13}C values differed between fish caught inshore and offshore, suggesting that animals spent significant amounts of time in the habitat where they were caught prior to capture and identified that δ^{13}C was able to differentiate between these habitat types. Abrantes & Barnett (2011) also reported that δ^{15}N values were lower in the offshore animals and suggested this was a result of a lower baseline δ^{15}N value in offshore food webs or a potentially shorter food web. This highlights the complications involved in disentangling δ^{13}C and δ^{15}N values of highly mobile animals (Hussey *et al*., 2011*a*). Female *N. cepedianus* also had different δ^{13}C values compared to males and this was attributed to males undertaking a northern migration observed in telemetry tracking data and higher catch rates of males in beach protection nets; females remained in coastal areas. Although not the overall focus of the study, SIA has been used to determine the principal feeding location (*i.e.* benthic *v*. pelagic feeding) in small rays and sharks (Botto *et al*., 2011; Vaudo & Heithaus, 2011) and to identify potentially variable inshore *v*. offshore feeding strategies in *I. oxyrinchus* (Estrada *et al*., 2003). Kerr *et al*. (2006), Estrada *et al*. (2006) and Hussey *et al*. (2012) describe a general trend of δ^{13}C decline with size in *C. carcharias* indicating proportionally more time spent foraging in offshore waters.

The potential to sample the incremental growth bands of shark vertebral centra (Estrada *et al*., 2006) and caudal thorns will provide a unique material for future SIA of movement and migration patterns of elasmobranchs, particularly through laser-based quantification of carbon and nitrogen stable isotopes. Furthermore, SIA of prenatal vertebral centra may prove an appropriate tool for identifying stock structure and variability in the foraging location of females prior to parturition. Although modern telemetry methods such as pop-up archival satellite tags provide detailed high resolution data on both vertical and horizontal movement behaviour, electronic tags are typically restricted in deployment time (<12 months) and cost-restricted in terms of the numbers of individuals that can be monitored. In contrast, SIA of tissues is a relatively simple, inexpensive process enabling the analysis of large field data sets. In addition, through SIA of archived collections of shark vertebral centra there is the real possibility to examine intra-individual variation in movement and migration patterns at the population level and to undertake retrospective analysis of potential trophic effects of overfishing and climate. As suggested by Hussey *et al*. (2011*a*), inexpensive ontogenetic δ^{13}C profiles may provide effective tools to direct the deployment of electronic tags, in terms of selecting suitable life-stages and sex. Combining acoustic and satellite tracking technologies with SIA also has the potential to validate SIA as a robust chemical tracer of movement and to further explore the behavioural factors that drive variation in stable-isotope values.

As noted by Hussey *et al*. (2011*a*), it is important, however, to consider that many sharks are highly mobile in both horizontal and vertical spatial planes, undertaking long distance return migrations (Bonfil *et al*., 2005) and oscillating deep diving profiles over short periods of time (Queiroz *et al*., 2010). The SIA profiles of mobile elasmobranch species may therefore represent a collective isotopic measure

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of isotopically distinct environments rather than discrete isotopic profiles, as previously identified for bowhead whales *Balaena mysticetus* (Schell *et al*., 1989) and Antarctic fur seals *Arctocephalus gazella* (Cherel *et al*., 2009). Tissue selection for a specific isotopic incorporation rate is critical for detecting residency and movement patterns at an appropriate scale. Future work should examine multiple elasmobranch species at one geographical locality, that have relatively well defined and distinct movement patterns, and where the isotopic baselines of the habitats are well characterized. This would enable a quantitative assessment of the difference in δ^{13}C values between species and tissues having different incorporation rates, to see how these correlate with various habitats.

INDIVIDUAL SPECIALIST–GENERALIST AND NICHE-WIDTH SIA ANALYSES

Ecological theory has traditionally viewed niche width as a population-level factor. There is a growing body of evidence, however, suggesting population niches are more complex, comprising individual specialists that utilize a sub-set of the total population niche (Bolnick *et al*., 2002, 2003). The prevalence of intra-population foraging has important implications for population biology, behavioural and evolutionary ecology, and ecosystem dynamics (Tinker *et al*., 2008). To date, most research examining niche width and individual specialization has been based on stomach-content analysis (Bolnick *et al*., 2002). Quantifying individual specialization through stomach contents is, however, hindered by (1) measuring the relative abundance of different prey items, (2) the snapshot aspect of recent feeding behaviour and (3) the inability to account for variation in prey assimilation rates (Bearhop *et al*., 2004). Moreover, stomach-content analysis is intrusive, often requiring sacrifice of the animal, and identification of stomach contents is labour intensive (Vinson *et al*., 2011). With continued advances in the understanding of stable-isotope dynamics, recent work has evolved from studies on diet, food web and trophic dynamics to examining niche width or individual level resource specialization (Bearhop *et al*., 2004; Newsome *et al*., 2007; Vander Zanden *et al*., 2010).

For elasmobranchs, few data are available to characterize individual feeding strategies and as a largely unexplored area of research, provides a promising avenue for understanding elasmobranch ecological roles in marine food webs. Matich *et al*. (2010*a*) undertook the only study to date examining individual diet specialization of two large species of generalist shark, *C. leucas* and *G. cuvier*. This study employed multiple tissues with different incorporation rates from single sampling points to examine dietary variation within individuals (WIC, within individual component of variation) and between individuals (BIC, between individual component of variation) following the quantitative methods of Bolnick *et al*. (2002). Matich *et al*. (2010*a*) reported that despite wide population-level isotopic niches for both species, population niche breadth of *G. cuvier* was explained predominantly by variation within individuals suggesting true generalist behaviour. In contrast, for *C. leucas*, stable-isotope values of individuals were more stable over time with the wide population-level niche breadth explained by variation among specialist individuals (Matich *et al*., 2010*a*). The authors suggested that differences in individual dietary specialization between these two species were driven by relative resource abundance and spatial variation in food-predation risk tradeoffs.

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The Matich *et al*. (2010*a*) study utilized a novel method to examine individual specialization in elasmobranchs, but two factors should be considered to advance and refine the use of this technique. Firstly, as discussed above, DTDFs differ between fish tissues (Pinnegar & Polunin, 1999) and shark tissues (Hussey *et al*., 2010*a*, 2011*b*; Kim *et al*., 2011). A major assumption of Matich *et al*. (2010*a*), similar to MacNeil *et al*. (2005), was that all analysed tissues had equal DTDFs; if violated, this could have major implications for the interpretation of the results, potentially altering the conclusions of both these studies. Furthermore, Matich *et al*. (2010*a*) used δ^{13}C values for the individual level diet analyses in contrast to Newsome *et al*. (2009) who used a combination of both δ^{13}C and δ^{15}N values to examine dietary specialization in sea otters *Enhydra lutris nereis*. In SIA, δ^{13}C is typically considered an indicator of the carbon fixing organisms at the base of the food chain, for example between benthic and pelagic systems (France, 1995). Because carbon isotopes do not vary substantially at each step in a food web ($\leq$1‰; Fry & Shear, 1989) they are primarily used to evaluate the source of primary productivity, providing a measure of foraging location rather than dietary intake. Further experimental work is needed to better understand SIA specialization in terms of both diet and habitat, respectively. While Matich *et al*. (2010*a*) used single samples from multiple tissues, there is also the potential to use incremental tissues such as vertebral centra to undertake this type of analysis (Bearhop *et al*., 2004; Newsome *et al*., 2009).

Metrics have recently been devised that utilize SIA to examine the niche width of a species or an ecological community (Layman *et al*., 2007; Jackson *et al*., 2011). Considering niche as the total of all interactions linking one species within a system, stable-isotope values in an animal's tissue can provide a time-integrated measure of niche width. Layman *et al*. (2007) described six community wide metrics for analysing niche width: δ^{15}N range (NR), δ^{13}C range (CR), total niche area (TA), the mean distance to centroid (CD), mean nearest neighbour distance (NND) and the standard deviation of nearest neighbour distance [SDNND; Layman *et al*. (2007)]. Vaudo & Heithaus (2011) used the Layman *et al*. (2007) metrics to examine the trophic niches of a nearshore elasmobranch community in Shark Bay, Western Australia. The authors found that although there were differences in mean isotopic values of species, there was large overlap in the niche space used by the community when examined at the individual level. They concluded that possible individual specialization within the groups examined resulted in high resource overlap of the community as a whole.

The application of community niche metrics holds promise for understanding the role of elasmobranchs in marine systems, but as Vaudo & Heithaus (2011) highlight, there is a need to consider (1) sample sizes and (2) ontogenetic shifts in stable-isotope profiles of species associated with diet switches. With this in mind, Jackson *et al*. (2011) reformulated the Layman *et al*. (2007) metrics in a Bayesian framework accounting for uncertainty in the sampled data and error arising from the sampling process. In addition, the new method proposed by Jackson *et al*. (2011) uses novel multivariate normal metrics that are unbiased with regard to sample size. This should markedly improve the application of these tools for examining the role of elasmobranch species within a community and for directly comparing isotopic niches across communities. This could in turn provide insight into how exploitation and removal of elasmobranch species affects community structure, composition and organization.

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ALTERNATIVE STABLE-ISOTOPE TRACERS

Given the applicability of $\delta^{13}C$ and $\delta^{15}N$ to a range of ecological and evolutionary questions, there are a number of additional stable-isotope tracers, such as sulphur, hydrogen and oxygen, as well as compound-specific stable-isotope analysis that have the potential to provide new opportunities for stable-isotope studies in elasmobranchs.

SULPHUR

Sulphur stable isotopes ($\delta^{34}S$) have found application in distinguishing terrestrial or freshwater *v*. marine organic matter source contribution to diet (Peterson & Howarth, 1987). Marine sulphate generally has higher $\delta^{34}S$ values (21‰ for phytoplankton) than terrestrial or freshwater derived sulphates (2–8‰ for upland plants) and therefore sulphur isotope analysis has been extensively used in species that use estuarine habitats (Peterson & Howarth, 1987; Connolly *et al*., 2004; Olin *et al*., 2012). In addition to directly tracing nutrient sources to an organism, $\delta^{34}S$ has been used to track migratory behaviours of birds, regarding overwintering and breeding habitats (Lott *et al*., 2003; Hebert *et al*., 2008), and to reconstruct Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) life history from the larval to adult stages (Weber *et al*., 2002).

Because $\delta^{34}S$ is not affected by trophic fractionation (*i.e.* fractionation <1‰), the use of $\delta^{34}S$ in combination with $\delta^{13}C$ provides added discriminatory power that is often necessary in estuarine and nearshore coastal environments, where there are a number of potential organic matter sources (Peterson & Fry, 1987). As such, $\delta^{34}S$ has been recently employed to track resident and transient fishes across an estuarine salinity gradient (Fry & Chumcal, 2011). This application of $\delta^{34}S$ is particularly relevant to elasmobranchs, as a number of species use estuarine environments during different life-history stages (Springer, 1967), and thus $\delta^{34}S$ has the potential to provide a fine scale view of a species movement among and within these habitats.

HYDROGEN AND OXYGEN

The stable isotopes of hydrogen (deuterium, δD) and oxygen ($\delta^{18}O$) have a long history of use in hydrology and palaeoclimatology, where they are used to trace the hydrological cycle from evaporation in the oceans to local precipitation and groundwater (Dansgaard, 1964). The fundamental control on the isotopic composition of precipitation is temperature. Varying proportions of ^{18}O:^{16}O and ^{2}H:^{1}H are dependent upon the temperature and isotope value of the water that the organism uses. With increasing temperature, precipitation becomes linearly enriched in the heavier ^{18}O and ^{2}H isotopes. Temperature affects fractionation at a rate of *c*. 0·5‰ for every $^{\circ}$ C for oxygen. Similar effects are shown with increasing elevation and increased distance from the equator. For tissues that are biochemically inert after synthesis (*e.g.* keratin, bone collagen and otoliths), these labels are used to estimate the location or origin of organisms. Much of the work to date has focused on estimating the natal or breeding latitudes of migrating birds (Hobson *et al*., 2004; Rubenstein & Hobson, 2004) or reconstructing movement patterns of fishes (Thorrold *et al*., 2001; Whitledge *et al*., 2006). Although useful in reconstructing behaviour and life history, these isotopes are being more widely applied in fisheries management. Rooker *et al*. (2008) using $\delta^{18}O$ of *T. thynnus* yearlings, demonstrated that U.S. fisheries are

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a mixture of recruits from both the eastern Atlantic Ocean and the Mediterranean Sea.

Deuterium and ^{18}O are also more enriched in marine *v*. terrestrial systems. Kerr *et al*. (2007) reported the successful application of $\delta^{18}O$ as a proxy for the salinity histories of juvenile white perch *Morone americana* (Gmelin 1789). Pruell *et al*. (2010) demonstrated that a strong correlation was found between collection site salinity and the $\delta^{18}O$ of juvenile winter flounder otoliths *Pseudopleuronectes americanus* (Walbaum 1792); −4·4‰ in lower salinity *v*.−1·2‰ at higher salinities. Similar to the application of $\delta^{34}S$, these tracers provide a number of pathways for understanding relative fine scale habitat use and movement, and large-scale migration of elasmobranch species.

COMPOUND-SPECIFIC STABLE-ISOTOPE ANALYSIS

Given the complexity of calculating elasmobranch trophic position using bulk SIA, the nitrogen isotope composition of individual amino acids provides a potentially more robust alternative (McClelland & Montoya, 2002; McClelland *et al*., 2003; Popp *et al*., 2007; Chikaraishi *et al*., 2009; Hannides *et al*., 2009). This method is based on the premise that there are large enrichments in ^{15}N in some amino acids (*e.g.* glutamic acid) with each trophic step, as a result of isotopic fractionation during metabolic transamination. For other amino acids (*e.g.* phenylalanine), there is minimal change in enrichment because the dominant metabolic processes neither forms or cleaves bonds related to nitrogen (Chikaraishi *et al*., 2009; Hannides *et al*., 2009). Amino acids that fractionate are commonly referred to as trophic amino acids, whereas those that show minimal change are referred to as source amino acids. By measuring the $\delta^{15}N$ values of these source and trophic amino acids it is possible to estimate the T_P of an individual animal without the requirement for external baseline organisms. The T_P using $\delta^{15}N$ values of individual amino acids is calculated as: $T_{Px/y} = (\delta^{15}N_x - \delta^{15}N_y - \beta_{x/y})(\Delta_x - \Delta_y)^{-1} + 1$, where $\beta_{x/y}$ represents the difference in isotopes between amino acids x and y in the primary producers and Δ_x and Δ_y represent the ^{15}N-enrichment factors with each trophic level for amino acids x and y [often referred to as trophic enrichment factors (TEF)]. The subscripts x and y indicate the trophic and source amino acids, respectively (Chikaraishi *et al*., 2009). Typically, investigators have used glutamic acid (Glu, trophic) and phenylalanine (Phe, source) with TEFs (Δ_{GLU} and Δ_{PHE}) of *c*. 7 and 0‰, respectively (Schmidt *et al*., 2004; Hannides *et al*., 2009); however other trophic and source amino acid combinations are possible (Popp *et al*., 2007; Chikaraishi *et al*., 2009).

To date, the amino acid-specific method has been successfully used to investigate the trophic position of various organisms ranging from primary and secondary copepod consumers (Hannides *et al*., 2009) to yellowfin tuna *Thunnus albacares* (Bonnaterre 1788) (Popp *et al*., 2007). The approach has been found to provide accurate T_P estimates even when the organisms are sampled from environments with spatially and temporally variable primary producers (Hannides *et al*., 2009). For two elasmobranch species, *D. lata* and *S. lewini*, Dale *et al*. (2011) found that the usual TEF of *c*.7‰ underestimated T_P of these species and that a TEF of 5‰ was more appropriate. Further controlled experimental work will be required to accurately estimate trophic position of elasmobranchs using nitrogen stable-isotope analysis of amino acids, ideally in conjunction with bulk SIA to determine species-specific TEFs.

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Recent work has also investigated carbon isotopes in amino acids in fish muscle finding that essential amino acids directly reflect a pure protein diet signature, with a fractionation near 0‰ that is distinct from non-essential amino acids (McMahon *et al*., 2010). Essential amino acids may prove highly useful for characterizing sources at the base of the food web with the potential to mitigate problems associated with sampling isotopically variable primary consumers. Given the small sample size required, there is also potential to examine $\delta^{15}N$ and $\delta^{13}C$ values of amino acids from collagen in vertebral centra. Compound-specific stable-isotope analysis is an extremely powerful tool that has immense scope for resolving questions over the complex trophic interactions of elasmobranchs within food webs, but sample preparation is laborious and analyses are currently costly.

CONCLUSION

Despite an increase in dedicated elasmobranch research, significant data gaps still exist for many species, limiting effective conservation and management of this potentially important functional group. SIA provides a tool that has the potential to assist in addressing many of these data deficiencies. There is, however, a need for extensive experimental work to validate the basic assumptions underlying the application of SIA. This is especially important for elasmobranchs, whose specific physiology creates unique stable-isotope dynamics. As Gannes *et al*. (1997), Martínez del Rio *et al*. (2009) and Wolf *et al*. (2009) have stated, more controlled experiments are needed to understand stable-isotope dynamics and this is particularly true for elasmobranchs. The challenges of large animal husbandry mean that innovative experiments are required to understand dynamics in large sharks. Moreover, it is important that investigators using SIA in elasmobranchs follow standardized SIA methodologies (*e.g.* lipid–urea extraction) and consider all relevant assumptions (tissue-specific DTDFs–TEFs and turnover rates) when examining field data. Validation of stable-isotope dynamics would vastly improve confidence in the interpretation of elasmobranch stable-isotope data and in the overall use of stable-isotope techniques by the broader research community.

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