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## REVIEWS

# Stable isotope analysis in deep-sea chondrichthyans: recent challenges, ecological insights, and future directions

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**Abstract** Deep-sea chondrichthyans are cryptic species subject to increasing anthropogenic exploitation. Defining their role in deep-water ecosystems is therefore crucial for predicting the ecosystem-wide effects of their removal. Stable isotope analyses (SIA) of carbon and nitrogen have been increasingly used in chondrichthyan studies as a non-lethal method to investigate aspects of their ecology. In recent years these methods have been applied to deep-sea chondrichthyans to investigate their trophic structure, niche width, and describe energy flow in the deep sea. Despite the increasing popularity of SIA in deep-sea chondrichthyan studies, methods rely on a multitude of assumptions, such as the need to determine accurate trophic discrimination and tissue turnover rates, which are currently lacking for most species. These uncertainties may preclude the reliability of isotope-based approaches, and as a result inferences from isotopic data must be viewed with relative caution. Due to the growing use of isotopic-based approaches in deep-sea chondrichthyans, we review the literature in the context of current methodological challenges and ecological inferences. We provide recommendations and novel approaches that may help develop and refine a rapidly growing field of study.

**Keywords** Vulnerable · Deep-water · Food web · Chemical tracer · Carbon · Nitrogen

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## Introduction

In recent decades, stable isotope analysis (SIA) has emerged as a useful tool to perform quantitative ecological assessments of food web structure and wider community interactions across all major marine biomes (Boecklen et al. 2011). SIA relies on determining the ratio of heavy to light isotopes present within animal tissues (reported in ‰) as these are transferred in a relatively predictable manner from primary producers or prey to consumer (defined as trophic discrimination; DeNiro and Epstein 1978, 1981). The isotope ratios of carbon ($\delta^{13}$C),

nitrogen ($\delta^{15}$N) and sulfur ($\delta^{34}$S) are most frequently used in marine ecosystem studies and combinations of these isotopic tracers can provide powerful insights into food-web dynamics. Examples include connectivity and systems (McCauley et al. 2012), community ecology (Reid et al. 2012, 2013), inter-species tropho-dynamics (Polunin et al. 2001; Post 2002; Pethybridge et al. 2012), and population level behaviours (e.g. Jackson et al. 2011).

The variable chemical characteristics of elemental isotopes can be utilised to examine numerous fundamental ecological questions (Gannes et al. 1997). Isotope ratios of carbon ($\delta^{13}$C) are used to infer sources of primary production supporting the base of marine food webs. This is possible because the trophic discrimination of carbon isotopes is typically small, averaging from approximately 0.6 to 1.0‰ per trophic level (Hussey et al. 2012), such that $\delta^{13}$C of a consumer is similar to that of prey or basal resource, especially given variation in basal resource $\delta^{13}$C (Melville and Connolly 2003). This applies even for specific taxa where trophic discrimination in $\delta^{13}$C is larger, particularly in fish (Caut et al. 2009). Sulfur isotopes ($\delta^{34}$S) have also been used to differentiate sources of production with low trophic discrimination, nominally less <1.0‰, although much less rigorously tested. $\delta^{34}$S does not show the same differentiation among primary production sources as $\delta^{13}$C, although there are discernible differences in $\delta^{34}$S between benthic and pelagic sources as well as terrestrial versus marine sources of primary production in food-webs. Chemosynthetic inputs (e.g. autotrophic hydrothermal vent bacteria) are also differentiated (Reid et al. 2012). Isotope analysis of $\delta^{15}$N has been more commonly used in derivation of trophic position, as nitrogen isotopes generally exhibit high trophic discrimination between prey and consumer (typically 3.0–3.4‰, Hussey et al. 2012), although discrimination factors vary across taxa and can be influenced by diet (Caut et al. 2009). The use of $\delta^{15}$N to determine trophic ecology, particularly relative or absolute trophic position, is by far the most common application in marine isotope ecology.

A significant advantage of isotope-based approaches compared to conventional diet-based methodologies, such as gut content analysis, includes non-lethal sampling of individuals. This becomes especially important when sampling more vulnerable, larger bodied marine vertebrates, which often exhibit more conservative life histories (Lewison et al. 2004). The extraction of a small mass (0.6–0.8 µg dry weight) of tissue, such as white muscle, blood, fin, scales, vertebrae or otoliths can provide powerful insight into feeding ecology and wider community dynamics of marine species. In addition, the differential metabolic turnover rate of specific tissues allows for a temporally resolute analysis of diet (Pinnegar and Polunin 1999). For example, the comparably slow turnover rate of white muscle compared to blood or liver, which exhibit high metabolic activity, provides both short and long-term indication of diet (Pinnegar and Polunin 1999; Estrada et al. 2006; Madigan et al. 2015). When lethal sampling is required, e.g. for sampling more solid structures such as otoliths and vertebrae, analysis can provide a powerful temporal profile of diet occurring across the entire life span of an animal (Estrada et al. 2006). SIA therefore offers a diverse suite of advantages, which often require a lower sampling effort, whilst providing insight into the feeding ecology and food web dynamics of marine species.

The deep sea remains the most data-deficient biome on the planet, yet is becoming increasingly exposed to extrinsic pressures such as commercial fishing (Norse et al. 2012) and deep-sea mining (Van Dover 2011), which pose a significant threat to ecosystem functioning (Mengerink et al. 2014). Keystone examples have highlighted the vulnerability of deep-sea fish taxa across the literature; for example the collapse of the orange roughy (*Hoplostethus atlanticus*) fishery has been widely reported (Koslow et al. 2000; Clark 2001; Roberts 2002; Norse et al. 2012). In more recent decades, an increased number of studies have reported the exponential declines of larger vertebrates, such as deep-sea chondrichthyans (sharks, skates, rays and chimaeras) (White and Kyne 2010; Graham and Daley 2011). Deep-sea chondrichthyans are among the most vulnerable extant deep-sea taxa due to their extremely conservative life histories (slow growing, late to mature, small number of young), and thus slow population rebound rates (Simpfendorfer and Kyne 2009); and in recent years catastrophic population declines have been observed across multiple species at numerous locales (Anderson and Ahmed 1993; White and Kyne 2010; Graham and Daley 2011; Norse et al. 2012; Barbier et al. 2014). Although deep-sea taxa make up approximately half (47.6%) of extant chondrichthyans, they are poorly represented in the

scientific literature (Cotton and Grubbs 2015). This is largely due to the inherent difficulty associated with studying remote, intrinsically inaccessible deep-sea habitats, which requires expensive equipment and rigorous logistical protocols (Brooks et al. 2015). The fundamental lack of biological and ecological data for these species raises concerns over the health of deep-sea chondrichthyan populations and creates ambiguity regarding potential effects of their removal on ecosystem structure and function.

Only in the last fifteen years have we begun thoroughly assessing ecological interactions and community structure of deep-sea chondrichthyan communities (e.g. Fisk et al. 2002; Pethybridge et al. 2010, 2012; Laxson et al. 2011; Navarro et al. 2014; Cresson et al. 2014; Churchill et al. 2015a). The relative scarcity of basic biological and ecological information creates difficulties when constructing appropriate and efficient management strategies (Simpfendorfer and Kyne 2009). Generally for chondrichthyans, SIA has been applied more readily to coastal and pelagic species, quantitatively assessing otherwise elusive aspects of their ecology, such as ontogenetic changes in feeding strategies, migration, and trophic structure (reviewed by Logan and Lutcavage 2010; Hussey et al. 2012). In addition, the development of advanced statistical analyses such as Bayesian mixing models has allowed scientists to assess the role played by chondrichthyans on a macroecological scale, such as outlining their role in facilitating connectivity across multiple ecosystems (McCauley et al. 2012; Papastamatiou et al. 2015). Such holistic approaches to chondrichthyan ecology are fundamental in ensuring more effective management, as the important ecological functions provided by these species, and thus potential effects of their removal, become increasingly recognised.

To date, SIA-based studies have significantly improved our understanding of deep-sea chondrichthyan community ecology; however, most have been restricted to a limited number of discrete marine ecoregions. Isotopic studies are dominated by Mediterranean sampling with only a few widely scattered examples from elsewhere, although studies from the Gulf of Mexico (Churchill et al. 2015a), Australasia (Pethybridge et al. 2012), and the Northeast Atlantic (Trueman et al. 2014) have quatitatively described ecologically significant community interactions that may be unique to deep-sea systems. The

importance of stable isotope techniques in deep-sea ecological research is illustrated in Table 1. In departure from patterns in wider ecological research, there appears to be under utilization of stable isotope techniques, particularly in deep-sea shark research, in favor of fatty acid or traditional stomach content analyses. However, when compared to wider deep-sea community ecology, studies on deep-sea sharks appear to represent an extremely low proportion of the scientific literature (Table 1).

Despite the advantages of SIA as an ecological tool, its specific application to deep-sea chondrichthyans comes with a significant number of caveats; specifically, little consideration has been applied to how ecological characteristics unique to deep-sea species may affect the application and interpretation of isotope-based approaches. For example, the potential variability in trophic discrimination between deep-sea and shallower species is rarely acknowledged, nor how this may change due to reduced metabolic rates typical of deep-sea organisms (Drazen and Seibel 2007; McClain et al. 2012). Furthermore, the unique mix of production sources in the deep sea may affect isotope values, including chemoautotrophic, benthic, pelagic, and fluvial production (Newman et al. 2011). These current challenges must be identified and addressed to enhance SIA-based inferences of deep-sea chondrichthyan ecology.

Herein, we address both the successes and the current challenges of SIA-based studies in deep-sea chondrichthyans. Specifically, we describe and evaluate the literature in the context of (1) methodological challenges, (2) ecological insights, and (3) future directions. We aim to provide a current conceptual and methodological framework to inform ongoing and future studies in a growing field of study.

## Methodological challenges

Despite the growing popularity of SIA, a thorough understanding of the underlying assumptions and caveats of this approach is important to put the current findings and inferences into context, as failure to do so may result in erroneous ecological interpretation of isotopic data. It is therefore imperative to the efficient evolution of SIA, and the application to deep-sea chondrichthyans, that such challenges are clearly identified in light of the current scientific literature.

**Table 1** Total number of returns from ISI Web if Science literature search based on key words "Deep Sea ⋯" and "***" for the period 1970 to September 2016

| *** | Deep Sea "⋯" | | | |
|---|---|---|---|---|
| | Community | Ecology | Food web | Shark |
| Isotope | 410 | 159 | 181 | 18 |
| Stable isotope | 297 | 134 | 167 | 12 |
| As % of isotope | 72.4 | 84.3 | 92.3 | 66.7 |
| Gut *and or* Stomach | 156 | 129 | 73 | 24 |
| Fatty acid | 174 | 59 | 64 | 34 |
| Metal | 164 | 31 | 13 | 9 |
| Genetic | 230 | 116 | 15 | 15 |
| Biomarker | 84 | 15 | 14 | 2 |
| Tracer | 100 | 17 | 32 | 0 |

Here we discuss current methodological challenges facing the use of SIA in deep-sea chondrichthyans: specifically (1) the identification and mitigation of polar compound effects; (2) the importance of using accurate diet-tissue discrimination factors; and (3) a need to understand maternal provisioning and related effects on stable isotope values of young-of-the-year individuals.

### Lipid effects

Lipids are approximately 6–8‰ depleted in $^{13}$C relative to pure protein (DeNiro and Epstein 1977). This lipid offset appears relatively robust across a wide range of species including numerous fishes (Sweeting et al. 2006) and, while not rigorously tested, is assumed to be similar in chondrichthyans (Li et al. 2016; Carlisle et al. 2016). In many samples, lipids and proteins represent the only non-negligible biochemical tissue fractions; therefore higher lipid content drives lower $\delta^{13}C$ values. The lipid protein-offset is sufficiently large enough to create bias in $\delta^{13}C$, e.g. even at relatively low lipid content of 5%, bias would be 0.4‰, assuming 8‰ difference (Sweeting et al 2006). In deep-sea chondrichthyans, there can be systematic variability in lipid content among species or within species across tissue types (Pethybridge et al. 2010), life history stage, or body condition from physiological extremes such as reproduction or health. Lipids therefore introduce error in ecological interpretations of $\delta^{13}C$ data, including basal production sources, resource mixing, size-based feeding, or isotopic niche variation. Lipid-biased $\delta^{13}C$ may potentially mask or create differences or trends in variables of interest (e.g. depth related $\delta^{13}C$) or alter variance patterns and data distributions, affecting statistical approaches and statistical power (Galván et al. 2010).

### Urea and TMAO effects

Urea concentrations are higher in chondrichthyan tissues compared to bony fishes, having a role in facilitating osmoconformation (maintaining cellular osmotic balance with the environment) (Laxson et al. 2011). However, elevated concentrations are thought to have a deleterious effect due to protein folding and binding effects (Laxson et al. 2011). As a result, deep-sea chondrichthyans must also synthesize and retain additional osmolytes to neutralise the effects of excess urea, the most common being trimethylamine N-oxide (TMAO) (Hussey et al. 2012). Most physiologically efficient ratios of urea:TMAO are approximately 2:1 for shallower species (Laxson et al. 2011), however ratios within deep-sea chondrichthyans are depth dependent. For example, across 13 species of deep-sea chondrichthyans caught off the coast of California, urea content decreased while TMAO content increased with depth (Laxson et al. 2011). Increased TMAO could be accredited to adaptive protein stabilisation with increased hydrostatic pressure, and an inability to produce large amounts of urea at increasing depths.

Urea and TMAO concentrations are of concern in isotopic analysis because both compounds are depleted in $^{15}$N relative to proteins (Hussey et al. 2012; Carlisle et al. 2016). Like lipids, variable quantities of these compounds have the potential to

influence interpretation of trophodynamics, such as trophic level or size-based feeding. Carlisle et al. (2016) estimated urea bias could result in a trophic shift of approximately 30–50%, highlighting the importance of accounting for this compound prior to ecological interpretation. Finally, both urea and TMAO are thought to show similar depletion in $^{15}$N relative to proteins (Churchill et al. 2015b), so the precise relative concentration of urea:TMAO is less important than the overall concentrations of both compounds.

Extraction of polar compounds

The need to account for polar compound bias within deep-sea chondrichthyans has been largely underexplored. Generally for marine taxa, two main methods are broadly accepted across the literature: (1) chemical extraction and (2) mathematically-normalized correction. Here we discuss both approaches in the light of their associated advantages and caveats.

In chondrichthyans, a broad range of chemical extraction techniques have been used to remove lipids and urea/TMAO from biological tissues (Hussey et al. 2012), and can improve data comparability among deep-sea species (e.g. Reid et al. 2012, 2013). Extraction approaches have been reviewed extensively elsewhere (e.g. Sweeting et al. 2006; Post et al. 2007; Logan et al. 2008; Hoffman and Sutton 2010a, b) and more recent evaluations include data on chondrichthyans (e.g. Reum 2011; Hussey et al. 2012; Churchill et al. 2015a, b; Li et al. 2016). Lipids have been extracted from Greenland shark (*Somniosus microcephalus*) using the highly non-polar compound toluene (Fisk et al. 2002), cyclohexane extractions were applied to *Centrophorus mollucensis* and *Squalus megalops* (Le Bourg et al. 2014) and others have used chloroform:methanol solution (2:1) (Churchill et al. 2015a, b) in line with favourable methods for coastal and pelagic species (Hussey et al 2012).

Since chemical lipid extraction can result in artefacts regarding $\delta^{15}$N data (Sweeting et al. 2006 and references therein), caution is necessary when comparing data resulting from different methods of chemical extraction. Many of the polar lipid extraction techniques also result in extraction of urea and TMAO with varying degrees of efficiency (Churchill et al 2015b), but are enhanced by de-ionized water rinsing

(Li et al. 2016). Two robust studies (Li et al. 2016; Carlisle et al. 2016) both concluded that both lipid and urea/TMAO extraction may produce the most reliable isotopic signatures for chondrichthyans, through removing both lipid and urea biases and their resultant effects on both $\delta^{13}$C and $\delta^{15}$N.

$^{15}$N enrichment has been attributed to the loss of urea- and TMAO-derived nitrogen during lipid extraction on chondrichthyans, though this effect appears highly variable between species (Hussey et al. 2012). Finally, post-extraction changes in $\delta^{15}$N have not notably differed from those for teleosts, which generally do not contain large concentrations of these nitrogenous compounds. Although the need to adopt chemical extraction is often contested in the literature, obvious differences are observed between treated vs. non-treated samples. These are shown here through exploration of literature derived C:N ratios from both extracted and non-extracted tissue which, despite species-specific variability, are generally higher for extracted tissue (Table 2).

Mathematically-normalized corrections

Due to lipid extraction effects on $\delta^{15}$N and the time, cost, and labour associated with performing chemical extractions, mathematical corrections are now being widely applied to marine species (Sweeting et al. 2006; Logan et al. 2008; Hoffman and Sutton 2010a, b). When direct quantification of lipid content is unavailable for correction, tissue C:N ratio has been used as a proxy (Sweeting et al. 2006; Post et al. 2007). C:N proxies for lipid content assume that variation in C:N results from addition of lipid-derived carbon, that lipids have negligible nitrogen, and that lipid and proteins are the only non-negligible biochemical fractions. Lipid-free fish muscle tissue, assumed to be pure protein, has C:N of approximately 3.1 (by elemental %). Relating variation in C:N to change in $\delta^{13}$C upon lipid extraction has suggested C:N <3.4 are mostly composed of protein, with only a small lipid bias such that lipid extraction is not required (e.g. Post et al. 2007).

Urea and TMAO complicate C:N-based lipid correction in elasmobranchs, including deep-sea chondrichthyans. Urea and TMAO are highly nitrogenous, adding elemental nitrogen to tissue samples and thus reducing C:N. Variable concentrations of urea and/or TMAO alter C:N independently of lipid

**Table 2** Literature-derived bulk versus lipid/urea + TMAO extracted C:N ratios for common deep-sea chondrichthyans

| Common name | Latin name | C:N$_{Bulk}$ | C:N$_{Extracted}$ | Reference |
|---|---|---|---|---|
| South China catshark | *Apristurus sinensis* | 3 | – | Pethybridge et al. (2012) |
| Silky shark | *Carcharhinus falciformis* | 2.8 | 3.1 | Churchill et al. (2015b) |
| Night shark | *Carcharhinus signatus* | 2.6 | 3.1 | Churchill et al. (2015b) |
| Gulper shark | *Centrophorus cf. granulosus* | 2.8 | 3.2 | Churchill et al. (2015b) |
| Small-fin gulper | *Centrophorus moluccensis* | 2.5 | – | Le Bourg et al. (2014) |
| Taiwan gulper shark | *Centrophorus niaukang* | 2.7 | 3.2 | Churchill et al. (2015b) |
| Portugese dogfish | *Centroscymnus coelopsis* | 3 | – | Pethybridge et al. (2012) |
| Roughskin dogfish | *Centroscymnus owstoni* | 3 | – | Pethybridge et al. (2012) |
| Longnose velvet dogfish | *Centroselachus crepidater* | 2.9 | – | Pethybridge et al. (2012) |
| Carpenter's chimaera | *Chimaera lignaria* | 2.7 | – | Pethybridge et al. (2012) |
| Kitefin shark | *Dalatias licha* | <3.5 | – | Navarro et al. (2014) |
| Kitefin shark | *Dalatias licha* | 2.9 | – | Pethybridge et al. (2012) |
| Birdbeak dogfish | *Deania calcea* | 2.9 | – | Pethybridge et al. (2012) |
| New Zealand lantern shark | *Etmopterus baxteri* | 2.9 | – | Pethybridge et al. (2012) |
| Blurred lantern shark | *Etmopterus bigelowi* | 2.8 | 3.2 | Churchill et al. (2015b) |
| Tiger shark | *Galeocerdo cuvier* | 2.8 | 3.1 | Churchill et al. (2015b) |
| Blackmount catshark | *Galeus melastomus* | 2.7 | – | Cresson et al. (2014) |
| Bluntnose sixgill shark | *Hexanchus griseus* | 5.1 | 3.2 | Churchill et al. (2015b) |
| Dusky smooth-hound | *Mustelus canis* | 2.7 | 3.1 | Churchill et al. (2015b) |
| Plunket's shark | *Proscymnodoms plunketi* | 2.9 | – | Pethybridge et al. (2012) |
| Pacific spookfish | *Rhinochimaera pacifica* | 3 | – | Pethybridge et al. (2012) |
| Small spotted catshark | *Scyliorhinus canicula* | 2.7 | – | Cresson et al. (2014) |
| Shortspine spurdog | *Squalus cf. mitsukurii* | 2.7 | 3.1 | Churchill et al. (2015b) |
| Cuban dogfish | *Squalus cubensis* | 2.6 | 3.2 | Churchill et al. (2015b) |
| Shortnose spurdog | *Squalus megalops* | 2.8 | – | Le Bourg et al. (2014) |
| | Average (S.D.) | 2.9 (0.5) | 3.2 (0.1) | |

content, invalidating assumptions of C:N as a diagnostic for lipid extraction. Similarly, other influences on tissue C:N invalidate the underpinning assumptions of current empirical or arithmetic lipid corrections used in Post et al. (2007) and Sweeting et al. (2006) that use C:N as a proxy for lipid content. C:N and %N have also been suggested for urea correction methods. The viability of urea correction models has yet to be robustly analysed; recent studies suggest that mathematical corrections cannot be applied homogenously across species. Churchill et al. (2015b) compared chemical-based chloroform:methanol extractions with a numeric lipid correction on $\delta^{15}$N for 10 species of deep-sea elasmobranchs from the Gulf of Mexico. Increases in $\delta^{15}$N were observed in tissue subject to a 2:1 chloroform:methanol extraction, highlighting the potential $\delta^{15}$N depletion in bulk tissue as a combined result of urea and TMAO. Furthermore, mathematical corrections were successfully applied to only two species, *Centrophorus* cf. *granulosus* (=*Centrophorus uyato*) and *Squalus* cf. *mistukurii*. This suggests that although corrections may be derived for some species, chemical extraction may provide greater comparability of isotope values between species with varying concentrations of lipids, urea, and TMAO in their tissues. Although species-specific mathematical corrections are likely to be generated through broader studies with increased sample sizes, and may reduce cost through simplistic sample preparation, the ambiguity in performing corrections across many species suggests a normalised chemical extraction will account for cumulative effects of lipid, urea and

TMAO, and provide increased uniformity within datasets.

## Applying accurate diet-tissue discrimination factors (DTDFs)

Diet-tissue discrimination factors (DTDFs) can be defined as the degree to which isotope values are enriched in a predator relative to its prey (DeNiro and Epstein [1978], [1981]). Such metrics are particularly useful for dietary analysis, such as the application of Bayesian mixing models when discriminating between prey-types, and inferences from such methods appear highly sensitive to variable DTDFs (Bond and Diamond [2011]; Olin et al. [2013]). For some chondrichthyans, DTDFs have been calculated through controlled feeding studies, whereby animals are fed a single prey source for an extended period of time, until isotope values plateau (Hussey et al. [2010]; Kim et al. [2012a], [b]). However, the difficulty associated with keeping chondrichthyans in captivity for long timeframes results in a limited number of DTDFs generated across the literature. Current evidence suggests that the general discrimination (3.4‰ for $\delta^{15}$N and 1.0‰ $\delta^{13}$C) proposed by DeNiro and Epstein ([1978], [1981]) does not provide accurate measures of DTDFs for chondrichthyans, which show high inter-taxon and diet-based variability (Hussey et al. [2012]). Kim et al. ([2012a], [b]) exemplified the complexity in deriving accurate DTDF estimates in leopard sharks (*Triakis semifasciata*) and highlighted factors such as prey species, prey tissue-type, consumer tissue-type, and natural conspecific variation can all influence DTDF values. This suggests a precautionary approach when applying such values in food-web studies, and multiple DTDFs should be explored prior to ecological interpretation.

Despite variability in DTDFs for chondrichthyans, the relatively low fractionation of $\delta^{13}$C observed across a range of species suggests an estimated $\delta^{13}$C fractionation value can tentatively be applied to deep-sea taxa. Although $\delta^{13}$C discrimination has not been specifically quantified for deep-sea chondrichthyans, a relatively small range of values has been applied to coastal species (see review by Hussey et al. [2012]). The generally higher trophic discrimination of $\delta^{15}$N can present larger error, especially regarding scaled discrimination, an important consideration for higher predators such as large chondrichthyans (Hussey et al. [2014]). Discriminative scaling was first described by Robbins et al. ([2005]), who suggested that greater $\delta^{15}$N discrimination factors (>3.0‰) are associated with lower trophic-level species, and are significantly lower (<3.0‰) in higher trophic level species. This is attributed to the greater dietary protein quality of higher trophic level predators, and has been illustrated in a number of chondrichthyans (see Olin et al. [2013]; Hussey et al. [2014]). It is therefore particularly difficult to apply accurate $\delta^{15}$N discrimination factors in any predatory fish species, which feed opportunistically on multiple prey items (Dunn et al. [2010]), across multiple trophic levels, and thus ingest variable protein content and quality. Discrimination is therefore likely to be variable, and may be influenced by environment, prey abundance, and prey quality. Extended study into the feeding ecology of deep-sea elasmobranchs through direct diet analyses could link stable isotope data to direct diet information, elucidate species-specific feeding strategies, and directly assess protein quality of different prey types.

Variability of species-specific metabolic rates may also reduce the reliability of literature-derived discrimination factors (Hussey et al. [2012]), particularly due to metabolic attributes of deep-sea chondrichthyans. Deep-sea fishes generally have lower metabolic rates than their coastal counterparts (Drazen and Seibel [2007]; McClain et al. [2012]), and there are few direct data to describe discrimination factors between different tissues in such fishes. Furthermore, the variability in metabolic rates between different tissues, e.g. white muscle (slower) vs. blood (faster), may also yield variable discrimination factors (Malpica-Cruz et al. [2012]), which are yet to be accounted for in the scientific literature. As a result, caution should be taken when applying metrics used to infer trophic discrimination, such as trophic level and food web linkages. In SIA studies of deep-sea chondrichthyans, we suggest a thorough and conservative approach in which multiple discrimination factors are applied, providing a range of potential outcomes from which probable scenarios can be derived and considered.

## Recognising mother-embryo isotope bias

A high variability of matrotrophy (maternal provisioning) is observed within chondrichthyans, with at least ten reproductive modes described, which range between strictly lecithotrophic (e.g. oviparous) and

extremely matrotrophic (e.g. placental and trophone-matic) (García et al. 2008). Very limited matrotrophy is exhibited across deep-sea chondrichthyan taxa (e.g. Cotton et al. 2015), and an extremely low number of species are highly matrotrophic. Several studies have used SIA to better understand the ecology of juvenile chondrichthyans, including young-of-the-year. It is a common assumption that isotope values of juveniles represent individual foraging history, which disregards isotopic effects of maternal provisioning (Le Bourg et al. 2014). This is particularly important for tissue signatures representative of long timeframes (months to years), such as white muscle (Hussey et al. 2012). For coastal chondrichthyans acquiring nutrients from an external yolk sac before receiving maternal nutrient contribution, isotope values are generally higher in embryos than in the mother (McMeans et al. 2009). This was observed in the Atlantic sharpnose shark (*Rhizopriondon terraeno-vae*) for $\delta^{15}N$ of liver, white muscle, and cartilage, and for $\delta^{13}C$ of muscle and liver (McMeans et al. 2009). Similar results were also observed for $\delta^{15}N$ and $\delta^{13}C$ in white muscle tissue of the scalloped hammerhead shark (*Sphyrna lewini*), and for $\delta^{15}N$ in blacktip shark (*Carcharhinus limbatus*) (Vaudo et al. 2010). It is noteworthy that placentotrophy is rare in the deep sea, only occurring in a few species of carcharhinid and triakid sharks that make marginal use of deep-sea habitats. Mother-embryo isotope discrimination data exist for only two species of lecithotrophic (yolk-sac live bearing) sharks: the shortnose spurdog (*Squalus megalops*) and smallfin gulper shark (*Centrophorus moluccensis*) (Le Bourg et al. 2014). Similar or lower isotopic values were observed in muscle tissue of embryos for both species, and liver tissue of the shortnose spurdog compared to the mother (Le Bourg et al. 2014).

Although deep-sea chondrichthyans employ a restricted range of reproductive modes dominated by lecithotrophy, the effects of maternal provisioning should be established prior to interpretation of isotope values of young-of-the-year individuals. Development of age and growth parameters would improve age assessment of field-sampled juveniles, and analysis of shorter-term tissues (e.g. liver) would complement longer-term tissue (e.g. muscle), ensuring correct time-integrated assessment of diet. Where such data are absent, extremes of bias from material influence can be bounded by considering dilution of maternal isotopic influences through growth of new tissue in the offspring. Tissue turnover will further reduce maternal bias (Hesslein et al. 1993) but is difficult to assess in such species.

## Ecological Inferences

Stable isotope approaches have begun to provide critical insights into the ecology of deep-sea chondrichthyans, at both intra- and inter-species levels. Here we broadly categorise and summarise current findings across this limited but growing field of study.

Sources of primary production for deep-sea chondrichthyans

Deep-sea primary production is largely attributed to pelagic phytoplankton in the form of 'marine snow' (Polunin et al. 2001). Churchill et al. (2015a) associated $\delta^{13}C$ values (−17 to −15.1‰) derived from muscle tissue of deep-sea elasmobranchs in the Gulf of Mexico to reflect a large reliance on surface phytoplankton production, as opposed to alternate sources such as chemosynthetic (−65 to −25‰; McAvoy et al. 2008) or terrestrial production (−23.8 to −26.8‰; Wang et al. 2004). A limited number of studies have begun exploring how exposure to multiple sources of primary production may influence the overall biomass of deep-sea chondrichthyans. For productive hydrothermal vent and cold seep communities, production largely originates from geothermal based sources, which have the ability to influence resource partitioning within deep-sea chondrichthyans (Newman et al. 2011). Newman et al. (2011) observed an influence of both geothermal and pelagic production on isotope values of the great lanternshark (*Etmopterus princeps*), highlighting use of multiple resource pathways. In the Exuma Sound deep-sea slope community, isotope values of upper-slope chondrichthyans reflected reliance upon a mix of potential production sources, which may derive from both open-ocean and coastal resource pools, whereas deeper mid-slope species were highly dependent on open-ocean sources (phytoplankton and sargassum) (Shipley et al. 2017b). Assessment of energy flow to and from deep-sea communities is a rich area for future work, and isotopic tracers provide a diagnostic tool for tracking productivity sources.

Size-based enrichment

Isotopic enrichment with increasing body size has been documented for a wide range of marine fishes for both $\delta^{15}N$ and $\delta^{13}C$ (Jennings 2005). Such relationships appear variable across deep-sea chondrichthyans, with only some species showing significant size-dependent shifts (Churchill et al. 2015a). In theory, a greater body size increases the capacity to ingest larger prey items, thus increasing the ingestion of prey that are more enriched in $^{15}N$ (Hussey et al. 2011). Churchill et al. (2015a) have provided the only current species-specific calculations between body size (pre-caudal length) and $\delta^{15}N$ in deep-sea sharks. For eight species captured in the Gulf of Mexico, significant size-dependent enrichment in $\delta^{13}C$ and $\delta^{15}N$ were found in one and four species, respectively. This was attributed to opportunistic scavenging in largely oligotrophic deep-sea ecosystems, in which prey sources are less reliable than in coastal or pelagic systems. However, intra-specific trends with size have been observed in both Cuban dogfish (*Squalus cubensis*) and shortspine spurdog, which appear to feed at higher trophic levels as body size increases. A negative relationship between body size and $\delta^{15}N$ was observed for the dusky smoothhound (*Mustelus canis*), a crab specialist, suggesting possible ontogenetic diet switches to lower trophic level prey resources. Sampling a greater volume of species, from regions of relatively high productivity such as deep-sea canyons (De Leo et al. 2010), insular slopes (Shipley et al. 2017b), and terrestrial outfalls (Churchill et al. 2015a) may provide greater insight into potential size-based isotopic enrichment in deep-sea chondrichthyans.

Defining isotopic shifts associated with size-based feeding in shallow water fishes has been hampered by limited statistical power and lack of *a priori* definitions of meaningful effect sizes (Galván et al. 2010). In deep-sea chondrichthyans, such challenges are exaggerated, where the logistics of sampling mobile and dispersed species with life-history dependent depth patterns makes reaching adequate sample sizes and size ranges difficult. Lack of significance due to power limitations appears likely in some of the above cases, suggesting that size-based enrichment could be more common than has been reported conclusively.

Effects of depth on isotope values

Mesopelagic communities often perform largescale diel vertical migrations through the water column, facilitating connectivity between epipelagic and deep benthic or bathyal communities (Trueman et al. 2014). Connectivity is also governed by the transport of nutrients from surface waters through physical vectors such as ocean currents, whereby proximity to additional sources of primary production are likely to influence isotope values of deep-water species (Shipley et al. 2017b). Churchill et al. (2015a) found significant relationships between depth and $\delta^{13}C$ for the Cuban dogfish, shortspine spurdog and little gulper shark, attributed to changing sources of food web production or dietary switches to specific prey items. Current observations suggest that species occupying greater depths generally have higher $\delta^{15}N$. Depth effects on $\delta^{13}C$ seem to be variable, which could be associated with different sources of primary production between distinct depth thresholds. However, the relative lack of data from chondrichthyans across depth gradients warrants further examination as to the effects of depth on isotope values.

Trophic level and niche width

Estimates of trophic level and isotopic niche width can provide indications of trophic overlap and associated foraging competition, both between and within species. Although determining trophic level allows limited ecological conclusions to be drawn, coupling both $\delta^{15}N$ and $\delta^{13}C$ through methods such as Bayesian ellipses (see Jackson et al. 2011) has provided a valuable indication of niche width, thus displaying a quantitative measure of unique niche space occupied by marine species. In recent years, such metrics have been applied to deep-sea chondrichthyans and have provided a relative measure of dietary overlap, and hence the degree of foraging competition, displayed between species.

Deep-sea chondrichthyans have been observed to occupy similar trophic levels to pelagic and coastal species (e.g. $\sim 3.5$–4.3, Musick and Cotton 2015, or 3.4–4.7, Pethybridge et al. 2012). In theory, low productivity food webs could result in high niche overlap between higher predators, as competition for resources is increased by prey limitation. However, for

the limited number of deep-sea chondrichthyan studies identifying niche width, the amount of unique niche space occupied by a species appears highly variable, both at intra- and inter-species levels. A number of methods to determine niche width have been proposed across the literature. Most derive from Hutchinson's hypervolume theory (for full description see Hutchinson 1978), and stable isotopes have been employed directly into niche metrics (see Layman et al. 2007; Jackson et al. 2011). Use of such approaches is limited for deep-sea chondrichthyans, but they may help delineate important unique trophic interactions exhibited within deep-sea food webs. Churchill et al. (2015a) highlighted a relatively large (>50%) percentage of unique niche space occupied by bluntnose sixgill sharks (*Hexanchus griseus*) (75.9%), gulper sharks (*Centrophorus* cf. *granulosus* (=*C. uyato*) and *Centrophorus* cf. *niaukang* (=*C. granulosus*)) (52.3 and 69.5%), and Cuban dogfish (*Squalus cubensis*) (52.1%). This could be attributed to the large vertical movements associated with some of these taxa (Rodríguez-Cabello et al. 2016; Comfort and Weng 2015; Nakamura et al. 2015; Shipley et al. 2017a), increasing the chance of encountering a broader range of prey items. Ontogenetic variability in trophic niche width has also been observed. Pethybridge et al. (2012) observed significant unique niche space between juvenile and adult New Zealand lantern sharks (*Etmopterus baxterii*) and the longnose velvet dogfish (*Centroselachus crepidater*). It is therefore likely similar patterns can be attributed to other deep-sea species; however this requires and warrants further study.

## Future directions

Despite the associated caveats, it is clear that stable isotope approaches are extremely useful for examining aspects of chondrichthyan ecology. However, a critical need exists to refine both methodological protocols and ecological interpretation of stable isotope data to continue propelling this rapidly growing field forward. Here we provide a suite of recommendations to help facilitate the evolution of future deep-sea chondrichthyan stable isotope studies.

### Multi-tissue approaches

Tissue-specific turnover rates have been acknowledged and utilised by isotope ecologists to provide increasing temporal resolution to food-web studies, by comparing isotope values from tissues with slow, longer term (e.g. white muscle) and fast, shorter term (e.g. liver or blood) turnover rates (Pinnegar and Polunin 1999; Madigan et al. 2015). Such analyses not only provide an insight into broad-scale movements (Hussey et al. 2011), but also highlight generalist vs. specialist feeding strategies (MacNeil et al. 2005). For deep-sea species, movement data are available for few species, therefore such approaches could provide a evidence of broad-scale movement patterns, as well as provide novel insights into potentially unique foraging strategies.

The isotopic distinction associated with marine isoscapes is increasingly used to help determine the movement of animals across broad spatial scales (Graham et al. 2010). As both carbon and nitrogen baselines vary based on a multitude of environmental factors, this often allows ecologists to determine potential migratory corridors of marine species. This approach therefore holds great potential if applied to more elusive deep-sea species, where conventional broad-scale tracking approaches, such as satellite telemetry, fail to offer insight due to the requirement of ambient light readings to triangulate geolocation estimates (Hammerschlag et al. 2011; Shipley et al. 2017a). Although environmental conditions associated with the deep-sea e.g. light and temperature, which are similar from region to region, the unique mix and isotopic signature of primary production sources may allow for distinction between unique bioregions, which is likely to vary across latitudes. Secondly, some deep-sea species perform large diel vertical migrations throughout the water column (Grubbs and Kraus 2010; Comfort and Weng 2015; Rodríguez-Cabello et al. 2016; Shipley et al. 2017a), which may increase the isotopic reciprocation of latitude-specific signatures of $\delta^{13}$C as such movements are likely to pertain to foraging. If broad-scale movements do exist, seasonal sampling of individuals using both fast and slow turnover tissue is likely to indicate the potential scale, as well as the potential migratory corridors utilised by deep-water chondrichthyan species.

Tissue-specific differences in isotopic turnover rates allow for higher resolution evaluation of diet in coastal and pelagic chondrichthyans, and can demonstrate diet-switching which may be an artefact of seasonality (MacNeil et al. 2005) or foraging mode (i.e. generalist vs. specialist foraging) (Matich et al. 2011). These facets have been exemplified in coastal chondrichthyans; for example Matich et al. (2011) analysed $\delta^{13}C$ and $\delta^{15}N$ of blood, plasma, white muscle, and fin tissue in tiger sharks (*Galeocerdo cuvier*) and bull sharks (*Carcharhinus leucas*), and suggested tiger sharks exhibit a highly generalist feeding strategy compared to more specialized feeding exhibited by bull sharks. Similarly, such approaches have been applied to pelagic sharks to explore potential diet-shifts in relation to known seasonal migrations. Madigan et al. (2015) analysed both slow turnover (white muscle) and fast turnover (blood plasma) tissues in oceanic white tip sharks (*Carcharhinus longimanus*) captured from Cat Island, The Bahamas, which exhibited diet shifts from large teleosts to mixed squid and teleost diets in association with known seasonal migrations (Howey-Jordan et al. 2013). Although similar approaches theoretically could be applied to deep-sea species to assess temporal variation of feeding, the lack of described turnover rates for chondrichthyans may preclude the use of such applications confidently. Therefore, direct seasonal sampling of diet coupled with an isotopic multi-tissue approach could elucidate more complex seasonal-based foraging, foraging strategy, and potentially allow quantification of tissue-specific isotopic turnover estimates.

## A call for greater sampling effort

Sample sizes for deep-sea chondrichthyan isotope studies are generally lower than studies of other marine fishes (e.g. Polunin et al. 2001; Jennings 2005; Valls et al. 2014; Reid et al. 2013). This can be mainly attributed to the intrinsic difficulty of robustly sampling deep-sea ecosystems (Sweeting et al. 2006), as the price of bulk analyses has become relatively low-cost (ca. $20 per sample, C. Sweeting pers. comms.). Future studies should increase sampling effort to generate the diversity and size of samples needed for robust and ecologically representative analyses. Currently, the ability to acquire large sample sizes is compromised by the difficulty and cost associated with sampling deep-sea regions, and this is likely to remain unchanged in the near future. Creative and opportunistic approaches such as sampling from fisheries landings and/or utilising more proximate deep-sea systems (e.g., steep island slopes) may help increase sample size in selected regions.

## Applying Bayesian mixing models

Bayesian mixing models have been used to quantitatively estimate the contribution of multiple prey types to the diet of predators (Boecklen et al. 2011; Phillips 2012; Madigan et al. 2015). Furthermore, Bayesian approaches have also been used to delineate potential sources of primary production by sampling isotopically distinct end-members (high trophic level organisms displaying high site fidelity), and thus highlight the importance of multiple basal production sources to highly mobile chondrichthyans (McCauley et al. 2012; Papastamatiou et al. 2015). Such methods have been applied to a number of coastal and pelagic species such as the grey reef shark (*Carcharhinus amblyrhynchos*), blacktip reef shark (*C. melanopterus*) (McCauley et al. 2012), and oceanic whitetip shark (Madigan et al. 2015) to illustrate how highly mobile sharks can be reliant upon a number of prey resource pools. Interestingly, the broad-scale vertical movements of deep-water chondrichthyans suggests some species may interact significantly with multiple prey resource pools (e.g. epipelagic vs. mesopelagic), therefore Bayesian approaches may provide an important insight into the degree to which deep-water species interact, and therefore potentially connect such habitats. This scope for future study is paramount to more holistic management approaches, and is therefore of conservation interest to managers.

Despite the growing popularity of Bayesian mixing models in recent years, a number of drawbacks have been identified. Interpretation of model outputs must therefore be viewed with caution, especially when used in the absence of complementary stomach content data. One major concern is the sensitivity of Bayesian mixing models to variable trophic discrimination factors, which are currently unknown for most chondrichthyan species. Model sensitivity was first demonstrated with seabird data, which assessed the impact of varying trophic discrimination factors on the estimated contribution of prey items to the diet of common terns (*Sterna hirundo*) and balaeric shearwaters (*Puffinus mauretanicus*) (Bond and Diamond

2011). Model outputs appeared highly sensitive to small changes to trophic discrimination factors, which precluded a reliable interpretation of predator diet. Although these studies do not relate directly to deep-sea chondrichthyans, this general uncertainty precludes the reliable use of such models currently, and such approaches have not yet been used for deep-water species. Directly investigating trophic discrimination in deep-sea species may encourage the wider use of Bayesian mixing models in deep-sea chondrichthyans and improve model efficacy.

Compound-specific stable isotope analysis

Compound specific stable isotopes offer a time-integrated reconstruction of diet through tracing the trophic transfer of individual amino acids (Popp et al. 2007; Bowes and Thorp 2015), and were initially used to examine biogechemical processes (Hayes et al. 1990) and organic contaminants and pollutants entering the environment (Hunkeler et al. 1999; Elsner et al. 2012). In ecology, this approach offers a finer scale resolution of diet compared to bulk tissue through deconstructing individual amino acid signatures, and is therefore becoming utilised more readily across the scientific literature (Popp et al. 2007, Comfort 2012). In amino acid-specific analyses, higher trophic discrimination is observed in 'trophic' amino acids (e.g. glutamic acid), while negligible shifts are observed in other 'source' amino acids (e.g. phenylalanine) (McClelland and Montoya 2002; Popp et al. 2007). Larger trophic discrimination has the potential to resolve trophic position better than bulk SIA, partially by removing the confounding influence of varying ratios of trophic versus source amino acids in predator tissue. Non-fractionating source amino acids provide internal basal nitrogen reference, particularly useful in deep-sea ecology where logistical constraints on sampling inhibit extensive temporal and spatial exploration of basal species, and where sources of nitrogen are often not readily identifiable (Comfort 2012). These approaches have become particularly useful when differentiating between basal production sources contributing to the biomass of a consumer (Fantle et al. 1999), and as a result can complement multisource analyses such as Bayesian mixing models (e.g. Parnell et al. 2010).

Thus far, compound specific stable isotope analysis of deep-sea chondrichthyans is scarce across the literature. Comfort (2012) used compound specific stable isotope analysis of eight amino acids to assess the trophic level and feeding dynamics of blunt nose sixgill sharks in relation to their specific capture locale (Puget Sound vs. Hawaii). The authors highlighted the limitations of bulk tissue $\delta^{15}N$ analysis, which did not illustrate strong nitrogen enrichment through ontogeny compared to compound specific analysis. Finally, the use of compound specific $\delta^{15}N$ highlighted significant differences in the extent of ontogenetic size-based enrichment between locales, which appeared greater in Hawaii than in Puget Sound. Therefore this method could provide vital insights into the variability in predator trophic levels, within and between species, in addition to estimates of offshore transport of primary production, which may influence overall deep-sea productivity.

Sulfur isotopes ($^{34}S$)

Sulfur isotopes have been generally under-utilised in deep-sea chondrichthyan studies, but have been particularly useful in other locales to distinguish between pelagic and chemoautotrophic production systems. Such analyses, complemented by $\delta^{13}C$, can provide insight into the primary production sources, which support chondrichthyan assemblages. Similar to $\delta^{13}C$, $\delta^{34}S$ shows minimal trophic discrimination (<1‰) (Reid et al. 2013) and therefore can reliably distinguish benthic, pelagic, freshwater alluvial, and shallow water production due to isotopically distinct $\delta^{34}S$ baselines between these systems. The use of sulfur isotopes becomes extremely useful in habitats where fauna reside within proximity to chemoautotrophic primary production, such as hydrothermal vent communities (Fry et al. 1983). Reid et al. (2013) applied $\delta^{13}C$ and $\delta^{34}S$ to fauna on the Charlie Gibbs fracture zone, outlining the importance of pelagic primary production in areas where communities may be exposed to both pelagic and hydrothermal vent-based sources of production. Use of $\delta^{34}S$ in future deep-sea chondrichthyan food web studies is recommended to help identify sources of production which concurrently influence basal energy flow.

Mercury isotopes ($^{199/202}HG$)

Stable isotopes of mercury (Hg) have recently emerged as potential tracers of nutrient flow to marine

predators (Bergquist and Blum 2007). Hg isotopes display both mass-dependent fractionation (MDF) and mass-independent fractionation (MIF), usually represented by $\delta^{202}$Hg and $\Delta^{199}$Hg, respectively (Bergquist and Blum 2007). While both fractionation pathways are influenced by multiple processes, these have been linked to microbial methylation and photochemical reactions in the pelagic environment (Blum et al. 2013). Thus $\delta^{202}$Hg can be linked to microbial methylation and demethylation in benthic or mesopelagic waters, while $\Delta^{199}$Hg is linked to photochemical reduction in well-lit waters (Bergquist and Blum 2007; Blum et al. 2013). As a result, Hg isotopes can provide insight into foraging depth (Blum et al. 2013), feeding guilds (Yin et al. 2016), and coastal versus offshore nutrient inputs (Senn et al. 2010). As tracers of multiple biogeochemical processes, Hg isotopes can provide valuable complementary information to $\delta^{13}$C, $\delta^{15}$N, and $\delta^{34}$S isotope approaches, and have been successfully applied to wild (Blum et al. 2013; Kwon et al. 2014; Balogh et al. 2015; Yin et al. 2016) and captive (Kwon et al. 2013, 2016) fish. Hg isotopes may be particularly useful in ascertaining production sources to chondrichthyans in deep-sea systems that typically have multiple productivity pathways, and may forage over large vertical distances (Comfort and Weng 2015; Nakamura et al. 2015).

## Conclusions

Despite the growing popularity and usefulness of isotope-based approaches in deep-sea chondrichthyan studies, significant methodological challenges exist, many of which are in agreement with those already identified for coastal and pelagic species. SIA approaches would therefore benefit greatly from determination of accurate discrimination factors and tissue turnover rates for a wider number of species. Identifying these key parameters would provide greater confidence and justification for the use of Bayesian mixing models, as well as allowing for improved determination of trophic metrics such as trophic level and niche width. Despite these limitations, SIA has provided a valuable first step into outlining basic food web structure, species interactions, and identifying the potential sources of energy underpinning deep-sea chondrichthyan food webs. The advancement of emerging tracers, such as mercury isotopes and compound-specific isotopic analyses, hold great promise for the field of SIA in general. When applied directly to deep-sea chondrichthyans, this suite of tools will provide previously unattainable, novel insights into aspects of their biology and ecology.

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