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A multi-tissue approach to assess the effects of lipid extraction on the isotopic composition of deep-sea fauna

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ABSTRACT

Stable isotope analysis (SIA) of carbon and nitrogen is now a common tool to investigate trophic relationships and food-web structure in aquatic ecosystems. However, species-specific and tissue-specific lipid content sometimes hinders the correct interpretation of $\delta^{13}C$ and $\delta^{15}N$ values because lipids are ^{13}C-depleted with respect to proteins, and thus tissue lipid extraction is generally invoked. We assessed the effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ compositions and C:N ratios of muscle, liver (or hepatopancreas) and gonads of three common Mediterranean deep-sea species with different locomotory activity, buoyancy mechanisms and feeding modes: the benthic-feeder shrimp *Aristeus antennatus*, the nektobenthic-feeder shark *Galeus melastomus* and the pelagic-feeder fish *Micromesistius poutassou* to evaluate both the effect of lipid extraction on SIA data and the validity of $\delta^{13}C$ lipid correction models. Results showed that the effect of lipid extraction is not unique but some common patterns can be identified. Lipid extraction resulted in increased $\delta^{15}N$ values in the liver and the muscle and in decreased $\delta^{15}N$ values in the gonads, in increased $\delta^{13}C$ values in all the tissues of the three species, except in the hepatopancreas of *A. antennatus*, and in decreased C:N ratios. The magnitude of the changes was species- and tissue-specific. We assessed the validity of $\delta^{13}C_{bulk}$ correction equations for lipid content in muscle and liver tissues available from literature in the species from this study. Such equations provided corrected $\delta^{13}C$ values equivalent to those obtained through lipid extraction when applied to a species with similar characteristics (i.e., taxon, behavior, etc.) to those for which the equations were designed. Our results for muscle tissue showed that not one of the equations tested was valid for the deep-sea shark *G. melastomus*, and we propose a species-specific model with a fairly feasible model efficiency. Besides, tissue-specific equations for liver in *G. melastomus* provided non-significant differences between $\delta^{13}C_{corrected}$ and $\delta^{13}C_{lipid\ free}$ values, but all model efficiencies were fairly low. Thus, lipid extraction trials on elasmobranch muscle and liver tissue to determine effects on $\delta^{13}C$ and $\delta^{15}N$ values on a species-by-species basis are recommended. Our research together with a comprehensive literature review on this topic, highlights that there is no accepted or mandated standard of treatment for lipids when using stable isotope analyses.

1. Introduction

In the last decades stable isotopes of carbon and nitrogen have been demonstrated to be promising trophic tracers in different research fields. They are frequently used to describe the origins and fate of organic matter in food webs or to trace migrations of both aquatic and terrestrial vertebrates, including human populations (Fry, 2006; Michener and Latjha, 2007 and references cited therein). The $\delta^{13}C$ and $\delta^{15}N$ ratios provide a time-integrating and average estimate of an organism's assimilated diet. Specifically, in aquatic food-web studies the $\delta^{13}C$ value discriminates between terrestrial vs. marine food sources (Hobson, 1987), and between different types of marine primary food sources (e.g., macroalgae, seagrasses or phytoplankton), as well as between the pelagic vs. benthic origin of food (France, 1995). Animal $\delta^{13}C$ is typically enriched by 1.1‰ relative to its diet (e.g., De Niro and Epstein, 1978; Michener and Latjha, 2007). On the other hand, the $\delta^{15}N$ signature can be used to define the trophic level of organisms, since $\delta^{15}N$ usually increases ca. 2.5 to 3.4‰ from food to consumer (Minagawa and Wada, 1984; Post, 2002).

The low cost and/or time consumption of stable isotope analysis in front of other techniques (e.g., gut content analysis, analysis of fatty acid markers) has spread the interest in applying stable isotope analysis

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(SIA) to food-web studies. However, different problems and limitations of stable isotope determination and interpretation have been highlighted in parallel to their more popular use. Those are linked to sample storage (type of preservatives and duration of preservation: Sarakinos et al., 2002; Fanelli et al., 2010; Rennie et al., 2012 and references cited therein), and to the content of inorganic carbonates (Carabel et al., 2006; Mateo et al., 2008; Mazumder et al., 2010; Pomerlau et al., 2014 and references cited therein) and lipids (Sweeting et al., 2006; Kiljunen et al., 2006; Bodin et al., 2007; Hoffman and Sutton, 2010) in tissues. Lipids are depleted in ^{13}C with respect to carbohydrates and proteins (De Niro and Epstein, 1977; Griffiths, 1991) and thus tissue lipid content can strongly influence its $\delta^{13}C$ (Lorrain et al., 2002) and may result in misleading interpretations of food-web structure. Lipid extraction is a useful alternative to avoid lipid effects on isotopic signatures. Yet, different studies have already evidenced effects of lipid removal on $\delta^{13}C$ (Focken and Becker, 1998; Schlechtriem et al., 2003), and such effects on $\delta^{15}N$ and C:N ratios are still unclear (Pinnegar and Polunin, 1999; Sotiropoulos et al., 2004; Sweeting et al., 2006; Bodin et al., 2007). This is especially true for deep-sea species, which still have large amounts of lipids in muscle, liver and gonads (Rosa and Nunes, 2003a, 2003b; Drazen, 2002; Drazen and Seibel, 2007) despite the general pattern of total-lipid content decrease with depth described in some studies (Bailey and Robison, 1986; Childress et al., 1990). As lipid extraction may alter $\delta^{15}N$ through the loss of some non-lipid compounds (Pinnegar and Polunin, 1999; Murry et al., 2006), separate analysis for $\delta^{13}C$ and $\delta^{15}N$ are required after lipid extraction. To avoid such sample duplication, mass balance (Fry et al., 2003; Logan et al., 2008; Hoffman and Sutton, 2010), lipid correction (Lesage et al., 2010), empirical relationships (Lorrain et al., 2002; Schmidt et al., 2003) and lipid normalization using C:N (Post et al., 2007, Bodin et al., 2007, Hoffmann et al., 2015 among others) models for correcting $\delta^{13}C_{bulk}$ values for lipid content have been proposed. Briefly, mass balance models require data on the isotopic composition of lipids and the C:N of pure protein, which is assumed to be a constant (3.7 according to Fry et al., 2003); lipid correction only requires lipid content data, but assumes lipids are on average 6‰-depleted in ^{13}C relative to protein and that the isotopic signature of the lipid-extracted tissue is the same as that for pure protein (Sweeting et al., 2006). Both empirical relationships and lipid normalization use an empirical relationship between C:N and lipid content, without any assumptions on isotopic values. Empirical relationships are commonly determined from one species and tissue type and are therefore case-specific. Instead, lipid normalization models estimate such relationship among many species and tissues, providing models applicable to an array of taxa or tissues.

In this context, we analyzed the effects of lipid extraction on carbon and nitrogen stable isotope signatures of three deep-sea megafaunal species with different locomotory activity, buoyancy mechanisms and feeding modes, factors responsible for interspecies variations in tissue lipid content (Phleger et al., 1998; Rosa and Nunes, 2003b; Drazen and Seibel, 2007; Pethybridge et al., 2010). The species selected were the red shrimp *Aristeus antennatus*, the blackmouth catshark *Galeus melastomus* and the blue whiting *Micromesistius poutassou*, all dominant in the Mediterranean slopes and also common in the eastern Atlantic waters. *Aristeus antennatus* is a benthic feeder (Cartes et al., 2008) with adults strongly linked to the substrate (Cartes and Sardà, 1989), *G. melastomus* is a nektobenthic feeder (Fanelli et al., 2009) showing bottom-associated semi-active foraging behavior (Uiblein et al., 2003; Cresson et al., 2014), and *M. poutassou* is a macroplankton feeder (Papiol et al., 2014; Cresson et al., 2014), highly mobile and undertaking both vertical migration and large horizontal displacements (Hátún et al., 2007).

Besides interspecific variability, lipid content may also vary within a species' tissues. Also, the levels of lipid in the different tissues vary within and among species in space and time (Pickett and Pawson, 1994; Rosa and Nunes, 2003a, 2003b). We therefore chose to analyze the effects of lipid content in three different tissues, the gonads, liver and muscle, that act as lipid storage reserves to a different extent. Muscle is the tissue most commonly used in studies of aquatic trophic ecology (Boecklen et al., 2011) because its low lipid content and ease of homogenization. Yet, other tissues are sometimes used because tissue-specific turnover rates and metabolic pathways (Tieszen et al., 1983; Lorrain et al., 2002) influence isotopic signatures and their analysis may aid in assessing nutrient allocation and nutritional status (Jardine et al., 2005) or can provide dietary information integrated over different time scales (Hesslein et al., 1993; Lorrain et al., 2002; Watanabe et al., 2005).

Thus the objectives of this work were 1) to assess the effects of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ compositions of muscle, liver (or hepatopancreas), and gonads of these three deep-sea species; 2) to test the applicability of three $\delta^{13}C$ – C:N ratio models specifically developed for decapod crustaceans, deep-sea fishes and aquatic organisms muscle tissue (as in Bodin et al., 2007; Hoffman and Sutton, 2010; Post et al., 2007, respectively) and of two tissue-specific equations provided by Logan et al. (2008) for liver and 3) to provide lipid-normalization equations to be used with non-treated (i.e., non lipid-extracted) samples of these and other deep-sea species with similar characteristics. This latter approach was tested for muscle and liver being the most common tissues utilized in aquatic food-web studies (Boecklen et al., 2011; Chen et al., 2012; Fanelli et al., 2016).

2. Materials and methods

2.1. Sample collection and preparation

The red shrimp *Aristeus antennatus*, the blackmouth catshark *Galeus melastomus* and the blue whiting *Micromesistius poutassou* were collected at 600–800 m depth by trawling the seafloor with a semi-balloon otter trawl (OTSB-14) within the framework of the BIOMARE project carried out in 2007–2008 off Catalonian coasts (Papiol et al., 2012). For this study only females were analyzed because of the greater weight of gonads available for Stable Isotope Analysis (SIA). Specimens were immediately frozen to − 20 °C and stored until processing. At laboratory, dorsal white muscle (M) tissue from *G. melastomus* and *M. poutassou* and caudal muscle (M) tissue from *A. antennatus*, liver (or hepatopancreas in the case of *A. antennatus*) (L) and gonads (G) were carefully dissected from the inner body, oven dried at 60 °C during 24 h and ground up. For each species, specimens in a similar nutritional state (according to their hepatosomatic index) and a similar gonad maturity stage (bare eye assessment) were selected (all species being at a late maturity stage, i.e., stages 3–4 for *A. antennatus*: ICES, 2009; stages 2–3 for *G. melastomus*: ICES, 2010 and stages 2–3 for *M. poutassou*: GFCM, 2016). Ground samples were divided into two subsamples: one for bulk analysis (considered as a control, without lipid extraction) and another for lipid extraction. For all species we selected specimens of the same size with no statistical differences (Kruskal-Wallis test $H_{2,41} = 3.53$, $p > 0.05$ for *A. antennatus*, size range: 37–45 mm of carapace length, Number of specimens = 14; $H_{2,40} = 0.42$, $p > 0.05$ for *G. melastomus*; size range: 43–59 cm of total length, $N = 13$; $H_{2,28} = 0.60$, $p > 0.05$ for *M. poutassou*; size range: 25–37 cm of total length, $N = 16$).

2.2. Lipid extraction method

Lipid extraction was performed using a method modified from Folch et al. (1957) by homogenizing the tissue in a 2:1 chloroform/methanol mixture of 20 times the tissue volume. The homogenate was sonicated for 10 min at 35 kHz, and then centrifuged at 12000*g* for 5 min to separate the remaining tissue. The supernatant was removed and the tissue washed in ultra-pure water (20 times tissue volume), sonicated and centrifuged again to remove remaining chloroform/methanol which may influence $\delta^{13}C$. The lipid-extracted tissue was oven dried at 60 °C to a constant weight. The sample lipid content was calculated as the percentage of total tissue dry weight (dry weight bulk tissue – dry