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The effect of lipid extraction on carbon and nitrogen stable isotope ratios in oyster tissues: Implications for glycogen-rich species

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RATIONALE: Extraction of lipids from tissues prior to carbon stable isotope analysis (SIA) has become a common practice, despite a lack of species-specific data to indicate when lipid extraction is needed. Marine invertebrates, including bivalves, are known to store carbon as glycogen and less in the form of lipids than other species, potentially reducing the need for lipid extraction even when C:N values are above 3.5, a value that previous studies suggest indicates a need for lipid extraction of animal tissues.
METHODS: We investigated the need for lipid extraction on individual tissues (adductor muscle, gut gland, gill) and whole tissue of a glycogen-storing species, the oyster *Crassostrea virginica*. Bulk and lipid-extracted samples were analyzed for their C and N stable isotope ratios by continuous flow isotope ratio mass spectrometry (IRMS). Samples were analyzed on a 20–20 isotope ratio mass spectrometer (PDZ Europa) after combustion in an elemental analyzer (PDZ Europa Automatic Analyzer-Gas Solid Liquid).
RESULTS: Although the C:N values for most bulk (unextracted) tissue samples were greater than 3.5, the lipid-extracted $\delta^{13}C$ values did not differ from the bulk values. Lipid extraction, however, affected $\delta^{15}N$ values in all tissue types except adductor muscle, indicating that separate SIA may be required when tissues are lipid extracted.
CONCLUSIONS: These data demonstrate that it is not necessary to lipid extract oyster tissues in all cases, and that C:N thresholds for lipid extraction in other species may not be reliable for organisms such as oysters that store glycogen. Our data indicate that minimizing unnecessary lipid extraction through preliminary testing will save researchers time and expense by avoiding superfluous sample handling, reducing concern over secondary effects on data quality, and reducing the costs of reagents and additional separate stable isotope analysis to ensure analytical accuracy.

To extract or not to extract lipids from animal tissue prior to stable isotope analysis is an ongoing debate in stable isotope ecology. This issue is based on the fact that lipids are isotopically 'light' in carbon compared with other tissue components, and this potentially confounds trophic and other stable isotope-based analyses.[1,2] The characteristically light $\delta^{13}C$ signature in lipids is due to temperature-dependent kinetic effects during the pyruvate dehydrogenase reaction when pyruvate is turned into acetyl-CoA or is separated for other reactions.[3,4] Several authors have reviewed the use of lipid extraction in the field of stable isotope ecology.[1,2,5] Post *et al.*[1] proposed that stable isotope analyses of aquatic organisms required lipid extraction when C:$N > 3.5$. This threshold for lipid extraction has been adopted by many authors and reviewers rather than verifying the need for lipid extraction in each species (e.g. [6–11]).

Lipid extraction increases the cost of stable isotope analysis and may compromise results. Not all organisms store carbon primarily in the form of lipids;[12,13] therefore, it is appropriate to evaluate the need for lipid extraction on a case-by-case basis. Some invertebrates, including protozoans, cnidarians, some annelids and nemerteans, and a variety of molluscs, have been documented to mainly use glycogen rather than lipid as their reserve storage product (Table 1[12–16]). These taxa may not require lipid extraction even when the C:$N > 3.5$. The tissue glycogen content varies considerably between species and tissue type even when the lipid values are similar (Tables 1 and 2). In *Crassostrea virginica* (Eastern oyster) and other oyster species, glycogen has been documented to range from ~1% to as high as 75%, with highest values in muscle or reproductive tissues (Tables 1 and 2). In studies where the glycogen content has also been reported, the C:N ratios measured in oysters ranged from 2 to 5 in whole tissues and from 3 to 6 in individual organs (Supplementary Table 1, Supporting Information). These data suggest that C:N values can be uncoupled from lipid content, particularly in glycogen-storing species. Unfortunately, few studies have measured C:N ratios in taxa that use glycogen as a storage reserve. The majority of ecological applications of stable isotope analysis have focused on vertebrate species that primarily store lipids (Fig. 1) and have assumed that the tissue composition is similar across broad categories of organisms (e.g. vertebrates, invertebrates, freshwater or marine).[1,5]

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Table 1. Summary of the range of literature values for % glycogen, % carbohydrate (where % glycogen was not reported, carbohydrate values are provided for comparison), and % lipid in whole tissue of various invertebrates taxonomic groups (data and references are provided in Supplementary Table 1(A), Supporting Information)

Taxonomic group	% Glycogen	% Carbohydrate	% Lipid
Molluscs			
Bivalves	1–75	1–53	1–30
Gastropods	1–6	24?	1–5
Sponges	~1	–	3–13
Cnidarians	1–7	–	10[a]
Annelids	1–6	–	6–9
Nemerteans	–	2–4	10–14
Tunicates	1–2	–	6–11

[a]Single data point.

While lipid extraction can be necessary to obtain accurate $\delta^{13}C$ values in some animal tissues, lipid extraction is known to alter $\delta^{15}N$ values. Many authors have observed a change in the $\delta^{15}N$ values of lipid-extracted samples (e.g. [17–19]) presumably because lipid extraction can result in loss of nitrogen-rich lipids and non-lipid material in both lipid and non-lipid fractions.[20–22] When using lipid extraction it is therefore necessary to separately analyze samples for each element, resulting in a doubling of tissue mass required (analyzing extracted and bulk tissues), increasing costs (for supplies and analyses), and adding time spent processing and analyzing samples.[23] In addition, application of a generalized C:N-based correction factor to account for lipids, if not verified, could lead to inaccurate values due to over- or underestimation.[5,24,25] Hence, substantial benefit can be derived from studies to better inform when species-specific lipid extraction is needed.

In this study we compared $\delta^{13}C$, $\delta^{15}N$ and C:N values in bulk and lipid-extracted tissues (adductor muscle, gut gland, gill) and whole tissue of the *C. virginica* to determine whether lipid extraction affected these values in the tissues of a glycogen-storing marine invertebrate. We hypothesized that the relatively low lipid content in oyster tissues would make lipid extraction unnecessary even when the C:N values are above the 3.5 threshold suggested for other aquatic species.[1] We therefore compared $\delta^{13}C$ values in lipid-extracted and bulk (unextracted) oyster tissues and determined the C:N value at which lipid extraction would be necessary in *C. virginica*. We compared published literature values for tissue composition (relative protein, carbohydrate, lipid content) in marine species to determine the differences between glycogen-storing and lipid-storing taxa (Supplementary Table 1, Supporting Information).

EXPERIMENTAL

Hatchery-reared oysters were obtained from the Auburn University Shellfish Laboratory on Dauphin Island (AL, USA) and were deployed under typical aquaculture conditions at two locations in Mobile Bay (AL, USA) during the summer of 2008 for up to 120 days (May–August) before dissection and analysis. A detailed description of rearing conditions and locations is provided by Patterson.[26] At collection, the oysters ranged in size from 28.4 to 71.1 mm. The oysters were stored at −20°C prior to analysis. The soft tissues were separated from the shell by tissue type (adductor muscle, gill, gut gland) or left whole, dried at 60°C and ground to a powder using a mortar and pestle. As a control, a sample of 'fatty' tissue surrounding the gut gland, expected to primarily comprise stored lipid, was collected and treated similarly.

Lipid extraction

Following a method modified from Folch *et al.*,[27] Bligh and Dyer,[28] and Sweeting *et al.*,[23] 0.01 ± 0.002 g of dried and ground tissue was placed in a 15-mL plastic, chemical-resistant, centrifuge tube (VWR International, Radnor, PA, USA) with 2 mL of chloroform (Acros Organics, 99.9% Extra Dry, Fisher Scientific, Waltham, MA, USA), 1 mL of methanol (Fisher Scientific, Optima LC/MS), and 1 mL of ultrapure water. The tubes were manually shaken for 30 s, and then spun at 3000 rpm for 10 min in an I.E.C. HN-S centrifuge (Damon/IEC Division, East Lyme, CT, USA). The tubes were carefully removed, so as not to disturb the layers, and the lower (chloroform/lipid layer) was removed by

Table 2. The ratio of % glycogen to % lipid summarized from the range of literature values in individual tissues or organs of various invertebrate taxonomic groups (data and references are provided in Supplementary Table 1(B), Supporting Information)

	% Glycogen/% Lipid			
Taxonomic group	Muscle	Mantle/gill	Digestive organs	Reproductive organs
Molluscs				
Bivalves	1–9	1–4	1–2	1–5
Gastropods	8	–	–	–
Cephalopods	1–2	–	–	–
Sponges	1–4	–	<1	<1
Polyplacophores	<1	<1	<1	≤5

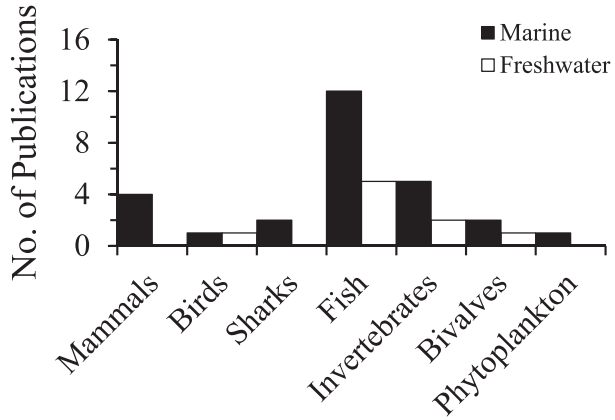

Figure 1. Published studies that investigated the need for lipid extraction of aquatic animal tissues for the purpose of stable isotope analysis (n = 26 sources; some sources presented data from studies of multiple taxa. If the citation contained both marine and freshwater species it was counted in both categories, but if the species spends time in both marine and freshwater environments it was counted as marine only). Bivalves were separated from other invertebrates. (References to published studies are provided in the Supporting Information.)

pipetting with gentle bubbling. This process was repeated three times until the lower layer was clear (providing visual evidence of lipid removal). The remaining upper layer was then filtered onto an ashed glass microfiber filter (grade GF/F) and rinsed with ultrapure water until no solvent remained. The filter was then dried at 60°C for 48 h. These methods were selected because they are widely used by ecologists to remove lipids before stable isotope analysis (e.g. [29–32]).

Stable isotope analysis

Lipid-extracted and bulk (unextracted) tissue samples (1.0 ± 0.2 mg) were packed in tin capsules (Elementar, Mt. Laurel, NJ, USA) and sent to the University of California Davis Stable Isotope Facility (Davis, CA, USA) for C and N stable isotope ratio determination by continuous flow isotope ratio mass spectrometry. The samples were analyzed on a 20–20 isotope ratio mass spectrometer (PDZ Europa, Sercon Ltd, Crewe, UK) after combustion in an elemental analyzer (PDZ Europa Automatic Analyzer-Gas Solid Liquid, ANCA-GSL). Ten percent of the samples were analyzed in duplicate to determine the effects of handling and instrument reproducibility; the variation in the values was within the range of instrument error (0.11 ± 0.06 ‰). A blank tin and acetanilide standard were included with each tray. The internal standards included G-11 (Nylon n = 39), G-13 (Bovine Liver n = 3), G-17 (USGS-41 Glutamic Acid n = 8) and G-9 (Glutamic Acid n = 12). All the stable isotope samples were analyzed relative to V-PDB (for carbon) and air (for nitrogen). The standard deviation for internal standards was ±0.08 ‰ (Nylon), ±0.07 ‰ (Bovine Liver), ±0.21 ‰ (USGS-41; USGS, Reston, VA, USA), and ±0.37 ‰ (Glutamic Acid) for carbon values and ±0.14 ‰ (Nylon), ±0.04 ‰ (Bovine Liver), ±0.15 ‰ (USGS-41), and ±0.26 ‰ (Glutamic Acid) for nitrogen. The $\delta^{13}C$ and $\delta^{15}N$ values, and the carbon-to-nitrogen (C:N) ratio by mass, were calculated for each sample before and after lipid extraction. The stable isotope ratios are expressed in delta notation as δX ‰ = $(R_{sample}/R_{standard} - 1)$, where R is the ratio of heavy to light isotope (e.g. $^{13}C/^{12}C$) and X is the element of interest (e.g. C).

Statistical analysis

All the tissue samples represent individuals (n = 7) and aggregates of 2–3 individuals (n = 31), except in the case of whole tissue where all the samples were from individual oysters. The concordance correlation coefficient[33,34] was calculated in R (version 2.15.2) using the epi.ccc package. In Minitab (version 15, State College, PA, USA; www.minitab.com) a paired sample t-test was used to compare the C:N ratios for extracted and bulk samples, as well as differences in the extracted and bulk $\delta^{13}C$ and $\delta^{15}N$ values. Regression analyses were performed in SigmaPlot (version 16, Systat Software, San Jose, CA, USA; www.systatsoftware.com). A significance value of $p < 0.05$ was used for all tests. The error is reported as standard error.

RESULTS

The mean C:N ratios ranged from 3.4 to 5.8 in bulk (unextracted) oyster tissues and 3.2 to 3.9 in lipid-extracted tissues, with the lowest values in adductor muscle and the highest in gut gland (all values >4.8; Fig. 2). In all bulk tissue samples except muscle, the C:N values were >3.5, the threshold for lipid extraction identified by Post *et al.*[1] (Fig. 2, dashed line), and remained >3.5 after extraction in gill and gut gland. The $\delta^{13}C$ values in tissues ranged from −20.4 to −24.6 ‰ and decreased significantly with increasing C:N (Fig. 2). The 'fatty' tissue sampled from surrounding the gut gland in oysters had a C:N value of 3.5 and a correspondingly low $\delta^{13}C$ (~ −24.5 ‰) value that were only comparable with the $\delta^{13}C$ values in gut gland and some gill tissues (Fig. 2).

The tissue C:N decreased significantly upon extraction (Table 3), with the greatest difference between bulk and extracted values in gut gland. Although the lipid-extracted $\delta^{13}C$ values tended to be lower than the $\delta^{13}C$ bulk values

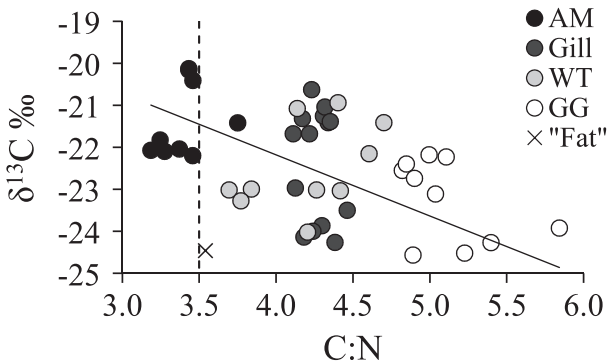

Figure 2. Bulk (unextracted) $\delta^{13}C$ values compared with C:N in adductor muscle (AM, n = 10), gill (Gill, n = 14), gut gland (GG, n = 15), whole tissue (WT, n = 10), and 'fat' (n = 1) from oysters (*C. virginica*). 'Fat' = Fatty tissue surrounding the gut gland. Dashed line represents the C:N value above which Post *et al.*[1] recommended lipid extraction for tissues from aquatic organisms. $y = -1.45x - 16.36$, $R^2 = 0.39$, $F_{reg\ (1,\ 48)} = 31.13$, $p < 0.001$.

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(Fig. 3), the mean values did not differ between treatments (Table 4). The $\delta^{13}C$ values in extracted tissues were correlated with the $\delta^{13}C$ values in bulk tissues and not significantly different from a 1:1 relationship (concordance correlation for all data: rho =0.87, Cd = 0.91; Fig. 3, Table 4). Adductor muscle showed the best correlation between the extracted and bulk treatments and the smallest deviation from the best-fit line, followed by whole tissue, gill, and gut gland (which showed the poorest correlation; Fig. 3, Table 4).

The mean $\delta^{15}N$ values in oyster tissues ranged from ~8.6 ‰ to 10.1 ‰ across both treatments and were significantly different between the bulk and lipid-extracted tissue samples for all tissues except adductor muscle (Table 4). Among the tissue that showed significant differences in $\delta^{15}N$ values, the mean differences between extracted and bulk samples was 0.45 ± 0.21 ‰ and they did not show a consistent shift due to lipid extraction.

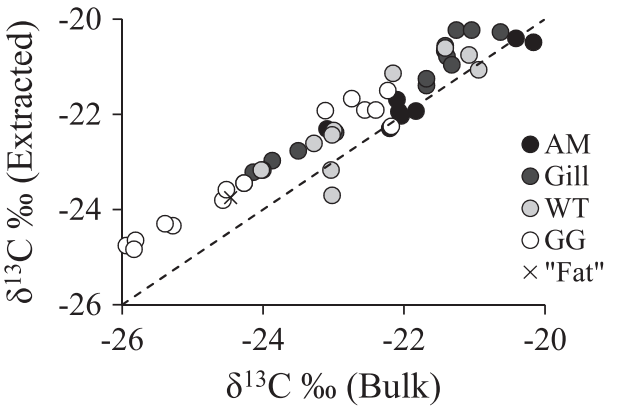

Figure 3. Concordance correlation for $\delta^{13}C$ values in lipid-extracted compared with bulk samples of oyster adductor muscle (AM, n = 10), gill (Gill, n = 14), whole tissue (WT, n = 10), gut gland (GG, n = 15) and 'fat' (n = 1). 'Fat' = Fatty tissue surrounding the gut gland. Dashed line represents the 1:1 relationship. Results of statistical analyses are shown in Table 3.

DISCUSSION

Our data demonstrate that the decision to use lipid extraction should be evaluated in light of the species-specific composition of the tissues to be analyzed. For *C. virginica*, lipid extraction prior to stable isotope analysis is not always necessary unless analyzing lipid-rich tissues such as gut gland. Kiljunen *et al.*[24] pointed out that the C:N in tissues from an organism that stores glycogen (as opposed to lipids) could be similar to the C:N in lipid-rich species, but lipid extraction or mathematical correction would not result in a substantial change in $\delta^{13}C$ values. Accordingly, our data showed relatively high C:N values in most oyster tissues (>3.5), but higher $\delta^{13}C$ values than in directly sampled fatty tissue (which had lower C:N) while the $\delta^{13}C$ values in lipid-extracted and bulk tissues were similar. The tissue-specific C:N and $\delta^{13}C$ values that we observed in bulk and extracted tissues are consistent with known differences in tissue composition. Gut gland contains higher concentrations of triglycerides (lipids)[35] and glycogen stores than muscle tissue,[36,37] accounting for the higher C:N than in adductor muscle and a ratio > 3.5 even after lipid extraction (Table 1). Although the values for the bulk and extracted tissues were not significantly different even for gut gland, our data suggest that analyses of gut gland tissues might benefit from lipid extraction prior to stable isotope analysis.

Our results suggest that previous C:N thresholds for lipid extraction may not be reliable for organisms such as oysters that store glycogen. The C:N ratios in all bulk tissues except gut gland were <4.7 but all were greater than or nearly equal to the 3.5 C:N threshold even after extraction (remaining between 3.2 and 3.9). These values are within the range of C:N values reported for *C. virginica* tissues across its range (e.g. 4.0–5.4 in NH, USA, 5.6 in NY, USA, 5.2–6.5 in VA, USA, and 2.9 in TX, USA[38–41]), suggesting that our conclusions are likely to be broadly applicable at least to *C. virginica*. Our findings suggest that a C:N ratio >3.5 may be more appropriate for lipid extraction of some tissues prior to stable isotope analysis among glycogen-storing species such as *C. virginica*. This approach would limit extraction to those tissues most likely to require some level of lipid correction, while minimizing the likelihood of unnecessary sample handling and treatment or over-correction (artificial increase) of $\delta^{13}C$ values. Because tissue composition in bivalves can be linked to food supply,[42,43] the high regional variation in tissue C:N values among oysters raises the question as to whether the need for lipid extraction may be to some extent location or study specific. It seems clear that broad application of lipid extraction thresholds or correction factors to major taxonomic groups such as 'all marine invertebrates' is likely to be at least imprecise.

We observed a small (<1 ‰) but significant shift in nitrogen stable isotope ratios with lipid extraction in all oyster tissues except adductor muscle (Table 3). Previous studies have reported $\delta^{15}N$ shifts large enough to affect food web assessments (2–4‰) and (as found in this study) the $\delta^{15}N$

Table 3. Mean ± standard error and paired t-test statistics for the difference between C:N in bulk and extracted tissues of oysters, including adductor muscle (AM, n = 10), gill (Gill, n = 14), whole tissue (WT, n = 10) and gut gland (GG, n = 15)

C:N Tissue	Bulk	Extracted	Difference	t-value	p-value
AM	3.40 ± 0.06	3.24 ± 0.02	0.16 ± 0.06	3.12	0.01
Gill	4.27 ± 0.03	3.70 ± 0.03	0.57 ± 0.04	23.10	<0.001
WT	4.20 ± 0.11	3.53 ± 0.04	0.67 ± 0.12	8.28	<0.001
GG	5.17 ± 0.07	3.85 ± 0.03	1.32 ± 0.08	15.33	<0.001

Table 4. Mean ± standard error $\delta^{13}C$ and $\delta^{15}N$ values in bulk and extracted tissues of oysters, results of a paired t-test comparing bulk and extracted samples for each tissue type (df = 1), and concordance correlation values (rho) and deviation (Cb; no deviation occurs when Cb = 1) from a 1:1 line comparing bulk and extracted $\delta^{13}C$ values. Adductor muscle (AM, n = 10), gill (Gill, n = 14), whole tissue (WT, n = 10) and gut gland (GG, n = 15)

	δX ‰		t-test statistics		Correlation statistics	
Tissue	Bulk	Extracted	T	p	rho	Cb
$\delta^{13}C$						
AM	–21.37 ± 0.26	–21.36 ± 0.26	–0.28	0.78	0.91	0.98
Gill	–22.37 ± 0.33	–21.69 ± 0.31	–1.39	0.17	0.86	0.87
WT	–22.50 ± 0.30	–22.10 ± 0.32	–0.82	0.43	0.81	0.93
GG	–24.05 ± 0.34	–23.03 ± 0.30	–1.90	0.07	0.75	0.79
$\delta^{15}N$						
AM	10.08 ± 0.11	9.88 ± 0.14	–1.09	0.29	—	—
Gill	9.55 ± 0.13	10.10 ± 0.08	–3.66	**0.001**	—	—
WT	9.86 ± 0.17	9.34 ± 0.17	–2.19	**0.04**	—	—
GG	8.58 ± 0.11	8.85 ± 0.05	–3.89	**0.001**	—	—

values were variable so no standard shift predictions could be made (e.g. [2,5,18,44,45]). In contrast, several studies that included zooplankton and tissues from mussels, birds, and fish found no effect of lipid extraction on $\delta^{15}N$ values.[46–48] Differences among studies could be due to differences in sample processing or handling such as the solvent type used,[20,21,47] but may also reflect variation due to tissue type or class of lipid being extracted (polar/non-polar).[49] To guarantee accurate values when lipid extraction is performed, in the case of oysters our data suggest that it will be necessary to separately determine the stable isotope ratios of carbon and nitrogen by performing $\delta^{15}N$ analysis on bulk tissues only. Because lipid extraction is not needed for most oyster tissues, however, stringency of extraction methods, resulting effects on $\delta^{15}N$ values, and separate isotope analysis should not be a concern.

Like oysters, other organisms that store glycogen and have low lipid content, especially other bivalve species, may not require lipid extraction before stable isotope analyses. Protein:lipid and carbohydrate:lipid comparisons among tissues in different groups of animals should aid in evaluating the need for the lipid extraction. For example, data from the literature shows that finfish, crustaceans, and molluscs have distinct taxon-specific relationships among tissue components (carbon content may be relatively high while lipid content is low; Fig. 4). If the relationship between tissue composition and extraction requirements in oysters that we documented can be generalized to other species, many molluscs may not require lipid extraction prior to isotope analysis. Among available studies, the bivalve lipid content ranged from 7.0 ± 0.4 to 13.6 ± 0.7% of ash-free dry weight (AFDW), and the carbohydrate content ranged from 8.2 ± 0.9 to 24.9 ± 1.2%,[13,50] and both sets of values were shown to vary with season, temperature, latitude, ontogeny, rate of growth, ploidy, sex, reproductive state, species and available food supply.[12,15,51–59] In organisms with different muscle types, the muscle group and function were also important (e.g. greater glycogen content in the tail than in the claw of American lobster[60]). Other authors have pointed out that C:N is not necessarily reflective of lipid content even in organisms that store lipids, such as fish.[61] To determine the net effects of lipid extraction on stable isotope ratios (correcting or confounding), thought should be given to tissue composition, tissue type, and the timing of tissue sampling during sample collection and preparation, as well as the absolute C:N in target tissues.

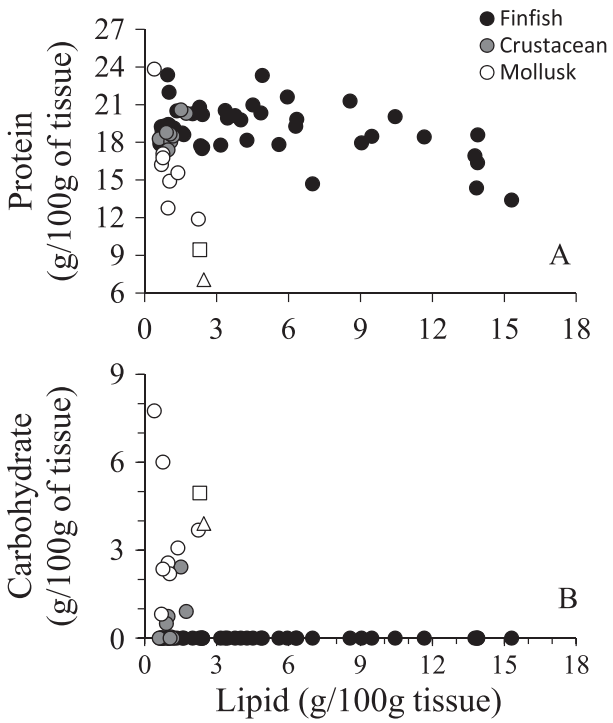

Figure 4. Protein (A) and carbohydrate (B) content compared with lipid content in tissues from finfish (n = 45), crustaceans (n = 8) and mollusks (n = 10). Data summarized from Exler[63] relative to the raw, edible portion of the organism. Carbohydrates were mainly glycogen. Oyster species are distinguished from other mollucs as triangles = *C. virginica*, squares = *C. gigas*.

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CONCLUSIONS

Overall, our data suggest that using generalized studies and large meta-analyses of multiple species from different locations, seasons, and ages (e.g. [18]) to set thresholds for lipid extraction is inappropriate and that these generalizations may not accurately gauge the effect of lipid extraction on individual tissues or species. We concur with Hoffman and Sutton,[62] who recommended using a species-specific approach because a generalized lipid correction model can introduce biases to data. Our data, coupled with the variation in findings previously published, leads us to recommend that authors examine the need for lipid extraction on a case-by-case, tissue-specific basis. For *C. virginica*, and other species that use glycogen as a primary storage substrate, lipid extraction may not be needed in some tissue types prior to SIA. Selective application of lipid extraction to high lipid content tissues such as gut gland may be sufficient. The literature values of C:N for many other invertebrates species are very similar to values that we found in oysters (Supplementary Table 1, Supporting Information). In these species energy storage commonly relies on glycogen/carbohydrate rather than lipids; therefore, lipid extraction may not be necessary for these taxa. Minimizing unnecessary lipid extraction through preliminary testing will save researchers time and expense by avoiding superfluous sample handling, reducing concern over secondary effects on data quality, and reducing costs of reagents and additional separate stable isotope analysis to ensure analytical accuracy.

Acknowledgements

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