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Effects of urea and lipid removal from *Carcharhinus leucas* and *Galeocerdo cuvier* white muscle on carbon and nitrogen stable isotope ratios

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Abstract

The analysis of stable isotope ratios of carbon and nitrogen is a tool commonly used in trophic ecology. However, the presence of nitrogen compounds and lipids in tissues of studied organisms can bias the ratio measurements. Treatments to eliminate problematic compounds have been highlighted in the literature. In this study the effects of two different treatments and their combination on the $\delta^{15}N$ and $\delta^{13}C$ ratio values of *Carcharhinus leucas* and *Galeocerdo cuvier* white muscle samples were tested. All sharks were caught along the west coast of Reunion Island (western Indian Ocean), within the framework of a shark-control programme. Deionized water rinsing proved to be the most effective treatment for nitrogen compound removal and the lipid extraction, using a 2:1 chloroform-methanol solution, the most effective treatment for lipid removal. The combination of both treatments was as effective as deionized water rinsing for nitrogen compound removal but produced an unexpected decrease of $\delta^{13}C$ ratio values. Deionized water rinsing caused a similar decrease on some $\delta^{13}C$ values in the bull shark. Some differences on the effects of the different treatments appeared when considering the sexes separately. Analytical normalization equations for the different treatments on the two stable isotope ratios are provided.

Keywords: ^{15}N, ^{13}C, TMAO, Lipid extraction, Shark, Reunion Island

Introduction

Sharks, as apex or mesopredators, play major roles in the functioning of ecosystems in which they evolve, affecting the dynamics of their prey populations directly through consumption and indirectly through risk avoidance behavior (Heithaus *et al.*, 2008; Roff *et al.*, 2016). Apex predators are usually the largest species, (Ferretti *et al.*, 2010; Heupel *et al.*, 2014) they can undertake large-scale movements and therefore transport energy, nutrients and other materials through the oceans, over long distances (Estes *et al.*, 2016). Recently, anthropogenic pressures have caused the decline of several shark populations, raising concerns about their conservation and the effect of their removal on the functioning of their ecosystems (Ferretti *et al.*, 2010). Top-down effects have been highlighted in certain shark species (Heithaus *et al.*, 2007; Myers *et al.*, 2007) but data are still lacking on the trophic dynamic of many others (Ferretti *et al.*, 2010).

One method to study trophic ecology is the analysis of stable isotopes, and more specifically the $^{15}N/^{14}N$ (expressed as $\delta^{15}N$) and $^{13}C/^{12}C$ (expressed as $\delta^{13}C$) ratios (Fry, 2006). Their use is based on the fact that the isotopic composition of a consumer is dependent of its diet, presenting a mix of the isotopic proportions of its prey plus a small increase due to fractionation throughout the food web (Fry, 2006; Layman *et al.*, 2011). In the case of $\delta^{15}N$, the increase from the prey to the predator is typically estimated to 2-5 ‰ per trophic

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level, allowing the determination of trophic positions. The fractionation is more conservative in the case of $\delta^{13}C$, usually with 0-1 ‰ per trophic level, and is typically used to identify the production at the base of the food chain and foraging location (Post, 2002; Martínez del Rio *et al.*, 2009; Hussey *et al.*, 2012). Accurate ecological interpretation of stable isotope data relies on confidence in a number of underpinning assumptions, including accounting for biasing effects of polar compounds, namely lipids, urea and trimethylamine oxide (Shipley *et al.*, 2017).

Of concern when measuring $\delta^{13}C$ values, is the presence of lipids in the samples. Indeed, lipids are ^{13}C-depleted compared to proteins and carbohydrates and introduce a bias in $\delta^{13}C$ values by lowering these (Newsome *et al.*, 2010). The presence of such a bias has been highlighted in certain studies of elasmobranches, but the low lipid proportion in some species suggests this bias is not systematic (Hussey *et al.*, 2010; Matich *et al.*, 2010; Kim and Koch, 2012; Li *et al.*, 2015). The C:N ratio is traditionally used to determine if a sample contains enough lipids to introduce a bias by assuming that ratios lower than 3.5 are mostly composed of proteins (Post, 2002; Pethybridge *et al.*, 2012). However, if this assumption is true in teleosts (Hoffman and Sutton, 2010), the use of nitrogenous compounds for osmoregulation in shark muscles imply that C:N ratios below 3.5 could still contain important lipid quantities (Shipley *et al*, 2017). Thus, it is recommended that lipids should be extracted from samples before stable isotope analysis to remove bias and standardize samples between species and across food webs (Hussey *et al.*, 2012; Shipley *et al.*, 2017).

The measurement of $\delta^{15}N$ ratios values can also be biased, especially in elasmobranches. Indeed, their tissues contain urea and trimethylamine oxide (TMAO) used to maintain osmotic balance. These nitrogenous compounds are ^{15}N depleted, which can lead to lowering $\delta^{15}N$ values when conducting stable isotope analyses. The removal of these compounds is necessary prior to analyses in elasmobranches (Kim and Koch, 2012; Hussey *et al.*, 2012). For lipids, although this bias is not systematic, it is recommended that elasmobranch samples are treated for urea to standardize samples.

Currently, lipids are commonly removed using a 2:1 chloroform methanol extraction following a modification of the Bligh and Dyer (1959) technique. Although nitrogenous compounds can be removed by the same technique in elasmobranch muscle, a deionized water rinsing has been shown to be the most effective technique to remove urea and TMAO from shark tissues. Combined lipid extraction and deionized water rinsing have also proven to be useful and even more effective than separated techniques in some instances (Li *et al*., 2015).

This study is part of a long-term project that is investigating the trophic ecology of bull (*Carcharhinus leucas*) and tiger (*Galeocerdo cuvier*) sharks in coastal ecosystems of Reunion Island (western Indian Ocean). Samples were collected from specimens caught in the local shark-control programme implemented by the French government and local authorities after a series of shark attacks on surfers and bathers since 2011. The main aim of the programme is to better understand the place and role of the two species in the functioning of coastal ecosystems, and how the removal of individuals could affect these ecosystems. A first description of the diet and position of the species in food chains has been conducted by Trystram *et al.* (2016), highlighting differences in feeding habits and resource use between the two studied species. Although preliminary tests conducted by Trystram *et al.* (2016) on the effect of lipids and urea removal on stable isotope ratios of carbon and nitrogen did not reveal significant effects of these components on isotopic values, a more systematic investigation of the lipid extraction and urea rinsing seemed necessary. Indeed, several recent studies suggested significant effects of these treatments, especially for large shark species (Li *et al.*, 2015; Carlisle *et al.*, 2016; Shipley *et al.*, 2017).

This study followed the protocol described in Li *et al.* (2015) to investigate the effect of lipid and urea removal on isotopic values of bull and tiger shark white muscle. Treatment-related differences were investigated both at the scale of the species and for the sexes separately. When a significant difference was observed between the control (no treatment) and treated samples, an analytical normalization was proposed to adjust the isotopic values of non-treated samples in the future.

Materials and methods

Sample collection

Samples were collected from individuals caught along the west coast of Reunion Island in the framework of the Reunion Island shark control programme, using both horizontal bottom longlines and smart drumlines (Guyomard *et al.*, 2019). Dead

individuals were stored at 4°C in a cold room shortly after their capture and dissected as soon as possible, and up to 36 h later. The total length (TL, cm) of each individual was measured to the nearest centimeter and the total weight (W, kg) of each individual was measured whenever possible, or otherwise derived from biometric equations (Pirog *et al.*, in press). A portion of white muscle was sampled from the back of each individual, from the front of the anterior dorsal fin, and frozen at -20°C shortly after sampling. Sixteen female and 15 male bull sharks and 14 male and 15 female tiger sharks, representative of the size range of the captures, were randomly selected for this study. All samples came from individuals caught in 2016 to limit possible effect of the year of catch on stable isotope values.

Sample preparation and analysis

All frozen white muscle samples were freeze-dried at -50 °C for 48 h using a CRIOS Cryotec freeze dryer. Dry samples were reduced by milling for 3 minutes to a homogeneous powder using a Mixer Mill Retsch MM400 at 30 Hz. Each powdered sample was divided into four equivalent subsamples and four different treatments were applied to each: Urea extraction (DW), lipid extraction (LE), lipid and urea extraction (LE+DW) and no treatment (control, C), following the methods of Li *et al.* (2015). In summary, deionized water was used to remove urea from muscle tissues and a 2:1 chloroform-methanol mixture was used to extract lipids (see supplementary materials for the detailed protocol). After each treatment all samples were dried again in an oven at 50 °C for 24 h.

0.3 to 0.9 mg of dry powdered material was put into a tin capsule for each sample for stable isotope analyses after completion of the treatment. The exact mass was weighed using a precision balance to the nearest 0.1 mg. The capsules were then folded into small spheres, placed in a 96-sink plate and sent to the IRMS platform at the University of La Rochelle for $\delta^{13}C$ and $\delta^{15}N$ measurements. $\delta^{13}C$ and $\delta^{15}N$ values were determined for each sample using a Thermo Scientific Flash EA 1112 elemental analyzer coupled with a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer with a Conflo IV interface. The machines were calibrated using the working standards USGS-61 (Caffeine) and USGS-62 (Caffeine). All results are expressed in the standard notation relative to the international standards Pee-Dee Belemnite for carbon and atmospheric N_2 for nitrogen. Replicate measurements of internal laboratory standards provided measurement errors <0.10 ‰ for both $\delta^{15}N$ and $\delta^{13}C$ values.

Statistical analysis

For each species, $\delta^{15}N$ and $\delta^{13}C$ values were statistically compared to test whether they differed between treatments. Parametric conditions were assessed using Bartlett's tests for homogeneity of variances and Shapiro's tests for normality. Pairwise paired t-tests with the Benjamini-Yekutieli p-value adjustment method were conducted when the data adhered to parametric assumptions. When this was not the case, a pairwise paired Wilcoxon rank sum test was conducted with the same p-value adjustment method, as the logarithmic and square-root data transformations did not allow parametric analyses. To assess for a sex-related response to treatments, the same statistical procedures were conducted for both sexes for each species. The differences between sexes for each treatment were determined using t-tests or Wilcoxon rank sum tests respectively, for parametric and non-parametric conditions.

When a significant effect of a treatment on stable isotope values was observed, an analytical normalization of non-treated samples was established with linear models. In order to test for species and sex-related differences in linear models, values observed and predicted by the models were statistically compared using either a t-test or Wilcoxon rank sum test, depending whether the dataset adhered to parametric assumptions.

Differences in C:N ratios between non-treated and treated samples were assessed for each species. As the data did not follow parametric assumptions, Kruskal-Wallis tests followed by Dunn post-hoc analyses with Bonferroni corrections were used.

All statistical analyses were performed using the software R version 3.4.3 with a significance level of 0.05.

Results

The DW treatment resulted in significantly higher $\delta^{15}N$ values but did not modify $\delta^{13}C$ values when compared to the control, except for *C. leucas* when considering both sexes together. In this case the DW treatment resulted in a significantly lower $\delta^{13}C$ value compared to the control. The LE treatment resulted in higher $\delta^{15}N$ values than the control except for *C. leucas* males where the value was significantly lower. The LE $\delta^{13}C$ values were higher than the control except for *C. leucas* males

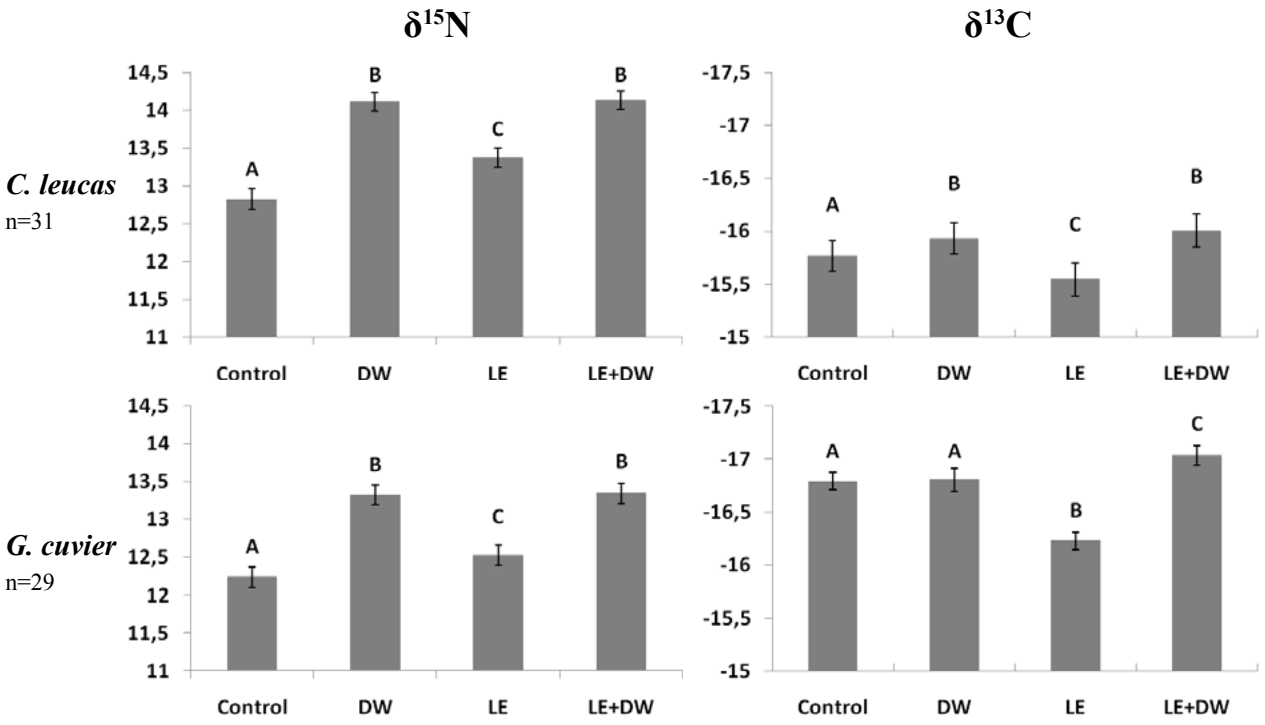

Figure 1. Histograms of the mean $\delta^{15}N$ and $\delta^{13}C$ values for *Carcharhinus leucas* and *Galeocerdo cuvier*. Significant results are indicated by different letters. Error bars are standard errors.

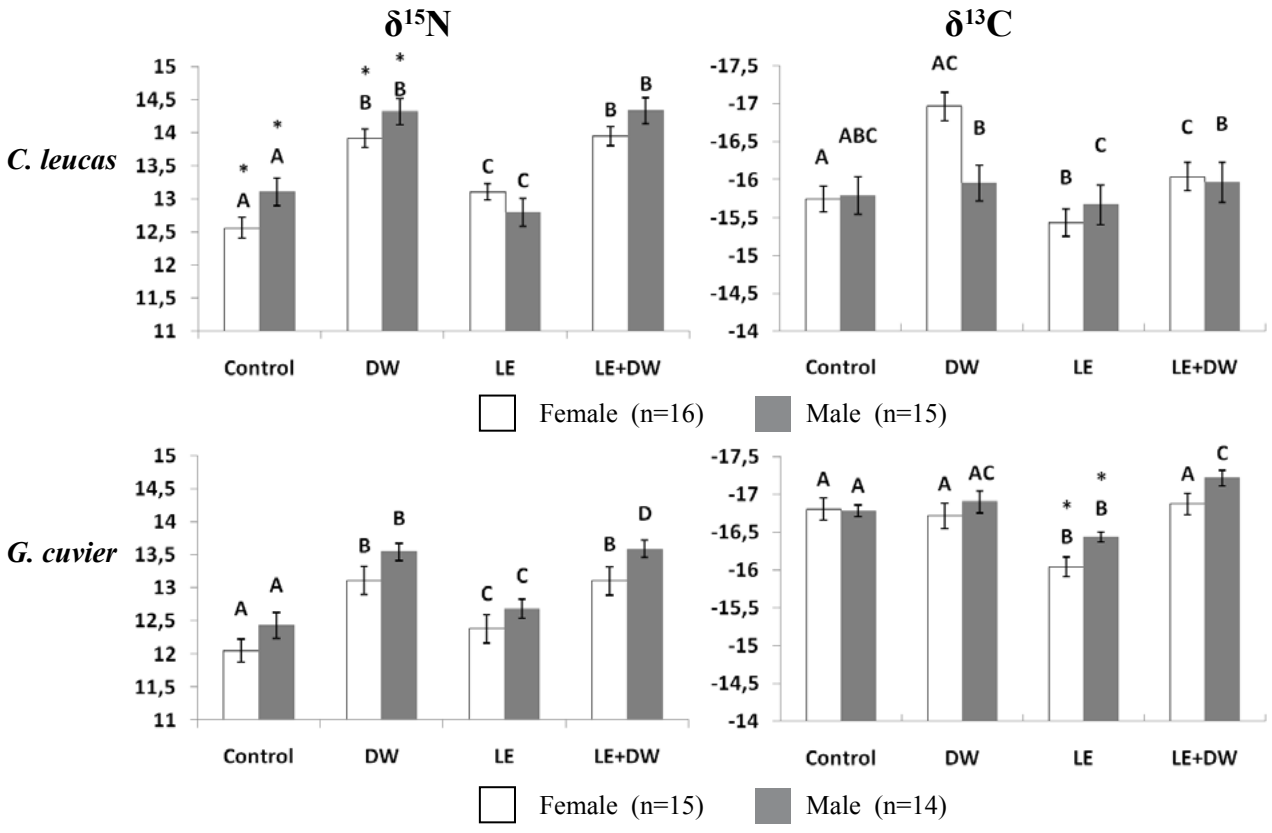

Figure 2. Histograms of the mean $\delta^{15}N$ and $\delta^{13}C$ values for male and female *Carcharhinus leucas* and *Galeocerdo cuvier*. Significant results within a sex are indicated by different letters. Asterisks indicate significant differences between two sexes for one treatment. Error bars are standard error.

where there was no significant difference. The LE+DW treatment resulted in an increase of $\delta^{15}N$ values and a decrease of $\delta^{13}C$ values compared to the control. The only exceptions were for the female *G. cuvier* and the male *C. leucas* $\delta^{13}C$ values, which were not significantly different between LE+DW treatment and control. LE samples always had significantly lower $\delta^{15}N$ values and higher $\delta^{13}C$ values than DW and LE+DW. These last two treatments generally did not significantly change $\delta^{15}N$ values, except for male *G. cuvier* for which the LE+DW treatment had a significantly higher value compared to the control. For the $\delta^{13}C$ values, the two treatments were significantly different for *G. cuvier* only, and LE+DW has the lowest value (Fig. 1). When comparing the means between sexes within a treatment, there were only significant differences for the control and DW treatment of $\delta^{15}N$ for *C. leucas* and the LE treatment of $\delta^{13}C$ for *G. cuvier* (Fig. 2).

Table 1. Regression equations displaying the relationship between the Control treatment and other treatments. The species column presents the species and the sexes. Sex equations are only presented when significantly different from the equations using both sexes. CL = *Carcharhinus leucas*. GC = *Galeocerdo cuvier*. The R^2 of the regression analyses are presented. All p-values <0.05. The equations recommended for the normalization values of non-treated samples (see discussion) are represented in grey.

Species	Parameter	Equation	R^2
CL	$\delta^{13}C$	$\delta^{13}C_{LE} = 1.043*\delta^{13}C_{Control} + 0.903$	0.94
Female	$\delta^{13}C$	$\delta^{13}C_{LE} = 1.075*\delta^{13}C_{Control} + 1,492$	0.93
Male	$\delta^{13}C$	$\delta^{13}C_{LE} = 1.022*\delta^{13}C_{Control} + 0.469$	*0.96*
CL	$\delta^{13}C$	$\delta^{13}C_{LE+DW} = 0.966*\delta^{13}C_{Control} - 0.779$	0.8
Female	$\delta^{13}C$	$\delta^{13}C_{LE+DW} = 0.993*\delta^{13}C_{Control} - 0.408$	0.79
Male	$\delta^{13}C$	$\delta^{13}C_{LE+DW} = 0.955*\delta^{13}C_{Control} - 0.886$	*0.8*
CL	$\delta^{13}C$	$\delta^{13}C_{DW} = 0.939*\delta^{13}C_{Control} - 1.131$	*0.86*
GC	$\delta^{13}C$	$\delta^{13}C_{LE} = 0.749*\delta^{13}C_{Control} - 3.66$	0.56
Female	$\delta^{13}C$	$\delta^{13}C_{LE} = 0.826*\delta^{13}C_{Control} - 2.162$	*0.88*
GC	$\delta^{13}C$	$\delta^{13}C_{LE+DW} = 0.772*\delta^{13}C_{Control} - 4.067$	0.44
Female	$\delta^{13}C$	$\delta^{13}C_{LE+DW} = 0.784*\delta^{13}C_{Control} - 3.703$	*0.62*
CL	$\delta^{15}N$	$\delta^{15}N_{DW} = 0.852* \delta^{15}N_{Control} + 3.192$	0.88
Female	$\delta^{15}N$	$\delta^{15}N_{DW} = 0.823* \delta^{15}N_{Control} + 3.576$	*0.85*
CL	$\delta^{15}N$	$\delta^{15}N_{LE+DW} = 0.834* \delta^{15}N_{Control} + 3.437$	0.82
Female	$\delta^{15}N$	$\delta^{15}N_{LE+DW} = 0.824* \delta^{15}N_{Control} + 3.595$	0.73
CL	$\delta^{15}N$	$\delta^{15}N_{LE} = 0.873* \delta^{15}N_{Control} + 2.176$	0.84
Female	$\delta^{15}N$	$\delta^{15}N_{LE} = 0.762* \delta^{15}N_{Control} + 3.54$	0.82
Male	$\delta^{15}N$	$\delta^{15}N_{LE} = 0.91* \delta^{15}N_{Control} + 1.735$	*0.8*
GC	$\delta^{15}N$	$\delta^{15}N_{DW} = 0.813* \delta^{15}N_{Control} + 3.37$	0.66
Female	$\delta^{15}N$	$\delta^{15}N_{DW} = 1.068* \delta^{15}N_{Control} + 0.235$	0.71
Male	$\delta^{15}N$	$\delta^{15}N_{DW} = 0.544* \delta^{15}N_{Control} + 6.781$	*0.66*
GC	$\delta^{15}N$	$\delta^{15}N_{LE+DW} = 0.832* \delta^{15}N_{Control} + 3.159$	0.67
Female	$\delta^{15}N$	$\delta^{15}N_{LE+DW} = 1.074* \delta^{15}N_{Control} + 0.157$	0.72
Male	$\delta^{15}N$	$\delta^{15}N_{LE+DW} = 0.559* \delta^{15}N_{Control} + 6.642$	*0.66*
GC	$\delta^{15}N$	$\delta^{15}N_{LE} = 0.858* \delta^{15}N_{Control} + 2.031$	0.73
Female	$\delta^{15}N$	$\delta^{15}N_{LE} = 1.099* \delta^{15}N_{Control} - 0.861$	0.74
Male	$\delta^{15}N$	$\delta^{15}N_{LE} = 0.659* \delta^{15}N_{Control} + 4.495$	0.81

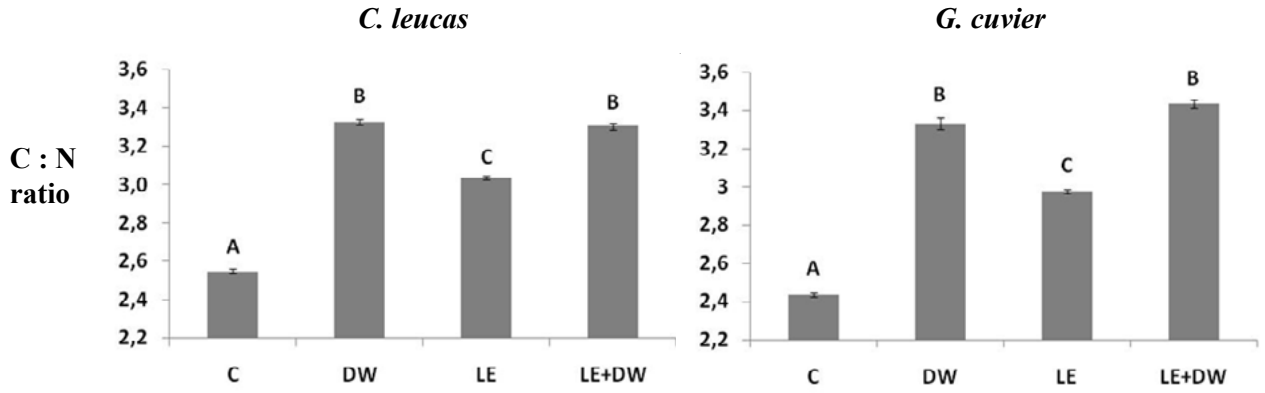

Figure 3. Histograms of the mean C:N ratios for *Carcharhinus leucas* and *Galeocerdo cuvier*. Significantly different results are indicated by different letters.

Equations of the linear models to normalize non-treated samples are shown in Table 1. When the general equation and the female and/or male equations produced significantly different datasets, all the equations are shown. The non-linear relations and the non-significant regressions (p-value >0.05) are not presented.

The C:N ratios increased between the control and the different treatments in both species (Fig. 3). The increase was more significant in DW and LE+DW treatments. The LE treatment also resulted in an increase, but this was less significant. The comparison of the results obtained and the results of Li *et al.* (2015) is shown in Table 2.

Table 2. Comparison of effects of the treatments on stable isotope values for the two studied species and other shark species (Li *et al.*, 2015). Different letters and colours indicate significant differences between control and treatments within each species. The comparison is shown for both the carbon and nitrogen analyses.

	Species	C	DW	LE	LE + DW
$\delta^{13}C$	*Carcharhinus leucas*	A	B	C	B
	Galeocerdo cuvier	A	A	B	C
	Carcharhinus falciformis	A	B	C	D
	Prionace glauca	A	B	B	C
	Sphyrna zygaena	A	B	B	C
	Sphyrna lewini	A	AB	B	B
	Carcharhinus longimanus	A	B	BC	C
	Isurus oxyrinchus	A	A	A	A
	Alopias pelagicus	A	A	B	B
$\delta^{15}N$	*Carcharhinus leucas*	A	B	C	B
	Galeocerdo cuvier	A	B	C	B
	Carcharhinus falciformis	A	B	C	B
	Prionace glauca	A	B	C	B
	Sphyrna zygaena	A	B	C	BC
	Sphyrna lewini	A	B	C	B
	Carcharhinus longimanus	A	B	C	B
	Isurus oxyrinchus	A	A	A	A
	Alopias pelagicus	A	B	C	B

Discussion

It is critically important to obtain correct stable isotope values in order to accurately analyze food webs. In this context, sample preparation using a deionized water rinsing and lipid extraction was deemed necessary in sharks (Fisk *et al.*, 2002; Hussey *et al.*, 2012) and the results of the present study support this. Indeed, significant changes in isotopic values of bull and tiger shark white muscle and C:N ratios were observed when applying the different treatments to extract lipids and/or urea, compared to non-treated samples (control samples).

When considering $\delta^{15}N$ values, all treatments resulted in a significant increase of the values compared to control values, which could lead to an underestimation of the trophic positions of the individuals. Such a result was expected for DW treatment as it is known that the presence of urea and trimethylamine oxide (TMAO) in the muscles of sharks result in lowering the $\delta^{15}N$ value and corresponding trophic level (Fisk *et al.*, 2002; Hussey *et al.*, 2012). The LE treatment, initially designed to remove lipids, also resulted in increasing $\delta^{15}N$ values, though this increase was lower than for DW or LE+DW treatments. Such a result was recently observed for several species of deep-sea sharks by Shipley *et al.* (2017), who also recommended that an additional DW rinse be performed to remove any remaining urea from shark muscle tissue. Hussey *et al.* (2010) suggested that lipid extraction removes soluble urea, and this is likely why this small increase in $\delta^{15}N$ values was observed. However, the water rinsing had a greater impact, which confirms that this treatment is more effective than lipid extraction for urea and TMAO removal. Interestingly, the combined treatment LE+DW had the same effect as the DW treatment, suggesting that water rinsing is sufficient to remove all the urea and TMAO present in samples, and that no additional lipid extraction is needed to produce accurate $\delta^{15}N$ values. The only exception was for the male tiger sharks where the combined treatments increased the $\delta^{15}N$ value even more than water rinsing only. However, this additional increase was marginal with a maximum of 0.04‰, a value close to the internal laboratory measurement error, which suggests that either the difference is an artifact that could disappear with additional replicates, or the difference is real, but weak enough to keep the DW treatment only.

In the case of *C.leucas* and *G.cuvier* $\delta^{15}N$ analysis, the DW treatment alone seems to be adequate to remove urea and TMAO. Li *et al.* (2015) suggest that the LE+DW treatment is the most effective because it reduces urea concentration in pelagic shark muscles to a greater extent than the DW treatment alone. Similarly, Dale *et al.* (2011) suggested that water rinsing may not be enough to remove all the influence of urea on $\delta^{15}N$ values for a sting ray (*Dasyatis lata*). The same kind of effect could be observed for the tiger and bull sharks, but the urea concentration was not measured in the samples in this study. However, the maximal difference of 0.04 ‰ between DW and LE+DW mean values for each species and sex in these results suggests that the DW treatment is sufficient.

It is known that lipids are depleted in ^{13}C compared to carbohydrates and proteins, and that lipid-rich samples cause the $\delta^{13}C$ values to decrease (Newsome *et al.*, 2010; Hussey *et al.*, 2012). Thus, lipid extraction is necessary in cases of high lipid content in samples and the $\delta^{13}C$ value is expected to increase with it. Such a significant increase in $\delta^{13}C$ values was observed in this study for the LE treatment. This result confirms the need to extract lipids from both tiger and bull shark muscles to result in correct $\delta^{13}C$ values. In addition, C:N ratios were under 3.5 for all the controls. Thus, assuming that these samples contained mainly proteins is incorrect, as lipid extraction caused significant ^{13}C changes. Nitrogenous compound washing also affected the C:N ratio, confirming previous research showing that the presence of these compounds make this ratio an unreliable proxy for lipid presence estimation in sharks (Shipley *et al.*, 2017).

For both DW and LE+DW treatments, the $\delta^{13}C$ value decreased in both species, a result which was not expected. The hypothesis of repeated manipulation error is not relevant here because of the number of replicates, the success of the LE treatment and the consistency in the effect in both species and for each sex. Therefore, this could result from an unknown aspect of the tiger and bull shark physiology causing water rinsing to decrease $\delta^{13}C$ in powdered muscle samples; for example, by an unidentified compound washed by deionized water and enriched in ^{13}C that would decrease the $\delta^{13}C$ value. Further research is needed to elucidate this unexpected effect.

Lipid extraction alone seems to have had the expected effect and successfully increased the $\delta^{13}C$ value. The only exception was for the male bull sharks where no significant effect was observed. This could be explained by the low percentage of lipids in muscles of male bull sharks. Differences in lipid quantity have

previously been observed between sexes of *Mustelus mustelus*, and the authors suggest that female fishes should have more lipids for maturation and embryo development (Bosch *et al.*, 2013). Furthermore, it has been highlighted that in some species of sharks, the quantity of lipids present in the muscles is very low and lipid extraction is not needed prior to SIA (Matich *et al.*, 2010; Trystram *et al.*, 2016). However, except for the male bull sharks, there was still an increase in $\delta^{13}C$ value, and this suggests that lipid extraction should still be undertaken in the two studied species.

Because of the confusing effect of water rinsing on $\delta^{13}C$ value, it is impossible to recommend one treatment for both $\delta^{13}C$ and $\delta^{15}N$ SIA for *C.leucas* and *G.cuvier*. Instead, water rinsing should be undertaken for $\delta^{15}N$ SIA and lipid extraction for $\delta^{13}C$ SIA on separated sub-samples. The combination of the two treatments, although usable for $\delta^{15}N$, is not recommended for $\delta^{13}C$. Such an effect of water rinsing on $\delta^{13}C$ values highlights the importance of assessing each species of shark separately when determining which sample treatment is necessary.

As these treatments are lengthy and costly, an alternative method used to result in correct values is the application of analytical normalization. For this purpose, a series of equations were produced which allowed the estimation of corrected isotopic values based on the values of non-treated samples. Considering the recommendation of treatments for carbon and nitrogen values in white muscle of tiger and bull sharks, the equations that should be used for the normalization of non-treated values are those highlighted in Table 1. When possible, the separated sex equations should be used. Interestingly, the models are less robust for tiger sharks compared to bull sharks (lower R^2 values). This suggests that tiger sharks display more variability in the lipid and urea contents in white muscles, and this could be linked to their life cycle.

When comparing the results from this study to those of Li *et al.* (2015), an interesting pattern appears for $\delta^{15}N$ values (Table 2). The effects of the different treatments are similar in each species except for *Isurus oxyrinchus* in which treatments had no significant effects. This indicates that the deionized water rinsing has the same outcome in various offshore pelagic species, as well as in the two coastal benthopelagic species studied, supporting the idea that this treatment is necessary at least in all large bodied shark species. The comparison of $\delta^{13}C$ results in more interspecific differences, underscoring once more the importance of species-specific tests in order to determine the most effective treatments. Again, *I. oxyrinchus* displays no difference between treatments. This species is believed to be the fastest and most active shark in the world (Ebert *et al.*, 2013) and could possess physiological attributes explaining the very low concentrations of both urea and lipids in its muscle. For the other species, the age of the individuals and their physiological and reproductive status could be factors explaining the differences in the results of treatments, as they might indicate different lipid contents in white mucles. Male bull sharks used in this study were, in particular, mostly caught outside of the reproductive period (pers. obs.), and this could explain the low lipid content of these individuals, which led to no significant effect of the LE treatment on $\delta^{13}C$ values. Further investigations could confirm or reject this hypothesis.

In conclusion, this research demonstrates the need to correct the stable isotope values of carbon and nitrogen in the white muscle of tiger and bull sharks, either by using a treatment or by analytical normalization. This conclusion is in accordance with previous studies conducted on other shark species (Li *et al.*, 2015; Carlisle *et al.*, 2016; Shipley *et al.*, 2017). A comparison of the results of treatments to extract lipids and urea in shark tissues from individuals from different locations could indicate whether analytical normalizations are specific to local individuals of a species, or to all specimens of the same species from any location.

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Supplementary material

Detailed protocol

Control

For stable isotope analysis, 0.3 to 0.9 mg of powdered material was put in a tin capsule for each sample. The exact mass was weighed using a precision balance. The capsules were then folded into small spheres, placed in a 96-sink plate and sent from Reunion Island to the University of La Rochelle. There, the $\delta^{13}C$ and $\delta^{15}N$ values were determined for each sample using a Thermo Scientific Flash EA 1112 and a Thermo Scientific Delta V Advantage with a Conflo IV interface. The machines were calibrated using the working standards USGS-61 (Caffeine) and USGS-62 (Caffeine).

Urea extraction

First, 1.8 ml of deionized water was added to each sample using a 2 ml scaled needle. The samples were then vortexed for 30 seconds. After that the closed tubes were left undisturbed at room temperature for 24 h. Following this, a Fugamix CM-50M centrifuge was used to sediment the material at 2000 rpm for 5 minutes. The water was then removed from the tube using a 1000 µL micropipette while being careful not to disturb the settled material. The described procedure was repeated 3 times in total. After that, the samples were placed in a dryer at 50 °C for 48 h. Finally, the samples were crushed in order to obtain a fine powder. Each sample then followed the steps described for the control.

Lipid extraction

The lipid extraction was carried out under a fume hood and with proper protective equipment. First, a 2:1 solution of chloroform-methanol was prepared using a scaled beaker. 1.8 mL of this solution was added to the tube of each sample using a scaled needle. The samples were then vortexed for 10 seconds. The closed tubes were then placed in a 30 °C water bath for 24 h. After that, the tubes were centrifuged at 2000 rpm for 6 minutes using a Fugamix CM-50M centrifuge. The chloroform-methanol solution was then poured off the tubes by tilting. 1.8 mL of a fresh 2:1 chloroform-methanol solution was then added to each sample. The tubes were again vortexed for 10 seconds and immediately centrifuged. The chloroform-methanol was again poured off the sample tubes. After that the sample tubes were left open under the fume hood for 24 h. Finally, the samples were crushed in order to obtain a fine powder. Each sample then followed the steps described for the control.

Urea and lipid extraction

For the urea and lipid extraction, the samples were subjected first to a lipid extraction and then urea extraction following the protocols described above. Each sample then followed the steps described for the control.