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1 # Interactive effects of urea and lipid content confound stable isotope analysis in
2 # elasmobranch fishes

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30 **Abstract**

31 Stable isotope analysis (SIA) is becoming a commonly used tool to study the ecology of elasmobranchs.
32 However, the retention of urea by elasmobranchs for osmoregulatory purposes may bias the analysis and
33 interpretation of SIA data. We examined the effects of removing urea and lipid on the stable isotope
34 composition of fourteen species of sharks, skates, and rays from the eastern North Pacific Ocean. While
35 effects were variable across taxa, removal of urea generally increased $\delta^{15}N$ and C:N. Urea removal had
36 less influence on $\delta^{13}C$, whereas extracting urea and lipid generally increased $\delta^{15}N$ and C:N while also
37 increasing $\delta^{13}C$. Because C:N values of non-extracted tissues are often used to infer lipid content and
38 adjust $\delta^{13}C$, shifts in C:N following urea extraction will change the inferred lipid content and bias any
39 mathematical adjustment of $\delta^{13}C$. These results highlight the importance of urea and lipid extraction and
40 demonstrate the confounding effects of these compounds, making it impossible to use C:N of non-urea-
41 extracted samples as a diagnostic tool to estimate and correct for lipid content in elasmobranch tissues.

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43 **Keywords:** Stable isotopes, urea, lipid, carbon, nitrogen, C:N, elasmobranch, mathematical lipid
44 correction, elasmobranch

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56 **Introduction**

57 Stable isotope analysis (SIA) uses the stable isotope composition of organismal tissue to
58 understand a diverse suite of biological and ecological processes. SIA is increasingly being used to
59 investigate the ecology of marine taxa (Peterson and Fry 1987, Michener and Kaufman 2007), including
60 sharks, skates, and rays (elasmobranchs) (Hussey et al. 2012b). Since SIA makes inferences based on the
61 chemical composition of tissues, certain compounds found in specific taxa can interfere with analysis and,
62 therefore, conclusions. Here, we investigate the effects of urea and lipid extraction on tissues from
63 fourteen elasmobranch species and report results that demonstrate the necessity to account for these
64 compounds when using SIA in elasmobranch studies.

65 The physiology and anatomy of elasmobranchs present unique challenges when applying SIA to
66 study their ecology. In particular, elasmobranchs retain urea ($(NH_2)_2CO$) and trimethylamine oxide
67 (TMAO (C_3H_9NO)) in their tissues for osmoregulatory processes (Ballantyne 1997, Olson 1999, Hazon et
68 al. 2003). This retention of urea can differentially bias stable isotope results depending upon the tissue
69 type examined (Hazon et al. 2008, Kim and Koch 2011, Hussey et al. 2012b, Churchill et al. 2015). As a
70 waste product, urea is expected to have low $\delta^{15}N$ values (Minagawa and Wada 1984, Balter et al. 2006)
71 because ^{14}N is preferentially concentrated in urea by deaminases and transaminases (Gannes et al. 1998).
72 We were unable to find any comparable data on TMAO, but as a waste product it also would be expected
73 to be depleted in ^{15}N. As a result, the relative concentrations of urea and TMAO in a tissue may influence
74 the $\delta^{15}N$ value of that tissue. As urea and TMAO (hereafter referred to together as urea) both contain
75 carbon, they could potentially affect $\delta^{13}C$. Kim and Koch (2011) reported that the carbon in urea is
76 enriched in $\delta^{13}C$ in some terrestrial taxa; however information on the isotopic composition of these waste
77 products, especially in aquatic taxa, remains lacking. Further complicating the effect of urea on SIA is its
78 varying concentration within organisms, which is influenced by a variety of factors including tissue type
79 (Ballantyne 1997), ambient salinity (Hazon et al. 2003, Pillans et al. 2005) and diet (Wood et al. 2010).
80 Information on how to address the effects of urea on SIA results is needed, both in terms of appropriate

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81 sample treatment methodology and data interpretation (Martinez del Rio et al. 2009, Logan and
82 Lutcavage 2010, Kim and Koch 2011, Hussey et al. 2012b, Li et al. 2016).

83 In addition to the potential effect of urea on the stable isotope composition of elasmobranchs, the
84 presence of lipids is known to influence the $\delta^{13}C$ values of tissues (Post et al. 2007, Martinez del Rio et al.
85 2009, Hussey et al. 2012a). Because lipids are depleted in ^{13}C relative to protein, the presence of lipid in
86 tissues can bias $\delta^{13}C$ values and increase the tissue carbon-to-nitrogen ratio (C:N) (Pinnegar and Polunin
87 1999, Post et al. 2007). Tissue samples with high lipid concentrations have lower $\delta^{13}C$ values than
88 samples of the same tissue with lipids removed (Post et al. 2007). To account for variation in lipids across
89 tissue types, researchers either chemically extract or mathematically correct for lipids based on the tissue
90 C:N, which has been used as a proxy for relative lipid content in tissues (Post et al. 2007).

91 The influence of lipid content on SIA data of elasmobranch tissues has been relatively well
92 studied (Kim and Koch 2011, Hussey et al. 2012a) compared to that of urea (Hussey et al. 2012b). Logan
93 and Lutcavage (2010) and Kim and Koch (2011) directly assess the effects of urea extraction on SIA data
94 of elasmobranchs. Logan and Lutcavage (2010) reported no effect of urea extraction on elasmobranch
95 tissues, whereas Kim and Koch (2011) reported a significant increase in $\delta^{15}N$ in urea-extracted tissues.
96 However, treatment methods differed between studies, with Kim and Koch (2011) using a more extensive
97 deionized water (DIW) extraction, which potentially resulted in more complete urea removal. Given that
98 lipid has a high C:N and urea has low C:N (0.5), removal of these compounds will influence tissue C:N.
99 Several studies examining the effect of lipid extraction on elasmobranch tissue noted increases in $\delta^{15}N$
100 and C:N following lipid extraction in a manner consistent with the removal of urea, suggesting that lipid
101 extraction may effectively remove urea as well as lipid (Hussey et al. 2010, Kim and Koch 2011, Hussey
102 et al. 2012a, Churchill et al. 2015, Li et al. 2016). However Kim and Koch (2011) reported that
103 elasmobranch tissues should have both urea and lipid-extracted to obtain the most reliable results. Li et al.
104 (2016) recently conducted the most thorough study of the interactive effects of urea and lipid to-date,
105 examining the effects of urea and lipid extraction on six species of pelagic sharks. They reported
106 significant increases in $\delta^{15}N$ and C:N following lipid extraction, urea extraction, and lipid and urea

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107 extraction, with δ^{13}C also increasing significantly in treatments with lipid extraction. Li et al. (2016)
108 supported the conclusion of Kim and Koch (2011) that both urea and lipid should be removed when
109 analyzing elasmobranch tissues for SIA.

110 Despite the reported shifts in C:N following urea and lipid extraction and recommendations to
111 make lipid extraction a standard practice when processing elasmobranch tissues for SIA, estimating lipid
112 content based on the C:N of unextracted bulk samples remains a common practice. Specifically, it is
113 typically assumed that tissues of aquatic organisms with C:N values < 3.3 – 3.5 have low lipid content
114 and do not warrant lipid extraction (Post et al. 2007). This is of potential concern since this does not
115 account for the influence of urea on C:N, which is then used to mathematically adjust δ^{13}C to account for
116 inferred (based on C:N) lipid content. As a result, the δ^{15}N and potentially δ^{13}C values of samples
117 processed without urea and/or lipid extraction may be biased, with any resulting analyses or ecological
118 interpretations being potentially based on inaccurate δ^{15}N and δ^{13}C values. Given the common use of δ^{15}N
119 to estimate trophic level and δ^{15}N and δ^{13}C to understand habitat use and trophic relationships in
120 elasmobranchs (Fisk et al. 2002, Estrada et al. 2003, Dale et al. 2011, Kim and Koch 2011, Vaudo and
121 Heithaus 2011, Carlisle et al. 2012, Hussey et al. 2012b), these biases may have important effects on the
122 ecological interpretation of SIA data.

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123 To examine how common this issue may be, we surveyed 50 recent scientific publications (2013-
124 present) that used SIA to study the ecology of elasmobranchs (Google scholar; search terms: "stable
125 isotope", "elasmobranch", "shark", "ray"; selected the first 50 pertinent results, Table S1). We found 28%
126 used low C:N values (< 3.5) of tissues containing urea to support not extracting lipid from their samples,
127 another 16% used mathematic corrections to adjust δ^{13}C based on lipid content estimates inferred from
128 C:N values of tissues containing urea, and 12% did not account for urea or lipid in any manner. Thus,
129 56% of the surveyed studies potentially had results biased due to not accounting for the combined effects
130 of urea and lipids. While it is not possible to know if the lack of urea or lipid extraction had any
131 meaningful effect on the isotopic results or their interpretation in these studies, it is clear previous

132 recommendations to make urea and lipid extraction the standard practice when analyzing elasmobranch
133 tissues for SIA (Kim & Koch 2011, Hussey et al. 2012a, Li et al. 2016) have not been fully adopted.
134 In this study, we expand upon previous work to better understand and account for the interactive
135 effects of urea and lipids on SIA in elasmobranch tissues. The importance of urea and lipid extraction has
136 been demonstrated in leopard sharks (*Triakis semifasciata*, Kim and Koch (2011) and a suite of pelagic
137 sharks (Li et al. 2016), yet published studies commonly do not appropriately account for the potential
138 effects of urea and lipid on elasmobranch tissues. In addition, the effects of applying multiple chemical
139 treatments to remove urea and lipid, and how to determine an appropriate methodological course for
140 individual species or taxa of interest, remain unaddressed for many elasmobranch taxa across broad
141 ranges of tissue compositions and habitats. In particular, while the interactive effects of urea and lipid
142 have been explored in sharks, they have not been investigated in batoids (skates and rays), a group that
143 comprises over 50% of extant elasmobranchs (Dulvy et al. 2014). Finally, it may not always be feasible or
144 desirable to perform lipid extraction (i.e. avoid the cost, chemical waste generated, and time associated
145 with chemical extraction or to preserve information on the movement of lipids through foodwebs). While
146 Li et al. (2016) provides species specific isotopic correction models to account for urea content in lipid
147 extracted samples, there is a lack of specific guidance in the literature on the appropriate development and
148 application of mathematical correction models for $\delta^{13}C$ based on inferred tissue lipid content (C:N) for
149 elasmobranchs that account for urea's effects on C:N, $\delta^{13}C$ and $\delta^{15}N$.
150 The goals of this study were to 1) assess the relative effects of urea and lipid extraction on the
151 stable isotope composition of muscle tissue from a variety of shark and batoid species, including pelagic,
152 demersal and benthic species, with variable lipid content, 2) address the utility of using C:N as a
153 diagnostic tool to understand and adjust for lipid content in elasmobranch tissue, particularly in the
154 context of the influence of urea on C:N, 3) develop models to mathematically adjust $\delta^{13}C$ of urea-
155 extracted samples to account for lipid content and 4) provide a conceptual framework to understand how
156 urea and lipid interact to influence SIA results in elasmobranchs in order to facilitate proper application of
157 the technique.

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158 **Materials and Methods**

159 To examine the effects of urea extraction and lipid extraction on elasmobranch tissues, we
160 collected white muscle samples from fourteen species, including six species of sharks from the families
161 Lamnidae, Carcharhinidae, Squalidae, and Triakidae, and eight species of batoids (skates, rays, and their
162 allies) from the families Arhynchobatidae, Rajidae, Myliobatidae, Gynmnuridae, Dasyatidae, and
163 Urolophidae. All samples were collected in the eastern North Pacific, primarily off California but ranging
164 as far north as the Gulf of Alaska. Samples were collected from juvenile salmon sharks (*Lamna ditropis*)
165 stranded on beaches in California and Oregon as described by Carlisle et al. (2015). Juvenile white sharks
166 (*Carcharodon carcharias*) caught as incidental bycatch in the coastal gillnet fisheries in southern
167 California were sampled as part of the Monterey Bay Aquarium juvenile white shark research program as
168 described in Mull et al. (2012). Samples from shortfin makos (*Isurus oxyrinchus)*, blue sharks (*Prionace*
169 *glauca*), and pelagic rays (*Pteroplatytrygon violacea*) were collected during the annual National Oceanic
170 and Atmospheric Administration (NOAA) Juvenile Shark Longline Survey off southern California.
171 Round stingray samples (*Urobatis halleri*), leopard shark (*Triakis semifasciata*), butterfly ray (*Gymnura*
172 *marmorata*), and bat rays (*Myliobatis californica*) were collected in southern California as described by
173 Lyons et al. (2014). Skates (*Bathyraja aleutica, Bathyraja interrupta, Beringraja binoculata, Raja rhina*)
174 were collected from the western Gulf of Alaska as described in Bizzarro et al. (2014). Spotted spiny
175 dogfish (*Squalus suckleyi*) were collected off central California during the National Marine Fisheries
176 Service Northwest Fisheries Science Center West Coast Groundfish Bottom Trawl Survey. All muscle
177 samples were collected from the dorsal musculature and stored frozen (-20°C) until processed and
178 analyzed.

179 Individual tissue samples were homogenized and subdivided into three parts for analysis, with
180 one as the control sample (Control), one for urea extraction (U), and one for both urea and lipid extraction
181 (UL). Methods to process tissues and extract lipids and urea generally followed Kim and Koch (2011). To
182 extract urea, tissue samples were placed in scintillation vials and rinsed three times in 10 mL of DIW
183 (Kim and Koch 2011). A rinse entailed sonication for 15 minutes and then decanting the supernatant.

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184 Lipids were extracted from all tissues except skate tissues using a 2:1 chloroform:methanol solution
185 (Bligh and Dyer 1959, Logan and Lutcavage 2010) by immersing tissues in the solution for 24 hours in
186 glass scintillation vials (Bligh and Dyer 1959, Logan and Lutcavage 2010). Following both treatments,
187 tissue samples were lyophilized and homogenized using a Spex/CertiPrep 5100 mill.

188 Skate samples were processed slightly differently than the other species as part of another study.
189 For urea extraction of skate tissue, 10 mL of DIW were added to each homogenized sample, and then the
190 samples were mixed using a vortex mixer (Fisher Scientific). After 30 minutes, the sample was
191 centrifuged and the supernatant was decanted. For samples that were lipid and urea-extracted, lipids were
192 extracted using Petroleum Ether (PE) following Kim and Koch (2011). Briefly, samples were immersed
193 in PE, mixed in a vortex mixer and left uncapped in a fume hood for 8 hours, centrifuged for 10 minutes,
194 and the supernatant decanted. The sample was then rinsed in DIW using the method described for urea
195 extraction of skate tissue. Following urea or urea and lipid extraction samples were dried in an oven at
196 60˚C for 24 hours.

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197 For each treatment, approximately 500 μg of tissue was weighed into tin boats and analyzed at
198 the Stable Isotope Laboratory at the University of California Santa Cruz (UCSC) using an elemental
199 analyzer coupled to an isotope ratio monitoring mass spectrometer (Delta XP-EA,Thermo- Finnagen
200 IRMS). For skate and dogfish samples, 500 μg of tissue was weighed into tin boats and analyzed at Idaho
201 State University (ISU) using an elemental analyzer coupled to an isotope ratio monitoring mass
202 spectrometer (Elemental Combustion System (ECS) 4010 interfaced with a Delta V Advantage mass
203 spectrometer through the ConFlo IV System). Isotopic composition is expressed using standard δ
204 notation, using Vienna Pee Dee Belemnite limestone as the standard for carbon and AIR for nitrogen. For
205 runs at UCSC, analytical precision, based on an internal lab standard (Pugel), was 0.11‰ for $\delta^{15}N$ and
206 0.07‰ for $\delta^{13}C$ across multiple runs. For runs at ISU, analytical precision, based on internal lab standards
207 of ISU Peptone, Costech Acetanilide, and DORM-3, was 0.08, 0.04, and 0.04 ‰ for $\delta^{15}N$ respectively and
208 0.05, 0.05, and 0.04 for $\delta^{13}C$, respectively. Where parametric assumptions were met (assessed with One-
209 Sample Kolmogorov-Smirnov and Levene's Tests and visual inspection of residuals) a single factor

210 ANOVA, followed by Tukey's post hoc tests, was used to test for differences in $\delta^{13}C$ and $\delta^{15}N$ among
211 treatments for species with sample sizes > 3. When assumptions were not met for $\delta^{13}C$, $\delta^{15}N$, or C:N
212 differences were tested using Mann-Whitney 2-sample tests with sequential Bonferroni adjustments (Rice
213 1989). To show the magnitude and direction of the effects of treatments U and UL, differences between
214 treatment and control samples (U – Control, UL – Control, UL - U) were calculated.

215 We considered four previously used lipid correction models (Post et al. 2007, Logan et al. 2008,
216 Reum 2011) to examine the utility of using C:N as a diagnostic tool to understand and adjust for lipid
217 content in urea-extracted elasmobranch tissue. Lipid correction models were used to characterize $\Delta^{13}C$,
218 the difference between lipid and urea (UL) and urea-extracted (U) $\delta^{13}C$ values ($\Delta^{13}C = \delta^{13}C_U - \delta^{13}C_{UL}$) as
219 a function of the C:N of urea-extracted tissue ($C{:}N_U$). The first (model 1) is a three-parameter model
220 derived by Logan et al. (2008) from McConnaughey and McRoy (1979): $\Delta^{13}C = (aC{:}N_U + b)(C{:}N_U + c)^{-1}$
221 , where a, the y-asymptote, corresponds to protein-lipid $\delta^{13}C$ discrimination and $-ba^{-1}$, the x-intercept, is
222 the urea and lipid free C:N value ($C{:}N_{UL}$), and bc^{-1}, the y-intercept, is the value of $\Delta^{13}C$ at $C{:}N_U = 0$. The
223 second (model 2) is a two parameter model (Fry 2002): $\Delta^{13}C = P - PF(C{:}N_U)^{-1}$, where P represents
224 protein-lipid $\delta^{13}C$ discrimination and F is $C{:}N_{UL}$. The third and fourth are linear models: (model 3, Logan
225 et al. 2008) $\Delta^{13}C = \beta_0 + \beta_1 \mathrm{Ln}(C{:}N_U)$ and (model 4, Post et al. 2007) $\Delta^{13}C = b + aC{:}N_U$, where
226 $e^{(-\beta_0 \beta_1^{-1})}$ and $-ba^{-1}$ are estimates of $C{:}N_{UL}$, respectfully.

227 We modeled the relationship between $C{:}N_U$ and $\Delta^{13}C$ for five groups: all species, batoids, all
228 sharks other than *S. suckleyi*, *S. suckleyi,* and *C. carcharias.* We modeled *S. suckleyi* independently since
229 its lipid content was higher than all other taxa and its urea-extracted samples had the widest range of C:N
230 values (Results). For *C. carcharias,* we wanted to attempt to develop a species-specific relationship and
231 this species had the largest sample size. To compare the performance of potential lipid correction models,
232 the corrected Akaike Information Criterion, AIC_c (Burnham and Anderson 2002), was calculated for each
233 model. The model with the lowest AIC_c is considered the best fit, but any model(s) with AIC_c values
234 within two units of the lowest value have strong support as well (Burnham and Anderson 2002). In

235 addition for those models that preformed best based on AIC_c, we calculated the mean and standard
236 deviation of the absolute values of the residuals errors, to further evaluate model fit, and compared
237 estimates of protein-lipid $\delta^{13}C$ discrimination and $C:N_{UL}$ (Logan et al. 2008, Reum 2011). All models
238 were fitted with least-squares procedures using R and the libraries nlme and AICcmodavg (www.r-
239 project.org).

240 **Results**

241 The removal of urea and lipid from elasmobranch tissue influenced $\delta^{15}N$, $\delta^{13}C$, and C:N in most
242 species, although the direction and magnitude of the effects varied by species and with the lipid content of
243 the sample (Table 1, Figures 1-2, Figure S1). The C:N of control samples was consistently low for all
244 species, with 13 of the 14 taxa having values < 3.5 (mean ± SD of all species 3.0 ± 0.4). The only
245 exception to this was *S. suckleyi*, which had a high C:N of 4.5 due to higher lipid content of its muscle.

246 Urea extraction (treatment U) generally increased $\delta^{15}N$ and C:N, but generally did not
247 significantly change $\delta^{13}C$, results that are consistent with the removal of isotopically light nitrogen present
248 in urea (Table 1, Figures 2a and 3a). In seven of the ten species (4 of 5 sharks, 3 of 5 batoids) that were
249 statistically tested, $\delta^{15}N$ increased significantly following urea extraction (mean 0.8‰ ± 0.2). This result
250 was very similar to the overall trend across all taxa, which showed an average increase of 0.7‰ ± 0.2.
251 Three taxa had increases in $\delta^{15}N$ greater than 1‰ (*B. aleutica* 1.1‰, *L. ditropis* 1.1‰ and *I. oxyrinchus*
252 1.0 ‰). $\delta^{13}C$ only changed significantly in *B. binoculata* (-0.9‰) and *U. halleri* (-0.5‰). Overall, there
253 was a consistent, though generally non-significant, decrease in $\delta^{13}C$ across the batoids that was not
254 evident in sharks (mean -0.4‰ for batoids, 0.0‰ for sharks, and -0.2‰ ± 0.3 for all taxa). C:N increased
255 significantly in nine of the ten taxa statistically tested (4 of 5 sharks, 5 of 5 batoids), with the exception
256 being *S. suckleyi*, which had a high initial C:N that increased from 4.5 to 5.6 (+ 1.1‰) following urea
257 extraction. Overall, C:N increased by an average of 0.7 (± 0.2) across all taxa following urea extraction.
258 C:N values increased to values above 3 (mean C:N of 3.6) in all species, and the C:N of *C. carcharias, L.*
259 *ditropis, G. marmorata, and U. halleri* increased to values above 3.5.

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260 Extracting urea and lipid (treatment UL) from samples consistently increased $\delta^{15}N$ and C:N in a
261 fashion similar to what was observed with urea extraction only, while also generally increasing $\delta^{13}C$
262 (Table 1, Figures 2b and 3b). Seven out of the ten taxa tested had significantly higher $\delta^{15}N$ values
263 following urea and lipid extraction, although all taxa showed some increase (mean 0.8‰ ± 0.2). *L.*
264 *ditropis* showed the largest increase in $\delta^{15}N$ following lipid extraction (1.1‰). Four of the five
265 statistically tested sharks had significantly higher $\delta^{13}C$ following urea and lipid extraction, with dogfish
266 (mean 2.0‰), salmon sharks (1.5‰) and white sharks (1.1‰) having the largest increases. Three batoids
267 showed a decrease in $\delta^{13}C$ following urea and lipid extraction, with *B. binoculata* exhibiting a significant
268 decrease (mean -0.9‰). Except for *S. suckleyi*, all taxa exhibited an increase in C:N (mean increase 0.3 ±
269 0.3, mean of species C:N 3.3), with nine of ten taxa tested statistically having significant changes. C:N of
270 *S. suckleyi* decreased, though non-significantly, following urea and lipid extraction (control C:N 4:5, UL
271 C:N 3.9).

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272 The differences between the U and UL treatments were more obvious in sharks than in the
273 batoids, in which the differences were relatively small (Table 1). All taxa showed an increase in $\delta^{13}C$ in
274 the UL treatment relative to the U treatment, although only three of the five sharks (*C. carcharias, L.*
275 *ditropis* and *S. suckleyi*), and one of five batoids (*U. halleri*), had significant increases. There were no
276 significant differences in $\delta^{15}N$ between U and UL treatments in any species examined ($p > 0.05$),
277 indicating that lipid extraction did not affect $\delta^{15}N$. C:N was generally lower in the UL treatment relative
278 to the U treatment, especially in the sharks. In the five shark species tested, all had significant decreases
279 in C:N in UL treatments relative to U treatments, whereas only two of the five tested batoids had
280 significant decreases.

281 Lipid correction models were created to adjust urea-extracted tissue $\delta^{13}C$ to account for lipid
282 content. Model performance varied across elasmobranch groups (Figure 3, Figure S2), with no single
283 model amongst those exhibiting the lowest AIC_c values across all groups (see supplementary Table S2 for
284 AIC_c Values, r^2 (linear models only) and model parameters). For all species pooled, models 1 and 2 (non-
285 linear models) had the lowest AIC_c, with identical mean ± SD of the absolute values of the residual errors

286 (MRE, 0.29 ± 0.24) and similar estimates of protein-lipid $\delta^{13}C$ discrimination (5.39 and 6.12) and $C:N_{UL}$
287 (3.20 and 3.18). All four models for sharks (excluding *S. suckleyi*) had similarly low AIC_c values (i.e.
288 within 2 units), MREs (0.20 – 0.25 ± 0.25 – 0.29) and estimates of $C:N_{UL}$ (3.10 – 3.13). However,
289 estimates of protein-lipid $\delta^{13}C$ discrimination for sharks varied widely between models 1 (1.94) and 2
290 (7.86, Table S2). The two linear models (3 and 4) performed equally for batoids (Table S2), with identical
291 MRE (0.21 ± 0.16) and estimates of $C:N_{UL}$ (3.27). Model performance varied between the two single
292 species groups. For *S. suckleyi*, which had both the widest range and highest C:N values, model 2
293 provided the singular best fit (MRE 0.26 ± 0.24) with estimates of protein-lipid $\delta^{13}C$ discrimination and
294 $C:N_{UL}$ of 6.31 and 3.35, respectively (Table S2) . In contrast, for *C. carcharias*, which had only two urea-
295 extracted C:N values > 4, models 2, 3 and 4 had similarly low AIC_c, MREs (0.12 – 0.13 ± 0.12 – 0.13)
296 and estimates of $C:N_{UL}$ (3.05 – 3.14). The estimate of urea extracted protein-lipid $\delta^{13}C$ discrimination for
297 *C. carcharias* was the highest in the study (8.30, Table S2).

298 **Discussion**

299 Our results provide further evidence of the substantial, direct affect that urea can have on $\delta^{15}N$.
300 We also show the important, and often unconsidered, indirect role urea plays in influencing $\delta^{13}C$ values
301 by lowering the C:N, effectively masking lipid content and leading to inaccurate assessments of lipid
302 content and inappropriate mathematical corrections (Figure 4). Our results indicate that urea must be
303 removed to obtain reliable $\delta^{15}N$ and C:N values, and that only with urea-extracted tissues can C:N be used
304 as a diagnostic tool for understanding and mathematically adjusting for lipid content.

305 Urea extraction resulted in an increase in $\delta^{15}N$ across all taxa, and a significant increase in 7 of 10
306 statistically tested taxa, ranging from ~0.5 to 1.1‰ (mean 0.7 or 0.8‰ for U and UL respectively), with
307 urea and lipid extraction producing similar changes. When using $\delta^{15}N$ to infer trophic level, this shift is
308 equivalent to an inferred trophic level difference of ~22 – 50% or ~15 – 30% assuming a trophic
309 discrimination factor for nitrogen of 2.3‰ (Hussey et al. 2010) or 3.7‰ (Kim et al. 2012), respectively.
310 This shift is similar to that reported by Hussey et al. (2012a) for elasmobranch tissues following lipid
311 extraction as well as by Li et al. (2016) following both urea extraction and urea and lipid extraction.

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312 Clearly the presence of urea in analyzed tissues will directly bias the use of $\delta^{15}N$ as a tracer in ecological
313 studies, whether it is being used to assess trophic level, reconstruct diet, habitat or migration patterns, or
314 even for simple qualitative comparisons.

315 The urea effects we describe here also have important implications for the use of C:N as a metric
316 of lipid content of elasmobranch tissue. The C:N of non-extracted elasmobranch tissues are consistently
317 very low (< 3) across studies (Logan and Lutcavage 2010, Matich et al. 2010, Dale et al. 2011, Kim and
318 Koch 2011, Vaudo and Heithaus 2011, Hussey et al. 2012a, Hussey et al. 2012b), and are often much
319 lower than would be expected of pure protein. Frequently, when tissues with C:N values < 3.5 are
320 assumed to have little lipid content (Post et al. 2007), the low C:N values are used to infer that lipid
321 extraction is not warranted. However, results from this study and previous work (Kim and Koch 2011,
322 Hussey et al. 2012a, Li et al. 2016) demonstrate that urea extraction generally increases the C:N as
323 nitrogen is removed (Figures 1 and 2). In this study, extracting urea through DIW rinses caused
324 significant increases in C:N in every species tested, increasing it by as much as 1.2‰ (mean 0.7‰).

325 Removal of the nitrogen contributed by urea will increase the C:N value of a sample, thereby
326 changing the estimated lipid content that are based on C:N (Figure 4). Following removal of urea, C:N
327 can increase from very low values to values above threshold levels that are used to indicate low lipid
328 content (e.g. 3.5). In effect, the presence of urea and its lowering of C:N has the potential to mask lipid
329 content. In four of the species examined in this study (*C. carcharias, L. ditropis, G. marmorata* and *U.*
330 *halleri*) the C:N value shifted from values < 3 to values > 3.5, and other species had C:N values of ~3.3
331 following DIW rinses, which is similar to pure protein values (Post et al. 2007). The interpretation of
332 these urea-extracted samples would then be that lipid extraction is warranted, and in two of these species
333 (*C. carcharias, L. ditropis)*, there was a significant increase in $\delta^{13}C$ following lipid extraction.
334 Importantly, despite having C:N values ~3.3 following urea extraction, most of the species exhibited an
335 increase in $\delta^{13}C$ following lipid extraction. These findings suggest that lipid extraction can significantly
336 affect $\delta^{13}C$ even when C:N is < 3.5. Hence, lipid extraction may be required even in a tissue that is
337 relatively lean, a result that is concordant with the findings of Li et al. (2016). Our results indicate that

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338 failure to lipid extract elasmobranch tissues based on low C:N values of untreated tissue, where urea has
339 not been removed, will result in biased δ^{15}N values, due to the inclusion of the isotopically light nitrogen
340 of urea, and potentially biased δ^{13}C values as well, due to inclusion of lipid content that was masked by
341 low C:N values.

342 An additional important, and generally unrecognized implication of the effect of urea on C:N is
343 that it will bias C:N based arithmetic corrections that are used to adjust δ^{13}C in lieu of lipid extracting
344 tissues. Any adjustment to δ^{13}C values that is based on C:N values from non-urea-extracted tissue will be
345 biased, although the magnitude of the effect will vary based on the urea and lipid content of the tissue.
346 Thus, failing to extract urea will not only bias δ^{15}N, but by affecting C:N it will lead to incorrect estimates
347 of inferred lipid content upon which mathematical correction models rely. As our results indicate,
348 however, it is possible to develop models to adjust urea-extracted tissues to account for lipid content
349 when lipid extraction is not feasible. For example, the model we developed for *C. carcharias*
350 demonstrates that even with a relatively small sample size (n = 19), we were able to generate a robust
351 species-specific simple linear model based on urea-extracted tissue C:N (model 4, r^2 = 0.92, with no
352 systematic prediction biases based on visual inspection of residuals). Deriving taxa-specific relationships
353 is always desirable, but for sharks (excluding dogfish) all four models examined seem to provide
354 potentially suitable lipid correction models. However, examination of model parameters reveals that for
355 model 1 the estimation of protein-lipid δ^{13}C discrimination is unrealistically low, ~3 times, or more, lower
356 than other estimates from this study and the generally reported range of 5–8‰ (Fry 2002, Post et al. 2007,
357 Logan et al. 2008, Reum 2011), demonstrating the need to consider other factors beyond AIC_c and fit (r^2
358 and residual distribution) when determining the suitability of a correction model. Sharks (excluding
359 dogfish) have C:N of urea-extracted tissues below ~4.5 and there appears to be a linear relationship
360 between C:N and Δ^{13}C. This suggests that linear correction models might be most appropriate for sharks
361 with relatively low C:N values (~4.5), which is concordant with the findings of Post et al. (2007) for
362 aquatic organisms over a similar C:N range. This relationship is likely non-linear when tissues span a
363 wide range of C:N values, such as with *S. suckleyi* (Logan et al. 2008, Reum 2011). It is therefore

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364 important to consider the range in lipid content in species and tissues of interest when developing and
365 applying lipid correction models. Batoids had a less clear C:N relationship relative to other taxa in the
366 study. While exhibiting a significant linear relationship between $C:N_u$ and $\Delta^{13}C$, the models explained a
367 relatively low proportion of the variability in $\Delta^{13}C$ (models 3 and 4, $r^2 = 0.56$ and 0.58) and may not
368 provide the same relative correction across all batoids (Figure 3). This emphasizes that the interaction
369 between urea and lipid content may change across disparate elasmobranch taxonomic groups.

370 Although our results indicate that urea directly influences $\delta^{15}N$ values and potentially indirectly
371 influences the $\delta^{13}C$ of elasmobranch tissues by affecting the C:N and inferred lipid content, the effects are
372 variable and species-dependent (Figures 1, 2). For species that have low lipid content in their muscle,
373 such as batoids (e.g. ~0.2% *in U. halleri*, (Lyons unpublished data), ~1-2% in *B. binoculata* and *R. rhina*
374 (Farrugia et al. 2015)), the effect on $\delta^{13}C$ will be minimal, but $\delta^{15}N$ may change substantially. In species
375 with higher lipid content, such as *L. ditropis*, which can have lipid content as high as 6.5 to 14.6% (mean
376 9%) in their muscle (data source: https://dec.alaska.gov/eh/vet/fish.htm), the effect on $\delta^{13}C$ and $\delta^{15}N$ will
377 be significant. The relatively low lipid content of batoid muscle compared to shark muscle may underlie
378 the observed general decrease in $\delta^{13}C$ of batoids following urea extraction while urea and lipid extraction
379 showed less overall change in $\delta^{13}C$ relative to the control samples. As described by Kim and Koch (2011),
380 the carbon in urea is enriched in ^{13}C relative to the diet in humans (enriched 3 – 5‰) and cattle (0 –
381 3.5‰), suggesting that its removal may reduce the $\delta^{13}C$ (Ivlev et al. 1996, Knobbe et al. 2006). In batoids
382 with low lipid content, removal of the ^{13}C enriched urea would reduce $\delta^{13}C$, while lipid extraction, by
383 removing a small amount of ^{13}C depleted lipids, would offset the removal of urea and result in little net
384 change in $\delta^{13}C$. This effect would vary based on both the concentration of urea as well as lipid content of
385 the tissue and again highlights the importance of understanding how these relationships change across
386 taxa. It is possible that other differences in the composition of batoid tissue may play a role as well, such
387 as differences in urea concentration or the presence of ceratotrichia, but this remains unclear.

388 Although lipid extraction by itself may remove lipids and much of the urea present in tissues
389 (Hussey et al. 2012a, Churchill et al. 2015), we reiterate the recommendations of Kim and Koch (2011)

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390 and Li et al. (2016) to make lipid and urea extraction the standard practice when analyzing elasmobranch
391 tissues for SIA. Since extracting urea is simple and inexpensive, there is no practical reason not to remove
392 it. In addition, our results indicate that $\delta^{13}C$ may change significantly following lipid extraction even in
393 apparently lean tissues with relatively low C:N, suggesting that lipid extraction may be warranted in all
394 situations as suggested by Li et al. (2016). However, in some taxa that are very lean, such as the batoids in
395 this study, lipid extraction may not be required. However, urea would still need to be extracted to evaluate
396 the need for lipid extraction or correction. In instances where it is not feasible or desirable to lipid extract
397 every sample, we demonstrate that it is possible to develop species- or group-specific correction curves to
398 adjust for lipid content in urea-extracted tissues. Though lipid extraction did not affect $\delta^{15}N$ in our study,
399 a potential benefit of using mathematical correction models is the ability to account for the effect of lipid
400 content on $\delta^{13}C$ while avoiding potential effects of chemical extraction on $\delta^{15}N$, which have been reported
401 previously in other taxa (Post et al. 2007). However, our results show that the confounding effects of urea
402 and lipids make it impossible to use C:N of non-urea-extracted samples as a diagnostic tool to determine
403 the proper method of tissue treatment, something that occurs regularly in the literature.

404 The changes in the stable isotope composition of elasmobranch tissue resulting from urea and
405 lipid extraction will be mediated by the relative concentration of those compounds (Figure 4), which vary
406 across taxa, and their differential effects on $\delta^{13}C$ and $\delta^{15}N$ (Figures 1 & 2). We conclude that at a
407 minimum, urea should be removed to evaluate lipid content and a species or group specific lipid
408 correction relationship created to account for lipid content. The most robust approach to most confidently
409 eliminate bias and to facilitate comparisons across studies will be to apply urea and lipid removal
410 techniques in SIA-based ecological studies of elasmobranchs.

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Tables

SHARKS

Species	ID	Treatment	$\delta^{13}C$ mean	sd	C test	U test	$\delta^{15}N$ mean	sd	C test	U test	C:N mean	sd	C test	U test	n	TL (cm) mean (SD)
Carcharodon	CC	C	-16.8	0.9	-	-	16.8	0.6	-	-	2.8	0.2	-	-	19	161 (21)
carcharias		U	-16.6	0.8	ns	-	17.3	0.7	*	-	3.6	0.3	*	-		
		UL	-15.6	0.5	*	*	17.3	0.7	*	ns	3.2	0.0	*	*		
Isurus	IO	C	-18.0	0.2	-	-	16.4	0.2	-	-	2.8	0.1	-	-	8	147 (22)
oxyrinchus		U	-17.8	0.7	ns	-	17.4	0.3	*	-	3.3	0.1	*	-		
		UL	-17.6	0.2	ns	ns	17.4	0.3	*	ns	3.2	0.1	*	*		
Lamna	LD	C	-19.4	0.4	-	-	14.4	0.3	-	-	2.9	0.2	-	-	10	106 (9)
ditropis		U	-19.0	0.4	ns	-	15.4	0.4	*	-	3.7	0.2	*	-		
		UL	-17.9	0.6	*	*	15.5	0.4	*	ns	3.2	0.1	*	*		
Prionace	PG	C	-18.0	0.2	-	-	16.1	0.6	-	-	2.6	0.1	-	-	10	116 (26)
glauca		U	-18.1	0.3	ns	-	16.9	0.5	*	-	3.2	0.0	*	-		
		UL	-17.8	0.2	*	ns	17.0	0.6	*	ns	3.1	0.0	*	*		
Squalus	SS	C	-19.4	1.1	-	-	14.0	0.7	-	-	4.5	1.1	-	-	9	73 (15)
suckleyi		U	-19.7	1.0	ns	-	14.5	0.6	ns	-	5.6	1.2	ns	-		
		UL	-17.4	0.2	*	*	14.9	0.6	*	ns	3.9	0.1	ns	*		
Triakis	TS	C	-16.0	0.3			15.3	0.2			2.9	0.3			2	U
semifasciata		U	-16.1	0.5			16.0	0.2			3.3	0.0				
		UL	-15.7	0.4			16.0	0.2			3.1	0.0				

BATOIDS

Species	ID	Treatment	$\delta^{13}C$ mean	sd	C test	U test	$\delta^{15}N$ mean	sd	C test	U test	C:N mean	sd	C test	U test	n	TL (cm) mean (SD)
Bathyraja	BA	C	-16.7	0.5	-	-	15.0	0.5	-	-	2.7	0.0	-	-	7	128 (20)
aleutica		U	-16.9	0.4	ns	-	16.1	0.5	*	-	3.4	0.0	*	-		
		UL	-16.7	0.4	ns	ns	15.9	0.5	*	ns	3.4	0.0	*	ns		
Bathyraja	BI	C	-16.3	0.4			15.7	0.4			2.9	0.0			3	72 (14)
interrupta		U	-16.6	0.3			16.2	0.5			3.4	0.0				
		UL	-16.5	0.4			16.1	0.2			3.3	0.0				
Gymnura	GM	C	-17.0	1.1			16.6	0.9			3.1	0.3			3	U
marmorata		U	-17.2	1.5			17.0	0.9			3.7	0.4				
		UL	-16.1	0.9			17.2	0.7			3.2	0.0				
Myliobatis	MC	C	-16.4	1.2			16.2	0.4			3.0	0.4			2	U
californica		U	-16.5	1.2			16.9	0.1			3.3	0.0				
		UL	-16.2	1.2			17.0	0.0			3.1	0.0				
Pteroplatytrygon	PV	C	-18.6	0.5	-	-	13.5	0.8	-	-	2.6	0.1	-	-	7	55 (9)
violacea		U	-18.8	0.5	ns	-	13.9	1.2	ns	-	3.4	0.1	*	-		
		UL	-18.5	0.4	ns	ns	14.1	0.9	ns	ns	3.2	0.1	*	*		
Beringraja	BB	C	-15.2	0.5	-	-	15.0	0.1	-	-	2.9	0.1	-	-	5	132 (24)
binoculata		U	-16.2	0.4	*	-	15.5	0.3	*	-	3.4	0.0	*	-		
		UL	-16.1	0.4	*	ns	15.5	0.3	*	ns	3.4	0.0	*	ns		
Raja	RR	C	-16.2	1.0	-	-	15.7	0.7	-	-	2.9	0.0	-	-	6	104 (11)
rhina		U	-16.8	0.6	ns	-	16.5	0.8	ns	-	3.4	0.0	*	-		
		UL	-16.7	0.6	ns	ns	16.4	0.7	ns	ns	3.4	0.1	*	ns		
Urobatis	UH	C	-15.0	0.3	-	-	15.7	0.3	-	-	2.7	0.1	-	-	11	15 (4)
halleri		U	-15.4	0.3	*	-	16.5	0.3	*	-	3.8	0.1	*	-		
		UL	-14.9	0.3	ns	*	16.5	0.4	*	ns	3.4	0.1	*	*		

Table 1: Effects of urea and lipid extraction on stable isotope composition of various sharks (top panel) and batoids (bottom panel). Treatments are C (control), U (urea-extracted), UL (urea & lipid-extracted). C test shows results of statistical comparisons between the control (C) and U and UL treatments, whereas U test shows comparison between U and UL (ns = not significant, * = significant, $p < 0.05$). Note that for taxa with low sample sizes (<=3) we did not test for statistical differences.

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573 **Figure Captions**
574
575 Figure 1: Differences in $\delta^{13}C$, $\delta^{15}N$, and C:N between urea-extracted and control samples (A) and urea-
576 and lipid-extracted and control samples (B) in sharks. Statistically significant differences between control
577 and U and UL treatments are indicated (* $p <= 0.05$). Species are *Prionace glauca* (PG), *Squalus suckleyi*
578 (SS), *Triakis semifasciata* (TS), *Isurus oxyrinchus* (IO), *Lamna ditropis* (LD), and *Carcharodon*
579 *carcharias* (CC). We did not test for differences in TS due to low sample size.
580
581 Figure 2: Differences in $\delta^{13}C$, $\delta^{15}N$, and C:N between urea-extracted and control samples (A) and urea-
582 and lipid- extracted and control samples (B) in batoids. Statistically significant differences between
583 control and U and UL treatments are indicated (* $p <= 0.05$). Species are *Bathyraja aleutica* (BA),
584 *Bathyraja interrupta* (BI), *Beringraja binoculata* (BB), *Raja rhina* (RR), *Myliobatis californica* (IO),
585 *Gymnura marmorata* (TS), *Pteroplatytrygon violacea* (PV), and *Urobatis halleri* (UH). We did not test
586 for differences in BI, MC and GM due to low sample sizes.
587
588 Figure 3: Relationships between the C:N of urea-extracted tissue (treatment U, $C{:}N_u$) and changes in $\delta^{13}C$
589 between the U and UL treatments ($\Delta\delta^{13}C = \delta^{13}C_U - \delta^{13}C_{UL}$). Lines show selected best fit modeled
590 relationship between $C{:}N_u$ and $\Delta\delta^{13}C$ using best fit models based on AIC_c, which for groups with
591 relatively low lipid content and $C{:}N_u$ (batoids and all sharks except for dogfish) was a linear model, but
592 for groups with a higher lipid content and $C{:}N_u$ was based on the two parameter model from Fry (2002).
593
594 Figure 4: Conceptual diagram showing relative effects of urea and lipid extraction on elasmobranch
595 muscle tissue. Axes show relative change in $\delta^{13}C$ and $\delta^{15}N$ following different treatments, and color bar
596 shows C:N. The secondary axes show the relative effect of urea and lipid removal on $\delta^{13}C$ and $\delta^{15}N$, with
597 the size of arrows indicating relative magnitude of effect. Urea extraction will generally increase $\delta^{15}N$ and
598 C:N, and potentially also affect $\delta^{13}C$ as ^{13}C enriched urea is removed. Lipid extraction does not influence
599 $\delta^{15}N$, but increases $\delta^{13}C$ and reduces C:N. The degree to which the different treatments affect $\delta^{13}C$, $\delta^{15}N$
600 (depicted by the magnitude and direction of the "Urea extraction" and "Urea & lipid extraction" arrows)
601 and C:N (depicted by the shading gradient within each treatment arrow) will vary based on the urea and
602 lipid (dashed arrow) content of the tissue.
603
604 Figure S1: Changes in $\delta^{13}C$ and $\delta^{15}N$ of urea-extracted (grey) and urea- & lipid-extracted samples (black)
605 relative to control samples in sharks (A) and batoids (B). Dotted lines show relative shift in values
606 between urea-extracted and urea & lipid-extracted samples. Shark species are *Prionace glauca* (PG),
607 *Squalus suckleyi* (SS), *Triakis semifasciata* (TS), *Isurus oxyrinchus* (IO), *Lamna ditropis* (LD), and
608 *Carcharodon carcharias* (CC). Batoid species are *Bathyraja aleutica* (BA), *Bathyraja interrupta* (BI),
609 *Beringraja binoculata* (BB), *Raja rhina* (RR), *Myliobatis californica* (IO), *Gymnura marmorata* (TS),
610 *Pteroplatytrygon violacea* (PV), and *Urobatis halleri* (UH).
611
612 Figure S2: Relationship between the C:N of urea extracted tissue (U, $C{:}N_u$) and changes in $\delta^{13}C$ between
613 the U and UL treatments ($\Delta\delta^{13}C = \delta^{13}CU - \delta^{13}C_{UL}$) for individual species. Inset: magnified view of data
614 with low C:N (< 4.4).
615
616
617

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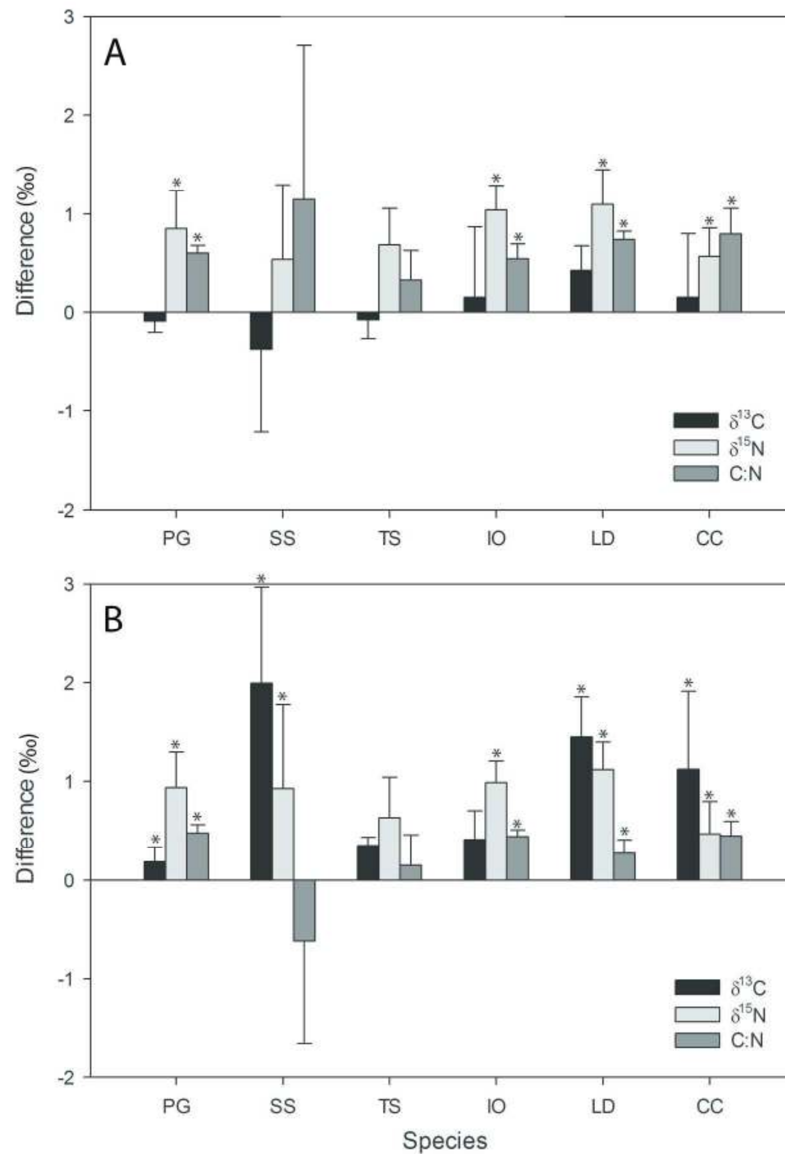

Figure 1: Differences in $\delta^{13}C$, $\delta^{15}N$, and C:N between urea-extracted and control samples (A) and urea- and lipid-extracted and control samples (B) in sharks. Statistically significant differences between control and U and UL treatments are indicated (* p <= 0.05). Species are *Prionace glauca* (PG), *Squalus suckleyi* (SS), *Triakis semifasciata* (TS), *Isurus oxyrinchus* (IO), *Lamna ditropis* (LD), and *Carcharodon carcharias* (CC).We did not test for differences in TS due to low sample size.

205x256mm (300 x 300 DPI)

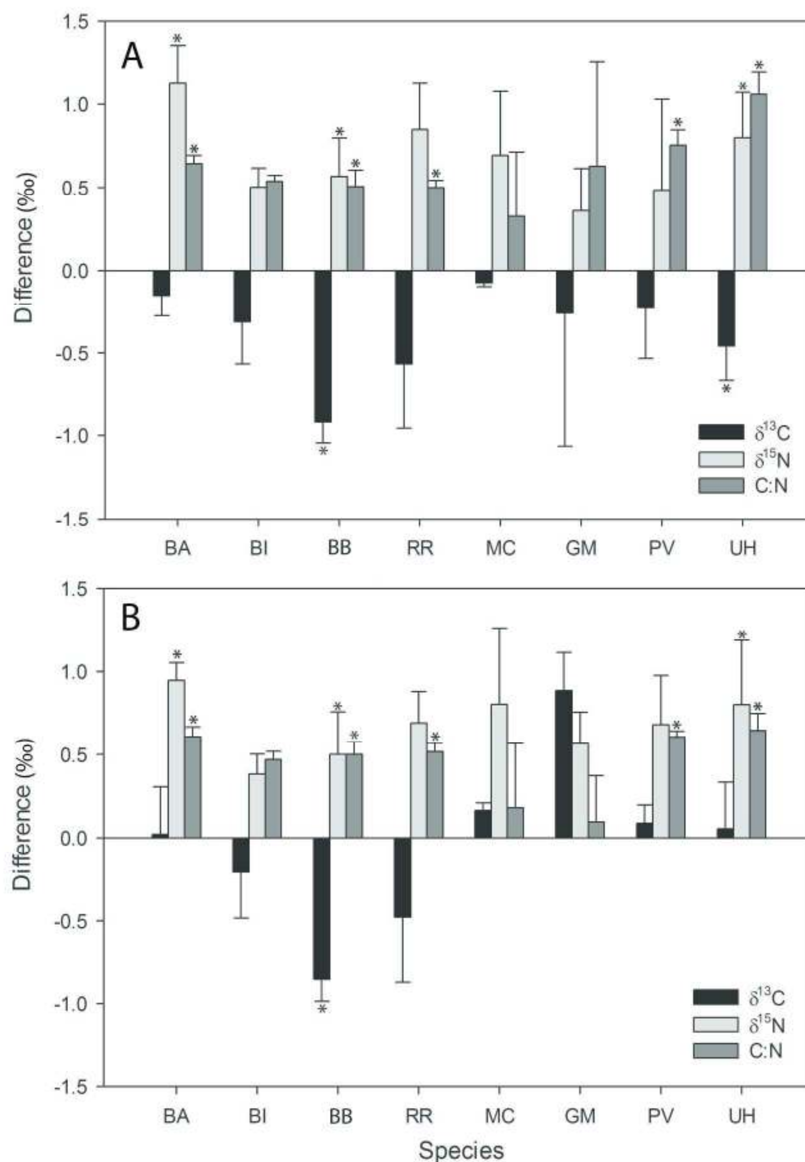

Figure 2: Differences in $\delta^{13}C$, $\delta^{15}N$, and C:N between urea-extracted and control samples (A) and urea- and lipid- extracted and control samples (B) in batoids. Statistically significant differences between control and U and UL treatments are indicated (* p <= 0.05). Species are *Bathyraja aleutica* (BA), *Bathyraja interrupta* (BI), *Beringraja binoculata* (BB),*Raja rhina* (RR), *Myliobatis californica* (IO), *Gymnura marmorata* (TS), *Pteroplatytrygon violacea* (PV), and *Urobatis halleri* (UH). We did not test for differences in BI, MC and GM due to low sample sizes.

207x261mm (300 x 300 DPI)

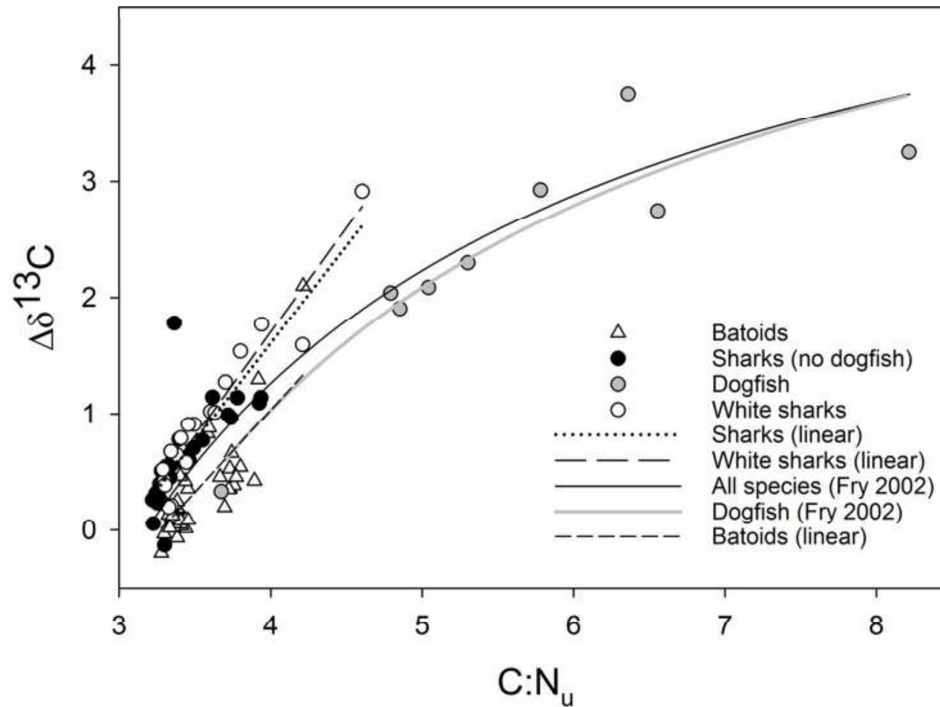

Figure 3: Relationships between the C:N of urea-extracted tissue (treatment U, $C:N_u$) and changes in $\delta^{13}C$ between the U and UL treatments ($\Delta\delta^{13}C = \delta^{13}C_U - \delta^{13}C_{UL}$). Lines show selected best fit modeled relationship between $C:N_u$ and $\Delta\delta^{13}C$ using best fit models based on AIC_c, which for groups with relatively low lipid content and $C:N_u$ (batoids and all sharks except for dogfish) was a linear model, but for groups with a higher lipid content and $C:N_u$ was based on the two parameter model from Fry (2002).

122x98mm (300 x 300 DPI)

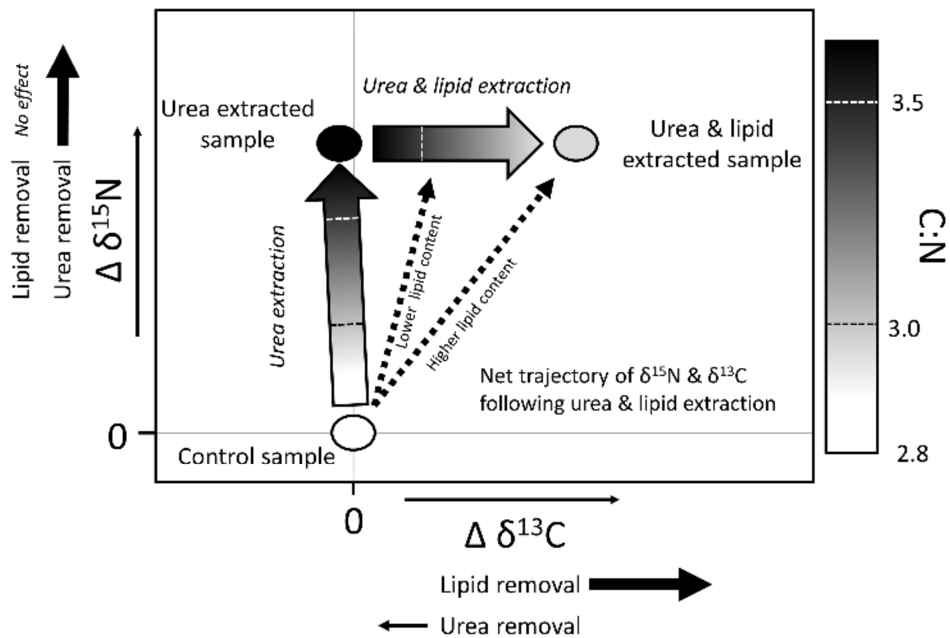

Figure 4: Conceptual diagram showing relative effects of urea and lipid extraction on elasmobranch muscle tissue. Axes show relative change in $\delta^{13}C$ and $\delta^{15}N$ following different treatments, and color bar shows C:N. The secondary axes show the relative effect of urea and lipid removal on $\delta^{13}C$ and $\delta^{15}N$, with the size of arrows indicating relative magnitude of effect. Urea extraction will generally increase $\delta^{15}N$ and C:N, and potentially also affect $\delta^{13}C$ as ^{13}C enriched urea is removed. Lipid extraction does not influence $\delta^{15}N$, but increases $\delta^{13}C$ and reduces C:N. The degree to which the different treatments affect $\delta^{13}C$, $\delta^{15}N$ (depicted by the magnitude and direction of the "Urea extraction" and "Urea & lipid extraction" arrows) and C:N (depicted by the shading gradient within each treatment arrow) will vary based on the urea and lipid (dashed arrow) content of the tissue.

246x171mm (300 x 300 DPI)

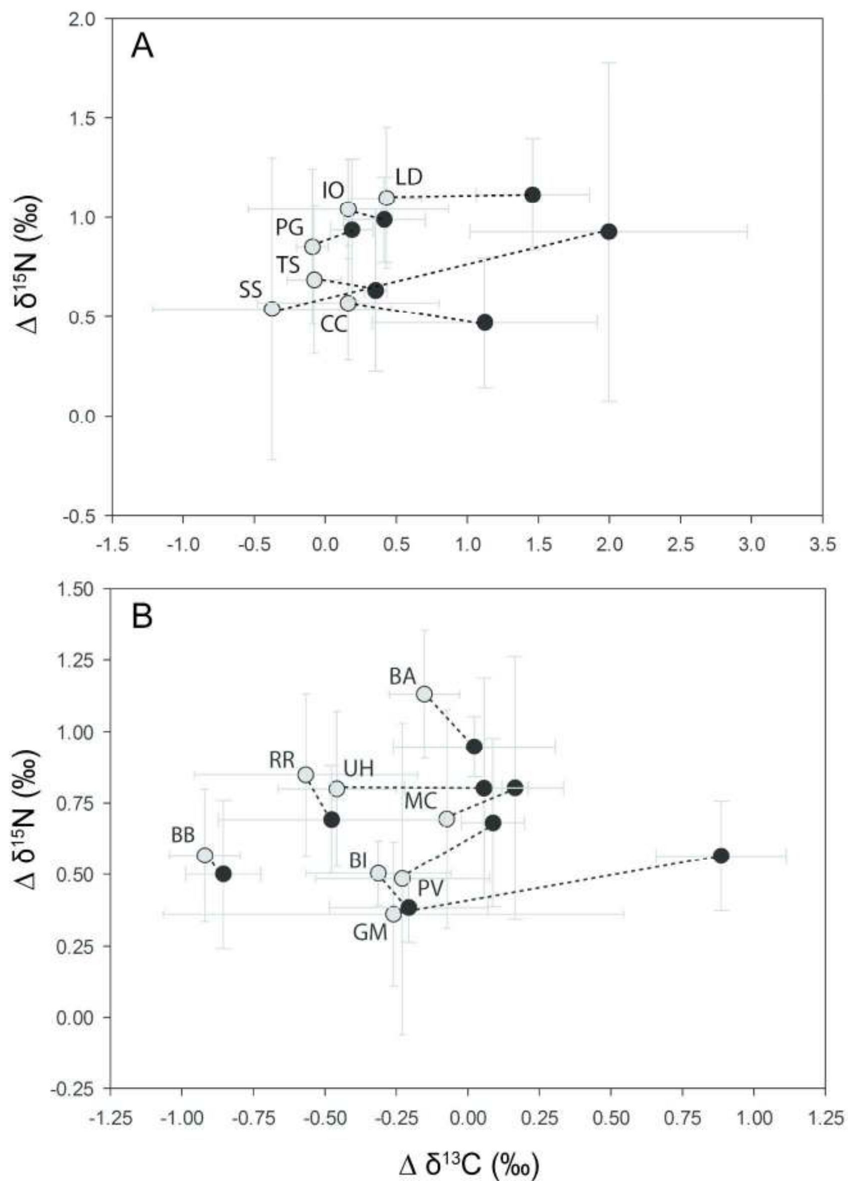

Figure S1: Changes in $\delta^{13}C$ and $\delta^{15}N$ of urea-extracted (grey) and urea- & lipid-extracted samples (black) relative to control samples in sharks (A) and batoids (B). Dotted lines show relative shift in values between urea-extracted and urea & lipid-extracted samples. Shark species are *Prionace glauca* (PG), *Squalus suckleyi* (SS), *Triakis semifasciata* (TS), *Isurus oxyrinchus* (IO), *Lamna ditropis* (LD), and *Carcharodon carcharias* (CC). Batoid species are *Bathyraja aleutica* (BA), *Bathyraja interrupta* (BI), *Beringraja binoculata* (BB), *Raja rhina* (RR), *Myliobatis californica* (IO), *Gymnura marmorata* (TS), *Pteroplatytrygon violacea* (PV), and *Urobatis halleri* (UH).

209x273mm (300 x 300 DPI)

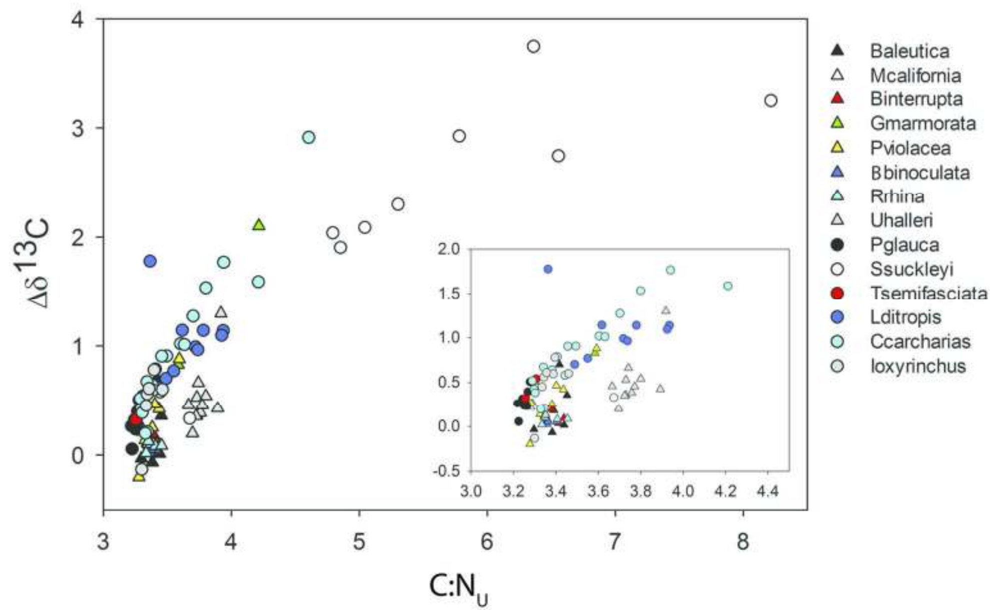

Figure S2: Relationship between the C:N of urea extracted tissue (U, $C:N_u$) and changes in $\delta^{13}C$ between the U and UL treatments ($\Delta\delta^{13}C = \delta^{13}C_U - \delta^{13}C_{UL}$) for individual species. Inset: magnified view of data with low C:N (< 4.4).

123x78mm (300 x 300 DPI)

Study #	C:N metric	Adjustment	Nothing	Lipid ext.	Urea ext.
1	0	1	0	0	0
2	1	0	0	0	0
3	0	0	0	1	0
4	1	0	0	0	0
5	0	0	0	1	0
6	0	0	1	0	0
7	1	0	0	0	0
8	0	0	0	1	1
9	0	0	1	0	0
10	0	0	0	1	0
11	1	0	0	0	0
12	1	0	0	0	0
13	0	0	0	1	0
14	0	0	0	1	0
15	0	0	0	1	0
16	0	0	0	0	1
17	0	0	0	1	0
18	0	0	0	1	1
19	0	0	0	1	0
20	0	0	0	1	0
21	0	1	0	0	0
22	0	0	0	1	0
23	0	0	1	0	0
24	0	0	0	0	1
25	1	0	0	0	0
26	1	0	0	0	0
27	1	0	0	0	0
28	1	0	0	0	0
29	0	1	0	0	0
30	0	1	0	0	0
31	0	1	0	0	0
32	0	0	0	1	1
33	0	0	1	0	0
34	0	1	0	0	0
35	0	0	0	0	1
36	0	0	1	0	0
37	0	0	0	1	0
38	1	0	0	0	0
39	0	1	0	0	0
40	0	0	0	0	1
41	1	0	0	0	0
42	1	0	0	0	0
43	0	0	0	1	0
44	0	0	0	1	0
45	0	0	0	1	0
46	0	0	0	1	0
47	1	1	0	0	0

48	0	0	0	1	0
49	1	0	0	0	0
50	0	0	1	0	0
Number	14	8	6	19	7

Table S2: Survey of 50 recent (2013-present) studies using stable isotope analysis tc
"stable isotope", "elasmobranch", "shark", "ray"; and selected the first 50 pertinent
indicates the study used the C:N of bulk, non-extracted samples (i.e. contain urea an
whether lipid extraction was required or not. "Adjustment" indicates the study used

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Study
Couturier et al. 2013. Stable Isotope and Signature Fatty Acid Analyses Suggest Reef Manta Rays Feed on Demersa
Matich & Heithaus 2014. Multi -tissue stable isotope analysis and acoustic telemetry reveal seasonal variability in
Hussey et al. 2014. Rescaling the trophic structure of marine food webs. Ecology Letters, 17(2), pp.239-250.
Daly et al. 2013. Comparative feeding ecology of bull sharks (Carcharhinus leucas) in the coastal waters of the Sout
McMeans et al. 2013. The role of Greenland sharks (Somniosus microcephalus) in an Arctic ecosystem: assessed vi
Meneses et al. 2016. Trophic overlap between blue sharks (Prionace glauca) and shortfin makos (Isurus oxyrinchus)
Heithaus et al. 2013. Apparent resource partitioning and trophic structure of large-bodied marine predators in a relat
Shiffman et al. 2014. Feeding ecology of the sandbar shark in south carolina estuaries revealed through δ13C and δ1
De Lecea et al. 2013. Processes controlling the benthic food web of a mesotrophic bight (KwaZulu-Natal, South Afr
Caut et al. 2013. Diet-and tissue-specific incorporation of isotopes in the shark Scyliorhinus stellaris, a North Sea m
Tilley et al. 2013. Diet reconstruction and resource partitioning of a Caribbean marine mesopredator using stable iso
Navarroet al. 2014. Short-and long-term importance of small sharks in the diet of the rare deep-sea shark Dalatias li
Polo-Silva et al. 2013. Trophic shift in the diet of the pelagic thresher shark based on stomach contents and stable is
Olin et al. 2013. Seasonal variability in stable isotopes of estuarine consumers under different freshwater flow regim
Munroe et al. 2015. Regional movement patterns of a small -bodied shark revealed by stable -isotope analysis. Jo
Li et al. 2014. Trophic ecology of sharks in the mid-east Pacific ocean inferred from stable isotopes. Journal of Oce
Churchill et al. 2015. Trophic interactions of common elasmobranchs in deep-sea communities of the Gulf of Mexic
Kiszka et al. 2015. Plasticity of trophic interactions among sharks from the oceanic south-western Indian Ocean rev
Espinoza et al. 2015. Feeding ecology of common demersal elasmobranch species in the Pacific coast of Costa Rica
McMean et al. 2015. Impacts of food web structure and feeding behavior on mercury exposure in Greenland Sharks
Malpica-Cruz et al. 2013. Tissue -specific stable isotope ratios of shortfin mako (Isurus oxyrinchus) and white (Car
Torres et al. 2014. Trophic ecology and bioindicator potential of the North Atlantic tope shark. Science of The Total
McCauley et al. 2014. Reliance of mobile species on sensitive habitats: a case study of manta rays (Manta alfredi) a
Papastamatiou et al. 2015. Movements and foraging of predators associated with mesophotic coral reefs and their p
de Moura et al. 2015. Assessment of trace elements, POPs, 210 Po and stable isotopes (15 N and 13 C) in a rare filte
Madigan et al. 2015. Diet shift and site-fidelity of oceanic whitetip sharks Carcharhinus longimanus along the Grea
Speed et al. 2012. Trophic ecology of reef sharks determined using stable isotopes and telemetry. Coral Reefs, 31(2)
Drymon et al. 2012. Trophic plasticity in the Atlantic sharpnose shark (Rhizoprionodon terraenovae) from the north
Fanelli et al. 2013. Trophic webs of deep-sea megafauna on mainland and insular slopes of the NW Mediterranean:
Courtney & Foy, R., 2012. Pacific sleeper shark Somniosus pacificus trophic ecology in the eastern North Pacific O
McCauley et al. 2012. Assessing the effects of large mobile predators on ecosystem connectivity. Ecological Applic
Lyons et al. 2013. Effects of trophic ecology and habitat use on maternal transfer of contaminants in four species of
Matich et al. 2015. Short-term shifts of stable isotope (δ 13 C, δ 15 N) values in juvenile sharks within nursery areas
Albo-Puigserver et al. 2015. Feeding ecology and trophic position of three sympatric demersal chondrichthyans in t
Rojas et al. 2014. Feeding grounds of juvenile scalloped hammerhead sharks (Sphyrna lewini) in the south-eastern
Reum & Marshall 2013. Evaluating δ15N–body size relationships across taxonomic levels using hierarchical model
Hussey et al. 2015. Expanded trophic complexity among large sharks. Food Webs, 4, pp.1-7.
Valls et al. 2014. Structure and dynamics of food webs in the water column on shelf and slope grounds of the weste
Teffer et al. 2014. Trophic influences on mercury accumulation in top pelagic predators from offshore New England
Kiszka et al. 2014. Trophic ecology of common elasmobranchs exploited by artisanal shark fisheries off south-weste
Cresson et al. 2014. Mercury in organisms from the Northwestern Mediterranean slope: Importance of food sources.
Barría et al. 2015. Unravelling the ecological role and trophic relationships of uncommon and threatened elasmobra
Lopez et al. 2013. Trophic ecology of the dusky catshark Bythaelurus canescens (Günther, 1878)(Chondrychthyes:
Freedman et al. 2015. Connectivity and movements of juvenile predatory fishes between discrete restored estuaries
Shaw et al. 2016. Trophic Ecology of a Predatory Community in a Shallow-Water, High-Salinity Estuary Assessed
Jaime-Rivera et al. 2014. Feeding and migration habits of white shark Carcharodon carcharias (Lamniformes: Lamn
Torres-Rojas et al. 2015. Diet and trophic level of scalloped hammerhead shark (Sphyrna lewini) from the Gulf of

Hernández-Aguilar et al. 2015. Trophic ecology of the blue shark (Prionace glauca) based on stable isotopes (δ
Raoult et al. 2015. Not all sawsharks are equal: species of co-existing sawsharks show plasticity in trophic consump
Frisch et al. 2016. Reassessing the trophic role of reef sharks as apex predators on coral reefs. Coral Reefs, pp.1-14.

› study the ecology of elasmobranchs. We conducted the search on Google scholar using the search terms:
results. We assessed each study to determine how they treated samples for urea and lipid. "C:N metric"
ıd lipid) to assess the lipid content of their samples and use that inferred lipid content to inform them
the C:N of non-extracted samples to mathematically adjust $\delta^{13}C$ to account for inferred lipid content.

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data	$\Delta^{13}C = (a\,C{:}N_U + b)(C{:}N_U + c)^{-1}$		$\Delta^{13}C = P - PF\,(C{:}N_U)^{-1}$		$\Delta^{13}C = \beta_0 + \beta_1 Ln(C{:}N_U)$		$\Delta^{13}C = b + a\,C{:}N_U$	
All species	AIC_c:	94.02	AIC_c:	92.53	AIC_c:	102.33	AIC_c:	121.93
	a =	5.39 ± 0.85	P =	6.12 ± 0.30	β_0 =	-4.89 ± 0.33	a =	0.88 ± 0.06
	b =	-17.25 ± 2.62	F =	3.18 ± 0.03	β_1 =	4.34 ± 0.25	b =	-2.57 ± 0.22
	c =	-0.68 ± 0.75			r^2:	0.76	r^2:	0.70
All sharks	AIC_c:	38.58	AIC_c:	38.97	AIC_c:	39.54	AIC_c:	40.50
(no Dogfish)	a =	1.94 ± 0.23	P =	7.86 ± 0.70	β_0 =	-7.69 ± 0.82	a =	1.81 ± 0.18
	b =	-6.27 ± 0.75	F =	3.15 ± 0.03	β_1 =	6.74 ± 0.66	b =	-5.61 ± 0.63
	c =	-2.98 ± 0.06			r^2:	0.69	r^2:	0.68
All batoids	AIC_c:	*nf*	AIC_c:	15.41	AIC_c:	13.87	AIC_c:	12.26
	a =	-	P =	5.63 ± 0.77	β_0 =	-6.15 ± 0.91	a =	1.45 ± 0.19
	b =	-	F =	3.28 ± 0.04	β_1 =	5.19 ± 0.73	b =	-4.74 ± 0.69
	c =	-			r^2:	0.56	r^2:	0.58
Dogfish	AIC_c:	22.46	AIC_c:	17.30	AIC_c:	20.93	AIC_c:	24.39
S. suckleyi	a =	4.76 ± 1.03	P =	6.31 ± 0.62	β_0 =	-4.08 ± 1.27	a =	0.62 ± 0.16
	b =	-16.96 ± 3.44	F =	3.35 ± 0.20	β_1 =	3.79 ± 0.74	b =	-1.18 ± 0.91
	c =	-1.90 ± 1.03			r^2:	0.76	r^2:	0.65
Great White	AIC_c:	-0.46	AIC_c:	-5.15	AIC_c:	-3.88	AIC_c:	-3.27
C. carcharias	a =	14.18 ± 16.56	P =	8.30 ± 0.52	β_0 =	-7.80 ± 0.61	a =	1.80 ± 0.13
	b =	-43.96 ± 50.48	F =	3.14 ± 0.03	β_1 =	6.90 ± 0.48	b =	-5.49 ± 0.46
	c =	3.22 ± 9.12			r^2:	0.92	r^2:	0.92

Table S1. Parameter estimates (± SE), r^2 (linear models only) and corrected Akaike Information Criteria valeus (AIC_c) for models of Δ13C, the difference between lipid and urea (UL) and urea (U) extracted $\delta^{13}C$ values ($\Delta^{13}C = \delta^{13}C_U - \delta^{13}C_{UL}$), fit to all species, batoids, all sharks other than *S. suckleyi* (dogfish), *S. suckleyi*, and *C. carcharias*. *nf* = model failed to converg.

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