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Effects of preservation methods of muscle tissue from upper-trophic level reef fishes on stable isotope values ($\delta^{13}C$ and $\delta^{15}N$)

Research that uses stable isotope analysis often involves a delay between sample collection in the field and laboratory processing, therefore requiring preservation to prevent or reduce tissue degradation and associated isotopic compositions. Although there is a growing literature describing the effects of various preservation techniques, the results are often contextual, unpredictable and vary among taxa, suggesting the need to treat each species individually. We conducted a controlled experiment to test the effects of four preservation methods of muscle tissue from four species of upper trophic-level reef fish collected from the eastern Gulf of Mexico (Red Grouper *Epinephelus morio*, Gag *Mycteroperca microlepis*, Scamp *Mycteroperca phenax*, and Red Snapper *Lutjanus campechanus*). We used a paired design to measure the effects on isotopic values for carbon and nitrogen after storage using ice, 95% ethanol, and sodium chloride (table salt), against that in a liquid nitrogen control. Mean offsets for both $\delta^{13}C$ and $\delta^{15}N$ values from controls were lowest for samples preserved on ice, intermediate for those preserved with salt, and highest with ethanol. Within species, both salt and ethanol significantly enriched the $\delta^{15}N$ values in nearly all comparisons. Ethanol also had strong effects on the $\delta^{13}C$ values in all three groupers. Conversely, for samples preserved on ice, we did not detect a significant offset in either isotopic ratio for any of the focal species. Previous studies have addressed preservation-induced offsets in isotope values using a mass balance correction that accounts for changes in the isotope value to that in the C/N ratio. We tested the application of standard mass balance corrections for isotope values that were significantly affected by the preservation methods and found generally poor agreement between corrected and control values. The poor performance by the correction may have been due to preferential loss of lighter isotopes and corresponding low levels of mass loss with a

substantial change in the isotope value of the sample. Regardless of mechanism, it was evident that accounting for offsets caused by different preservation methods was not possible using the standard correction. Caution is warranted when interpreting the results from specimens stored in either ethanol or salt, especially when using those from multiple preservation techniques. We suggest the use of ice as the preferred preservation technique for muscle tissue when conducting stable isotope analysis as it is widely available, inexpensive, easy to transport and did not impart a significant offset in measured isotopic values. Our results provide additional evidence that preservation effects on stable isotope analysis can be highly contextual, thus requiring their effects to be measured and understood for each species and isotopic ratio of interest before addressing research questions.

Effects of preservation methods of muscle tissue from upper-trophic level reef fishes on stable isotope values ($\delta^{13}C$ and $\delta^{15}N$)

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INTRODUCTION

The application of stable isotope analysis (SIA) has been one of the most important innovations in the field of ecology in the last 50 years. SIA has been used across ecological sub-disciplines, providing a powerful tool to answer once intractable questions (DeNiro & Epstein 1981; Fry 2006; Peterson & Fry 1987). Stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) are innate components of all biological material, and the ratio of heavy to light isotopes observed in organisms is controlled by a confluence of biological and physical factors that fractionate the isotopes by differences in mass. These values are set by autotrophs and incorporated into the ecosystem as primary production is consumed. (O'Leary 1988). Carbon is typically used to identify primary production sources. For example, plants that use the C3 photosynthetic pathway have carbon isotope values depleted in the heavy isotope (-28 ‰) relative to grasses that use the C4 pathway (-12‰) (O'Leary 1988). This difference has been used to determine when ancient cultures switched from gathering to farming (Schoeninger & Moore 1992) and when brewers skirted Bavarian Purity Laws (Brooks et al. 2002). In contrast, nitrogen isotopes are often used to establish trophic position (Post 2002). After food is consumed, metabolic processes preferentially cleave the bonds in proteins made with the lighter ^{14}N isotope. These waste products of metabolism are converted to urea and excreted leaving behind tissues made with the enriched ^{15}N amino acids (Wright 1995). Typically, organisms are enriched approximately 3‰ relative to their food (Hussey et al. 2014; Post 2002).

Research that uses stable isotope analysis often involves a delay between sample collection in the field and laboratory processing, therefore requiring preservation to prevent or reduce tissue degradation and associated isotopic compositions. Methods used to preserve soft tissues, such as muscle, can present issues in the interpretation of the observed isotope values (Sarakinos et al.

2002). Although there is a growing literature describing the effects of various preservation techniques, the results are often unpredictable and vary among taxa, suggesting the need to treat each species individually (Arrington & Winemiller 2002; Correa 2012; Kelly et al. 2006; Sarakinos et al. 2002). When a systematic offset in isotope values is detected, a mass balance correction can be employed using the variation in C/N ratio to correct the isotope values of the preserved tissue (Fry et al. 2003; Ventura & Jeppesen 2009). The underlying assumption of this method is that the preservation technique removes substances from the whole tissue (e.g., hydrolyzed lipids), altering the isotope value of the whole tissue and this can be accounted for by relating the change in isotope value to the change in C/N ratio. However, these corrections are not always successful and there are still open questions about the mechanisms that alter tissue isotope values after preservation (Kelly et al. 2006).

In the current study, we conducted a controlled experiment to test the effects of four preservation methods of muscle tissue from four species of upper trophic-level reef fish (Red Grouper *Epinephelus morio*, Gag *Mycteroperca microlepis*, Scamp *Mycteroperca phenax*, and Red Snapper *Lutjanus campechanus*). We used a paired design to measure the effects on isotopic values for carbon and nitrogen after storage using ice, 95% ethanol, and sodium chloride (table salt), against that in a liquid nitrogen control. Additionally, we tested the application of standard mass balance corrections for isotope values that were significantly affected by the preservation methods.

METHODS

Collection and Preservation of Samples

Red Grouper, Gag, Scamp, and Red Snapper are co-occurring, essential members of their reef ecosystems. They are ecologically important, mid- to upper-level predators that have also been among the most highly targeted fishes by commercial and recreational fishermen in the region. Specimens were collected using hook-and-line from reef habitats in the eastern Gulf of Mexico as part of an ongoing fishery-independent study (Fig.1). Collections of fishes were conducted in accordance with ethics policies followed by the University of South Florida Institutional Animal Care and Use Committee (approval no. W4193) and permits from the Florida Fish and Wildlife Conservation Commission (Special Activity License SAL-13-1244-SRP-2) and the US National Oceanic and Atmospheric Administration (Letter of Acknowledgment and Exempted Fishing Permit). A total of 78 individuals were collected for this study, across a range of sizes commonly observed for each species (Table 1). White muscle tissue ventral to the dorsal fin was removed from each specimen and cut into four, equal-sized pieces. Each piece was then subjected to one of four preservation techniques – liquid nitrogen (control), ice, 95% ethanol, or salt – all placed in uniquely-labeled, 2 ml microcentrifuge tubes. Control samples were frozen instantaneously by being placed in liquid nitrogen in a 4 liter vacuum flask. Liquid nitrogen served as a control as it neither effects existing isotopic values, nor does it allow bacterial degradation of the tissue to occur (Michener & Lajtha 2007). Samples preserved with liquid nitrogen and those placed on ice were transferred to a -20°C freezer after 48 hours, representing a likely sequential scenario commonly used by field ecologists for tissue preservation. For the other preservatives, samples were placed in microcentrifuge tubes with 1 ml of either 95% ethanol (CH_3CH_2OH) or table salt (NaCl), and were kept at ambient room

102 temperature (22°C). All samples were held for 30 days prior to processing for stable isotope
103 analysis.
104
105 **Analytical Procedures**
106 At the conclusion of the preservation period, all tissues were rinsed with deionized water, and
107 placed in glass vials in a drying oven (55-60°C) for 48 hours. Each desiccated muscle sample
108 was then ground to a fine powder using a mortar and pestle to ensure even combustion during
109 mass spectroscopy. The mortar and pestle, as well as additional tools and work surfaces, were
110 cleaned with 99.5% ethanol and Kimwipes® between individual processing to prevent cross-
111 contamination of samples. Ground samples with a dry weight of 200-1000µg were placed in tin
112 capsules and sealed for combustion and isotopic analysis. Using a Carlo-Ebra NA2500 Series II
113 elemental analyzer coupled to a continuous-flow ThermoFinnigan Delta+XL isotope ratio mass
114 spectrometer we measured $^{13}C/^{12}C$, $^{15}N/^{14}N$ and C/N at the University of South Florida College
115 of Marine Science in St. Petersburg, Florida. The lower limit of quantification for this
116 instrumentation was 12 µg C or N. We used calibration standards NIST 8573 and NIST 8574 L-
117 glutamic acid standard reference materials. Analytical precision, obtained by replicate
118 measurements of NIST 1577b bovine liver, was ±0.19‰ for $\delta^{15}N$ and ±0.11‰ for $\delta^{13}C$. Results
119 are presented in standard notation (δ, in ‰) relative to international standards Pee Dee Belemnite
120 (PDB) and air.
121
122 **Mass Balance Corrections**
123 We used an arithmetic correction based on changes in C/N and preserved vs control stable
124 isotope values (Fry et al. 2003; Smyntek et al. 2007; Ventura & Jeppesen 2009). This method

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assumes the preservation method alters the isotope values of the original tissue by leaching material into the preservative, specifically through the loss of hydrolyzed proteins or lipids. The assumption is that the loss of protein or lipid will be expressed by changes in the C/N of the preserved tissue and can be corrected by relating changes in isotope value of the preserved tissue to the change between the control and preserved tissue C/N as:

$$\delta_{control} = \delta_{preserved} - \Delta\delta_{(preserved-control)} \quad (1)$$

$$\Delta\delta_{(preserved-control)} = X\left(\frac{C/N_{control} - C/N_{preserved}}{C/N_{preserved}}\right) \quad (2)$$

where the $\delta_{control}$ is the isotope value of the unpreserved tissue and $\delta_{preserved}$ is the isotope value of the preserved tissue. $\Delta\delta_{(preserved-control)}$ is the net effect of preservation of the isotope value of the preserved tissue. X is the difference between the isotope value of the preserved and control tissue.

Statistical Analysis

We provide mean (SE) offset values for preservative – control both across and within species. For each species, we used paired *t*-tests to determine whether $\delta^{15}N$ and $\delta^{13}C$ isotopic values from preserving samples with ice, ethanol, and salt were statistically different from control samples preserved in liquid nitrogen. We also use linear regression with 95% confidence intervals of corrected against control isotopic values to determine efficacy of the mass balance corrections.

148 **RESULTS**

149 Across species, mean offsets for both $\delta^{13}C$ and $\delta^{15}N$ values from controls were lowest for
150 samples preserved on ice, and highest for those preserved with ethanol (Table 2). Offsets for
151 $\delta^{15}N$ were generally higher than those for $\delta^{13}C$. For $\delta^{13}C$, salt imparted a 21% offset and ethanol
152 a 50% offset compared to ice. For $\delta^{15}N$, salt imparted a 135% offset compared to ice and ethanol
153 a 180% offset.

154

155 Within species, the effects of the different preservatives ranged in both magnitude and statistical
156 significance (Figure 2, Table 3). Ethanol preservation significantly affected $\delta^{15}N$ values in all
157 four species, and $\delta^{13}C$ values in all three groupers. Salt preservation significantly affected $\delta^{15}N$
158 values in three species (Red Grouper, Scamp and Red Snapper), and $\delta^{13}C$ in only Red Grouper.
159 Ice preservation did not impart a strong or statistically significant offset in either isotope ratio of
160 any species measured.

161

162 C/N corrections

163 Overall, there was poor agreement between corrected and control values using the mass balance
164 approach. Although all regressions were within 95% confidence of a 1:1 slope (i.e., slope = 1,
165 intercept = 0), with the exception of ethanol-preserved $\delta^{13}C$ for Red Snapper, the fit was low for
166 both corrected ethanol- (mean $R^2 = 0.23$; R^2 range = < 0.01-0.39; Figs. 3-6 A,B) and salt-treated
167 samples (mean $R^2 = 0.23$; range = < 0.01-0.55; Figs. 3-6 C,D) across all four species. Corrected
168 values for both preservatives fell on both sides of the 1:1 line, thus our correction did not tend to
169 systematically under or overestimate the change in nitrogen isotope values after preservation.
170 The poor correction values were a direct consequence of the small change and small degree of

171 correlation between the change in the C/N ratio of the control and preserved tissues relative to
172 the change in isotope values (Table 4).

173

174 **DISCUSSION**

175 Using a controlled experiment, we have demonstrated that three techniques used to preserve
176 muscle tissue can have varying effects on measured isotope values for four species of reef fish.
177 Both ethanol and salt caused significant changes to the measured isotope values, but the effects
178 were contextual on species and the isotope being measured. Conversely, preservation of muscle
179 tissue on ice for 48 hours, followed by storage in a -20°C freezer for 28 days, did not impart a
180 significant offset in the isotopic values of either carbon or nitrogen for any of our focal species.
181 Because ice is widely available, inexpensive, and easy to transport relative to liquid nitrogen, we
182 suggest its use as a preservation technique for muscle tissue from Red Grouper, Gag, Scamp and
183 Red Snapper when conducting stable isotope analysis.

184

185 There is a substantial and growing number of studies on the effects of various preservatives and
186 methods on carbon and nitrogen stable isotope values in animal tissues (Barrow et al. 2008;
187 Sarakinos et al. 2002; Ventura & Jeppesen 2009). Despite the large body of work on the topic,
188 there is little consensus on the effect of preservation techniques on stable isotope values with a
189 near even number of studies finding significant and non-significant shifts (Kelly et al. 2006;
190 Sweeting et al. 2004; Ventura & Jeppesen 2009). When significant differences between control
191 and preserved tissues have been observed, researchers most often opt to develop a correction
192 curve based on the variation in the C/N in the preserved tissues (Fry et al. 2003; Sarakinos et al.
193 2002). However, our results show that even very small changes in C/N can co-occur with
194 significant enrichment in nitrogen stable isotope values (Fig 2). This indicates there was a

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195 significant loss of the light isotope from tissue upon preservation with little change in mass from
196 the sample itself. Therefore, we conclude there were some critical fractionation processes
197 associated with the loss of amino acids linked with the breakdown of proteins. This resulted in
198 low levels of mass loss but a substantial change in the isotope value of the sample.
199
200 There are a two mechanisms that may be responsible for the observed results. Ethanol is known
201 to denature proteins and form new bonds between ethanol and the protein side chains (Herskovits
202 et al. 1970; Nozaki & Tanford 1971). The free energy required to conduct these reactions is high
203 and therefore likely favors the cleaving of ^{14}N-^{14}N bonds. Thus such reactions may explain the
204 very high fractionation yet low mass loss observed in this study with preservation in ethanol. We
205 also observed a strong fractionation of the nitrogen isotope values of the preserved tissues with
206 salt. Salt is highly effective at extracting proteins from tissue samples, removing as much as 91%
207 of the available protein (Dyer et al. 1950). Because our samples were stored at a low
208 temperature, the extraction was likely less efficient but none-the-less preferentially removed the
209 light nitrogen bonds, resulting in little mass loss with high fractionation. It is our suggestion that
210 given both preservatives are known to extract proteins and amino acids with the potential for an
211 unknown amount of fractionation to occur, caution be used when interpreting the results from
212 specimens stored in either ethanol or salt.
213
214 To further illustrate why caution is warranted for interpreting isotope values for specimens
215 preserved in ethanol or salt, we provide a standard biplot with mean (SE) values of $\delta^{13}C$ and
216 $\delta^{15}N$ for each species-by-preservation method (Fig. 7). In $\delta^{13}C$ - $\delta^{15}N$ space, samples preserved
217 in liquid nitrogen and on ice are indistinguishable from each other. However, a strong departure

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from control values is evident especially in $\delta^{15}N$ space for salt and even more so for ethanol. While the ecological importance of these statistically significant offsets would be dependent upon the questions being asked, it is clear from the biplot that both quantitative and qualitative conclusions would be hindered by ethanol and salt preservation. This observation could be further exacerbated by comparing stable isotope values from samples preserved with multiple techniques in the same study. With an increased focus on marine systems of the Gulf of Mexico following the Deep Water Horizon oil rig blowout in 2010, researchers using stable isotope analysis on these species, and any others involving soft tissues, should either use a common preservation method or at a minimum understand the potential effects of different preservation methods, before making cross-study comparisons. Our results provide additional evidence that preservation effects on stable isotope analysis can be highly contextual, thus requiring their effects to be measured and understood for each species and isotopic ratio of interest before addressing research questions.

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Table 1. Sample sizes and size ranges for the study species.

Species	No. collected	Mean TL (mm) ± 1 SE	Min TL (mm)	Max TL (mm)
E. morio	24	569 ± 26	360	764
M. microlepis	19	841 ± 38	500	1090
M. phenax	15	569 ± 13	512	664
L. campechanus	20	677 ± 16	546	794

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Table 2. Mean (se) offset (‰) for nitrogen and carbon isotopes across four focal species based on absolute values of preservative - control.

Preservative	**$\delta^{15}N$**	**$\delta^{13}C$**
Ice	0.20 (0.02)	0.28 (0.03)
EtOH	0.56 (0.04)	0.42 (0.04)
NaCl	0.47 (0.04)	0.34 (0.05)

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Table 3. Summary of paired *t*-tests and two-sided *P*-values across species and preservation method for δ15N and δ13C isotopes. Significant *P*-values are bold-typed.

Species	Preservation (df)	$\delta^{15}N$		$\delta^{13}C$	
		***t*-value**	***P*-value**	***t*-value**	***P*-value**
E. morio					
	ice (23)	-1.494	0.149	-0.885	0.385
	ethanol (22)	-9.956	**< 0.001**	-7.446	**< 0.001**
	salt (23)	-7.400	**< 0.001**	-2.472	**0.021**
M. microlepis					
	ice (18)	-1.276	0.218	-0.418	0.681
	ethanol (18)	-11.077	**< 0.001**	2.438	**0.025**
	salt (15/18)	-1.569	0.134	-0.939	0.360
M. phenax					
	ice (13)	-0.498	0.627	-0.533	0.603
	ethanol (12)	-6.906	**< 0.001**	-5.794	**< 0.001**
	salt (13)	-5.464	**< 0.001**	-0.892	0.389
L. campechanus					
	ice (19)	-1.318	0.203	-0.802	0.432
	ethanol (18)	-5.040	**< 0.001**	-1.269	0.221
	salt (18)	-6.246	**< 0.001**	-0.189	0.852

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Table 4. Mean (SE) change in C/N due to ethanol and salt preservation methods.

Species	EtOH	NaCl
E. morio	0.06 (0.19)	0.03 (0.15)
M. microlepis	0.02 (0.20)	-0.18 (0.57)
M. phenax	0.04 (0.20)	-0.02 (0.14)
L. campechanus	-0.09 (0.22)	-0.07 (0.24)

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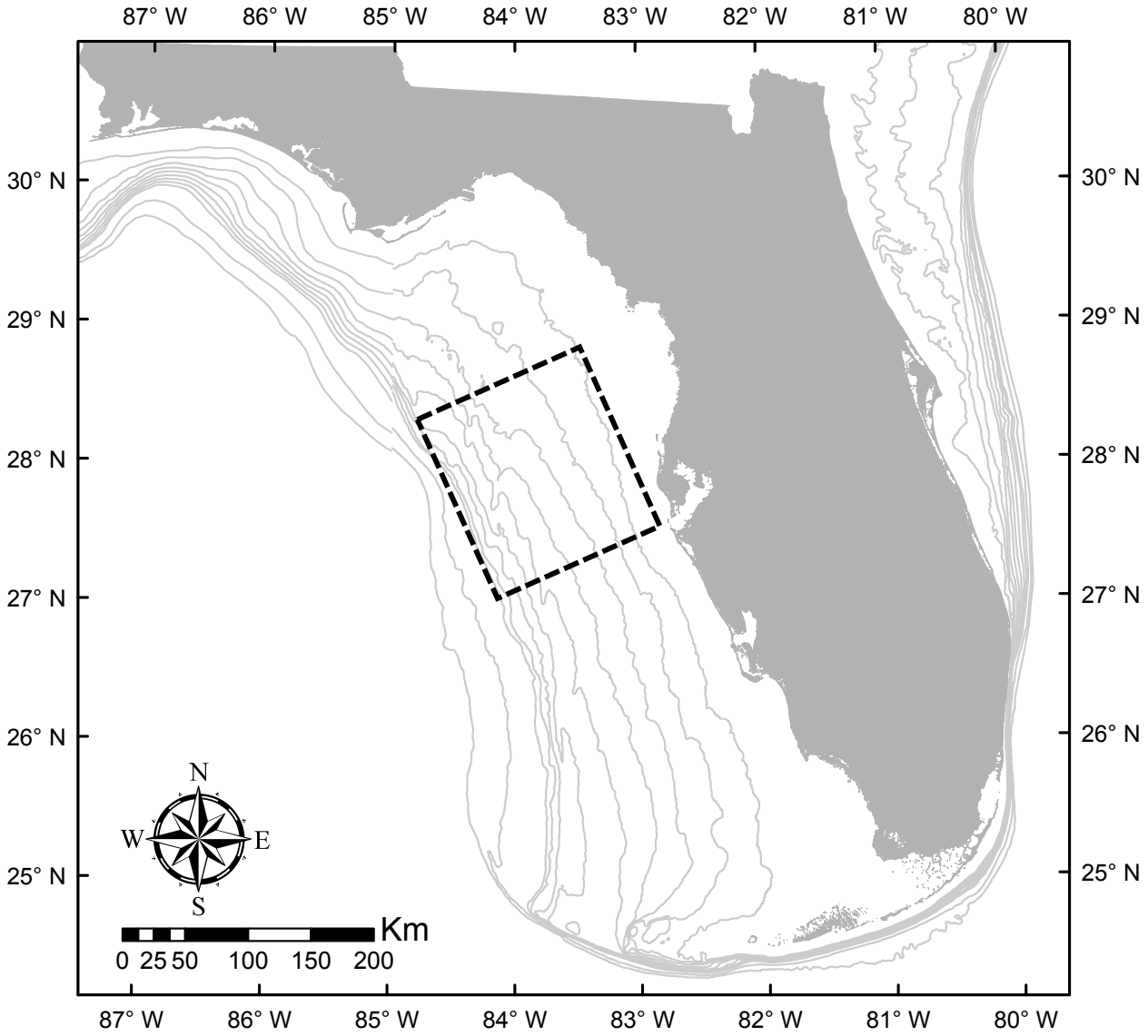

Figure 1. Study region in the eastern Gulf of Mexico where samples were collected (inside dashed line box). 10-m isobaths are shown from 10-100m.

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Figure 2.

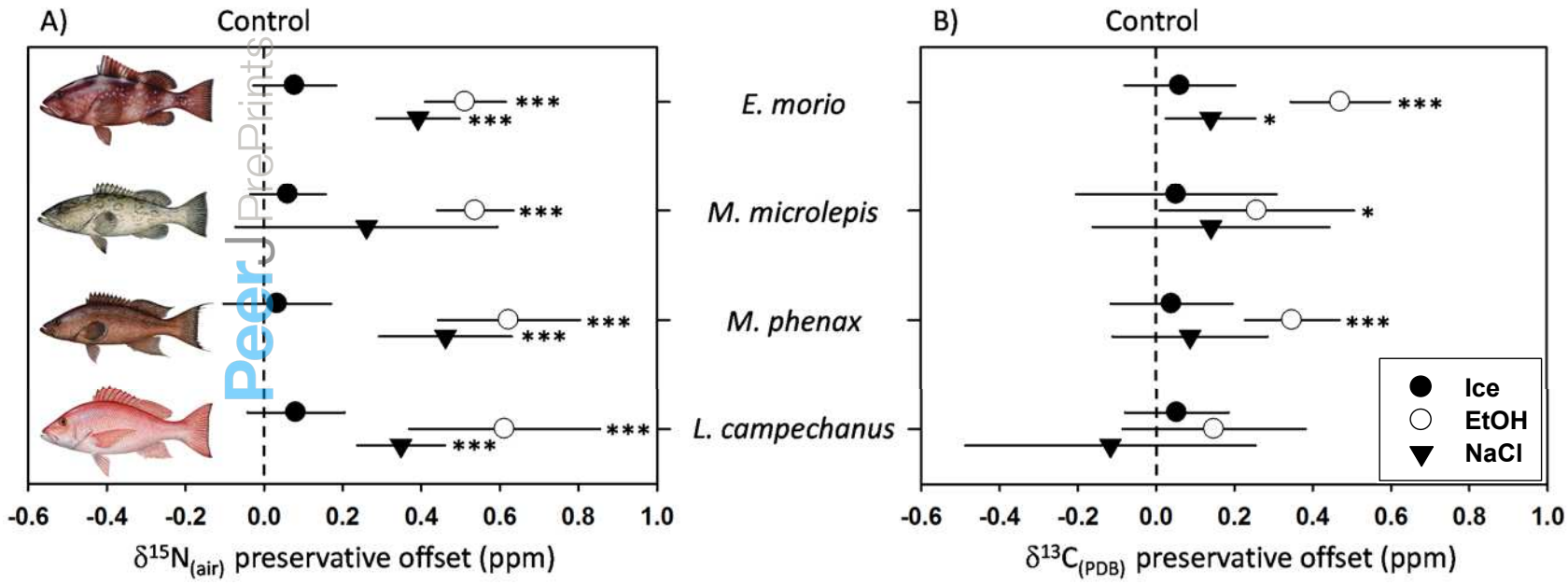

Fig. 2. Offsets (preservative – control) in isotopic values due to preservation technique (mean ± 2 S.E.). Offsets for preservatives that were statistically different from the liquid nitrogen controls are noted as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Fish illustrations courtesy of Diane Peebles.

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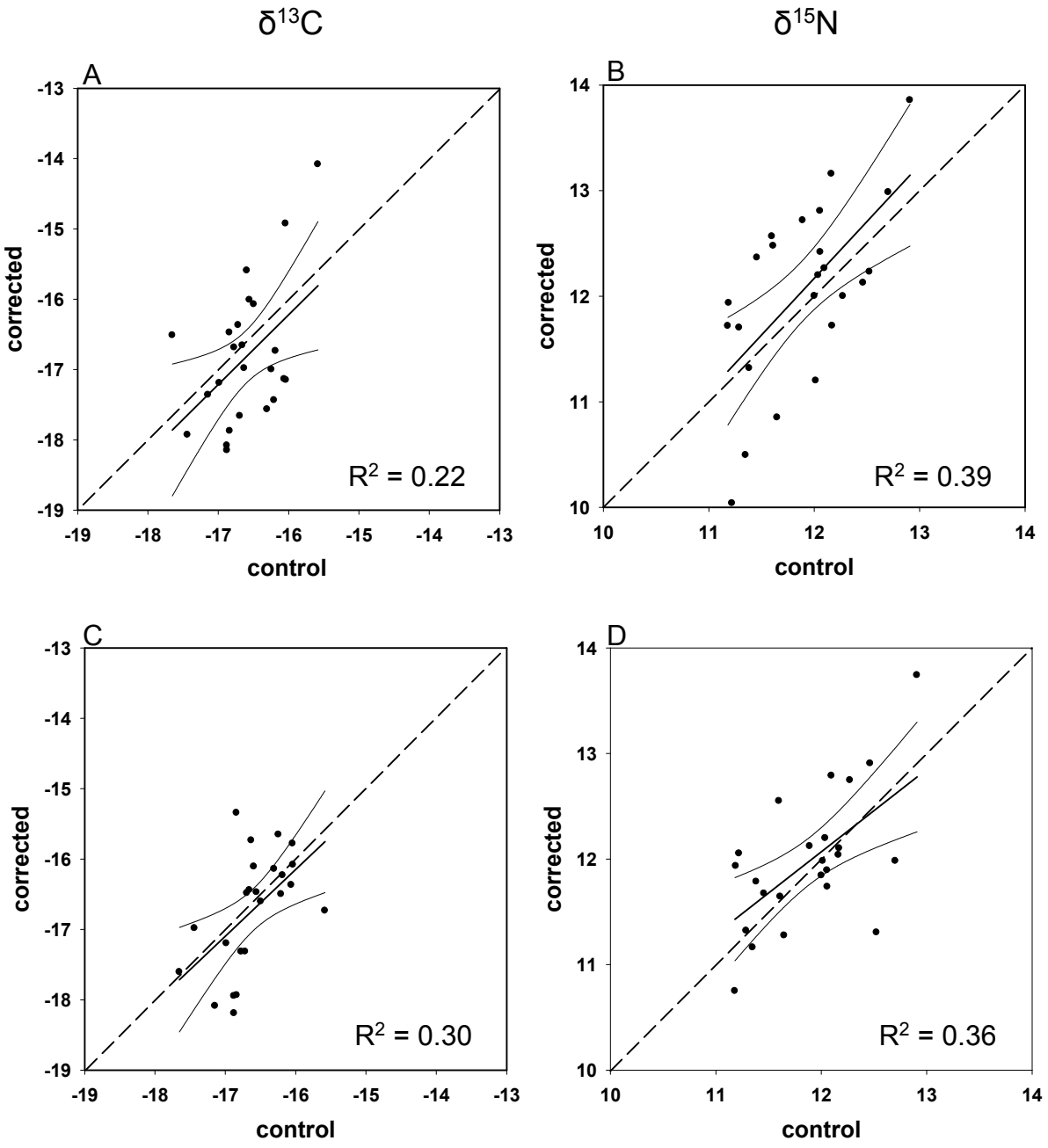

Figure 3. Corrected values against control values for Red Grouper. The 1:1 line (hashed), predicted (solid), and 95% confidence intervals are given for A) ethanol $\delta^{13}C$, B) ethanol $\delta^{15}N$, C) salt $\delta^{13}C$, and D) salt $\delta^{15}N$.

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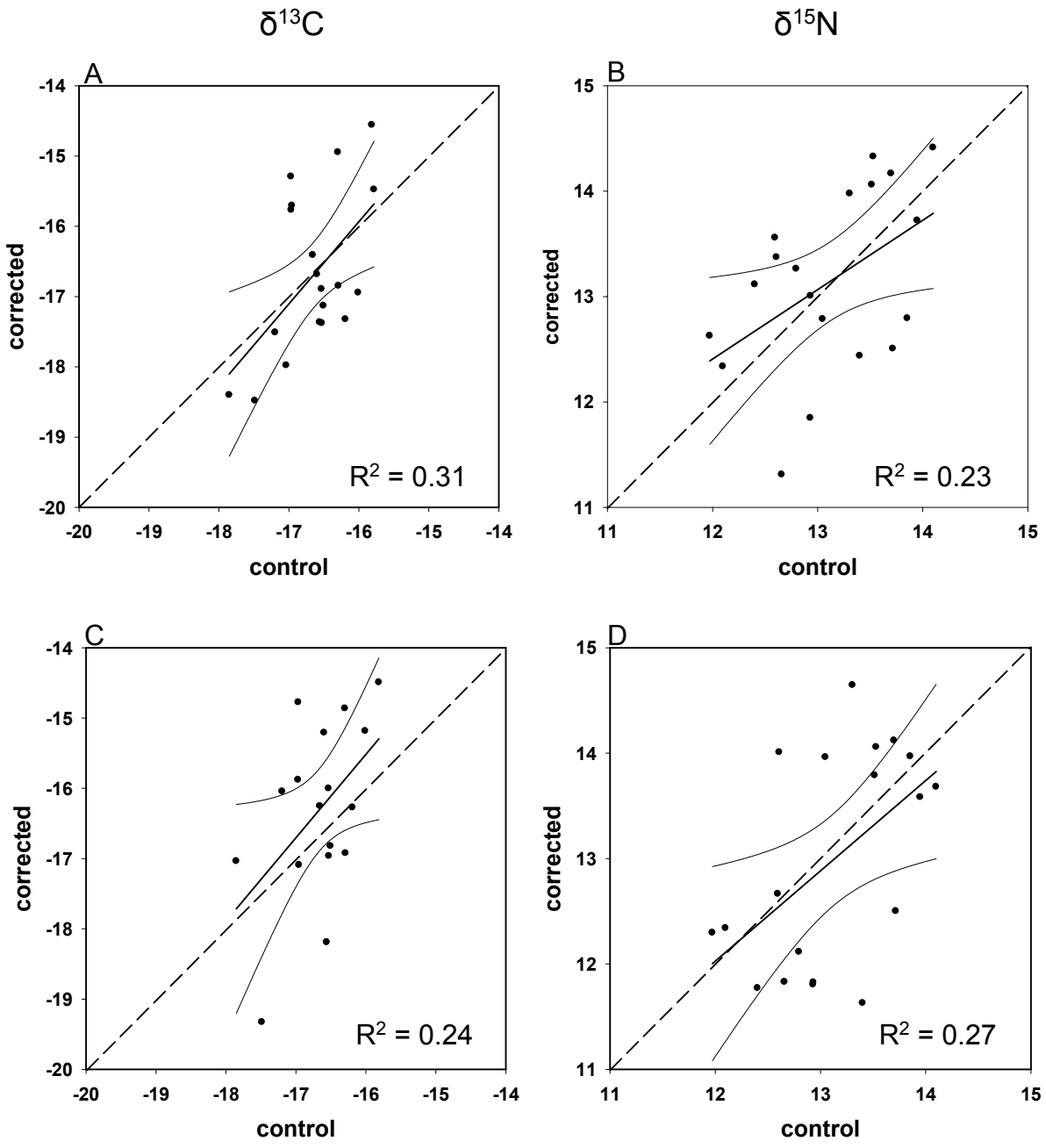

Figure 4. Corrected values against control values for Gag. The 1:1 line (hashed), predicted (solid), and 95% confidence intervals are given for A) ethanol $\delta^{13}C$, B) ethanol $\delta^{15}N$, C) salt $\delta^{13}C$, and D) salt $\delta^{15}N$.

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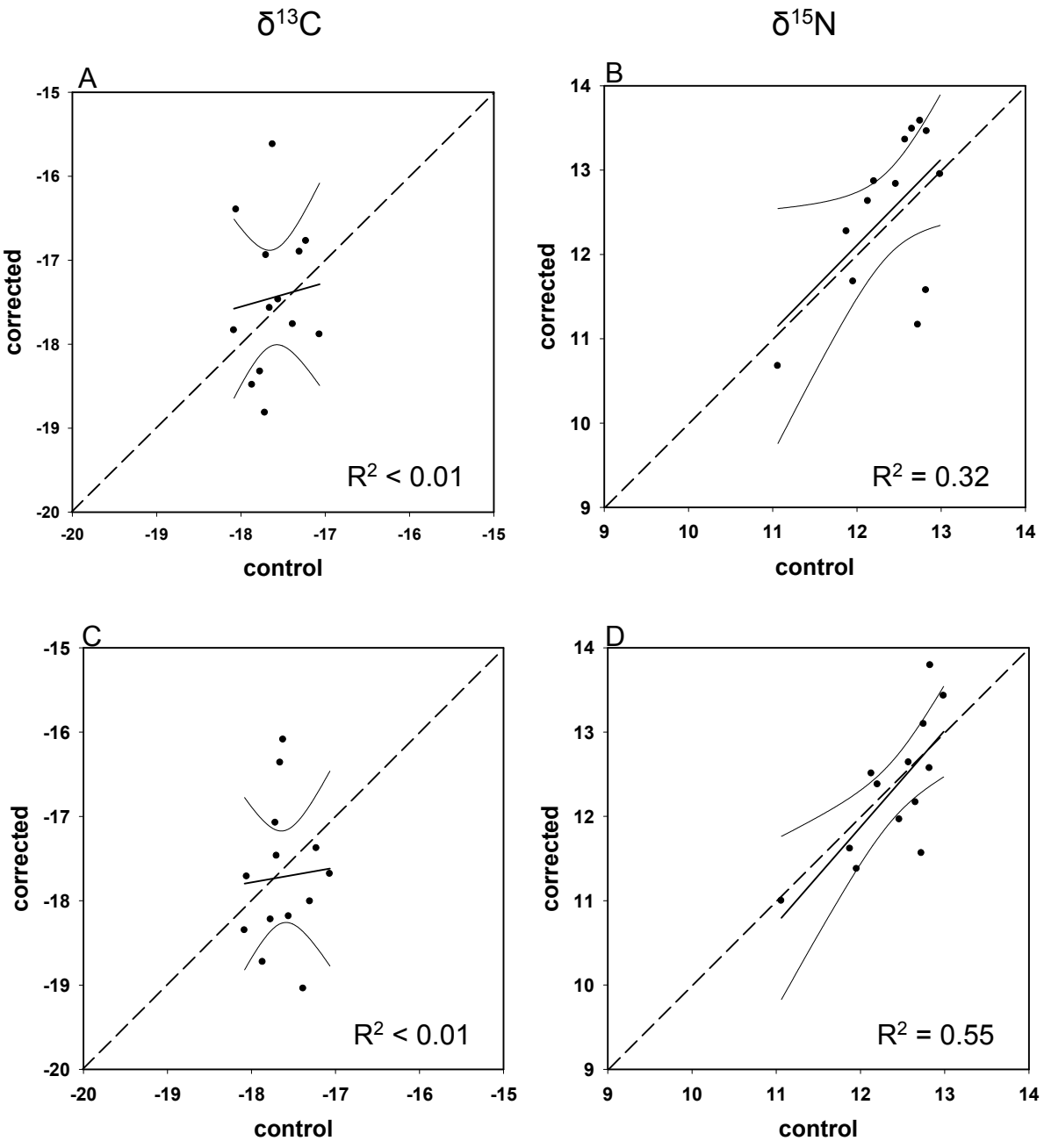

Figure 5. Corrected values against control values for Scamp. The 1:1 line (hashed), predicted (solid), and 95% confidence intervals are given for A) ethanol $\delta^{13}C$, B) ethanol $\delta^{15}N$, C) salt $\delta^{13}C$, and D) salt $\delta^{15}N$.

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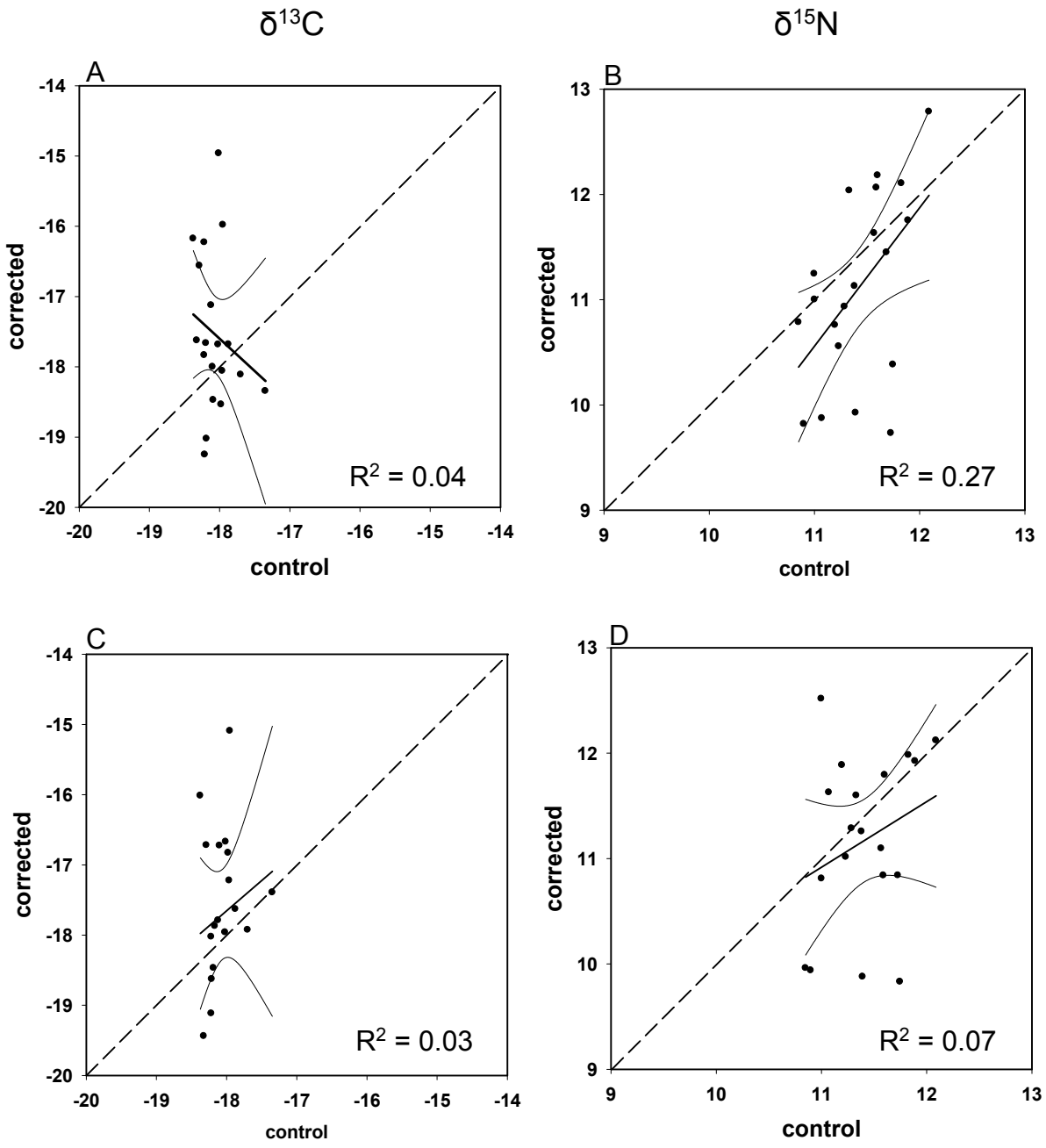

Figure 6. Corrected values against control values for Red Snapper. The 1:1 line (hashed), predicted (solid), and 95% confidence intervals are given for A) ethanol $\delta^{13}C$, B) ethanol $\delta^{15}N$, C) salt $\delta^{13}C$, and D) salt $\delta^{15}N$.

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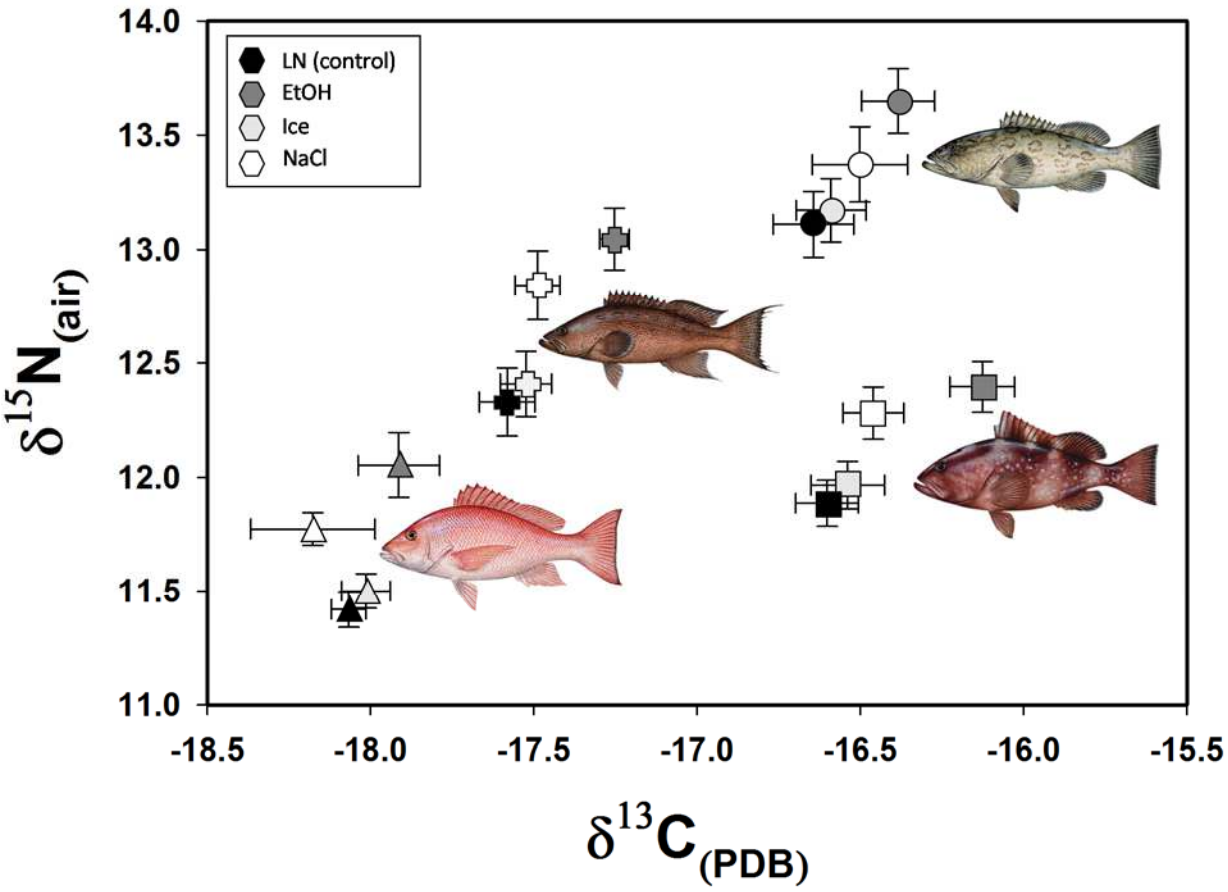

Figure 7. Carbon and nitrogen isotope values for the four study species showing the relative trophic positions and the effects of different preservation methods. Fish illustrations courtesy of Diane Peebles.