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Preservation Effects on Stable Isotope Values of Archived Elasmobranch Fin Tissue: Comparisons between Frozen and Ethanol-Stored Samples

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NOTE

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Abstract

Elasmobranch fin tissue has been sampled and archived for decades to support genetics research. However, these collections have the potential to provide additional information on the trophic ecology of and habitat use by elasmobranch species. The use of fin tissue is especially attractive considering the threatened status of many elasmobranchs and the call for limiting mortalities. However, the use of fin samples for stable isotope analysis requires either that (1) storage methods do not alter tissue isotope values or (2) any alterations in isotope composition that occur during storage are predictable. In this study, paired fin tissues sampled from Smalltooth Sawfish *Pristis pectinata* and cownose rays *Rhinoptera* spp. were stored frozen and in ethanol and were subsequently analyzed for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios. Fin $\delta^{13}C$ and $\delta^{15}N$ values were highly correlated between treatments for both taxa ($r^2 \geq 0.80$). For Smalltooth Sawfish, ethanol storage significantly increased fin $\delta^{13}C$ values by 0.5 $\pm$ 0.1‰ (mean $\pm$ SE) and decreased fin $\delta^{15}N$ values by 0.1 $\pm$ 0.1‰ relative to frozen samples; differences were similar for cownose rays ($\delta^{13}C$: 0.2 $\pm$ 0.2‰; $\delta^{15}N$: 0.2 $\pm$ 0.1‰) but were not significant. A range of approximately 3‰ for $\delta^{13}C$ between treatments could have effects on data interpretation, suggesting the use of regressions for ethanol correction of $\delta^{13}C$ values, although trends were comparable between frozen and ethanol-preserved samples without correction. Given the low variability in $\delta^{15}N$ values, a correction was not warranted. For endangered species such as the Smalltooth Sawfish, stable isotope analysis of ethanol-archived fin samples can provide important information regarding habitat use and trophic ecology, with potential significance for conservation and management strategies. The general uniformity in isotope ratio shifts observed for archived samples between the two taxa suggests that these findings can be generalized across elasmobranch species.

The stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in consumer tissues reflect the isotope ratios of the diet and trophic hierarchy in a predictable manner and can thus be used to infer species or community trophic ecology at the time and location of tissue synthesis (DeNiro and Epstein 1978; Minagawa and Wada 1984). Specifically, the marked discrimination of $\delta^{15}N$ between prey and consumer is used to examine diet, trophic position, and food web structure, whereas $\delta^{13}C$, which exhibits a lesser degree of discrimination, indicates the isotopic composition of primary production sources and provides a tool for examining animal habitat use and movement–migration patterns (Vander Zanden and Rasmussen 2001; Post 2002). However, the successful application of stable isotope analyses to address ecological questions is dependent on several assumptions, such as tissue preparation, tissue turnover rates, and diet tissue discrimination factors, our understanding of which is continually evolving.

Nondestructive sampling to gain insights into animal ecology is of particular importance with regard to

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elasmobranch species given the threatened status of many elasmobranchs and the need to limit mortalities (Dulvy et al. 2008; Heupel and Simpfendorfer 2010; Hammerschlag and Sulikowski 2011). Moreover, there is increased interest in using archived materials for food web analysis based on stable isotopes (Vander Zanden et al. 2003; Rennie et al. 2012). Fin tissue is attractive to sample as it is relatively easy to obtain nonlethally from elasmobranchs (Hussey et al. 2011), and archived fin clip or fin punch tissue libraries now exist from many species that were originally sampled for genetic analyses (e.g., Chapman et al. 2009). The archiving of samples therefore enables access to large sample numbers and permits analysis without additional field sampling. Likewise, remote or challenging fieldwork often necessitates chemical preservation of samples, particularly in the absence of electricity for freezing or when transport of samples is unreliable. Collectively, archived samples have the potential to provide information on trophic ecology of and habitat use by elasmobranch species.

Existing studies related to assessing the effects of chemical storage on fish tissues have examined these effects in a variety of chemicals (see Sarakinos et al. 2002; Sweeting et al. 2004; Kelly et al. 2006), with little consensus (Barrow et al. 2008). For elasmobranchs, limited experimental work has been undertaken to examine the effects of chemical storage methods. To date, only one study has examined the effects of ethanol storage on elasmobranch tissue; ethanol-stored muscle tissue of the Longnose Skate *Raja rhina* had significantly higher $\delta^{13}C$ values than frozen samples, whereas storage method exerted minimal effects on $\delta^{15}N$ values (Kim and Koch 2012).

Considering the potential for analysis and the contrasting results of chemical storage reported for other taxa, an understanding of storage effects on stable isotope values in elasmobranch fin tissue is warranted. Use of stored tissue samples requires either that (1) storage does not alter the isotopic composition of the tissue or (2) changes in isotopic composition are predictable and can be incorporated into subsequent analysis. The objectives of this study were (1) to investigate the effect of ethanol storage on $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C:N values of paired fin tissues; (2) to examine how the effects of ethanol storage varied over time; and (3) given variability among species, to test whether the relationship between frozen and ethanol-stored fin samples is similar across species. Fin tissue from two batoid taxa, the endangered Smalltooth Sawfish *Pristis pectinata* and cownose rays *Rhinoptera* spp., were selected for the analyses.

METHODS

Smalltooth Sawfish ($n = 50$; stretch TL range = 754–1,859 mm) and cownose rays ($n = 5$; disk width range = 417–920 mm) were sampled from Charlotte Harbor, Florida, between May 2011 and September 2012 (see Poulakis et al. 2011 and Poulakis 2013 for sampling methods). Using scissors, a sample (~2 g) of fin tissue was excised from the free rear tip of the first or second dorsal fin of each Smalltooth Sawfish or from either pelvic fin of each cownose ray. Each fin clip was divided in half for storage; one half was stored in a 95% solution of ethanol, and the other half was frozen at −20°C.

Samples were preserved for a period of between 220 and 700 d. Ethanol was evaporated from fin tissue samples in a fume hood for 48 h. All samples were then rinsed in distilled water, dried in an oven at 60°C for 72 h, and homogenized using scissors. The relative abundances of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) were determined for approximately 1,350–1,550-μg subsamples analyzed on a Thermo-Finnigan DeltaPlus mass spectrometer coupled with a Costech elemental analyzer. The analytical precision based on the SD of two standards (bovine muscle and internal fish laboratory standard: $n = 71$) ranged from 0.08‰ to 0.09‰ for both $\delta^{13}C$ and $\delta^{15}N$. Lipid extraction was not undertaken on the fin samples based on the premise of low lipid content (Hussey et al. 2011). This was verified by nonsignificant relationships between $\delta^{13}C$ and either C:N or %C of the frozen and ethanol-stored fin tissue samples.

Differences in $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C:N (elemental percentage) values between frozen and ethanol (EtOH) treatments (e.g., $\delta^{13}C_{diff} = \delta^{13}C_{Frozen} - \delta^{13}C_{EtOH}$) were calculated for both elasmobranch taxa. To assess whether the treatment differences for fin tissue $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C:N values varied with fish size (stretch TL; mm) or number of days stored (220–700 d), least-squares linear regressions were used for Smalltooth Sawfish only. Least-squares linear regression analysis was then used to examine the relationship between the $\delta^{13}C$ values of frozen versus ethanol-preserved Smalltooth Sawfish samples and between the $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C:N values of cownose ray fin samples from the two treatments. Multiple linear regression analysis was used to examine the relationships between the %C, $\delta^{15}N$, %N, and C:N values of Smalltooth Sawfish fin samples from the two treatments and to account for the effect of storage time (see Results). An examination of probability plots showed that Smalltooth Sawfish and cownose ray data were generally described by normally distributed errors and were equal in variance. A slope analysis was then performed to examine whether these relationships differed from a 1:1 relationship for each species. We performed ANCOVA to examine whether the relationship between treatments differed between the two elasmobranch taxa. To examine whether the differences in $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C:N were significant, paired *t*-tests were used for Smalltooth Sawfish samples and Wilcoxon's signed rank tests were used for cownose ray samples. For Smalltooth Sawfish only, ANOVA was

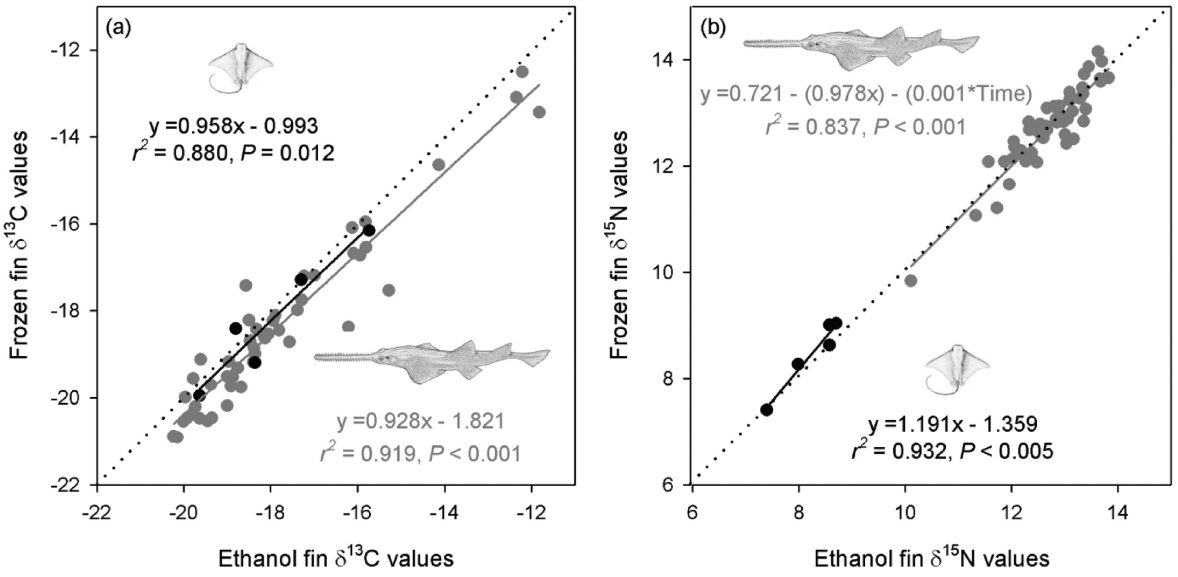

FIGURE 1. Comparison of **(a)** δ^{13}C (‰) and **(b)** δ^{15}N (‰) values between paired frozen and ethanol-stored fin tissue samples from Smalltooth Sawfish ($n = 50$; gray points) and cownose rays *Rhinoptera* spp. ($n = 5$; black points). The dotted line represents a 1:1 relationship (i.e., no effect of ethanol storage). (Fish illustration credits: Sarah Erickson [Smalltooth Sawfish] and the Food and Agriculture Organization of the United Nations [cownose ray].)

performed to examine the effect of sex on the δ^{13}C and δ^{15}N treatment differences. All analyses were conducted in R version 2.13.0 (R Development Core Team 2011); a significance level α of 0.05 was used for all statistical tests.

RESULTS

There was no significant effect of fish size on the treatment differences for δ^{13}C, %C, δ^{15}N, %N, or C:N values of Smalltooth Sawfish fin tissue (Figure A.1). The duration of storage for Smalltooth Sawfish tissue did not influence δ^{13}C values but had a significant negative effect on δ^{15}N, %C, and %N values and a positive effect on C:N values, although correlations were weak ($r^2 < 0.14$; Figure A.2). Specifically, the difference in δ^{15}N, %C, and %N values between frozen and ethanol-preserved samples decreased with time in storage, while the difference in C:N between frozen and ethanol samples increased with time in storage.

Regression analysis of δ^{13}C and δ^{15}N in frozen versus ethanol-stored fin samples showed significant positive relationships for both taxa (Figure 1a, b); other paired comparisons were not significant ($P > 0.05$, $r^2 < 0.19$). The δ^{13}C and δ^{15}N values from ethanol-preserved and frozen samples did not significantly deviate from a 1:1 relationship for either taxon (Smalltooth Sawfish, δ^{13}C: $\beta = 1.03$, $P = 0.42$; Smalltooth Sawfish, δ^{15}N: $\beta = 0.94$, $P = 0.31$; cownose rays, δ^{13}C: $\beta = 0.99$, $P = 0.98$; cownose rays, δ^{15}N: $\beta = 0.82$, $P = 0.21$). The ANCOVAs showed a significant effect of treatment (ethanol storage) on δ^{13}C and δ^{15}N values, but the main effect of taxon and the treatment × taxon interaction effect were not significant (Table 1), indicating that the slopes of regressions between treatments were similar for the two taxa. Examination of differences between frozen and ethanol-treated fin tissue samples from Smalltooth Sawfish indicated significant increases in δ^{13}C and C:N values and significant decreases in δ^{15}N, %N, and %C values (Table 2). Differences between

TABLE 1. Analysis of covariance results for δ^{13}C and δ^{15}N data describing the relationship between frozen storage and ethanol storage (i.e., treatments) of fin samples from Smalltooth Sawfish and cownose rays *Rhinoptera* spp. (SS = sum of squares; MS = mean square; statistical significance is indicated by bold italics).

		δ^{13}C (‰)				δ^{15}N (‰)			
Effect	df	SS	MS	*F*	*P*	SS	MS	*F*	*P*
Treatment	1	180.79	180.79	584.72	***0.000***	107.68	107.68	1,140.88	***0.000***
Taxon	1	0.41	0.41	1.32	0.255	0.08	0.08	0.85	0.360
Treatment × taxon	1	0.01	0.01	0.02	0.877	0.04	0.04	0.44	0.509
Residuals	51	15.77	0.31			4.81	0.09		

frozen and ethanol-treated fin tissue samples from cownose rays showed increasing trends in $\delta^{13}C$, %C, and C:N values and decreasing trends in $\delta^{15}N$ and %N values, although the differences were not significant (Table 2). There was no statistical effect of sex on the ethanol–frozen relationship for $\delta^{13}C$ ($F_{1, 48} = 0.16$, $P = 0.62$) or $\delta^{15}N$ ($F_{1, 48} = 0.32$, $P = 0.57$) in Smalltooth Sawfish fin tissue.

DISCUSSION

Our understanding of stable isotope dynamics in commonly used tissues (e.g., muscle) has improved considerably in recent years (Martinez del Rio et al. 2009); however, use of additional tissues requires testing before broad application in examining aspects of animal ecology (Hussey et al. 2011). This study was an assessment of ethanol storage effects on elasmobranch fin tissues; our findings are in general agreement with the single existing study of elasmobranch muscle tissue ($\delta^{13}C = -0.4$‰, $\delta^{15}N = 0.2$‰; Kim and Koch 2012) with respect to increased $\delta^{13}C$ values (mean = −0.2 to −0.5‰) and decreased $\delta^{15}N$ values (mean = 0.1–0.2‰) in both taxa between treatments. Our results also generally agree with findings from several studies of muscle tissues from marine teleosts with respect to shifts in $\delta^{13}C$ values between treatments (Kaehler and Pakhomov 2001; Kelly et al. 2006). However, the range in $\delta^{13}C$ values and, to a lesser extent, $\delta^{15}N$ values observed in this study highlights the importance of considering chemical storage effects on stable isotope values to ensure the most accurate interpretation of data. The present findings are important for advancing the application of stable isotope analyses to trophic ecology and movement–migration studies of elasmobranchs, as inferences regarding a species' ecological role in its community will be influenced.

Ethanol storage resulted in a greater magnitude of isotopic shifts for $\delta^{13}C$ than for $\delta^{15}N$, particularly in Smalltooth Sawfish, with values (−2.3 to 1.2‰) ranging well above analytical error between exact duplicate samples (0.01–0.05‰) and above the generally accepted 1–2‰ for stepwise trophic discrimination (Post 2002). Despite this variability, the observed shifts in $\delta^{13}C$ would largely be insignificant for ecological comparisons between production source end members with divergent carbon isotope values (Arrington and Winemiller 2002), such as C_3 versus C_4 plants (Smith and Epstein 1971; Fry and Sherr 1984), marine versus terrestrial sources (Chanton and Lewis 2002), or inshore versus offshore habitats (Caut et al. 2008). However, many elasmobranch species are highly mobile and commonly feed on diverse prey species across multiple food webs or inhabit areas of mixed production resources (e.g., estuaries) during specific life stages, thereby incorporating multiple production resources into their tissues and confounding separation based on $\delta^{13}C$. In such cases (e.g., to detect subtle differences in $\delta^{13}C$ values), variability of up to 3‰ could provide marked effects on the interpretation of data, specifically with respect to fine-scale studies that are designed to characterize diet resources or habitat use by elasmobranchs. For example, a 3‰ range for enrichment in ^{13}C for Smalltooth Sawfish could be interpreted as an indication of feeding in coastal or seagrass habitats (−15‰) rather than in estuarine habitats (−18‰). This result suggests the need for a correction of fin carbon isotope ratios before their use in such applications. At a minimum, the variability in $\delta^{13}C$ suggests that studies using $\delta^{13}C$ values from ethanol-stored samples should consider this as a source of uncertainty.

The average effect of ethanol preservation on $\delta^{15}N$ values was relatively small in both taxa—0.1 ± 0.1‰ for Smalltooth Sawfish and 0.2 ± 0.2‰ for cownose rays—relative to

TABLE 2. Results of paired *t*-tests for Smalltooth Sawfish ($n = 50$) and Wilcoxon's signed rank tests for cownose rays *Rhinoptera* spp. ($n = 5$). Tests compare $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C:N values between frozen and ethanol-stored fin tissue (i.e., treatments). Mean (±SE) is presented for each treatment; mean (±SE) and range are presented for differences between treatments (e.g., $X_{diff} = X_{Frozen} - X_{EtOH}$, where X corresponds to $\delta^{13}C$, %C, $\delta^{15}N$, %N, or C:N). Results of statistical analysis are listed for each comparison, with statistical significance indicated by bold italics ($P < 0.05$).

Variable	Frozen mean (±SE)	Ethanol mean (±SE)	Difference mean (±SE)	Difference range	Test statistic	*P*
			Smalltooth Sawfish			
$\delta^{13}C$ (‰)	−18.4 ± 0.3	−17.9 ± 0.3	−0.5 ± 0.1	−2.3–1.2	$T_{49} = -4.328$	***<0.0001***
%C	33.0 ± 0.7	30.1 ± 0.9	2.9 ± 1.0	−9.3–18.9	$T_{49} = 2.793$	***0.007***
$\delta^{15}N$ (‰)	12.7 ± 0.1	12.6 ± 0.1	0.1 ± 0.1	−0.5–0.8	$T_{49} = 2.048$	***0.045***
%N	11.0 ± 0.3	9.7 ± 0.3	1.4 ± 0.4	−2.8–7.4	$T_{49} = 3.608$	***0.001***
C:N	3.0 ± 0.02	3.1 ± 0.01	−0.1 ± 0.02	−0.5–0.2	$T_{49} = -5.697$	***<0.0001***
			Cownose Rays			
$\delta^{13}C$ (‰)	−18.2 ± 0.7	−18.0 ± 0.7	−0.2 ± 0.2	−0.8–0.4	$V = 4$	0.438
%C	36.6 ± 2.1	37.6 ± 0.8	−1.0 ± 2.7	−11.4–4.0	$V = 8$	0.729
$\delta^{15}N$ (‰)	8.5 ± 0.3	8.3 ± 0.2	0.2 ± 0.1	0.01–0.4	$V = 10$	0.100
%N	12.8 ± 0.9	12.5 ± 0.3	0.4 ± 1.1	−3.5–3.3	$V = 10$	0.625
C:N	2.9 ± 0.05	3.0 ± 0.02	−0.2 ± 0.1	−0.4–0.03	$V = 1$	0.104

observed ecological variation (Vander Zanden and Rasmussen 2001). The range in $\delta^{15}N$ values of fin tissues was lower (Smalltooth Sawfish: −0.5 to 0.8‰; cownose rays: 0.01–0.4‰) than the range for $\delta^{13}C$. More importantly, the overall shift due to ethanol storage was small relative to trophic-level shifts, which are routinely reported as 3–4‰ (Post 2002). Although $\delta^{15}N$ variability between treatments has potential consequences for fine-scale isotope analysis with respect to estimating trophic position or the use of mixing models for diet studies, the variability identified between treatments in this study is exceeded by uncertainty in diet tissue discrimination factors (Olin et al. 2013) and isotope turnover of fin tissues (Willis et al. 2013). Accordingly, the between-treatment variability in nitrogen isotopic shifts identified in Smalltooth Sawfish and cownose rays is unlikely to be a significant concern for using fin tissue to answer ecological questions.

Relative to the freezing of samples, storage in ethanol does alter the fin tissue isotope values, raising the question of whether or not ethanol-stored fin tissues should be corrected for this effect. There is no consensus in the literature regarding the use of correction factors to allay chemical storage effects on stable isotope values. Vander Zanden et al. (2003) applied a correction factor for both $\delta^{13}C$ and $\delta^{15}N$ values based on mean differences derived from a number of experimental studies of freshwater fish muscle tissue; those authors found that the use of a correction did not add a major source of bias because it was less than the error associated with trophic discrimination. Alternatively, Kim and Koch (2012) advocated that a correction for $\delta^{13}C$ should not be applied because the difference between frozen and ethanol-preserved muscle tissue samples from Longnose Skate was too variable among individuals. In our study, $\delta^{13}C$ and $\delta^{15}N$ values of fin samples from Smalltooth Sawfish and cownose rays were highly correlated between treatments despite the significant differences found between mean values, the high interspecific variability, and the low sample size (i.e., in the case of cownose rays). Additionally, in Smalltooth Sawfish, treatment differences in $\delta^{13}C$ and $\delta^{15}N$ did not vary with size. Given that fin tissue stored in ethanol provided strong estimates of isotopic values relative to frozen samples in both taxa, a developed correction factor (principally for $\delta^{13}C$) may be applicable across elasmobranch taxa. Considering the range in treatment differences, correction using a regression may be more applicable than subtracting the mean value. However, additional studies will be necessary to confirm this application.

Estimation of trophic position and application of mixing models for characterizing dietary resources are vulnerable to chemical storage effects, with consequences not only for ecological investigations but for management decisions. Overall, the results documented here are similar to those from a number of studies focused on ethanol storage of fish muscle tissues—particularly, there was a larger effect of ethanol storage on $\delta^{13}C$ values than on $\delta^{15}N$ values. The general uniformity in isotope ratio shifts associated with ethanol storage between the two taxa studied here implies that comparisons and ecological interpretations can be made, provided that (1) the materials to be compared are handled in the same manner or (2) in the case of comparing tissues from different storage methods, a correction is considered, especially for $\delta^{13}C$. Given the minimal effects of time in storage on isotope values, especially with variability decreasing with duration in storage, fin tissue stored in ethanol can serve as a useful material for ecological analyses. This is especially relevant with regard to the Smalltooth Sawfish, for which the endangered status limits the sampling of other tissues and requires the use of archived samples for understanding components of the species' ecology. However, it is important to note the need for further species-specific evaluations of chemical effects on stable isotope values of fin tissues to assess whether these findings can be generalized across elasmobranch species.

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APPENDIX

Q4

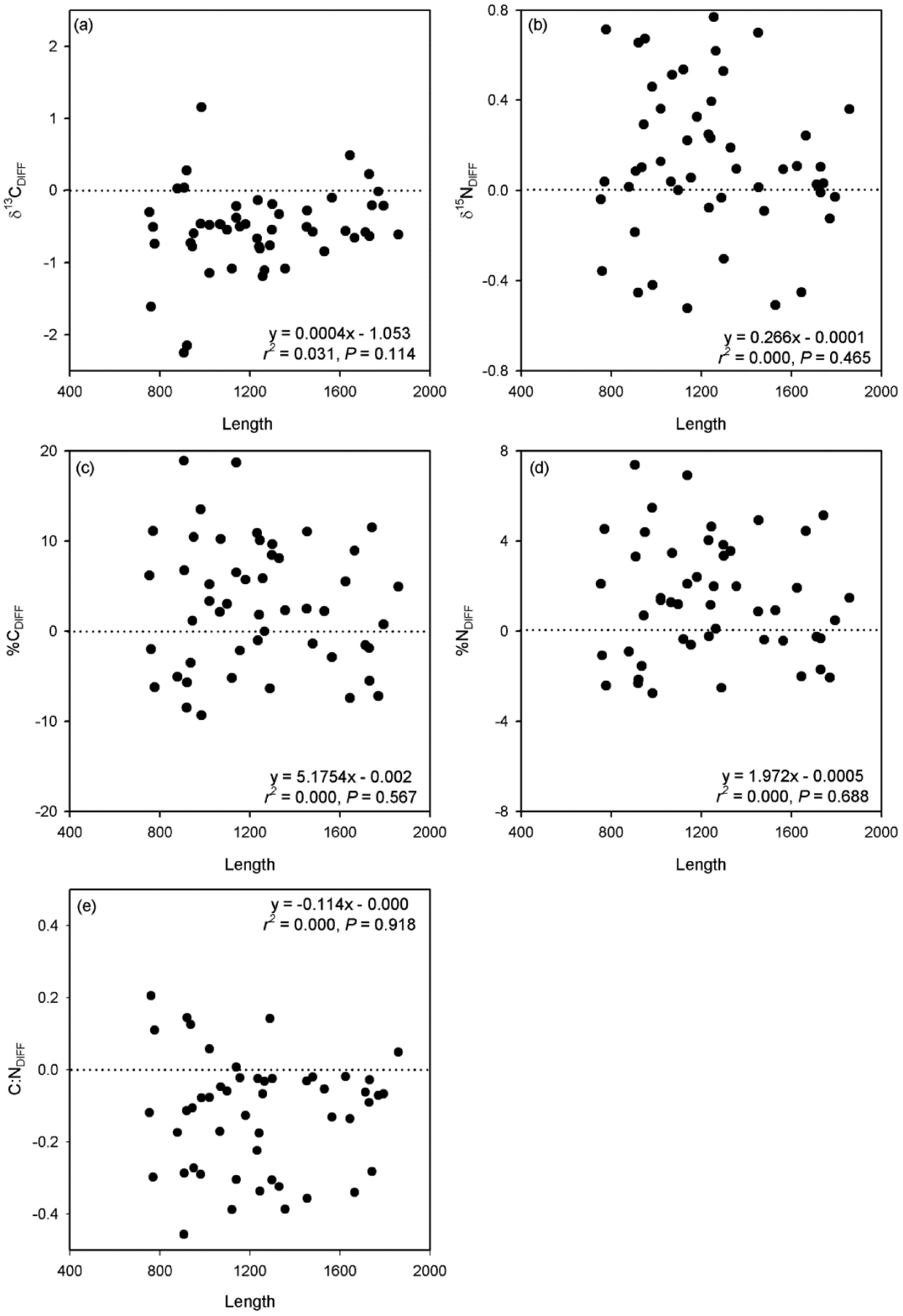

FIGURE A.1. Differences in **(a)** $\delta^{13}C$, **(b)** $\delta^{15}N$, **(c)** %C, **(d)** %N, and **(e)** C:N values between paired fin tissue samples (e.g., $X_{diff} = X_{Frozen} - X_{EtOH}$, where X corresponds to $\delta^{13}C$, %C, $\delta^{15}N$, %N, or C:N) with increasing stretch TL (mm) of Smalltooth Sawfish. Dotted line at 0.0 indicates no effect of ethanol storage.

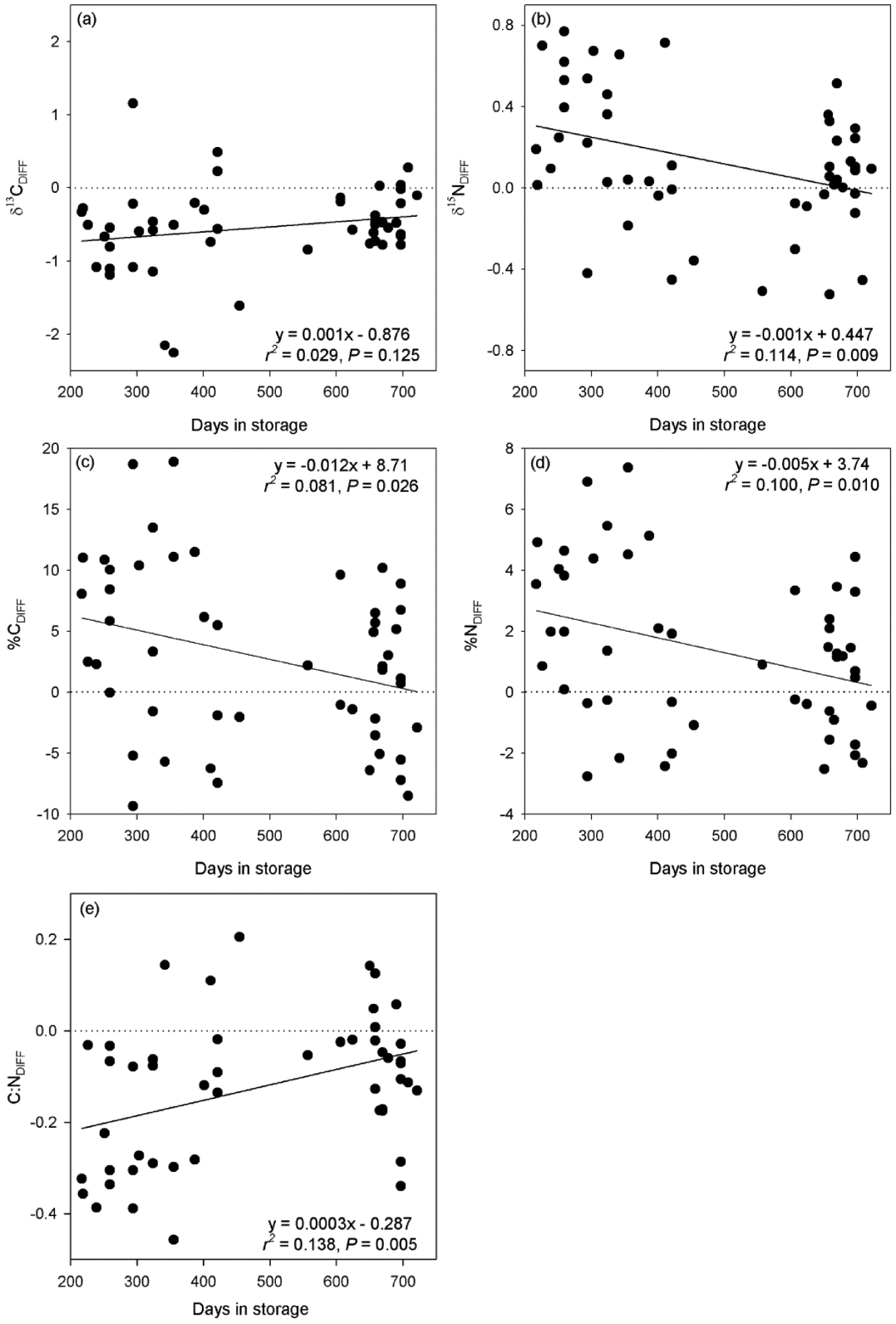

FIGURE A.2. Differences in **(a)** $\delta^{13}C$ (‰), **(b)** $\delta^{15}N$ (‰), **(c)** %C, **(d)** %N, and **(e)** C:N values (e.g., $X_{diff} = X_{Frozen} - X_{EtOH}$, where X corresponds to $\delta^{13}C$, %C, $\delta^{15}N$, %N, or C:N) for Smalltooth Sawfish fin tissue versus the number of days of storage in ethanol. Dotted line at 0.0 indicates no effect of ethanol storage.