

Type of the Paper: Article

Effects of tissue preservation on carbon and nitrogen stable isotope signatures in syngnathid fishes and prey

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Simple Summary: Stable isotope analysis (SIA) was used to assess the influence of various preservative methods (drying, freezing, ethanol and formaldehyde) on syngnathid (seahorses and pipefishes) fins, seahorse newborns (seahorses), and prey (copepods and *Artemia*). The first available conversion models are provided, enabling their application to isotopic studies in the field and in the laboratory.

Abstract: Isotopic stable analysis (SIA) is a powerful tool in the assessment of different types of ecological and physiological studies. For that, different preservative methods for the samples are commonly used prior to isotopic analysis. The effects of various preservative methods (drying, freezing, ethanol and formaldehyde) have been analyzed for C:N ratio, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ on a variety of tissues including dorsal fins (three seahorse and two pipefish species), seahorse newborns (three seahorses species), and prey (copepods and different stages of *Artemia*) commonly used to feed the fishes in rearing conditions. The aims of the study were to: (i) evaluate isotopic effects of preservation methods across tissues; and (ii) construct the first conversion models available in syngnathid fishes. The preservation in ethanol and to a lesser extend in formaldehyde significantly affected $\delta^{13}\text{C}$ values, whereas $\delta^{15}\text{N}$ signatures were not affected significantly. Due to their low lipid content, the isotopic signals in fish fins were almost unaffected, supporting the suitability of dorsal fins as a convenient tool in isotopic studies on vulnerable species such as syngnathids. The regression equations provided permit the successful conversion of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between preservative treatments. The conversion models can be applied to isotopic studies in the field and in the laboratory.

Keywords: stable isotopes; preservation; syngnathids; seahorses; pipefishes; conversion models

1. Introduction

Isotopic analyses for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures are used in a wide variety of studies, including tracing trophic chains, estimation of trophic enrichment factors, diet reconstruction, turnover in tissues, discrimination between hatchery-reared and wild spawned individuals, or migrations, among others [1-7]).

The isotopic composition of animal tissues reflects the dietary isotopic composition, especially for C and N, with a difference of a few units (discrimination factor) [8,9]. Isotopic signatures are species- and tissue-specific [10,11] and might vary depending on several factors such as dietary isotopic values or developmental stage [12-14]. However, there are some important issues that need to be properly addressed in stable isotope analysis (SIA). Most concern regarding SIA accuracy refers to type of tissue, sample preservation method, and lipid correction.

Whenever possible, SIA is performed on muscle tissue due to its relative isotopic stability [10]. Where lethal sampling is not desirable, other fish tissues such as fins and scales are non-lethal alternatives for muscle [13, 15-18], especially in threatened and endangered species [19-22]. The isotopic signals from those surrogate tissues can be converted to muscle values by means of mathematical corrections obtained from inter-tissue comparisons.

Methods used to preserve tissues include drying, freezing, ethanol or formaldehyde, depending on the objectives and limitations of the study and sampling conditions [23-26]. However, the results of inter-methodological comparisons might be unpredictable and vary among taxa, suggesting the need to treat each species individually [25].

Lipids are depleted (more negative values) in $\delta^{13}\text{C}$ compared to other biochemical compounds (i.e. proteins, carbohydrates), affecting to bulk tissue signatures [8-9, 27-28]. For that, raw samples exceeding 3.5 in C:N ratios (about 5% of lipids) are generally submitted to a lipid extraction procedure prior to SIA [29]. Alternatively, mathematical normalization techniques may be applied to standardize lipid content when C:N ratios are high. Ideally, corrections should be used from estimates on the same or similar type of organisms. Even though lipids mainly affect the heavier ^{13}C isotope, $\delta^{15}\text{N}$ values might result also impaired [30-31] due to artifacts in the extraction of lipids [6].

In the present study, we carried out SIA in syngnathid fishes, including adults (fin clipping) and newborn (bulk specimens) as well as in prey (copepods and different stages of *Artemia*) commonly used to raise those species. Syngnathids are a group of fishes including endangered and vulnerable species [32]. For that reason, the use of lethal sampling should be avoided whenever possible, and fin tissue results the most adequate tissue for isotopic analyses [20]. Since tissue conversion models specific for syngnathid fishes were lacking, the main aim of this study was to generate for the first time mathematical corrections for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in syngnathid species considering different types of specimens and preservation techniques.

2. Materials and Methods

2.1. Live prey

We analyzed different sources of prey commonly used in the rearing of syngnathids: calanoid copepods (*Acartia tonsa*) and *Artemia* (nauplii, enriched metanauplii, enriched adults and unenriched adults). Microalgae (*Phaeodactylum tricornutum*, *Rhodomonas lens* and *Isochrysis galbana*) were cultivated to feed copepods and *Artemia* until the adult stage. Microalgae cultures were grown at 22 ± 1 °C in 80 L plastic bags containing sterilized seawater supplemented with F2P media (100 g L⁻¹) (VarAqua). Additionally, silicates were added to *P. tricornutum* cultures, and 200 µl F2P media to *R. lens* culture flasks.

The copepod *Acartia tonsa* was cultivated in 700 L cylindrical conic tanks at 26-27 °C and 38 salinity, with an initial density of 1 copepod mL⁻¹. Copepods were fed every two days on the microalgae *R. lens* (10³ cells mL⁻¹). Siphoning of the culture tanks and water renewals (10% of the total volume) were carried out three times per week. Only copepods collected on a 180 µm mesh (copepodites and adults) were analyzed.

Artemia nauplii and enriched metanauplii are produced to feed seahorse juvenile, whereas adult *Artemia* are delivered to adult seahorses. *Artemia* cysts (EG MC450 and AF; Ocean Nutrition, USA) were hatched at 28 °C for 20 h in 20 L units. Newly hatched nauplii were gently rinsed with tap-water, collected on a 125µm mesh, rinsed and transferred to 5 L buckets to produce enriched metanauplii (from AF cysts) and adults (from EG cysts). For metanauplii, the nauplii were enriched (2-3 days at 100 *Artemia* mL⁻¹) twice daily on a mixture including live microalgae (*P. tricornutum* 10⁷ cells mL⁻¹), Red Pepper (0.015 g L⁻¹) (Bernaqua, Belgium) and dried *Spirulina* (0.03 g L⁻¹, Iberfrost, Spain).

For the production of adult *Artemia*, the nauplii were hatched from EG MC450 cysts (Ocean Nutrition, USA) and grown in 100 L units, at 26–28 °C with gentle aeration and constant light. Adult *Artemia* was long-time enriched (3-6 days) or unenriched. The enrichment was carried out from day 16 onwards on a mixture consisting on live microalgae *P. tricornutum* and *I. galbana* (10⁷ cells mL⁻¹), Red Pepper (0.015 g L⁻¹) and dried *Spirulina* (0.03 g L⁻¹) [33].

All samples were rinsed with distilled water, preserved according to the established procedure (see below), dried for 48 h (60 °C) and manually homogenized in a mortar.

2.2. Fishes

The following five species of Syngnathidae were assayed: pipefishes *Syngnathus acus* Linnaeus, 1758 and *S. typhle* Linnaeus, 1758, and seahorses *Hippocampus guttulatus* Cuvier, 1829, *H. hippocampus* (Linnaeus, 1758) and *H. reidi* Ginsburg, 1933.

The pipefishes were captured in Arcade Cove (Ría de Vigo, NW Spain) in March-April 2016, transferred to the laboratory and fin clipped for further sampling [28]. The seahorses were reared in captivity and sampled for dorsal fin tissue (fin clipping) and bulk newborn. The adults were fed on mixtures of adult *Artemia* and mysidaceans (frozen or captured in the wild). Further details on the maintenance and rearing conditions for the three seahorse species are provided in [34-37].

A variable number (n>5) of bulk juveniles were sampled and pooled prior to conservation. Sampled seahorse juveniles were taken after male's pouch release (prior to first feeding) and euthanized with Tricaine MS-222 (0.1 mg L⁻¹, Sigma Aldrich).

2.3. Experimental preservation procedures

Direct drying (50 °C for 48 h) and three preservative procedures were compared for δ¹³C and δ¹⁵N in prey samples. Preservation treatments included freezing at -80 °C, 95% ethanol and 4% formaldehyde (Merck, Germany).

Three preservative procedures (freezing at -80 °C, 95% ethanol and 4% formaldehyde) were compared for δ¹³C and δ¹⁵N values in dorsal fins in five species of adult syngnathids (*H. guttulatus*, *H. hippocampus*, *H. reidi*, *S. acus* and *S. typhle*).

Bulk newborn of three species of seahorses (*H. abdominalis*, *H. guttulatus* and *H. reidi*) were preserved as for dorsal fin and analyzed for δ¹³C and δ¹⁵N values. Seahorse breeders were fed on different types of prey and enrichment. Consequently, we obtained a wide range in isotopic values (especially for δ¹³C) and C:N ratios both in fins and newborn samples.

All collected samples were filtered, rinsed with distilled water and stored for 3-4 months prior to analysis.

2.4. Isotope ratio mass spectrometry

Samples for stable isotope analyses (SIA) were homogenized and aliquots were transferred to preweighted tin capsules (ø 3.3 x 5 mm, 0.03 ml) (Lüdiswiss, Switzerland). The analyses were made on sub-samples of 0.5 - 1 mg dry weight biomass. δ¹³C and δ¹⁵N values and elemental composition (total C and N percentage) were analyzed at Servicios de Apoyo a Investigación (SAI) of the University of A Coruña (Spain) [20]. The samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyser (ThermoFinnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface. Carbon and nitrogen stable isotope abundance was expressed as permil (‰) relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air, according to the following equation:

$$\delta X = (R_{\text{sample}} / R_{\text{reference}}) - 1,$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. As part of an analytical batch run, a set of international reference materials for $\delta^{15}\text{N}$ values (IAEA-N-1, IAEA-N-2, IAEA-NO-3) and $\delta^{13}\text{C}$ values (NBS 22, IAEA-CH-6, USGS24) were analyzed. The precision (standard deviation) for the analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the laboratory standard (acetanilide) was $\pm 0.15\%$ (1-sigma, $n=10$). Standards were run every 10 biological samples.

2.5 Data analysis

The data were submitted to t-test pairwise comparisons. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of different treatments were regressed on type of preservation method using least-squares linear regression of corrected against control isotopic values to determine efficacy of the conversion models. The significance level was set at $P < 0.05$. Statistical tests were conducted using Statistica 8.0 software package (Statsoft, USA).

2.6 Bioethical Approval

Animal capture, handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (Real Decreto 1201/2005, 10th October) and the Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01 and ES360570202001/16/EDU-FOR07/MPO01).

3. Results

3.1. Live prey

Isotopic values in prey ranged from -21.1 to -14.7% for $\delta^{13}\text{C}$ and from 1.6 to 11.1% for $\delta^{15}\text{N}$, with mean values of $-18.7 \pm 2.5\%$ and $6.2 \pm 3.6\%$, respectively (Figure 1). C:N ratios ranged from 6.3 to 3.8 , with mean values of 4.8 ± 0.8 .

Direct drying and freezing were used as control treatments. Both treatments performed similarly for mean $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C:N values (Table 1, Figure 2). The most noticeable differences across treatments were observed in C:N ratios and EtOH preserved samples, whereas CH₂O treatment performed similarly to controls, except for $\delta^{13}\text{C}$ (depleted compared to controls). Samples preserved in EtOH resulted enriched in $\delta^{13}\text{C}$ compared to all other treatments. The effects of preservation method on $\delta^{15}\text{N}$ values were negligible.

3.2. Fish fins and newborn

Due to the limited availability of samples, direct drying was not tested on fish fins nor newborn. Hence, frozen samples were used as controls.

Except for $\delta^{15}\text{N}$ signatures, the treatments in dorsal fins significantly affected to $\delta^{13}\text{C}$ and C:N values (Table 2, Figure 3). The later resulted particularly affected, especially in samples preserved in EtOH. Similarly to prey, $\delta^{15}\text{N}$ values were very similar across treatments, whereas $\delta^{13}\text{C}$ signatures were slightly overestimated compared to frozen and CH₂O samples.

Broadly, the effects of preservation methods in newborn seahorses resulted similar to those in fins (Table 2, Figure 4). However, the effects were amplified, especially for C:N ratios. Besides, a significant effect was noticed for $\delta^{15}\text{N}$ signals, with a slight overestimation in EtOH and CH₂O samples compared to controls (frozen samples).

4. Discussion

The present study demonstrates for the first time the effects of three preservation methods on isotopic signals ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and C:N ratios in syngnathid fishes, including adults (dorsal fin) and newborn and prey (also included direct drying of samples) generally used to feed marine fishes in rearing systems. Among the preservation methods tested, ethanol caused significant changes in $\delta^{13}\text{C}$ signals and C:N (loss of lipids depleted in $\delta^{13}\text{C}$ from tissues), and an insignificant enrichment in $\delta^{15}\text{N}$ compared to controls. Conversely, dried and frozen samples performed similarly. In spite of the

global effects observed for each preservation method tested, there were important differences across treatments depending on the type of sample analyzed. Such differences seemed to rely mainly but not only on the C:N ratios (i.e. lipid content) of samples.

The diverse tissues of an animal differ in turnover rates due to inter-tissue differences in isotopic fractionation [8-10, 38-39]. In most isotopic studies, muscle is the focal tissue due to its turnover rate. The disadvantage of sampling fish muscle is that the animal must be sacrificed [40]. However, there are alternative tissues that do not entail fish killing, providing isotopic signatures highly correlated with those in muscle [13, 20, 41]. Fish fins are excellent tissues for that, especially convenient in studies involving threatened or endangered species [15,20]. In syngnathids, fin clipping has the advantage that the clipped tissue regenerate completely in 1-2 months [34]. In the present study, samples of fin tissue were analysed for seahorses and pipefishes and both performed similarly (data not shown). Fin clipping has been successfully used on syngnathids in isotopic field [21] and laboratory studies [22], both in seahorses and pipefishes.

The analyses of the data across treatments and tissues (prey, fins and newborn) revealed that linear relationships with slopes significantly different from 1 were present for several regressions, particularly for $\delta^{13}\text{C}$ in dorsal fins and C:N in seahorse newborns. There is a wide number of isotopic studies on the effects of preservation methods and lipid normalization in fish tissues [27, 29, 42]. It is not the aim of the present study to discuss on the reasons underlying those effects but to provide practical mathematical models for the conversion of isotopic signals in live prey and syngnathid adults and newborns preserved frozen, in ethanol or in formaldehyde. The regression models might be applied to field studies where samples cannot be analysed immediately, requiring any type of preservative until further analysis. In addition, in spite of further assessment on the effect of long-term storage on isotopic signals [23], the models might be applied to syngnathid specimens in historic collections.

We did not assess the effects of lipid extraction usually applied to samples with C:N ratios above 3.5 [29, 43]. A previous study carried out in seahorses compared dorsal fin, muscle, and liver tissues [20]. The authors reported similarities between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in dorsal fin and muscle tissue, significant effects of lipid extraction for $\delta^{13}\text{C}$ values in muscle and liver, and concluded that lipid removal is not necessary in dorsal fin tissues due to their low lipid content (2.6% dry weight compared to 7.1% in muscle tissue). The low lipid content in fin tissues of syngnathids was confirmed by C:N values in the present study (2.88 – 3.19 in *Hippocampus* spp; 2.93 – 3.53 in *Syngnathus* spp.). Accordingly, fin clipped samples could be submitted to SIA without the need to apply lipid extraction nor mathematical lipid corrections. For $\delta^{13}\text{C}$, the regression models given in Table 2 could be applied.

5. Conclusions

The results achieved indicate that the preservation method used have different influences on isotopic signatures and C:N ratios in syngnathid fishes (dorsal fin in adults and bulk newborn) and prey commonly used to feed fishes in the laboratory. Frozen and dried samples performed similarly, whereas the impact of ethanol was high compared to formaldehyde, especially for $\delta^{13}\text{C}$ signals and C:N ratios. The influence of preservatives on $\delta^{15}\text{N}$ values was almost negligible. The first conversion models for the mathematical correction of data across the tested preservatives were constructed specifically for syngnathids. Those models could be applied to field collected samples and historical collections as well. Further work should be conducted to determine the isotopic effect of lipid extraction and duration of preservation, except for dorsal fins.

Table 1. Summary of paired t-tests and two-sided P-values across preservation methods for C:N ratios, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. Significant P-values are given in bold. The regression models with slopes different from 1 are given in bold. DR – dried; FR – frozen; ET – ethanol; FO – formaldehyde.

	Treatments		t-Test		Conversion model (Regression)				
	y-x	t	P	n	F	P	β	Model	R ²
Prey									
C:N	DR-FR	-0.11	0.918	12	69.1	<0.001	0.935	y=0.969x	0.874
	DR-ET	6.76	<0.001	12	31.9	<0.001	0.873	y=0.805x + 1.646	0.762
	DR-FO	-0.92	0.377	12	36.6	<0.001	0.886	y=1.110x - 0.646	0.785
	FR-ET	6.05	<0.001	12	22.9	<0.001	0.834	y=0.742x + 1.902	0.696
	FR-FO	-1.28	0.229	12	104.3	<0.001	0.955	y=1.154x - 0.854	0.912
	FO-ET	6.61	<0.001	12	22.5	<0.001	0.998	y=1.130x	0.692
$\delta^{13}\text{C}$	DR-FR	0.83	0.423	12	1042.6	<0.001	0.995	y=0.996x	0.991
	DR-ET	-7.31	<0.001	12	360.6	<0.001	0.986	y=1.116x + 1.036	0.973
	DR-FO	17.05	<0.001	12	2009.2	<0.001	0.998	y=1.006x + 1.221	0.995
	FR-ET	-7.13	<0.001	12	281.4	<0.001	0.982	y=1.111x + 0.897	0.966
	FR-FO	21.67	<0.001	12	1111.8	<0.001	0.996	y=1.004x	0.991
	FO-ET	-16.76	<0.001	12	408.0	<0.001	0.998	y=1.108x	0.976
$\delta^{15}\text{N}$	DR-FR	-2.69	0.423	12	1977.2	<0.001	0.997	y=0.971x	0.995
	DR-ET	-3.06	0.011	12	400.7	<0.001	0.988	y=0.991x - 0.438	0.976
	DR-FO	-2.15	0.055	12	423.3	<0.001	0.988	y=0.994x	0.977
	FR-ET	-1.91	0.083	12	523.4	<0.001	0.991	y=1.021x - 0.425	0.981
	FR-FO	-0.88	0.399	12	576.5	<0.001	0.991	y=1.025x - 0.288	0.982
	FO-ET	-2.26	0.045	12	2195.7	<0.001	0.998	y=1.000x	0.995

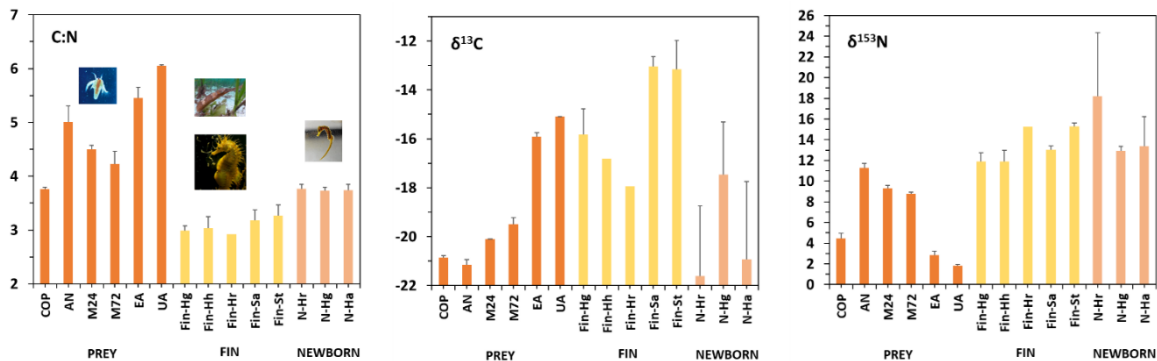


Figure 1. Values (means \pm SD) of C:N ratios, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for prey, dorsal fin in adult syngnathids and newborn seahorses. CO – copepods; NA - *Artemia nauplii*, M24 and M72 – *Artemia metanauplii* enriched for 24 and 72 h, respectively; EA and UA – enriched and unenriched adult *Artemia*, respectively.

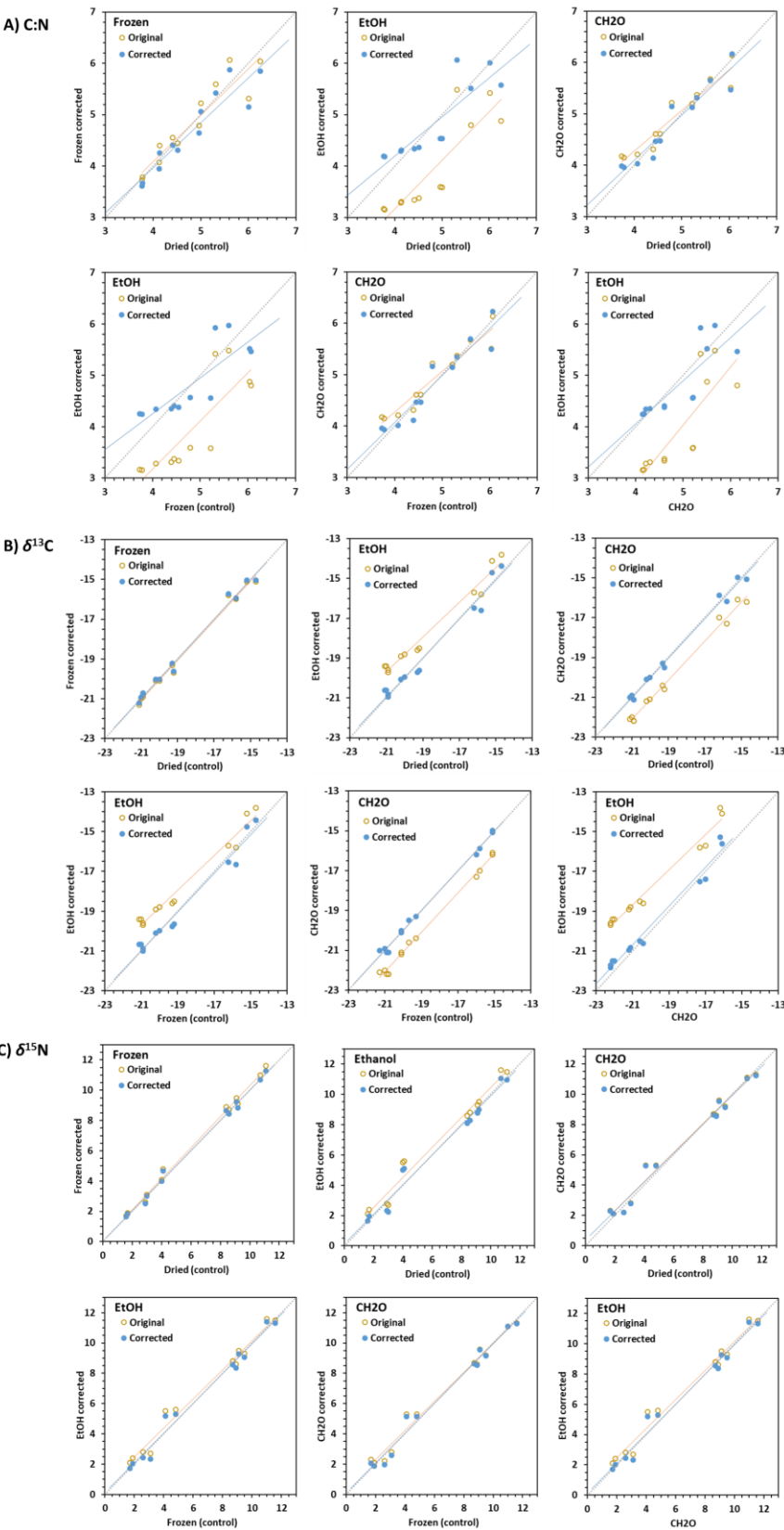


Figure 2. Original (open symbols) and corrected (solid symbols) values for (A) C:N ratios, (B) $\delta^{13}\text{C}$ and (C) $\delta^{15}\text{N}$ in prey. Dried and frozen samples were used as controls. The 1:1 dashed line and regression lines are given for dried, frozen, EtOH (etanol) and CH2O (formaldehyde) comparisons.

Table 2. Summary of paired t-tests and two-sided P-values across organisms (dorsal fin in adult syngnathids and bulk newborn seahorses) and preservation methods for C:N values $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. Significant P-values are given in bold. The regression models with slopes different from 1 are given in bold. FR – frozen; ET – ethanol; FO – formaldehyde.

Treatments		t-Test			Conversion model (Regression)				
	y-x	t	P	n	F	P	β	Model	R ²
Dorsal fin									
C:N	FR-ET	4.76	0.002	17	7509.6	<0.001	0.999	y=1.059x	0.998
	FR-FO	-1.76	0.097	18	70.6	<0.001	0.908	y=1.067x - 0.242	0.824
	FO-ET	3.69	0.004	18	3480.6	<0.001	0.998	y=1.067x	0.997
$\delta^{13}\text{C}$	FR-ET	-5.61	<0.001	14	865.0	<0.001	0.993	y=0.918x - 1.617	0.986
	FR-FO	5.83	<0.001	16	30.169	<0.001	0.999	y=0.969x	0.999
	FO-ET	-4.01	0.002	18	4.421	<0.001	0.998	y=1.049x	0.998
$\delta^{15}\text{N}$	FR-ET	-0.72	0.482	15	107259	<0.001	0.999	y=0.997x	0.999
	FR-FO	-0.17	0.870	16	50677	<0.001	0.999	y=0.999x	0.999
	FO-ET	-1.19	0.269	18	38065	<0.001	0.999	y=0.994x	0.999
Newborn									
C:N	FR-ET	34.61	<0.001	20	32847	<0.001	0.999	y=1.226x	0.999
	FR-FO	-11.11	<0.001	21	23795	<0.001	0.999	y=0.927x	0.999
	FO-ET	26.17	<0.001	20	10842	<0.001	0.999	y=1.324x	0.999
$\delta^{13}\text{C}$	FR-ET	-19.54	<0.001	20	2045	<0.001	0.996	y=0.961x - 2.181	0.991
	FR-FO	13.90	<0.001	21	4622	<0.001	0.998	y=1.024x + 1.166	0.996
	FO-ET	-26.56	<0.001	21	2215	<0.001	0.996	y=0.938x - 3.281	0.992
$\delta^{15}\text{N}$	FR-ET	-6.39	<0.001	20	146531	<0.001	0.999	y=0.984x	0.999
	FR-FO	-7.80	<0.001	21	12356	<0.001	0.999	y=1.014x - 0.528	0.999
	FO-ET	1.14	0.270	20	176197	<0.001	0.999	y=1.001x	0.999

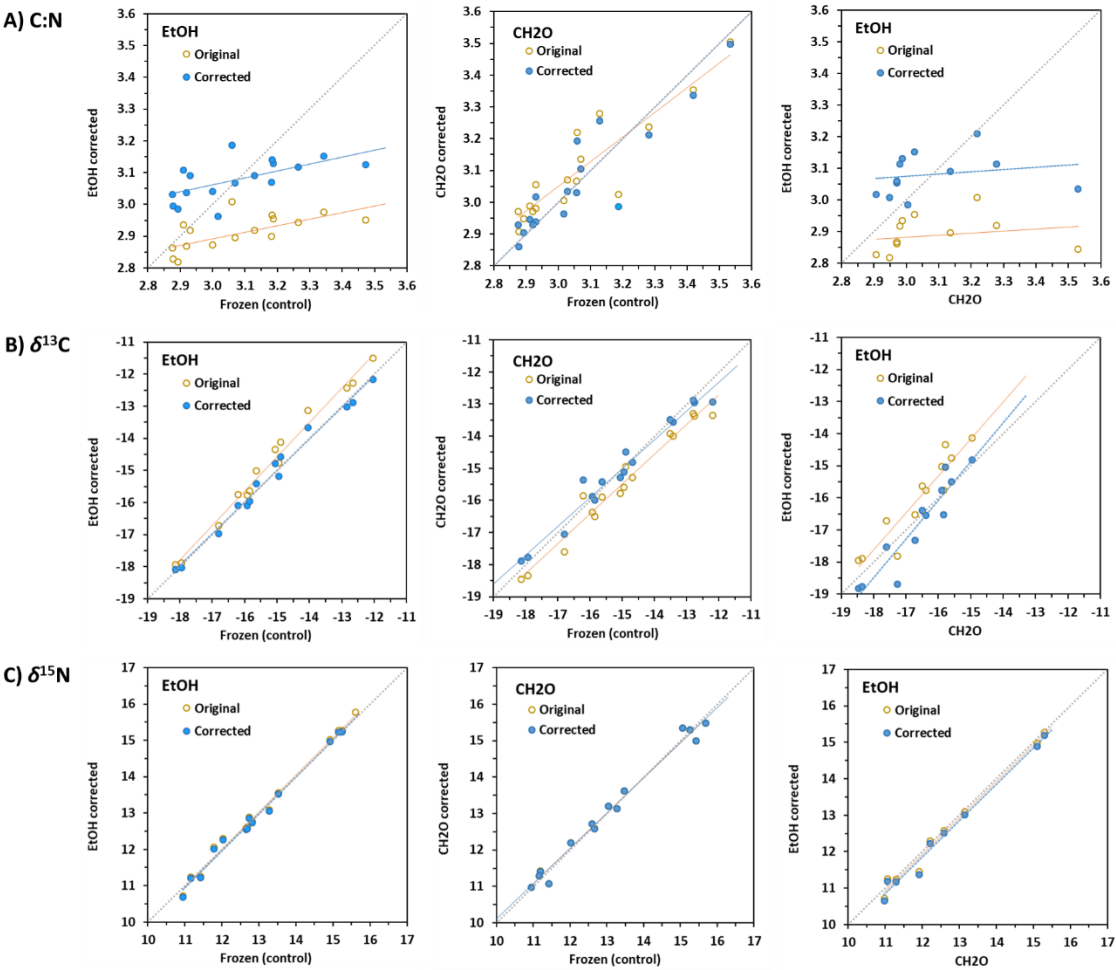


Figure 3. Original (open symbols) and corrected (solid symbols) values for (A) C:N ratios, (B) $\delta^{13}\text{C}$ and (C) $\delta^{15}\text{N}$ in dorsal fin of adult syngnathids. Dried and frozen samples were used as controls. The 1:1 dashed line and regression lines are given for dried, frozen, EtOH (ethanol) and CH₂O (formaldehyde) comparisons.

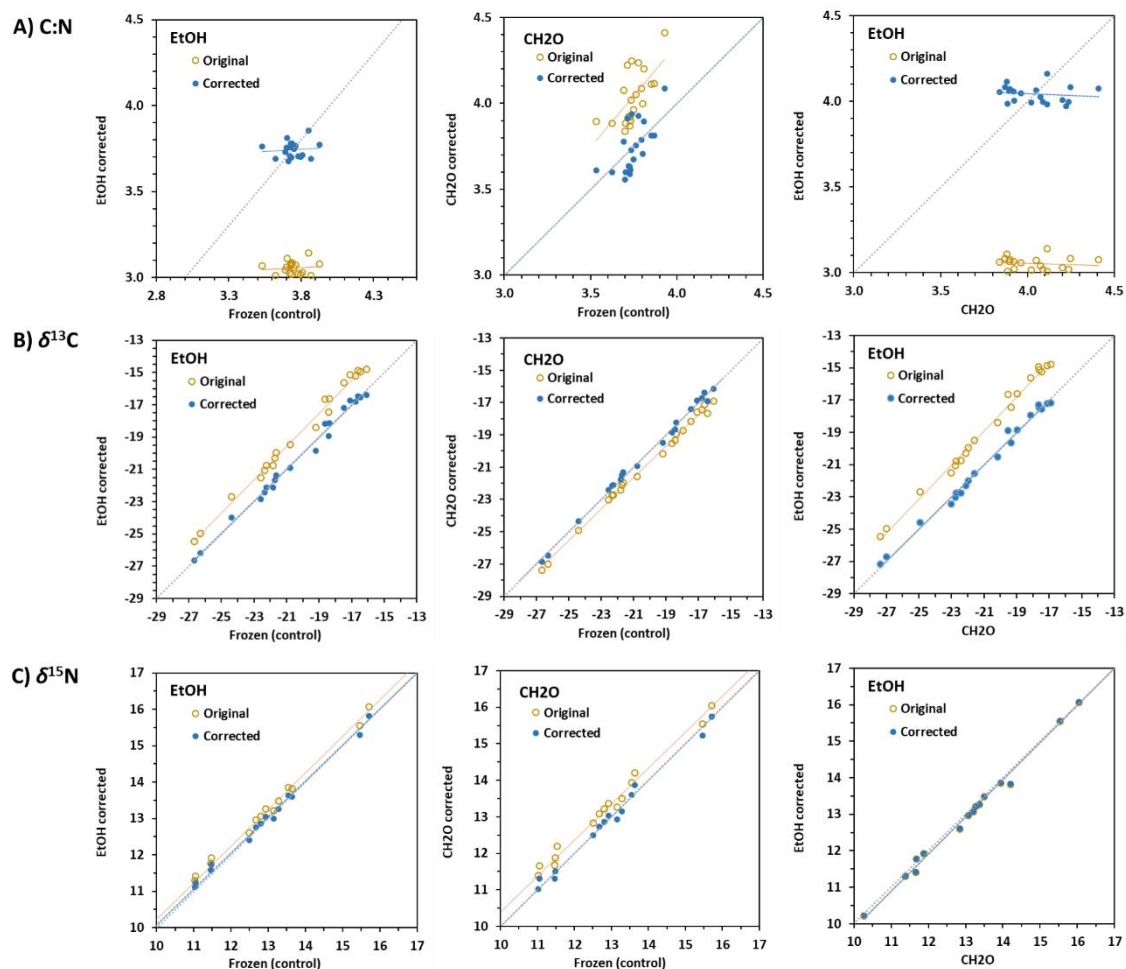


Figure 4. Original (open symbols) and corrected (solid symbols) values for (A) C:N ratios, (B) $\delta^{13}\text{C}$ and (C) $\delta^{15}\text{N}$ in newborn seahorses. Dried and frozen samples were used as controls. The 1:1 dashed line and regression lines are provided for dried, frozen, EtOH (etanol) and CH2O (formaldehyde) comparisons..

Author Contributions: Conceptualization, M.P.; Formal analysis, M.P., A.P, M.D.D.C. and J.H.U. Investigation, M.P., A.P, M.D.D.C. and J.H.U.; Methodology, M.P., A.P, M.D.D.C. and J.H.U.; Project administration, M.P.; Resources, M.P.; Supervision, M.P.; Validation, M.P.; Original draft preparation, M.P.; and Review and editing of the manuscript, M.P., A.P, M.D.D.C. and J.H.U.

Funding: This study was financially supported by the Spanish Government with projects Hippoeco (Ref. CGL2015-68110-R, Ministerio de Ciencia, Innovación y Universidades and Fondos FEDER) and Hippoparques (Ref. 1541S/2015, Organismo Autónomo de Parques Nacionales de España, Ministerio para la Transición Ecológica). J.H.U. was supported by a Juan de la Cierva's post-doc research grant (FJCI-2016-30990; Ministerio de Ciencia, Innovación y Universidades, Spain), A.P. was supported by Erasmus+ funding programme (Università Politecnica delle Marche, Italy) and M.D.D.C. was granted by Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (PDSE 88881.187275/2018-01; Ministério da Educação – MEC, Brazil).

Acknowledgments: The authors are grateful to Cofradía de Pescadores de Arcade (Ría de Vigo, NW Spain) for providing *Syngnathus* spp. specimens, Alexandro Chamorro for fish rearing technical support and Antía González Barreiro for sampling assistance.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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