Acta Limnologica Brasiliensia

Publication of the Brazilian Association of Limnology

Thematic Section: Methods
Acta Limnologica Brasiliensia, 2025, vol. 37, e32
https://doi.org/10.1590/S2179-975X7624
ISSN 2179-975X on-line version

Effects of freezing on the carbon and nitrogen stable isotopic compositions of fish tissues

Efeitos do congelamento nas composições isotópicas estáveis de carbono e nitrogênio nos tecidos de peixes

Luciana Mulero Beloto[1], Thiago Simon Marques[2], Barbara Protocevich[2*], Ronnie Von Mateus Ferreira[2] and Plinio Barbosa de Camargo[1]

[1]Laboratório de Ecologia Isotópica, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Av. Centenário, 303, São Dimas, 13416-903, Piracicaba, SP, Brasil

[2]Laboratório de Ecologia Aplicada, Universidade de Sorocaba, Rod. Raposo Tavares, Km 92,5, Vila Artura, 18023-000, Sorocaba, SP, Brasil

*e-mail: barbarappina@gmail.com

Cite as: Beloto, L.M. et al. Effects of freezing on the carbon and nitrogen stable isotopic compositions of fish tissues. *Acta Limnologica Brasiliensia*, 2025, vol. 37, e32. https://doi.org/10.1590/S2179-975X7624

Abstract: Stable isotope analysis is a vital tool for elucidating nutrient flow within food chains. Studies utilizing this methodology must consider potential variables that could influence the $\delta^{13}C$ and $\delta^{15}N$ values of the species being examined. One such variable is the preservation of samples in a frozen state during field studies, particularly when immediate sample processing is not possible. **Aim:** The objective of this study is to investigate the long-term effects of freeze-thaw at intervals of 1, 2, 7, 15, 30, 90 and 180 days on the $\delta^{13}C$, $\delta^{15}N$, and C:N ratios in biological samples from three fish species. **Methods:** Tissues from three estuarine fish species (*Cathorops spixii, Genidens barbus* and *Chloroscombrus chrysurus*) were frozen and aliquots were subjected to thawing for sample collection aimed at isotopic determination. **Results:** The discrepancies observed in this study were related to the $\delta^{13}C$ and C:N ratios in the skin samples of *C. spixii* and *G. barbus*, as well as the muscle samples of *C. chrysurus*. These variations seem to be more closely linked to the potential volatility of the ^{12}C isotope within the samples, resulting in $\delta^{13}C$ enrichment and an increase in C:N values. **Conclusions:** This suggests that these changes are not solely attributable to the freeze-thaw process, but may also be influenced by other factors like the lipid oxidation. Our findings suggest that this process significantly influences the $\delta^{13}C$ and C:N ratios of skin and muscle tissues. Furthermore, we advocate for prompt processing of samples to minimize the impacts of freeze-thaw events.

Keywords: marine fish; experiment; preservation of sample; ratios; isotopes.

Resumo: A análise de isótopos estáveis é uma ferramenta essencial para elucidar o fluxo de nutrientes nas cadeias alimentares. Estudos que utilizam essa metodologia devem considerar as variáveis potenciais que podem influenciar os valores de $\delta^{13}C$ e $\delta^{15}N$ das espécies analisadas. Uma dessas variáveis é a preservação das amostras em estado congelado durante os estudos de campo, especialmente quando o processamento imediato das amostras não é possível. **Objetivo:** O objetivo deste estudo é investigar os efeitos a longo prazo o congelamento e descongelamento em intervalos de 1, 2, 7, 15, 30, 90 e 180 dias sobre os valores de $\delta^{13}C$, $\delta^{15}N$ e as razões C:N em amostras biológicas de três espécies de peixes. **Métodos:** Tecidos de três espécies de peixes estuarinos (*Cathorops spixii*, *Genidens barbus* e *Chloroscombrus chrysurus*) foram congelados e alíquotas foram submetidas ao descongelamento para coleta de amostras destinadas à determinação isotópica. **Resultados:** As discrepâncias observadas neste estudo estavam relacionadas aos valores de $\delta^{13}C$ e às razões C:N nas amostras de pele de *C. spixii* e *G. barbus*, bem como nas amostras de músculo de *C. chrysurus*. Essas variações parecem estar mais fortemente ligadas à potencial volatilidade do isótopo ^{12}C nas amostras, resultando em um enriquecimento de $\delta^{13}C$ e um aumento nos valores de C:N.

Conclusões: Isso sugere que essas alterações não são exclusivamente atribuíveis ao processo de congelamento e descongelamento, mas também podem ser influenciadas por outros fatores presentes nas amostras. Nossos resultados indicam que esse processo influencia significativamente os valores de $\delta^{13}C$ e as razões C:N nos tecidos de pele e músculo. Além disso, recomendamos o processamento imediato das amostras para minimizar os efeitos do congelamento e descongelamento.

Palavras-chave: peixes marinhos; experimento; preservação de amostras; razões; isótopos.

1. Introduction

The stable isotope methodology is a pivotal tool for unveiling nutrient flow within food chains (Fry, 2006). Its utilization in animal ecology studies has enhanced the ability to trace trophic relationships and discern consumption patterns in wild animals (Bosley et al., 2002; Martinez Del Rio et al., 2009; Ramos & González-Solís, 2012). Consequently, there has been a substantial increase in the inclusion of this methodology in scientific research over the recent decades (Ben-David & Flaherty, 2012; Layman et al., 2012; Cobain et al., 2024; Mhande et al., 2025).

This methodology enables the identification of various food sources contributing to the composition of a particular tissue (Fry, 2006), such as C_3 and C_4 plants in the diet (Magnusson et al., 1999; Cerling et al., 2006). The substantial disparity in $\delta^{13}C$ values between photosynthetic cycles (C_3: –35 to –22 ‰; C_4: –16 to –9 ‰) serves as a distinctive marker (Farquhar et al., 1989). Furthermore, the nitrogen isotope ratio ($\delta^{15}N$) indicates an organism's trophic level, with nitrogen values progressively increasing along the food chain (Minagawa & Wada, 1984). In simple or less complex food chains, the biomagnification effect of this isotope unveils an individual's trophic position. Overall, stable isotope analysis can provide insights into resource utilization across various timescale (Dalerum & Angerbjörn, 2005), facilitating the evaluation of long-term ecological patterns essential for effective management and conservation strategies (Newsome et al., 2007).

Studies employing isotopic methodology must consider the potential variables that may interfere with the carbon and nitrogen isotopic values of the tissue's species. These variations can be attributed to factors such as age, size, temperature, lipid concentration, reproduction, migration, seasonal variations influenced by environmental factors, and the processing of biological samples (Durako & Hall, 1992; Grice et al., 1996; Hemminga & Mateo, 1996; Riera & Richard, 1997; Lecea et al., 2011).

Given the multitude of potential influences on data, it is crucial to consider sample preservation methods during analysis, especially in field studies where immediate processing may not be feasible, necessitating the need to freeze samples (Bosley & Wainright, 1999; Feuchtmayr & Grey, 2003; Lecea et al., 2011; Syväranta et al., 2011). Although freezing is a practical method for preserving samples, it can alter the carbon and nitrogen isotope values, which may lead to errors and misinterpretation of the data (Bosley & Wainright, 1999; Feuchtmayr & Grey, 2003; Lecea et al., 2011; Syväranta et al., 2011). Several studies investigate the long-term effects of freezing, finding no changes in carbon and nitrogen isotopic values in marine species (Bosley & Wainright, 1999; Syväranta et al., 2011). None of the studies have explored the impact of freezing followed by thawing over time.

Lecea et al. (2011) discovered changes in $\delta^{13}C$ and $\delta^{15}N$ in the invertebrates and marine fish subjected to freezing followed by thawing and drying in the oven at 60°C, thought C:N values remained conserved. During the thawing process, changes can occur in biological tissues, either chemical (e.g., protein in solubilization, lipid oxidation) or physical (e.g., recrystallization, volume change), as well as microbial growth (Benjakul et al., 2003; Colla & Hernandez-Prentice, 2003; Sriket et al., 2007). The "DRIP" phenomenon which is the exudate liquid of organic tissues during their thawing process, deserves attention. When organic tissues freeze, dissolved substances in the cell liquid concentrate (Benjakul et al., 2003; Colla & Hernandez-Prentice, 2003; Sriket et al., 2007). These substances, whether linked either to protein or carbohydrate, do not return to their original state during thawing, becoming free and forming the DRIP (Benjakul et al., 2003; Colla & Hernandez-Prentice, 2003; Sriket et al., 2007). Uncontrolled and prolonged thawing can lead to condensation, microbial growth, and tissue breakdown (Colla & Hernandez-Prentice, 2003; Arannilewa et al., 2006).

Consequently, investigating the standardization and conservation implications of freezing and subsequent at intervals of 1, 2, 7, 15, 30, 90 and 180 days on stable isotope aliquots holds critical significance. Fieldwork logistics often mandate the use of freezing techniques, prompting this study to scrutinize potential changes occurring during 180 days of freezing and subsequent thawing

in biological tissue samples. Thus, our aim is to investigate the influence of successive freeze-thaw cycles on the stable isotopic composition ($\delta^{13}C$, $\delta^{15}N$, and C:N ratio) of different tissues from estuarine fish species. We hypothesize that repeated cycles over 180 days may cause isotopic shifts due to chemical and physical degradation of the samples. These altered data may lead to erroneous interpretation of isotopic data in ecology.

2. Materials and Methods

2.1. Study species

Tissues from three estuarine fish species (*Cathorops spixii, Genidens barbus* and *Chloroscombrus chrysurus*) sampled in the city of Ubatuba, on the northern coast of São Paulo State, were utilized in this study. *Cathorops spixii* (spotted sea catfish) and *Genidens barbus* (marine catfish) belong to the Ariidae family and exhibit generalist feeding habits (Marceniuk & Ferraris Junior, 2003; Gurgel et al., 2004; Beneditto et al., 2018). *Chloroscombrus chrysurus* (pompano) is a member of the Carangidae family and primarily displays carnivorous feeding behavior (Silva & Lopes, 2002). These demersal fish species are frequently targeted by both commercial and recreational fishermen and represent a crucial food source for numerous communities (Freire et al., 2021).

2.2. Sampling methods and preservation

Five individuals of each species were sampled the in the coastal region of Lagoinha beach, situated on the North Coast of the State of São Paulo, Brazil (23°31′4”S, 45°11′4”W, in March 2019). Individuals were acquired directly from local fishermen who use gill-nets (mesh size 4; 10 m in height; 60 m in length) for their catches.

The captured individuals were transported on ice to the laboratory (approximately a 10-min journey), where biometric data was collected, and tissues from the control group were extracted. The samples in the control group are fresh and recently collected. They are extremely important in representing the isotopic values of carbon and nitrogen. The three fish species exhibited distinct total lengths and body masses. *C. spixii* and *G. barbus* showed similar mean total lengths (280.60 ± 0.94 mm and 280.60 ± 0.56 mm, respectively) and body masses (148.80 ± 1.30 g and 148.00 ± 2.35 g, respectively). In contrast, *C. chrysurus* presented lower values for both total length (210.70 ± 0.94 mm) and body mass (99.17 ± 1.33 g). These values were not subjected to statistical comparison.

Following this, each specimen was individually placed in labeled zip-top bags and frozen at –20°C to prevent contamination during freezing and thawing processes.

After the initial freezing at –20 °C, tissue aliquots were taken from the original frozen fish at specific intervals (1, 2, 7, 15, 30, 90, and 180 days). For each time point, a new aliquot was extracted while the specimen remained frozen, and only the portion designated for analysis was thawed at room temperature for 30 min. This approach prevented repeated freeze-thaw cycles on the same tissue. A control group consisting of fresh, unfrozen samples was analyzed to establish baseline isotopic values. This protocol simulates fieldwork conditions in which samples are stored for extended periods and accessed gradually for subsampling or transport. The tissues used for *C. spixi* were muscle, liver, and skin; for *G. barbus*, muscle and skin; for *C. chrysurus*, muscle and scale. It is relevant to carry out this experiment with several tissues of biological and ecological importance in order to verify whether the isotopic composition can change over time with the freezing and thawing process.

2.3. Sample analysis

A 5g tissue sample was taken. Muscle, liver, and skin samples were dried in an oven at 60°C for 48 h until reaching a constant mass. The resulting material was then macerated using a mortar. Scale tissues were washed three times in distilled water, dried in an oven at a constant temperature of 60°C for 2 h, and cut using scissors. The tissues were placed in tin capsules and weighed (0.8-1.0 mg). The isotopic compositions of carbon and nitrogen were determined through the online combustion of the samples using CF-IRMS (Continuous Flow Isotope Ratio Mass Spectrometry) in a Carlo Erba elemental analyzer (CHN-1110) coupled to the Thermo Scientific mass spectrometer (Delta Plus) at the Isotopic Ecology Laboratory, Center for Nuclear Energy in Agriculture (LEI-CENA), University of São Paulo (USP).

The calculation of the isotopic composition of carbon and nitrogen was performed using the Equation 1:

$$\delta^{13}C \text{ ‰ } or \text{ } \delta^{15}N \text{ ‰} = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) \quad (1)$$

where R denotes the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ molar ratio in the sample and standard, and the results reported in delta (δ) per thousand (‰). Typical isotopic and elemental compositions for fish tissues (such as *C. spixii, G. barbus*, and *C. chrysurus*, or ecologically

similar estuarine species) are as follows: $\delta^{13}C$: generally, ranges from –22‰ to –16‰, depending on lipid content and primary carbon sources; $\delta^{15}N$: usually ranges from 9‰ to 13‰, influenced by trophic position; C:N ratios: Muscle: ~3.2–3.5%; Liver: ~3.5–4.0%; Skin: ~4.0–5.0%. These values may vary according to species, diet, and environmental conditions but serve as reliable reference ranges for fish tissue analyses. Repeated measurements of the internal standard (sugarcane) were used to determine the reference material.

The certified isotopic standards used to determine the analytical error were Vienna-PeeDee Belemnite (VPDB) for carbon ($\delta^{13}C$) and atmospheric air (AIR) for nitrogen ($\delta^{15}N$). These standards were used in accordance with international conventions for stable isotope analyses. The analytical error of isotopic measurements in LEI-CENA was estimated at 0.3 ‰ and 0.5 ‰ for carbon and nitrogen, respectively.

2.4. Data analysis

Normality data and homoscedasticity were tested prior to statistical analyses by Anderson Darling's test and Levene's test, respectively. The $\delta^{13}C$, $\delta^{15}N$ and C:N values of the tissues for each species (*C. spixii*, *G. barbus* and *C. chrysurus*) at each time point of the freezing and thawing protocol were compared using analysis of variance (ANOVA). When a significant difference was found, Tukey's mean comparison test ($P < 0.05$) was employed. Values were reported as mean ± one standard deviation (SD). Statistical analyses were performed using Origin 2016- © software (OriginLab Corporation).

3. Results

Over the course of 180 days of freezing and thawing, no significant differences was found in $\delta^{13}C$, $\delta^{15}N$, and C:N ratios between muscle and liver samples of *C. spixii*. However, skin samples exhibited differences in $\delta^{13}C$ and C:N values, showing an increase from 1 to 180 days. $\delta^{15}N$ of skin showed no variation over time (Table 1; Table 2).

Muscle samples of *G. barbus* showed no significant differences in $\delta^{13}C$, $\delta^{15}N$, and C:N values throughout the 180 days of freezing and thawing. However, the skin samples exhibited differences over time for $\delta^{13}C$ (stronger from 90 days onwards) and the carbon and nitrogen ratio (increased from 30 days onwards). $\delta^{15}N$ of skin showed no variation over time (Table 1; Table 3).

Muscle samples from *C. chrysurus* individuals showed increased $\delta^{13}C$ values after the 15th day. C:N values notably rose after 30 days, while $\delta^{15}N$ remained unchanged over the 180-day treatment. Scales exhibited no significant differences in $\delta^{13}C$, $\delta^{15}N$, or C:N values throughout the 180-day treatment (Table 1; Table 4).

In contrast, the skin samples of *C. spixii* and *G. barbus*, as well as the muscle samples of *C. chrysurus*, exhibited a depletion of ^{12}C at $\delta^{13}C$, leading to an increase in the C:N value. After 1 day, the skin samples of *C. spixii* showed an enrichment at $\delta^{13}C$ (–1.57 ‰), which persisted up to 180 days. The C:N values were higher from 1 day until the conclusion of the 180-day freeze-thaw protocol. *G. barbus* displayed a slight variation, with an increase of –1.37 ‰ in $\delta^{13}C$ only after 90 days, which persisted up to 180 days. However, the C:N

Table 1. Model of effects of the freezing and thawing treatment over time at $\delta^{13}C$, $\delta^{15}N$ and C:N of three fish species.

Cathorops spixii	**$\delta^{13}C$**			**$\delta^{15}N$**			**C:N**		
	df	***F*-value**	***p* - value**	**df**	***F*-value**	***p* - value**	**df**	***F*-value**	***p* - value**
Muscle	7	0.36	0.918	7	1.01	0.444	7	1.01	0.443
Liver	7	1.25	0.307	7	0.95	0.479	7	1.33	0.269
Skin	7	17.90	<0.001	7	1.00	0.449	7	6.64	<0.001
Genidens barbus	**$\delta^{13}C$**			**$\delta^{15}N$**			**C:N**		
	df	***F*-value**	***p* - value**	**df**	***F*-value**	***p* - value**	**df**	***F*-value**	***p* - value**
Muscle	7	1.80	0.123	7	0.73	0.645	7	2.10	0.07
Skin	7	4.48	<0.001	7	0.56	0.784	7	6.64	<0.001
Chloroscombrus chrysurus	**$\delta^{13}C$**			**$\delta^{15}N$**			**C:N**		
	df	***F*-value**	***p* - value**	**df**	***F*-value**	***p* - value**	**df**	***F*-value**	***p* - value**
Muscle	6	11.78	<0.001	6	2.00	0.090	6	8.82	<0.001
Scale	7	1.43	0.219	7	1.12	0.370	7	0.87	0.536

*df = degrees of freedom (graus de liberdade); **F*-value = statistical value obtained from ANOVA; **p*-value = probability level indicating statistical significance ($P < 0.05$).

Table 2. Mean and standard deviation (SD) of the values of carbon and nitrogen stable isotope composition and C:N ratio of muscle, liver and skin of *Cathorops spixii* sampled on the North Coast of the State of São Paulo, Brazil. Values in the line accompanied by the same letter do not differ by Tukey test ($P < 0.05$). $\Delta^{13}C$, $\Delta^{15}N$ and ΔC:N represents the difference between mean values of control and thawing intervals (days) samples.

Muscle										
Time	**N**	**$\delta^{13}C$ (‰)**	**SD**	**$\Delta\delta^{13}C$**	**$\delta^{15}N$ (‰)**	**SD**	**$\Delta\delta^{15}N$**	**C:N**	**SD**	**ΔC:N**
Control	5	−16.87[a]	0.18		14.75[a]	0.56		3.06[a]	0.10	
1 day	5	−16.69[a]	0.34	0.18	14.34[a]	0.52	0.41	2.89[a]	0.25	0.17
2 days	5	−17.01[a]	0.45	−0.14	14.46[a]	0.62	0.29	3.30[a]	0.25	−0.24
7 days	5	−17.17[a]	0.89	−0.30	14.60[a]	0.20	0.15	3.36[a]	0.63	−0.03
15 days	5	−17.13[a]	0.80	−0.26	14.23[a]	0.44	0.52	3.37[a]	0.47	−0.31
30 days	5	−17.17[a]	0.82	−0.30	14.46[a]	0.20	0.29	3.31[a]	0.45	−0.25
90 days	5	−17.15[a]	0.83	−0.28	14.14[a]	0.38	0.61	3.33[a]	0.44	−0.27
180 days	5	−16.90[a]	0.56	0.03	14.47[a]	0.39	0.28	3.20[a]	0.27	−0.14
Liver										
Time	**N**	**$\delta^{13}C$ (‰)**	**SD**	**$\Delta\delta^{13}C$**	**$\delta^{15}N$ (‰)**	**SD**	**$\Delta\delta^{15}N$**	**C:N**	**SD**	**ΔC:N**
Control	5	−17.22[a]	0.30		13.35[a]	0.28		3.71[a]	0.24	
1 day	5	−16.86[a]	0.31	0.36	13.19[a]	0.19	0.16	3.68[a]	0.14	0.03
2 days	5	−17.24[a]	0.53	−0.02	13.18[a]	0.18	0.17	3.91[a]	0.21	−0.20
7 days	5	−17.51[a]	0.61	−0.29	13.24[a]	0.23	0.11	3.90[a]	0.29	−0.19
15 days	5	−17.12[a]	0.19	0.10	13.27[a]	0.22	0.08	3.69[a]	0.11	0.02
30 days	5	−17.23[a]	0.27	−0.01	13.13[a]	0.09	0.22	3.80[a]	0.02	−0.09
90 days	5	−17.20[a]	0.24	0.02	13.09[a]	0.08	0.26	3.69[a]	0.10	0.02
180 days	5	−17.37[a]	0.36	−0.15	13.15[a]	0.15	0.20	3.81[a]	0.22	−0.10
Skin										
Time	**N**	**$\delta^{13}C$ (‰)**	**SD**	**$\Delta\delta^{13}C$**	**$\delta^{15}N$ (‰)**	**SD**	**$\Delta\delta^{15}N$**	**C:N**	**SD**	**ΔC:N**
Control	5	−15.84[a]	0.54		12.87[a]	0.35		3.16[a]	0.34	
1 day	5	−14.00[b]	0.28	1.84	12.74[a]	0.59	0.13	3.88[b]	0.25	−0.72
2 days	5	−14.37[b]	0.18	1.47	12.71[a]	0.48	0.16	4.04[b]	0.33	−0.88
7 days	5	−13.83[b]	0.57	2.01	12.86[a]	0.25	0.01	4.08[b]	0.22	−0.92
15 days	5	−13.99[b]	0.11	1.85	12.42[a]	0.31	0.45	4.06[b]	0.30	−0.90
30 days	5	−14.14[b]	0.44	1.70	12.50[a]	0.41	0.37	4.11[b]	0.32	−0.95
90 days	5	−14.04[b]	0.08	1.80	12.68[a]	0.57	0.19	4.14[b]	0.25	−0.98
180 days	5	−13.91[b]	0.12	1.93	12.39[a]	0.23	0.48	4.14[b]	0.26	−0.98

Table 3. Mean and standard deviation of the values of carbon and nitrogen stable isotope composition and C:N ratio of muscle and skin of *Genidens barbus* sampled on the North Coast of the State of São Paulo, Brazil. Values in the line accompanied by the same letter do not differ by Tukey test ($P < 0.05$).

Muscle							
Time	**N**	**$\delta^{13}C$ (‰)**	**SD**	**$\delta^{15}N$ (‰)**	**SD**	**C:N**	**SD**
Control	5	−17.34[a]	0.26	13.73[a]	0.38	3.42[a]	0.12
1 day	5	−17.14[a]	0.26	13.70[a]	0.47	3.31[a]	0.17
2 days	5	−17.12[a]	0.08	13.67[a]	0.41	3.16[a]	0.06
7 days	5	−17.40[a]	0.39	13.50[a]	0.57	3.37[a]	0.14
15 days	5	−17.20[a]	0.30	13.33[a]	0.39	3.20[a]	0.17
30 days	5	−17.37[a]	0.17	13.83[a]	0.30	3.26[a]	0.10
90 days	5	−17.72[a]	0.54	13.49[a]	0.50	3.24[a]	0.23
180 days	5	−17.34[a]	0.31	13.48[a]	0.45	3.16[a]	0.12
Skin							
Time	**N**	**$\delta^{13}C$ (‰)**	**SD**	**$\delta^{15}N$ (‰)**	**SD**	**C:N**	**SD**
Control	5	−15.97[a]	0.28	11.95[a]	0.38	3.26[a]	0.18
1 day	5	−15.83[a]	0.74	12.00[a]	0.41	3.00[a]	0.22
2 days	5	−15.93[a]	0.40	11.95[a]	0.26	2.87[a]	0.22
7 days	5	−15.84[a]	0.86	12.21[a]	0.43	3.23[a]	0.35
15 days	5	−15.70[a]	0.50	12.30[a]	0.46	2.88[a]	0.25
30 days	5	−15.39[a]	0.11	11.91[a]	0.44	3.52[b]	0.08
90 days	5	−14.60[b]	0.51	11.95[a]	0.34	3.38[b]	0.29
180 days	5	−14.82[b]	0.49	11.93[a]	0.65	3.59[b]	0.15

Table 4. Mean and standard deviation of the values of carbon and nitrogen stable isotope composition and C:N ratio of muscle and scale of *Chloroscombrus chrysurus* sampled on the North Coast of the State of São Paulo, Brazil. Values in the line accompanied by the same letter do not differ by Tukey test ($P < 0.05$).

Muscle							
Time	**N**	**$\delta^{13}C$ ‰**	**SD**	**$\delta^{15}N$ ‰**	**SD**	**C:N**	**SD**
Control	6	−17.71[a]	0.48	13.30[a]	0.19	3.28[a]	0.17
1 day	6	−17.69[a]	0.25	13.20[a]	0.27	3.34[a]	0.14
2 days	6	−17.71[a]	0.19	13.18[a]	0.20	3.49[a]	0.02
7 days	6	−17.47[a]	0.13	13.20[a]	0.20	3.42[a]	0.09
15 days	6	−16.91[b]	0.38	13.03[a]	0.14	4.30[a]	0.70
30 days	6	−16.68[b]	0.40	13.03[a]	0.10	5.26[b]	0.85
90 days	6	−16.93[b]	0.23	13.05[a]	0.08	4.47[b]	1.21
Scale							
Time	**N**	**$\delta^{13}C$ ‰**	**SD**	**$\delta^{15}N$ ‰**	**SD**	**C:N**	**SD**
Control	6	−15.22[a]	0.29	13.99[a]	0.38	2.73[a]	0.03
1 day	6	−15.20[a]	0.12	13.57[a]	0.82	2.68[a]	0.12
2 days	6	−15.45[a]	0.47	13.85[a]	0.40	2.74[a]	0.18
7 days	6	−15.26[a]	0.38	13.56[a]	0.33	2.74[a]	0.17
15 days	6	−14.89[a]	0.20	13.61[a]	0.32	2.55[a]	0.07
30 days	6	−15.01[a]	0.46	13.92[a]	0.27	2.70[a]	0.25
90 days	6	−14.99[a]	0.32	13.99[a]	0.19	2.73[a]	0.18
180 days	6	−15.13[a]	0.51	13.62[a]	0.33	2.75[a]	0.22

was higher from 30 days onwards. For *C. chrysurus*, the muscle samples exhibited a loss of ^{12}C, with the heaviest $\delta^{13}C$ at –0.81 ‰ after 15 days, maintaining this level up to 90 days. Nevertheless, the C:N was higher from 30 days until the end of the experiment.

4. Discussion

Stable isotope analyses have been employed to elucidate ecological issues due to their broad applicability across various fields of knowledge. However, it is necessary to establish a proper protocol for collecting animal tissues to ensure that the preservation method does not interfere with their isotopic compositions. An important alternative for samples that cannot be dried in an oven would be lysis, but this type of equipment is not easily available, especially during field work. This study found that both *C. spixii* and *G. barbus* demonstrated no significant variations in the values of $\delta^{13}C$, $\delta^{15}N$, and C:N in muscle samples throughout the 180-day freeze-thaw protocol. This consistency was also evident in the liver samples of *C. spixii*, which is in agreement with the findings for Bosley & Wainright (1999), where no variations were detected in $\delta^{13}C$ and $\delta^{15}N$ during the freezing process in shrimp samples.

Although some $\delta^{13}C$ and C:N variations were statistically significant, they were generally small (<1.5‰) and must be interpreted cautiously. However, in ecological studies where fine-scale isotopic discrimination is essential (e.g., to infer diet shifts or trophic position), even minor alterations may lead to incorrect conclusions about resource use or food web structure.

Several aspects of our findings are consistent with those reported in others studies (Lecea et al., 2011; Syväranta et al., 2011), although it should be noted that these studies were limited to muscle samples. These studies observed a loss of ^{12}C, which resulted in stronger $\delta^{13}C$ values, and a loss of ^{14}N, leading to elevated $\delta^{15}N$ values. However, these studies did not report any changes in the C:N ratio during the freezing and thawing of muscle samples. In contrast, Sweeting et al. (2004) did not find any differences in $\delta^{13}C$, $\delta^{15}N$, and C:N ratios in frozen copepods. The observed change in the C:N ratio in copepods preserved in formalin was attributed to a greater loss of %N compared to %C, which resulted in an increased C:N ratio. In our current study, we observed an increase in the C:N ratio during the 4-month freezing and thawing process of skin and muscle samples. This appears to be related to an increase in %C, which may be due to the preferential loss of ^{12}C relative to %N.

The influence on carbon data during the freezing process may be due to the negative relationship between $\delta^{13}C$ and lipids in tissues. Lipids $\delta^{13}C$ is depleted by ^{13}C compared to proteins (Parker, 1964; Deniro & Epstein, 1977), attributed to the stable isotopes kinetics that occur during lipid biosynthesis on the formation of Acetyl-COA by pyruvate dehydrogenase (Deniro & Epstein, 1977;

Monson & Hayes, 1992). Lecea et al. (2011) associated the depletion of ^{13}C and ^{15}N in their samples with two factors. First, the drying process at 60°C may induce the volatility of the ^{12}C, resulting in stronger $\delta^{13}C$. However, this alone does not explain the increase in $\delta^{15}N$, which requires a loss of ^{14}N. The disruption of cells, possibly due to the crystallization during freezing (Benjakul et al., 2003; Colla & Hernandez-Prentice, 2003; Sriket et al., 2007; Lecea et al., 2011), may be responsible for this. Crystallization can lead to cell disruption, causing the loss of exudate liquid rich in carbon and nitrogen. However, no increase in $\delta^{15}N$ was observed in our study.

Lipid oxidation is another factor that may influence $\delta^{13}C$ and C:N in skin and muscle samples. Oxidation, associated with undesirable odor development, is more likely in fish due to lipid unsaturation and low endogenous antioxidants. Differences in freezing, thawing levels, and drying processes can contribute to lipid oxidation, causing a potential ^{12}C loss and enriching $\delta^{13}C$ and C:N at 60°C (Silva et al., 1999; Kolakowska et al., 2002; Lecea et al., 2011; Maqsood & Benjakul, 2011).

In contrast, *C. chrysurus* scales exhibited no significant differences in $\delta^{13}C$, $\delta^{15}N$, and C:N during the 180-day freezing and thawing process. This resilience is likely attributed to the composition of teleost scales, comprising a mineralized portion of calcium phosphate and a collagen portion (Fouda, 1979; Zylberberg & Nicolas, 1982; Zylberberg, 2004). Similar to the isotopic displacement in muscle collagen and bone, carbon in scales is predominantly in protein, with minimal mineral content (Lee-Thorp et al., 1989). Scales, resistant to rupture and cellular liquid leakage are also impervious to lipid oxidation and carbon loss through volatilization, making them a promising tissue for field studies requiring long-term freezing techniques.

5. Conclusion

Our study underscores the need for further research to analyze the effects of the freezing and thawing process on samples. Our findings suggest that this process significantly influences the $\delta^{13}C$ and C:N ratios of skin and muscle tissues. Additionally, we recommend immediate sample processing to minimize the effects of freezing and thawing. These effects include lipid oxidation, which can lead to the loss of ^{12}C through volatilization at high temperatures. By implementing these recommendations, future studies can ensure more accurate and reliable results.

The observed alterations in $\delta^{13}C$ and C:N values in skin and muscle samples throughout the freeze-thaw cycles are likely related to lipid oxidation, which promotes the loss of ^{12}C and alters isotopic integrity. This finding directly addresses the study's objective of evaluating the effects of freeze-thaw process on the isotopic composition of fish tissues, emphasizing the importance of tissue-specific preservation strategies to ensure data reliability in ecological research.

Acknowledgments

Coordenação de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) for the PROEX grant from the Academic Excellence Program.

This study was approved by the ethics committee of CENA-USP (number 2019.5.19.64.7 - CEUA 001-2019). Licenses for field activities were issued by the technical-scientific committee (COTEC - 260108-001.758/2019) and by the Biodiversity Authorization and Information System (SISBIO - 66825-3).

Data availability

The entire data set supporting the results of this study has been published in the article itself.

References

Arannilewa, S.T., Salawu, S.O., Sorungbe, A.A. & Ola-Salawu, B.B., 2006. Effect of frozen period on the chemical, microbiological and sensory quality of frozen Tilapia fish *Sarotherodun galiaenus*. Nutr. Health 18(2), 185-192. PMid:16859181. https://doi.org/10.1177/026010600601800210.

Ben-David, M. & Flaherty, E.A., 2012. Theoretical and analytical advances in mammalian isotope ecology: an introduction. J. Mammal., 93(2), 309-311. https://doi.org/10.1644/11-MAMM-S-315.1.

Beneditto, A.P.M.D., Tavares, M.T.M. & Monteiro, L.R., 2018. Isotopic niche of the catfishes *Bagre bagre* and *Genidens barbus* in a coastal area of south-eastern Brazil. Biota Neotrop., 18(3), e20180527. https://doi.org/10.1590/1676-0611-bn-2018-0527.

Benjakul, S., Visessanguan, W., Thongkaew, C. & Tanaka, M., 2003. Comparative study on physicochemical changes of muscle proteins from some tropical fish during frozen storage. Food Res. Int., 36(8), 787-795. https://doi.org/10.1016/S0963-9969(03)00073-5.

Bosley, K.L. & Wainright, S.C., 1999. Effects of preservatives and acidification on the stable isotope's ratios (^{15}N:^{14}N, ^{13}C:^{12}C) of two species of marine animals. Can. J. Fish. Aquat. Sci., 77(12), 2181-2185. https://doi.org/10.1139/f99-153.

Bosley, K.L., Witting, D.A., Chambers, R.C. & Wainright, S.C., 2002. Estimating turnover rates of carbon and nitrogen in recently metamorphosed winter flounder *Pseudopleuronectes americanus* with stable isotopes. Mar. Ecol. Prog. Ser., 236, 233-240. https://doi.org/10.3354/meps236233.

Cerling, T.E., Wittemyer, G., Rasmussen, H.B., Vollrath, F., Cerling, C.E., Robinson, T.J. & Douglas-Hamilton, I., 2006. Stable isotopes in elephant hair document migration patterns and diet changes. Proc. Natl. Acad. Sci. USA 103(2), 371-373. PMid:16407164. https://doi.org/10.1073/pnas.0509606102.

Cobain, M.R., McGill, R.A. & Trueman, C.N., 2024. Stable isotopes demonstrate seasonally stable benthic-pelagic coupling as newly fixed nutrients are rapidly transferred through food chains in an estuarine fish community. J. Fish Biol., 105(5), 1406-1420. PMid:35099820. https://doi.org/10.1111/jfb.15005.

Colla, L.M. & Hernandez-Prentice, C., 2003. Congelamento e descongelamento – sua influência sobre os alimentos. Vetor (Online), 13, 53-66. Retrieved in 2024, August 29, from https://periodicos.furg.br/vetor/article/view/428

Dalerum, F. & Angerbjörn, A., 2005. Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. Oecologia 144(4), 647-658. PMid:16041545. https://doi.org/10.1007/s00442-005-0118-0.

Deniro, M.J. & Epstein, S., 1977. Mechanism of carbon isotope fractionation associated with lipid syntheses. Science 197(4300), 261-263. PMid:327543. https://doi.org/10.1126/science.327543.

Durako, M.J. & Hall, M.O., 1992. Effect of light on the stable carbon isotope composition of the seagrass *Thalassia testudinum*. Mar. Ecol. Prog. Ser., 87(1), 99-101. https://doi.org/10.3354/meps086099.

Farquhar, G.D., Ehleringer, J.R. & Hubick, K.T., 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol., 40(1), 503-537. https://doi.org/10.1146/annurev.pp.40.060189.002443.

Feuchtmayr, H. & Grey, J., 2003. Effect of preparation and preservation procedures on carbon and nitrogen stable isotope determination from zooplankton. Rapid Commun. Mass Spectrom., 17(23), 2605-2610. PMid:14648896. https://doi.org/10.1002/rcm.1227.

Fouda, M.M., 1979. Studies on scale structure in the common goby *Pomatoschistus microps* Krøyer. J. Fish Biol., 15(2), 173-183. https://doi.org/10.1111/j.1095-8649.1979.tb03581.x.

Freire, K.M.F., Almeida, Z.S., Amador, J.R.E.T., Aragão, J.A., Araújo, A.R.R., Ávila-da-Silva, A.O., Bentes, B., Carneiro, M.H., Chiquieri, J., Fernandes, C.A.F., Figueiredo, M.B., Hostim-Silva, M., Jimenez, É.A., Keunecke, K.A., Lopes, P.F.M., Mendonça, J.T., Musiello-Fernandes, J., Olavo, G., Primitivo, C., Rotundo, M.M., Santana, R.F., Sant'Ana, R., Scheidt, G., Silva, L.M.A., Trindade-Santos, I., Velasco, G. & Vianna, M., 2021. Reconstruction of marine commercial landings for the Brazilian industrial and artisanal fisheries from 1950 to 2015. Front. Mar. Sci., 8, 659110. https://doi.org/10.3389/fmars.2021.659110.

Fry, B., 2006. Stable isotope ecology. Springer Sci., 521, 308. https://doi.org/10.1007/0-387-33745-8.

Grice, A.M., Loneragan, N.R. & Dennison, W.C., 1996. Light intensity and the interaction between physiology, morphology, and stable isotope ratios in five species of seagrass. J. Exp. Mar. Biol. Ecol., 195(1), 91-110. https://doi.org/10.1016/0022-0981(95)00096-8.

Gurgel, H.D., Souza, D.D. & Albuquerque, C.Q., 2004. Alimentação de *Cathorops spixii* (Agassiz, 1829) (Pisces, Ariidae) do estuário do rio Potengí, Natal, Rio Grande do Norte. Arq. Fundacao Apadec 8(2), 9-11. https://doi.org/10.4025/arqmudi.v8i2.20590.

Hemminga, M.A. & Mateo, M.A., 1996. Stable carbon isotopes in seagrass: variability in ratios and use in ecological studies. Mar. Ecol. Prog. Ser., 140, 285-298. https://doi.org/10.3354/meps140285.

Kolakowska, A., Olley, J. & Dunstan, G.A., 2002. Fish lipids. In Sikorski, Z.E., & Kolakowska, A., eds. Chemical and functional properties of food lipids. Boca Raton: CRC Press, 221-264.

Layman, C.A., Araujo, M.S., Boucek, R., Peyer, C.M., Hammerschlag, N., Harrison, E., Jud, Z.R., Matuch, P., Rosenblatt, A.E., Vando, J.J., Yeager, L.A., Post, D.M. & Bearhop, S., 2012. Applying stable isotopes to examine food-web structure: an overview of analytical tools. Biol. Rev. Camb. Philos. Soc., 87(3), 545-562. PMid:22051097. https://doi.org/10.1111/j.1469-185X.2011.00208.x.

Lecea, A.M., Smit, A.J. & Fennessy, S.T., 2011. The effects of freeze/thaw periods and drying methods on isotopic and elemental carbon and nitrogen in marine organisms, raising questions on sample preparation. Rapid Commun. Mass Spectrom., 25(23), 3640-3649. PMid:22095513. https://doi.org/10.1002/rcm.5265.

Lee-Thorp, J., Sealy, J.C. & Van Der Merwe, N.J., 1989. Stable carbon isotope ratio differences between bone collagen and bone apatite, and their relationship to diet. J. Archaeol. Sci., 16(6), 585-599. https://doi.org/10.1016/0305-4403(89)90024-1.

Magnusson, W.E., Araújo, M.C., Cintra, R., Lima, A.P., Martinelli, L.A., Sanaiotti, T.M., Vasconcelos, H.L. & Victoria, R.L., 1999. Contributions of C3 and C4 plants to higher trophic levels in an Amazonian savanna. Oecologia 119(1), 91-96. PMid:28308164. https://doi.org/10.1007/PL00008821.

Maqsood, S. & Benjakul, S., 2011. Retardation of haemoglobin–mediated lipid oxidation of Asian sea bass muscle by tannic acid during ice storage. Food Chem. 124(4), 1056-1062. https://doi.org/10.1016/j.foodchem.2010.07.077.

Marceniuk, A.P. & Ferraris Junior, C.J., 2003. Ariidae (Sea catfishes). In Reis, R.E., Kullander, S.O. & Ferraris Junior, C.J., eds. Checklist of the freshwater fishes of South and Central America. Porto Alegre: EDIPUCRS, 447-455. https://doi.org/10.1111/j.0022-1112.2004.00504c.x.

Martinez Del Rio, C., Wolf, N., Carleton, S.A. & Gannes, L.Z., 2009. Isotopic ecology ten years after a call for more laboratory experiments. Biol. Rev. Camb. Philos. Soc., 84(1), 91-111. PMid:19046398. https://doi.org/10.1111/j.1469-185X.2008.00064.x.

Mhande, Z., Mihale, M.J., Hellar-Kihampa, H., Brion, N. & Baeyens, W., 2025. Deciphering the carbon and nitrogen cycling in selected tropical coastal fish: revelations from stable isotope fluctuations and feeding patterns. Environ. Monit. Assess., 197(3), 237. PMid:39904889. https://doi.org/10.1007/s10661-025-13625-2.

Minagawa, M. & Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. Geochim. Cosmochim. Acta 48(5), 1135-1140. https://doi.org/10.1016/0016-7037(84)90204-7.

Monson, K.D. & Hayes, J.M., 1992. Carbon isotopic fractionation in the biosynthesis of bacterial fatty acids. Ozonolyses of unsaturated fatty acids as a means of determining the intramolecular distribution on carbon isotopes. Geochim. Cosmochim. Acta 46(2), 139-149. https://doi.org/10.1016/0016-7037(82)90241-1.

Newsome, S.D., Martinez Del Rio, C., Bearhop, S. & Phillips, D.L., 2007. A niche for isotope ecology. Front. Ecol. Environ., 5(8), 429-436. https://doi.org/10.1890/1540-9295(2007)5[429:ANFIE]2.0.CO;2.

Parker, P.L., 1964. The biogeochemistry of the stable isotopes of carbon in marine bay. Geochim. Cosmochim. Acta 28(11), 1155-1164. https://doi.org/10.1016/0016-7037(64)90067-5.

Ramos, R. & González-Solís, J., 2012. Trace me if you can: the use of intrinsic biogeochemical markers in marine top predators. Front. Ecol. Environ., 10(5), 258-266. https://doi.org/10.1890/110140.

Riera, P. & Richard, P., 1997. Temporal variation of $\delta^{13}C$ in particulate organic matter and oyster *Crassostrea gigas* in Marennes-Oléron Bay (France): effect of freshwater inflow. Mar. Ecol. Prog. Ser., 147, 105-115. https://doi.org/10.3354/meps147105.

Silva, F.A.M., Borges, M.F.M. & Ferreira, M.A., 1999. Métodos para avaliação do grau de oxidação lipídica e da capacidade antioxidante. Quim. Nova 22(1), 94-103. https://doi.org/10.1590/S0100-40421999000100016.

Silva, J.T.A. & Lopes, P.R.D., 2002. Notas sobre a alimentação e morfologia do aparelho digestivo de *Chloroscombrus chrysurus* (Linnaeus, 1766) (Actinopterygii, Carangidae) na Praia de Ponta da Ilha (Ilha de Itaparica, Bahia). Rev. Bras. Zoociencias (Online), 4(2), 179-192. Retrieved in 2024, August 29, from https://periodicos.ufjf.br/index.php/zoociencias/article/view/24237

Sriket, P., Benjakul, S., Visessanguan, W. & Kijroongrojana, K., 2007. Comparative studies on chemical composition and thermal properties of black shrimp *Penacus monodon* and white shrimp *Penacus vannamei* meats. Food Chem., 103(4), 1199-1207. https://doi.org/10.1016/j.foodchem.2006.10.039.

Sweeting, C.J., Polunin, N.V.C. & Jennings, S., 2004. Tissue and fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved ecological material. Rapid Commun. Mass Spectrom., 18(21), 2587-2592. PMid:15468144. https://doi.org/10.1002/rcm.1661.

Syväranta, J., Martino, D., Kopp, D., Céréghino, R. & Santoul, F., 2011. Freezing and chemical preservatives alter the stable isotope values of carbon and nitrogen of the Asiatic clam *Corbicula fluminea*. Hydrobiologia 658(1), 383-388. https://doi.org/10.1007/s10750-010-0512-4.

Zylberberg, L. & Nicolas, G., 1982. Ultrastructure of scales in a teleost *Carassues auratus* after use of rapid freeze-fixation and freeze substitution. Cell Tissue Res., 223(2), 349-367. PMid:7066978. https://doi.org/10.1007/BF01258495.

Zylberberg, L., 2004. New data on bone matrix and its proteins. C. R. Palevol 3(6-7), 591-604. https://doi.org/10.1016/j.crpv.2004.07.012.

Received: 29 August 2024
Accepted: 14 October 2025

Associate Editor: Fernando Mayer Pelicice.