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Effect of alcohol and formaldehyde on the $\delta^{13}C$ and $\delta^{15}N$ isotopic composition of *Plagioscion squamosissimus* and *Hypophthalmus edentatus* (Pisces, Osteichthyes)

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ABSTRACT. The present study investigates the $\delta^{13}C$ and $\delta^{15}N$ isotopic composition in frozen samples (control), samples in alcohol and in formaldehyde of *Plagioscion squamosissimus* and *Hypophthalmus edentatus*. From each individual we extracted a strip of muscle from the region above the lateral line, in the dorsal fin base, that was divided into three equal parts, each one was submitted to one type of treatment: freeze – control group (-15ºC), conservation in alcohol 70% and fixation in formaldehyde 4%. Samples were kept under those treatments for 30 days, washed and submerged in distilled water for 4 hours. Afterwards, they were dried up in air oven at 60ºC for 48 hours and macerated until the obtaining of a fine powder. A significant difference was found in isotopic values of carbon and nitrogen, between the control and the samples in alcohol and formaldehyde, except for $\delta^{13}C$ from the *H. edentatus* samples in formaldehyde. The carbon isotopic values of samples in alcohol were mostly enriched compared to control, whereas the samples in formaldehyde presented depleted values in relation to the control. The nitrogen isotopic values for both samples preserved in alcohol and formaldehyde were enriched when compared to the values of frozen samples, independently of used preservatives. Therefore, the isotopic correction should be accomplished according to the isotope and preservative employed for species of freshwater fish.

Keywords: stable isotope, preservative, fishes, *Plagioscion squamosissimus*, *Hypophthalmus edentatus*.

RESUMO. Efeito do álcool e formol sobre a composição isotópica de $\delta^{13}C$ e de $\delta^{15}N$ em *Plagioscion squamosissimus* e *Hypophthalmus edentatus* (Pisces, Osteichthyes). O presente estudo investiga a composição isotópica de $\delta^{13}C$ e $\delta^{15}N$ entre as amostras congeladas (controle), em álcool e em formol de *Plagioscion squamosissimus* e *Hypophthalmus edentatus*. De cada indivíduo foi extraída uma faixa de músculo localizada na região acima da linha lateral, na base da nadadeira dorsal, a qual foi subdividida em três partes iguais, sendo cada uma delas submetida a um tipo de tratamento: congelamento - grupo control (-15ºC), conservação em álcool 70% e fixação em formol 4%. As amostras foram mantidas nos referidos tratamentos por 30 dias, enxaguadas e submersas em água destilada por 4h. Em seguida, foram secas em estufa de ventilação a 60ºC por 48h e maceradas até a obtenção de pó. Identificou-se diferença significativa nos valores isotópicos de carbono e de nitrogênio, entre o controle e as amostras de álcool e as de formol, com exceção do $\delta^{13}C$ das amostras mantidas em formol de *H. edentatus*. Constatou-se que os valores isotópicos de carbono das amostras conservadas em álcool foram, na sua maioria, enriquecidos quando comparados com as controle, ao passo que, as amostras em formol tiveram os valores deplecionados em relação ao controle. Os valores isotópicos de nitrogênio, tanto para as amostras conservadas em álcool, quanto em formol, foram enriquecidos quando comparados aos valores daquelas congeladas, sendo estes independentes dos conservantes utilizados. Assim, a correção isotópica deve ser realizada de acordo com o isótopo e o conservante empregado para espécies de peixe de água doce.

Palavras-chave: isótopos estáveis, conservantes, peixes, *Plagioscion squamosissimus*, *Hypophthalmus edentatus*.

Introduction

The stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ are the most commonly employed in ecology for the identification of energy sources and trophic position, respectively (ARAUJO-LIMA et al., 1986; FRY; SHERR, 1984, VANDER ZANDEN; RASMUSSEN, 1999). It is essential to recognize the spatial and temporal patterns of energy flow in the ecosystems to better understand its functioning as well as plan and manage the impacts.

In this way, time and space series of sampling are frequently necessary but not always available. The

use of samples preserved in formaldehyde or alcohol allows such investigation. Therefore, the knowledge about the changes caused by the preservatives and possible corrections of isotopic values of carbon and nitrogen is indispensable in the reconstruction of food chains, especially ecosystems submitted to human disturbances (PEASE et al., 2006; VANDER ZANDEN et al., 2003).

Vander Zanden et al. (2003), using samples kept in museums, reconstructed the food chain of native fish community from the Lake Tahoe (California, USA) to compare with the current and identify changes in the energy flow caused by invasive species. Likewise, Rau et al. (2003), between 1951 and 2001, using preserved samples, identified the $^{15}N/^{14}N$ variability of zooplankton, in the coast of Central California. Moreover, Pease et al. (2006) analyzed preserved larvae and juvenile of fish from the New Mexico and identified the nutrient sources and the trophic positions of these organisms.

Thus, aiming to increase the reliability of the results obtained with the studies involving preserved samples, some studies have evaluated the effect of the preservatives on the isotopic values from zooplankton (RAU et al., 2003), marine invertebrates and fishes (ARRINGTON; WINEMILLER, 2002; BOSLEY; WAINRIGHT, 1999; KAEHLER; PAKHOMOV, 2001; SARAKINOS et al., 2002). Regarding the specific physiological attributes of freshwater species related to body pH, gas transport, ionic and osmotic balance (RANDALL et al., 2000), there is still need for a detailed survey about the effect of preservatives on these species.

The analysis of stable isotopes requires samples free of impurities, and without any contamination that may interfere with their actual isotopic value. In general, ecological studies require samplings in environments far from the location where the infrastructure is suitable for the maintenance of biological material. Besides that, frequently, the use of preservative substances is fundamental to achieve samples free of microorganisms.

In this way, the present study investigates the $\delta^{13}C$ and $\delta^{15}N$ isotopic composition in frozen samples (control), samples in alcohol and in formaldehyde of *Plagioscion squamosissimus* and *Hypophthalmus edentatus*.

Material and methods

We collected eight adult individuals of *P. squamosissimus* (Scianidae) and four of *H. edentatus* (Hypophthalmidae), during November 2004, in the upper Paraná river floodplain. From each individual, we extracted a strip of muscle from the region above the lateral line, in the dorsal fin base, that was divided into three equal parts; each one was submitted to one type of treatment: freeze - control group (-15°C), conservation in alcohol 70% and fixation in formaldehyde 4%. The samples were kept under those treatments for 30 days, washed and submerged in distilled water for 4 hours. Afterwards, they were dried up in air oven at 60°C for 48 hours and macerated until obtaining of a fine powder.

The isotopic ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ were determined in the Centro de Isótopos Estáveis do Instituto de Biociências from UNESP (Botucatu, São Paulo State). The values of isotopic ratios were expressed in parts per thousand (‰), relative to international standard *PeeDee Belemnite* (PDB) for ^{13}C and the atmospheric nitrogen for ^{15}N, according to the following equation:

$$\delta^{13}C \text{ and } \delta^{15}N = [(R_{sample}/R_{standard})-1]\star 10^3$$

where:

R is the ratio between the least and the most abundant isotope, in particular $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

Differences in isotopic values of carbon and nitrogen between the control and preserved samples were evaluated through the Student's t-test, using Statistica Software version 7.1 (STATSOFT, 2005).

Results

Effect of preservatives on $\delta^{13}C$

The mean values of $\delta^{13}C$ from the samples in formaldehyde for both fish species were lower than those recorded for the control. An inverse pattern was verified for the samples preserved in alcohol (Table 1, Figure 1 and 2). Significant differences were observed for the carbon isotopic values between the control and the samples in alcohol, both for *P. squamosissimus* (T_{Ps} = 0.39; $p < 0.05$), and *H. edentatus* (T_{He} = -5.66; $p < 0.05$). Nevertheless, for those samples preserved in formaldehyde, we detected significant differences for *P. squamosissimus* (T_{Ps} = 0.39; $p < 0.05$), unlike the observed for *H. edentatus* (T_{He} = 2.73; $p > 0.05$).

Table 1. Carbon isotopic value, mean and standard deviation of samples control, preserved in alcohol and in formaldehyde of *P. squamosissimus* and *H. edentatus* (* = values depleted in relation to the control).

Plagioscion squamosissimus		
Control	Alcohol	Formaldehyde
-29.55‰	-28.99‰	-29.92‰*
-27.52‰	-27.20‰	-28.45‰*
-31.57‰	-30.92‰	-31.80‰*
-26.58‰	-26.81‰*	-26.51‰
-28.33‰	-27.64‰	-28.69‰*
-25.57‰	-25.85‰*	-26.21‰
-30.37‰	-29.91‰	-31.00‰*
-29.35‰	-28.84‰	-30.27‰*
$\overline{X}$ = -28.60 ± 1.99	-28.27 ± 1.69	-29.10 ± 2.02
Hypophthalmus edentatus		
-29.61‰	-29.31‰	-30.35‰*
-30.85‰	-30.42‰	-31.57‰*
-29.19‰	-28.80‰	-30.01‰*
-36.36‰	-35.69‰	-36.31‰
$\overline{X}$ = -31.50 ± 3.31	-31.05 ± 3.16	-32.06 ± 2.91

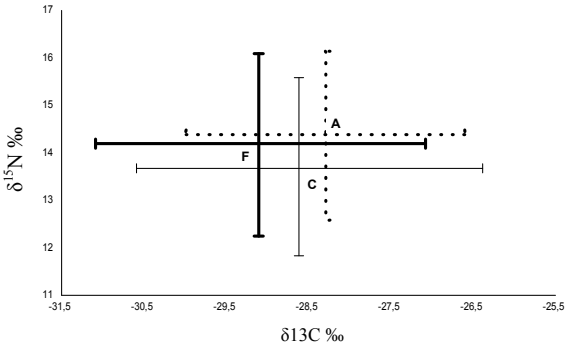

Figure 1. Mean isotopic values of carbon and nitrogen and standard deviation of samples control (C), in alcohol (A) and in formaldehyde (F) of *Plagioscion squamosissimus.*

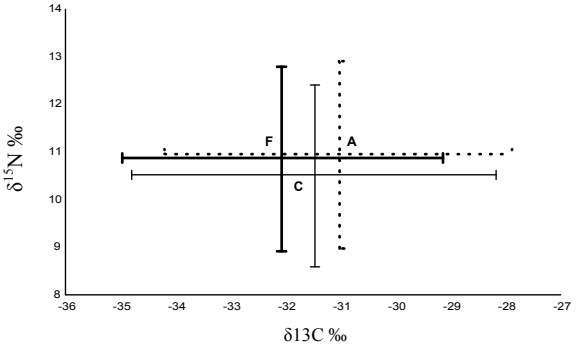

Figure 2. Mean isotopic values of carbon and nitrogen and standard deviation of samples control (C), in alcohol (A) and in formaldehyde (F) of *Hypophthalmus edentatus*.

For *P. squamosissimus,* the mean isotopic difference of carbon, and the standard deviation, between the control and the samples preserved in alcohol was 0.33 ± 0.36‰ and in formaldehyde was 0.50 ± 0.34‰. In the same way, for *H. edentatus*, we registered a mean difference of 0.45 ± 0.16‰ for samples in alcohol and 0.56 ± 0.41‰ for those in formaldehyde.

Effect of preservatives on $\delta^{15}N$

The isotopic values of nitrogen from the preserved muscle samples of *P. squamosissimus* and *H. edentatus*, both in formaldehyde and alcohol, were enriched in $\delta^{15}N$ in relation to the control (Table 2; Figure 1 and 2).

Significant differences were recorded for *P. squamosissimus* and *H. edentatus* regarding the nitrogen isotopic values from the samples control and preserved in alcohol (T_{Ps} = -12.62; $p < 0.05$); (T_{He} = -4.62; $p < 0.05$), and in formaldehyde (T_{Ps} = -17.03; $p < 0.05$), (T_{He} = -9.35; $p < 0.05$), respectively.

Table 2. Nitrogen isotopic value, mean and standard deviation of samples frozen, preserved in alcohol and in formaldehyde of *P. squamosissimus* and *H. edentatus*.

Plagioscion squamosissimus		
Frozen	Alcohol	Formaldehyde
12.50‰	13.27‰	12.98‰
13.26‰	13.88‰	13.71‰
11.87‰	12.86‰	12.47‰
16.63‰	17.47‰	17.31‰
12.94‰	13.61‰	13.64‰
16.65‰	17.14‰	17.28‰
12.21‰	12.79‰	12.85‰
13.33‰	14.01‰	13.82‰
$\overline{X}$ = 13.67± 1.89	14.38 ± 1.86	14.25 ± 1.93
Hypophthalmus edentatus		
10.25‰	10.41‰	10.53‰
13.30‰	13.86‰	13.73‰
9.07‰	9.55‰	9.42‰
9.42‰	9.98‰	9.88‰
$\overline{X}$ = 10.51 ±1.92	10.95 ± 1.97	10.89 ± 1.94

Mean isotopic differences of nitrogen for *P. squamosissimus,* between the control and the samples in alcohol was 0.64 ± 0.26‰, while for the samples in formaldehyde was 0.58 ± 0.19‰. For *H. edentatus*, this isotopic difference was 0.44 ± 0.19‰ for the alcohol and 0.40 ± 0.08‰ for the formaldehyde.

Discussion

Most of the carbon isotope values obtained from samples preserved in alcohol, for both analyzed species, was enriched in comparison to the values verified in the control samples. On the other hand, the samples maintained in formaldehyde presented depleted isotopic values of carbon. Thus, the values of $\delta^{13}C$ changed differently of the used preservative. While the nitrogen isotopic values from preserved samples, both in alcohol and formaldehyde, were enriched in relation to the control, independently of the preservative.

Studies have verified that the carbon isotopic values are depleted, whereas those of nitrogen are enriched when the samples of fish muscle are maintained in formaldehyde followed by alcohol

(ARRINGTON; WINEMILLER, 2002; BOSLEY; WAINRIGHT, 1999); in formaldehyde (BOSLEY; WAINRIGHT, 1999; EDWARDS et al., 2002); and in salt (ARRINGTON; WINEMILLER, 2002). In this way, regardless of the substance used to preserve the tissues, the isotopic values of carbon become enriched and those of nitrogen, depleted. Otherwise, when analyzing only the samples preserved in alcohol as the case of the present study and of Sarakinos et al. (2002), the carbon isotopic values become enriched. One of the reasons to explain the effect of preservative formalin-ethanol on the carbon isotopic signal in the tissue is the lower quantity of lipids in the tissue preserved in alcohol and of proteins in those preserved in formaldehyde. This information is also emphasized by Benedito-Cecílio and Morimoto (2002).

After the observation that the preservatives modify the values of the stable isotopes, some authors as Vander Zanden et al. (2003) and Pease et al. (2006) corrected the isotopic values of carbon and nitrogen for fish muscles kept in formalin or in formaldehyde followed by alcohol, considering the depletion of $\delta^{13}C$ (mean ± SE; -1.12‰ ± 0.23) and the enrichment of $\delta^{15}N$ (0.53‰ ± 0.15). Nevertheless, when the samples were preserved in alcohol the mean of enrichment for $\delta^{13}C$ was 0.45 ± 0.20 and for $\delta^{15}N$ was 0.07 ± 0.10 (VANDER ZANDEN et al., 2003).

For both fish species analyzed in the present study, the differences found in the carbon isotopic values, between the control and the samples in alcohol (0.4‰) were alike those from Vander Zander et al. (2003), indicating that this value is suitable to correct samples in this preservative. However, for the formaldehyde, the mean values (0.5 ‰) were lower than those suggested by the same authors. This difference may be related to the preservation methodology used by the authors, immersing the samples in two different substances: fixation in formaldehyde followed by preservation in alcohol.

Conclusion

In summary, the muscles from adult freshwater fish species analyzed presented modified isotopic values of carbon and nitrogen. In this way, the maintenance of biological material, in alcohol 70% or formaldehyde 4%, significantly interfere with the carbon and nitrogen isotopic values, except the samples of $\delta^{13}C$ from *H. edentatus*, maintained in formaldehyde. We must consider that for the correction, the carbon values are dependent of used preservatives, whereas for the nitrogen isotopic values, these are independent. Therefore, when using a given preservative, the correction should be carried out according to the used isotope and preservative employed.

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