Diet-tissue discrimination factors of three neotropical freshwater fishes and a comparison of the trophic position

Gislaine Iachstel Manetta,[1,2] Matheus Maximilian Ratz Scoarize,[2,3,4*] Driele Delanira-Santos,[2,3] Patricia Almeida Sacramento,[2,3] Vinícius de Andrade Urbano,[2] Evanilde Benedito[1,2,3]

[1]Graduate Program in Compared Biology (PGB), State University of Maringá, Brazil; [2]Research Nucleus in Limnology, Ichthyology and Aquaculture (NUPELIA), State University of Maringá, Brazil; [3]Graduate Program in Ecology of Inland Water Ecosystems (PEA), State University of Maringá, Brazil; [4]Australian Rivers Institute (ARI), Griffith University, Brisbane, Australia

ABSTRACT

The trophic discrimination factor (TDF) is a key parameter for stable isotope analysis and due to a lack of species-specific TDFs, mean universal values have been used, resulting in uncertainties about the trophic position of species and a call for more experiments. In this study, we have addressed the lack of experimental species-specific TDFs conducting three experiments of 128 days each to determine the TDF (muscle and liver) of three species, the piscivore *Pseudoplatystoma corruscans* (Spix & Agassiz, 1829), and the omnivores *Piaractus mesopotamicus* (Holmberg, 1887) and *Astyanax lacustris* (Lütken, 1875), tropical fishes native to the La Plata River basin. Then, we calculated the trophic position (TP) using the mean universal TDF from literature and the species-specific TDF produced in this study for *Pseudoplatystoma corruscans*. We estimated the TDFs for the three species through experiment and the values found differed from the mean universal TDF in the literature. Moreover, the TP was lower when using the species-specific TDFs. The TP is important for several analyses, including its use in functional diversity. Therefore, we recommend using species-specific TDF values for calculating TP once it differs from the results calculated with mean universal TDF.

Corresponding author: maxscoarize@gmail.com

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INTRODUCTION

Stable isotope analysis (SIA) has been used in ecological studies regarding diet composition, trophic position (TP), habitat use, pollution (contaminant sources) (Canseco *et al.*, 2022) and food webs structure (Peterson and Fry, 1987). Food webs are ecological macro-descriptors of the trophic relationships in a biological community (Jepsen and Winemiller, 2002) and in neotropical environments, there are a multitude of trophic groups feeding on diverse food items. Thus, identifying and quantifying these relationships with conventional diet analysis is challenging. Therefore, SIA is a solid method to properly assess the importance of producers and consumers as sources of matter and energy. The mainly stable isotopes used were the $\delta^{13}C$ and $\delta^{15}N$ because these isotopes may investigate the energy sources and the TP, respectively. Like consumers, primary producers may have distinct carbon isotope proportions ($\delta^{13}C$) depending on their photosynthetic pathways (*e.g.*, C_3 and C_4 plants) (Smith and Epstein, 1971; Deleens *et al.* 1974). While the $\delta^{15}N$ has a trend in fractionation between each trophic level that allows investigating TP (Post, 2002).

Although there are many possibilities for the SIA to

analyse different estimates for different groups, there is one parameter that seems to be vital for the analysis: the TDF. Despite its importance, due to several experiment-related difficulties, only a derisory number of species (compared to the worldwide species richness) had their TDF estimated. The TDF is the difference in isotopic values between consumer and diet and a δ value is the ratio between heavier and lighter isotopes (*e.g.*, $^{13}C/^{12}C$ or $^{15}N/^{14}N$) in a sample (Canseco *et al.*, 2022). The use of appropriate TDF is a common problem due to the lack of determination of species-specific TDF (Kadye *et al.*, 2020). Thus, most studies use mean universal TDF values determined for a handful of species to calculate TP. The TP is the continuous measure of a specimen's position, regarding the transfer of energy, in the trophic structure of a food chain and it is used to measure average trophic function, *i.e.* the mean length of the path over which a specimen acquires energy from a source (Levine, 1980). Examples of universal TDF for fish are +0.39±1.3‰ (mean±SD) for $\Delta^{13}C$ and +3.4±0.98‰ for $\Delta^{15}N$ (Post, 2002), and +0.4±0.17‰ for $\Delta^{13}C$ and +2.3±0.28‰ for $\Delta^{15}N$ (McCutchan *et al.*, 2003). Recent studies agree that using these mean TDF values is not the most adequate approach (Canseco *et al.*, 2022) and consequently the TDF is the Achilles' heel in stable isotope mixing models in ecological studies (Nahon *et al.*, 2020), since they can present multiple sources of variation. Some factors can affect the TDF, such as the nutritional value of the protein (Mill *et al.*, 2007; Robbins *et al.*, 2005, 2010), growth rate (Reich *et al.*, 2008), the starting $\delta^{15}N$ value of the tissue (Caut *et al.*, 2009; Dennis *et al.*, 2010). Besides, the taxon, the type of tissue, type of diet, and the sample treatment (elimination of lipids) and characteristics of habitat (Phillipsen and Benedito, 2013; Canseco *et al.*, 2022) have an influence on precise TP estimates (Nawrocki *et al.*, 2020).

Considering the many uses SIA may have, the aquatic ecology field has been using this analysis in the last four decades (Canseco *et al.*, 2022), thus there is great potential for development in the field. The first studies on energy flow in food webs determining the trophic discrimination factor (TDF) between trophic levels were performed in marine environment (McConnaughey and Macroy, 1979). A study conducted in the 80s with aquatic organisms led to the establishment of a mean value for $\Delta^{15}N$ (nitrogen TDF) (Minagawa and Wada, 1984); widely used in mixing models even though based on a small number of individuals ($\Delta^{13}C$=+0.39±1.3‰; $\Delta^{15}N$=+3.4±0.98‰) (Barnes *et al.*, 2007).

This need for species-specific TDF, or at least taxonomic group-specific or trophic guild TDF instigated a call for more laboratory experiments in the animal ecology field (Gannes *et al.*, 1997), which was reviewed years later (Martínez del Rio *et al.*, 2009) and this need remains to this decade (Canseco *et al.*, 2022). This study addresses specifically this lack of experiments with three species. But more than that, the increase in the experimentally calculated species-specific TDF means that future estimations of TP and other parameters will be less biased for these species, even with this small step towards the improvement of stable isotope ecology.

Thus, the aims of this study were to: i) determine the TDF ($\Delta^{13}C$ and $\Delta^{15}N$) of muscle and liver of three fish species of different trophic guilds, one being piscivore and the other two omnivores; ii) provide evidence for the effects of using species-specific TDFs vs universal TDF on trophic position estimates in one wild population, determining the TP. Considering that universal TDF from literature are averages from a pool of different fishes, we hypothesised that the TP calculated with the species-specific TDF differs from the TP calculated with the universal mean TDF.

METHODS

Study species

Three species were selected in this study: *Pseudoplatystoma corruscans* (Spix & Agassiz, 1829); *Piaractus mesopotamicus* (Holmberg, 1887) and *Astyanax lacustris* (Lütken, 1875).

Pseudoplatystoma corruscans known as spotted sorubim (in Portuguese: pintado), is a piscivore species (Hahn *et al.*, 2002), whose distribution covers the drainage basins of the Paraná-Paraguay and São Francisco River basins (Ota *et al.*, 2018).

Piaractus mesopotamicus, known as pacu, has been described as an omnivore species (Hahn *et al.*, 2002), with a tendency to herbivory (consumes fruits, other plants and insects) (Hoeinghaus *et al.*, 2009), whose distribution covers the drainage basins of the Paraná-Paraguay River system (Ota *et al.*, 2018).

Astyanax lacustris known as lambari-tambiú, was described as an omnivore specie (Vidotto-Magnino *et al.*, 2021), which occur in important hydrographic basins of South America, covers the drainage basins of Río de la Plata, laguna dos Patos, Tramandaí River, São Francisco River, Tocantins-Araguaia River (Ota *et al.*, 2018).

Diet-switch experiments

The individuals (of each species) submitted to controlled experiments were juveniles that came from captivity (*P. corruscans* and *P. mesopotamicus*) or obtained from spawning (*A. lacustris*), in order to standardise the stage of life. Although the species have specific metabolic rates, the pace of the life history (which varies between experimental environments that do not have interspecific competition or predation, and natural environments) of a population influences its individuals (Auer *et al.*, 2018).

The experiments were carried out after the university's Animal Ethics Committee approval: CEUA number 7319260215.

Before the beginning of the experimental period, the fish were acclimated in aquariums, submitted for 10 days to a prophylactic treatment with sodium chloride (4 g/L of water). During these 10 days, the fish were fed the same rations from their aquaculture farms. The water to supply the aquariums was previously oxygenated and rested for chlorine evaporation for at least 24 hours before use. The aquariums (in this study containing 30 L water) and tanks (in this study containing 1000 L) were syphoned (excretes accumulated in the bottom removed by syphon) daily, to prevent the accumulation of faeces during the whole experiment. The aquariums were used for the smaller species and the tanks for the largest. The withdrawn water was replaced daily (with previously oxygenated and rested water). The limnological parameters dissolved oxygen concentration (mg/L) and water temperature (°C) were measured weekly using an oximeter and thermometer (YSI® 550A). The tanks of both acclimatisation and the experiment were in the experimental area of the Research Nucleus in Limnology, Ichthyology and Aquaculture (NUPELIA) (T-10), on the main campus of the State University of Maringá (UEM) (23°24'11.8"S 51°56'31.6"W).

The experiment consisted of treatments referencing the administration of different rations named as C3.C4 and C3/C4. These rations differ in isotopic values of $\delta^{13}C$ and $\delta^{15}N$ (Tab. 1), and are constituded with different ingredients concerning the lipid, protein and carbohydrate compositions (Tab. S1), (Faria and Benedito, 2011).

The experiments differed regarding a few characteristics, such as number of treatments and number of aquariums/tanks (please see description below), but in general, have the same basis: juvenile individuals; total duration of 128 days, with nine withdrawals; during autumn/winter in the Southern Hemisphere. The duration of the experiment (128 days) was established because other studies have shown that the time elapsed for the determination of TDF and turnover rates was under 128 days (Busst and Britton, 2016; Maruyama *et al.*, 2016; Sacramento *et al.*, 2016).For *Pseudoplatystoma corruscans* and *Piaractus mesopotamicus* three treatments were administered (C3, C4 and C3/C4). For *A. lacustris*, two treatments (C_4 and C_3/C_4) were administered because the specimens obtained from spawning were not enough to perform the three treatments. The diets had lipid, protein and carbohydrate composition similar to the natural diet consumed by the species (Tab. S1 and Tab. 1). Each (two per day) ration fed made up approximately 5% of the fish live weight. A conceptual design of the experiment can be found in Fig. 1.

Tab. 1. Mean isotopic values and standard deviation (mean±SD) of ^{13}C and ^{15}N of the rations C_3, C_4, and C_3/C_4.

Treatment	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Ration C_3	-25.78±0.11	+1.46±0.22
Ration C_4	-13.19±0.20	+4.48±0.07
Ration C_3/C_4	-15.88±0.28	+4.24±0.04

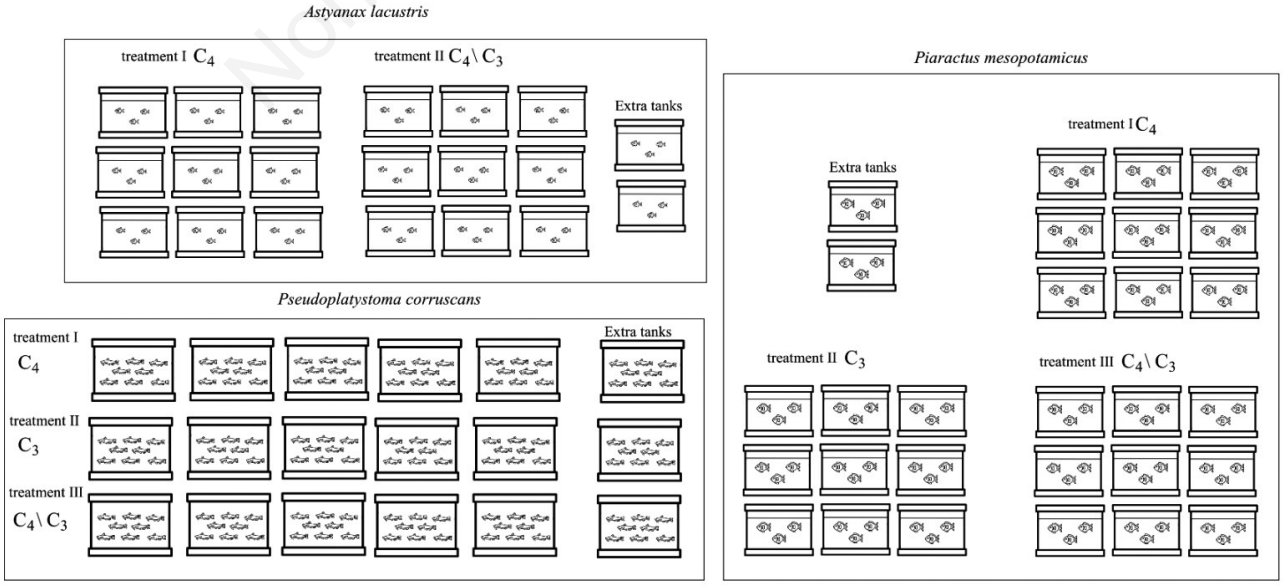

Fig. 1. Conceptual design of the experiments.

Experimental design for each species

Pseudoplatystoma corruscans

The juveniles were acquired from the fish farming center *Centro de Piscicultura do Mato Grosso do Sul*. Before the experiment, the fish were acclimated in 1000 L tanks for 10 days and fed with the commercial ration to which they were accustomed. After acclimatisation, the specimens were separated into 15 tanks of 1000 L, each contained eight individuals. Three extra tanks were prepared (total of 18 tanks: 15+3), one for each treatment (ration), with about 20 fish in total for the three tanks. These extra tanks were added if any complications occurred with the fish for possible replacement. The tanks had oxygenation (oxygen pump) and temperature (thermostat) maintained with low variation (1 to 3 mg L^{-1} 1 to 2°C). The experiment took place from March to July (autumn/winter in the Southern Hemisphere) 2016. There were nine times for fish sampling, corresponding to the following days of experiment duration: 0, 2, 4, 8, 16, 32, 64, 90 and 128; 1 to 2°C). The experiment took place from March to July (autumn/winter in the Southern Hemisphere) 2016. There were nine times for fish sampling, corresponding to the following days of experiment duration: 0, 2, 4, 8, 16, 32, 64, 90 and 128; time 0 reflects the isotopic value of the old diet (control). The 15 tanks were submitted to three treatments (C_3, C_4 and C_3/C_4 rations) with five replicates per treatment. At each time, one fish from each tank (5 tanks per treatment) was collected to obtain liver and muscle samples, totaling five individuals per treatment. The fish were fed (twice a day: around 8 am and 5 pm) with ration formulated with isotopic values described in Tab. 1, for more information see Tab. S1 (Food composition of the diets formulated for the experiments, in g $10g^{-1}$).

Piaractus mesopotamicus

The juveniles were acquired from the fish farming *Centro de Piscicultura Piracema* (http://www.pisciculturapiracema.com.br; Reproduction Laboratory located in Estrada Água da Areia, Munhoz de Mello – PR, Brazil). Before the experiment, the specimens were acclimated in 30 L aquariums for 10 days and fed with the commercial ration to which they were accustomed. After acclimatisation, the fish were separated into 27 aquariums of 30 L, each tank containing three individuals. Three extra tanks were prepared (total of 30 tanks: 27+3), one of each treatment (ration), with nine fish in total for the three tanks. These extra tanks were added if any complications occurred with the fish for possible replacement. The tanks had oxygenation (oxygen pump) and temperature (thermostat) maintained with low variation (1 to 3 mg L^{-1}; 1 to 2°C). The experiment took place from March to June (autumn in the Southern Hemisphere) 2015. There were nine times for fish sampling, corresponding to the following days of experiment duration: 0, 2, 4, 8, 16, 32, 64, 90 and 128; time 0 reflects the isotopic value of the old diet (control). The 27 aquariums were submitted to three treatments (C_3, C_4 and C_3/C_4 rations) with nine replicates per treatment. At each time, three fishes from the aquarium were collected (eliminating all three fishes from the aquarium) to obtain liver and muscle samples, totalizing three individuals per treatment. The fish were fed (twice a day: around 8 am and 5 pm) with ration formulated with isotopic values described in Tab. 1, for more information see Tab. S1.

Astyanax lacustris

The juveniles resulted from induced spawning performed by the Nupélia's Ichthyoplankton Ecology Laboratory (https://www.nupelia.uem.br/laboratórios/ ictioplâncton; located on UEM main campus). Before the experiment, the fish were acclimated in 30 L aquariums for 10 days and fed with the commercial ration to which they were accustomed. After acclimatisation, the fish were separated into 18 aquariums of 30 L, each aquarium containing three individuals. Two extra tanks were prepared (total of 20 tanks: 18+2), one for each treatment (ration), with six fish in total for the two tanks. These extra tanks were added if any complications occurred with the fish for possible replacement. The tanks had oxygenation (oxygen pump) and temperature (thermostat) maintained with low variation. The experiment took place from May to July (autumn/winter in the Southern Hemisphere) 2015. There were nine times for fish sampling, corresponding to the following days of experiment duration: 0, 2, 4, 8, 16, 32, 64, 90 and 128; time 0 reflects the isotopic value of the old diet (control). The 18 aquariums were submitted to two treatments (C_4 and C_3/C_4 rations) with nine replicates per treatment. At each time, three fishes from the aquarium were collected (eliminating all three fishes from the aquarium) to obtain liver and muscle samples, totalizing three individuals per treatment. The fish were fed (twice a day: around 8 am and 5 pm) with ration formulated with isotopic values described in Tab. 1, for more information see Tab. S1.

Sample processing

Immediately after each experimental period (nine sampling times) specimens were anesthetised by benzocaine solution (250 mg L^{-1}). Liver and muscle were removed, washed with distilled water, frozen and stored at -20°C (freezer). Carcasses and other components (skin and viscera), not used in this experiment, were frozen and sent to the UEM Central Bioterium (http://www.bit.uem. br/localizacao; located on UEM main campus) for incineration.

Subsequently, the frozen samples were removed from the freezer and dried in a ventilation oven for 72 hours at

50°C. All samples were powdered and only the larger samples (muscle) were powdered in a ball mill until fine powder was obtained. Finally, each sample was weighed (approximately 0.35 to 0.45 mg) on an analytical scale at the Research Support Centre Complex (http://www.comcap.uem.br:8080/comcap/estatico/home/; located on UEM main campus), placed in a tin capsule and sent to the isotopic determination of ^{13}C and ^{15}N at the Hoeinghaus Laboratory of Ecosystem Ecology and Aquatic Communities, University of North Texas (http://biol.unt.edu/~djhoeinghaus/Facilities.html, Denton, TX, USA).

The design was carried out for these species in order to determine in experiments with different treatments, TDF and turnover rate for carbon and nitrogen in liver and muscle tissues, which present different metabolic rates (Fry, 1981; Pereira and Benedito, 2007; Philippsen and Benedito, 2013). The TDF was determined using the following equation:

$$\Delta=\delta_{tissue} - \delta_{diet} \quad \text{(eq. 1)}$$

Where: $\Delta^{13}C/\Delta^{15}N$: carbon and nitrogen TDF; δ_{tissue}: tissue isotopic value (liver and muscle); δ_{diet}: diet isotopic value.

The turnover rate and the other parameters were estimated using the model proposed by Hobson and Clark (1992):

$$Y=Ya+a \times e^{(-\lambda t)} \quad \text{(eq. 2)}$$

Where: Y: ^{13}C or ^{15}N in time t; Ya: the asymptotically approximate value; a: difference between the initial and equilibrium conditions; λ= turnover rate; t: time since the diet was altered.

The turnover rate was expressed in terms of half-life. The half-life refers to the amount of time (days) required for the stable isotope signal of the consumer tissue to reach the mean value between the observed original diet and what will be verified in the new diet in equilibrium, as demonstrated by the equation:

$$t_{1/2}=\frac{ln(2)}{\lambda} \quad \text{(eq. 3)}$$

Where: $t_{1/2}$= is the time required to replace 50% of the initial tissue. Equations can be found in Martínez del Rio *et al.* (2009).

Field sampling

To compare the TP calculation with mean TDF values from the literature and the experimental species-specific TDF values, we selected one species (*Pseudoplatystoma corruscans*) with data from the Atlantic Forest in the La Plata River basin, from 2010 and 2020. The studied area was selected due to its relevance for worldwide biodiversity as a hotspot (Myers *et al.*, 2000) and for its economic importance for Latin America, once the La Plata River basin is the fifth largest in the world and more than one hundred million people rely on its resources (Villar *et al.*, 2018).

The samplings took place through the PELD project (acronym in Portuguese for Long Term Ecological Research – Site 6, 2010 process number: 403686/2012-1/CNPq, 2020 process no. 441356/2020-6/CNPq, 4854/2021/SGP-UEM. Scientific research permits 71/000666/2021/IMASUL, 52596-5/ICMBio) in the Ivinhema River system from the Upper Paraná River Floodplain, Brazil. This river system was chosen due to its environmental quality as a conserved and protected river, since it is a free-from-dam river that provides permanent water flow and high habitat heterogeneity (Garcia *et al.*, 2020). To calculate the TP, it is necessary to obtain data from the consumer (fish) and the isotopic baseline, or probable energy sources (primary producers or preys with known TP), from the same environment.

For the fish samples, muscle (approximately 2 cm^3) was collected from the base of the dorsal fin insertion of *Pseudoplatystoma corruscans*. The specimens were collected using gill nets, with different types of meshes (2 to 16 cm between nodes) exposed for a period of 24 hours and searched every 8 hours. After sampling, specimens were transported and sacrificed according to AVMA guidelines (American Veterinary Medical Association; Underwood *et al.*, 2013) and with the permit from the Ethics Committee for Animal Use (1420221018/CEUA-UEM). The species were identified according to Graça and Pavanelli (2007) and deposited in the Ichthyological Collection of the NUPELIA, State University of Maringá.

For the probable energy sources, three samples of each primary producer were collected. The primary producers were selected for the baseline because they are the first trophic level. They consisted of periphytic biofilm, seston (suspended particles, including planktonic organisms, particulate organic carbon, and inorganic substances, according to Urabe, 1995), riparian vegetation, and aquatic macrophytes. The periphytic biofilm was obtained by scraping the petiole of aquatic plants that were cut and stored in dark flasks in the field. The seston was obtained with direct storage of near superficial water in 500 mL pots for further filtration. The biofilm and seston were filtered and retained on 47 mm aperture (Whatman GFC) glass filters, pre-calcined at 450ºC for four hours. From the riparian vegetation (represented by *Inga vera* Willd. subsp. *affinis* (DC.) TD Pennington) and the aquatic macrophytes [represented by *Pontederia azurea* Sw (= *Eichhornia azurea* (Sw.) Kunth)], each sample consisted of three leaves, at least. The leaves were collected directly from the plants with pods or scissors and stored.

All samples were oven dried at 60ºC for 72 hours.

After drying, fish and leaves (riparian vegetation and aquatic macrophytes) were powdered until a fine and homogeneous powder was obtained. The filters' surfaces were scraped to reduce the amount of glass fibres on the samples. The powdered samples and the scraped filters containing biofilm and seston samples were stored in tin capsules and sent for the determination of stable isotope ratios of carbon and nitrogen.

In 2010, the samples were sent to UC Davis Facility Stable Isotope Laboratory. The determination of isotopic ratios for the powdered samples was performed on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) and the filtered samples on a Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) and also interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd.). The 2020 samples were sent to the Thünen Institute Stable Isotope Analysis Laboratory (Braunschweig, Germany). The determination of isotopic ratios was performed on a Thermo Finnigan DeltaPlus Advantage isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific, Waltham, MA, USA) coupled to an ECS 4010 EA elemental analyser (Costech Analytical Technologies, Inc., Valencia, CA, USA).

Stable isotope analysis

The analytical protocol with the description of carbon and nitrogen isotopic analysis follows that establishes in Fry (2006).

Statistical analysis

Discrimination factors and turnover rates

To address the first aim, the isotopic tissue data were analysed by non-parametric estimation using nonlinear regression using the least squares method. Within this method, Levenberg-Marquardt method was selected. The decay curves were calculated by nonlinear regression, when all the estimated parameters of the model were significant. The analyses were performed in the Statistica 7.0® software, using the aforementioned model. For all estimated parameters, the level of significance was $p<0.05$.

Trophic position calculation with different TDF

To address the second aim and test the hypothesis, we calculated the TP using different TDF (universal from literature and experimental species-specific TDF). From the isotopic data collected in 2010 and 2020, we used the discrimination factors of this experiment for *P. corruscans* to calculate TP. The TP was calculated in software R (R Core Team, 2022) using the 'tRophicPosition' package (Quezada-Romegialli *et al.*, 2018). This method uses Bayesian approach, incorporating individual variability and propagating sampling error of trophic discrimination, isotopic baselines and consumers, and posterior estimates of parameters. The TP was modelled using the Markov chain Monte Carlo (MCMC) method with 10,000 interactions and 10,000 adaptive samples in JAGS 4.3.0 for the model with one baseline. The universal mean TDF from the literature used to compare with the species-specific (S-TP) TDF were Post's (P-TP) TDF +0.39±1.3 ‰ (mean±SD) for $\delta^{13}C$ and +3.4±0.98‰ for $\delta^{15}N$ from Post (2002), and McCutchan's (M-TP) TDF +0.4±0.17‰ for $\delta^{13}C$ and +2.3±0.28‰ for $\delta^{15}N$ from *McCutchan et al.* (2003). As a trophic baseline we used all the probable primary energy sources available as we were unsure of which source was the main one for the fish.

RESULTS

Diet-tissue discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$)

The mean and standard deviations (±SD) of temperature and oxygen during the experiment of *Pseudoplatystoma corruscans* were 22.28±2.04°C; 7.10 mg/L±2.11, respectively. We observed that there was an increase for both mean weight and standard length of the specimens sampled at the beginning and at the end of the experiment (Tab. S2), showing that the change in weight and growth probably contributed to tissue replacement and leading to its isotopic exchange in each treatment. The highest mean weight and standard length were in the treatment with C_4, 21.24 g and 142.22 mm (Tab. S2). The initial mean isotopic values (±SD) of carbon and nitrogen for muscle and liver showed different isotopic values in the final time (128 days) (Tab. S3). For ^{13}C, isotopic values varied more until the isotopic balance (for treatments C_3 and C_4. For ^{15}N, this variation was much less evident (Tab. 2). The isotopic balance (obtained for all tissues and treatments was significant and therefore it was possible to obtain the TDF for them. The parameters a e λ were estimated in the model, but were not statistically significant and therefore were not included in the results. As the turnover rate (λ) was not significant, it was not possible to measure the period of time in which isotopic tissue replacement occurred through diet administration. For ^{13}C, the TDF ranged from -1.91 ‰ to +8.36 ‰ for the muscle, between -1.46 ‰ and +4.65 ‰ for the liver (Tab. 2). While for ^{15}N the values were higher, ranging from +4.49 ‰ to +6.28 ‰ for liver and between +3.81 ‰ and +5.7 ‰ for muscle (Tab. 2). The decay curves were not presented due to the non-significance of all estimated parameters. The mean and standard deviations (±SD) of temperature and oxygen during the experiment of *P. mesopotamicus* were 26.31±1.41°C and 5.20±0.58, respectively. The mean weight was differ-

ent for each food administered and exceeded 100% of the initial weight. The highest values of weight and standard length were in the treatment with C_3 ration. It was possible to observe that there was an increase in mean weight and in the standard length of the specimens sampled at the beginning and at the end of the experiment (Tab. S4). In general, the isotopic values of $\delta^{13}C$ and $\delta^{15}N$ were changing from the beginning (T0) to the end of the experiment (T128), both for the muscle and for the liver (Tab. S5). The asymptotes () of the regression equations did not have significant values ($p<0.05$) for the liver ^{13}C isotope of the treatments C_4 and C_3/C_4, so it was not possible to calculate the TDF for this tissue (Tab. 3). However, for the other treatments and tissues the isotopic values of $\delta^{13}C$ and $\delta^{15}N$ varied within the times established for the experiment, obtaining the asymptote () and calculating the TDF. The $\Delta^{13}C$ varied between treatments (C_3 ration, C_4 ration, C_3/C_4 ration) being higher for the liver of C_3 ration ($\Delta^{13}C$=+6.25‰) (Tab. 3). The $\Delta^{15}N$ varied between +3.81‰ and +2.39‰ for the muscle, between +4.79‰ and +3.62‰ for the liver (Tab. 3).The estimated turnover parameter (λ) in the regression was significant only for the $\delta^{13}C$ value for the muscle (ration C_4), and the $\delta^{15}N$ value for the liver of the C_3 ration treatment, so the half-life ($t_{1/2}$) for these tissues and diets were approximately 7 days (Tab. 3). In the *A. lacustris* experiment, temperature and dissolved oxygen were maintained on average 23.45±0.71°C and 6.96±0.59, respectively. The highest mean weight and standard length were in the treatment with C_4 (Tab. S6). The isotopic values of ^{13}C and ^{15}N were replaced throughout the experiment according to the ration. At the end of the experiment the values are different for muscle and liver (Tab. S7). For $\delta^{13}C$, the TDF varied between treatments (muscle: $\Delta^{13}C$=+0.19 to +1.78‰;

Tab. 2. Initial isotopic signatures of the tissue's $\delta^{13}Ci$ (±SD) and $\delta^{15}Ni$ (±SD), parameters calculated (±SE) through the decay curve (all with $p<0.05$). The discrimination factor ($\Delta^{13}C$ and $\Delta^{15}N$) of muscles and liver of *Pseudoplatystoma corruscans* for three treatments.

Treatment	Tissue	$\delta^{13}Ci$‰ (±SD)	Yα‰ (asymptote)	Diet ‰	$\Delta^{13}C$ (‰)	$t_{1/2\ (days)}$
C_3	Muscle	-15.50 (±0.16)	-17.42 (±0.62)	-25.78	+8.36	_
	Liver	-16.20 (±0.01)	-21.13 (±1.12)	-25.78	+4.65	_
C_4	Muscle	-15.50 (±0.16)	-15.10 (±0.15)	-13.19	-1.91	_
	Liver	-16.20 (±0.01)	-14.65 (±0.23)	-13.19	-1.46	_
C_3/C_4	Muscle	-15.50 (±0.16)	-15.23 (±0.07)	-15.88	+0.65	_
	Liver	-16.20 (±0.01)	-15.50 (±0.23)	-15.88	+0.38	_
Treatment	**Tissue**	**$\delta^{15}Ni$‰ (±SD)**	**Yα‰ (asymptote)**	**Diet ‰**	**$\Delta^{15}N$**	**$t_{1/2\ (days)}$**
C_3	Muscle	+8.10 (±0.17)	+7.62 (±0.24)	+1.46	+6.16	_
	Liver	+9.90 (±0.24)	+7.74 (±0.40)	+1.46	+6.28	_
C_4	Muscle	+8.10 (±0.17)	+8.29 (±0.03)	+4.48	+3.81	_
	Liver	+9.95 (±0.2)	+8.97 (±0.19)	+4.48	+4.49	_
C_3/C_4	Muscle	+8.10 (±0.17)	+8.30 (±0.08)	+4.24	+4.06	_
	Liver	+9.90 (±0.24)	+9.03 (±0.25)	+4.24	+4.79	_

Tab. 3. Initial isotopic signatures of $\delta^{13}Ci$ (±SD), $\delta^{15}Ni$ (±SD), parameters calculated (±SE) through the decay curve (all with $p<0.05$). The discrimination factor ($\Delta^{13}C$ and $\Delta^{15}N$) of muscles and liver of *Piaractus mesopotamicus* for three treatments.

Treatment	Tissue	$\delta^{13}Ci$ ‰ (±SD)	Yα‰ (asymptote)	Diet ‰	$\Delta^{13}C$ ‰	λ	$t_{1/2\ (days)}$
C_3	Muscle	-18.70 (±0.41)	-21.32 (±0.47)	-25.78	+4.46		-
	Liver	-19.70 (±0.89)	-19.25 (±8.00)	-25.78	+6.25		-
C_4	Muscle	-18.70 (±0.41)	-14.52 (±0.33)	-13.19	-1.33	+0.10 (±0.03)	6.93
	Liver	-19.70 (±0.89)	-	-	-		-
C_3/C_4	Muscle	-18.70 (±0.41)	-16.44 (±0.39)	-15.88	-0.56		-
	Liver	-19.70 (±0.89)	-	-	-		-
Treatment	**Tissue**	**$\delta^{15}Ni$ ‰ (±SD)**	**Yα‰ (asymptote)**	**Diet ‰**	**$\Delta^{15}N$ ‰**	**λ**	**$t_{1/2\ (days)}$**
C_3	Muscle	+6.90 (±0.17)	+5.27 (±0.20)	+1.46	+3.81		-
	Liver	+7.80 (±0.22)	+6.25 (±3.00)	+1.46	+4.79	+0.10 (±0.04)	6.93
C_4	Muscle	+6.90 (±0.17)	+8.29 (±0.03)	+4.48	+3.81		-
	Liver	+7.80 (±0.22)	+8.10 (±1.17)	+4.48	+3.62		-
C_3/C_4	Muscle	+6.90 (±0.17)	+6.63 (±0.13)	+4.24	+2.39		-
	Liver	+7.80 (±0.22)	-	-	-		-

liver: $\Delta^{13}C$=+2.68‰). For $\delta^{15}N$, the TDF ranged from +4.1 ‰ to +4.52 ‰ for the muscle, between +3.07‰ and +5.02% for the liver (Tab. 4). The estimated turnover parameter (λ) in the regression was not significant for the values of $\delta^{13}C$ and $\delta^{15}N$ of muscle and liver. Therefore, it was not possible to calculate the half-life ($t_{1/2}$) for these tissues and diets. Considering the $\Delta^{13}C$ of the trophic guilds together, the piscivore had the highest value of the muscle and the omnivore *P. mesopotamicus* of the liver tissue. However, for the $\Delta^{15}N$, the piscivore presented a higher value of both muscle and liver (Tab. 5).

Trophic position: literature means and species-specific discrimination factors

The comparison of species-specific TDF (experimental values) with Post's TDF and McCutchan's TDF (mean universal TDF) found lower TP for *Pseudoplatystoma corruscans* with species-specific TDF (Tab. 6, Fig. 2) in both analyses (2010 and 2020 data). But Post's values were closer to the species-specific values.

DISCUSSION

The TP of *Pseudoplatystoma corruscans* (piscivore) was lower when the TDF values were determined by species-specific experiments than Post's and McCutchan's TDF. Hence, we have accepted our hypothesis. When using only $\Delta^{15}N$ TDF, Sacramento *et al.* (2016) had the same reduction in the TP and change in trophic level for *Prochilodus lineatus* (detritivore). Thus, the ideal scenario for TP determination is to use species-specific experimental TDF values, otherwise it could be significantly biased.

Tab. 4. Initial isotopic signatures of $\delta^{13}Ci$ (±SD), $\delta^{15}Ni$ (±SD), parameters calculated (±SE) through the decay curve (all with $p<0.05$). The discrimination factor ($\Delta^{13}C$ and $\Delta^{15}N$) of muscles and liver of *Astyanax lacustris* for two treatments.

Treatment	Tissue	$\delta^{13}Ci$ ‰ (±SD)	Yα‰ (asymptote)	Diet ‰	$\Delta^{13}C$ (‰)	$t_{1/2\ (days)}$
C_3/C_4	Muscle	-16.00	-14.10 (±4.00)	-15.88	+1.78	-
	Liver	-15.98 (±0.97)	-13.20 (±4.00)	-15.88	+2.68	-
C_4	Muscle	-16.00	-13.00 (±5.00)	-13.19	+0.19	-
	Liver	-15.98 (±0.97)	-	-	-	-
Treatment	**Tissue**	**$\delta^{15}Ni$ ‰ (±SD)**	**Yα‰ (asymptote)**	**Diet ‰**	**$\Delta^{15}N$(‰)**	**$t_{1/2\ (days)}$**
C_3/C_4	Muscle	+8.35 (±0.78)	+8.34 (±1.7)	+4.24	+4.10	-
	Liver	+8.60	+7.31 (±2.1)	+4.24	+3.07	-
C_4	Muscle	+8.35 (±0.78)	+9.00 (±2.2)	+4.48	+4.52	-
	Liver	+8.60	+9.50 (±2.6)	+4.48	+5.02	-

Tab. 5. Discrimination factor ($\Delta^{13}C$ and $\Delta^{15}N$) of muscle and liver, for *Piaractus mesopotamicus*, *Astyanax lacustris* and *Pseudoplatystoma corruscans* and mean (±SD) by guild and total, calculated for 128 days experiments. Specimens were fed distinct rations according to the isotopic signal (ration C_3, ration C_4, and ration C_3/C_4).

	Discrimination factor			
	Muscle		Liver	
	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)
P. corruscans C_3	+8.36	+6.16	+4.65	+6.28
P. corruscans C_4	-1.91	+3.81	-1.46	+4.49
P. corruscans C_3/C_4	+0.65	+4.06	+0.38	+4.79
Mean±SD	+2.34±5.34	+4.68±1.29	+1.19±3.13	+5.19±0.96
P. mesopotamicus C_3	+4.46	+3.81	+6.25	+4.79
P. mesopotamicus C_4	-1.33	+3.81	-	+3.62
P. mesopotamicus C_3/C_4	-0.56	+2.39	-	-
Mean±SD	+0.86±3.14	+3.33±0.82	+6.25	+4.20±0.83
A. lacustris C_3	-	-	-	-
A. lacustris C_4	+0.19	+4.52	-	-
A. lacustris C_3/C_4	+1.78	+4.10	+2.68	+5.02
Mean±SD	+0.98±1.12	+4.31±0.30	+2.68	+5.02
Mean±SD	+1.45±3.42	+4.08±1.04	+2.50±3.12	+4.83±0.86

Although different from the species-specific, Post's resulted in a TP closer to the species-specific than McCutchan's.

Considering the experiments performed, the mean $\Delta^{13}C$ values were different between the guilds and between the diets, for muscle and liver. Similarly, $\Delta^{15}N$ values differed, but with lower amplitude than $\Delta^{13}C$ values (Tab. 5). Both $\Delta^{13}C$ and $\Delta^{15}N$ presented values close to or higher than those recorded in the literature ($\Delta^{13}C$=+0.39±1.3‰; $\Delta^{15}N$=+3.4±0.98‰, Deniro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002). In comparison with other experiments (Sacramento *et al.*, 2016; Colborne *et al.*, 2017; García-Pérez *et al.*, 2018; Winter *et al.*, 2019; Kadye *et al.*, 2020; Sawada *et al.*, 2021), $\Delta^{13}C$ muscle values of carnivore/piscivore species were similar (numerically), while omnivore species were distinct (numerically). The species *Pseudoplatystoma corruscans* (carnivore/piscivore), *Prochilodus lineatus* (detritivore), *Astyanax lacustris* (omnivore), *Cyprinus carpio* (omnivore) and *Plecoglossus altivelis* (omnivore) presented higher TDF than the mean TDF presented for $\Delta^{15}N$ (Vander Zanden and Rasmussen, 2001; Post, 2002; McCutchan *et al.*, 2003), most of them tropical species.

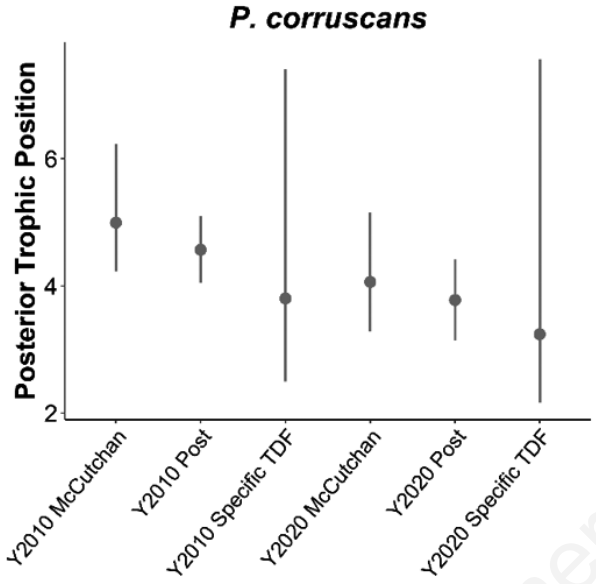

Fig. 2. Trophic position of *Pseudoplatystoma corruscans* calculated with different discrimination factors (TDF). Posterior mode of trophic position (middle point) and 95% credibility interval (lines) of *P. corruscans* population [sampled in 2010 (Y2010) and 2020 (Y2020)] calculated with different TDF (species-specific TDF, Post's TDF, and McCutchan's TDF).

Regarding the protein quality hypothesis (*i.e.*, isotopic discrimination between consumers and their prey increases as the protein quality decreases (Roth and Hobson, 2000), Winter *et al.* (2019) suggest that fishes that feed on plant material normally assimilate proteins with lower quality, compared to carnivores. It would result in decreasing TDF the higher the trophic level. However, our results suggest the opposite for muscles; *Astyanax lacustris*, which mainly feeds on plant material in lotic environments (Vidotto-Magnoni *et al.*, 2021) and *Piaractus mesopotamicus*, presented lower $\Delta^{13}C$ and $\Delta^{15}N$ values than the piscivore *Pseudoplatystoma corruscans*. Thus, the piscivore fish had a higher $\Delta^{15}N$ than the omnivores, similar to the result of Vanders Zanden and Rasmussen (2001), in which the piscivore had a higher $\Delta^{15}N$ value.

The turnover parameter of the regression was not significant, but the duration of the experiments (128 days) was close to the average days of recent experiments, ~126

Tab. 6. Number of samples (N), mean and SD of the carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$, respectively, represented as parts per thousand ‰) of the consumers and baseline groups from 2010 and 2020, and consumers' posterior TP mode values with the species-specific (S-TP), Post's (P-TP) and McCutchan's (M-TP) TDF based calculations.

	N	$\delta^{13}C$±SD	$\delta^{15}N$±SD	S-TP	P-TP	M-TP
2010						
P. corruscans	11	-26.6±2.92	11.9±2.2	3.799	4.57	4.99
Arboreous	4	-29.9±0.366	2.25±0.078	BL	BL	BL
Biofilm	16	-28.8±2.88	4.18±1.51	BL	BL	BL
Macrophyte	4	-31.1±0.125	5.03±0.467	BL	BL	BL
Seston	16	-28.2±4.84	1.83±0.947	BL	BL	BL
2020						
P. corruscans	6	-28.2±2.8	11.5±1.61	3.23	3.78	4.06
Arboreous	9	-29.7±1.08	2.25±1.58	BL	BL	BL
Biofilm	18	-26.1±2.73	6.18±1.64	BL	BL	BL
Macrophyte	12	-30.6±1.16	6.72±1.86	BL	BL	BL
Seston	18	-28.2±2.16	5.66±2.6	BL	BL	BL

BL, baseline.

(Heady and Moore, 2013; Busst and Britton, 2016; Maruyama *et al.*, 2016; Sacramento *et al.*, 2016; Britton and Busst, 2017; Colborne *et al.*, 2017; García- *et al.*, 2018; Winter *et al.*, 2019; Kadye *et al.*, 2020; Nahon *et al.*, 2020; Zhou and Gu, 2020; Maitland *et al.*, 2021; Sawada *et al.*, 2021; Scharnweber *et al.*, 2021). *Piaractus mesopotamicus* was the only one for which it was possible to calculate the half-life, for some tissue and diet. Interestingly, omnivores and herbivores are more efficient in metabolising carbohydrates as an energy source than carnivores (Takahashi *et al.*, 2017) and *P. mesopotamicus* juveniles are also capable of efficiently metabolising lipids as an energy source as well (Abimorad and Carneiro, 2007). These aptitude of *P. mesopotamicus* could explain part of the results. However, the pace of a population's life history influences the metabolic rates of individuals (Auer *et al.*, 2018). Therefore, it is possible that the experiments, carried out without predators and other aspects that could accelerate the pace of life history, favour a slower pace of development by specimens, contributing to the decrease of metabolic rates. This could explain the insufficient time to calculate the turnover rate.

It was confirmed that the turnover rate inherent to the species (specific to each species) can vary greatly in this study. For instance, for *Piaractus mesopotamicus*, the ^{13}C $t_{0.5}$ was seven days for muscle (ration C_4) and the ^{15}N $t_{0.5}$ for liver was seven days (ration C_3) as well. But in a similar experiment with the freshwater carnivore/piscivore *Oncorhynchus mykiss* (Walbaum, 1792), specimens were kept for 210 days and the turnover rate was: ^{15}N $t_{0.5}$ of 39 days (Heady and Moore, 2013). In another experiment with the marine carnivore *Seriola dorsalis* (Gill, 1863), specimens were kept for more than 700 days and presented much longer turnover rates: ^{13}C $t_{0.5}$ of 341 days and ^{15}N $t_{0.5}$ of 181 days (Madigan *et al.*, 2021). In a third example, the marine carnivore *Plectropomus leopardus* (Lacepède, 1802) specimens were kept for 196 days and the ^{15}N $t_{0.5}$ was 126 days. Thus, likely due to this high variability of the turnover rate according to the species, we could not determine it for more than one species and in different tissues and rations. Unfortunately, we could not calculate the turnover rates for the freshwater piscivore studied in this experiment due to the short time, and thus we were not able to compare its values with the freshwater piscivore *O. mykiss*. Therefore, regarding this high variability, we suggest that future experiments, targeting to calculate the turnover rates of freshwater tropical species should be conducted for more than 200 days.

CONCLUSIONS

The TDF of ^{13}C differed between trophic guilds for both muscle and liver. The mean TDF of ^{13}C and ^{15}N for the different guilds and the general for taxon can be used in the determination of the food chains of freshwater fish. Intriguingly, our results did not support the protein quality hypothesis, showing the opposite trend. The use of different TDF (species-specific defined by experiment and the universal adopted in the literature) resulted in different values when used to calculate the TP. Thus, we must use the species-specific TDF to calculate TP and other parameters, related to SIA application to trophic ecology, to avoid bias. The main contribution of this study to the freshwater ecology field is to maintain the call for more experiments that calculate species-specific TDF. There is also a need for a systematic review of fish TDF experiments, addressing the differences between regions (tropical, subtropical, temperate); ecosystems (freshwater, marine and estuarine), regarding different isotopes, tissues, rations, age and experimental conditions.

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Online supplementary material:

Tab. S1. Food composition of the diets formulated for the experiments, in g $10g^{-1}$.

Tab. S2. Values of standard length and total weight at the initial and final times, in the treatments for Pseudoplatystoma corruscans.

Tab. S3. Mean of the isotopic values of carbon and nitrogen for muscles and livers of Pseudoplatystoma corruscans.

Tab. S4. Values of standard length and total weight at the initial and final times, in the treatments for for Piaractus mesopotamicus.

Tab. S5. Mean of the isotopic values of carbon and nitrogen for muscles and livers of Piaractus mesopotamicus *fed different diets.*

Tab. S6. Values of standard length and total weight at the initial and final times, in the treatments for Astyanax lacustris.

Tab. S7. Mean of the isotopic values of carbon and nitrogen for muscles and livers of Astyanax lacustris *fed different diets.*