

Are you what you eat? Effects of trophic discrimination factors on estimates of food assimilation and trophic position with a new estimation method



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ARTICLE INFO

Article history:

Received 10 May 2016

Received in revised form 10 October 2016

Accepted 5 December 2016

Keywords:

Carnivory

Diet shift

Herbivory

Isotopic fractionation

Omnivory

Trophic enrichment

ABSTRACT

A key factor for estimates of assimilation of resources and trophic position based on stable isotope data is the trophic discrimination factor (TDF). TDFs are assumed based on literature reviews, but may vary depending on a variety of factors, including the type of diet. We analyzed effects of alternative TDFs on estimates of assimilated resources and trophic positions for an omnivorous fish, *Jenynsia multidentata*, that reveals dietary variation among locations across a salinity gradient of a coastal lagoon in southern Brazil. We also compared estimates of foods ingested vs. foods assimilated. Food assimilation was estimated using carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope ratios of food sources and consumer muscle tissue and an isotopic mixing model (SIAR); consumer trophic position (TP) was estimated from consumer and production source $\delta^{15}\text{N}$ values. Diet was estimated using an index of relative importance based on frequency of occurrence and volumetric and numeric proportions of food items from stomach contents. The effect of variation in TDF on food assimilation and TP was tested using three alternative TDFs reported in review papers. We then created a new method that used food source-specific TDFs (reported separately for herbivores and carnivores) weighted in proportion to estimated assimilation of resources according to mixing model estimates to estimate TP (hereafter TP_{WAR}). We found that plant material was not assimilated in a proportion similar to its importance in the diet of fish at a freshwater site, and the new method yielded best assimilation estimates. Animal material made greatest contributions to fish biomass irrespective of TDFs used in the mixing model. The new method produced TP estimates consistent with differences in estimated food assimilation along the salinity gradient. Our findings support the idea that food source-specific TDFs should be used in trophic studies of omnivores, since the method improved our ability to estimate trophic position and resource assimilation, two important ecological indicators.

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1. Introduction

Analysis of elemental stable isotopes is widely used for estimation of flows of organic material in food webs (DeNiro and Epstein, 1981, 1978), with carbon ($^{13}\text{C}/^{12}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$) particularly useful owing to (1) their abundance in organic material and (2) relatively predictable shifts in isotopic ratios between

tissues of foods and consumers (Fry, 2006). This shift during the process of food digestion, incorporation and assimilation has been termed *trophic fractionation*, and an estimate of its magnitude is called the trophic discrimination factor (TDF) (Post, 2002). TDF is a critical parameter used for estimation of both food assimilation and consumer trophic position. Most researchers now estimate food assimilation using isotopic mixing models that adopt Bayesian statistical approaches that can incorporate uncertainty associated with TDF and other inputs (Bessa et al., 2014; Bond and Diamond, 2011; Parnell et al., 2013). Computation of trophic position based on isotopic data relies on values assigned for TDFs as well as appropriate isotopic references (i.e., trophic position baselines) (Post, 2002; Qu et al., 2016).

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Almost 20 years after a call for more laboratory experiments (Gannes et al., 1997; Wolf et al., 2009), studies have revealed multiple factors affecting TDFs, including variation in food quality (e.g., protein content, amino acid profile), metabolic state (e.g., anabolic vs. catabolic), food intake rate, developmental stage, body mass, sex and even controversial ones, such as isotopic composition (Caut et al., 2009; Florin et al., 2011; Gaye-Siessegger et al., 2007; Kelly and Martínez del Rio, 2010; McMahon et al., 2010; Newsome et al., 2011; Poupin et al., 2011; Robbins et al., 2005; Wessels and Hahn, 2010). The trophic discrimination factor for nitrogen (TDF_N) can vary considerably between trophic levels, with values often greater for primary consumers and smaller for higher trophic levels (Hussey et al., 2014). Several reviews of TDF_N showed inconsistent results when comparing consumers fed plant material with those fed foods derived from animal tissue (Caut et al., 2009; Post, 2002; Vander Zanden and Rasmussen, 2001; Vanderklift and Ponsard, 2003). Studies involving fish have revealed different TDF_N for herbivores and carnivores, with the latter generally having lower values (Madigan et al., 2012; Varela et al., 2011) and herbivores having higher TDF_N compared to values reported in the literature (Lujan et al., 2011; Mill et al., 2007). However, laboratory experiments can test only a few parameters simultaneously, and trophic ecology is influenced by multiple factors. Moreover, captive studies often provide food ad libitum, which increases excretion rate, a factor that strongly influences TDF_N (Mill et al., 2007; Olive et al., 2003; Ponsard and Averbuch, 1999).

To the best of our knowledge, no prior study has addressed the relationship between TDF and food quality (i.e. animal origin vs. plant origin) for animals that change diet along temporal or spatial environmental gradients. To investigate the influence of TDF on estimation of trophic position and food assimilation, we analyzed isotopic and dietary variation of an omnivorous fish in relation a salinity gradient in a coastal ecosystem. The one-sided livebearer, *Jenynsia multidentata* (Jenyns, 1842), a dominant species in fresh and brackish waters along the coast of southern South America (Bastos et al., 2014; Garcia et al., 2004), feeds on both plants (e.g., algae and seagrass) and animals (e.g., microcrustaceans, insects, polychaetes) (Aranha and Caramaschi, 1999; Mai et al., 2006). Plant and animal material differ in nutritional quality, with animal tissue generally containing more protein, and plant tissues containing large fractions of cellulose and other compounds that are difficult or expensive for most animals to digest. Based on our findings, we propose a new approach for estimating trophic position that uses outputs from stable isotope mixing models and takes into account food-specific TDFs.

2. Methods

2.1. Field collections and sample processing

Samples were obtained monthly from April 2008 to May 2009 at Lagoa do Peixe National Park (LPNP) located on the coastal plain of Rio Grande do Sul state, Brazil (Fig. 1). Three sites were surveyed in Lagoa do Peixe: (1) lagoon mouth (LM) – the narrow channel that intermittently connects the main lagoon with the sea, (2) estuarine zone (EZ) – a mixohaline area located between the mouth and upper freshwater reaches, and (3) freshwater wetland (FW) fringing the upstream boundary of the lagoon (Fig. 1).

Jenynsia multidentata specimens were captured using a beach seine (9-m long, 2.4-m high, mesh size = 13 mm in wings and 5 mm in center) and beam trawl (0.9 × 0.9 m opening, with size mesh = 5 mm). Captured specimens were immediately euthanized in an ice bath, transported to the lab on ice, and then stored in a freezer. After thawing, each specimen was measured (total length, TL, mm), weighed (g) and dissected to remove the digestive tract

for stomach contents analysis. Approximately 5 g of muscle tissue was extracted from the dorso-lateral region of each specimen for isotopic analysis. For specimens <30 mm TL, a composite sample of muscle tissue from 2 to 5 individuals was obtained in order to have sufficient material for analysis of isotopic composition.

In order to estimate trophic positions based on stable isotope ratios of nitrogen ($\delta^{15}\text{N}$), tissue samples were obtained for basal production sources at each survey location (leaves from floating, emergent, and submerged macrophytes; filamentous algae; periphyton; suspended particulate organic matter (POM)). Additionally, major dietary items of *J. multidentata*, such as polychaete worms, amphipods and insects, were collected manually from sediments and macrophytes, and tissue was obtained for isotopic analysis.

2.2. Estimates of ingested resources

A total of 121 stomachs were analyzed to quantify the relative importance of food items ingested by *J. multidentata*. A stereoscopic binocular microscope was used to identify food items to the lowest feasible taxonomic level. Inorganic material and partially digested, unidentifiable organic matter were recorded as present or absent and excluded from subsequent analyses. The relative importance of each food category was calculated by the Index of Relative Importance (IRI) (Pinkas et al., 1970). We recorded the frequency of occurrence (F) of food categories in stomachs as percentages of total stomachs examined (Hyslop, 1980). For each stomach sample, we recorded the number of items or major fragments of each food category (N) and the area (mm²) (A) of each item or category when material was spread evenly over a Petri dish at a depth of approximately 1 mm. If an item was thicker than 1 mm, the item was broken into smaller pieces to achieve a thickness of 1 mm (Hellawell and Abel, 1971). When thickness was <1 mm, thickness was estimated visually (e.g., 0.25, 0.5, or 0.75 mm). The volume (V) of each food category then was calculated as thickness × A. IRI was calculated using the formula: $\text{IRI} = \%F \cdot (\%N + \%V)$, where %N was the ratio between the total number of a given food category and the total number among all categories items from all stomachs in the sample, and %A was the ratio between the total area occupied by a given food item or category and the total area occupied by all food items from all stomachs in the sample. Finally, IRI was expressed as a percentage (%IRI) obtained from the ratio between the calculated IRI for a given food category and the total sum of the IRI calculated for all food categories.

2.3. Effects of TDFS on estimates of food assimilation and trophic position

Muscle tissue samples were obtained from the flanks of *J. multidentata* specimens captured from the three survey sites (Table S1, supplementary material). Muscle samples and whole bodies of invertebrates (n = 48), samples of filamentous algae (n = 7), periphyton (n = 32) and macrophytes (n = 59) were rinsed with distilled water to remove foreign material. POM samples (n = 42) were obtained by filtering water through a pre-combusted (450 °C, 4 h) Whatman glass fiber filter (GF/F) with the aid of a manual vacuum pump (Table S1, supplementary material). Samples were placed in sterile Petri dishes, and dried in an oven at 60 °C for a minimum of 48 h. Dried samples were ground to a fine powder with a mortar and pestle and stored in clean Eppendorf tubes. Sub-samples were pressed into Ultra-Pure tin capsules (Costech, Valencia, CA) and sent to the Analytical Chemistry Laboratory, Institute of Ecology, University of Georgia, for measurement of stable isotope ratios (¹³C/¹²C and ¹⁵N/¹⁴N). Stable isotope ratios (R = ¹⁵N/¹⁴N or ¹³C/¹²C) were compared to internal laboratory standards and then reported as parts per thousand (‰) relative to the correspond-

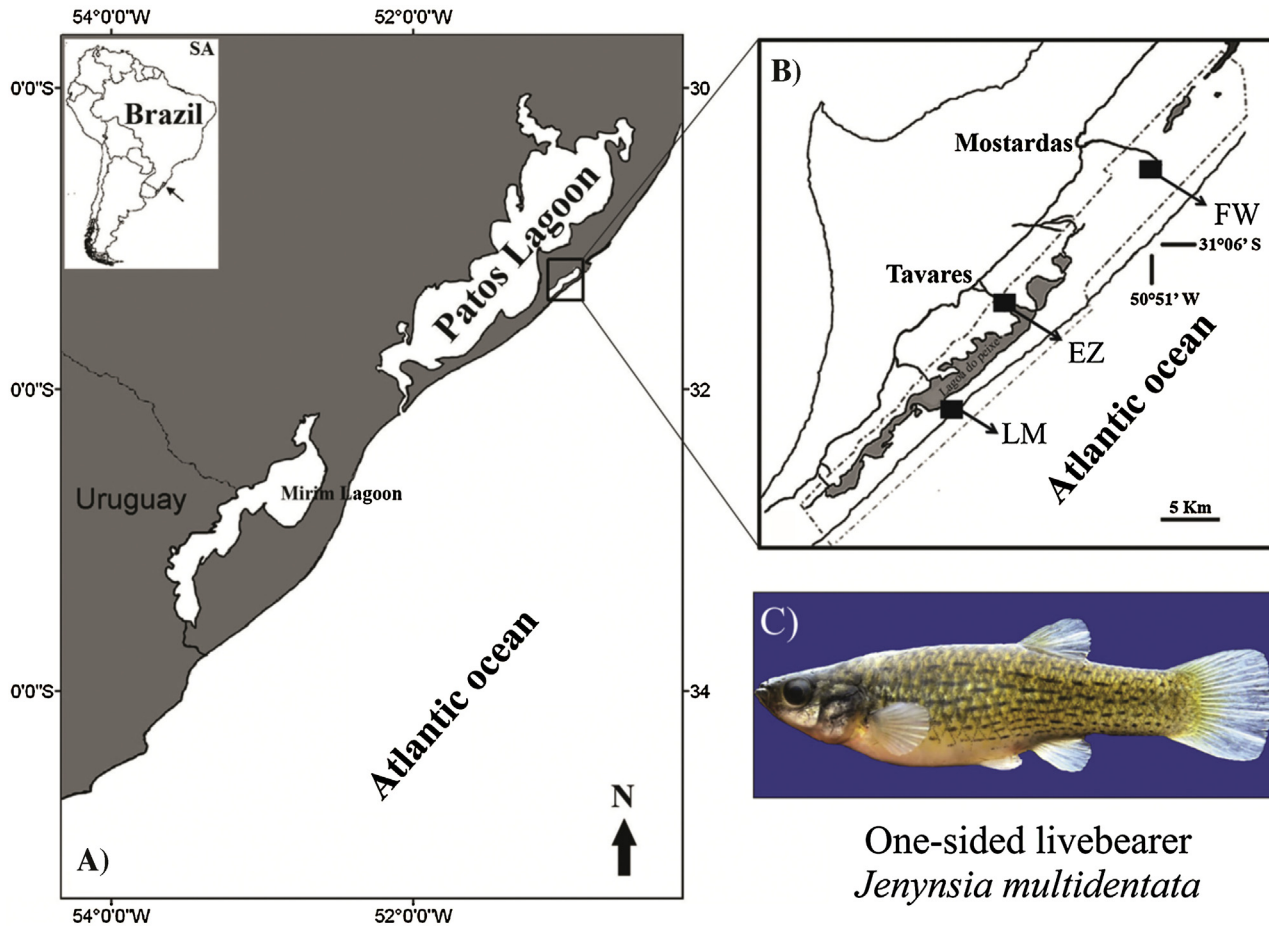


Fig. 1. Map showing the Patos-Mirim lagoon complex in Brazil, South America (SA) (A) and the coastal lagoon studied inside Lagoa do Peixe National Park limits (dashed line), located between Mostardas and Tavares cities (B). Species studied, the one-sided livebearer, *Jenynsia multidentata* (C).

ing international standards for $\delta^{13}\text{C}$ (PeeDee Belemnite) and $\delta^{15}\text{N}$ (atmospheric Nitrogen) = $[(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$. Standard deviations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ based on analysis of internal standard replicates were 0.14‰ and 0.13‰, respectively. Organisms were grouped by studied location (LM, EZ, FW), and bi-plots of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of sampled tissues and organisms were compared across sites.

To estimate relative contributions of food resources to consumers, we used the Bayesian stable isotope mixing model in R (SIAR, version 3.2.1) (Parnell et al., 2008). To achieve higher resolution and ecological meaning in mixing models, Phillips et al. (2005) recommended *a posteriori* pooling of production sources with similar isotopic composition and similar ecological roles. Based on results from stomach contents analysis, and considering the nutritional quality differences between primary producers and animals, we considered two main food resource categories for the mixing model: (1) algae (filamentous algae and periphyton) and (2) animals (amphipods, polychaete worms and insects).

Trophic position (TP) of *J. multidentata* was estimated using the following equation (Post, 2002):

$$\text{TP} = \lambda + ((\delta^{15}\text{N}_{\text{Jenynsia}} - \delta^{15}\text{N}_{\text{Baseline}})/\text{TDF}_\text{N})$$

where λ is the baseline trophic level which is set at 1 for primary producers; $\delta^{15}\text{N}_{\text{Jenynsia}}$ is the $\delta^{15}\text{N}$ value of a fish specimen; $\delta^{15}\text{N}_{\text{Baseline}}$ is the average $\delta^{15}\text{N}$ value from primary producers at the corresponding site; and TDF_N is the trophic discrimination factor for $\delta^{15}\text{N}$.

TDFs is a strongly influential variable in multiple source mixing models and trophic position estimates (Caut et al., 2009; Hussey et al., 2014; Parnell et al., 2013). To account for potential bias from error associated with choice of TDF values, four combinations of TDFs for both carbon and nitrogen isotopic ratios were used to estimate source assimilation and trophic positions. Considering that TDF could be associated with differences in food digestibility, food quality, trophic level, assimilation and excretion (Caswell et al., 1973; Fantle et al., 1999; Hussey et al., 2014; Olive et al., 2003), omnivores could span a range of TDF values depending on relative amounts of plant and animal material in their diets. Therefore, an appropriate model to estimate source assimilation for omnivores would be one that assumes a different TDF for each plant and animal sources. We computed mixing models and TP estimates using four different combinations of TDF values. We used the average of TDF values reported in literature reviews to produce three combinations of TDF for Nitrogen (TDF_N) and for Carbon (TDF_C), respectively (combination 1 = 2.75 and 0.40, combination 2 = 2.9 and 0.47, and combination 3 = 3.4 and 0.75) (Table S2, supplementary material). The SIAR mixing model allows potential food sources to have unique values for average and standard deviation of TDF_C and TDF_N . Therefore, we used the mean of published TDFs for carnivorous fishes (1.77 for N; 0.82 for C) for animal food categories, and the mean of published TDFs for herbivorous fishes (4.78 for N) for algae food categories (Table S2, supplementary material) on the combination number 4 (Jennings et al., 1997; Keegan and DeNiro, 1988; Pinnegar and Polunin, 2000). We were unable to find a literature report for TDF_C of strictly herbivorous fish; therefore, we

used the mean of values reported in the literature fish irrespective of trophic niche (0.54). Calculation of TP requires a single TDF_N value, and thereby ignores differences in trophic discrimination associated with different sources. In order to address such issues, a novel method was created based on the assumption that foods with different qualities implies different TDF_N values. Proportional contributions (95% credibility) to *J. multidentata* tissue from alternative sources (animal preys vs. algae) estimated from SIAR were used to produce specific TDF_N ($TDF_{N\text{carnivorous}}$, $TDF_{N\text{herbivorous}}$) as follow:

$$TDF_N = ((TDF_{N\text{carnivorous}} \times C_{\text{animal}}) + (TDF_{N\text{herbivorous}} \times C_{\text{algae}})) / (C_{\text{animal}} + C_{\text{algae}})$$

where TDF_N is the effective TDF; $TDF_{N\text{carnivorous}}$ is the specific TDF_N for the animal source; C_{animal} is the proportional contribution of animal material to fish tissue; $TDF_{N\text{plant}}$ is the specific TDF_N for the algae source; C_{algae} is the proportional contribution of algae to fish tissue. In this way, the TP estimate method proposed here (TP_{WAR}) is weighted by proportional assimilation of resources. Because the SIAR model output (95% credibility) is an interval of feasible contributions, we generated three TP_{PA} estimates ($TP_{\text{WAR-low}}$, $TP_{\text{WAR-med}}$ and $TP_{\text{WAR-high}}$) using separately the lowest, median, and highest values of C_{animal} combined with the highest, median and lowest values of C_{algae} , respectively.

Non-parametric analysis of variance (Kruskal-Wallis ANOVA) was used to compare $\delta^{15}\text{N}$ of *J. multidentata* and primary producers (isotopic baseline) among sites as well as TP estimates based on the four different combinations. When a significant main effect was observed, multiple comparisons were performed (Zar, 2010).

3. Results

3.1. Ingested resources

Examination of 121 *J. multidentata* specimens yielded 22 food categories within 116 stomachs that contained food. The diet of *J. multidentata* was comprised of both plant (e.g., algae) and animal matter (Table 1, Fig. 2A), with composition differing among locations along the longitudinal fluvial gradient (Fig. 2A). Filamentous algae and other plant material dominated the diet of fish from the freshwater wetland (FW, 70.65%), but invertebrates such as amphipods (15.7%) also were ingested. In the estuarine zone (EZ) fish ingested mostly invertebrates (86.3%), including polychaetes (29.4%), amphipods (23.2%) and cladocerans (7.5%). Fish from the lagoon mouth (LM) had fed exclusively on animal prey, especially polychaete worms (96.4%). Amorphous particulate organic matter was more frequently ingested in the FW (80.0%) compared to EZ (47.1%) and LM (57.1%) sites. Sand grains were more frequent in stomachs of individuals from the LM (78.6%) than other sites (17.65% at EZ, absent at FW).

3.2. Spatial variation of $\delta^{15}\text{N}$

Tissue samples from 89 *J. multidentata* specimens produced 49 samples for stable isotope analysis (27 individual samples and 22 composite samples) from the three survey sites (Table 2). Nitrogen isotopic ratios increased significantly from freshwater to lagoon mouth (Kruskal-Wallis, $H = 22.10$, $p < 0.05$) (Fig. 2B). Post-hoc tests showed statistically significant differences ($p < 0.05$) between $\delta^{15}\text{N}$ mean values for fish from FW (6.8‰) and EZ (10.07‰), and FW vs. LM (11.78‰), but not EZ vs. LM (Table S2, Fig. 2B). Mean $\delta^{15}\text{N}$ of the primary producers isotopic baseline also varied spatially, with a trend of ^{15}N enrichment observed from upstream to downstream locations (FW = 1.66‰, EZ = 4.76‰ and LM = 6.21‰) (Table S2, Fig. 2B). Post-hoc tests revealed statistically significant differences among sites ($p < 0.05$) (Table S2, Fig. 2B).

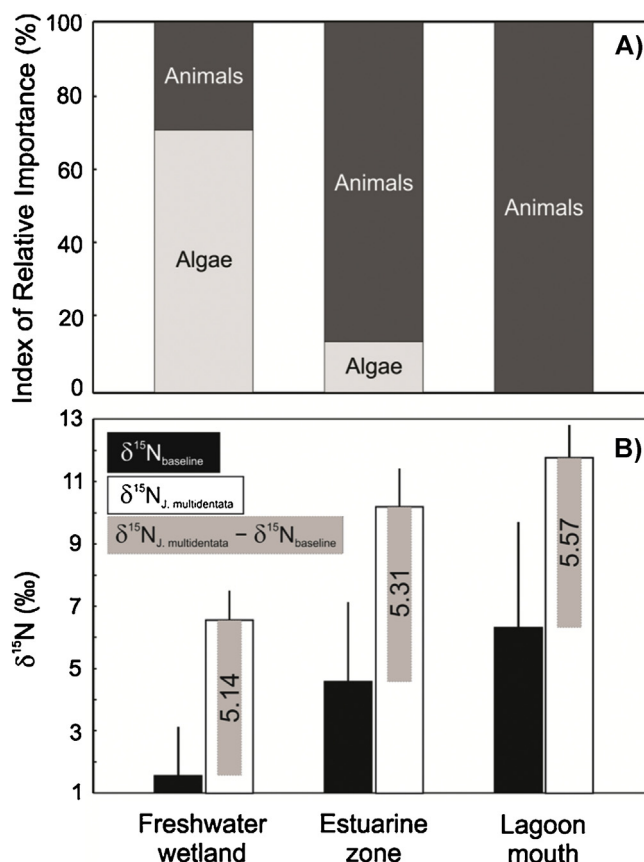


Fig. 2. (A) Index of relative importance (IRI%) of major food categories (pooled as filamentous algae (light gray bars) vs. animal prey (dark gray bars)) from stomach contents of *Jenynsia multidentata* from three sites along a freshwater-estuarine gradient. (B) Average $\delta^{15}\text{N}$ of tissues from the primary producer isotopic baseline (dark bars), *Jenynsia multidentata* (white bars), and the difference between them (grey bars) at the three sites along the freshwater-estuarine gradient. Vertical lines denotes 1 standard deviation.

3.3. Effects of TDFS on mixing model estimates

Mixing models outputs, regardless of the TDF values employed, indicated that *J. multidentata* at all three locations had assimilated material derived mostly from animal food resources (Table 1). Considering 95% credibility intervals, the contribution of filamentous algae to *J. multidentata* tissue ranged from 0% to 38%, whereas assimilation of animal food categories ranged from 68% to 100% (Fig. 3). Compared to the combinations that used a single (unweighted) TDF value for each element, the new mixing model method that considered food-specific TDFs for each source yielded greater contributions of algae to fishes from FW site (0–38%, Fig. 3), lowest contributions of algae to EZ (0–14%) and LM fish (0–21%), and greatest contribution of animal food categories to EZ (86–100%) and FW fish (79–100%). The proposed method tended to produce narrower 95% credibility intervals, which indicates higher resolution for estimates compared to those obtained from the other three combinations of C and N TDFs (Fig. 3).

3.4. Effects of TDFS on trophic position estimates

The effective TDF_N estimated based on proportional assimilation of resources were 2.40, 2.16 and 2.43 for the $TP_{\text{WAR-low}}$, and 2.30, 1.98 and 2.09 for $TP_{\text{WAR-med}}$ estimates in FW, EZ and LM sites, respectively. For $TP_{\text{WAR-high}}$ estimate, effective TDF_N were the same (1.77) as for strictly carnivorous fishes, since mixing model estimate number 4, which considered carnivorous vs. herbivorous

Table 1

Index of relative importance (IRI) of food items found in the stomach contents of *Jenynsia multidentata* caught at the freshwater wetland (FW), estuarine zone (EZ) and lagoon mouth (LM). The index was calculated for three grouping levels (1) bold font indicates food items grouped in a first level and first analyzed just as plants or animals; (2) Italic fonts indicates food items grouped in a second level and analyzed as plants and other lower animal level of classifications (*Crustacea*, *Insecta*, *Other Arthropods*, *Polychaeta* and *Fish*); (3) Normal fonts indicates food items grouped and analyzed at the higher level of classification possible.

Food items	Index of Relative Importance IRI%		
	Freshwater Wetland FW	Estuarine Zone EZ	Lagoon Mouth LM
Plants	70.65	13.68	–
<i>Plants</i>	<i>81.03</i>	<i>21.71</i>	–
Filamentous algae	80.95	32.09	–
Macrophyte fragments	–	0.76	–
Animals	29.35	86.32	100.00
<i>Crustacea</i>	<i>15.65</i>	<i>61.12</i>	–
Amphipoda	15.69	23.17	–
Ostracoda	–	0.45	–
Cladocera	–	7.49	–
Copepoda	–	3.32	–
<i>Insecta</i>	<i>1.98</i>	<i>1.09</i>	–
Aquatic Insects	–	–	–
Diptera larvae	1.99	–	–
Diptera pupae	–	0.02	–
Ephemeroptera larvae	–	1.61	–
Trichoptera larvae	–	0.08	–
Insect larvae (unidentified)	–	0.39	–
Insect larvae remains	–	0.05	–
Terrestrial Insects	–	–	–
Coleoptera	–	0.10	–
Hymenoptera	–	0.02	–
Insect (unidentified)	–	0.05	–
<i>Other Arthropods</i>	<i>–</i>	<i>–</i>	<i>3.61</i>
Aranae	–	0.04	–
Arthropoda (unidentified)	–	–	1.66
<i>Polychaeta</i>	<i>1.34</i>	<i>15.93</i>	<i>96.39</i>
<i>Heteromastus similis</i>	<i>1.37</i>	<i>0.04</i>	<i>–</i>
<i>Laeonereis acuta</i>	–	28.86	74.61
Polychaeta (unidentified)	–	0.50	–
Polychaeta remains	–	0.02	23.73
<i>Fish</i>	<i>–</i>	<i>0.45</i>	<i>–</i>
Eggs	–	0.41	–
Fish remains	–	0.04	–

Table 2

Trophic position (TP) (mean – μ , and standard deviation – s.d.) for *Jenynsia multidentata* from three locations calculated according to four combinations of TDFs.

Sampling site	TP combination 1		TP combination 2		TP combination 3		TP _{WAR} method					
							Low		Median		High	
	μ	s.d.	μ	s.d.	μ	s.d.	μ	s.d.	μ	s.d.	μ	s.d.
Freshwater Wetland	2.87	0.35	2.77	0.33	2.51	0.28	3.14	0.40	3.24	0.41	3.91	0.54
Estuarine Zone	2.93	0.42	2.83	0.40	2.56	0.34	3.46	0.53	3.68	0.58	4.00	0.65
Lagoon Mouth	2.76	0.78	2.67	0.74	2.42	0.63	2.99	0.88	3.32	1.03	3.73	1.21

TDFs, revealed 100% of animal contribution at the upper limit of estimate for all sites.

Fish TP varied significantly between locations (Kruskal-Wallis, $p < 0.05$) only when the TP_{PA} method was used, with post-hoc tests revealing a significant difference between FW and EZ sites ($p < 0.05$) and a marginally significant difference between FW and LM sites ($p = 0.06$) (Table 2). The four different combinations resulted in significantly different TP estimates ($p < 0.05$) (Fig. 4), with TP_{WAR} method producing values approximately one trophic level higher than other combinations (Fig. 4).

4. Discussion

Ingested resources analysis of *J. multidentata* in Lagoa do Peixe revealed a major shift from a diet dominated by plants to one dominated by animal prey along a longitudinal gradient from freshwater

marsh to the estuary and its connection to the sea. Shifts in trophic position across habitats with different primary producers at the baseline is frequently observed for omnivorous species (Agrawal and Klein, 2000; Gibb et al., 2011). The four combinations used for isotopic analysis of food assimilation produced estimates that were discordant with estimates based on dietary analysis. Choice of trophic discrimination factors (TDFs) affected estimates on trophic position estimates more than it affected estimates of food assimilation from isotopic mixing models. Despite the herbivorous to carnivorous diet shift along the environmental gradient, $\delta^{15}\text{N}$ differences between the consumer and primary producers isotopic baseline were similar across locations.

Even though algae were the dominant item ingested by fish at the freshwater site, all of our isotopic mixing models estimated that animal prey were the most important resource assimilated. At least four factors could explain this apparent discrepancy between

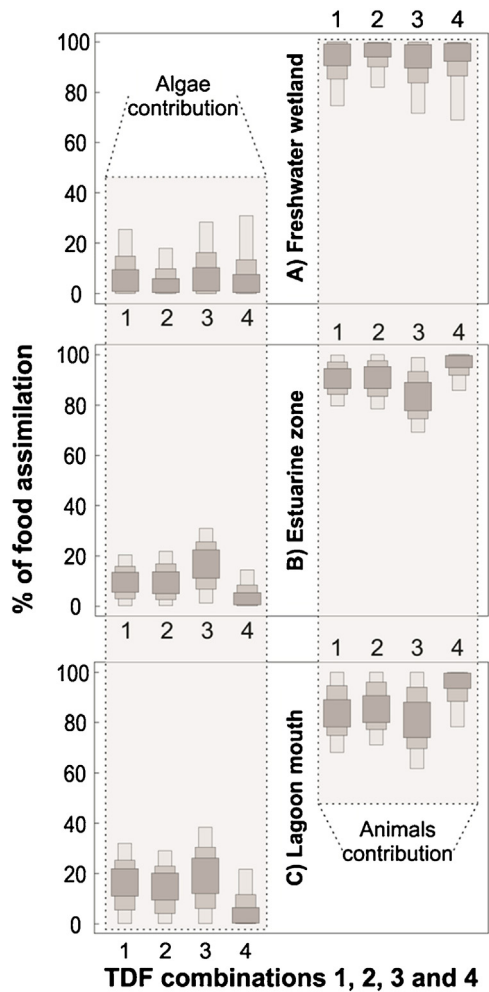


Fig. 3. Estimated relative contributions of algae (left column) and animal prey (polychaets, amphipods and insects combined; right column) assimilated by *Jenynsia multidentata* at three locations along the estuarine freshwater gradient – A) Freshwater wetland, B) Estuarine zone, C) Lagoon mouth. Numbers 1, 2, 3 and 4 refer to mixing model estimates generated using different trophic discrimination factors (see Table S2). Each plot shows 50% (dark grey), 75% (medium grey) and 95% (light grey) Bayesian credibility intervals of feasible contributions of each source. See Materials and Methods section and Table S2 for details about the 4 combinations.

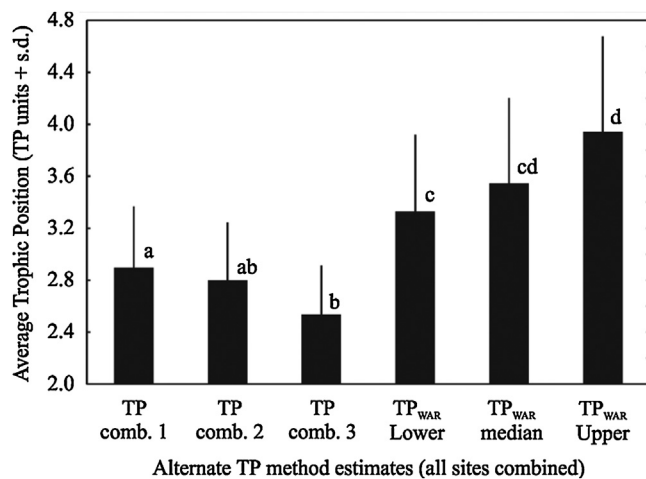


Fig. 4. Average trophic position (TP) estimated obtained by each of 4 combinations with data combined for all survey sites. Bars denoted by the same letter are not significantly different according to multiple comparisons of p values. Vertical lines denotes 1 standard deviation.

diet composition from SCA and food assimilation inferred from SIA. First, differential digestibility and nutritional quality (protein content) of food items affect their assimilation (Bowen et al., 1995). SCA and SIA data reveal trophic processes that operate on different time scales. SCA generally provides a snapshot of food ingested over preceding minutes or hours (Hellawell and Abel, 1971; Hyslop, 1980), whereas SIA integrates assimilation of food resources over weeks to months (Heady and Moore, 2013; Hesslein et al., 1991; Madigan et al., 2012; Weidel et al., 2011). Second, foods such as algae and macrophytes digest more slowly and, therefore, remain within stomachs longer than animal prey, which would result in their overestimation by SCA (Hellawell and Abel, 1971; Hyslop, 1980). Third, some of the amorphous particulate organic matter in stomachs of fish from the freshwater habitat could have derived from easily digested animal material rather than plant material as generally assumed. Fourth, some food items in fish stomachs could have been ingested incidentally during foraging bouts that targeted invertebrate prey (Baeta et al., 2006; Colombini and Chelazzi, 2003; Conдини et al., 2014, 2011). Polychaete worms were the most important item within stomachs of fish from the lagoon mouth, and sand also was frequently ingested at this location. Given that sand has no direct nutritional value, this material had been ingested incidentally, and we speculate that algae, at least part of, within stomachs of fish from the freshwater site had been ingested incidentally during capture of invertebrate prey. Finally, bias in TDF values employed in isotopic mixing models could influence accuracy assimilation estimates (Layman et al., 2012; Martínez del Rio et al., 2009).

Additional evidence supporting the hypothesis that fish ingest but do not assimilate significant amount of plant material is the lack of spatial variation in fish trophic position. Average trophic position was not lower at the freshwater wetland where greater fractions of plant material had been ingested (IRI ca. 70%). Because the $\delta^{15}\text{N}$ difference between fish and local basal production sources was nearly the same at all sites, it seems unlikely that isotopic variation in basal sources or TDFs influenced this finding.

Among the four combinations used to estimate trophic position, only the TP_{PA} method revealed differences among sites. This method considers food-specific TDFs rather than a single TDF based on a reported average. In some cases, use of reported TDFs resulted in TP estimates for *J. multidentata* around 2.0, which would indicate an almost entirely herbivorous diet. A recent study of a marine food web found that use of a single TDF value to estimate consumer TP can bias results (Hussey et al., 2014). Use of a single TDF value regardless of the quality of ingested food types may be responsible for discrepancies between TP estimates from SCA and SIA (Rybczynski et al., 2008; Winemiller et al., 2011). The use of TDFs based on assimilation of resources estimates for plant vs. animal foods (TP_{PA} method) yielded TP estimates for *J. multidentata* indicative of a carnivorous rather than omnivorous diet. SCA indicated that algae, crustaceans and polychaete worms were the major dietary components in FW, EZ and LM respectively. A strictly herbivorous diet would yield $\text{TP}=2$, and a strictly carnivorous diet would yield a TP above 3, because most of invertebrates preys are also omnivorous or even carnivorous, which will lead fish to be considered at some level between secondary ($\text{TP}=3$) and tertiary ($\text{TP}=4$) consumer. $\text{TP}_{\text{WAR-med}}$ produced an average TP estimate of 3.54, which is consistent with assimilation of polychaetes and other omnivorous invertebrate prey. The other three combinations produced TP values of 2.89, 2.79 and 2.53, which are inconsistent with our findings for assimilation of food sources.

Trophic position should vary according to dietary composition, food quality and assimilation dynamics, and the TP_{WAR} method should improve TP estimates, especially for omnivorous species. TP estimates are known to be highly sensitive to choice of TDF as well as variation in estimates of proportional assimilation of food types

(Caut et al., 2009; Hussey et al., 2014; Parnell et al., 2013). The TP_{WAR} method not only employs food-specific TDFs, but also produces a range of feasible TPs (95% credibility intervals). The method should improve estimates not only for omnivores, but any consumer that has dietary shifts involving food types having different assimilation dynamics. Research on trophic ecology that combines SCA and SIA greatly increases precision and accuracy of biomass assimilation and TP estimates. More experimental studies that manipulate diets to track assimilation dynamics are needed to improve and refine the TP_{WAR} method proposed here.

Acknowledgements

We thank João Paes Vieira, Daniel Loebmann, Sônia Huckembeck, Marlucy Coelho Claudino and Mario Vinicius Lopes Condini for field and laboratory work assistance and also to Lagoa do Peixe National Park staff for support during fieldwork. This research was financial supported by CNPq Universal 15/2007 and International Foundation for Science – IFS (grant no. A/4419-1). Fabiano Correa thanks CAPES, Rodrigo Ferreira Bastos thanks IFS (grant no. A/4419-1) and Alexandre Miranda Garcia thanks CNPq (grant no. 305888/2012-9) for scholarships. This research was permitted by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA under authorization number 14443-1.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecolind.2016.12.007>.

References

- Agrawal, A.A., Klein, C.N., 2000. What omnivores eat: direct effects of induced plant resistance on herbivores and indirect consequences for diet selection by omnivores. *J. Anim. Ecol.* 69, 525–535, <http://dx.doi.org/10.1046/j.1365-2656.2000.00416.x>.
- Aranha, J.M.R., Caramaschi, E.P., 1999. Estrutura populacional, aspectos da reprodução e alimentação dos Cyprinodontiformes (Osteichthyes) de um riacho do sudeste do Brasil. *Rev. Bras. Zool.* 16, 637–651, <http://dx.doi.org/10.1590/S0101-81751999000300005>.
- Baeta, A., Cabral, H.N., Marques, J.C., Pardal, M.A., 2006. Feeding ecology of the green crab, *Carcinus maenas* (L., 1758) in a temperate estuary, Portugal. *Crustaceana* 79, 1181–1193, <http://dx.doi.org/10.1163/156854006778859506>.
- Bastos, R.F., Calliari, L.J., Garcia, A.M., 2014. Marine intrusion and freshwater discharge as opposite forces driving fish guilds distribution along coastal plain streams. *Hydrobiologia* 726, 245–258, <http://dx.doi.org/10.1007/s10750-013-1771-7>.
- Bessa, F., Baeta, A., Marques, J.C., 2014. Niche segregation amongst sympatric species at exposed sandy shores with contrasting wrack availabilities illustrated by stable isotopic analysis. *Ecol. Indic.* 36, 694–702, <http://dx.doi.org/10.1016/j.ecolind.2013.09.026>.
- Bond, A.L., Diamond, A.W., 2011. Recent Bayesian stable-isotope mixing models are highly sensitive to variation in discrimination factors. *Ecol. Appl.* 21, 1017–1023, <http://dx.doi.org/10.1890/09-2409.1>.
- Bowen, S.H., Lutz, E.V., Ahlgren, M.O., 1995. Dietary protein and energy as determinants of food quality: trophic strategies compared. *Ecology* 76, 899–907, <http://dx.doi.org/10.2307/1939355>.
- Caswell, H., Reed, F., Stephenson, S.N., Werner, P.A., 1973. Photosynthetic pathways and selective herbivory: a hypothesis. *Am. Nat.* 107, 465–480, <http://dx.doi.org/10.1086/282851>.
- Caut, S., Angulo, E., Courchamp, F., 2009. Variation in discrimination factors $\Delta^{15}\text{N}$ and $\Delta^{13}\text{C}$: the effect of diet isotopic values and applications for diet reconstruction. *J. Appl. Ecol.* 46, 443–453, <http://dx.doi.org/10.1111/j.1365-2664.2009.01620.x>.
- Colombini, I., Chelazzi, L., 2003. Influence of marine allochthonous input on sandy beach communities. *Oceanogr. Mar. Biol. Annu. Rev.* 41, 115–159.
- Condini, M.V., Seyboth, E., Vieira, J.P., Garcia, A.M., 2011. Diet and feeding strategy of the dusky grouper *Mycteroperca marginata* (Actinopterygii: epinephelidae) in a man-made rocky habitat in southern Brazil. *Neotrop. Ichthyol.* 9, 161–168, <http://dx.doi.org/10.1590/S1679-62252011005000006>.
- Condini, M.V., Hoehinghaus, D.J., Garcia, A.M., 2014. Trophic ecology of dusky grouper *Epinephelus marginatus* (Actinopterygii: epinephelidae) in littoral and neritic habitats of southern Brazil as elucidated by stomach contents and stable isotope analyses. *Hydrobiologia* 743, 109–125, <http://dx.doi.org/10.1007/s10750-014-2016-0>.
- DeNiro, M.J., Epstein, S., 1978. Influence of diet on the distribution of carbon isotopes in animals*. *Geochim. Cosmochim. Acta* 42, 495–506, [http://dx.doi.org/10.1016/0016-7037\(78\)90199-0](http://dx.doi.org/10.1016/0016-7037(78)90199-0).
- DeNiro, M.J., Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* 45, 341–351, [http://dx.doi.org/10.1016/0016-7037\(81\)90244-1](http://dx.doi.org/10.1016/0016-7037(81)90244-1).
- Fantle, M.S., Dittel, A.I., Schwalm, S.M., Epifanio, C.E., Fogel, L., 1999. A food web analysis of the juvenile blue crab, *Callinectes sapidus*, using stable isotopes in whole animals and individual amino acids. *Oecologia* 120, 416–426, <http://dx.doi.org/10.1007/s004420050874>.
- Florin, S.T., Felicetti, L.A., Robbins, C.T., 2011. The biological basis for understanding and predicting dietary-induced variation in nitrogen and sulphur isotope ratio discrimination. *Funct. Ecol.* 25, 519–526, <http://dx.doi.org/10.1111/j.1365-2435.2010.01799.x>.
- Fry, B., 2006. *Stable Isotope Ecology*. Springer, New York, <http://dx.doi.org/10.1007/0-387-33745-8>.
- Gannes, L.Z., O'Brien, D.M., Martínez Del Rio, C., 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology* 78, 1271–1276, [http://dx.doi.org/10.1890/0012-9658\(1997\)078\[1271:SIIAEA\]2.0.CO;2](http://dx.doi.org/10.1890/0012-9658(1997)078[1271:SIIAEA]2.0.CO;2).
- Garcia, A.M., Vieira, J.P., Winemiller, K.O., Raseira, M.B., 2004. Reproductive cycle and spatiotemporal variation in abundance of the one-sided livebearer *Jenynsia multidentata*, in Patos Lagoon, Brazil. *Hydrobiologia* 515, 39–48, <http://dx.doi.org/10.1023/B:HYDR.0000027316.59258.a0>.
- Gaye-Siessegger, J., Focken, U., Abel, H., Becker, K., 2007. Starvation and low feeding levels result in an enrichment of ^{13}C in lipids and ^{15}N in protein of Nile tilapia *Oreochromis niloticus* L. *J. Fish Biol.* 71, 90–100, <http://dx.doi.org/10.1111/j.1095-8649.2007.01469.x>.
- Gibb, H., Cunningham, S.A., 2011. Habitat contrasts reveal a shift in the trophic position of ant assemblages. *J. Anim. Ecol.* 80, 119–127, <http://dx.doi.org/10.1111/j.1365-2656.2010.01747.x>.
- Heady, W.N., Moore, J.W., 2013. Tissue turnover and stable isotope clocks to quantify resource shifts in anadromous rainbow trout. *Oecologia* 172, 21–34, <http://dx.doi.org/10.1007/s00442-012-2483-9>.
- Hellawell, J.M., Abel, R., 1971. A rapid volumetric method for the analysis of the food of fishes. *J. Fish Biol.* 3, 29–37, <http://dx.doi.org/10.1111/j.1095-8649.1971.tb05903.x>.
- Hesslein, R.H., Capel, M.J., Fox, D.E., Hallard, K.A., 1991. Stable isotopes of Sulfur, Carbon, and Nitrogen as indicators of trophic level and fish migration in the Lower Mackenzie river basin, Canada. *Can. J. Fish. Aquat. Sci.* 48, 2258–2265, <http://dx.doi.org/10.1139/f91-265>.
- Hussey, N.E., Macneil, M.A., Mcmeans, B.C., Olin, J.A., Dudley, S.F.J., Cliff, G., Wintner, S.P., Fennessy, S.T., Fisk, A.T., 2014. Rescaling the trophic structure of marine food webs. *Ecol. Lett.* 17, 239–250, <http://dx.doi.org/10.1111/ele.12226>.
- Hyslop, E.J., 1980. Stomach contents analysis—a review of methods and their application. *J. Fish Biol.* 17, 411–429, <http://dx.doi.org/10.1111/j.1095-8649.1980.tb02775.x>.
- Jennings, S., Reñones, O., Morales-Nin, B., Polunin, N.V.C., Moranta, J., Coll, J., 1997. Spatial variation in the ^{15}N and ^{13}C stable isotope composition of plants, invertebrates and fishes on Mediterranean reefs: implications for the study of trophic pathways. *Mar. Ecol. Prog. Ser.* 146, 109–116, <http://dx.doi.org/10.3354/meps146109>.
- Keegan, W.F., DeNiro, M.J., 1988. Stable carbon- and nitrogen-isotope ratios used to study coral-reef and terrestrial components of prehistoric bahamian diet. *Am. Antiq.* 53, 320–336, <http://dx.doi.org/10.2307/281022>.
- Kelly, L.J., Martínez del Rio, C., 2010. The fate of carbon in growing fish: an experimental study of isotopic routing. *Physiol. Biochem. Zool.* 83, 473–480, <http://dx.doi.org/10.1086/649628>.
- Layman, C.A., Araujo, M.S., Boucek, R., Hammerschlag-Peyer, C.M., Harrison, E., Jud, Z.R., Matic, P., Rosenblatt, A.E., Vaudo, 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, 545–562, <http://dx.doi.org/10.1111/j.1469-185X.2011.00208.x>.
- Lujan, 1883 Lujan, N.K., German, D.P., Winemiller, K.O., et al., 2011. Do wood-grazing fishes partition their niche?: Morphological and isotopic evidence for trophic segregation in Neotropical Loricariidae. *Funct. Ecol.* 25, 1327–1338, <http://dx.doi.org/10.1111/j.1365-2435.2011.01883.x>.
- Madigan, D.J., Litvin, S.Y., Popp, B.N., Carlisle, A.B., Farwell, C.J., Block, B.A., 2012. Tissue turnover rates and isotopic trophic discrimination factors in the endothermic teleost, pacific bluefin tuna (*Thunnus orientalis*). *PLoS One* 7, e49220, <http://dx.doi.org/10.1371/journal.pone.0049220>.
- Mai, A.C.G., Garcia, A.M., Vieira, J.P., 2006. Ecologia alimentar do barrigudinho *Jenynsia multidentata* (Jenyns, 1842) (Pisces: Cyprinodontiformes) no estuário da Laguna dos Patos, Rio Grande do Sul, Brasil. *Comun. do Mus. Ciências e Tecnol. da PUCRS* 19, 3–18.
- Martínez 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.* 84, 91–111, <http://dx.doi.org/10.1111/j.1469-185X.2008.00064.x>.
- McMahon, K.W., Fogel, M.L., Elsdon, T.S., Thorrold, S.R., 2010. Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein. *J. Anim. Ecol.* 79, 1132–1141, <http://dx.doi.org/10.1111/j.1365-2656.2010.01722.x>.
- Mill, A.C., Pinnegar, J.K., Polunin, N.V.C., 2007. Explaining isotope trophic-step fractionation: why herbivorous fish are different. *Funct. Ecol.* 21, 1137–1145, <http://dx.doi.org/10.1111/j.1365-2435.2007.01330.x>.

- Newsome, S.D., Fogel, M.L., Kelly, L., Martínez Del Rio, C., 2011. Contributions of direct incorporation from diet and microbial amino acids to protein synthesis in Nile tilapia. *Funct. Ecol.* 25, 1051–1062, <http://dx.doi.org/10.1111/j.1365-2435.2011.01866.x>.
- Olive, P.J.W., Pinnegar, J.K., Polunin, N.V.C., Richards, G., Welch, R., 2003. Isotope trophic-step fractionation: a dynamic equilibrium model. *J. Anim. Ecol.* 72, 608–617, <http://dx.doi.org/10.1046/j.1365-2656.2003.00730.x>.
- Parnell, A.C., Inger, R., Bearhop, S., Jackson, A., 2008. *SIAR: Stable isotope analysis in R*.
- Parnell, A.C., Phillips, D.L., Bearhop, S., Semmens, B.X., Ward, E.J., Moore, J.W., Jackson, A.L., Grey, J., Kelly, D.J., Inger, R., 2013. Bayesian stable isotope mixing models. *Environmetrics*, 387–399, <http://dx.doi.org/10.1002/env.2221>.
- Phillips, D.L., Newsome, S.D., Gregg, J.W., 2005. Combining sources in stable isotope mixing models: alternative methods. *Oecologia* 144, 520–527, <http://dx.doi.org/10.1007/s00442-004-1816-8>.
- Pinkas, L., Oliphant, M.S., Iverson, I.L.K., 1970. Food habits of albacore, bluefin tuna, and bonito in California waters. *Fish. Bull.* 152, 1–105.
- Pinnegar, J.K., Polunin, N.V.C., 2000. Contributions of stable-isotope data to elucidating food webs of Mediterranean rocky littoral fishes. *Oecologia* 122, 399–409, <http://dx.doi.org/10.1007/s004420050046>.
- Ponsard, S., Averbuch, P., 1999. Should growing and adult animals fed on the same diet show different $\delta^{15}\text{N}$ values? *Rapid Commun. Mass Spectrom.* 13, 1305–1310, [http://dx.doi.org/10.1002/\(SICI\)1097-0231\(19990715\)13:13<1305:AID-RCM654>3.0.CO;2-D](http://dx.doi.org/10.1002/(SICI)1097-0231(19990715)13:13<1305:AID-RCM654>3.0.CO;2-D).
- Post, D.M., 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology* 83, 703–718, [http://dx.doi.org/10.1890/0012-9658\(2002\)083\[0703:USITET\]2.0.CO;2](http://dx.doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2).
- Poupin, N., Bos, C., Mariotti, F., Huneau, J.F., Tomé, D., Fouillet, H., 2011. The nature of the dietary protein impacts the tissue-to-diet ^{15}N discrimination factors in laboratory rats. *PLoS One* 6, e28046, <http://dx.doi.org/10.1371/journal.pone.0028046>.
- Qu, P., Wang, Q., Pang, M., Zhang, Z., Liu, C., Tang, X., 2016. Trophic structure of common marine species in the Bohai Strait, North China Sea, based on carbon and nitrogen stable isotope ratios. *Ecol. Indic.* 66, 405–415, <http://dx.doi.org/10.1016/j.ecolind.2016.01.036>.
- Robbins, C.T., Felicetti, L.A., Sponheimer, M., 2005. The effect of dietary protein quality on nitrogen isotope discrimination in mammals and birds. *Oecologia* 144, 534–540, <http://dx.doi.org/10.1007/s00442-005-0021-8>.
- Rybczynski, S.M., Walters, D.M., Fritz, K.M., Johnson, B.R., 2008. Comparing trophic position of stream fishes using stable isotope and gut contents analyses. *Ecol. Freshw. Fish* 17, 199–206, <http://dx.doi.org/10.1111/j.1600-0633.2007.00289.x>.
- Vander Zanden, M.J., Rasmussen, J.B., 2001. Variation in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ trophic fractionation: implications for aquatic food web studies. *Limnol. Oceanogr.* 46, 2061–2066, <http://dx.doi.org/10.4319/lo.2001.46.8.2061>.
- Vanderklift, M.A., Ponsard, S., 2003. Sources of variation in consumer-diet ^{15}N enrichment: a meta-analysis. *Oecologia* 136, 169–182, <http://dx.doi.org/10.1007/s00442-003-1270-z>.
- Varela, J.L., Larrañaga, A., Medina, A., 2011. Prey-muscle carbon and nitrogen stable-isotope discrimination factors in Atlantic bluefin tuna (*Thunnus thynnus*). *J. Exp. Mar. Bio. Ecol.* 406, 21–28, <http://dx.doi.org/10.1016/j.jembe.2011.06.010>.
- Weidel, B.C., Carpenter, S.R., Kitchell, J.F., Vander Zanden, M.J., 2011. Rates and components of carbon turnover in fish muscle: insights from bioenergetics models and a whole-lake ^{13}C addition. *Can. J. Fish. Aquat. Sci.* 68, 387–399, <http://dx.doi.org/10.1139/F10-158>.
- Wessels, F.J., Hahn, D.A., 2010. Carbon 13 discrimination during lipid biosynthesis varies with dietary concentration of stable isotopes: implications for stable isotope analyses. *Funct. Ecol.* 24, 1017–1022, <http://dx.doi.org/10.1111/j.1111/j.1365-2435.2010.01716.x>.
- Winemiller, K.O., Zeug, S.C., Robertson, C.R., Winemiller, B.K., Honeycutt, R.L., 2011. Food-web structure of coastal streams in Costa Rica revealed by dietary and stable isotope analyses. *J. Trop. Ecol.* 27, 463–476, <http://dx.doi.org/10.1017/S0266467411000277>.
- Wolf, N., Carleton, S.A., Martínez Del Rio, C., 2009. Ten years of experimental animal isotopic ecology. *Funct. Ecol.* 23, 17–26, <http://dx.doi.org/10.1111/j.1365-2435.2008.01529.x>.
- Zar, J.H., 2010. *Bioestatistical Analysis*, Fifth. ed. Prentice Hall Upper Saddle River, New Jersey, USA.