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Diet-tissue discrimination factors and turnover of carbon and nitrogen stable isotopes in tissues of an adult predatory coral reef fish, *Plectropomus leopardus*

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RATIONALE: Stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ values) provide a unique perspective into the ecology of animals because the isotope ratio values of consumers reflect the values in food. Despite the value of stable isotopes in ecological studies, the lack of species-specific experimentally derived diet-tissue discrimination factors (DTDFs) and turnover rates limits their application at a broad scale. Furthermore, most aquatic feeding experiments use temperate, fast-growing fish species and few have considered medium- to large-sized adults with low growth rates from tropical ecosystems.
METHODS: A controlled-diet stable isotope feeding trial was conducted over a 196-day period for the adult predatory reef fish leopard coralgrouper (*Plectropomus leopardus*). This study calculated $\delta^{13}C$ and $\delta^{15}N$ DTDFs and turnover rates in five tissues (liver, plasma, red blood cells (RBC), fin, and muscle) using a continuous flow isotope ratio mass spectrometer equipped with an elemental analyzer. In addition, the effect of chemical lipid extraction (LE) on stable isotope values was examined for each tissue.
RESULTS: Turnover was mainly influenced by metabolism (as opposed to growth) with LE $\delta^{15}N$ half-life values lowest in fin (37 days) and plasma (66 days), and highest in RBC (88 days) and muscle (126 days). The diet-tissue discrimination factors for $\delta^{15}N$ values in all tissues ($\Delta^{15}N$: –0.15 to 1.84‰) were typically lower than commonly reported literature values. Lipid extraction altered both $\delta^{15}N$ and $\delta^{13}C$ values compared with untreated samples; however, for the $\delta^{15}N$ values, the differences were small (mean $\delta^{15}N_{LE\text{-}Bulk}$ <0.46‰ in all tissues).
CONCLUSIONS: This study informs future interpretation of stable isotope data for medium- to large-sized fish and demonstrates that DTDFs developed for temperate fish species, particularly for $\delta^{15}N$ values, may not apply to tropical species. Sampling of muscle and/or RBC is recommended for a relatively long-term representation of feeding habits, while plasma and/or fin should be used for a more recent indication of diet.

The application of stable isotope analysis (SIA) in ecosystem studies is a powerful tool that uses biogeochemical markers to explore the relationship between animals, their diet, and their environment.[1] The use of carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) stable isotope ratios in ecological research has increased significantly over the last 25 years.[2,3]

SIA has been used to track the bioaccumulation of contaminants in Arctic marine megafauna,[4,5] determine residency and movement patterns of tropical fish,[6,7] identify ontogenetic niche shifts of Antarctic organisms,[8,9] and quantify the dietary/energetic pathways in food webs of whole ecosystems.[10,11]

Despite its widespread application, there are a number of caveats that must be considered to properly interpret and apply stable isotopes in ecology (see Gannes *et al*.[12] and Post[13] for reviews). For example, one of the main applications of $\delta^{13}C$ and $\delta^{15}N$ values is to calculate the trophic position of organisms as a quantitative tool to measure the hierarchical role that each organism has in a food web.[13,14] In addition, $\delta^{13}C$ and $\delta^{15}N$ values are often used to infer the proportional contribution of different prey items in the diet, typically via statistical mixing models.[3] However, both applications are heavily biased by a user-defined input parameter, the diet-tissue discrimination factor (DTDF). Diet-tissue discrimination factors represent the difference in $\delta^{13}C$ (or $\delta^{15}N$) values between the consumer and its food ($\Delta^{13}C = \delta^{13}C_{consumer} - \delta^{13}C_{food}$; $\Delta^{15}N = \delta^{15}N_{consumer} - \delta^{15}N_{food}$). This metric is informative because it is a quantitative tool to estimate trophic pathways via mixing models, which can account for variation in parameter estimates. Most studies rely on experimentally derived DTDFs found in the literature, and often use values that have been determined from species with different life history traits or that inhabit dissimilar environments.[15] However, there can be considerable inter- and intra-specific variability in DTDFs caused by a number of factors such as diet quality,[16,17] tissue type,[18,19]

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growth/size,[20,21] and temperature and feeding rates.[22] Furthermore, applying fixed DTDFs based on constant ^{15}N enrichment at each trophic level (e.g., 3.4‰ is commonly used) may bias top predator trophic position/DTDF estimates because the dietary $\delta^{15}N$ value is inversely related to $\Delta^{15}N$.[14,15,23,24] Therefore, instead of using potentially inaccurate and inappropriate values, DTDFs characterized by relevant trophic interactions (including meaningful variation associated with estimates) to interpret isotopic data are necessary.[25,26]

There are several advantages to using SIA to study trophic dynamics over traditional techniques such as gut content analysis, which only provides a short-term snapshot of often highly degraded prey. First, because different tissues metabolize proteins and carbohydrates at different rates, food is incorporated into consumer tissues at rates (and DTDFs) specific to each tissue–turnover rate. By sampling multiple tissues, it is possible to obtain dietary information over a range of time periods.[18] Second, in addition to $\delta^{13}C$ and $\delta^{15}N$ values providing information on trophic structure (described above), they also indicate the baseline source of carbon or nitrogen in a particular food chain, after accounting for DTDFs at each trophic exchange.[27,28] For example, in aquatic environments, consumers that feed on benthically linked dietary pathways often have higher $\delta^{13}C$ values than those using pelagic pathways.[29,30] Another advantage of SIA is that non-lethal approaches can be used.[31]

As part of SIA, there are several considerations regarding tissue preparation (see Newsome *et al.*[2] for review); one of the most influential is the decision whether to extract lipids prior to analysis. The common basis for this decision is that lipids are depleted in ^{13}C (lower $\delta^{13}C$ values) compared with proteins and carbohydrates and that there is inherent lipid variability among individuals and species, as well as among tissue types within an individual.[32,33] This can lead to bias when comparing the same tissues of different individuals, and different tissues from the same individual. Removing lipids chemically to reduce this bias is not always feasible because it is expensive and time-consuming, and can influence $\delta^{15}N$ values of a sample by preferentially removing isotopically lighter nitrogenous compounds.[34] Adjusting stable isotope values using mathematical normalizations is an alternative method to account for lipids but remains largely untested across ecosystems. Therefore, standardized protocols to deal with bias associated with lipids are encouraged at a species and tissue level.

The goal of this study was to determine DTDFs and turnover rates for several tissues of an economically and ecologically important coral reef fish, the leopard coralgrouper (*Plectropomus leopardus*), in a captive feeding trial. The leopard coralgrouper is a large (up to ~65 cm; 4 kg) predatory epinephelid with broad distribution on the Great Barrier Reef, Australia and throughout the Indo-Pacific region.[35,36] Recently, its future role in fisheries has received increased attention due to concerns relating to climate change (e.g., reduced habitat, altered prey distribution, and metabolic costs due to warmer temperatures).[37] As a result, a few pilot studies have used $\delta^{13}C$ and $\delta^{15}N$ values to begin to understand their trophic relationships in the reef environment.[38,39] However, no study has determined DTDFs or turnover rates for the leopard coralgrouper or any other coral reef fish species, notwithstanding a preliminary study using four individual gag grouper (*Mycteroperca microlepis*).[40] Given concerns about coral reef food webs and the role of key predators such as the leopard coralgrouper, there is need to understand stable isotope dynamics for predatory coral reef fish species. The explicit aims of this study were to (1) quantify accurate DTDFs and turnover rates in a predatory reef fish and reveal the best tissues for inclusion in ecological studies using stable isotopes, (2) investigate the utility of non-lethal sampling, and (3) evaluate the need for lipid correction approaches for specific tissues.

EXPERIMENTAL

Fish collection

Forty-seven leopard coralgrouper were collected from John Brewer Reef, Australia (18°37′52.05"S, 147° 3′21.40"E), during 19–20 August 2013. Individuals were captured using barbless hook (10/0) and line, and immediately placed in a ~50 L container filled with fresh seawater. Each individual was unhooked, vented to avoid barotrauma, and externally tagged (PDS; Hallprint©; Hindmarsh Valley, South Australia, Australia) in the dorsal musculature for identification. Following this, fish were moved to a live well (~350 L) with continuous seawater flow. All fish were transported to the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University (<48 h from initial capture). Individual fish were measured (fork length, mm) and weighed (total mass, g), then placed in one of four 2000-L holding tanks. These tanks constantly received re-circulated filtered seawater, and were aerated by at least one air stone per tank. The study was conducted under Ethics Approval Number A1933 (Animal Ethics Committee, James Cook University).

Feeding trial

Fish were left for 2 days to acclimate to the holding tanks prior to commencing the feeding trial. Initially, 10 of the 47 individuals were sacrificed and their tissues sampled to provide a baseline for $\delta^{13}C$ and $\delta^{15}N$ values (Day 0). Ideally, DTDFs are calculated by measuring the isotope change between two distinct end-members (food items) when both are at equilibrium with consumer tissues.[41] However, due to anticipated difficulties keeping this large predatory reef fish alive for enough time for two end-members to reach equilibrium, Day 0 samples were used as the initial end-member.[24] The ten Day 0 individuals were sampled to account for potential variation in feeding in the wild. Moreover, to reduce isotopic variation among individuals, leopard coralgrouper were only captured from one reef over a short period. The remaining leopard coralgrouper were fed only one food item (*Nemipterus theodorei*) for the duration of the trial and turnover rates were calculated by comparing Day 0 samples with subsequent sampling periods. After the initial sampling (Day 0), tissues were lethally and non-lethally sampled intermittently over a 196-day period (see Table 1 for sampling schedule and sample sizes). Due to the relatively small number of individuals obtained for this

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Table 1. Summary of sampling regime for *Plectropomus leopardus* during experimental feeding trial, including mean (± SE) size (length range in brackets), final mass, mass change, hepatosomatic index (HSI), and condition factor (CF) for each sampling period

Sampling day	n	Type	Tissues sampled	Fork length (mm)	Final mass (g)	Relative mass change	HSI	CF
0	10	Lethal	Fin, liver, muscle, plasma, RBC	441 ± 16 (365–527)	1444 ± 199		9.4 ± 1.0	1.58 ± 0.04
7	5	Lethal	Fin, liver, muscle, plasma, RBC	439 ± 22 (385–514)	1382 ± 224	0.98 ± 0.04	9.7 ± 1.4	1.58 ± 0.08
15	5	Lethal	Fin, liver, muscle, plasma, RBC	429 ± 22 (368–500)	1319 ± 223	1.05 ± 0.03	11.0 ± 1.0	1.61 ± 0.08
21	5	Lethal	Fin, liver, muscle, plasma, RBC	441 ± 17 (377–481)	1376 ± 173	1.02 ± 0.02	10.0 ± 1.4	1.56 ± 0.04
28	5	Non-lethal[a]	Fin, plasma, RBC	427 ± 21 (392–496)	1262 ± 208	1.02 ± 0.01		1.58 ± 0.03
42	4	Non-lethal[b]	Fin, plasma, RBC	402 ± 9 (390–430)	1049 ± 82	1.10 ± 0.03		1.60 ± 0.03
49	5	Lethal[a]	Fin, liver, muscle, plasma, RBC	444 ± 20 (395–495)	1385 ± 192	1.00 ± 0.03	8.9 ± 0.7	1.53 ± 0.04
63	4	Non-lethal[b]	Fin, plasma, RBC	406 ± 9 (391–434)	1099 ± 87	1.15 ± 0.06		1.63 ± 0.05
70	5	Non-lethal[c]	Fin, plasma, RBC	441 ± 24 (366–511)	1449 ± 256	1.05 ± 0.02		1.61 ± 0.03
77	4	Non-lethal[b]	Fin, plasma, RBC	408 ± 9 (394–434)	1135 ± 99	1.19 ± 0.05		1.65 ± 0.03
92	5	Non-lethal[c]	Fin, plasma, RBC	440 ± 23 (366–504)	1435 ± 251	1.04 ± 0.03		1.60 ± 0.04
98	4	Lethal[b]	Fin, liver, muscle, plasma, RBC	409 ± 9 (395–434)	1175 ± 92	1.24 ± 0.08	12.9 ± 1.3	1.71 ± 0.06
147	4	Lethal	Fin, liver, muscle, plasma, RBC	446 ± 31 (388–507)	1379 ± 290	1.13 ± 0.04	12.9 ± 2.7	1.50 ± 0.09
196	5	Lethal[c]	Fin, liver, muscle, plasma, RBC	449 ± 22 (387–519)	1634 ± 253	1.21 ± 0.07	11.3 ± 0.8	1.75 ± 0.02

Note: three fish died (as described in the Results section) and were not included in study, nor was one individual that showed declining weight and liver condition. Relative weight change was calculated as final weight (during that period)/initial weight. Hepatosomatic index (HSI) was calculated as $(W_{liver}/W_{total}) * 100$ and condition factor (CF) was $(W_{total} * 10^5)/L_{fork}^3$, where W_{liver} and W_{total} are liver and total fish weight (g), respectively, and L_{fork} is fork length (cm). [a,b,c]represent repeated sampling of tissues from the same individuals.

experiment, some individual fish were repeatedly sampled (non-lethally) prior to the final lethal sampling (Table 1). The minimum time between repeat sampling of the same individual was 14 days.

Leopard coralgrouper were fed pieces of thawed threadfin bream (*N. theodorei*) (excluding the head) to satiation every Monday, Wednesday, and Friday throughout the experiment. *Nemipterus theodorei* was selected because of its success as a feed for leopard coralgrouper in the past (A. Tobin, personal observation; Johansen *et al.*[37]). This food was purchased in bulk prior to the commencement of the feeding trial to reduce variation in prey isotope signatures. *Nemipterus theodorei* is found near sand or muddy bottoms in offshore waters of the Great Barrier Reef, feeding on crustaceans, molluscs, and small fish.[42] A random subsample (n = 15) of *N. theodorei* (excluding the head) was kept aside (frozen) and homogenized for SIA of the food item.

Tissue sampling

Lethal and non-lethal sampling was conducted as outlined in Table 1. The non-lethal approach sampled fin, red blood cells (RBC), and plasma, while liver and muscle tissues (in addition to fin, RBC, and plasma) were collected during lethal sampling. The protocol for tissue sampling was similar for both lethal and non-lethal approaches. First, an individual was moved from the holding tank into a ~50-L container filled with an anesthetic solution (1:10000 Aqui-S® (Lower Hutt, New Zealand)/seawater) using a dip net. Once the animal lost equilibrium, it was weighed and measured. Next, a small segment (~2 cm × 1 cm) of caudal fin membrane along the exterior margin was removed with scissors, washed with distilled water, and stored in a sterile 2-mL vial. Since many of the individuals were sampled on multiple occasions during the experiment, fin tissue collection was alternated between the lower and upper portions of the caudal fin (minimum of 35 days between sampling fin from the same portion). Between 1 and 2 mL of blood was taken from the 2nd or 3rd gill arch of each individual using a 23-gauge sterile needle. This method was chosen over sampling from the haemal arch because it was more efficient and a short trial revealed no lasting damage to the gills. Similar to fin tissues, the left and right gill arches were alternated when individuals were repeatedly sampled. Whole blood was immediately transferred to a sterile 2-mL vial and centrifuged for 4–8 min using a PC100 micro centrifuge (Imbros Pty Ltd, Cambridge, Tasmania, Australia). The plasma component was pipetted (Eppendorf Research® plus 10–100 µL; North Ryde, NSW, Australia) into a sterile 2-mL vial. The remaining plasma layer and the top layer of RBC (including white blood cells) were then discarded leaving only RBC in the vial. Vials containing fin, RBC, and plasma were immediately placed on ice after collection until they could be moved to a –20 °C freezer (within the hour). If non-lethal sampling was scheduled for that particular day, fish were moved to a seawater-filled container to recover from the anesthetic before being returned to their holding tank. If lethal sampling was required, the gills of anesthetized fish were severed and the fish was placed in an ice-slurry. Within the hour, the liver was excised, weighed, and a small portion (~4 cm^3) removed with scissors, rinsed in ethanol then distilled water, and placed into a sterile 2-mL vial. Similarly,

a piece of dorsal muscle (no skin/scales attached) was removed and placed into a vial. Liver and muscle tissues were then frozen.

Stable isotope analysis

The tissues were freeze-dried for 48 h, and ground into a fine powder with a mortar and pestle, except for fin tissues, which were cut into small pieces with scissors. To compare the influence of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values, SIA was performed for untreated (bulk) tissues and the same tissues after lipid extraction (LE). For LE tissues, lipids were removed following McMeans *et al.*[43] by adding 5 mL 2:1 chloroform/methanol to a <1 g subsample, vortexed for 30 s, and left for 24 h in a 30 °C water bath. Afterwards, another 5 mL of solvent was added, vortexed, then poured out, and the tissue was left to dry for 24 h. Bulk and LE tissues (400-800 µg) were weighed into tin capsules, and $\delta^{13}C$ and $\delta^{15}N$ values were determined using a continuous flow isotope ratio mass spectrometer (Finnigan MAT Deltaplus, ThermoFinnigan, San Jose, CA, USA) equipped with an elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA). Stable isotope ratio values are expressed following the equation:

$$\delta X = \left[\left(R_{Sample}/R_{Standard}\right) - 1\right] \quad (1)$$

where X is ^{13}C or ^{15}N, R_{Sample} is the ratio ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample, and $R_{Standard}$ is the ratio in the standard. The standard reference material was PeeDee Belemnite carbonate and atmospheric N_2 for carbon and nitrogen samples, respectively. Every 12th sample was run in triplicate to assess precision, where the standard deviations (SD) of the $\delta^{13}C$ and $\delta^{15}N$ values were generally <0.2 and <0.1 ‰, respectively. Further, laboratory and National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) standards were analyzed every 12 samples. The analytical precision (standard deviation) for NIST standard 8414 (bovine liver, n = 130) and an internal laboratory standard (tilapia muscle, n = 130) for $\delta^{13}C$ values was 0.05 and 0.07 ‰, respectively, and for $\delta^{15}N$ values was 0.16 and 0.13 ‰, respectively. The accuracy was checked monthly using certified urea (n = 120) and was within 0.16 and 0.05 ‰ of the mean calculated values for $\delta^{13}C$ and $\delta^{15}N$ values.

Statistical analysis

For tissues that demonstrated a transition in isotope values toward equilibrium during the feeding trial (i.e., $\delta^{15}N$ values), turnover rates were estimated for LE and bulk tissues by fitting a nonlinear least-squares regression model using the following equation:[44]

$$\delta_t = \delta_f + \left(\delta_i - \delta_f\right)e^{(-vt)} \quad (2)$$

where δ_t is the stable isotope ($\delta^{15}N$) value at time t; δ_f is the asymptotic stable isotope value at equilibrium with the new diet; δ_i is the initial value for that tissue (Day 0), v is the fractional rate of isotopic incorporation into the tissue, or turnover rate;[45] and t is the sampling day. The primary influences of tissue turnover rates are growth and metabolism.[44] Thus, the parameter v was further defined as the sum of tissue net growth (k_g) and tissue catabolic turnover (m):[41]

$$v = k_g + m \quad (3)$$

The parameter k_g was estimated by fitting nonlinear least squares to an exponential growth model:[46]

$$W_f = W_i \; e^{k_g t} \quad (4)$$

where W_f is the final wet mass of an individual at time of sampling; W_i is the initial mass; and k_g and t are defined as before.

Therefore, m was the unknown solved with this approach providing tissue turnover rates (day^{-1}) independent of growth.

The turnover rate for both growth and metabolism (v) was also presented as a half-life ($T_{0.5}$) to assist interpreting wild tissue samples in future studies:[47]

$$T_\alpha = \ln(1-\alpha)/-v \quad (5)$$

where T_α is the length of time (in days) needed to achieve a target transition state α (e.g., 50%) from initial stable isotope values (Day 0) to equilibrium values. Similarly, 95% ($T_{0.95}$) transition periods were calculated for each tissue.

Diet-tissue discrimination factors were calculated as:[48]

$$\Delta\delta = \delta_f - \delta_d \quad (6)$$

where δ_f is the tissue-specific stable isotope value of *P. leopardus* at equilibrium with the new diet; and δ_d is the mean value of the *N. theodorei* diet. Standard errors (SE) for the DTDFs were calculated using the SE associated with model estimate δ_f and the SE of *N. theodorei* values:[49]

$$SE_{\Delta\delta} = \sqrt{SE_{\delta f}{}^2 + SE_{\delta d}{}^2} \quad (7)$$

For those tissues whose turnover/equilibrium could not be estimated (i.e., unable to fit with Eqn. (2) – $\delta^{13}C$ values), mean DTDFs were estimated by subtracting the mean *N. theodorei* stable isotope values (δ_d) from the leopard coralgrouper values (δ_t) sampled between Days 98 and 196. This approach was selected because the $\delta^{13}C$ values were relatively consistent throughout the feeding trial for each tissue, especially after Day 98, indicating that the consumer values had reached equilibrium with the prey.

The effect of lipid extraction on the $\delta^{13}C$, $\delta^{15}N$, %C, %N, and C:N (%C/%N) values was evaluated in the different tissues by examining differences between the LE and the bulk values. Paired t-tests were then performed to determine if the LE values differed from the bulk values for each tissue sampled. The effectiveness of using lipid-normalizing models for bulk $\delta^{13}C$ values was examined by comparing observed LE values with corresponding predicted values from three correction models.[33,50,51] The accuracy of these models was determined by calculating the percentage of estimates that fell within 0.1‰ ($P_{0.1}$) and 0.5‰ ($P_{0.5}$) of LE values. In addition, r^2 and Akaike's Information Criterion corrected for small sample sizes (AIC_c) were determined to evaluate the precision and fit of correction models.[52] The resultant linear

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model was used to re-estimate the model values in order to standardize them, and adjusted $P_{0.1}$ and $P_{0.5}$ were determined.

All the modelling and data analyses were conducted in the R environment[53] and results were considered significant when $p < 0.05$. Assumptions relating to normality of dependent variables and homogeneity of variances were verified using Q-Q plots and visual inspection of residual plots, respectively.

RESULTS

The feeding trial lasted 196 days, during which lethal and non-lethal sampling of muscle, liver, fin, plasma, and RBC was conducted at designated intervals (Table 1; Supplementary Table S1, Supporting Information). After an initial acclimation period of a few days, all individuals began feeding and displayed limited signs of stress. Three individuals died during the experiment: one after 10 days, and the other two after more than a month. The first may have been stress-induced, while the two latter died after propelling themselves out of the tank through a mesh cover. On a few occasions, an individual became externally infected with bacterial/fungal growth. Infected individuals were bathed in freshwater <2 min and Betadine® was applied to the infected area, after which they recovered fully. One of the larger individuals (sampled on Days 42, 63, 77, and 98) fed less than all others and decreased in mass by ~15% compared with initial measurements. Consequently, data from this individual were removed from all analyses to avoid bias associated with fasting/nutritional stress.[54] In addition, examination of Cook's D (identifies outliers) was used to remove four $\delta^{13}C$ values (in plasma, RBC, and fin tissues) and three $\delta^{15}N$ values (in plasma and RBC). The mean fork length and mass of leopard coralgrouper at each sampling period ranged between 402 and 449 mm, and 1049 and 1634 g, respectively (Table 1). The general health of individuals throughout the experiment was good, and most demonstrated increased mass and liver condition (HSI) (Table 1).

Tissue turnover for $\delta^{15}N$ and $\delta^{13}C$ values

Lipid-extracted and bulk fin, liver, plasma, muscle, and RBC stable isotope parameters were estimated for the time-based $\delta^{15}N$ model (Eqn. (2)) (Fig. 1; Table 2). For muscle $\delta^{15}N$, the initial values were elevated and could not be fitted to the model above. Under the assumption that prey tissues take >15 days to be incorporated into consumer muscle tissues,[40,49] and acknowledging inherent isotopic variability in wild-caught fish, the model was adapted to only incorporate sampling periods between Days 21 and 196 (Fig. 1). The nonlinear model described changes in $\delta^{15}N$ values over time relatively well for muscle and RBC ($r^2 = 0.70$–0.76; Table 2). Tissue-specific metabolic turnover rates (v) were calculated after the exponential growth model estimated the net growth constant (k_g) to be 0.00084 day^{-1} (Table 2; individuals ranged between –0.00786 and 0.00906 day^{-1}). The half-lives ($T_{0.5}$) for LE and the bulk $\delta^{15}N$ values of liver, fin, plasma, RBC, and muscle ranged between 10 and 126 days, and 95% incorporation rates ($T_{0.95}$) were between 43 and 543 days (Table 2). None of the $\delta^{13}C$ tissue values could be fitted to the time-based nonlinear model to estimate turnover rates.

Diet-tissue discrimination factors for $\delta^{15}N$ and $\delta^{13}C$ values

The food (*N. theodorei*) $\delta^{15}N$ values varied slightly for LE (n: 15; mean ± SE: 10.9‰ ± 0.1; range: 9.8 to 11.7 ‰) and untreated (n: 15; mean ± SE: 10.3‰ ± 0.1; range: 9.5 to 11.1 ‰) samples. The range in mean DTDFs for LE and bulk $\delta^{15}N$ values among tissues was 0.0–1.8 and –0.2–1.7, respectively (Table 2).

The LE $\delta^{13}C$ values of food (n: 15; mean ± SE: –16.5‰ ± 0.1; range: –17.1 to 15.7 ‰) were less variable than those of the untreated samples (n: 15; mean ± SE: –17.8‰ ± 0.3; range: –20.3 to –16.0‰). The range in mean DTDFs among tissues for LE and bulk $\delta^{13}C$ values was 0.1–3.2 and 0.4–3.9, respectively (Table 2).

Bulk vs lipid-extracted tissues

The t-tests comparing LE and bulk values of $\delta^{13}C$, $\delta^{15}N$, %C, %N, and C:N showed that lipid extraction produced generally different outputs from untreated/bulk samples. Only the $\delta^{15}N$ values of RBC ($t = 0.34$, $df = 71$, $p = 0.73$) and %C in fin ($t = -1.34$, $df = 61$, $p = 0.19$) were similar for both LE and bulk. Differences between LE and bulk parameters also showed marked differences (Fig. 2(a)), although the values were consistent for some tissues, particularly $\delta^{15}N_{LE\text{-}Bulk}$ (mean ± SD) for muscle (0.5 ± 0.1), plasma (0.1 ± 0.2), and RBC (0.0 ± 0.2); and $\delta^{13}C_{LE\text{-}Bulk}$ (mean ± SD) for muscle tissue (–0.1 ± 0.2) (Fig. 2(b)). Lipid extraction reduced C:N for all tissues and food; however, the C:N in LE liver tissue remained relatively high (mean ± SD: 6.9 ± 1.7; Table 3) even after multiple extractions. The lipid-normalizing models that were examined produced relatively similar outputs (Table 4). Based on r^2, the ΔAIC (values ≤2 show strongest support for model fitting)[52] and adjusted $P_{0.1}$ and $P_{0.5}$, the best models varied for each tissue (indicated in Table 4). Overall, muscle and RBC were the tissues best described by the correction models (Table 4).

DISCUSSION

The 196-day feeding trial that consisted of sampling five tissues lethally and non-lethally from 43 individual leopard coralgrouper revealed expected variation in stable isotope dynamics and associated metrics, which have implications for their use in studies with this species. Overall, RBC and muscle tissues produced the least variable and most reliable estimates of DTDFs and turnover rates associated with the captive diet, as well as comparisons between LE and bulk C:N, and accounting for lipid-related bias. By contrast, stable isotope trends in lipid-rich liver were variable independent of lipid extraction suggesting caution is needed when used in future work with this species, and others like it. Stable isotope values in plasma and fin, both non-lethal sampling methods, reflected short-term dietary patterns (half-life <70 days), while diet-assimilation was slowest in muscle and RBC (half-life >80 days). Diet-tissue discrimination factors for $\delta^{15}N$ values were <2 ‰ for all tissues – lower than values

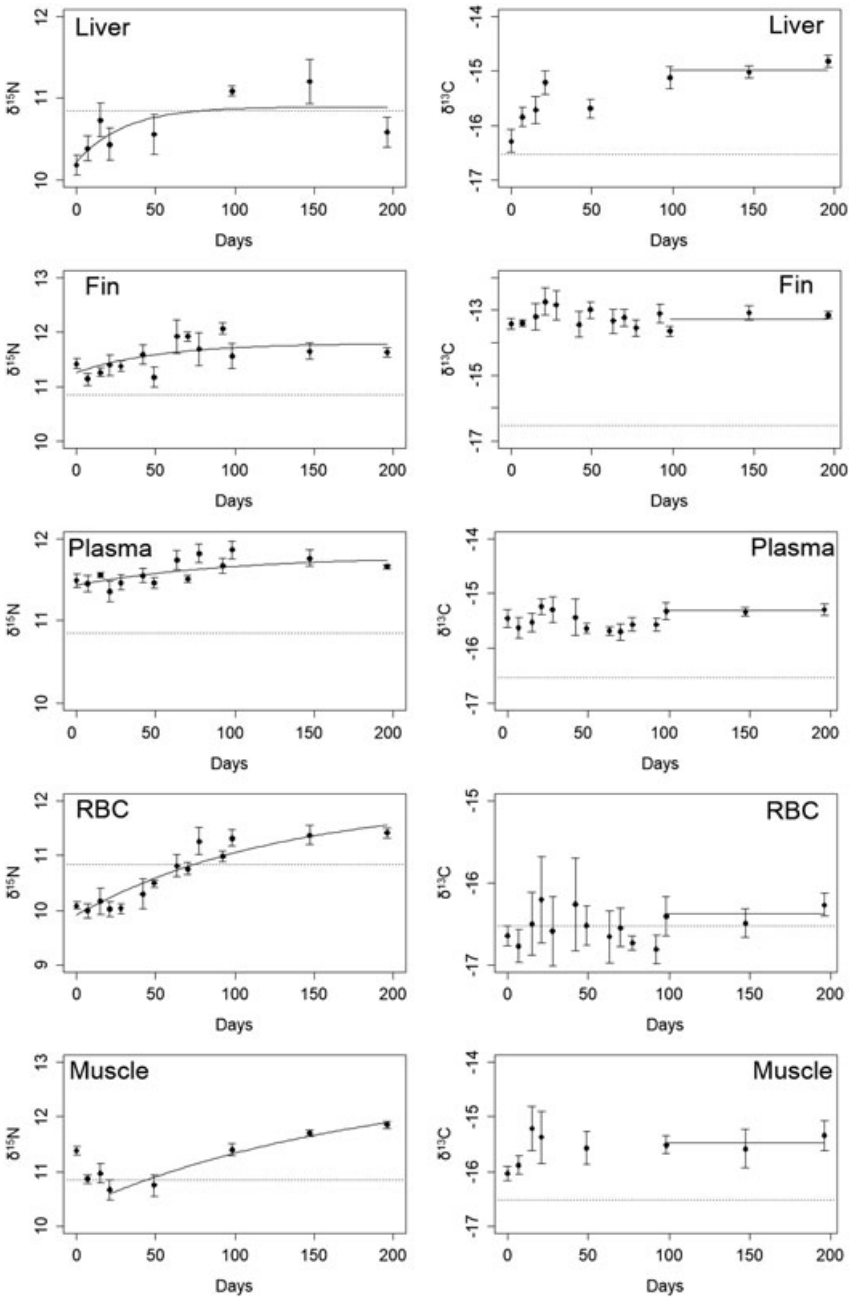

Figure 1. Mean (±SE) $\delta^{15}N$ and $\delta^{13}C$ estimates for lipid-extracted tissues during feeding trial. Plots for $\delta^{15}N$ values (left) contain the least-squares regression from the time-based isotope models for liver, fin, plasma, RBC, and muscle tissues. Solid horizontal lines in $\delta^{13}C$ plots (right) represent the mean $\delta^{13}C$ values of *Plectropomus leopardus* tissues between Days 98 and 196. The dotted horizontal lines on each plot represent the mean value of *Nemipterus theodorei* ($\delta^{15}N = 10.9‰$; $\delta^{13}C = -16.5‰$).

commonly reported in the literature (e.g., ~3.4 ‰).[13] By contrast, DTDFs for $\delta^{13}C$ values ranged between 0 and 4‰, demonstrating that stepwise enrichment in ^{13}C was not negligible for some tissues.

Tissue turnover for $\delta^{15}N$ and $\delta^{13}C$ values

As expected, given that the leopard coralgrouper in this study were medium- to large-sized adults (mature at ~36 cm)[55] with slow growth rates compared with juveniles,[56] most ^{15}N incorporation was driven by metabolism, as opposed to growth. Growth contributed <10% of turnover in the metabolically slower tissues such as muscle and RBC, and ~1% in tissues with fast turnover such as liver. A few studies have examined the contribution of growth to isotope incorporation in larger slow-growing species and also found that metabolic processes such as tissue catabolism and protein synthesis were the main drivers of turnover rates.[40,57,58] By contrast, growth contributed more to turnover rates in smaller juveniles with faster relative

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Table 2. Parameter estimates from nonlinear least-squares time-based lipid-extracted (LE) and untreated (Bulk) $\delta^{15}N$ models for liver, fin, plasma, RBC, and muscle tissues, including the initial $\delta^{15}N$ value for that tissue (i.e., Day 0; δ_i, ‰), equilibrium value (δ_f; ‰), turnover rate constant (v; day^{-1}), tissue catabolic turnover (m; day^{-1}), proxy to model fit (r^2), tissue half-life and 95% incorporation time ($T_{0.5}$, $T_{0.95}$; days), and mean diet-tissue discrimination factor (DTDF or Δ_{tissue}; ‰; ± SE, SD*; also estimated for LE and bulk $\delta^{13}C$)

Isotope	Type	Tissue	δ_i	δ_f	v	m	r^2	$T_{0.5}$	$T_{0.95}$	Δ_{tissue}
$\delta^{15}N$	LE	Liver	10.2	10.9	0.034	0.033	0.27	21	89	0.0 ± 0.2, 0.4
		Fin	11.3	11.8	0.019	0.018	0.17	37	158	0.9 ± 0.2, 0.2
		Plasma	11.4	11.8	0.011	0.010	0.20	66	283	0.9 ± 0.2, 0.1
		RBC	9.9	12.0	0.008	0.007	0.70	88	380	1.1 ± 0.5, 0.2
		Muscle	10.3	12.7	0.006	0.005	0.74	126	543	1.8 ± 1.5, 0.2
	Bulk	Liver	10.1	10.2	0.069	0.068	0.01	10	43	−0.2 ± 0.2, 0.2
		Fin	11.3	11.5	0.016	0.015	0.05	44	191	1.2 ± 0.2, 0.3
		Plasma	11.3	11.7	0.018	0.017	0.18	39	170	1.3 ± 0.2, 0.1
		RBC	10.0	12.0	0.008	0.007	0.74	90	388	1.7 ± 0.4, 0.1
		Muscle	9.8	11.8	0.008	0.007	0.76	83	360	1.5 ± 0.6, 0.2
$\delta^{13}C$	LE	Liver								1.5 ± 0.1, 0.3
		Fin								3.2 ± 0.1, 0.4
		Plasma								1.2 ± 0.1, 0.2
		RBC								0.1 ± 0.1, 0.4
		Muscle								1.1 ± 0.2, 0.5
	Bulk	Liver								0.4 ± 0.2, 0.6
		Fin								3.9 ± 0.1, 0.4
		Plasma								1.0 ± 0.1, 0.4
		RBC								1.6 ± 0.1, 0.3
		Muscle								2.3 ± 0.2, 0.5

Note: Tissue catabolic turnover (m) was estimated by subtracting a tissue net growth (k_g) value of 0.00084 day^{-1} from the turnover rate constant (v). To calculate Δ_{tissue} the mean stable isotope values of *Nemipterus theodorei* (LE: $\delta^{15}N = 10.9$‰; $\delta^{13}C = -16.5$‰, Bulk: $\delta^{15}N = 10.3$‰; $\delta^{13}C = -17.8$‰) were subtracted from equilibrium (δ_f) estimates for $\delta^{15}N$ and mean $\delta^{13}C$ values of *Plectropomus leopardus* tissues between Days 98 and 196. $\delta^{15}N$ values from Days 0, 7, and 15 were not included in muscle estimates. *For $\delta^{15}N$ Δ_{tissue}, SE was calculated using Eqn.(7); SD (and SE for $\delta^{13}C$ Δ_{tissue}) was calculated by subtracting *N. theodorei* $\delta^{13}C/\delta^{15}N$ values from *P. leopardus* values (between Days 98 and 196 for $\delta^{13}C$; Day 196 for $\delta^{15}N$) for use in isotopic mixing models.

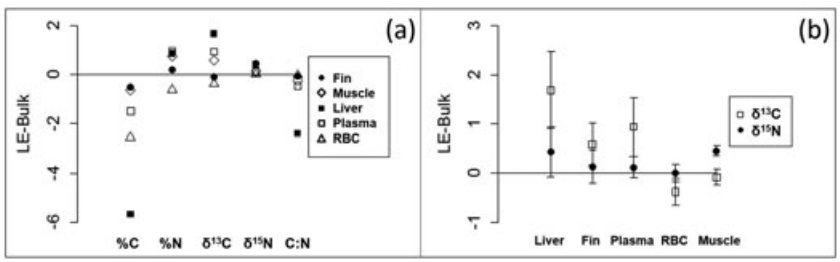

Figure 2. Comparison of mean %C, %N, $\delta^{15}N$, $\delta^{13}C$, and C:N values of several tissues after subtracting untreated (Bulk) values from lipid-extracted (LE) values (a). Mean (±SD) $\delta^{15}N$ and $\delta^{13}C$ values are plotted again at a finer scale (b).

growth.[45,59,60] For example, in hatchery-reared juvenile summer flounder (*Paralichthys dentatus*) with growth rates of 0.00816 day^{-1} (compared with 0.00084 day^{-1} in this study), growth contributed ~11% in liver and >50% in blood and muscle.[49] Since leopard coralgrouper have a minimum retain size of 38 cm in commercial and recreational fisheries, only adults were examined to address stable isotope ecology in the context of fisheries management.

In LE tissues, the ^{15}N turnover rates from quickest to slowest were liver, fin, plasma, RBC, and muscle with half-lives between 21 and 126 days. In bulk tissues, the order was liver, plasma, fin, muscle, and RBC, with half-lives between 10 and 90 days. The differences in turnover rates and estimated half-lives between tissues, independent of tissue treatment approach, match relatively well with the few studies using medium- to large-sized fish (Table 5). For example, the half-lives of $\delta^{15}N$ in plasma and fin were relatively short in the adult catfish *Pterygoplichthys disjunctivus* (<35 days)[58] and similar rates have been determined in liver for juvenile species of goby (*Pomatoschistus minutus*) and flounder (*Paralichthys dentatus*).[49,61] Plasma and liver are hypothesized to have

Table 3. Mean (±SD) ratio of %Carbon to %Nitrogen (C:N) from stable isotope analysis conducted for lipid-extracted (LE) and untreated tissues (Bulk)

Tissue	n	C:N (LE)	C:N (Bulk)	p
Liver	44	6.9 ± 1.7	9.3 ± 2.8	<0.001
Fin	62	2.9 ± 0.1	3.2 ± 0.1	<0.001
Plasma	69	3.6 ± 0.1	4.1 ± 0.3	<0.001
RBC	72	3.4 ± 0.1	3.5 ± 0.1	<0.001
Muscle	45	3.2 ± 0.1	3.3 ± 0.1	<0.001
Food *(Nemipterus theodorei)*	15	3.2 ± 0.1	4.0 ± 0.7	<0.001

Note: p-values were calculated from paired t-tests between LE and Bulk samples

similar turnover rates because plasma proteins are mainly synthesized in the liver.[45,62,63] However, in this study, for LE and bulk treatments $\delta^{15}N$ turnover in liver was quicker than in plasma (i.e., half-life up to 45 days earlier in liver), and may indicate different catabolic processes involved, although the large amount of liver $\delta^{15}N$ variation throughout the feeding trial may have confounded the estimate. Muscle and RBC $\delta^{15}N$ values fitted the turnover rate models best for both LE and bulk tissues. Not surprisingly, estimates of RBC $\delta^{15}N$ turnover in this study (half-life ~90 days) were considerably higher than determined for smaller and faster growing adult *P. disjunctivus* (half-life ~10 days, $k_g = 0.0017$ day^{-1}).[58] Nevertheless, $\delta^{15}N$ incorporation rates in RBC are commonly slower than in plasma solutes and faster than (or similar to) in muscle.[18,64,65] Turnover rates of $\delta^{15}N$ in muscle vary between studies but are slower than in other tissues because protein synthesis and degradation rates are slow (Table 5).[66–68] For example, the estimated $\delta^{15}N$ half-life in the muscle of leopard shark (*Triakis semifasciata*) is ~225 days whereas those for RBC and plasma are ~100 and 40 days, respectively.[40] Muscle is also the tissue commonly sampled for isotopic studies because values are less variable within and between individuals.[69,70] A longer sampling period would have improved turnover estimates, particularly for tissues with slower turnover such as muscle but logistically this was not possible.

Tissue turnover for $\delta^{13}C$ values could not be determined due to the lack of consistent temporal trends in these values. There are a few reasons why this may have been the case. First, variation in dietary *N. theodorei* $\delta^{13}C$ values may have resulted in a variable exposure to ^{13}C in leopard coralgrouper. Second, there appeared to be more inherent $\delta^{13}C$ variability in tissues than for $\delta^{15}N$ values, particularly in plasma, RBC, and muscle, which made fitting models more difficult (see also Post[13] and Pinnegar and Polunin[69]). Finally, the most likely reason that turnover could not be calculated was because the $\delta^{13}C$ value of *N. theodorei* was similar to the $\delta^{13}C$ values of prey consumed on the reef in the wild. Hence no significant isotopic change was found over time because the $\delta^{13}C$ values in the wild did not vary sufficiently from aquarium values.

Table 4. Linear relationship between LE $\delta^{13}C$ and lipid-normalized $\delta^{13}C$ values from three predictive models for each tissue. Output includes the following metrics to interpret the best fitting models: the percent of predicted $\delta^{13}C$ values that fall within 0.1‰ ($P_{0.1}$) and 0.5‰ ($P_{0.5}$) of LE $\delta^{13}C$ values (%); the linear model equation comparing LE $\delta^{13}C$ and lipid-normalized $\delta^{13}C$ values; r^2 of the linear model; and AIC_c and ΔAIC_c for model selection

Tissue	Lipid correction approach	$P_{0.1}$	$P_{0.5}$	Equation	r^2	AIC_c	ΔAIC_c	$P_{0.1}$ (adjusted)	$P_{0.5}$ (adjusted)
Muscle	Post *et al.*[33]*	55.6	97.8	y = 0.984x – 0.270	0.943	−32.8	0	57.8	100
	McConnaughey & McRoy[50]*	0	2.2	y = 0.992x + 0.757	0.941	−31.0	1.8	51.1	100
	Kiljunen *et al.*[51]	44.4	97.8	y = 0.994x – 0.148	0.939	−29.7	3.1	51.1	100
	This study (LE vs bulk)	44.4	97.8	y = 0.971x – 0.541	0.946	−32.2	0.6	51.1	100
Fin	Post *et al.*[33]*	4.8	29.0	y = 0.722x – 3.121	0.692	47.6	1.7	17.7	88.7
	McConnaughey & McRoy[50]*	0	0	y = 0.738x – 2.145	0.699	46.0	0.1	16.1	88.7
	Kiljunen *et al.*[51]*	3.2	14.5	y = 0.742x – 2.775	0.700	45.9	0	14.5	88.7
	This study (LE vs bulk)	6.5	43.5	y = 0.686x – 3.736	0.664	52.9	7	27.4	87.1
Plasma	Post *et al.*[33]	27.5	71.0	y = 0.569x – 6.505	0.375	65.8	5.9	23.2	89.9
	McConnaughey & McRoy[50]	2.9	5.8	y = 0.595x – 5.707	0.399	63.1	3.2	24.6	89.9
	Kiljunen *et al.*[51]*	21.7	85.5	y = 0.624x – 5.934	0.426	59.9	0	24.6	89.9
	This study (LE vs bulk)	1.4	21.7	y = 0.341x – 9.836	0.200	82.8	22.9	24.6	87.0
RBC	Post *et al.*[33]*	2.8	59.7	y = 0.974x – 0.888	0.841	15.4	0	23.6	97.2
	McConnaughey & McRoy[50]*	15.3	65.3	y = 0.974x – 0.077	0.839	16.6	1.2	25.0	97.2
	Kiljunen *et al.*[51]	0	34.7	y = 0.973x –1.057	0.837	17.5	2.1	27.8	95.8
	This study (LE vs bulk)	8.3	72.2	y = 0.967x – 0.902	0.840	16.1	0.5	20.8	95.8
Liver	Post *et al.*[33]	0	6.8	y = 0.153x – 13.816	0.370	74.0	10	11.4	63.6
	McConnaughey & McRoy[50]*	6.8	36.4	y = 0.485x – 8.347	0.498	64.0	0	18.2	72.7
	Kiljunen *et al.*[51]*	0	2.3	y = 0.449x – 9.564	0.488	64.9	0.9	18.2	72.7
	This study (LE vs bulk)	0	2.3	y = 0.395x – 8.750	0.249	81.7	17.7	13.6	63.6

Note: adjusted $P_{0.1}$ and $P_{0.5}$ are taken after the linear equation was used to standardize lipid-normalized $\delta^{13}C$ values.
*represent best models for each tissue. Results for 'this study' are based on regressions between LE $\delta^{13}C$ and bulk $\delta^{13}C$ values for comparison.

Table 5. Summary of previously published nitrogen turnover (reflecting growth and metabolic incorporation) and diet-tissue discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$) in different fish tissues

Source	Species	Tissue	Temperature (°C)	Maturity	Mass (g)	Length (mm)	$\delta^{15}N$ Turnover rate (day^{-1})	$\delta^{15}N$ Half life (days)	$\Delta^{15}N$	$\Delta^{13}C$
Herzka and Holt[97]	Red drum *(Sciaenops ocellatus)*	Whole	24,28	Larvae	<0.1	<7	0.25	2.8[a]	1.5–4.2	0.2–1.9
Herzka *et al.*[98]	Red drum *(Sciaenops ocellatus)*	Whole	16–30	Larvae	<0.1	<7	>0.058	<12[a]	6	
Vander Zanden *et al.*[99]	Smallmouth bass *(Micropterus dolomieu)*	Whole		Larvae/ juvenile	<0.1	<50	0.14–0.23[a]	3–5[a]		
Bosley *et al.*[78]	Winter flounder *(Pseudopleuronectes americanus)*	Whole	13,18	Juvenile	<0.1		0.18,0.22	3.9,3.1	−0.3–2.2	2–2.5
Witting *et al.*[100]	Summer flounder *(Paralichthys dentatus)*	Whole	13,22	Larvae	<0.1		0.09, 0.22[a]	8,3[a]	3.8,2.9	0.2,0.6
		Whole	13,22	Larvae	<0.1		0.05,0.11[a]	14,6[a]	2.8,3.1	0.5,0.9
		Whole	13,22	Juvenile	0.34,0.43	37.8–61.3	0.01,0.01[a]	63,99[a]	12.2,3.7	−0.2,0.2
Maruyama *et al.*[101]	Goby *(Rhinogobius* sp.*)*	Muscle	13,22	Juvenile	0.1–0.9		0.007–0.021[a]	33–99[a]	5.1	
Logan *et al.*[102]	Mummichog *(Fundulus heteroclitus)*	Muscle	18	Juvenile	0.84–1.75		−2.33[b]		−1.0,0.2	
		Liver	18	Juvenile	0.84–1.75		−5.85[b]		0,1.2	
McIntyre and Flecker[103]	Armoured catfish *(Ancistrus triradiatus)*	Muscle	20–27	Juvenile	0.24–2.64		0.038	18.2		
		Blood	20–27	Juvenile	0.24–2.64		0.041	16.9		
		Fin	20–27	Juvenile	0.24–2.64		0.057	12.2		
Guelinckx *et al.*[60]	Sand goby *(Pomatoschistus minutus)*	Muscle	17	Juvenile	~5–15	>42	0.025	27.8	3.4	1
		Heart	17	Juvenile	~5–15	>42	0.026	26.6	1	0.84
		Liver	17	Juvenile	~5–15	>42	0.251	2.8	1.35	−3.92
Sweeting *et al.*[104]	European sea bass *(Dicentrarchus labrax)*	Muscle	4–17	Juvenile	8.0–48.5		0.0140, 0.0215	49.5, 32.2		
		Heart	4–17	Juvenile	8.0–48.5		0.0202, 0.0507	34.3, 13.7		
		Liver	4–17	Juvenile	8.0–48.5		0.0193, 0.0196	35.9, 35.4		
German and Miles[58]	Catfish *(Pterygoplichthys disjunctivus)*	Fin	25	Adult	>51	>128	0.021	33[c]	1.29	−0.93
		RBC	25	Adult	>51	>128	0.0715	9.7[c]	5.17 ± 0.13[e]	0.24 ± 0.56[e]
		Plasma	25	Adult	>51	>128	0.0905	7.7[c]	4.39 ± 0.05[e]	0.06 ± 0.08[e]
Suzuki *et al.*[60]	Japanese temperate bass (*Lateolabrax japonicus*)	Fin	23	Juvenile	9.87–94.1	84.0–178.0	0.031	22.4	2.21	3.66
		Muscle	23	Juvenile	9.87–94.1	84.0–178.0	0.036	19.3	2.41	2.4
		Liver	23	Juvenile	9.87–94.1	84.0–178.0	0.048	14.4	0.59	0.34
Hesslein *et al.*[41]	Broad whitefish (*Coregonus nasus*)	Muscle	10	Juvenile	5.1–325	51–210	0.032–0.072	9–22	3.8	2
		Liver	10	Juvenile	5.1–325	51–210	0.033–0.073	9–22	3.8	2
MacAvoy *et al.*[71]	Channel catfish *(Ictalurus punctatus)*	Muscle	11–19	Spawning adult			0.017–0.023	30–39		1.3
		Blood	11–19	Spawning adult			0.022–0.027	25–32		1.5
		Barbel	11–19	Spawning adult			0.039–0.044	15–18		

(Continues)

Table 5. (Continued)

Source	Species	Tissue	Temperature (°C)	Maturity	Mass (g)	Length (mm)	$\delta^{15}N$ Turnover rate (day^{-1})	$\delta^{15}N$ Half life (days)	$\Delta^{15}N$	$\Delta^{13}C$
MacNeil *et al.*[19]	Ocellate stingray (*Potamotrygon motoro*)	Cartilage	26	Juvenile/ adult	>106		0.005	133.3		
		Muscle	26	Juvenile/ adult	>106		0.007	97.6		
		Blood	26	Juvenile/ adult	>106		0.011	61.3		
		Liver	26	Juvenile/ adult	>106		0.018	38.5		
Harvey *et al.*[106]	Lake trout (*Salvelinus namaycush*)	Muscle	10.6	Juvenile	55–196		0.0005[a]	69[a]	~ –0.7	~3.0
Trueman *et al.*[21]	Atlantic salmon (*Salmo salar*)	Liver		Juvenile	48.5–341.7	132–334	22[d]	2.25[d]	0.0 ± 0.3[f]	1.6 ± 0.3[f]
		Muscle		Juvenile	48.5–341.7	132–334	40[d]	1[d]	2.3 ± 0.3[f]	2.1 ± 0.1[f]
Tarboush *et al.*[106]	Zebra danio (*Danio rerio*)	Muscle	28.5	Adult			0.0047	147	7.4	~ 2
Buchheister and Latour[49]	Summer flounder (*Paralichthys dentatus*)	Muscle	20	Juvenile/ early adult	26.3–446.0	130–325	0.0082,0.0065	84.9,106.5	2.13 ± 0.12[e]	3.10 ± 0.51, 4.79 ± 0.36[e]
		Blood	20	Juvenile/ early adult	26.3–446.0	130–325	0.0158, 0.021	43.8,33.0	2.26 ± 0.32, 3.86 ± 0.29[e]	3.11 ± 0.17[e]
		Liver	20	Juvenile/ early adult	26.3–446.0	130–325	0.0632	10	1.45 ± 0.10, 2.23 ± 0.09[e]	2.86 ± 0.09[e]
This study (lipid extracted)	Leopard coralgrouper (*Plectropomous leopardus*)	Muscle	27–29	Adult	660–2730	365–527	0.006	126	1.8 ± 1.5[e]	1.1 ± 0.2[e]
		RBC	27–29	Adult	660–2730	365–527	0.008	88	1.1 ± 0.5[e]	0.1 ± 0.1[e]
		Plasma	27–29	Adult	660–2730	365–527	0.011	66	0.9 ± 0.2[e]	1.2 ± 0.1[e]
		Fin	27–29	Adult	660–2730	365–527	0.019	37	0.9 ± 0.2[e]	3.2 ± 0.1[e]
		Liver	27–29	Adult	660–2730	365–527	0.034	21	0.0 ± 0.2[e]	1.5 ± 0.1[e]
Kim *et al.*[65]	Leopard shark (*Triakis semifasciata*)	Muscle	13–17	Juvenile/ adult	1000–4250	600–1000	0.00307	225.8[c]	5.5 ± 0.4[f]	3.5 ± 0.6[f]
		RBC	13–17	Juvenile/ adult	1000–4250	600–1000	0.00687	100.9[c]	4.6 ± 0.3[f]	2.8 ± 0.6[f]
		Plasma	13–17	Juvenile/ adult	1000–4250	600–1000	0.0172	40.3[c]	4.2 ± 0.3[f]	3.7 ± 0.4[f]

[a]Represents estimates calculated by McIntyre and Flecker.[104]
[b]Calculation derived from a growth-based model, where if c = –1 growth is entirely responsible for turnover and lower values have increasingly greater metabolic contribution.
[c]Half-life value estimated from turnover rate using $T_\alpha = \ln(1 - \alpha)/-v$.
[d]Study estimates were monthly instead of daily.
[e]Standard error (SE).
[f]Standard deviation (SD).

Although equilibrium values could not be confirmed for $\delta^{13}C$ in different tissues, the mean values throughout the trial remained similar across sampling dates, especially after Day 98 of the experiment; thus $\Delta^{13}C$ values appear to be suitable for most tissues. Furthermore, $\delta^{13}C$ turnover is commonly faster than $\delta^{15}N$ turnover in fish tissues,[49,60,61,71] suggesting that the elapsed time before calculating $\Delta^{13}C$ was more than adequate to represent equilibrium values.

Diet-tissue discrimination factors for $\delta^{15}N$ and $\delta^{13}C$ values

Diet-tissue discrimination factors of $\delta^{15}N$ values varied between tissues. Muscle and RBC had the highest DTDFs, followed by plasma and fin, and liver had the lowest $\Delta^{15}N$ values – between –0.2 and 0.0 ‰ – indicating relatively little change in $\delta^{15}N$ values between consumer and prey. This order in tissue $\Delta^{15}N$ matches well with other studies (Table 5). For example, previous work has found that liver $\delta^{15}N$ and $\Delta^{15}N$ values are usually lower than in muscle for fish.[19,49,72] Pinnegar and Polunin[69] hypothesized that fish muscle is typically more ^{15}N-enriched because of the high abundance of the non-essential amino acid taurine. By contrast, fish liver contains less taurine and more essential amino acids, which fractionate less during tissue catabolism.[69,73,74] Similarly, the order of $\Delta^{15}N$ values in liver, fin, and muscle of juvenile bass (*Lateolabrax japonicas*)[60] followed the present study (i.e., $\Delta^{15}N_{liver} < \Delta^{15}N_{fin} < \Delta^{15}N_{muscle}$). Although few studies have compared DTDFs in blood components with other tissues in fish, variation in biochemical composition, specifically the relative abundance of amino acids, appears to be the main factor responsible for different DTDFs among tissues.[69,75]

In general, the $\delta^{15}N$ DTDFs in this study were lower (range: –0.2 to 1.8‰) than commonly used or reported values in fish, particularly for muscle (~2–5‰) (Table 5; see also Sweeting *et al.*[76]). Muscle $\Delta^{15}N$ values had reduced precision in DTDF estimates due to the relatively large standard errors associated with the consumer $\delta^{15}N$ values not being equilibrated to the diet (see also Buchheister and Latour[49]). Consequently, DTDFs for muscle may have been underestimated, as demonstrated by predicted $\Delta^{15}N$ values (2.1–2.8‰[15] and 3.0‰[14]) from linear relationships with dietary $\delta^{15}N$ for muscle/whole fish tissue in the literature. A longer sampling period would have increased the precision of muscle $\delta^{15}N$ equilibrium estimates but that was beyond the scope of this study. Plasma and RBC $\Delta^{15}N$ values from this study were within the lower range estimated by Buchheister and Latour[49] for whole blood in *P. dentatus* (1.1–2.8‰) and lower than the plasma (4.4‰) and RBC (5.2‰) values determined for the herbivore *P. disjunctivus*. Fin $\Delta^{15}N$ values of leopard coralgrouper were also lower than those of juvenile bass (*Lateolabrax japonicas*; 2.2–2.5‰).[60] Despite these differences, variation in $\Delta^{15}N$ is common within the same tissues of different fish species (see Appendix A in Sweeting *et al.*[76]), largely because dietary protein content and quality affect $\Delta^{15}N$.[16,77] Also, most DTDF estimates are based on temperate species, and several studies have found a significant relationship between decreasing $\Delta^{15}N$ and increasing water temperature.[21,78,79] Therefore, DTDFs of tropical species may not be readily comparable with those in temperate ecosystems.

Diet-tissue discrimination factors for LE and bulk $\delta^{13}C$ values were between 0 and 4‰ among tissues. Removing lipids chemically altered the $\delta^{13}C$ and $\Delta^{13}C$ values compared with untreated samples (see below). Lipid extraction also changed the order of enrichment between tissues, probably in response to the adjusted lipid content in relation to other biochemical fractions.[69] Similar to the $\Delta^{15}N$ tissue order determined for *L. japonicas*),[60] $\Delta^{13}C$ also matched this study ($\Delta^{13}C_{liver} < \Delta^{13}C_{muscle} < \Delta^{13}C_{fin}$) for untreated samples. The high lipid content in liver resulted in lower $\delta^{13}C$ values and hence lower $\Delta^{13}C$ than in other tissues. By contrast, fin tissue, which consists of mainly collagen,[80] had the highest $\Delta^{13}C$ for both LE and bulk samples. Fin tissue is often ^{13}C-enriched because of its protein content,[31] and this is unrelated to lipid effects – C:N ratios were low for LE and bulk samples (see Post *et al.*[33] and Hanisch *et al.*[81]).

Commonly, $\delta^{13}C$ DTDFs are assumed to be <1‰ because of limited fractionation between diet and consumer.[13,27,32] However, Sweeting *et al.*[82] found that $\Delta^{13}C$ values in fish tissues such as liver, muscle, heart, and whole body are often between 1‰ and 2‰ (see also Table 5). Based on the negative linear relationship between $\Delta^{13}C$ and dietary $\delta^{13}C$ values,[15] the $\Delta^{13}C$ values for all LE tissues in this study were predicted to be ~0.7‰; however, this estimate is based only on liver, muscle and whole body tissues. Nevertheless, only a few tissues had $\Delta^{13}C$ values larger than 2‰ (i.e., LE and bulk fin and bulk muscle) in this study, demonstrating that the $\Delta^{13}C$ estimates were consistent with other studies. Compared with $\Delta^{13}C$ values in fin of *L. japonicas* (bulk: 3.1–3.7‰), the findings of this study (LE: 3.2‰; bulk: 3.9‰) were similar. The sampling of fin membranes resulted in relatively consistent $\delta^{13}C$ values and improves on other studies where fin tissues were composed of varying tissue elements (e.g., bone, hard spines, and soft rays) which differ in fractionation.[31,57] Plasma (LE: 1.2‰; bulk: 1.0‰) and RBC (LE: 0.1‰; bulk: 1.6‰) $\Delta^{13}C$ estimates were lower than the values in leopard sharks (*Triakis semifasciata*) (plasma: 2.8–3.7‰, RBC: 2.3–2.8‰),[65,83] and whole blood $\Delta^{13}C$ values in *P. dentatus* (bulk: 2.3–3.3‰),[49] yet were similar to published values in marine mammals (see Caut *et al.*[84]). Further studies are necessary to understand these contrasts; however, they may be related to how differences in amino acids affect $\delta^{13}C$ values in different blood components and organisms.[84,85]

Bulk vs lipid-extracted tissues

Tissues that are rich in lipids are often ^{13}C-depleted, resulting in lower $\delta^{13}C$ estimates than in tissues high in proteins or carbohydrates.[50,86] In addition, there can be considerable heterogeneity in lipid content among species, individuals, and tissues.[87,88] To reduce bias associated with tissue lipid content, chemical removal of lipids is common; however, this may cause fractionation in $^{15}N/^{14}N$ and it is more laborious to process tissues.[69,89] In this study, it was evident that removing lipids affected both $\delta^{13}C$ and $\delta^{15}N$ values (i.e., only RBC $\delta^{15}N$ values did not significantly change). This was surprising because the bulk C:N of three of the five tissues was <3.5, an amount which is considered to produce negligible lipid bias for $\delta^{13}C$ values.[33] Other studies have also detected higher muscle $\delta^{15}N$ values after removing lipids,[26,90,91] and proposed that leaching of nitrogenous

metabolites or waste occurs during lipid extraction.[34,89] Yurkowski *et al.*[92] found that lipid extracts contained small amounts of ^{15}N-depleted nitrogen compounds in liver and muscle tissues of Arctic marine mammals, correlating to higher δ^{15}N values after chemical lipid extraction. Nevertheless, for δ^{15}N values, the difference between LE and bulk samples was small (mean $\delta^{15}N_{LE\text{-}Bulk}$ <0.5‰ in all tissues) and often varied little (e.g., muscle, plasma, and RBC), signifying limited influence of lipid extraction on δ^{15}N values in these tissues; lipid extraction should still be considered depending on the specific study. The large change in liver %C, δ^{13}C, and C:N values indicated a high amount of lipids in liver, and this tissue should be treated with caution (see below); while lipid extraction may not be necessary for RBC and fin which had low lipid content based on small δ^{13}C and C:N differences, consistent with other studies.[26,93]

Lipid-normalizing models for δ^{13}C values, specifically those proposed by Post *et al.*[33] and McConnaughey and McRoy,[50] were useful at predicting LE δ^{13}C values in muscle in leopard coralgrouper. All three models are derived from various temperate and sub-arctic aquatic invertebrate and vertebrate organisms.[33,50,51] To a large extent, these models are based on measurements from fish muscle tissue, which provides reasoning for the strong correlation with LE muscle δ^{13}C values in this study. It also explains why other lean tissues such as RBC and fin were well supported by models due to the small variation in lipid-free C:N ratios in these tissues.[33] For most tissues, corrections using regression models from this study were as informative as other models. Also, muscle tissue does not necessarily require lipid correction as bulk and LE δ^{13}C values were relatively similar, although the accuracy was marginally better using the correction model suggested by Post *et al.*[33]

CONCLUSIONS

The main purpose of this experiment was to better understand species- and tissue-specific δ^{15}N and δ^{13}C values and patterns of tropical coral reef fish so that future ecological studies can interpret isotopic data meaningfully. In general, decisions relating to tissue preparation (e.g., lipid extraction) and tissue selection should be based on the specific goals of the study. For example, if research questions are addressing a particular time period or season, sampling must account for temporal variation in tissue turnover. Also, the feasibility of lethal/non-lethal sampling needs to be considered, especially for species that are facing or are at risk of population declines. Muscle and RBC provided the most reliable ^{15}N turnover estimates and represented similar isotopic incorporation periods. In addition, LE δ^{15}N, δ^{13}C, and C:N values in muscle and RBC had little variation compared with bulk values and these tissues worked well with lipid-normalizing models. Therefore, for a relatively long-term representation of feeding habits, RBC or muscle should be used. Both tissues can be sampled non-lethally; however, if lethal approaches are deemed necessary, muscle is often more amenable because it can be sampled post-mortem. Similarly, if chemical lipid extraction is deemed too expensive or time-consuming, the lipid-normalizing techniques described here can easily be utilized with comparable success.

For future work interested in determining short-term feeding ecology, we suggest using plasma or fin, both non-lethal approaches. Both performed similarly in non-linear δ^{15}N equilibrium models with relatively quick turnover periods (half-life <70 days). Also, bulk δ^{15}N and C:N values changed little when these tissues were lipid extracted, and correction models predicted LE δ^{13}C values adequately. Liver is often used in stable isotope studies due to its quick turnover;[49,69,72] however, this study demonstrated that, at least for this species, it is not likely to be a suitable selection for most studies. Its high lipid content confounded isotopic interpretation, as has been seen in other marine fish.[26] For example, when C:N values are >3.5, the presence of lipids will probably bias δ^{13}C estimates.[33] Even after chemical lipid extraction, liver C:N values remained high, suggesting that the lipid extraction methods used were not effective for high lipid content. Previous studies have also found high C:N values in fish liver after chemical lipid extraction and cautioned about the difficulty in effectively standardizing high lipid content tissues.[26,88,91]

Estimates of δ^{13}C and δ^{15}N DTDFs for many tissues were within the range of previous studies, despite some variation within sampling periods. The finding that muscle Δ^{15}N was less than the commonly used range of 3 to 4‰[1,29,76] is important for estimating trophic position and prey proportions in tropical ecosystems more accurately in the future. Based on the estimated $T_{0.95}$, an experimental period of at least twice as long as used in this study would have improved Δ^{15}N estimates. Nevertheless, isotopic mixing models can account for deviation in parameter estimates to simply provide more conservative outputs.[94] Tissue-specific estimates of this kind are not readily available, especially for tropical species, and are necessary to interpret isotope data in feeding ecology studies.

There are limited studies that have calculated DTDFs and turnover rates for medium- to large-sized or adult fish, particularly for tropical reef fish. Fewer still have additionally sampled numerous different tissues or explored the utility of lipid correction techniques. It is not known how applicable the patterns and estimates from this study are to wild individuals or other species and locations. For example, the composition of macromolecules (i.e., proteins, lipids, carbohydrates) in prey, as well as prey itself (i.e., multiple diet items), will vary for leopard coralgrouper in the wild, which could lead to differential DTDFs.[95] Some studies have found differences between laboratory and field isotopic estimates,[27,49] while other values appear to be robust and applicable in the field.[76,82]

There is also concern that 'unrestricted' laboratory feeding rates may bias stable isotope signatures because they are not representative of natural conditions (e.g., reduced prey availability and increased competition can lead to restricted feeding and growth rates).[76] However, during this study, growth was comparable with that of wild individuals;[56] they were fed at similar intervals as in the wild, and wild adult leopard coralgrouper feed almost exclusively on fish (i.e., high protein diet).[96] This study is one of the first to provide experimentally derived stable isotope data for an adult tropical fish and is an important step towards validating metrics to understand the ecology of this and similar species, as well as reef trophic structure.

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