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RESEARCH ARTICLE

Tissue-specific turnover and diet-tissue discrimination factors of carbon and nitrogen isotopes of a common forage fish held at two temperatures

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Rationale: The application of stable isotopes to foraging ecology is dependent on understanding life-history and environmental factors unrelated to diet that may influence isotopic composition. Diet-tissue discrimination factors (DTDFs) and turnover rates will increase the accuracy of isotope-based studies. Furthermore, little consideration has been given to the effects of temperature or life-history stage on isotopic ratios despite the prevalence of variation in temperature and growth rates throughout life.

Methods: We measured $\delta^{13}C$ and $\delta^{15}N$ values with an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer. These values were used to estimate turnover and DTDFs for Emerald Shiners (*Notropis atherinoides*), a common North American freshwater forage fish. Fish were assigned to a temperature treatment, either 10°C (Low) or 20°C (High), and provided one of three diets (commercial pellet, *Artemia salina*, or *Hemimysis anomala*). At regular intervals fish were sampled and the isotopic compositions of whole body and liver tissues were determined.

Results: Tissue turnover rates for fish fed *Artemia* were faster for liver than for whole body, but were also influenced by temperature. Turnover occurred faster at higher temperatures for body and liver $\delta^{15}N$ values, but not for $\delta^{13}C$ values. The pellet and *Hemimysis* treatments were in isotopic equilibrium from the start of the experiment and estimated DTDFs based on these treatments were lower than assumed for $\Delta^{15}N$ (+0.6 to 2.7‰) and variable, but within expected ranges for $\Delta^{13}C$ (−1.9 to +1.5‰).

Conclusions: The results for Emerald Shiners differed from commonly made assumptions for applying stable isotopes to ecological questions, possibly related to a bias in the use of juveniles in studies of turnover and DTDFs and assumptions regarding thermal-independence of isotopic relationships. The species-specific DTDF and tissue turnover estimates provided here will inform interpretations of stable isotope data for smaller fish species and improve food-web studies.

1 | INTRODUCTION

Unlike stomach contents that represent only the most recently consumed food items, stable isotopes are a longer-term indicator of diet reflecting energy assimilated into the tissues of consumers.[1-3] Stable isotope analysis (SIA) has expanded the ability to make long-term inferences of diet from fewer sampling events[4-6] as well as to inform general ecology and life history of terrestrial and aquatic organisms.[7-9] Among the naturally occurring stable isotopes frequently used, carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) are the most commonly applied for diet-related questions because the distinct properties of these elements relating consumers to their resources often provide complementary information. Carbon, often referred to as the 'source isotope', changes by relatively small amounts, e.g. + 0.4 ± 1.3 ‰, between consumers and their resources.[10] Importantly, the $\delta^{13}C$ value differentiates among habitats based on variation in photosynthetic pathways among primary producers, e.g. freshwater littoral and pelagic primary producers.[3,10,11] Nitrogen is referred to as a 'trophic isotope' because it increases with each trophic level, e.g. +3.4 ± 1.0 ‰.[10,12] However, these assumptions are not universal and variation has been noted around these mean values,[10,13,14] leading to research efforts focused on variation in these relationships.[15-17]

Among factors that need to be considered in the application of SIA, two have been identified as key in diet studies: (i) the rates at which tissues change with diet (turnover rate), and (ii) the isotopic relationships between consumers and their prey (diet-tissue

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discrimination factor; DTDF). Stable isotope studies often assume that the study group is in isotopic equilibrium with their diet, but knowledge of species-specific tissue turnover rates will improve the accuracy of inferences using stable isotopes and potentially influence the choice of tissue type to be sampled.[18] Turnover rates can be broken down into two primary contributors; (i) the formation of tissues using energy derived from recently consumed resources, and (ii) metabolic turnover as cellular synthesis replaces older cells.[18-20] Combined, these two factors have been linked to variation in turnover rates between life stages, e.g. rapidly growing juveniles and slower growing adults,[21,22] and among tissue types.[17,23,24] The variation among tissue types is of interest because if tissues can be reliability divided into three broad categories: (i) slow turnover tissues, e.g. white muscle; (ii) fast turnover tissues, e.g. liver and blood; or (iii) inert tissues that do not change after formation, e.g. hair and scales, it is possible to ask temporal questions about diet.[25,26]

In addition to tissue turnover rates, assumptions are frequently made about the isotopic relationships between consumers and diet once they have reached isotopic equilibrium. At equilibrium, the isotopic difference between a consumer and its diet is the diet-tissue discrimination factor (DTDF); this reflects the differential fractionation that occurs between the heavier and lighter isotopes of both carbon and nitrogen ($\Delta X = \delta X_{consumer} - \delta X_{diet}$ where X is typically ^{13}C or ^{15}N).[17,27] Studies of wild populations frequently assume increases of approximately 0.4 ‰ and 3.4 ‰ for $\delta^{13}C$ and $\delta^{15}N$ values,[10,12] respectively, between consumers and their resources.[28,29] These mean DTDFs for $\delta^{13}C$ and $\delta^{15}N$ values derived across species, life stages, and tissue types provide a starting point for studies that do not have species-specific information available, but it is also known that there is variation in DTDFs related to both dietary[30] and non-dietary factors.[10] Dietary variation in protein content[31] and in the bulk isotopic composition of foods, e.g. diet-dependent discrimination factors,[30] can influence DTDFs. Non-dietary variables linked to DTDF variation include the type of tissue that is sampled[19,32] and temperature, e.g. increased $\Delta^{13}C$ and decreased $\Delta^{15}N$ with increasing temperatures.[33,34] These sources of DTDF variation may introduce biases to isotope mixing models that include DTDFs as a predictor parameter (e.g. SIAR – Stable Isotope Analysis in R),[35,36] and more accurate DTDFs will improve the information obtained from SIA of diet.

Stable isotope ecology has provided numerous insights into the foraging relationships and community dynamics of wild populations, but it is based on the ability to accurately infer relationships within food webs that may contain an array of taxa, size ranges, and life-history stages. The increased recognition of a bias in isotope turnover and DTDF studies to using larval or juvenile fishes, see review by Matley et al.,[17] and the absence of species-specific data for relatively small bodied species, e.g. shiners and minnows,[29] are a critical gap in the assumptions made about both turnover and DTDFs in many isotope studies. The objectives of this study were to bring the adult stage of a small-bodied wild-caught fish into the laboratory to estimate tissue-specific turnover rates and DTDFs under different diet and temperature treatments. We chose Emerald Shiners (*Notropis atherinoides*), a common freshwater fish species often consumed as prey and frequently included in multi-species studies in the stable isotope literature.[37-39] Emerald Shiners, typically consumers of zooplankton,[40] are widespread in eastern North America, including the Laurentian Great Lakes region, and experience seasonal shifts in their thermal environment.[41] Based on our objectives we identified three primary predictions for Emerald Shiners brought into the laboratory. First, as fish incorporate novel diet sources into their tissues the isotopic composition should change over time, i.e. turnover, to reflect the isotope values of the different food sources; however, given that turnover rates have been shown to vary among tissue types,[26] we expected liver to turn over more quickly than whole body. Second, once fish tissues are in isotopic equilibrium with their diets we predicted that they would show elevated isotope ratios, i.e. carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values), relative to their food for both carbon and nitrogen, i.e. positive DTDF values, but that these increases would be greatest for $\delta^{15}N$ values. Third, temperature has been associated with variation in both turnover rates[18] and DTDFs.[33,34] We expected slower turnover in the lower temperature treatment. We also predicted that there would be isotope-specific variation in DTDFs, with higher $\Delta^{13}C$ and lower $\Delta^{15}N$ values in the higher temperature treatment.

2 | EXPERIMENTAL

2.1 | Fish collection and feeding trials

A total of 300 Emerald Shiners were collected by beach seine from central Lake Ontario near Cobourg, ON, Canada (43°57′16.81″N, 78°10′01.45″W), on 4 November 2011. All the Emerald Shiners selected for this study were between 45 and 70 mm (total length), consistent with age 1 fish that represent the peak abundance of Emerald Shiner size distributions.[42] Fish were transported to the Glenora Fisheries Station (Picton, ON, Canada) in 75-L insulated coolers filled with lake water and provided with supplemental aeration. At the lab, fish were randomly assigned to one of three diets (*Artemia salina* – hereafter Artemia; *Hemimysis anomala* – hereafter Hemimysis; or commercial fish feed pellets – hereafter Pellet) and two biologically relevant temperature treatments (Low: 10°C; High: 20°C)[41] for a total of six treatment groups. Each treatment group was replicated across two tanks with 50 fish per treatment tank (114-L insulated glass aquaria) for a total of 100 fish per diet/temperature group. An additional five Emerald Shiners were sampled and euthanized on the day of collection using an overdose of MS-222 (tricaine methanesulfonate), and were used to set baseline isotope ratios for the fish.

After being collected, wild-caught fish were monitored and provided a diet of TetraMin fish flakes (Tropical fish ‘Rich Mix’; crude protein (46.0%), crude fat (8.0%), fiber (2.0%), moisture (6.0%), phosphorus (1.3%), ascorbic acid (193 mg/kg)) for 10 days (4–13 November, 2011) to observe the transition of these wild-caught fish into the laboratory setting while temperatures were adjusted to treatment temperatures. After this acclimation period, the experimental period began, at which point fish were switched to one of three diets and one of two temperature treatments (six treatment groups in total). Fish assigned to the Artemia treatment received a diet of commercially available frozen *Artemia salina* (Premium Brine Shrimp – San Francisco Bay brand; crude protein 5.0%, fiber 0.3%,

moisture 90.0%). For each new batch of *Artemia salina*, a representative sample was taken for stable isotope analysis. Fish assigned to the Hemimysis treatment were provided with *Hemimysis anomala* sampled directly from the Port of Montreal using vertical hauls of a 0.5 m, 650 μm 3:1 plankton net. *Hemimysis anomala* were collected on one of four dates (3, 10, 21 November or 13 December 2011). The Pellet diet treatment groups were provided with commercial fish feed (Corey Nutrition Co., Fredericton, NB, Canada; 1.0 GR Optimum Fish Feed – lot #11115; protein 53%, fat 17.0%, fiber 1.0%, calcium 2.7%, phosphorus 1.6%, sodium 0.8%, vitamin A 3750 IU/kg, vitamin D 3600 IU/kg, vitamin E 400 IU/kg) for the duration of the experimental period. Fish were fed to satiation on a daily basis, except for the Hemimysis treatment, which was fed to satiation over the first 12 days (19–30 November 2011) to establish a mean amount of food consumed. Following this period, a food ration per fish was provided on each day of the experiment. Leftover food was removed from all tanks on a daily basis, at the same time as the general fish condition and water temperature were monitored.

At regular intervals (experimental days 0, 2, 6, 9, 15, 22, 29, 35, 46, 57, 71, 85) three to five fish were removed from each treatment group, but not all treatment groups were sampled throughout the 85-day period. The Hemimysis group (both temperatures) ended after sampling on day 46 because the supply of wild-caught *Hemimysis anomala* was depleted and could not be replenished. All experimental treatments were designed to run for 57 days, but due to lower than expected mortality for fish transitioning from the wild to a lab environment there were sufficient fish and food supply to extend the Artemia and Pellet (cool temperature) treatments to 85 days.

On a given sampling day fish were removed from each treatment and immediately euthanized after which the liver was dissected from each fish. The remainder of the fish, minus the head and internal organs, was then used as a whole body tissue sample. Both tissues were stored at −20°C for later stable isotope analysis at the Fisk Trophic Ecology Lab (Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON, Canada).

2.2 | Stable isotope analysis

All tissue samples were freeze-dried for 48 h at −50°C and then ground into a fine powder using a mortar and pestle. Due to the potential for lipids to affect $\delta^{13}C$ values,[26,43] lipid extraction was performed on all samples using a 2:1 chloroform/methanol solution that was vortexed, left for 24 h in a 30°C water bath and drained from the vial.[44] This process was repeated and each sample was then dried and re-mixed into a fine powder. The lipid-extracted samples were then weighed into tin capsules (400–800 μg) and processed for $\delta^{13}C$ and $\delta^{15}N$ values using an elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) coupled to a continuous flow isotope ratio mass spectrometer (ThermoFinnigan, San Jose, CA USA). Stable isotope ratios were determined using the equation:

$$\delta X = \left[\left(R_{sample}/R_{standard}\right) - 1\right]$$

where X is either ^{13}C or ^{15}N, R_{sample} is the ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in the sample, and $R_{standard}$ is the ratio in a standard material ($\delta^{13}C$ values – PeeDee Belemnite; $\delta^{15}N$ values – atmospheric nitrogen) and is expressed in ‰ notation. The analytical precision of the mass spectrometer using replicate samples of two internal laboratory standards, bovine muscle and tilapia muscle, analyzed after every 10 samples was ≤0.10 ‰ ($\delta^{15}N$ values; n = 62) and ≤0.11 ‰ ($\delta^{13}C$ values; n = 62) for both standards. The accuracy was monitored using two NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) standards for $\delta^{15}N$ values (NIST 8573 and 8549; n = 18 per standard) and two $\delta^{13}C$ NIST standards (NIST 8542 and 8573; n = 18 per standard). The mean differences from the certified values were +0.20 ‰ (NIST 8573), +0.24 ‰ (NIST 8549), −0.09 ‰ (NIST 8542) and −0.07 ‰ (NIST 8573); therefore, all accuracy and precision measures were within acceptable variation for stable isotope analysis.

2.3 | Statistical analysis

The isotopic compositions of the experimental diets provided to the Emerald Shiners were compared using Kruskal-Wallace models and Wilcoxon *post hoc* comparisons for non-parametric models (dependent variable: $\delta^{13}C$ or $\delta^{15}N$ value; independent variable: diet – Artemia, Hemimysis, Pellet) because the diet groups did not meet the assumption of equal variances among groups to use analysis of variance (ANOVA) models. Emerald Shiners assigned to different treatment groups and holding tanks were compared on day 0 to assess if there was baseline isotopic variation at the beginning of the experiment using separate ANOVA models for each tissue type (whole body or liver; dependent variable: $\delta^{13}C$ or $\delta^{15}N$; independent variable: tank).

Measurements of isotopic turnover were taken only for groups that were found to have significant differences between Emerald Shiner isotopic composition and the respective experimental diet types on day 0. The turnover rate was modelled using an asymptotic exponential non-linear least-squares regression based on the equation:[17,18]

$$\delta_t = \delta_f + (\delta_i - \delta_f) \times e^{(-vt)} \quad (1)$$

where δ_t represents the stable isotope ratio of interest (either $\delta^{13}C$ or $\delta^{15}N$ value), δ_f is the asymptotic stable isotope ratio (i.e. isotopic equilibrium with diet), δ_i is the mean day 0 isotope ratio for that tissue and treatment, v is the turnover rate (see below), and t represents time (i.e. experiment day).

The isotopic tissue turnover rate is determined by both the net growth of a tissue (k_g), e.g. the formation of new tissue as organisms grow in overall size, and the metabolic turnover (m) of existing tissues, and is represented by the equation:

$$v = k_g + m \quad (2)$$

where the net growth parameter (k_g) can be estimated using an non-linear exponential growth model:

$$W_f = W_i \times e^{k_g t} \quad (3)$$

where W_f is the final relative wet mass of an individual (i.e. day t compared with mean day 0 mass), W_i is the mean wet mass of the Emerald Shiners sampled on day 0, k_g represents the net tissue growth and t represents the experiment day. Using the equations above it is possible to determine the total turnover rate (v) and the contribution of growth (k_g) and therefore the metabolic contribution to turnover (m) by rearranging Equation 2.

The overall tissue turnover rate (v) was used to estimate the number of experimental days required to achieve 50% ($T_{0.5}$) and 95% ($T_{0.95}$) turnover:

$$T_{\alpha} = \frac{\ln(1-\alpha)}{-v} \quad (4)$$

where T_{α} is the time (i.e. days) required to reach the target (α) turnover percentage (50% or 95%).

Given the role of growth in the models above (net growth component of turnover, k_g) the change in mass, a proxy for overall growth, over the experimental period was also examined using linear regression analysis (dependent variable: fish mass; independent variable: experiment day). Separate models were used for each diet and temperature treatment, resulting in six regression relationships that covered the entire sampling period of each treatment from day 0 to the final day that the fish were sampled (up to 85 days; see above).

For Emerald Shiner groups that were already in isotopic equilibrium with the diets provided, i.e. the isotope ratios of the Emerald Shiners on day 0 did not differ significantly from the $\delta^{13}C$ or $\delta^{15}N$ values of the experimental foods, we estimated the DTDF values.

TABLE 1 Isotopic compositions of the three prey groups used as diet treatments and the isotope ratios of Emerald Shiners (*Notropis atherinoides*) sampled from each diet treatment on day 0 of the experimental period

				Body tissue				Liver tissue			
Prey type	n	$\delta^{13}C$	$\delta^{15}N$	Day 0 $\delta^{13}C$	$\Delta^{13}C$ Fish - Food	Day 0 $\delta^{15}N$	$\Delta^{15}N$ Fish - Food	Day 0 $\delta^{13}C$	$\Delta^{13}C$ Fish - Food	Day 0 $\delta^{15}N$	$\Delta^{15}N$ Fish - Food
Artemia	5	−30.4 ± 0.2^{A}	19.2 ± 0.2^{A}	−21.5 ± 0.3	+9.0	12.3 ± 0.1	−6.9	−22.9 ± 0.4	+7.5	12.7 ± 0.2	−6.5
Hemimysis	4	−21.0 ± 0.8^{B}	10.2 ± 0.7^{B}	−21.2 ± 0.3	+0.2	12.4 ± 0.1	+2.2	−21.7 ± 0.4	+0.7	12.6 ± 0.2	+2.4
Pellet food	4	−20.8 ± 1.1^{B}	9.1 ± 1.2^{B}	−21.7 ± 0.3	−0.9	12.3 ± 0.1	+3.2	−22.1 ± 0.4	−1.3	12.2 ± 0.2	+3.1

Diet groups identified by the same letter indicate similar isotopic compositions based on separate Kruskal-Wallis rank sum models for $\delta^{13}C$ and $\delta^{15}N$ values. The differences in mean (± SD) Emerald Shiner isotopic composition and the food type for each treatment are displayed separately for each isotope ratio and for each fish tissue sampled.

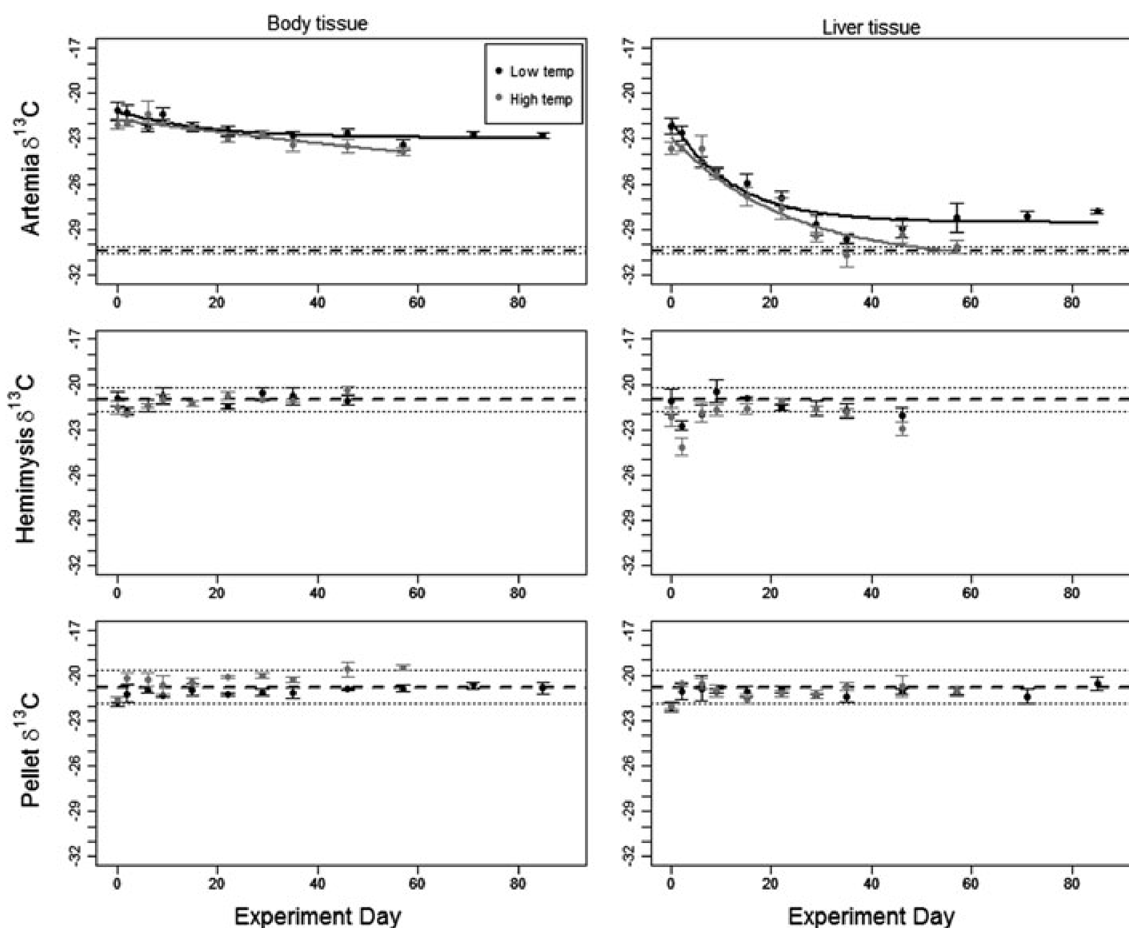

FIGURE 1 Mean (± 1 SE) $\delta^{13}C$ values of Emerald Shiner (*Notropis atherinoides*) body (left column) and liver tissues (right column) collected at regular sampling intervals for up to 85 days (see section 2 for sampling details). Plots with lines include the line of fit for the asymptotic exponential equation (see section 2) used to estimate the turnover rate of tissues following a change to one of three diet types (Artemia, Hemimysis, or Pellet). Groups without lines did not show a transition in isotope ratios towards equilibrium with the provided diets and, therefore, could not be fitted with an asymptotic relationship. The line in each plot represents the mean (± 1 SE) of the $\delta^{13}C$ values for each diet treatment

Based on this criterion DTDF values were determined for the Pellet and Hemimysis treatments only (see below). Diet-tissue discrimination factors were estimated by subtracting the mean isotope ratio of the food type (δ_d value) from the mean value of the Emerald Shiners for that diet and temperature treatment for all fish sampled after a minimum of 46 days on the experimental diets (δ_t value).[17] The DTDFs were then determined for each diet and temperature group by calculating the mean (± 1 standard deviation) difference between fish and their food across all sampling days within the time range.

All analyses were completed in either JMP (version 12.0; SAS Institute, Cary, NC, USA) or R-Studio (version 0.98; R Development Core Team, Vienna, Austria) with statistical significance determined at α = 0.05 for all analyses.

3 | RESULTS

The food types used as the three experimental diets differed significantly for both δ^{13}C values (Kruskal-Wallis; X^2 = 8.60, P = 0.01) and δ^{15}N values (Kruskal-Wallis; X^2 = 9.10, P = 0.01; Table 1). *Post hoc* comparisons of the diet treatments indicated that the Hemimysis and Pellet treatments were not significantly different for both isotope ratios (Wilcoxon δ^{13}C, P = 0.89; δ^{15}N, P = 0.31), but both food groups had significantly higher δ^{13}C values (Wilcoxon δ^{13}C, both P = 0.02) and lower δ^{15}N values (Wilcoxon δ^{15}N, both P = 0.02) than the Artemia food group.

On day 0 of the experimental period the Emerald Shiners had body tissue isotope ratios that were similar across all holding tanks (δ^{13}C: $F_{11,10}$ = 0.66, P = 0.75; δ^{15}N: $F_{11,10}$ = 0.82, P = 0.63). Liver tissue samples on day 0 also showed non-significant differences among all tanks (δ^{13}C: $F_{11,10}$ = 1.77, P = 0.19; δ^{15}N: $F_{11,10}$ = 1.34, P = 0.33). Based on these day 0 results, tank replicates of the same diet and temperature treatment were not considered a primary factor in subsequent analyses.

There was limited isotopic difference between Emerald Shiners on day 0 and either the wild-caught *Hemimysis anomala* (δ^{13}C ≤ 0.7 ‰, δ^{15}N ≤ 2.4 ‰) or the commercial pellet feed (δ^{13}C ≤ 1.3 ‰, δ^{15}N ≤ 3.2 ‰; Table 1); thus, these two diet treatments were excluded from the analysis of tissue turnover rate. The Shiners did differ from the *Artemia salina* on day 0 for both isotope ratios of interest (δ^{13}C ≥ 7.5 ‰, δ^{15}N ≥ 6.5 ‰) and were included in asymptotic models of tissue turnover (Figures 1 and 2). The predicted equilibrium isotope ratios (δ_f values) of body tissue samples in the low-temperature diet treatment did shift towards those of the Artemia diet, but had predicted asymptote values that were +7.4 ‰ (δ^{13}C values) and −4.4 ‰ (δ^{15}N values) compared with the food. In comparison, liver

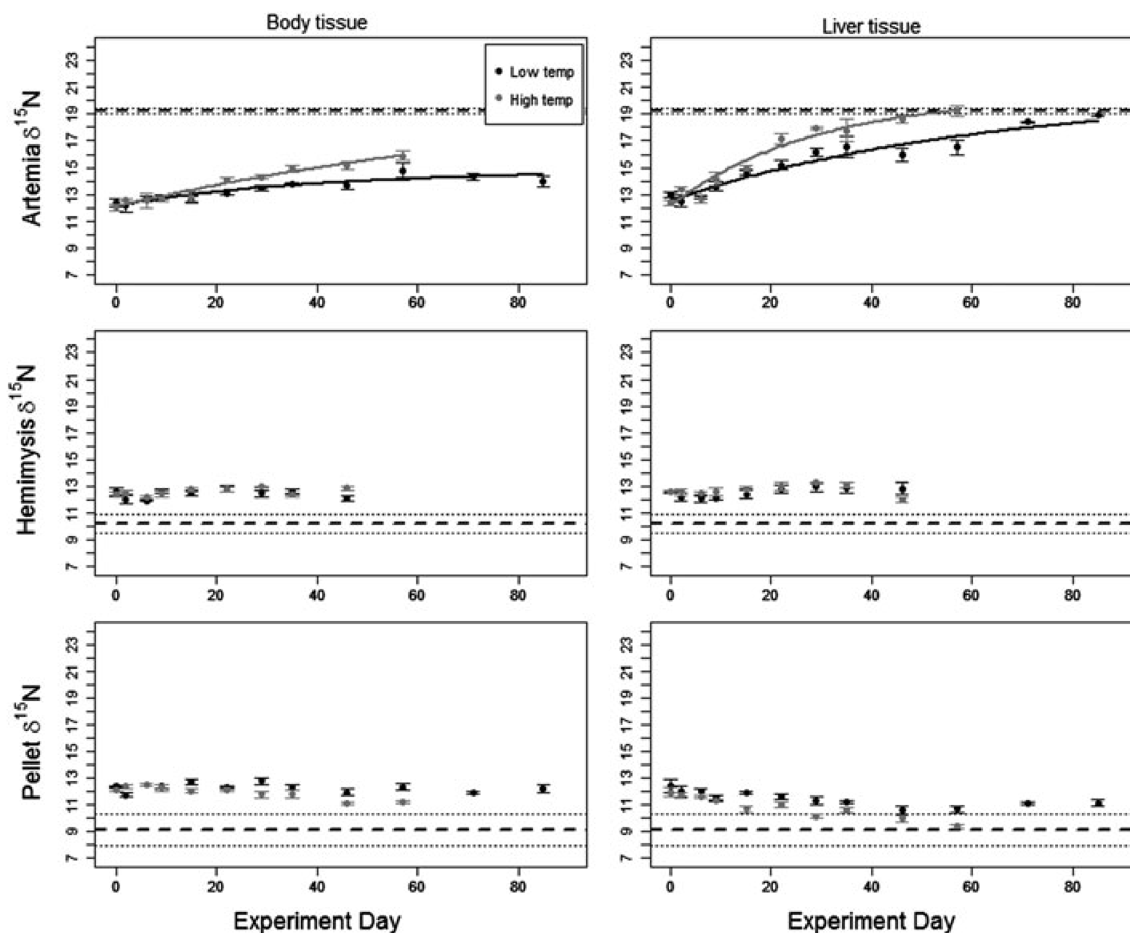

FIGURE 2 Mean (± 1 SE) δ^{15}N values of Emerald Shiner (*Notropis atherinoides*) body (left column) and liver tissues (right column) collected over a period of up to 85 days after being switched to one of three diet treatments (Artemia, Hemimysis, or Pellet). Also shown are the mean (± 1 SE) values of each diet treatment using the dashed lines. For the groups that showed a transition towards the new diet treatment (see section 2) the line of fit for the asymptotic exponential relationship that estimates tissue turnover parameters is shown

tissue samples from the same fish in the low-temperature Artemia treatment had predicted equilibrium isotope ratios within ±1.2 ‰ of those of the food provided (Table 2; Figures 1 and 2). The high-temperature treatment group had predicted equilibrium ratios within 1.6 ‰ of that of the Artemia food for both δ^{13}C and δ^{15}N values (Table 2). The estimated time to achieve both 50% and 95% turnover were greater for body tissues (high temperature $T_{0.5}$ = 68–166 days; $T_{0.95}$ = 295–719 days) than for liver tissues (high temperature $T_{0.5}$ = 16–20 days; $T_{0.95}$ = 69–85 days; see Table 2 for details).

Emerald Shiners in the Artemia diet treatment had non-significant changes in mass over the course of the experimental period (Figure 3; Table 3). In the Hemimysis and Pellet treatments, the Shiner mass remained stable at low temperatures and increased in the high-temperature treatment (Table 3). The stable masses of Emerald Shiners fed *Artemia salina* throughout the experiment resulted in low growth constants (mean k_g = 0.002) relative to the catabolic contributions to turnover rate (mean m = 0.030; Table 2).

Estimates of DTDFs were made for the Hemimysis and Pellet diet treatments because for these groups the δ^{13}C and δ^{15}N values in the fish were similar to those of the food provided in the diet treatments on day 0 and had unresolvable asymptotic models for tissue turnover (see above; Figures 1 and 2). The estimated δ^{13}C DTDFs (Δ^{13}C) for the Emerald Shiners, regardless of diet group, temperature treatment, or the tissue sampled, were within ±2 ‰ of those of their food source (Δ^{13}C range − 1.9 to +1.5 ‰; Table 4). In comparison, the Δ^{15}N values showed that Emerald Shiners had consistently higher δ^{15}N values than their food (Δ^{15}N range + 0.6 to 2.7 ‰; Table 4).

4 | DISCUSSION

The experimental manipulations of Emerald Shiner diets in this study resulted in variable measures of turnover rates and DTDFs of δ^{13}C and δ^{15}N values between two temperature treatments, providing insights into non-diet-related variation in the isotopic composition of a widespread small-sized North American fish. Isotopic turnover, modelled using *Artemia salina*, the only diet type that significantly differed from the Emerald Shiners on day 0, showed different rates of turnover between tissue types (liver turnover > whole body turnover) and holding temperatures (high temperature > low temperature). Samples of body tissue in the low-temperature treatment produced unrealistic DTDFs relative to those of the food provided (δ^{13}C = +7.4 ‰; δ^{15}N = −4.4 ‰) although values for both isotope ratios shifted in the expected direction, possibly related to the duration of the experiment and the rate of turnover for body tissues. The isotopic similarity between Emerald Shiners and both Pellet feed and *Hemimysis anomala* allowed for estimates of DTDFs and showed that the Δ^{13}C and Δ^{15}N values fell within the ranges reported for fishes, but had variation sufficient to affect the interpretation of smaller forage fish species and estimates of trophic structure in populations, assuming constant DTDFs across trophic levels.

The rate of tissue turnover is broken down into two primary drivers; (i) turnover related to metabolic processes, i.e. replacement of old cells, and (ii) the formation of new tissues, i.e. growth.[18] Given the relatively constant mass of fish in the *Artemia* diet group, measures of isotopic turnover rate in this study are related to metabolic functions rather than growth (Table 3). Indeed, the metabolic Δ^{15}N values in this study (range 0.016–0.033 day^{-1}) are consistent with those found in other laboratory feeding experiments, including those for a tropical fish, the Leopard Coralgrouper (*Plectropomus leopardus*; 0.005–0.068 day^{-1}),[17] and a freshwater stingray (*Potamotrygon motoro*; 0.0041–0.15 day^{-1}).[22] Furthermore, within the *Artemia* diet group the numbers of days to reach 50% and 95% equilibrium (i.e. $T_{0.5}$ and $T_{0.95}$) for δ^{13}C and δ^{15}N values were lower for liver than for the whole body from the same temperature treatment. The slower turnover of body tissues, our proxy for white muscle,[45] than for liver is consistent with patterns across fish species and has been linked to the higher metabolic activity of liver.[26,46] Liver has been described as a regulatory tissue with faster isotopic turnover[46] due to the presence of proteins and essential amino acids that act in various body functions, processes that are likely to be necessary across a wide range of temperatures.[47-49] In comparison,

TABLE 2 Tissue turnover estimates based on asymptotic exponential non-linear regression analysis of δ^{13}C and δ^{15}N values for body and liver tissues samples of Emerald Shiners (*Notropis atherinoides*) held on a diet of Artemia for an 85-day period at two temperature levels

Tissue	Isotope	Temp	δ_i	δ_f	γ	v	k	m	r^2	$T_{0.5}$	$T_{0.95}$
Body	δ^{13}C	Low	−21.1	−23.0*	−2.841	0.058	0.000	0.058	0.44	12*	51*
		High	−22.1	−31.9	−5.481	0.004	0.003	0.001	0.59	166	719
	δ^{15}N	Low	12.3	14.8*	−3.702	0.025	0.000	0.025	0.49	28*	121*
		High	12	20.8	−4.588	0.010	0.003	0.007	0.83	68	295
Liver	δ^{13}C	Low	−22.1	−28.6	−2.519	0.081	0.000	0.081	0.72	9	37
		High	−23.6	−31.2	−3.139	0.043	0.002	0.041	0.80	16	69
	δ^{15}N	Low	12.9	20.6	−4.146	0.016	0.000	0.016	0.80	44	189
		High	12.4	20.4	−3.345	0.035	0.002	0.033	0.86	20	85

Both the *Hemimysis* and the pellet diet treatment groups have been omitted from this table because they did not show indications of turnover towards the diet treatment provided and were subsequently excluded from the turnover rate analysis.

Treatment groups marked with an asterisk (*) probably have inaccurate estimates of the turnover rates based on the predicted δX value for that group, see section 3 for details.

Parameter estimates include the initial (Day 0) δ^{13}C/δ^{15}N values (δ_i; ‰), predicted equilibrium value (δ_f; ‰), turnover rate constant (v; day^{-1}), tissue growth rate (k; day^{-1}), tissue metabolic turnover (m; day^{-1}), measure of model fit (r^2), and incorporation time estimates for 50% ($T_{0.5}$) and 95% ($T_{0.95}$) turnover (days).

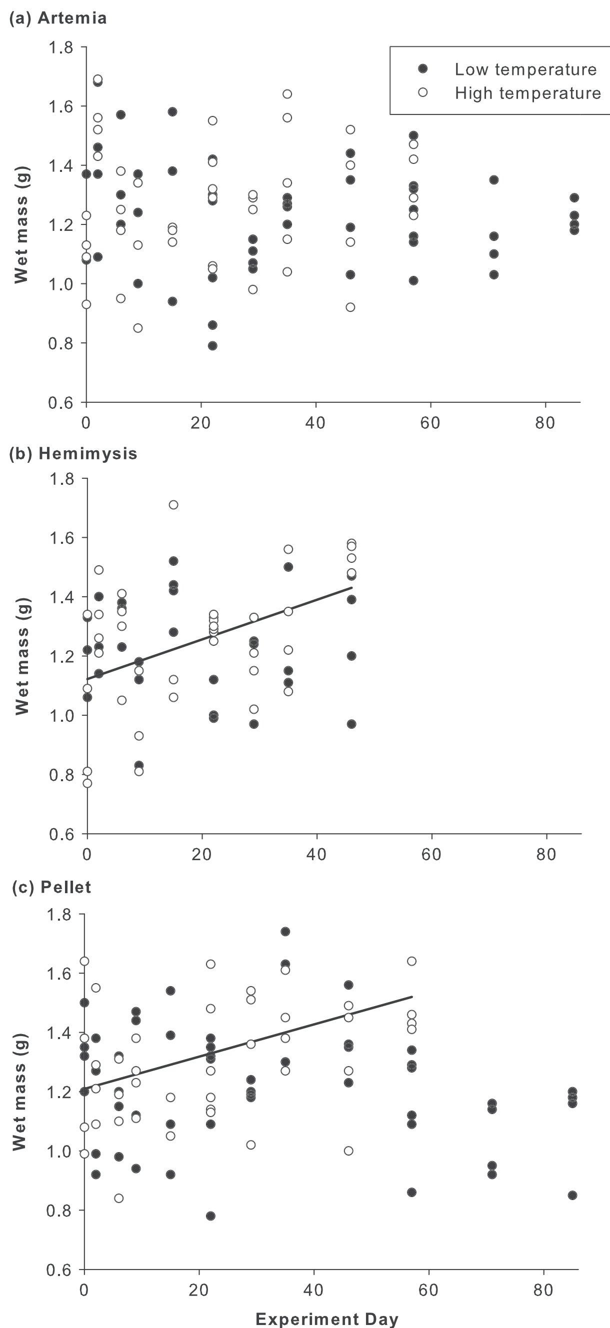

FIGURE 3 Change in Emerald Shiner (*Notropis atherinoides*) wet mass (g) when held at one of two temperatures and provided one of three diet treatments (Artemia, Hemimysis, or Pellet) over a period of up to 85 days

white muscle tissue has a largely protein storage function, experiencing the greatest isotopic change during periods of growth when new tissue is being formed.[46,49] Given the different physiological roles of the tissues and the maintenance of Emerald Shiner size during the experimental period, the faster turnover rates for liver tissue across temperatures were consistent with the increased metabolic role of liver even in the absence of growth.

Temperature had variable effects on the estimates of turnover between the tissue types sampled. Lower temperatures were generally associated with slower turnover, except for liver δ^{13}C values, and this may be related to decreased growth and general rates of ecotherm biochemical processes at lower temperatures.[18] Across taxa and temperatures metabolically active tissues, including liver, have lower $T_{0.5}$ than structural tissues, i.e. muscle.[50] Furthermore, variability in ectotherm tissue turnover rates has been related to the application of simplified models of isotopic incorporation that are better suited to structural tissues and endotherms, but which do not account for the unique metabolic roles and structural variation among ectotherm tissues.[50,51] It is beyond the scope of this study to establish the mechanisms of variation in turnover rate with temperature, but the rates observed here are consistent with liver and body tissues responding to temperature differently. Indeed, the δ^{13}C values of liver turned over fastest in the low-temperature treatment, possibly related to the functional role of this organ across a gradient of temperatures, and this is consistent with fish hepatosomatic index values (measures of total liver mass to total body mass) that have been found to increase at lower temperatures.[52,53] Therefore, physiological responses to a reduction in holding temperature on day 0 of the experiment may have contributed to more rapid turnover than with the high-temperature treatment. Selection of tissues for sampling in isotope studies is generally made based on assumptions of consistent variation in turnover rates among tissue types, but the variable effects of temperature on body and liver tissues found here support the added consideration of thermal environment when choosing a tissue type to sample.

Diet-tissue discrimination factors of δ^{13}C values are commonly expected to be lower than those of δ^{15}N values, e.g. +0.4 ± 1.3 ‰.[12,54] Across the Hemimysis and Pellet diets, holding temperatures, and tissue types sampled, the mean (± SD) Δ^{13}C value of Emerald Shiners was +0.03 ± 1.0 ‰, consistent with general expectations for Δ^{13}C values. However, it is the range of Δ^{13}C values (−1.9 to +1.5 ‰) that has the potential to affect isotope-based inferences. Between the diet types (Hemimysis and Pellet) the Δ^{13}C values were consistently lower for fish fed Hemimysis, the wild prey type, when comparing the same tissue and holding temperature. In addition, a positive relationship between temperature and Δ^{13}C values has been identified in multiple studies[33,34] and we observed this pattern in our treatments (mean low temperature Δ^{13}C = −0.1 ± 0.7 ‰; high temperature = +0.3 ± 1.4%). Despite mean Δ^{13}C values consistent with variation observed previously, we have demonstrated that these values can differ between food sources, possibly related to diet-dependent effects on DTDFs,[30] and temperatures relevant to temperate fishes both of which are relevant to studies of wild temperate fishes.

In contrast to carbon, the Δ^{15}N values of Emerald Shiners (mean = +1.7 ± 0.7 ‰; range + 1.7–3.2 ‰) included values that were lower than the frequently assumed +3.4 ± 1.0 ‰,[10,12,54] but fall within ranges reported for multiple fish species and developmental stages (Δ^{15}N range: 0 and +3.8 ‰, liver; −0.3 to +6 ‰, whole body samples).[17] Similar Δ^{15}N values to those reported here have been found for a larger-sized fish (e.g. Leopard Coralgrouper Δ^{15}N = −0.15 to +1.84 ‰).[17] Furthermore, temperature was expected to have a negative relationship with Δ^{15}N.[33,34] The Emerald Shiners here did show declining Δ^{15}N values with temperature (mean low temperature Δ^{15}N = +1.9 ± 0.6 ‰; high temperature = +1.2 ± 0.8 ‰), but this variation was not as distinct as previously reported (e.g. *Daphnia*

TABLE 3 Linear regression models of Emerald Shiner (*Notropis atherinoides*) mass (g) over an experimental period of up to 85 days during which fish were assigned to a diet (Artemia, Hemimysis, or Pellet) and temperature (low or high) treatment

Diet	Temperature	Initial mass (mean ± SD)	Final mass (mean ± SD)	r^2	F	P	Line equation
Artemia	Low	1.20 ± 0.07	1.23 ± 0.02	0.02	0.78	0.38	Mass (g) = 1.25–0.0008 × Day
	High	1.08 ± 0.05	1.47 ± 0.08	0.07	3.01	0.09	Mass (g) = 1.21 + 0.003 × Day
Hemimysis	Low	1.20 ± 0.06	1.26 ± 0.11	<0.01	0.02	0.89	Mass (g) = 1.23 + 0.0003 × Day
	High	1.00 ± 0.13	1.54 ± 0.02	0.21	9.33	0.004	Mass (g) = 1.12 + 0.007 × Day
Pellet	Low	1.34 ± 0.06	1.10 ± 0.08	0.04	1.87	0.18	Mass (g) = 1.27–0.001 × Day
	High	1.27 ± 0.15	1.62 ± 0.14	0.14	6.75	0.01	Mass (g) = 1.21 + 0.005 × Day

TABLE 4 Estimates of diet-tissue discrimination factors (DTDFs, mean +/− SD) for Emerald Shiner (*Notropis atherinoides*) maintained on Hemimysis (*Hemimysis anomala*) or Pellet (Corey Nutrition Optimum Fish Feed) diet groups under either low (10°C) or high (20°C) temperature conditions

				Body tissue			Liver tissue		
Isotope	Diet	Temperature	Mean δ_{diet}	n	δX_{fish}	Δ value	n	δX_{fish}	Δ value
$\delta^{13}C$	Hemimysis	Low	−21.0	4	−21.1 ± 0.6	−0.1 ± 0.6	4	−22.0 ± 0.9	−1.0 ± 0.9
		High		4	−20.4 ± 0.5	+0.6 ± 0.5	4	−22.9 ± 0.9	−1.9 ± 0.9
	Pellet	Low	−20.8	9	−20.8 ± 0.5	+0.2 ± 0.5	9	−21.0 ± 0.7	−0.2 ± 0.7
		High		9	−19.5 ± 0.7	+1.5 ± 0.7	9	−20.9 ± 1.0	−0.1 ± 1.0
$\delta^{15}N$	Hemimysis	Low	10.2	4	12.1 ± 0.3	+1.9 ± 0.3	4	12.8 ± 0.8	+2.6 ± 0.8
		High		4	12.9 ± 0.3	+2.7 ± 0.3	4	12.0 ± 0.4	+1.8 ± 0.4
	Pellet	Low	9.1	9	12.1 ± 0.5	+1.9 ± 0.5	9	10.9 ± 0.5	+1.8 ± 0.5
		High		9	11.2 ± 0.2	+1.0 ± 0.2	9	9.7 ± 0.5	+0.6 ± 0.5

The DTDFs are represented by the mean difference between the $\delta^{13}C$ or $\delta^{15}N$ values of Emerald Shiner body and liver tissues based on the food type provided ($\Delta^{13}C$ or $\Delta^{15}N$) and were calculated for Emerald Shiners sampled after a minimum of 46 days on the experimental diets.

magna $\Delta^{15}N$ = + 5.0 ‰ (13°C) vs. $\Delta^{15}N$ = + 2.7 ‰ (27°C)).[33] Furthermore, between the two diet types the $\Delta^{15}N$ values were lower for fish fed commercial pellets ($\Delta^{15}N$ = +1.5 ± 0.7 ‰) than for wild-caught *Hemimysis* ($\Delta^{15}N$ = +2.2 ± 0.6). The lower than expected $\Delta^{15}N$ values and variable effects of both temperature and diet type not only have implications for studies of Emerald Shiners and other small-bodied fish, but also for inferences about high trophic level consumers due to the frequent use of trophic position models that do not incorporate variable DTDFs across trophic levels.

5 | CONCLUSIONS

In this study, even when growth was removed as a factor, tissue-specific turnover rates still occurred, probably related to the physiological functions of tissue types and the effect that this has on metabolic turnover rates. These tissue-specific dynamics should not be viewed only as a negative aspect of using stable isotope analysis because they provide opportunities to answer questions by comparing between tissue types.[55,56] Temperature variation also had an effect on turnover, but this variation was not consistent between tissue types. Indeed, liver may be the most accurate tissue to sample across temperature gradients, e.g. when interested in diet during cold periods, because this metabolically active tissue may continue assimilating resources at similar rates despite temperature shifts. We found that the DTDFs in this study were largely consistent with the findings from other studies, and independent of tissue or temperature, but the variation that we observed from multi-species average $\Delta^{15}N$ values supports efforts to establish species-specific values.

Experimental determination of turnover and DTDFs with wild-caught fish and food sources may face limitations compared with experiments using laboratory-reared fish and commercially available food sources. The length of the study period to assess turnover and DTDFs can be challenging when using fish with relatively small growth rates and may be a driving factor of biases to experimental studies of larval and juvenile fishes.[17] The availability of commercial food products provides ample supplies of food for diet experiments, but the artificial manufacturing process of these feeds may miss isotopic variation related to the consumption of wild-type resources, e.g. *Hemimysis anomala* in this study. The similar isotopic compositions of the wild-caught Emerald Shiners to two of the three food types eliminated the possibility of measuring turnover for all our diet treatments and added uncertainty to DTDFs based on tissues that may not have fully assimilated the experimental feeds. We consider this unlikely, due to the maintenance or growth of fish from all diet treatments, i.e. they did not decline in mass over time, and the physiological role of liver that made an absence of turnover unlikely. Despite these caveats regarding the use of wild-caught fishes and prey sources, studies such as this one are tackling concerns about how turnover and DTDF occur in wild populations provided more natural-type resources to consume.

Finally, understanding foraging dynamics of wild populations typically involves sampling a community composed of many taxa, species, and life stages that experience temporal and spatial variation in a multitude of environmental variables. As such, we need to establish the isotopic relationships between consumers and their food sources at multiple trophic levels and environmental conditions. The general acknowledgement that small-bodied fishes, e.g. Shiners and darters, are largely absent from experimental studies of diet and turnover must be addressed not only to understand these fishes but also higher trophic level species due to the frequency of isotope studies that do not consider variation in DTDFs between trophic levels.[17,29] Stable isotope analysis continues to be an evolving technique for inferring diets and habitat use over extended time periods, but we must conduct experiments to understand the influence of environmental and dietary variables on isotopic relationships for multiple functional groups within a community if we are to maximize the accuracy of our inferences.

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