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RESEARCH ARTICLE

High variability in stable isotope diet–tissue discrimination factors of two omnivorous freshwater fishes in controlled *ex situ* conditions

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ABSTRACT

Diet–tissue discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) are influenced by variables including the tissues being analysed and the taxon of the consumer and its prey. Whilst differences in $\Delta^{13}C$ and $\Delta^{15}N$ are apparent between herbivorous and piscivorous fishes, there is less known for omnivorous fishes that consume plant and animal material. Here, the omnivorous cyprinid fishes *Barbus barbus* and *Squalius cephalus* were held in tank aquaria and exposed to three diets that varied in their constituents (plant based to fishmeal based) and protein content (13% to 45%). After 100 days and isotopic replacement in fish tissues to 98%, samples of the food items, and dorsal muscle, fin tissue and scales were analysed for $\delta^{13}C$ and $\delta^{15}N$. For both species and all diets, muscle was always enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$ compared with fin tissue and scales. Across the different diets, $\Delta^{13}C$ ranged between 2.0‰ and 5.6‰ and $\Delta^{15}N$ ranged between 2.0‰ and 6.9‰. The diet based on plant material (20% protein) always resulted in the highest discrimination factors for each tissue, whilst the diet based on fishmeal (45% protein) consistently resulted in the lowest. The discrimination factors produced by non-fish diets were comparatively high compared with values in the literature, but were consistent with general patterns for some herbivorous fishes. These outputs suggest that the diet–tissue discrimination factors of omnivorous fishes will vary considerably between animal and plant prey, and these specific differences need consideration in predictions of their diet composition and trophic position.

KEY WORDS: *Barbus barbus*, *Squalius cephalus*, Stable isotope analysis, Bayesian mixing models, Discrimination factors, Omnivorous fishes

INTRODUCTION

The application of stable isotope analysis to ecology provides considerable insight into many aspects of species' interactions (Boecklen et al., 2011). Natural variations in the ratios of ^{13}C to ^{12}C ($\delta^{13}C$) and ^{15}N to ^{14}N ($\delta^{15}N$) have been applied widely to trophic and food web studies in aquatic environments and, for example, have revealed the impacts of non-native fishes in native fish communities (Cucherousset et al., 2012; Tran et al., 2015), and the movements and ecology of endangered species (Seminoff et al., 2012; Hamidan et al., 2015). Critical to the interpretation of the stable isotope ratios is the stepwise enrichment that occurs along trophic levels between the consumer species and its prey resources (Boecklen et al., 2011), otherwise known as the isotopic fractionation or discrimination factor (Martínez del Rio and Wolf, 2005).

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An increasingly important application of stable isotope discrimination factors is within Bayesian mixing models that predict the proportional composition of consumer diets from data on their putative food resources (Jackson et al., 2011). These models have been applied widely in recent years, including application to questions relating to the use of allochthonous and autochthonous food resources in freshwater consumers (Grey and Jackson, 2012) and the relative contributions of native and non-native taxa to consumer diet (Britton et al., 2010). A fundamental requirement of these models is robust estimates of the stable isotope discrimination factors between the prey resources and consumer tissue being analysed (Bond and Diamond, 2011; Phillips et al., 2014). In general, they assume discrimination factors are constant across the size range of the consumer being studied and their diet spectrum, overlooking potential differences that might occur through, for example, diet changes through ontogeny (Mill et al., 2007; Phillips et al., 2014).

This general application of constant discrimination factors across consumers within mixing models is potentially problematic, as studies increasingly suggest they can vary between species, ages, diet compositions, body sizes, sample preparations and tissue types (Locke et al., 2013; Brush et al., 2012; Busst et al., 2015). The commonly cited values of 3.4±0.98‰ for $\delta^{15}N$ and 0.39±1.3‰ for $\delta^{13}C$ (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002) could thus be inappropriate for use in many models, resulting in dietary predictions whose variability within and between species could be due more to inappropriate discrimination factors than actual dietary variation (Busst et al., 2015). The specific food items that contribute to the overall diet of a consumer can substantially influence their stable isotope discrimination factors (Caut et al., 2008). For example, McCutchan et al. (2003) suggested that discrimination factors of $\delta^{15}N$ were lower in consumers with invertebrate-based diets (1.4±0.21‰) than in consumers with diets containing higher protein contents (3.3±0.26‰); mixed diets provided values between these (2.2±0.30‰). In herbivorous fishes, discrimination factors for $\delta^{15}N$ have been recorded as high as 5.25‰ (Mill et al., 2007), with Carassou et al. (2008) suggesting they have distinct stable isotope discrimination factors that distinguish them from piscivorous fishes.

Correspondingly, understanding the relationships between the long-term composition of the diet of consumer species and their stable isotope discrimination factors with their dietary items is a pre-requisite for obtaining robust diet predictions from models (Parnell et al., 2013; Phillips et al., 2014). Whilst there are often clear discrimination differences apparent between herbivorous, insectivorous and piscivorous fishes, this potentially becomes more complex for omnivores, with their diets potentially comprising of a wide range of food resources with contrasting discrimination factors (Caut et al., 2008; Florin et al., 2011). This variability could thus be incorporated into models and whilst this could be done via use of their weighted averages (Florin et al., 2011), Robbins et al.

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(2010) suggested erroneous estimates of fishmeal and soybean meal occurred in the dietary nitrogen of assimilated mixed diets when these were used in mixing models.

The aim of this study was to therefore quantify and assess the extent to which stable isotope discrimination factors were significantly affected by diet composition in two omnivorous fishes and across three tissue types, dorsal muscle, pelvic fin tissue ('fin-clip') and scales. The model species were selected to be representative of omnivorous freshwater fishes and were of the Cyprinidae family, European barbel *Barbus barbus* (Linnaeus) and chub *Squalius cephalus* (Linnaeus). Although both tend to be rheophilic, they also tolerate lentic conditions (Britton and Pegg, 2011), and are relatively long lived (>15 years; Britton, 2007). They are also present across much of Eurasia and have socio-economic importance as an angler-target species. Importantly, in the wild, both are highly omnivorous, with diets comprising a wide range of plant and animal taxa, including insect larvae, crustaceans, fish and macrophytes (Mann, 1976; Britton and Pegg, 2011). Thus, when estimations of their diet compositions are based on stable isotope data, these analyses could require due cognisance of differences in discrimination factors between common items in their diet. The first objective was to test the hypothesis that diet–tissue discrimination for the three tissues of each species varies according to their exposure to three constant diets of variable protein composition and concentration. The second objective was to assess how the diet–tissue discrimination values relate to the difference in pre- and post-experimental stable isotope values of tissues and the change in body mass of individual fishes.

MATERIALS AND METHODS

Experimental design

The model fishes were sourced from pond aquaculture where their diets were a mix of natural foods (zooplankton and macro-invertebrates), supplemented with some formulated feeds. Their starting lengths in the experiment were approximately 60–80 mm fork length (mean 69.4±0.9 mm) and their body mass was 2–7 g (mean 3.8±0.2 g). All experimental procedures and ethical regulations were completed under UK Home Office project licence PPL30/3094.

The experiment exposed the fish to three fixed diets (Table 1). These were fed *ad libitum* for 100 days. The duration of the experiment was balanced between feeding fish a single food source for an extended period and their tissues reaching an isotopic steady state with their new diet, i.e. turnover leading to equilibrium. Ethically, the issue limiting the duration of the experiment to 100 days was that considerable changes in body condition, lipid and mineral composition can occur when fish are fed fixed diets, with these changes potentially impacting their welfare (e.g. Fontagné et al., 1998; Kamler et al., 2012; Böhm et al., 2014). Consumers are generally considered to have equilibrated to their food resources in 4–5 half-lives, i.e. 94–97% isotopic replacement in their tissues (Hobson and Clark, 1992). Estimates of half-lives and isotopic replacement for the fishes over 100 days are provided here by literature and calculated estimates. The estimated half-life for consumers of 1 g at 20°C is 23 days for $\delta^{13}C$ (100 days=4.3 half-lives or 95% replacement) and 25 days for $\delta^{15}N$ (100 days=4.0 half-lives or 94% replacement) (Thomas and Crowther, 2015). Estimates using the mean starting mass of the fishes and equations from Vander Zanden et al. (2015) provided a half-life for both isotopes as low as 17 days (5.9 half-lives or 98.3% replacement). Estimates from equations of Thomas and Crowther (2015) suggested the half-life for $\delta^{13}C$ was 30 days (so 3.4 half-lives or 90% replacement in 100 days) and for $\delta^{15}N$ was 32 days (3.1 half-lives or 88% replacement).

The three diets used in the experiment were all based on pelletized feeds; these were preferred to natural foods given that the experiment was based on exposing the species to these diets over a set time period and thus variability in the stable isotope values of the feeds would vary little through use of single, homogeneous batches, and arguably less than if natural food sources were utilized (Busst et al., 2015). They also enabled fish to be exposed to set levels of the food over the course of the experiment. Correspondingly, the first diet was 'Red krill pellets', henceforth referred to as 'krill', comprising 13.7% protein, 11.5% fat, 6.5% crude fibre and 3.8%

Table 1. Number, mean fork starting and end lengths and stable isotope values per diet and species for each tissue type

Diet	Species	*N*	Mean starting and end length (mm)	Tissue	Mean $\delta^{13}C$ (‰)	95% CI	Mean $\delta^{15}N$ (‰)	95% CI
1					−24.62±0.11	−24.83 to −24.42	5.03±0.18	4.67 to 5.39
2					−24.94±0.26	−25.45 to −24.43	4.35±0.15	4.04 to 4.65
3					−22.15±0.06	−22.32 to −21.99	8.51±0.07	8.33 to 8.70
1	*B. barbus*	9	70±2, 90±2	Muscle	−21.79±0.08	−21.96 to −21.63	11.03±0.12	10.79 to 11.27
				Fin	−20.65±0.08	−20.82 to −20.49	10.03±0.12	9.79 to 10.27
				Scales	−20.23±0.08	−20.40 to −20.07	9.25±0.12	9.01 to 9.49
2		8	76±2, 91±1	Muscle	−20.98±0.09	−21.16 to −20.80	11.22±0.12	11.00 to 11.45
				Fin	−19.49±0.09	−19.67 to −19.31	10.78±0.12	10.56 to 11.01
				Scales	−19.16±0.09	−19.33 to −18.98	9.89±0.12	9.66 to 10.12
3		9	87±2, 118±2	Muscle	−19.59±0.04	−19.66 to −19.51	10.92±0.04	10.84 to 10.99
				Fin	−18.31±0.04	−18.38 to −18.24	10.60±0.04	10.52 to 10.68
				Scales	−17.41±0.04	−17.49 to −17.34	10.70±0.04	10.63 to 10.78
1	*S. cephalus*	10	69±1, 90±3	Muscle	−22.26±0.07	−22.41 to −22.11	10.09±0.13	9.83 to 10.34
				Fin	−21.88±0.08	−22.04 to −21.73	9.62±0.14	9.35 to 9.89
				Scales	−20.26±0.07	−20.40 to −20.11	9.12±0.13	8.86 to 9.37
2		10	69±2, 83±2	Muscle	−21.48±0.09	−21.66 to −21.30	11.02±0.10	10.82 to 11.22
				Fin	−20.70±0.09	−20.88 to −20.52	11.14±0.10	10.94 to 11.33
				Scales	−19.93±0.09	−20.11 to −19.75	10.04±0.10	9.84 to 10.23
3		11	97±6, 128±8	Muscle	−20.16±0.05	−20.26 to −20.06	10.86±0.05	10.78 to 10.95
				Fin	−18.91±0.05	−19.01 to −18.81	10.79±0.05	10.71 to 10.88
				Scales	−17.25±0.05	−17.35 to −17.15	10.48±0.05	10.39 to 10.56

Diet 1, krill; diet 2, wheatgerm; diet 3, fishmeal.
Data are means±s.e.m. for *Barbus barbus* and *Squalius cephalus*. CI, confidence interval.

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Table 2. Differences in $\delta^{13}C$ and $\delta^{15}N$ between diets per fish tissue and species

Species	Tissue	Diet comparison	Difference in $\delta^{13}C$ (‰)	Difference in $\delta^{15}N$ (‰)
B. barbus	Muscle	Fishmeal vs wheatgerm	1.52±0.17*	−0.31±0.18
		Fishmeal vs krill	2.21±0.17*	−0.11±0.17
		Wheatgerm vs krill	0.69±0.17*	0.20±0.18
	Fin	Fishmeal vs wheatgerm	1.33±0.17*	−0.18±0.18
		Fishmeal vs krill	2.34±0.17*	0.57±0.17
		Wheatgerm vs krill	1.02±0.17*	0.75±0.18*
	Scales	Fishmeal vs wheatgerm	1.88±0.17*	0.81±0.18*
		Fishmeal vs krill	2.82±0.17*	1.45±0.17*
		Wheatgerm vs krill	0.94±0.17*	0.64±0.18
S. cephalus	Muscle	Fishmeal vs wheatgerm	1.32±0.10*	−0.16±0.15
		Fishmeal vs krill	2.10±0.16*	0.18±0.16*
		Wheatgerm vs krill	0.78±0.10*	0.93±0.16*
	Fin	Fishmeal vs wheatgerm	1.79±0.10*	−0.34±0.15
		Fishmeal vs krill	2.97±0.16*	1.18±0.16*
		Wheatgerm vs krill	1.18±0.11*	1.52±0.16*
	Scales	Fishmeal vs wheatgerm	2.68±0.10*	0.44±0.15
		Fishmeal vs krill	3.01±0.16*	1.36±0.16*
		Wheatgerm vs krill	0.33±0.10*	0.92±0.16*

Differences (means±s.e.m.) were calculated according to pairwise comparisons from general linear models, where comparisons have undergone Bonferroni adjustments for multiple comparisons and significant differences are indicated (*P<0.05). Data for the fishmeal diet are from Busst et al. (2015).

ash, and whose base ingredient was krill oil (order Euphausiacea, no further details available; Dynamite Baits, available from http://www.dynamitebaits.com/products/p/swim-stim-red-krill-carp-pellets, last accessed 28 October 2015). The second diet was 'Wheatgerm pellets', hereafter referred to as 'wheatgerm', comprising 20% protein, 6% fat (as oil), 2.5% crude fibre and 2.5% ash, and whose base ingredient was plant based (Nishikoi Aquaculture, available from http://www.nishikoi.com/pond-food/nishikoi-wheatgerm, last accessed 28 October 2015). The third diet was crushed pelletized fishmeal, referred to here as 'fishmeal', comprising 45% protein, 10% fat, 1.4% crude fibre and 5.8% ash, and whose base ingredient was marine fish (Busst et al., 2015). Note that data for the fishmeal diet were generated in a previous experiment (Busst et al., 2015) and this was not repeated here to avoid unnecessary use of live fish in experiments.

For the krill and wheatgerm diets, at the commencement of the experiment, the fish were anaesthetized (MS-222) and a small incision made to the abdomen to allow for the insertion of a passive integrated transponder tag (PIT tag) into the stomach cavity to enable individual identification, with a sample of pelvic fin tissue taken for subsequent stable isotope analysis that was immediately frozen. This tagging had not been completed for the fish fed the fishmeal diet (Busst et al., 2015). For all diets, the fish were measured (fork length, to the nearest mm) and weighed (to the nearest 0.01 g). Following their recovery in oxygenated water, they were randomly allocated into experimental tanks. The fish were released into four 90 l tanks at 20°C on a 16 h:8 h light:dark cycle, with a maximum of 11 fish per tank, with the species held separately. Water quality was maintained through a flow-through filtration system. The diets were assigned to each tank, and thus across the four tanks, each species was exposed to each diet with daily *ad libitum* feeding. At the end of the experimental period, the fish were removed from the tanks, identified according to their PIT tag, re-measured and weighed, killed (anaesthetic overdose, MS-222), and samples taken of dorsal muscle, pelvic fin tissue (the unclipped fin) and scales (removed from the region

Table 3. Differences in $\delta^{13}C$ and $\delta^{15}N$ per fish tissue and per species for each diet

Diet	Species	*N*	Tissue comparison	Difference in $\delta^{13}C$ (‰)	Difference in $\delta^{15}N$ (‰)
Fishmeal	*B. barbus*	9	Muscle vs fin	−1.28±0.17*	0.32±0.17
			Muscle vs scales	−2.17±0.17*	0.21±0.17
			Fin vs scales	−0.89±0.17*	−0.10±0.17
	S. cephalus	11	Muscle vs fin	−1.25±0.15*	0.07±0.15
			Muscle vs scales	−2.91±0.15*	0.39±0.15
			Fin vs scales	−1.66±0.15	0.32±0.15
Wheatgerm	*B. barbus*	8	Muscle vs fin	−1.47±0.18*	0.44±0.18
			Muscle vs scales	−1.81±0.18*	1.33±0.18*
			Fin vs scales	−0.34±0.18	0.89±0.18*
	S. cephalus	10	Muscle vs fin	−0.77±0.10*	−0.12±0.16
			Muscle vs scales	−1.55±0.10*	0.98±0.16*
			Fin vs scales	−0.78±0.10*	1.10±0.16*
Krill	*B. barbus*	9	Muscle vs fin	−1.14±0.17*	1.00±0.17*
			Muscle vs scales	−1.56±0.17*	1.78±0.17*
			Fin vs scales	−0.42±0.17	0.78±0.17*
	S. cephalus	9	Muscle vs fin	−0.38±0.16	0.47±0.17
			Muscle vs scales	−2.00±0.16*	0.97±0.16*
			Fin vs scales	−1.62±0.16*	0.50±0.17

Differences (means±s.e.m.) were calculated according to pairwise comparisons from general linear models, where comparisons have undergone Bonferroni adjustments for multiple comparisons and significant differences are indicated (*P<0.05). Data for the fishmeal diet are from Busst et al. (2015).

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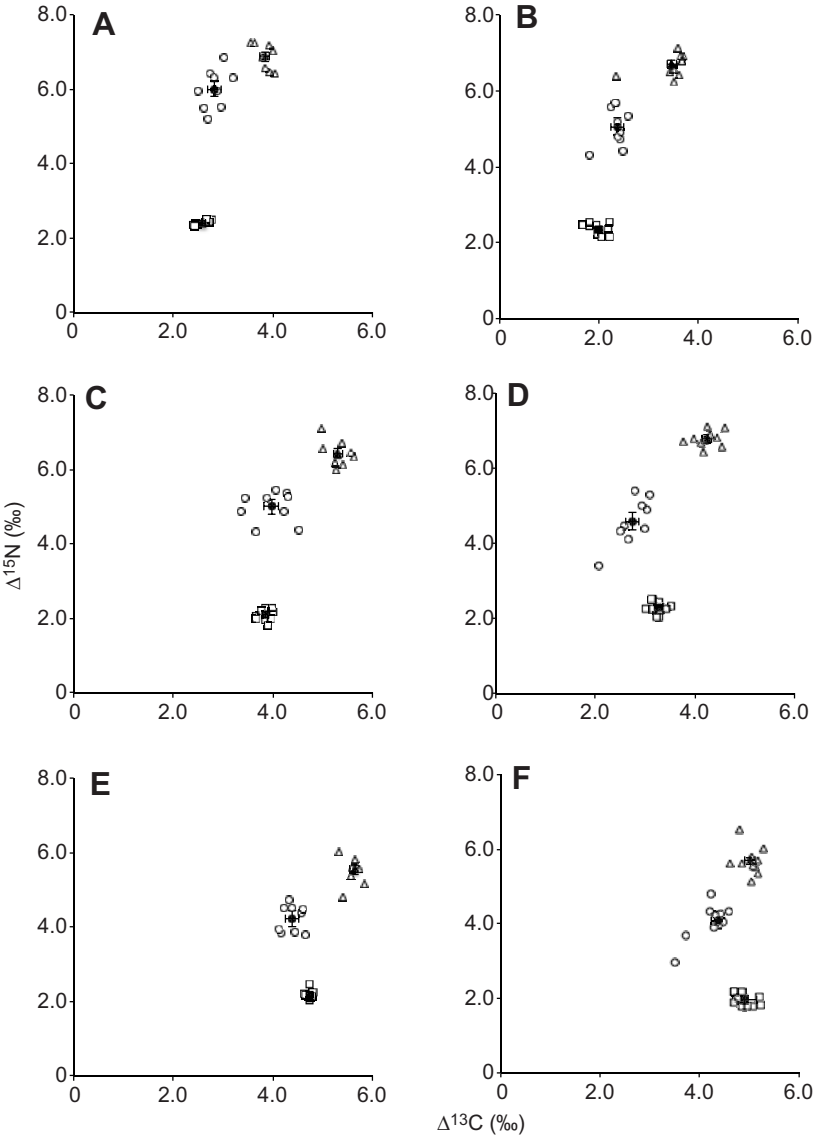

Fig. 1. Stable isotope discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$). Data are for *Barbus barbus* (A,C,E) and *Squalius cephalus* (B,D,F), and for muscle (A,B), fin tissue (C,D) and scales (E,F). Open triangles, wheatgerm diet; open squares, fishmeal diet; open circles, krill diet. Filled symbols are as per open symbols, except they represent mean values and s.e.m.

below the dorsal fin and above the lateral line). As the fish had not been PIT tagged for the fishmeal diet, individual changes in their body masses could not be determined. Other than the PIT tagging procedure, the fish exposed to the fishmeal diet were subjected to the same experimental conditions as the fish on the other diets, including the same tank sizes, filtration systems, water temperature and chemical parameters, light:dark cycle and feeding regime.

Stable isotope analysis

For the stable isotope analysis, fin clips and dorsal muscle were rinsed with distilled water and dried. Scales were lightly cleaned with distilled water to remove mucus, with their outer portion removed and used as this represents the most recent growth and thus their stable isotope values represent the most recent diets of the fish (Grey et al., 2009; Bašić et al., 2015). All samples were oven dried at 60°C to constant mass prior to analysis. Lipids were not extracted from samples as C:N ratios were <3.5%, indicating low lipid content and thus extraction or normalization would have little effect on $\delta^{13}C$ (Post et al., 2007). The tissues were then ground to powder and weighed precisely to ∼1000 µg in tin capsules and analysed on a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, USA) interfaced to a NC2500 elemental analyser (CE Elantach Inc., USA). Accuracy verification was against a range of international reference materials, including air and Vienna Pee Dee Belemnite (International Atomic Energy Agency, Vienna, Austria). The accuracy and precision of sample runs were tested every 10 samples using a standard mink sample to compensate for possible machine drift and quality control. Overall standard deviation was 0.11‰ for $\delta^{15}N$ and 0.09‰ for $\delta^{13}C$. Linearity correction was completed to account for differences in peak amplitudes between sample and reference gases (N_2 or CO_2); analytical precision of the $\delta^{15}N$ and $\delta^{13}C$ sample runs was 0.42‰ and 0.15‰, respectively.

Relationships between $\delta^{13}C$ and $\delta^{15}N$ and diet–tissue discrimination factors

Following stable isotope analysis (completed as described above), the $\delta^{13}C$ and $\delta^{15}N$ data for each species were tested for the extent of their discrimination (Δ) between each tissue and diet using general linear models (GLMs). The dependent variable was $\delta^{13}C$ or $\delta^{15}N$ versus the independent variables of diet (as krill, wheatgerm or

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Table 4. Comparison of stable isotope data and diet discrimination factors of fin tissue between the start and end of the experimental period

Diet	Species	Fin clip	Mean $\delta^{13}C$ (‰)	Mean $\Delta^{13}C$ (‰)	Mean $\delta^{15}N$ (‰)	Mean $\Delta^{15}N$ (‰)
Krill	*B. barbus*	Start	−20.96±0.09		12.27±0.13	
		End	−20.65±0.08	3.97±0.14	10.03±0.12	5.00±0.21
	S. cephalus	Start	−21.85±0.07		12.53±0.13	
		End	−21.88±0.08	2.74±0.13	9.62±0.14	4.59±0.23
Wheatgerm	*B. barbus*	Start	−20.82±0.09		12.34±0.12	
		End	−19.49±0.09	5.31±0.09	10.78±0.12	6.43±0.13
	S. cephalus	Start	−21.97±0.09		12.53±0.10	
		End	−20.70±0.08	4.24±0.10	11.14±0.10	6.79±0.10

Data are means±s.e.m.

fishmeal) and tissue type (as muscle, fin or scales). Testing of the mean differences in the $\delta^{13}C$ or $\delta^{15}N$ values between the diets and each tissue per species used the estimated marginal means generated by the GLMs and their pairwise comparisons with Bonferroni adjustments for multiple comparisons. The pairwise comparisons provided the species and diet-specific discrimination factors through the mean difference between the adjusted values for $\delta^{13}C$ and $\delta^{15}N$ for each diet and each tissue type, as well as the significance of the difference in $\delta^{13}C$ and $\delta^{15}N$ between each tissue, per species and per diet. All statistical analyses were completed using IBM SPSS Statistics (v22).

Relationships between change of $\delta^{13}C$ and $\delta^{15}N$ and change in fish mass

To test the relationship between the extent of change in $\delta^{13}C$ and $\delta^{15}N$ in fin tissue and the growth of the fish, isotopic data were calculated by deducting the values at the end of the experiment from those gained from tissue samples taken at its commencement. The individual fish were identified via their PIT tag code, enabling their increase in mass to be determined over the course of the experiment. Univariate linear regressions were then completed separately for each isotope ($\delta^{13}C$ and $\delta^{15}N$) and for each species and diet (krill and wheatgerm), where the independent variable was the increase in mass and the dependent variable was the difference in isotope value of the fin tissue between the start and end of the experiment.

RESULTS

Stable isotope discrimination factors between diets and tissues

The stable isotope values of the diets indicated some considerable differences between them. Fish fed on fishmeal had the highest $\delta^{13}C$ values, those fed on wheatgerm had the highest $\delta^{15}N$, and those fed on krill had the lowest $\delta^{15}N$ and the lowest $\delta^{13}C$ values (Table 1).

The muscle, fin and scale tissues were also distinct in their isotopic signatures, with highest $\delta^{15}N$ in muscle, followed by fin tissue and then scales, with the converse relationship between the tissues for $\delta^{13}C$ (Table 1). These relationships were consistent in both species and across each diet. These differences in stable isotope data per tissue and diet (Table 1) were then tested in GLMs. These were significant ($\delta^{13}C$: $F_{17,155}=164.76$, $P<0.01$; $\delta^{15}N$: $F_{17,155}=30.56$, $P<0.01$) and revealed some significant differences across the tissues and diets (Tables 2, 3).

These differences in the isotopic values then translated into considerable variation in the discrimination factors between the three diets. The largest differences in discrimination factors for each species were between wheatgerm and fishmeal (Fig. 1). For wheatgerm, the highest discrimination factor recorded for $\Delta^{13}C$ in *B. barbus* and *S. cephalus* was 5.6 and 5.0, respectively (both in scale tissue); for $\Delta^{15}N$, the highest discrimination factor recorded for *B. barbus* and *S. cephalus* was 6.9 and 6.8, respectively (Fig. 1). In contrast, for fishmeal, the highest discrimination factor recorded for $\Delta^{13}C$ in *B. barbus* and *S. cephalus* was 4.7 and 4.9, respectively (again, both in scale tissue); for $\Delta^{15}N$, the highest discrimination factor recorded for both species was only 2.4 (Fig. 1).

Changes in stable isotope data in fin tissues (krill and wheatgerm only)

The stable isotope data from fin clips taken before and after the experiment from the individual fish revealed the extent of isotopic change that occurred over the experimental period (Table 4), with GLMs testing the significance of the differences between these data. The overall models were significant ($\delta^{13}C$: $F_{11,93}=59.77$, $P<0.01$; $\delta^{15}N$: $F_{11,93}=122.46$, $P<0.01$), with pairwise comparisons revealing significant decreases in the $\delta^{15}N$ of fin tissues between the start and end of the experimental period for both diets ($P<0.01$), but for $\delta^{13}C$, differences were only significant for wheatgerm ($P<0.01$) (Table 5).

Across the experimental period, an increase in body mass was evident in all fishes fed on krill and wheatgerm (Table 6). Outputs of univariate linear regressions testing the extent of change in stable isotopes in the fin tissues of individual fish with their change in body mass over the experiment revealed the lack of a consistent pattern across species and diets (Table 6). There were, however, some significant relationships between isotopic change and increase in mass for both species on the krill diet ($P<0.05$; Table 6).

DISCUSSION

Diet–tissue discrimination factors

There was considerable variation in the stable isotope discrimination factors produced by the three diets in the tissues of the two fishes, as per the hypothesis. Across the diets, $\Delta^{13}C$ ranged between 2.0‰ and 5.6‰ and $\Delta^{15}N$ ranged between 2.0‰ and 6.9‰. For each tissue, the wheatgerm diet (20% protein) always had the highest discrimination factors, whilst the fishmeal diet (45% protein) had the lowest. These diet–tissue discrimination factors contribute to a growing knowledge base on the general

Table 5. Pairwise comparisons from general linear models of stable isotope data for fin tissue between the start and end of the experimental period

Diet	Species (*N*)	Difference in $\delta^{13}C$ (‰)	Difference in $\delta^{15}N$ (‰)
Krill	*B. barbus* (*N*=9)	−0.30±0.18	2.24±0.18*
	S. cephalus (*N*=10)	0.03±0.17	2.92±0.17*
Wheatgerm	*B. barbus* (*N*=8)	−1.28±0.19*	1.55±0.19*
	S. cephalus (*N*=10)	−1.27±0.12*	1.40±0.14*

Comparisons have undergone Bonferroni adjustments for multiple comparisons and significant differences are indicated (*$P<0.05$). Data are means±s.e.m.

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Table 6. Relationship between increase in fish mass over the experimental period and shift in $\delta^{13}C$ and $\delta^{15}N$ determined by individual univariate linear regressions

Diet	Species (*N*)	Mean increase in mass (g)	Stable isotope	R^2	*F*	*P*
Krill	*B. barbus* (*N*=8)	4.2±0.7	$\delta^{13}C$	0.05	0.29	0.61
			$\delta^{15}N$	0.58	8.21	**0.03**
Wheatgerm	(*N*=7)	4.2±0.1	$\delta^{13}C$	0.05	0.27	0.62
			$\delta^{15}N$	0.22	1.43	0.29
Krill	*S. cephalus* (*N*=9)	5.8±0.8	$\delta^{13}C$	0.49	6.60	**0.04**
			$\delta^{15}N$	0.64	12.47	**0.01**
Wheatgerm	(*N*=9)	3.8±0.2	$\delta^{13}C$	0.07	0.54	0.49
			$\delta^{15}N$	0.24	2.24	0.18

Data are means±s.e.m. Bold indicates significance.

patterns of how diet and feeding behaviour of fishes affects their stable isotope data. For example, whilst the $\Delta^{15}N$ values produced by the krill and wheatgerm diets were high compared with those of most other fishes, the fishmeal values were relatively consistent with those of Sweeting et al. (2007a), who suggested that white muscle of piscivorous European sea bass *Dicentrarchus labrax* had a $\Delta^{15}N$ of 3.15‰. Their suggestion that this could be used for fishes generally was, however, not consistent with our mean $\Delta^{15}N$ of 5.1–6.9‰ for the krill and wheatgerm diets. Nevertheless, our elevated discrimination factors were largely in line with those of Mill et al. (2007), who recorded $\Delta^{15}N$ in herbivorous fishes to be 5.25‰. They are also in general agreement with those of Carassou et al. (2008), who suggested that herbivorous fishes have distinct stable isotope discrimination factors that distinguish them from piscivorous fishes. Thus, we would suggest that an omnivorous fish with a strong proportion of plant material in their diet would be distinguishable from a conspecific with a fish-based diet through $\Delta^{15}N$.

For $\Delta^{13}C$, the literature generally suggests values for dorsal white muscle of fishes of between 0‰ and 1‰ (e.g. Peterson and Fry, 1987; Phillips et al., 2005; Cucherousset et al., 2012), with Post (2002) suggesting a mean of 0.39±1.3‰, which has recently been applied to mixing models to estimate fish diet composition (e.g. Bašić et al., 2015). However, Sweeting et al. (2007b) suggested that 1.5‰ was more appropriate for $\Delta^{13}C$ for fish muscle and samples that had not undergone lipid treatment could have higher values, such as 2.27‰. This latter inference is consistent with our outputs for all diets, where the lowest $\Delta^{13}C$ for dorsal muscle was 2.0‰ and highest was 3.8‰, and where no sample had undergone lipid treatment because of the low C:N ratios.

Correspondingly, whilst there are some general patterns apparent in $\Delta^{13}C$ and $\Delta^{15}N$ across herbivorous and piscivorous species and diets, the basis of these differences remains uncertain. The outputs of this study suggest that the primary reason behind the differences between the three diets was their contrasting sources of dietary protein (McClelland et al., 2003). These differences could then relate to one of two opposing hypotheses. Firstly, the protein quality hypothesis suggests that discrimination in the stable isotopes of consumers and their prey will increase as protein quality decreases, and thus as carnivores tend to assimilate higher quality protein than herbivores, discrimination factors decrease with trophic level (Roth and Hobson, 2000). Our results support this hypothesis, as fishmeal had the highest percentage of protein (45%), derived from marine fishes, and had consistently low discrimination factors. Wheatgerm, although having the mid-range protein content of the three diets (20%), was likely to have had the lowest quality protein as it was derived from plant material, and consistently produced the highest discrimination factors. This is also generally consistent with Macko et al. (1986), who suggested that as dietary protein increases, the percentage of nitrogen in the diet increases and more amino acids are catabolized for energy, potentially reducing discrimination factors in protein-rich diets. Secondly, the protein quantity hypothesis suggests that discrimination increases with dietary nitrogen concentration (i.e. decreasing C:N ratios), and thus as carnivores assimilate more protein than other fishes, discrimination increases with trophic level (Pearson et al., 2003). This is contrary to our findings, as the fishmeal diet had the lowest discrimination factors.

When the two fish species were compared, the discrimination factors tended to be higher in *B. barbus* than in *S. cephalus*, irrespective of diet and despite the species being of the same family. The reasons for these differences in discrimination factors between the species were not clear. Although somatic growth differences can result in differences in the isotopic enrichment of tissues (Thomas and Crowther, 2015; Vander Zanden et al., 2015) and have been used as an explanation for differences in discrimination factors between sexes (Kurle et al., 2014), the growth increments of each species per diet were similar in our study. Whilst the difference might have related to species-specific differences in the metabolic activity of tissues and isotopic routing as these can both influence the extent of discrimination (Caut et al., 2008; Vander Zanden et al., 2015), this is speculative and was not explored further. They do demonstrate both the underlying complexity of understanding discrimination factors across and within species and the importance of determining species-specific discrimination factor differences across different prey items.

Resolution of the differences in discrimination factors between food items composed of varying protein sources and content is required in studies that predict trophic level and diet composition using, for example, Bayesian mixing models (Parnell et al., 2013; Phillips et al., 2014). The outputs of this study indicate the difficulty of estimating discrimination factors from consumers with mixed diets. Whilst a final diet comprising a 50:50 mix of the wheatgerm and fishmeal feed might have indicated a discrimination factor between their values when fed in isolation that could have utility in subsequent analyses, this was not completed because of both ethical and logistical concerns. As the fishes both show natural shoaling behaviours, especially in smaller sizes (Britton and Pegg, 2011), holding them individually for extended periods in order to tightly control their food intake can represent an unnatural and highly stressful environment. Whilst this can be overcome by holding a number of fish together, it is then difficult to control the food intake of individual fish, with the potential for substantial deviations from the 50:50 food ratio through selective feeding. Greer et al. (2015) suggested a mathematical method for calculating unified discrimination factors based on known diet composition of a captive parrot. However, this approach might have limited

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applicability in natural situations as it loses the isotopic variability that is often inherent in wild diets. For example, diet composition can vary considerably between individuals of the same species as a result of ontogenetic dietary shifts (Byström et al., 2012). Moreover, in omnivorous fishes, whilst stomach contents data can indicate high proportions of algae and plant-based material in diet, this does not mean that a high proportion will actually be assimilated into tissues, making it difficult to determine the relative importance of plant-based materials as a food and energy source (e.g. Hamidan et al., 2015). In combination, these issues of varying diet–tissue discrimination differences between different food resources suggest that in predictive models for estimating the diet composition of omnivores, this variability must be captured within the model, rather than relying on the use of a single discrimination factor covering all putative food items (Phillips et al., 2014).

The discrimination factors supplied here, produced in controlled conditions with fixed diets, thus provide strong evidence that tissue- and diet-specific discrimination factors need further consideration in fishes and, potentially, other taxa. Nevertheless, general issues surrounding experimentally derived discrimination factors using formulated feeds have provoked debate in the literature. For example, Caut et al. (2008) also reported marked alterations in discrimination factors between fixed diets using formulated feeds in rats; it was argued by Perga and Grey (2010) that these were artefacts of experimental design and the effects of isotopic routing that caused differences in the preferential routing of amino acids to proteinaceous tissues between the diets. Moreover, as noted by Busst et al. (2015), the extent of isotopic routing may vary considerably in prevalence and magnitude between ecotherms and endotherms (Kelly and Martínez del Rio, 2010). Indeed, there is strong evidence to suggest that the rate at which stable isotopes turnover is significantly different between ecotherms and endotherms, with the half-lives in ectotherms being considerably longer (Thomas and Crowther, 2015; Vander Zanden et al., 2015). There remains, however, some uncertainty in how different discrimination between food item types will play out within omnivorous diets in the wild as it is likely that they would consume foods that vary extensively in both protein quality and protein quantity, which may alter temporally. Where diet shifts occur regularly, the likelihood of tissues reaching equilibrium with each diet before a shift occurs is reduced; furthermore, food items may have different discrimination factors, which may not be additive and linear and may vary with the degree of amino acid complementation determined by the entire diet (McClelland et al., 2003; MacNeil et al., 2006; Robbins et al., 2010), thus reducing the reliability of subsequent estimations of the assimilated diet where these interactions have not been factored into analyses within mixing models. Evidently, further work is required in order to reconcile some of the differences identified here.

Stable isotope values across different tissues

It was also apparent in the outputs that there was variability in the stable isotope values between the tissue types, with a general pattern of muscle always being enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$ compared with fin tissue and scales. This was consistent with the findings of Busst et al. (2015), who reported the same relationships for wild and laboratory populations of the two model species used here, plus eight other species of the Cyprinidae family. These general patterns were also consistent with those between muscle and fin tissue for chinook salmon *Oncorhynchus tshawytscha*, brook trout *Salvelinus fontinalis*, brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* (Pinnegar and Polunin, 1999; Jardine et al., 2005; Sanderson et al., 2009; Graham et al., 2013). Tronquart et al. (2012) also showed similar relationships between these tissues for 14 European freshwater fishes.

These differences in isotopic enrichment between the tissues may relate to variation in amino acid profiles (Reich et al., 2008). The $\delta^{15}N$ and $\delta^{13}C$ of individual amino acids can vary significantly (McClelland and Montoya, 2002; Fogel and Tuross, 2003), and so differences in the amino acid composition of a tissue can lead to differences in isotopic discrimination between tissues (Howland et al., 2003), although we were not able to test this here. The differences are also likely to have been influenced by factors that determine isotopic turnover in tissues, somatic growth and metabolic replacement (Martínez del Rio et al., 2009; Weidel et al., 2011; Thomas and Crowther, 2015; Vander Zanden et al., 2015). Because of the varying metabolic activity of tissues, the rate of isotopic turnover found in each often differs, with a strong relationship between turnover and metabolism; in fishes, isotopes in internal organs and blood plasma have shorter half-lives, and therefore faster turnover, than those in whole blood and muscle (Thomas and Crowther, 2015; Vander Zanden et al., 2015). Furthermore, Weidel et al. (2011) state that many fish diet-switch studies conclude that growth is primarily responsible for $\delta^{13}C$ change, and found metabolic replacement had a negligible effect on turnover.

Summary of implications for omnivorous fishes

The outputs of this study reveal that for two different omnivorous fishes, there were differences across diet–tissue discrimination factors by diet and by species. Although further work is required to disentangle some of the processes involved in this, they nevertheless demonstrate that the dietary analysis of omnivorous fishes using stable isotope analysis requires considerable attention to discrimination factors according to the consumer species and their putative prey resources. They also reveal that the destructive sampling of fishes to take samples of dorsal muscle can be replaced by non-destructive sampling by use of a ‘fin clip’ or scale as the tissue to be analysed, thus facilitating a shift towards the use of non-destructive sampling methods for stable isotope tissue collection.

Competing interests

The authors declare no competing or financial interests.

Author contributions

G.M.A.B. and J.R.B. designed the study; G.M.A.B. collected and analysed the data; G.M.A.B. and J.R.B. wrote the manuscript.

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