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ECOSYSTEM ECOLOGY - ORIGINAL RESEARCH

Fractionation of the stable carbon isotope ratio of essential fatty acids in zebrafish *Danio rerio* and mud snails *Bellamya chinensis*

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Abstract Fractionation of stable carbon (C) isotopes in the essential fatty acids 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3 was investigated in the zebrafish *Danio rerio* and the mud snail *Bellamya chinensis* fed the same two diets. These diets differed in essential fatty acid compositions: (1) TetraMin contained all five fatty acids, and (2) *Chlorella* contained only two, 18:2n-6 and 18:3n-3. On average, the isotopic fractionation was -0.5 ± 0.9 ‰ for 18:2n-6 and 18:3n-3 for all experiments, indicating that the fractionation of these essential fatty acids was negligible. However, the isotopic fractionation of 20:4n-6, 20:5n-3, and 22:6n-3 varied greatly between species and between diets. The isotopic fractionation of the *Chlorella* diet was -0.2 and -6.9 ‰ for zebrafish and mud snail, but 4.2 and -1.3 ‰, respectively, when these consumers were fed TetraMin. This variation could be explained by the different amount of assimilation and the biosynthesis of these fatty acids from their precursors (i.e., 18:2n-6 and 18:3n-3). These results indicate that the isotopic composition of C20 and C22 essential fatty acids was strongly influenced by the fatty acid composition in the diets. Thus the stable C isotope ratios of C18 essential fatty acids in consumers are more useful as dietary tracers in food web studies.

Keywords Lipids · Biomarker · Food chain · Trophic relationships · Elongation

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Introduction

Understanding the energy flow and carbon (C) transfer in ecosystems is necessary to understand interactions between food web organisms and ecosystem functions. Therefore, ecologists try to elucidate the origin and fate of assimilated food sources of consumers in aquatic and terrestrial ecosystems.

Bulk stable C and nitrogen (N) isotope analyses have been widely applied to food web studies because the isotope value of animals reflects that of their assimilated food sources with a slight increase. This difference in the isotope value between animals and their diets is defined as 'fractionation.' A fractionation of about 0–1 ‰ in C (DeNiro and Epstein 1978; Peterson and Fry 1987; Vander Zanden and Rasmussen 2001) and 3.4 ‰ in N (Minagawa and Wada 1984) has been widely accepted. However, isotopic fractionation is variable among species (e.g., McCutchan et al. 2003) and can be affected by the isotope value of the diet (Caut et al. 2009) and the C:N ratio of animals (Semenina and Tiunov 2011). These inconsistencies in isotopic fractionation can make trophic relationships difficult to estimate (but see Bunn et al. 2013).

Isotopic fractionation is the result of differences in the reactivity of lighter and heavier isotopes during metabolism (Hayes et al. 1990); a lighter isotope is theoretically more reactive. Thus, animals tend to be isotopically heavier than their diet because lighter reactive isotopes are rapidly consumed and released as metabolic products (e.g., CO_2). However, Klein Breteler et al. (2002) detected isotopically heavier respired CO_2 in copepods compared with their diet, and assumed that an isotopically heavy molecule (e.g., a carbohydrate) was used for respiration. This indicates that the isotopic fractionation of bulk carbon and nitrogen is

reflected in all of metabolism which can be affected by animal species and molecular composition of diets. Therefore, isotopic fractionation of bulk carbon and nitrogen may show wide range.

Compound-specific stable isotope ratios have only been used recently in food web studies. For example, stable N isotope ratios (δ^{15}N) of amino acids are used for the estimation of trophic positions with an isotopic fractionation of 7.6 ‰, which is defined as the difference in the δ^{15}N of glutamic acid and phenylalanine (e.g., Chikaraishi et al. 2009). This isotopic fractionation value is more constant among animals than bulk N because reactions that have the potential to induce isotopic fractionation are limited in amino acids.

For C source estimation, it is expected that stable C isotope ratios (δ^{13}C) of essential fatty acids such as 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3 may show no or small isotopic fractionation because they are primarily derived from diets with no or little modification. Budge et al. (2008) and Graham et al. (2014) analyzed the trophic links in food webs by using δ^{13}C of fatty acid biomarkers (e.g., 20:5n3) with the assumption that there is no isotopic fractionation between animals and their food sources.

However, there are some reactions within essential fatty acids. For example, 18:2n6 and 18:3n3 are metabolized as energy sources and are precursors of other essential fatty acids such as 20:4n6, 20:5n3, and 22:6n3. During these reactions, the remaining 18:2n6 and 18:3n3 are isotopically heavier because the lighter 18:2n6 and 18:3n3 may preferentially react during these reactions.

Although it is still unclear whether these reactions drive isotopic fractionation, a gradual depletion in the δ^{13}C of 18:2n-6 and 18:3n-3 through higher trophic level animals was reported for the Yensei river (Gladyshev et al. 2012), implying that the isotopic fractionation occurred in both 18:2n-6 and 18:3n-3 through the food chain. However, estimating isotopic fractionation in the field is difficult due to ambiguities in assimilated food sources and uncertainties regarding whether isotope values in diets are constant.

Similar to the C18 essential fatty acids, 20:4n6, 20:5n3, and 22:6n3 are metabolized as energy sources and precursors of hormones (Bell et al. 1986), which may also induce isotopic fractionation. Moreover, 20:4n-6, 20:5n-3, and 22:6n-3 can be synthesized from the precursors 18:2n-6 and 18:3n-3 in an animal's body if the animal possesses adequate desaturases (Bell et al. 1986). If these newly synthesized products (i.e., from dietary C18 fatty acids) accumulate in animals, the isotope values of 20:4n-6, 20:5n-3, and 22:6n-3 will not directly reflect those of the assimilated food sources of each counterpart. The magnitude of this new synthesis may depend on the dietary fatty acid composition, e.g., in fish the activities of lipogenic enzymes decreased when sufficient lipids were provided (Gaye-Siessegger et al. 2004). Therefore, if animals are supplied

with sufficient 20:5n-3 from their diet, the contribution of synthesized 20:5n-3 from 18:3n-3 may be reduced or negligible. However, this hypothesis has not been evaluated.

To evaluate the isotopic fractionation through the food chain, controlled-feeding experiments with a diet of constant isotope values have been conducted for bulk C and N isotopes in a wide range of animals (e.g., Tieszen et al. 1983; Suzuki et al. 2005; Yokoyama et al. 2005). Changes in the isotope values of animals can be expressed with exponential functions where the isotope values approach a certain asymptotic value. The difference of this asymptotic value and the isotope value of the diet is defined as 'isotopic fractionation' (e.g., Suzuki et al. 2005). However, to our knowledge few feeding experiments have reported the isotopic fractionation of fatty acids. Chamberlain et al. (2004) reported temporal changes of isotope values of collembolans after they switched food sources, but fatty acid biomarkers were not included (e.g., 18:2n-6, 20:5n-3, etc.).

The objective of this study was to elucidate the isotopic fractionation of fatty acid biomarkers in freshwater animals using a freshwater fish and snail species. The fatty acid composition of the diet could influence the metabolism of animals, and also affect the essential fatty acid isotope values. Therefore, we prepared two isotopically homogeneous diets, one containing five essential fatty acids and the other only 18:2n-6 and 18:3n-3, that were fed to the animals over a period of time.

Materials and methods

Controlled-feeding experiments

The zebrafish *Danio rerio* and mud snail *Bellamya chinensis* were used for the controlled-feeding experiments. *D. rerio* was obtained at an aquarium shop in May 2011, for the experiment with TetraMin, and in May 2012, for the experiment with *Chlorella*. *B. chinensis* was collected from a paddy field in Miyagi prefecture, Japan in May 2012. Before the feeding experiment, algae attached to the shells of *B. chinensis* were removed to prevent the ingestion of algae from the shells of other individuals. All species were kept in a tank with dechlorinated tap water for 2 days to allow the ejection of stomach contents and feces.

Each species was divided into two groups with equivalent size ranges. *D. rerio* was ca. 120 mg (dry weight), *B. chinensis* was ca. 65 mg (dry weight). During the experiments, one group was given the commercial diet, TetraMin (Tetra), and the other group was given *Chlorella* (Chlorella King, Chlorella Industry), which is a processed commercial diet derived from *Chlorella* sp.. The TetraMin used for *D. rerio* and *B. chinensis* was different because the experiments were not carried out at the same time. The

experiment with *D. rerio* was conducted 1 year earlier than the *B. chinensis* experiment. The TetraMin contained all essential fatty acids, whereas *Chlorella* contained only 18:2n-6 and 18:3n-3. Both diets were fed to the animals 1–2 times daily until the end of the experiment. The feeding experiments were conducted for 100 days. Dechlorinated water was used for the feeding experiments and exchanged weekly. The water temperature was maintained at around 20 °C during the experiment under constant room temperature. Water tanks for *B. chinensis* were covered with thick paper to shade them from light to prevent algal growth on the shells and tank wall.

At least five individuals of each species were sampled on days 0, 14, 28, 56, and 100 after the start of the feeding experiments. Animal samples were stored in the freezer at −30 °C until further analysis. To determine whether the isotope values of the diets were constant during the experiment, both diets were sampled on days 0, 56, and 100.

Extraction, derivatization, and identification of fatty acids

Lipid extraction and derivatization for freeze-dried samples of animal tissues and diets were conducted by the one-step method described by Abdulkadir and Tsuchiya (2008). Briefly, 100–200 mg of the freeze-dried sample was placed in a 50-ml glass tube with 1 ml of internal standard (0.1 mg 23:0/ml hexane), 4 ml hexane, and 2 ml of 14 % boron trifluoride (BF$_3$)–methanol, and N$_2$ gas for filling the head space. The glass tubes were put in a 100 °C water bath for 2 h. After cooling down to room temperature, 1 ml of hexane and 2 ml of ultrapure water were added to the tubes. After shaking vigorously, the tubes were centrifuged for 3 min at 2500 r.p.m. The upper layer of hexane, which contains the fatty acid methyl esters (FAME), was poured into a 1.5-ml gas chromatography (GC) vial. Solid residues of the animals were used for the analysis of bulk δ^{13}C as described below.

One microliter of a FAME solution was injected into a GC (GC-2014; Shimadzu) equipped with a capillary column (100 m × 0.25 mm internal diameter; Select FAME; Agilent Technologies). The column temperature was programmed from 150 °C (with a 5-min hold) to 230 °C (4 °C/min) and held for 10 min. Then, the temperature was increased to 250 °C at 4 °C/min and held for 10 min. Helium gas was used as a carrier. The temperatures of the injection and flame ionization detector were 270 and 280 °C, respectively.

Fatty acids in samples were identified by comparison with the retention time of commercial authentic standard mixtures (Supelco). In the present study, we only focused on 16:0 and five essential fatty acids, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n3. The fatty acids 18:2n-6 and 18:3n-3 are generally used as biomarkers for green algae,

cyanobacteria, and terrestrial plants (Napolitano et al. 1997; Mfilinge et al. 2005; Xu and Yang 2007), 20:5n-3 are used for diatoms (Mfilinge et al. 2005), 20:4n-6 are used for bryophytes (Torres-Ruiz et al. 2007), and 22:6n-3 are used for dinoflagellates (Napolitano 1999).

Stable C isotope analysis of fatty acids

The FAMEs in the hexane solution were also used for the analysis of δ^{13}C of fatty acids. The FAMEs were injected into a GC-isotope-ratio mass spectrometer (TRACE GC ULTRA, Delta-V advantage; Thermo), equipped with the same capillary column as for the GC-flame ionization detector analysis. The column temperature was programmed from 80 to 180 °C (5 °C/min), then to 205 °C (0.5 °C/min), then to 250 °C (3 °C/min), and finally 5 min at 250 °C. Peak identification was conducted in the same way as for the GC-FID analysis (see above).

Stable C isotope analysis of bulk samples

The freeze-dried diets and the extracted residues of animals from the analysis of fatty acids (see above) were used for the analysis of bulk δ^{13}C after drying them for 24 h at 60 °C. All samples were weighed in microcapsules and injected into an elemental analyzer (NA2500; CE Instruments) linked to a mass spectrometer (DELTA plus; FinniganMAT).

Data analysis

The δ^{13}C of bulk and fatty acids are expressed as:

$$\delta^{13}C(‰) = (R_{sample}/R_{standard} - 1) \times 1000$$

where R_{sample} is $^{13}C/^{12}C$ of the sample, $R_{standard}$ is $^{13}C/^{12}C$ of the international isotopic standard, Vienna Pee Dee belemnite. For the stable isotope ratio of fatty acids, the following equation was used to correct the effects of incorporated C during methylation from BF$_3$–methanol:

$$\delta^{13}C_{FA} = ((n + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH})/n$$

where $\delta^{13}C_{FAME}$ is the measured value of FAME and $\delta^{13}C_{MeOH}$ is the δ^{13}C value of BF$_3$–methanol that was measured in advance. $\delta^{13}C_{FA}$ is the δ^{13}C value of FA which does not include the C from BF$_3$–methanol, and *n* is the number of C atoms in the non-methylated FA.

The isotopic fractionation was defined as the difference between the asymptotic value of the animal and the value of its food source (Tieszen et al. 1983):

$$FAi\delta^{13}C_X = FA\delta^{13}C_{sample} - FA\delta^{13}C_{diet}$$

where $FAi\delta^{13}C_X$ is the isotopic fractionation of EFA, $FA\delta^{13}C_{sample}$ is the asymptotic value of the animal and $FA\delta^{13}C_{diet}$ is the value of the food source. $FA\delta^{13}C_{sample}$

Table 1 Fatty acid composition (% of total fatty acids) and isotope value of essential fatty acids

	TetraMin1				TetraMin2				Chlorella			
	%	SD	δ^{13}C (‰)	SD	%	SD	δ^{13}C (‰)	SD	%	SD	δ^{13}C (‰)	SD
14:0	3.0	0.2			3.2	0.1			0.5	0.0		
15:0	0.1	0.1			0.1	0.2			1.2	0.1		
16:0	18.3	0.6	−22.8	0.6	19.1	0.0	−26.7	0.2	23.8	1.6	−17.8	0.4
17:0	0.0	0.0			0.1	0.2			0.0	0.0		
18:0	9.2	0.2			8.5	0.1			1.4	0.1		
20:0	2.3	0.2			2.0	0.0			0.0	0.0		
16:1n-7	3.8	0.4			4.2	0.0			2.6	0.2		
18:1n-9	18.2	0.4			17.8	0.3			2.6	0.2		
18:1n-7	2.5	0.1			2.3	0.0			1.9	0.1		
18:2n-6[a]	23.1	0.9	−25.0	0.5	22.6	0.1	−27.2	0.6	37.0	2.4	−17.5	0.2
18:3n-6	0.0	0.0			0.0	0.0			3.5	0.3		
18:3n-3[a]	2.5	0.1	−25.8	0.8	2.3	0.0	−28.2	0.6	23.6	1.6	−15.8	0.2
18:4n-3	1.9	0.1			2.5	0.1			1.8	0.1		
20:4n-6[a]	3.2	0.1	−25.5	0.7	3.0	0.1	−25.1	1.2	0.0	0.0		
20:5n-3[a]	4.7	0.3	−23.6	0.7	5.0	0.1	−24.3	0.5	0.0	0.0		
22:5n3	1.2	0.0			1.1	0.0			0.0	0.0		
22:6n-3[a]	6.1	0.1	−24.0	0.6	6.1	0.1	−23.0	0.4	0.0	0.0		
ΣFAME	100	0			100	0			100	0.00		

[a] Essential fatty acids

FAME Fatty acid methyl ester

was calculated by the following exponential curve with c expressing the asymptotic value:

$$Y = a\exp^{(-bT)} + c.$$

Since *Chlorella* does not contain 20:4n-6, 20:5n-3, or 22:6n-3, isotopic fractionation of these fatty acids was not defined for this food source. In this study, the isotope value of 18:2n-6 in *Chlorella* was used to calculate the isotopic fractionation of 20:4n-6 as 20:4n-6 synthesized from 18:2n-6. Likewise, the isotope value of 18:3n-3 in *Chlorella* was used to calculate the fractionation of 20:5n-3 and 22:6n-3. Moreover, the difference in the isotope value of diets and animals at the end of experiment was also calculated to estimate the further isotopic fractionation of the fatty acids because it was expected that some results would not be explained sufficiently by the exponential curves. *t*-tests were used to test for significant differences between the isotope values of animals at the end of the experiment and that of a given food source.

Results

Fatty acid composition of diets and their assimilation in animals

TetraMin contained all essential fatty acids, and the most abundant essential fatty acid was 18:2n-6, which comprised
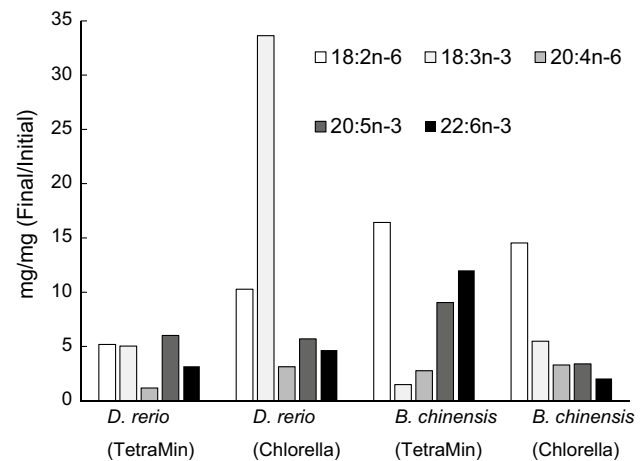
Fig. 1 The ratio of the amount of each essential fatty acid under initial and final conditions [$n = 10$, *Danio rerio* (TetraMin); $n = 5$, the others]. *B. chinensis Bellmaya chinensis*

23.1 % of the total fatty acids (Table 1). Corresponding to the fatty acid composition of TetraMin, the amount of 18:2n-6 increased during the feeding experiment in both animals on this diet (Fig. 1). At the end of the feeding experiment, the amount of 18:2n-6 was five times higher than initially in the tissue of both animals. The amounts of the other fatty acids also increased during the experiment, especially that of 20:5n-3 in *D. rerio* (Fig. 1) and 22:6n-3 in *B. chinensis* (Fig. 1).

Table 2 Body weight and fatty acid concentration of each animal at the beginning and end of the feeding experiment

	DR (TM) (0 day)		DR (TM) (100 days)		DR (CH) (0 day)		DR (CH) (100 days)		BC (TM) and BC (CH) (0 day)		BC (TM) (100 days)		BC (CH) (100 days)	
	mg/g	SD	mg/g	SD	mg/g	SD	mg/g	SD	mg/g	SD	mg/g	SD	mg/g	SD
14:0	0.3	0.4	1.8	0.4	0.2	0.2	0.4	0.2	0.4	0.0	0.4	0.0	0.3	0.0
15:0	0.1	0.2	0.3	0.1	0.0	0.1	0.2	0.0	0.4	0.0	0.3	0.0	0.3	0.0
a-15:0	0.6	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:0	13.7	6.9	19.7	4.2	9.3	4.0	20.5	2.6	3.1	0.3	3.0	0.3	3.2	0.5
17:0	0.6	0.4	0.5	0.1	0.3	0.2	0.3	0.1	0.6	0.1	0.4	0.1	0.4	0.2
18:0	5.7	1.4	8.0	0.9	5.4	1.3	6.2	0.8	2.2	0.2	1.8	0.1	2.0	0.2
20:0	0.0	0.1	0.0	0.0	0.0	0.0	0.2	0.1	0.2	0.1	0.1	0.0	0.2	0.1
24:0	0.2	0.1	0.0	0.0	0.4	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:1n-7	0.7	1.2	3.6	0.8	0.9	0.4	2.5	0.5	0.8	0.1	0.8	0.1	0.8	0.2
18:1n-9c	13.3	7.2	25.1	5.6	11.4	6.0	11.8	4.1	0.6	0.1	2.2	0.4	0.5	0.1
18:1n-7	1.4	0.7	2.6	0.5	1.0	0.3	1.6	0.2	0.4	0.1	0.7	0.1	0.5	0.1
20:1n-9	0.2	0.1	1.8	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18:2n-6	5.5	2.5	17.5	3.1	4.4	2.0	10.0	1.5	0.9	0.1	3.0	0.4	3.2	0.8
20:2n-6	0.2	0.1	0.0	0.0	0.1	0.2	0.3	0.0	0.5	0.0	1.4	0.2	1.3	0.2
18:3n-6	0.1	0.1	0.2	0.2	0.1	0.1	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.1
18:3n-3	0.5	0.3	1.5	0.3	0.4	0.2	2.9	0.7	0.7	0.1	0.2	0.0	1.0	0.2
18:4n-3	0.0	0.1	0.0	0.0	0.0	0.0	0.5	0.1	0.0	0.0	0.7	0.1	0.2	0.0
20:3n-6	0.9	0.4	0.8	0.4	0.7	0.2	2.1	0.6	0.0	0.0	0.1	0.0	0.2	0.1
20:4n-6	4.4	0.9	3.1	0.8	4.5	0.9	3.1	1.0	4.2	0.4	2.4	0.2	3.5	0.3
20:5n-3	0.9	0.7	3.4	0.4	0.7	0.2	0.9	0.2	0.9	0.1	1.7	0.1	0.8	0.1
20:4n-3	0.0	0.0	0.4	0.1	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:5n3	0.6	0.2	1.5	0.2	0.5	0.1	0.5	0.1	0.5	0.1	0.4	0.1	0.2	0.0
22:6n-3	7.0	1.5	13.5	1.8	7.1	0.5	7.3	2.7	0.3	0.0	0.7	0.1	0.1	0.0
ΣFAME	57.0	21.8	105.3	16.9	47.4	14.8	72.6	5.8	16.8	1.5	20.1	1.8	18.6	2.4
Weight (g)	0.14	0.05	0.23	0.09	0.10	0.02	0.45	0.19	0.07	0.01	0.32	0.06	0.26	0.07

DR Danio rerio, *BC Bellmaya chinensis*, *TM TetraMin*, *CH Chlorella*

The other food source, *Chlorella,* contained only 18:2n-6 and 18:3n-3 as essential fatty acids (Table 1). The amount of both 18:2n-6 and 18:3n-3 increased during the feeding experiment in both animals (Fig. 1). The amounts of the other essential fatty acids also increased during the feeding experiment, even though these fatty acids were absent in the food source. The amount of 20:5n-3 in *D. rerio* and *B. chinensis* increased by 3.4 and 5.7 times, respectively, compared with the initial amounts (Fig. 1). In addition, at the end of the feeding experiment, the individual dry weight of *D. rerio* fed with TetraMin and *Chlorella* increased 1.8- and 4.6-fold from the initial weight, respectively, and that of *B. chinensis* increased 4.9- and 4.0-fold, respectively (Table 2).

Isotopic fractionation of the bulk C

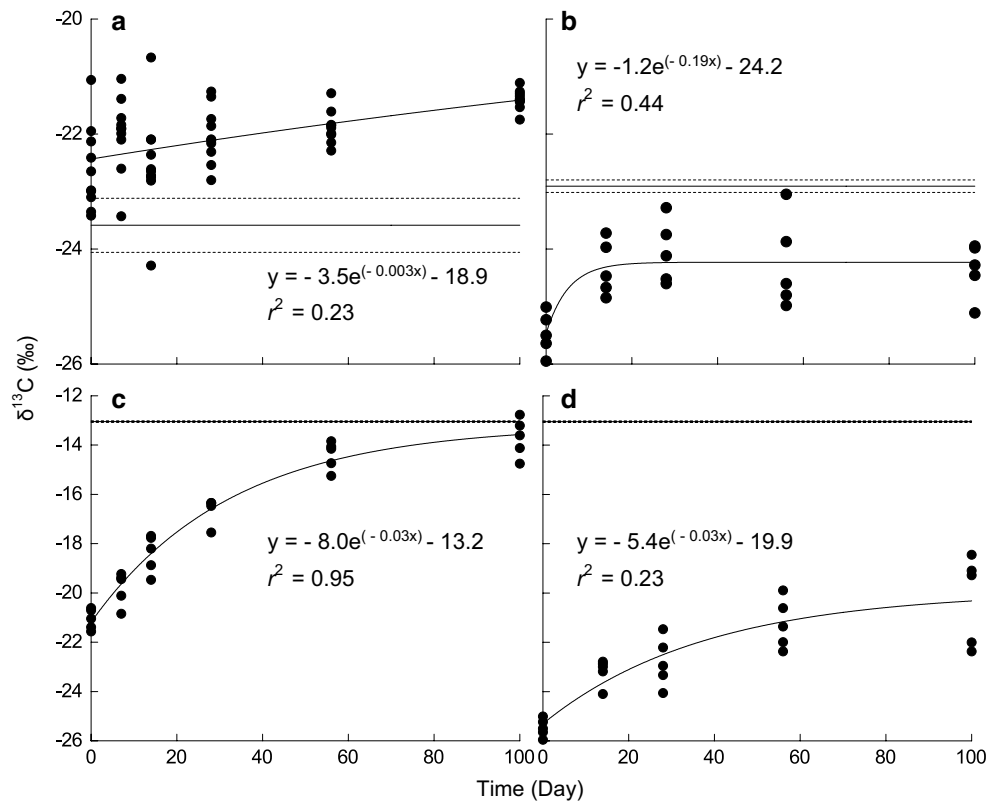Isotopic fractionation of bulk δ^{13}C of *D. rerio* with TetraMin and *Chlorella* was calculated as 4.2 ‰ and −0.2 ‰, respectively (Fig. 2a, c; Table 3). The values of *B. chinensis* varied widely, and were −1.3 ‰ for TetraMin and −6.9 ‰ for *Chlorella* (Fig. 2b, d; Table 3). The isotopic fractionation calculated by the difference in the diet and final isotope value of animals was almost the same, but *D. rerio* fed with TetraMin showed a 1.7 ‰ fractionation, which varied largely with the bulk stable C isotopic fractionation, as calculated by the difference in asymptotical values of animal and food sources (Table 3).

Isotopic fractionation on the fatty acids

The isotope value of each essential fatty acid did not change during the experiment (ANOVA, $p > 0.05$) in any of the diets. Thus, mean isotope values on days 0, 56, and 100 (Table 1) were used for further data analysis.

The isotopic fractionation calculated from the asymptotical values of 18:2n-6 and 18:3n-3 ranged from −2.1 to 0.6 ‰ in both animals and diet type (Figs. 3, 4; Table 3).

Fig. 2 Temporal change in the isotope value of bulk carbon (C) in tested animals during feeding experiment. **a** *D. rerio* (TetraMin) ($n = 10$), **b** *B. chinensis* (TetraMin) ($n = 5$), **c** *D. rerio* (*Chlorella*) ($n = 5$), **d** *B. chinensis* (*Chlorella*) ($n = 5$). *Horizontal solid lines* represent an average of the given diet, and the *dotted lines* represent the SD

a $y = -3.5e^{(-0.003x)} - 18.9$
$r^2 = 0.23$

b $y = -1.2e^{(-0.19x)} - 24.2$
$r^2 = 0.44$

c $y = -8.0e^{(-0.03x)} - 13.2$
$r^2 = 0.95$

d $y = -5.4e^{(-0.03x)} - 19.9$
$r^2 = 0.23$

There were no significant differences between the isotope value of animals at the end of experiment and those of diets except for 18:2n-6 in *B. chinensis* on the *Chlorella* diet (*t*-test, $p < 0.05$) (Table 3).

For 20:4n-6, only the isotope value of *D. rerio* fed on *Chlorella* approached asymptotic values (Fig. 5). The fractionation was calculated as 0 ‰, but the isotope values of *Chlorella* and *D. rerio* at the end of the experiment were significantly different (*t*-test, $p > 0.05$) (Table 3).

For 20:5n3 and 22:6n3, the isotope values of animals approached the asymptotic values of diets during the experiment, and their isotopic fractionation ranged from −1.3 ‰ (*B. chinensis* fed TetraMin) to 0.3 ‰ (*D. rerio* fed TetraMin), except for *B. chinensis* fed on *Chlorella* (Figs. 6, 7; Table 3). Neither essential fatty acid showed significant differences between isotope values at the end of the experiment and in the diet of *D. rerio* fed on TetraMin (Table 3).

For 16:0, exponential fitting failed in *B. chinensis* fed with TetraMin (Fig. 8); for *D. rerio* fed on TetraMin it also did not fit well. *D. rerio* and *B. chinensis* fed on *Chlorella* diets approached asymptotic values of the diets during the experiment, and the isotopic fractionation was calculated as 2.3 and −0.6 ‰, respectively.

Discussion

Isotopic fractionation of the bulk C and 16:0

The isotopic fractionation of bulk C has been widely reported to be 0–1 ‰ (DeNiro and Epstein 1978; Peterson and Fry 1987; Vander Zanden and Rasmussen 2001). However, the magnitude of the isotopic fractionation differed between *D. rerio* and *B. chinensis*, and between food sources in this study. These findings are consistent with those of a previous study on sea urchins, which showed different isotopic fractionation among food sources (Prado et al. 2012). Caut et al. (2009) also found a negative relationship between the isotope value of diets and isotopic fractionation, which is confirmed by the present study. Altogether, these results suggest that the isotopic fractionation value of bulk C is affected by diet and animal species.

Animals can biosynthesize 16:0 from acetyl-coenzyme A (Dalsgaard et al. 2003), thus the isotope value of 16:0 in animals is not always the same as that of 16:0 in their diet. Thus, the isotope value of 16:0 in animals may reflect an admixture of their diet and the synthesized 16:0s. In the present experiment, the concentration of 16:0 was higher in *Chlorella* than in TetraMin. This may explain why the exponential

Table 3 Isotopic fractionation calculated as the difference between the isotope value of the diet and the asymptotic value (*Asymptotic*) and isotope values at the end of the feeding experiment (*Final day*)

	Asymptotic	Final day
Bulk		
DR (TM)	4.2	1.7[a]
DR (CH)	−0.2	−1.4
BC (TM)	−1.3	−1.4[a]
BC (CH)	−6.9	−7.2[a]
18:2n6		
DR (TM)	0	0.2
DR (CH)	0.3	0.6
BC (TM)	−0.7	0.0
BC (CH)	−2.1	−1.6[a]
18:3n3		
DR (TM)	−0.3	−0.2
DR (CH)	0.2	0.6
BC (TM)	−1.0	0.5
BC (CH)	−0.2	−0.5
20:4n6		
DR (TM)	0.7	−0.7
DR (CH)	0	−3.2[a]
BC (TM)	−4.4	−3.8[a]
BC (CH)	>10	−6.4[a]
20:5n3		
DR (TM)	0	−0.2
DR (CH)	−0.3	−1.3[a]
BC (TM)	−1.3	−0.8[a]
BC (CH)	>10	−5.1[a]
22:6n3		
DR (TM)	0.3	0.6
DR (CH)	−1.2	−2.6[a]
BC (TM)	−1.1	−1.0[a]
BC (CH)	>10	−10.7[a]
16:0		
DR (TM)	<−10	−1.6[a]
DR (CH)	2.3	0.8
BC (TM)	–	1.1[a]
BC (CH)	−0.6	−2.3[a]

For abbreviations, see Table 2

[a] Siginificant difference ($p < 0.05$, *t*-test) with isotope value of given diet

regressions of *D. rerio* and *B. chinensis* with TetraMin did not fit well, but with *Chlorella* they did. Thus, it appears that 16:0 is not a useful marker for food web studies.

Isotopic fractionation of 18:2n-6 and 18:3n-3

Animals do not possess δ-15 and δ-12 desaturases, which are the enzymes required to synthesize 18:2n-6 and 18:3n-3 from 18:1n-9 (Hastings et al. 2001). In freshwater ecosystems, these fatty acids are mainly synthesized by green algae and cyanobacteria. Thus, when 18:2n-6 and 18:3n-3 are found in animal tissues, they must have been assimilated from the diet, thus the isotope values of these essential fatty acids in animals should be consistent with those in their diet. However, 18:2n-6 is a precursor of 20:4n-6, and 18:3n-3 is a precursor of 20:5n-3 and 22:6n-3. To synthesize these C20 and C22 essential fatty acids, δ-5 and δ-6 desaturases are required (Bell et al. 1986; Hastings et al. 2001). The occurrence of these enzymes depends on the animal species, and *D. rerio* has these enzymes (Hastings et al. 2001). The amount of 20:4n-6, 20:5n-3, and 22:6n-3 in *D. rerio* fed on *Chlorella* increased during the experiment, even though *Chlorella* did not contain these fatty acids. This indicates that *D. rerio* could biosynthesize C20 and C22 essential fatty acids from their precursors, 18:2n-6 and 18:3n-3, respectively. There are currently no published data on fatty acid biosynthesis in *B. chinensis*. However, we observed that *B. chinensis* also has the ability to biosynthesize 20:4n-6, 20:5n-3, and 22:6n-3 from dietary 18:2n-6 and 18:3n-3, as the content of these highly unsaturated fatty acids increased with time when the animals were reared on *Chlorella*. This also indicates that some of the assimilated dietary 18:2n-6 and 18:3n-3 was used to biosynthesize 20:4n-6 and 20:5n-3, respectively. It is also theoretically assumed that lighter isotopes show higher reactivity than heavier isotopes (Hayes et al. 1990). Therefore, it is expected that lighter 18:2n-6 and 18:3n-3 were selectively used in this reaction, and the remaining 18:2n-6 and 18:3n-3 in the animal may be heavier than in the diet.

The effect of this isotopic fractionation in the animals seems to be associated with the balance of reacted and stored unreacted 18:2n-6 and 18:3n-3 abundance. If dietary accumulation of these fatty acids in animals greatly exceeded the amount they synthesized from precursors, the isotopic enrichment would be small or negligible. While in this study we cannot determine the accurate balance of unreacted and reacted 18:2n-6 and 18:3n-3, the incremental change of each fatty acid during feeding experiments was evaluated (Fig. 1). In most cases, these fatty acids increased by five times compared to their initial concentrations. However, 18:3n3 in *B. chinensis* fed on TetraMin only increased by 1.5 times at the end of the feeding experiment, even though no substantial isotopic fractionation occurred. Moreover, the average mass increase of 18:3n-3 per individual during the experiment was about 1.3 mg (Table 2). However, the increments of both 20:5n-3 and 22:6n-3 were about 2.9 mg (Table 2). This indicates that the amount of consumed (metabolized) 18:3n-3 was higher than that of the unreacted stored 18:3n-3. However, isotopic fractionation was not confirmed. It is suggested that the isotopic fractionation related to reactions with 18:2n-6 and 18:3n-3 was small or negligible.
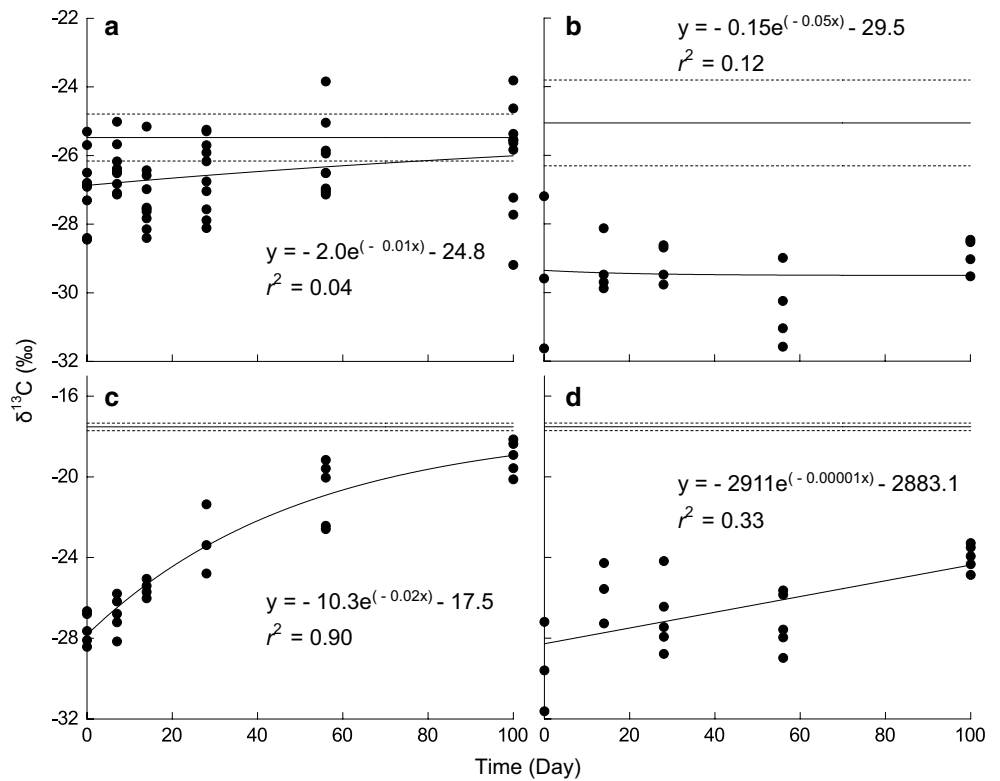
Fig. 3 Temporal change in the isotope value of 18:2n-6. **a** *D. rerio* (TetraMin) ($n = 10$), **b** *B. chinensis* (TetraMin) ($n = 5$), **c** *D. rerio* (*Chlorella*) ($n = 5$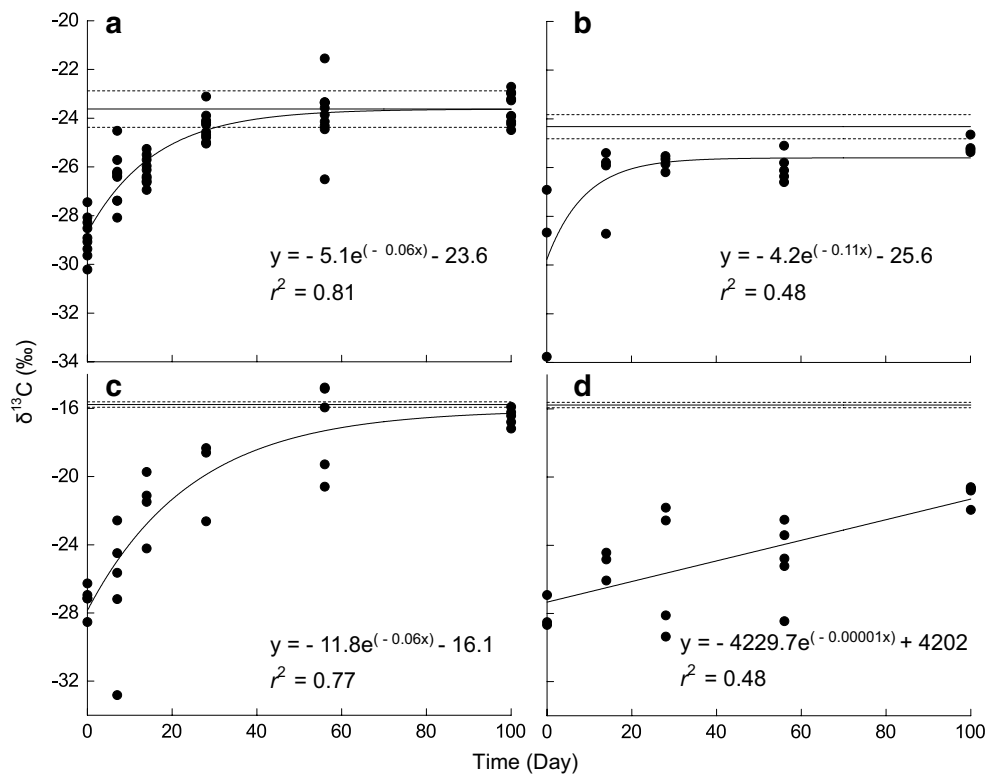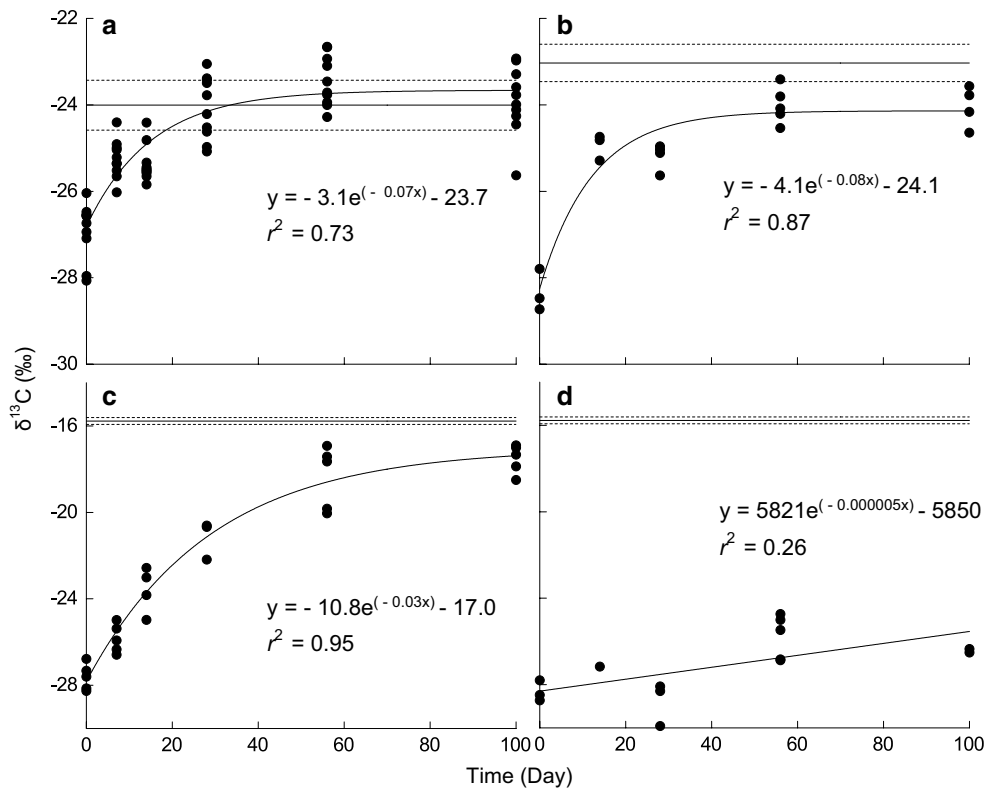), **d** *B. chinensis* (*Chlorella*) ($n = 5$). *Horizontal solid line* represents the average of a given diet, and the *dotted line* represents the SD
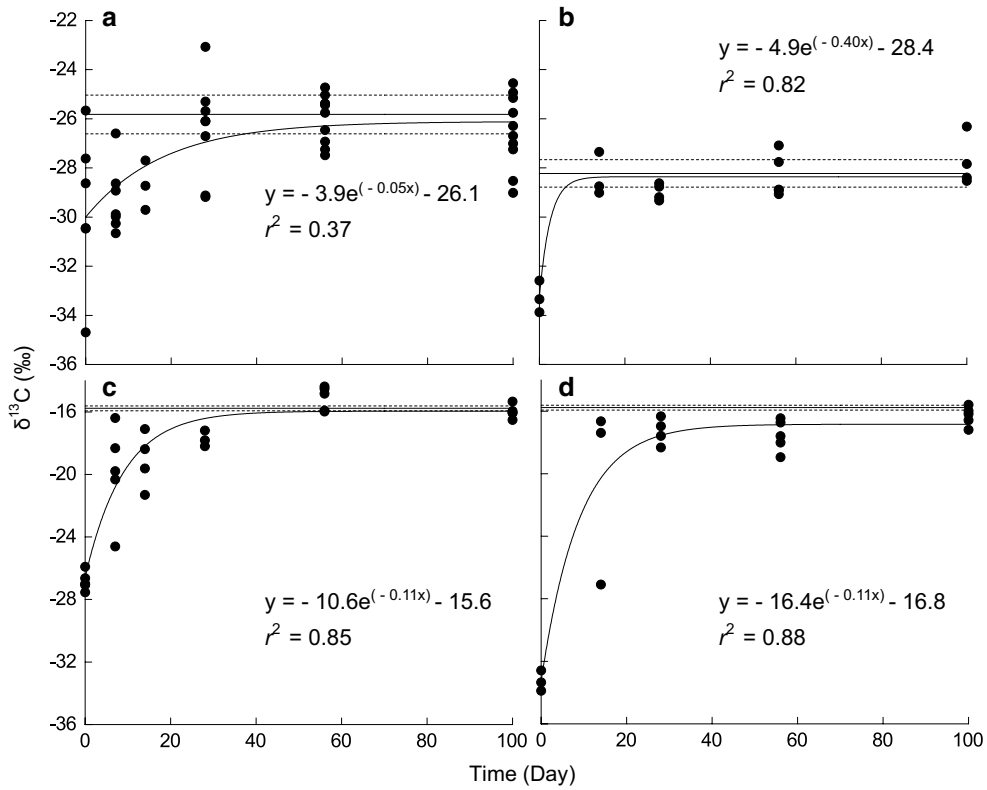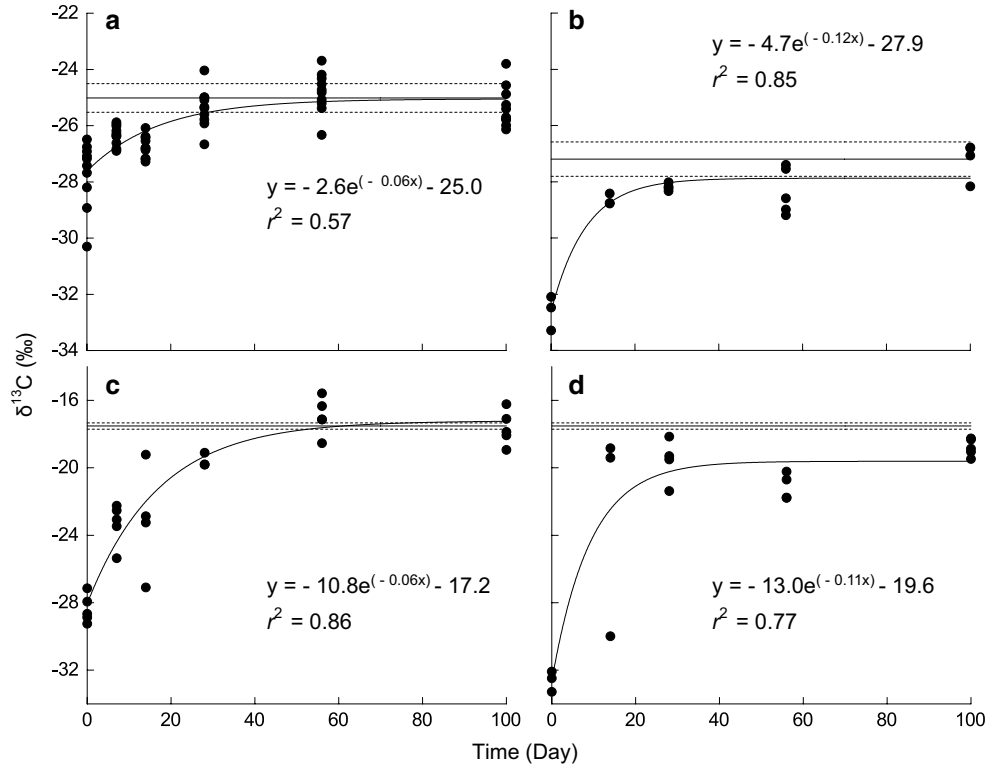
Fig. 4 Temporal change in the isotope value of 18:3n-3. **a** *D. rerio* (TetraMin) ($n = 10$), **b** *B. chinensis* (TetraMin) ($n = 5$), **c** *D. rerio* (*Chlorella*) ($n = 5$), **d** *B. chinensis* (*Chlorella*) ($n = 5$). *Horizontal solid line* represents an average of a given diet, and the *dotted line* represents the SD

Fig. 5 Temporal change in the isotope value of 20:4n-6. **a** *D. rerio* (TetraMin) (*n* = 10), **b** *B. chinensis* (TetraMin) (*n* = 5), **c** *D. rerio* (*Chlorella*) (*n* = 5), **d** *B. chinensis* (*Chlorella*) (*n* = 5). *Horizontal solid line* represents an average of a given diet, and the *dotted line* represents the SD

Fig. 6 Temporal change in the isotope value of 20:5n-3. **a** *D. rerio* (TetraMin) (*n* = 10), **b** *B. chinensis* (TetraMin) (*n* = 5), **c** *D. rerio* (*Chlorella*) (*n* = 5), **d** *B. chinensis* (*Chlorella*) (*n* = 5). *Horizontal solid line* represents an average of a given diet, and the *dotted line* represents the SD

Fig. 7 Temporal change in the isotope value of 22:6n-3. **a** *D. rerio* (TetraMin) (*n* = 10), **b** *B. chinensis* (TetraMin) (*n* = 5), **c** *D. rerio* (*Chlorella*) (*n* = 5), **d** *B. chinensis* (*Chlorella*) (*n* = 5). *Horizontal* *solid line* represents an average of a given diet, and the *dotted line* represents the SD

Fig. 8 Temporal change in the isotope value of 16:0. **a** *D. rerio* (TetraMin) (*n* = 10), **b** *B. chinensis* (TetraMin) (*n* = 5), **c** *D. rerio* (*Chlorella*) (*n* = 5), **d** *B. chinensis* (*Chlorella*) (*n* = 5). *Horizontal solid line* represents an average of a given diet, and the *dotted line* represents the SD
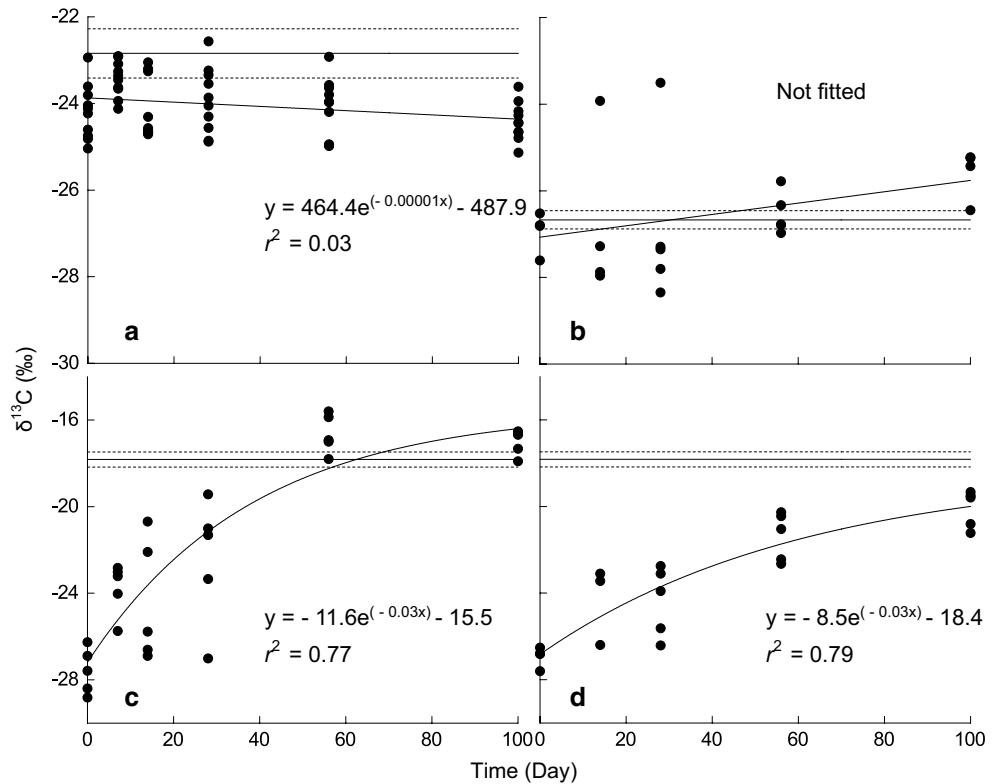
Isotopic fractionation of 20:4n-6, 20:5n-3, and 22:6n-3

As 20:4n-6, 20:5n-3, and 22:6n-3 were absent from *Chlorella*, the increment of these fatty acids in animals resulted from their biosynthesis from 18:2n-6 and 18:3n-3 which were present in *Chlorella*. Thus, the isotope values of 20:4n6, 20:5n3 and 22:6n3 could have been similar to those of 18:2n-6 and 18:3n-3 in *Chlorella*. However, the isotope values of these fatty acids were all small compared with those of their precursors. During synthesis, two carbons are added to 18:2n-6 and 18:3n-3 to synthesize 20:4n-6 and 20:5n-3, respectively, and a further two carbons are added to synthesize 22:6n-3 (Dalsgaard et al. 2003). Thus, if these biosyntheses occur, the isotope values of 20:4n-6, 20:5n-3, and 22:6n-3 may be highly affected by those of the external carbons. Generally, lipids are depleted in ^{13}C compared with other organic matter because lighter carbon tends to be used for lipid synthesis (DeNiro and Epstein 1977, 1978). Thus, these results may be explained by the preferential addition of ^{12}C to 18:2n-6 and 18:3n-3 for the biosynthesis of 20:4n-6 and 20:5n-3, respectively. The greatest depletion observed for 22:6n-3 may be attributed to the further addition of four ^{12}C. For *D. rerio*, the concentrations of 20:5n-3 and 22:6n-3 were also lower than those of their dietary precursors, but the isotopic fractionation was much smaller than that in *B. chinensis*. This may be explained by the difference in increments of these fatty acids during feeding experiments. The amounts of 20:5n-3 and 22:6n-3 increased by around five times in *D. rerio* fed on *Chlorella* after the experiment. However, *B. chinensis* showed only a two to threefold increase in these fatty acids. Fry and Arnold (1982) reported that isotopic equilibrium with new diets was observed after fourfold increases in weight. While this does not pertain to fatty acid isotope values, we hypothesize *B. chinensis* did not accumulate enough fatty acids from their diet to attain isotopic equilibrium. Moreover, the initial isotope value of bulk C in *B. chinensis* was lower than that in *D. rerio*. It seems that additional carbons, which were used for the biosynthesis of 20:4n-6, 20:5n-3, and 22:6n-3, were smaller in *B. chinensis* than in *D. rerio*. This is another reason to explain why the isotope values of *B. chinensis* were different from those of their precursors.

For TetraMin, the isotope values of 20:4n-6, 20:5n-3, and 22:6n-3 in animals were interpreted by the mixing of those of assimilated fatty acids, the effect of enrichment-related metabolism, and newly biosynthesized products from their precursors. Thus, to determine the abundance of new production is important to understand the isotope value of the fatty acids in animals. It is expected that if one precursor is much more abundant than another precursor, then biosynthesis from the latter will be suppressed because the synthesis of 20:4n6 from 18:2n6 and the synthesis of 20:5n3 from 18:3n3 utilize the same enzymes

(Ahlgren et al. 2009). TetraMin contained 18:2n-6 at levels ≈10 times higher than 18:3n-3. It is likely that biosynthesis from 18:2n-6 to 20:4n-6 was dominant and biosynthesis from 18:3n-3 to 20:5n-3 was relatively limited. Thus, the majority of 20:5n-3 and 22:6n-3 in animals can be considered to come from dietary fatty acids directly. This is a possible reason why the isotopic fractionation of 20:5n-3 and 22:6n-3 and its variations were substantially small. However, 20:4n-6 in *B. chinensis* was depleted in ^{13}C by 4.7 ‰ compared to that in the assimilated diet. This suggests that 20:4n-6 was affected by biosynthesis from 18:2n-6 in TetraMin, which had a lower isotope value than 20:4n-6. For *D. rerio*, 20:4n-6 showed high isotopic variation and was not fitted well by the exponential curve. At the end of the feeding experiment, 20:4n-6 only increased by 1.2 times. Thus, it is considered that most of the 20:4n-6 in *D. rerio* at the end of the feeding experiment comes from the initial experimental conditions, which may explain why 20:4n-6 did not show a clear trend. Moreover, the effect of the enrichment in ^{13}C through metabolism tends to be manifested when the contribution of accumulation from the diet is small. In addition, the effect of biosynthesis from 18:2n-6 was also included in the isotope value of 20:4n-6. The existence of multiple factors that can affect isotope values may explain why the variation of the 20:4n-6 isotope value was large.

Implications for food web analysis

Our results show that the isotopic fractionations of 18:2n-6 and 18:3n-3 were small for two animal species and two diets. This may be useful findings for tracing trophic relationships. However, the isotopic fractionation of 20:4n-6, 20:5n-3, and 22:6n-3 seemed to differ widely between the animals and diets, presumably due to the occurrence of bioconversions. The extent of 20:4n-6, 20:5n-3, and 22:6n-3 bioconversions from 18:2n-6 or 18:3n-3 is possibly an important factor governing the isotopic fractionation of these fatty acids. For animals without desaturases, small isotopic fractionation values for 20:4n-6, 20:5n-3, and 22:6n-3 can be expected. Marine animals generally lack the ability to biosynthesize 20:4n-6, 20:5n-3, and 22:6n-3 from the respective precursors (Tocher 2010); therefore, isotopic fractionations of 20:4n-6, 20:5n-3, and 22:6n-3 may be negligible in marine animals.

Author contribution statement M. F. and M. O. originally formulated the idea; M. F. and M. O. conducted all experiments and chemical analyses; O. N. gave advice on the experimental design; M. F. wrote the manuscript; O. N. and M. O. provided editorial advice.

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