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Stable isotope fractionation of fatty acids of *Daphnia* fed laboratory cultures of microalgae

Michail I. Gladyshev[a,b,*], Olesia N. Makhutova[a], Elena S. Kravchuk[a], Olesia V. Anishchenko[a], Nadezhda N. Sushchik[a,b]

[a] Institute of Biophysics of Siberian Branch of Russian Academy of Sciences, Akademgorodok, Krasnoyarsk 660036, Russia
[b] Siberian Federal University, Svobodny av. 79, Krasnoyarsk 660041, Russia

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ABSTRACT

We tested a comparatively new method of tracing of natural food webs, compound-specific isotope analysis (CSIA) of fatty acids (FA), using laboratory culture of *Daphnia galeata* fed *Chlorella vulgaris* and *Cryptomonas* sp. In general, *Daphnia* had significantly lighter carbon stable isotope composition of most fatty acids, including essential, than those of their food, microalgae. Thus, our results did not support the pivotal premise of the FA-CSIA application for food web analysis, i.e., transmitting the isotope 'signal' of essential FAs to consumers from their food without any modification. Moreover, the values of isotope fractionation of particular FAs in the consumer relative to its food were not constant, but varied from 1.35‰ to 7.04‰. The different isotope fractionation (depletion) values of diverse FAs in consumer were probably caused by different processes of their synthesis, catabolism and assimilation. More work is evidently to be done for correct interpretation of results of FA-CSIA during field studies for tracing of natural food webs.

Introduction

One of the pivotal tasks of ecology is study of origin and transfer of organic carbon in natural food webs. In aquatic ecosystems, carbon fluxes at present are traced using biomarkers (primarily fatty acids) and stable isotopes (e.g., Lu et al., 2014). Usually stable isotope ratio of bulk carbon is measured, while in last decades a new powerful tool, compound specific isotope analysis (CSIA), appeared, which combines biomarker and isotope approaches. For instance, the combination of fatty acid and isotope analyses (FA-CSIA) was found to be important for tracing of carbon fluxes in the food webs that might have been overlooked otherwise (Budge et al., 2008). Specifically, FA-CSIA is essential in three cases: (1) when studied organisms cannot be physically isolated from each other (e.g., phyto- and bacterioplankton); (2) if we need to trace quantitatively minor but qualitatively important component; (3) when different food sources have similar bulk carbon isotope and FA signatures (Gladyshev et al., 2012).

The key premise of the method of FA-CSIA is that the isotope 'signal' of essential FAs is transmitted to consumers from their food without any modification, since these FAs are not synthesized de novo by consumers (Budge et al., 2008; Koussoroplis et al., 2010; Bec et al., 2011; Wang et al., 2015). However, a number of authors reported significant changes of stable isotope composition of essential FAs in consumers' tissues, which occurred probably during metabolism (trophic fractionation) of these dietary FAs (Jim et al., 2003; Budge et al., 2011; Gladyshev et al., 2012, 2014a). The trophic fractionation of essential FAs might constitute a major fence to the use of FA-CSIA to trace natural food webs (Bec et al., 2011). Thereby, the important questions about isotopic fractionation of essential FAs should be studied in controlled feeding experiments before FA-specific isotope analysis is used to estimate diets of consumers in the field (Budge et al., 2011; Wang et al., 2015).

Very important controlled feeding experiment with conventional model planktonic consumer, *Daphnia*, was carried out recently by Bec et al. (2011). The animals were fed three food sources: diatom and flagellate algae and heterotrophic protist (Bec et al., 2011). Studying isotope ratios in neutral lipids and in phospholipids of *Daphnia*, the authors found out a significant isotope fractionation (namely depletion) of the consumer's essential fatty acids compared to their food, which contradicted to many conventional ideas on FA synthesis and transmission (Bec et al., 2011). However, there were some inevitable experimental biases in this

* Corresponding author at: Institute of Biophysics of Siberian Branch of Russian Academy of Sciences, Akademgorodok, Krasnoyarsk 660036, Russia. Tel.: +7 3912494517.
E-mail address: glad@ibp.ru (M.I. Gladyshev).

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study, for instance, related to FA turnover in *Daphnia* (Bec et al., 2011), and to the limited number of kinds of food sources which may result in a specific fractionation pattern. Indeed, the interpretation of stable isotopes even in comparatively simple laboratory experiments is complex, but essential to apply FA-CSIA to natural field systems (Pond et al., 2006). Thereby, further researches are deserved to interpret isotope patterns of fatty acids in *Daphnia* (Bec et al., 2011), especially taking into account conflicting results on the isotope fractionation (Wang et al. 2015).

Thus, the aim of our study was to test the findings of Bec et al. (2011) on the isotope fractionation of fatty acids in *Daphnia* compared to that of their food using a different experimental protocol, and to estimate a potential importance of the putative fractionation for interpretation of field FA-CSIA data for zooplankton. Specifically, we aimed to answer following questions: (1) does the isotope fractionation occurred in total FAs, which are often used in field measurements; (2) are there differences between the fractionation of the physiologically important eicosapentaenoic acid (20:5n-3, EPA), synthesized by *Daphnia* and obtained from food; (3) are there quantitative differences in the isotope fractionation of different FAs, including essential and non-essential?

Materials and methods

Cultivation of organisms

The stock culture of a clone of *Daphnia galeata* Sars, originally isolated from the Bugach Reservoir in 2000, was maintained in tap water at 20–26 °C and fed with the chlorophyte *Chlorella vulgaris* (culture collection of Institute of Biophysics SB RAS). In experiments, *Ch. vulgaris* and *Cryptomonas* sp. (culture collection of I.D. Papanin Institute for Biology of Inland Waters RAS) were used as food for *D. galeata*. We used batch cultures of the algae, like in the similar experiment of Bec et al. (2011). The batch cultures of *Ch. vulgaris* and *Cryptomonas* sp. were grown at 18–22 °C and an illumination of 6000 lx (16:8 h light:dark cycle). *Ch. vulgaris* was cultivated in aerated 1-L flasks in Tamiya medium. *Cryptomonas* sp. was cultivated in WC medium in 250-mL flasks without aeration.

Preparation of food

Algae from batch cultures were concentrated and washed from the medium by centrifugation. The conditions of centrifugation: for *Chlorella*—4000 × *g*, 6 min., for *Criptomonas*—1000 × *g*, 8 min. The concentrated algae were kept at +4 °C. An aliquot of concentrated algae were diluted by tap water to obtain concentration ~1 mg L^{-1} of organic carbon, like in similar experiment of Bec et al. (2011). To obtain the given concentration, the process of dilution was controlled by measurements of chlorophyll DCMU-fluorescence (Gaevsky et al., 2005) using fluorometer FL-303 (Siberian Federal University, Krasnoyarsk, Russia) with light beams 410 and 540 nm. Calibration curves for the DCMU-fluorescence vs. organic carbon content (using elemental analyzer Flash EA 1112 NC Soil/MAS 200, ThermoQuest, Italy) in each culture of algae were obtained before the experiment (data are not shown).

Experiments

The experiment was conducted under dim light (16:8 h light:dark cycle) at 18–22 °C and consisted of two stages. The first stage was an adaptation of the animals from stock culture to the given food. The adaptation was performed to overcome probable bias of the previous experiment of Bec et al. (2011), where the absence of adaptation might affect estimation of differences in $\delta^{13}C$ between dietary and *Daphnia* FA because of FA turnover in *Daphnia*. The adaptation was carried out 7 days, because it takes ~1 week for *Daphnia* and many other zooplankton species to turn over their FA pool (Taipale et al., 2009; Gladyshev et al., 2010). During the adaptation, animals were held in six 3-L glass jars with the food suspensions. In each jar 339 ± 34 ind., 33.2 ± 1.7 mg (wet weight) of *D. galeata* of different ages and sizes were placed to simulate natural populations. Every day, 10% of medium (food suspensions) in each jar were replaced by fresh portion from the batch cultures of algae.

At the start of the second stage of the experiment that lasted for 3 days, all the animals, adapted to the given food, from each 3-L jar were transferred into 1-L jars with newly prepared suspensions of the same food. Six 1-L jars were placed into a 'plankton wheel' (diameter, 38 cm, 0.2 rpm, Gladyshev et al., 1993). The 'plankton wheel' was used to prevent sedimentation of algae providing homogeneous 'plankton' conditions and to avoid probable effect of heterogeneity (crowding of some part of population of *Daphnia* near walls to obtain more food) on FA isotope fractionation. Every day, 50% of medium in each 1-L jar was replaced by a new portion of food suspensions.

Two runs of the above two-stage experiment were done. In the first run, in 5 jars the food was *Chlorella*, and in 1 jar the food was *Cryptomonas*. In the second run in 5 jars the food was *Cryptomonas*, and in 1 jar the food was *Chlorella*. Below, *D. galeata* fed *Ch. vulgaris* is designated as *Daphnia* (Chl), and *D. galeata* fed *Cryptomonas* sp. is designated as *Daphnia* (Cry).

Samples of algae for following FA and CSIA analyses were taken from the batch cultures, which were used for feeding. Although the batch cultures were kept under the same stable conditions during all the experiment, and thereby were believed to be similar in FA and isotope compositions, samples (replicates) were distributed through the period of experiment. Finally, 9 samples (replicates) of *Ch. vulgaris* were obtained: 3 samples at the end of the first run (10th day), 3 samples at 7th day of the second run, and per 1 sample at 8th, 9th and 10th day of the second run. For *Cryptomonas* sp. 6 samples were obtained: 3 samples at 1st day of the second run, and per 1 sample at 7th, 8th and 9th days of the second run.

Samples of *Daphnia* for FA and CSIA were taken from the 1-L 'plankton wheel' jars, at the end of the first and the second runs: 1st run, 5 samples of *Daphnia* (Chl), while 1 sample of *Daphnia* (Cry) was lost because of a technical accident; 2nd run, 1 sample of *Daphnia* (Chl) and 5 samples of *Daphnia* (Cry). Finally, number of samples of *Daphnia* (Chl), $n = 6$, and for *Daphnia* (Cry), $n = 5$. All the samples of each alga and *Daphnia* were treated as replicates in following statistical analyses.

Fatty acid sampling and analyses

To analyze fatty acids, samples of both algae cultures were collected onto precombusted Whatman GF/F filters. Each sample corresponded to 2–5 mg of organic carbon range. Filters loaded with algae biomass were placed in chloroform:methanol (2:1, v:v) and stored at −20 °C for later fatty acid analysis. At the end of each experiment, all *Daphnia* alive individuals from each jar were placed in a volume of tap water for 3 h to empty their guts. Then, they were collected as separate samples for fatty acid analysis. The collected animals were gently wiped with filter paper, weighed, placed in chloroform:methanol mixture (2:1, v/v), and kept at −20 °C for later analysis.

Lipid extraction and subsequent preparation of fatty acid methyl esters (FAMEs) were the same as in our previous works (e.g., Gladyshev et al., 2015). A gas chromatograph equipped with a mass spectrometer detector (model 6890/5975C; Agilent Technologies, Santa Clara, USA) and with a 30 m long, 0.25 mm internal diameter capillary column HP-FFAP was used for FAME analysis. Each sample of fatty acids was analyzed as a single replicate. Replicate injections of available authentic FAME standards (Sigma, USA) indicated