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Title: Stable isotope fractionation of fatty acids of Daphnia fed laboratory cultures of microalgae

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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 particular of 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.

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Cover Letter

Dear Editor,

Send you our revised paper. Our reply to Reviewers' comments, point by point, are attached in separate file.

Sincerely, Michail

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Reply to Reviewers' comments

Reviewer #1: *This study investigates whether fatty acids isotope ratios of Daphnia reflect those of its food. The authors have clearly explicited why it is a critical issue in trophic ecology. To my knowledge there is only one paper dealing with this issue (Bec et al). Contrary to the assumed hypothesis, Bec et al observed that even fatty acids signatures of Daphnia reflect of those of its food, trophic transfer could induce a significant fractionation (even for essential fatty acids). The present study (which has eliminated one of the potential experiemental bias cited in Bec et al) confirms this conclusion and that is why I think this paper deserves to be published. However some minor corrections should be done before publication and will improve the clarity of the ms.*

First the methods should be more clearly explicited. The experiment is divided in different stages and different runs. The authors should better explain why different stages and different runs have been conducted.

- The first stage of the experiment, adaptation to the food, was performed to overcome probable bias of the previous experiment of Bec et al. (2011), where the absence of adaptation might affect estimation of changes in $\delta^{13}C$ between dietary and *Daphnia* FA because of FA turnover in *Daphnia*. As recommended, this explanation is added in Methods (lines 114-116 of the revised MS).

The second stage of the experiment consisted in placing of the adapted animals and the food, to which they were adapted, in 'plankton wheel' to provide homogenous conditions and thereby to avoid probable effect of heterogeneity (crowding of some part of population near walls to obtain more food) on FA isotope fractionation. As recommended, this explanation is added in Methods (lines 126-129 of the revised MS).

Secondly there is only one figure of results. Where do these results come from? It is quiet difficult to link the methods ' to the results part.

- As recommended, sampling protocol is given in details in Methods (lines 136-149 of the revised MS).Besides, numbers of samples, *n*, are given in Table 1 heading.

Endly I think discussion could be improved. For instance the authors could mention which cautions are required for interpreting CSIA results in field studies.

- As proposed by the Reviewer, some recommendations on interpretation of FA-CSIA field results are given in Conclusion (lines 380-387 of the revised MS).

Below are some questions which could help the authors to improve their discussion. They found large fractionations especially for fatty acids which were in low proportions in the diet (18:2(n-6) in Cryptomonas for instance). Do such large fractionations should be taken into account?

- We found large trophic fractionation of the essential FAs when they occurred in food both in low and high proportions. We proposed the explanation of different fractionation of the absolutely essential FA (18:2n-6 and 18:3n-3) in *Daphnia* as resulted from their necessity for following conversion in long-chain polyunsaturated FA. In turn, the conversion rate depended on a presence and level of the end-products (20:4n-6 and 20:5n-3) in food (lines 332-344 of the revised MS).

Fatty acids isotope ratios of Daphnia do not necessary match those of its food but do fatty acids signatures of Daphnia reflect those of its food?

- Yes, it is very good remark of the Reviewer. We added in the Discussion the proposed comment, that although the signals were not equal, the isotope ratios of essential FA of *Daphnia* were lower, rather than higher, than the isotope ratios in the microalgae, and thereby reflected those of its food (lines 365-368 of the revised MS).Recommendations on interpretation of FA-CSIA field results, which are added in Conclusion (see above), also are based at this comment of the Reviewer.

Reviewer #2*: This interesting study on the fatty acid (non-)metabolism of Daphnia presents some potentially very important findings that deserve further study. In particular, the authors' conclusion that Daphnia may synthesize EPA (20:5n-3) contradicts the current paradigm that Daphnia, like most animals, cannot synthesize polyunsaturated fatty acids (PUFA) de novo. The approach is sound and the study seems well executed.*
The presentation of the results needs some more clarity. For instance, I understand that the FA composition were analyzed on two sampling days each; it is not clear if the results shown in Tab. 1 and Fig. 1 were averaged over both sampling dates.

- As recommended, sampling protocol is given in details in Methods (lines 136-149 of the revised MS).

The analytical limits should also be reported. This is important because the FA composition was presented in relative units (% of total), i.e. analytical errors for one particular FA may affect all results.To this end, I recommend to calculate the FA content on a per dry weight basis for both prey and predator.

- We gave the analytical error for FA measurement in Methods and also showed the detection limits (as percentages of the total FA) for FA measuring by GS-MS (lines 165-169). We must emphasize that analytical limit of a peak detection doesn’t depend on FA content per dry weight, but relates namely with FA percentages. We can not give in this MS the FA content per dry weight since we intentionally measured only FA percentages in the algae and *Daphnia*. We suppose that comparison of FA content per mass unit between a prey and a predator are necessary mostly for ecological trophic transfer studies. In contrast, most studies of FA metabolism are based on FA percents in various lipid classes or total lipids. We aimed in the MS to study isotope trophic fractionation in a consumer organism that results from kinetics of metabolic reactions. Therefore, we chose FA percentages as a base of our study.

Isotope fractionation exceeding 5‰ deserve some explanation.

- We found large trophic fractionation of the essential FAs when they occurred in food in both low and high proportions. We proposed the explanation of different fractionation of the absolutely essential FA (18:2n-6 and 18:3n-3) in *Daphnia* as resulted from their necessity for following conversion in long-chain polyunsaturated FA. In turn, the conversion rate depended on a presence and level of the end-products (20:4n-6 and 20:5n-3) in food (lines 332-344 of the revised MS).

The previous similar study by Bec et al (2011) used an unspecified Daphnia sp.; the question arises to what an extent the results are species- or strain-specific. Accordingly, the authors could make a much stronger claim for de novo synthesis of PUFA's in Daphnia if they would verify their results with a second species.

- We absolutely agree with the Reviewer. Unfortunately, at present it is impossible to answer these questions. More work should be done in future. Nevertheless, as recommended, we arise the above issues in Discussion (lines 275-278 of the revised MS).

The language requires some editing by a native speaker.

- We tried to do our best to improve English.

Minor: Please report irradiance in SI units (Methods) p.4 and label Y-axis in Fig. 1

- As recommended, the irradiance is given in SI units and Y-axis in Fig. 1 is labeled.

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

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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 particular of 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.

Keywords: Fatty acids; Compound-specific isotope analysis; Stable isotope fractionation; 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, 2014). 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 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 butch cultures, which were used for feeding. Although the butch 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 hours 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 analysed as a single replicate. Replicate injections of available authentic FAME standards (Sigma, USA) indicated that analytical precision was <0.6%. The limit of FAME detection, i.e., the minimum percentage at which distinct peaks could still be discerned above the baseline noise, was accounted for 0.02 % of the total FA. Detailed description of chromatographic and mass-spectrometric conditions was given elsewhere (Gladyshev et al., 2014).

Compound specific isotope analyses

The compound specific isotope analyses of fatty acids (CSIA-FA) were done according to the protocol described by Gladyshev et al. (2012). Briefly, $\delta^{13}C$ of samples (expressed in ‰) were analyzed from FAME sub-samples using the same chromatographic column and similar temperature conditions as for GC-MS analyses of fatty acid composition. Carbon isotopic composition of an individual FAME was determined with a Trace GC Ultra (Thermo Electron) gas-chromatograph which was interfaced with a Delta V Plus isotope ratio mass spectrometer

(Thermo Fisher Scientific Corporation) via a type-III combustion interface. The isotopic values of the chromatographic peaks produced by the combustion of all separated compounds were calculated using CO_2-spikes of known isotopic composition, introduced directly into the source three times at the beginning and end of every run. The alkane references mixture of known isotopic composition (C15, C20, C25, Chiron, Norway) was run after every three-four samples to check the accuracy of the isotopic ratios determined by the GC-IRMS. Stable carbon isotope ratios for individual fatty acids were recalculated from FAME data by correcting for one carbon from the methyl group added during the methanolic transesterification. The isotopic composition of the used methanol was determined by the same GC-IRMS system working isothermally at 65 ^{o}C.

Not all FAs were present in sufficient quantities to determine their respective δ^{13}C FA values. δ^{13}C FA values were determined for 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3 in all samples. In *Chlorella* and *Daphnia* (Chl) also 16:2n-6, 16:3n-3 and 16:4n-6 gave enough large peaks to determine their δ^{13}C values. In samples of *Cryptomonas* we additionally determined the δ^{13}C values of 18:5n-3, 20:5n-3 and 22:6n-3. In samples of *Daphnia* (Cry) and *Daphnia* (Chl) the δ^{13}C values of 20:4n-6, 20:4n-3, 20:5n-3 and 22:6n-3, and 20:4n-6 and 20:5n-3, respectively, were measured.

Statistical analysis

Standard errors (SE), Student's *t*-test and one-way ANOVA with Tukey HSD *post hoc* tests were calculated conventionally, using STATISTICA software, version 9.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

43 fatty acids were identified in all samples. Quantitatively prominent FAs are given in Table 1. *Ch. vulgaris* and *Cryptomonas* sp. contained typical fatty acid composition for green algae and cryptophytes, respectively. *Chlorella* was characterized by high percent of 18:3n-3, 16:0, 16:3n-3 and 18:2n-6, while typical FAs for *Cryptomonas* were 16:0, 18:4n-3, 18:3n-3 and 20:5n-3 (Table 1). There were several groups of fatty acids concerning ratios of their average percentages in food source versus *Daphnia* biomass. In the first group, there were FAs, 15:0, 16:1n-9, 16:1n-7, i17:0, ai17:0, i17:1, 17:0, 18:0, 18:1n-9, 20:0, 20:4n-6, 20:3n-3 and 22:0, which had significantly higher percentage in biomass of *Daphnia* (Chl) and *Daphnia* (Cry), than those in biomass of their food, *Chlorella* and *Cryptomonas*, respectively (Table 1). 18:1n-7 also tended to belong to the first group, although the increase in *Daphnia* (Chl) was statistically insignificant by Tukey test (Table 1), but it was significant ($p<0.05$) according to Student's test, $t = 16.29$, degree of freedom, d.f. = 13.

In the second group, 16:1n-13tr had significantly lower percentage in biomass of *Daphnia* (Chl) and *Daphnia* (Cry), than those in biomass of their food, *Chlorella* and *Cryptomonas*, respectively (Table 1). 16:2n-4 also tended to belong to the second group, although the decrease in *Daphnia* (Chl) was statistically insignificant by Tukey test (Table 1), but it was significant according to Student's test, $t = 3.08$, d.f. = 13.

In the third group, there were FAs, 14:0, i15:0 and 20:5n-3, which had significantly higher percentage in biomass of *Daphnia* (Chl) than in *Chlorella*, but nearly similar or significantly lower percentages in *Daphnia* (Cry) than in *Cryptomonas* (Table 1).

In the fourth group, there were FAs, 16:3n-3, 16:4n-3 and 18:2n-6, which had significantly higher percentage in biomass of *Daphnia* (Cry) than in *Cryptomonas*, but nearly similar or significantly lower percentages in *Daphnia* (Chl) than in *Chlorella* (Table 1).

In the fifth group, there were FAs, 16:2n-6 and 18:3n-3, which had significantly lower percentage in biomass of *Daphnia* (Chl) than in *Chlorella*, but nearly similar or significantly higher percentages in *Daphnia* (Cry) than in *Cryptomonas* (Table 1).

In the sixth group, there were FAs, 12:0, 16:0, 18:4n-3, 18:5n-3, 20:2n-6, 22:5n-6 and 22:6n-3, which had significantly lower percentage in biomass of *Daphnia* (Cry) than in *Cryptomonas*, but nearly similar or significantly higher percentages in *Daphnia* (Chl) than in *Chlorella* (Table 1).

Two FAs, ai15:0 and 20:4n-3, had nearly similar percentages in both *Daphnia* and in their food (Table 1).

All fatty acids, taken for CSIA, were significantly more depleted in *Daphnia* than in their food, except 16:4n-3 and 18:0 for *Daphnia* – *Chlorella*, and 22:6n-3 for *Daphnia* – *Cryptomonas* (Fig. 1). In *Daphnia* (Chl), 20:5n-3 was significantly depleted compared to its precursor, the essential 18:3n-3: difference $\delta^{13}C_{18:3n-3} - \delta^{13}C_{20:5n-3} = 5.55‰$, $t = 7.80$, d.f. = 10. In *Daphnia* (Cry) this difference was also significant, but comparatively small: $\delta^{13}C_{18:3n-3} - \delta^{13}C_{20:5n-3} = 1.60‰$, $t = 3.07$, d.f. = 8. In contrast to the pair 20:5n-3 and 18:3n-3, in *Daphnia* (Chl), 20:4n-6 was insignificantly ($p>0.05$) enriched compared to its precursor, the essential 18:2n-6: $\delta^{13}C_{18:2n-6} - \delta^{13}C_{20:4n-6} = –1.10‰$, $t = 1.38$, d.f. = 10. In turn, in *Daphnia* (Cry) a significant depletion occurred: $\delta^{13}C_{18:2n-6} - \delta^{13}C_{20:4n-6} = 2.93‰$, $t = 2.39$, d.f. = 8.

Isotope ratios of two fatty acids, 16:0 and 18:3n-3, in *Chlorella* were significantly higher, than those in *Cryptomonas*, $t = 13.90$ and $t = 2.64$, respectively, d.f. = 13, while that of 18:1n-9 was significantly lower, $t = 4.19$, d.f. = 13, and $\delta^{13}C$ values of 18:0 and 18:2n-6 differed insignificantly (Fig. 1). The isotope ratios of 18:1n-9 in *Daphnia* (Chl) was significantly lower, than that in *Daphnia* (Cry), $t = 3.62$, d.f. = 9, reflecting the difference between algae (Fig. 1). In turn, $\delta^{13}C$ value of 18:3n-3 was significantly higher in *Daphnia* (Chl) than in *Daphnia* (Cry), $t = 7.56$, d.f. = 9 (Fig. 1) and also resulted from the corresponding difference between algae. There

were no statistically significant differences in isotope ratios of the other FAs between these two experimental populations of *Daphnia* (Fig. 1).

Discussion

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, evidently was not supported by the results of our experiments. Indeed, average $\delta^{13}C$ values of 18:2n-6, 18:3n-3 in *Daphnia* (Chl) and in *Daphnia* (Cry) were lower by 5.02‰, 1.35‰ and 7.04‰, 4.18‰, respectively, than those in their food. The isotope ratio of 20:5n-3, which also may be regarded as essential for daphnids (Bec et al., 2011), was lower by 1.42‰ in *Daphnia* (Cry) than in their food. However, it is worth to note, that in spite of the absence of 20:5n-3 in *Chlorella*, there was a considerable level of this FA, 0.65 ± 0.10%, in *Daphnia* (Chl). Thus, the studied population of *D. galeata* evidently was capable of synthesis of small amounts of 20:5n-3 from 18:3n-3, which appeared to be sufficient for survival of this species in the laboratory monoculture. However, survival of populations of *Daphnia* with such low level of 20:5n-3 in natural ecosystems is quite questionable. Indeed, the lowest level of 20:5n-3, published for *Daphnia* in an ecosystem, was 2.5% (Gladyshev et al., 2015). It should be noted, that at present we do not know to what an extent the fractionation and the ability to synthesize 20:5n-3 from 18:3n-3 are species- or strain-specific. Evidently, more work should be done in future to specify these issues.

Thereby, using FA-CSIA to trace food sources of *Daphnia* in natural conditions, one would made the misleading conclusion, that these animals had another food sources, than *Chlorella* and *Cryptomonas*, since these alga and *Daphnia* had different $\delta^{13}C$ values of essential FAs. It was supposed earlier, that there could be a 'fractionation constant' for essential FAs in

consumers vs. their food, which could give an opportunity to make an appropriate correction of FA-CSIA data and thereby enable their usage for tracing of food webs (Gladyshev et al., 2014). However, data of present experiments did not support this hypothesis, because the fractionation, i.e., difference between $\delta^{13}C$ values of the essential FAs of *Daphnia* and their food, as mentioned above, evidently was not constant, but varied from 1.35‰ to 7.04‰.

What processes could cause different values of fractionation of fatty acids isotope signatures in consumers? Conventionally, the fractionation may occur during FA synthesis (kinetic isotope effect, KIE, during elongation and desaturation), catabolism (β-oxidation) and digestive assimilation (hydrolysis, esterification, re-esterification) (DeNiro and Epstein, 1977; Abrajano et al., 1994; Rhee et al., 1997; Koussoroplis et al., 2010; Budge et al., 2011; Bec et al., 2011; Gladyshev et al., 2012; Hixson et al., 2014).

As to FA depletion due to KIE during their synthesis and further elongation and desaturation, an opposite effect occurred in many cases in our experiment. Indeed, in *Chlorella* 16:2n-6 was heavier, not lighter, than 16:0, 18:2n-6 was heavier than 18:1n-9, and 18:3n-3 was heavier, than 18:2n-6. In *Cryptomonas* 18:2n-6 also was heavier than 18:1n-9, and 18:0 was heavier, than 16:0. In *Daphnia* (Chl) 20:4n-6 was heavier, than 18:2n-6. However, isotope ratios of many other FAs, e.g., 20:5n-3 vs. 18:3n-3 in *Daphnia* (Chl), were in a good agreement with KIE.

The diverse processes, synthesis, catabolism and assimilation, seemed to result in different values of isotope fractionation of different fatty acids. Basing on comparison of percentages of FAs in *Daphnia* and their food, fatty acids were subdivided in several groups, probably controlled by different processes. For instance, in the first group, 18:0 was accumulated (had significantly higher level) in *Daphnia*, than in the algae. However, the higher levels were probably provided by different mechanisms in two cultures, which resulted in different isotope signatures. Indeed, *Chlorella* had very low level of 18:0, and *Daphnia* (Chl) probably had to synthesize this important acid *de novo*. Since the carbon source for the synthesis and the

pathways were similar in the algae and the animals, there were no differences in isotope signatures between 18:0 in *Chlorella* and in *Daphnia* (Chl). In contrast, *Cryptomonas* had comparatively high level of 18:0, and *Daphnia* (Cry) could assimilate and accumulate this FA from food, rather than synthesize it *de novo*. Thereby, due to putative digestive fractionation, 18:0 in *Daphnia* (Cry) had significantly lighter isotope composition, than that in *Cryptomonas*.

Values of $\delta^{13}C$ of FA from another 'percent group', 20:5n-3, were nearly similar in *Daphnia* (Chl), which evidently synthesized this acid, and in *Daphnia* (Cry), which obtained it from food. However, in some cases it may be better to analyze the relative changes in carbon isotopic compositions of the physiologically important FA and its biochemical precursor rather than compare their absolute values of this FA (Schouten et al., 1998). Thus, comparing isotope ratios of 20:5n-3 with those of 18:3n-3 in both cultures of *Daphnia*, one can see, that in *Daphnia* (Chl) 20:5n-3 was significantly more depleted than its biochemical precursor, 18:3n-3. Meanwhile, in *Daphnia* (Cry) the difference in $\delta^{13}C$ between 20:5n-3 and 18:3n-3 was comparative very small, probably due to the fact, that *Daphnia* (Cry) did not synthesize 20:5n-3, but accumulated it from food.

Most FAs of *Chlorella* were heavier, than those of *Cryptomonas*. This fact could be explained by twice higher value of specific growth rate of the culture of *Chlorella* compared to that of *Cryptomonas* (Kravchuk et al., 2014), since $\delta^{13}C$ values of algae are known to increase with increase of growth rate (e.g., Pel et al., 2003; Tolosa et al., 2004). Nevertheless, in contrast to many other FAs, isotope ratio of 18:1n-9 in *Chlorella* was significantly lower, than that of *Cryptomonas*. Accordingly, many fatty acids of *Daphnia* (Chl) were isotopically heavier than those of *Daphnia* (Cry), except the significantly lighter 18:1n-9. Thus, there was no uniform pattern in isotope fractionation of all fatty acids, including that of essential and non-essential.

Indeed, the fractionation of absolutely essential 18:2n-6 was relatively large in both cases, i.e., in *Daphnia* (Cry), and in *Daphnia* (Chl). In turn, value of trophic fractionation of 18:3n-3 was prominent (4.18 ‰) only in *Daphnia* (Cry). In *Daphnia* (Chl), 18:3n-3 was

evidently used as the precursor for 20:5n-3 synthesis, since *Chlorella* contained no 20:5n-3 at all. Thus, we suppose that *Daphnia* likely assimilated 18:3n-3 from *Chlorella* food with a high rate, and, according to KIE, this resulted in relatively low fractionation of this essential FA. Moreover, for synthesis of 20:5n-3, the lighter part of 18:3n-3 pool was used, hence, the rest of the pool would become heavier. In contrast, 20:4n-6 presented in the both types of alga food (Table 1), therefore, conversion of 18:2n-6 → 20:4n-6 was probably limited in both *Daphnia* (Chl) and *Daphnia* (Cry). This resulted in low assimilation rates and high fractionation of 18:2n-6, especially in *Daphnia* (Cry), 7.04‰, since *Cryptomonas* was moderately richer in 20:4n-6, than *Chlorella*. Hence, we hypothesized that the less an essential FA is necessary as a precursor for following synthesis, the more fractionation during assimilation from food happens.

In our study, we confirmed the main finding of the seminal experiment of Bec et al. (2011), that FA in *Daphnia* lipid classes were generally ^{13}C-depleted compared with their counterpart in the corresponding diet. We confirmed this result, using total fatty acids, which are often used for CSIA in field food web studies (Budge et al., 2008; Lau et al., 2009; Gladyshev et al., 2012; Wang et al., 2015). It was worth to test if the fractionation, found by Bec et al. (2011) in the laboratory culture for neutral and polar lipids, was also prominent for total lipids. Moreover, Bec et al. (2011) noted, that their estimates of changes in δ^{13}C between dietary and *Daphnia* FA might be affected by experimental biases related to FA turnover in *Daphnia*. In our experiment, we used *Daphnia*, pre-adapted to FA composition of given food during a week (Taipale et al., 2009; Gladyshev et al., 2010). Nevertheless, the main result on the isotope fractionation was confirmed.

However, in addition to the differences mentioned above, i.e., analyses of total FA and usage of pre-adapted cultures, there was another peculiarity in protocol of our experiment compared to that of Bec et al. (2011). Indeed, we used food, *Chlorella*, which contained no 20:5n-3. Thereby, we revealed synthesis of 20:5n-3 by *Daphnia*, and measured the relevant value of isotope fractionation of this fatty acid. In contrast, Bec et al. (2011) studied only

accumulation of this important FA from different food sources. Thus, in our experiment, we found that even the same fatty acid, e.g., 20:5n-3, can have different values of isotope fractionation in consumer, if it is obtained by different mechanisms, synthesis and accumulation. Besides 20:5n-3, the same was true also for other FAs, for instance for 18:0.

Thus, isotope ratios of essential fatty acids of *Daphnia*, did not match of its food, i.e., their values were not equal. However, since isotope ratios of all the essential acids in the animals were lower, not higher, than the isotope ratios in the microalgae, it may be concluded that the isotope signals of these FAs of *Daphnia* generally reflected those of its food.

Conclusions

Using analyses of total fatty acids and laboratory cultures of *Daphnia*, pre-adapted to given food, we confirmed significant fractionation of isotope content of fatty acids in the consumer compared to those in their food. However, values of the fractionation were not constant, but varied significantly between different FAs, both essential and non-essential, and likely depended on way of obtaining of this or that FA, e.g., by synthesis or by accumulation. Thus, the common way of interpretation of results of FA-CSIA for tracing of natural food chains, based on unmodified transmission of isotope 'signal' of essential fatty acids or on a constant of their fractionation, may give misleading results. More work is evidently to be done for correct application of FA-CSIA during field studies. However, basing on the present results, we can give some recommendations for interpretation of field FA-CSIA data: 1) if values of $\delta^{13}C$ of essential FA of a zooplankton species is lower, than that of a microalga, it does not necessary mean, that this microalga is not consumed by this zooplankton; 2) if values of $\delta^{13}C$ of essential FA of a zooplankton species is equal to that of a microalga, it does not necessary mean, that this microalga is consumed by this zooplankton; 3) if values of $\delta^{13}C$ of essential FA of a zooplankton

species is higher, than that of a microalga, it likely means, that this microalga is not consumed by this zooplankton and an alternative food item should be considered.

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Table 1

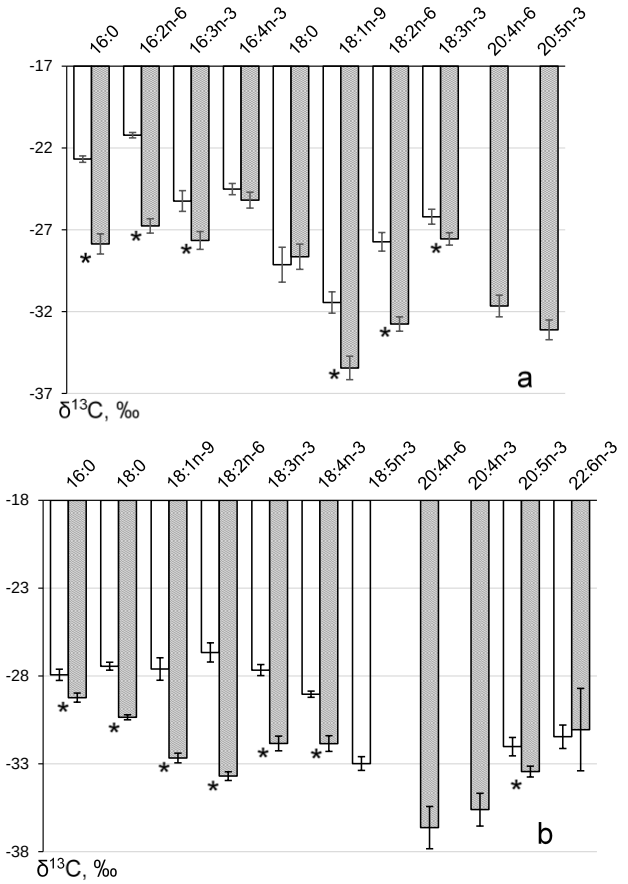
Mean values (± SE) of percentages of quantitatively prominent fatty acids (% of total FA) in biomass of *Chlorella vulgaris* (numbers of samples, *n* = 9), *Cryptomonas* sp. (*n* = 6), and in biomass of *Daphnia galeata*, fed *Ch. vulgaris*, *Daphnia* (Chl) (*n* = 6), and *D. galeata* fed *Cryptomonas* sp., *Daphnia* (Cry) (*n* = 5). Means labelled with the same letter are not significantly different at $P< 0.05$ after Tukey HSD *post hoc* test.

Fatty acid	*Chlorella*	*Cryptomonas*	*Daphnia* (Chl)	*Daphnia* (Cry)
12:0	0.16 ± 0.01^{A}	0.86 ± 0.14^{B}	0.19 ± 0.01^{A}	0.26 ± 0.02^{A}
14:0	0.40 ± 0.01^{A}	1.04 ± 0.11^{B}	1.35 ± 0.16^{B}	1.05 ± 0.05^{B}
i15:0	0.03 ± 0.00^{A}	0.41 ± 0.14^{B}	0.38 ± 0.02^{B}	0.62 ± 0.05^{B}
ai15:0	0.02 ± 0.01^{A}	0.15 ± 0.06^{B}	0.11 ± 0.01^{AB}	0.21 ± 0.01^{B}
15:0	0.25 ± 0.01^{A}	0.45 ± 0.03^{B}	1.40 ± 0.04^{C}	0.85 ± 0.04^{D}
16:0	19.20 ± 0.21^{A}	18.21 ± 0.87^{A}	17.83 ± 1.04^{A}	14.48 ± 0.47^{B}
16:1n-9	0.69 ± 0.02^{A}	0.51 ± 0.06^{B}	1.38 ± 0.02^{C}	1.46 ± 0.05^{C}
16:1n-7	0.89 ± 0.02^{A}	3.16 ± 0.87^{B}	3.05 ± 0.41^{B}	5.48 ± 0.18^{C}
16:1n-13tr	4.33 ± 0.06^{A}	0.63 ± 0.06^{B}	0.00 ± 0.00^{C}	0.00 ± 0.00^{C}
i17:0	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	0.65 ± 0.01^{B}	0.71 ± 0.02^{C}
16:2n-6	4.81 ± 0.08^{A}	0.00 ± 0.00^{B}	3.33 ± 0.23^{C}	0.96 ± 0.06^{D}
ai17:0	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	0.11 ± 0.01^{B}	0.29 ± 0.02^{C}
16:2n-4	0.09 ± 0.00^{A}	1.22 ± 0.05^{B}	0.07 ± 0.00^{A}	0.09 ± 0.00^{A}
i17:1	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	1.10 ± 0.07^{B}	1.02 ± 0.05^{B}
17:0	0.38 ± 0.04^{A}	0.37 ± 0.03^{A}	1.32 ± 0.03^{B}	1.28 ± 0.04^{B}
16:3n-3	14.02 ± 0.19^{A}	0.00 ± 0.00^{B}	8.41 ± 0.58^{C}	2.09 ± 0.11^{D}
16:4n-3	3.92 ± 0.08^{A}	0.05 ± 0.02^{B}	1.77 ± 0.13^{C}	0.60 ± 0.04^{D}
18:0	0.62 ± 0.04^{A}	2.62 ± 0.16^{B}	4.94 ± 0.19^{C}	5.76 ± 0.12^{D}
18:1n-9	1.10 ± 0.03^{A}	1.09 ± 0.11^{A}	6.40 ± 0.90^{B}	6.50 ± 0.12^{B}
18:1n-7	1.73 ± 0.05^{A}	7.42 ± 1.33^{B}	3.64 ± 0.11^{A}	10.54 ± 0.17^{C}
18:2n-6	12.45 ± 0.21^{A}	0.49 ± 0.04^{B}	12.05 ± 0.58^{A}	4.73 ± 0.08^{C}
18:3n-3	34.02 ± 0.32^{A}	16.27 ± 0.92^{B}	26.22 ± 1.39^{C}	15.56 ± 0.36^{B}
18:4n-3	0.00 ± 0.00^{A}	17.92 ± 1.30^{B}	0.00 ± 0.00^{A}	7.07 ± 0.31^{C}
20:0	0.07 ± 0.00^{A}	0.01 ± 0.01^{B}	0.22 ± 0.02^{C}	0.20 ± 0.02^{C}
18:5n-3	0.00 ± 0.00^{A}	2.23 ± 0.21^{B}	0.00 ± 0.00^{A}	0.22 ± 0.01^{A}
20:2n-6	0.02 ± 0.01^{A}	0.73 ± 0.05^{B}	0.06 ± 0.00^{AC}	0.13 ± 0.00^{C}
20:4n-6	0.22 ± 0.02^{A}	0.34 ± 0.04^{A}	1.20 ± 0.24^{B}	1.86 ± 0.08^{C}
20:3n-3	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}	0.34 ± 0.01^{B}	0.17 ± 0.02^{B}
20:4n-3	0.00 ± 0.00^{A}	0.51 ± 0.04^{B}	0.00 ± 0.00^{A}	0.58 ± 0.02^{B}
20:5n-3	0.00 ± 0.00^{A}	13.95 ± 0.86^{B}	0.65 ± 0.10^{A}	11.53 ± 0.54^{C}
22:0	0.03 ± 0.01^{A}	0.00 ± 0.00^{A}	0.25 ± 0.02^{B}	0.40 ± 0.02^{C}
22:5n-6	0.00 ± 0.00^{A}	2.06 ± 0.22^{B}	0.00 ± 0.00^{A}	0.16 ± 0.01^{A}
22:6n-3	0.00 ± 0.00^{A}	3.35 ± 0.27^{B}	0.05 ± 0.02^{A}	0.31 ± 0.02^{A}

Figure legend

Fig. 1. Average isotope composition of fatty acids in algae (open bars) and *Daphnia galeata* (dashed bars), fed given algae: a) *Chlorella vulgaris*, b) *Cryptomonas* sp. Horizontal bars represent standard errors; * - difference between algal and animal FA is statistically significant ($p < 0.05$) after Student's *t*-test (number of samples, see Table 1 heading).

Fig. 1


16:0
16:2n-6
16:3n-3
16:4n-3
18:0
18:1n-9
18:2n-6
18:3n-3
20:4n-6
20:5n-3
-17
-22
-27
-32
-37
δ13C, ‰
a
16:0
18:0
18:1n-9
18:2n-6
18:3n-3
18:4n-3
18:5n-3
20:4n-6
20:4n-3
20:5n-3
22:6n-3
-18
-23
-28
-33
-38
δ13C, ‰
b