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

Michail I. Gladyshev[1,2,*], Olesia N. Makhutova[1], Elena S. Kravchuk[1], Olesia V. Anishchenko[1], Nadezhda N. Sushchik[1,2]

[1]*Institute of Biophysics of Siberian Branch of Russian Academy of Sciences, Akademgorodok, Krasnoyarsk, 660036, Russia*

[2]*Siberian Federal University, Svobodny av. 79, Krasnoyarsk, 660041, Russia*

*Corresponding author. *E-mail addresses*: glad@ibp.ru

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

103 controlled by measurements of chlorophyll DCMU-fluorescence (Gaevsky et al., 2005) using
104 fluorometer FL-303 (Siberian Federal University, Krasnoyarsk, Russia) with light beams 410
105 and 540 nm. Calibration curves for the DCMU-fluorescence vs. organic carbon content (using
106 elemental analyzer Flash EA 1112 NC Soil/MAS 200, ThermoQuest, Italy) in each culture of
107 algae were obtained before the experiment (data are not shown).
108
109
110 *Experiments*
111
112 The experiment was conducted under dim light (16:8 h light:dark cycle) at 18-22°C and
113 consisted of two stages. The first stage was an adaptation of the animals from stock culture to the
114 given food. The adaptation was performed to overcome probable bias of the previous experiment
115 of Bec et al. (2011), where the absence of adaptation might affect estimation of differences in
116 $\delta^{13}C$ between dietary and *Daphnia* FA because of FA turnover in *Daphnia*. The adaptation was
117 carried out 7 days, because it takes ~1 week for *Daphnia* and many other zooplankton species to
118 turn over their FA pool (Taipale et al., 2009; Gladyshev et al., 2010). During the adaptation,
119 animals were held in six 3-L glass jars with the food suspensions. In each jar 339±34 ind.,
120 33.2±1.7 mg (wet weight) of *D. galeata* of different ages and sizes were placed to simulate
121 natural populations. Every day, 10% of medium (food suspensions) in each jar were replaced by
122 fresh portion from the batch cultures of algae.
123 At the start of the second stage of the experiment that lasted for 3 days, all the animals,
124 adapted to the given food, from each 3-L jar were transferred into 1-L jars with newly prepared
125 suspensions of the same food. Six 1-L jars were placed into a 'plankton wheel' (diameter, 38 cm,
126 0.2 rpm, Gladyshev et al., 1993). The 'plankton wheel' was used to prevent sedimentation of
127 algae providing homogeneous 'plankton' conditions and to avoid probable effect of
128 heterogeneity (crowding of some part of population of *Daphnia* near walls to obtain more food)

129 on FA isotope fractionation. Every day, 50% of medium in each 1-L jar was replaced by a new
130 portion of food suspensions.

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

136 Samples of algae for following FA and CSIA analyses were taken from the batch
137 cultures, which were used for feeding. Although the batch cultures were kept under the same
138 stable conditions during all the experiment, and thereby were believed to be similar in FA and
139 isotope compositions, samples (replicates) were distributed through the period of experiment.
140 Finally, 9 samples (replicates) of *Ch. vulgaris* were obtained: 3 samples at the end of the first run
141 (10th day), 3 samples at 7th day of the second run, and per 1 sample at 8th, 9th and 10th day of
142 the second run. For *Cryptomonas* sp. 6 samples were obtained: 3 samples at 1st day of the
143 second run, and per 1 sample at 7th, 8th and 9th days of the second run.

144 Samples of *Daphnia* for FA and CSIA were taken from the 1-L 'plankton wheel' jars, at
145 the end of the first and the second runs: 1st run, 5 samples of *Daphnia* (Chl), while 1 sample of
146 *Daphnia* (Cry) was lost because of a technical accident; 2nd run, 1 sample of *Daphnia* (Chl) and
147 5 samples of *Daphnia* (Cry). Finally, number of samples of *Daphnia* (Chl), $n = 6$, and for
148 *Daphnia* (Cry), $n = 5$. All the samples of each alga and *Daphnia* were treated as replicates in
149 following statistical analyses.

150

151 *Fatty acid sampling and analyses*

152

153 To analyze fatty acids, samples of both algae cultures were collected onto precombusted
154 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).

Isotope signatures of fatty acids, taken for CSIA in all samples in different days of both runs of the experiment, are given in Fig. 1, except samples of *Daphnia* (Cry), which were obtained at the end of the second run only. There were modest variations between the stable isotope values of each FA in each object during the experiment, except that of 18:0 in *Chlorella* (Fig. 1a). The relatively high variations of the isotope values of 18:0 evidently were due to its low quantity in *Chlorella* (Table 1) and therefore by relatively higher measurement error.

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. 2). 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. 2). 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. 2). 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. 2) 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. 2).

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 certain 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

283 18:3n-3 are species- or strain-specific. Evidently, more work should be done in future to specify
284 these issues.

285 Thereby, using FA-CSIA to trace food sources of *Daphnia* in natural conditions, one
286 would made the misleading conclusion, that these animals had another food source, than
287 *Chlorella* and *Cryptomonas*, since these alga and *Daphnia* had different $\delta^{13}C$ values of essential
288 FAs. It was supposed earlier, that there could be a 'fractionation constant' for essential FAs in
289 consumers vs. their food, which could give an opportunity to make an appropriate correction of
290 FA-CSIA data and thereby enable their usage for tracing of food webs (Gladyshev et al., 2014).
291 However, data of present experiments did not support this hypothesis, because the fractionation,
292 i.e., difference between $\delta^{13}C$ values of the essential FAs of *Daphnia* and their food, as mentioned
293 above, evidently was not constant, but varied from 1.35‰ to 7.04‰.

294 What processes could cause different values of fractionation of fatty acids isotope
295 signatures in consumers? Conventionally, the fractionation may occur during FA synthesis
296 (kinetic isotope effect, KIE, during elongation and desaturation), catabolism (β-oxidation) and
297 digestive assimilation (hydrolysis, esterification, re-esterification) (DeNiro and Epstein, 1977;
298 Abrajano et al., 1994; Rhee et al., 1997; Koussoroplis et al., 2010; Budge et al., 2011; Bec et al.,
299 2011; Gladyshev et al., 2012; Hixson et al., 2014).

300 As to FA depletion due to KIE during their synthesis and further elongation and
301 desaturation, an opposite effect occurred in many cases in our experiment. Indeed, in *Chlorella*
302 16:2n-6 was heavier, not lighter, than 16:0, 18:2n-6 was heavier than 18:1n-9, and 18:3n-3 was
303 heavier, than 18:2n-6. In *Cryptomonas* 18:2n-6 also was heavier than 18:1n-9, and 18:0 was
304 heavier, than 16:0. In *Daphnia* (Chl) 20:4n-6 was heavier, than 18:2n-6. However, isotope ratios
305 of many other FAs, e.g., 20:5n-3 vs. 18:3n-3 in *Daphnia* (Chl), were in a good agreement with
306 KIE.

307 The diverse processes, synthesis, catabolism and assimilation, seemed to result in
308 different values of isotope fractionation of different fatty acids. Basing on comparison of

309 percentages of FAs in *Daphnia* and their food, fatty acids were subdivided in several groups,
310 probably controlled by different processes. For instance, in the first group, 18:0 was accumulated
311 (had significantly higher level) in *Daphnia*, than in the algae. However, the higher levels were
312 probably provided by different mechanisms in two cultures, which resulted in different isotope
313 signatures. Indeed, *Chlorella* had very low level of 18:0, and *Daphnia* (Chl) probably had to
314 synthesize this important acid *de novo*. Since the carbon source for the synthesis and the
315 pathways were similar in the algae and the animals, there were no differences in isotope
316 signatures between 18:0 in *Chlorella* and in *Daphnia* (Chl). In contrast, *Cryptomonas* had
317 comparatively high level of 18:0, and *Daphnia* (Cry) could assimilate and accumulate this FA
318 from food, rather than synthesize it *de novo*. Thereby, due to putative digestive fractionation,
319 18:0 in *Daphnia* (Cry) had significantly lighter isotope composition, than that in *Cryptomonas*.
320 Naturally, we used non-axenic cultures of *Chlorella* and *Cryptomonas* for *Daphnia*
321 feeding that lead to bacterial signatures in fatty acid profiles. Although the percentages of gram-
322 positive bacterial FA, branched odd-chain acids, were relatively low in both algae cultures and in
323 *Daphnia*, the percentages of 18:1n-7 which is considered as gram-negative bacterial marker,
324 were prominent. However, gram-negative bacteria are able to synthesize only saturated and some
325 monounsaturated FA, such as 16:1n-7 and 18:1n-7. Therefore, bacterial contamination might
326 change isotopic signatures only for 16:0 and 18:0, but did not affect those of PUFA, such as
327 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, which were the main objects of our study.
328 Values of $\delta^{13}C$ of FA from another 'percent group', 20:5n-3, were nearly similar in
329 *Daphnia* (Chl), which evidently synthesized this acid, and in *Daphnia* (Cry), which obtained it
330 from food. However, in some cases it may be better to analyze the relative changes in carbon
331 isotopic compositions of the physiologically important FA and its biochemical precursor rather
332 than compare their absolute values of this FA (Schouten et al., 1998). Thus, comparing isotope
333 ratios of 20:5n-3 with those of 18:3n-3 in both cultures of *Daphnia*, one can see, that in *Daphnia*
334 (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

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

370 However, in addition to the differences mentioned above, i.e., analyses of total FA and
371 usage of pre-adapted cultures, there was another peculiarity in protocol of our experiment
372 compared to that of Bec et al. (2011). Indeed, we used food, *Chlorella*, which contained no
373 20:5n-3. Thereby, we revealed synthesis of 20:5n-3 by *Daphnia*, and measured the relevant
374 value of isotope fractionation of this fatty acid. In contrast, Bec et al. (2011) studied only
375 accumulation of this important FA from different food sources. Thus, in our experiment, we
376 found that even the same fatty acid, e.g., 20:5n-3, can have different values of isotope
377 fractionation in consumer, if it is obtained by different mechanisms, synthesis and accumulation.
378 Besides 20:5n-3, the same was true also for other FAs, for instance for 18:0.

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

383

384 **Conclusions**

385

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.

Acknowledgments

This work was supported by grant of Russian Foundation for Basic Research No. 14-04-00053 and by Russian Federal Tasks of Fundamental Research (project No. 51.1.1). We are grateful to two anonymous Reviewers for their helpful comments to improve the manuscript.

References

Abrajano, Jr T.A., Murphy, D. E., Fang, J., Comet, P., Brooks, J.M., 1994. 13C/12C ratios in individual fatty acids of marine mytilids with and without bacterial symbionts. Org. Geochem. 21, 611-617.

Bec, A., Perga, M.-E., Koussoroplis, A., Bardoux, G., Desvilettes, C., Bourdier, G., Mariotti, A., 2011. Assessing the reliability of fatty acid–specific stable isotope analysis for trophic studies. Methods Ecol. Evol. 2, 651–659.

Budge, S.M., Wang, S.W., Hollmen, T.E., Wooller, M.J., 2011. Carbon isotopic fractionation in eider adipose tissue varies with fatty acid structure: implications for trophic studies. J. Exp. Biol. 214, 3790-3800.

Budge, S.M., Wooller, M.J., Springer, A.M., Iverson, S.J., McRoy, C.P., Divoky G.J., 2008. Tracing carbon flow in an arctic marine food web using fatty acid-stable isotope analysis. Oecologia 157, 117–129.

DeNiro, M.J., Epstein, S., 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197, 261-263.

Gaevsky, N.A., Kolmakov, V.I., Anishchenko, O.V., Gorbaneva, T.B., 2005. Using DCMU-fluorescence method for identification of dominating phytoplankton groups. J. Appl. Phycol. 17, 483–494.

Gladyshev, M.I., Sushchik, N.N., Dubovskaya, O.P., Buseva, Z.F., Makhutova, O.N., Fefilova, E.B., Feniova, I.Y., Semenchenko, V.P., Kolmakova, A.A., Kalachova, G.S., 2015. Fatty acid composition of Cladocera and Copepoda from lakes of contrasting temperature. Freshwater Biol. 60, 373–386.

Gladyshev, M.I., Sushchik, N.N., Kalachova, G.S., Makhutova, O.N., 2012. Stable isotope composition of fatty acids in organisms of different trophic levels in the Yenisei River. PLoS ONE 7(3), e34059.

Gladyshev, M.I., Sushchik, N.N., Makhutova, O.N., Dubovskaya, O.P., Kravchuk, E.S., Kalachova, G.S., Khromechek, E.B., 2010. Correlations between fatty acid composition of seston and zooplankton and effects of environmental parameters in a eutrophic Siberian reservoir. Limnologica 40, 343-357.

Gladyshev, M.I., Sushchik, N.N., Makhutova, O.N., Kalachova, G.S., 2014. Trophic fractionation of isotope composition of polyunsaturated fatty acids in the trophic chain of a river ecosystem. Dokl. Biochem. Biophys. 454, 4−5.

Gladyshev, M.I., Sushchik, N.N., Gubanenko, G.A., Makhutova, O.N., Kalachova, G.S., Rechkina, E.A., Malyshevskaya, K.K., 2014. Effect of the way of cooking on contents of essential polyunsaturated fatty acids in filets of zander. Czech J. Food Sci. 32, 226–231.

Gladyshev, M.I., Temerova, T.A., Degermendzhi, A.G., Tolomeev, A.P., 1993. Kinetic characteristics of growth of zooplankton in flowing and closed cultivators. Dokl. Akad. Nauk 33, 795–797.

Hixson, S.M., Parrish, C.C., Anderson, D.M., 2014. Changes in tissue lipid and fatty acid composition of farmed rainbow trout in response to dietary camelina oil as a replacement of fish oil. Lipids 49, 97–111.

Jim, S., Ambrose, S.H., Evershed, R.P., 2003. Natural abundance stable carbon isotope evidence for the routing and *de novo* synthesis of bone FA and cholesterol. Lipids 38, 179–186.

Koussoroplis, A.-M., Bec, A., Perga, M.-E., Koutrakis, E., Desvilettes, C., Bourdier, G., 2010. Nutritional importance of minor dietary sources for leaping grey mullet *Liza saliens* (Mugilidae) during settlement: insights from fatty acid $\delta^{13}C$ analysis. Mar. Ecol-Prog. Ser. 404, 207–217.

Kravchuk, E.S., Sushchik, N.N., Makhutova, O.N., Trusova, M.Y., Kalacheva, G.S., Gladyshev, M.I., 2014. Differences in carbon isotope signatures of polyunsaturated fatty acids of two microalgal species. Dokl. Biochem. Biophys. 459, 183–185.

462 Lau, D.C.P., Leung, K.M.Y., Dudgeon, D., 2009. Evidence of rapid shifts in the trophic base of
463 lotic predators using experimental dietary manipulations and assimilation-based analyses.
464 Oecologia 159, 767–776.
465 Lu, Y.H., Canuel, E.A., Bauer, J.E., Chambers, R.M., 2014. Effects of watershed land use on
466 sources and nutritional value of particulate organic matter in temperate headwater streams.
467 Aquat. Sci. 76, 419–436.
468 Pel, R., Hoogveld, H., Floris, V., 2003. Using the hidden isotopic heterogeneity in phyto- and
469 zooplankton to unmask disparity in trophic carbon transfer. Limnol. Oceanogr. 48, 2200–
470 2207.
471 Pond, D.W., Leakey, R.J.G., Fallick, A.E., 2006. Monitoring microbial predator–prey
472 interactions: an experimental study using fatty acid biomarker and compound-specific
473 stable isotope techniques. J. Plankton Res. 28, 419–427.
474 Rhee, S.K., Reed, R.G., Brenna, J.T., 1997. Fatty acid carbon isotope ratios in humans on
475 controlled diets. Lipids 32, 1257–1263.
476 Schouten, S., Klein Breteler, W.C.M., Blokker, P., Schogt, N., Rijpstra, W.I.C., Grice, K., Baas,
477 M., Sinninghe Damste, J.S., 1998. Biosynthetic effects on the stable carbon isotopic
478 compositions of algal lipids: Implications for deciphering the carbon isotopic biomarker
479 record. Geochim. Cosmochim. Acta 62, 1397–1406.
480 Taipale, S., Kankaala, P., Hamalainen, H., Jones, R.I., 2009. Seasonal shifts in the diet of lake
481 zooplankton revealed by phospholipid fatty acid analysis. Freshwater Biol. 54, 90–104.
482 Tolosa, I., Vescovali, I., LeBlond, N., Marty, J.-C., de Mora, S., Prieur, L., 2004. Distribution of
483 pigments and fatty acid biomarkers in particulate matter from the frontal structure of the
484 Alboran Sea (SW Mediterranean Sea). Mar. Chem. 88, 103–125.
485 Wang, S.W., Budge, S.M., Iken, K., Gradinger, R.R., Springer, A.M., Wooller, M.J., 2015.
486 Importance of sympagic production to Bering Sea zooplankton as revealed from fatty acid-
487 carbon stable isotope analyses. Mar. Ecol-Prog. Ser. 518, 31–50.

488

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}

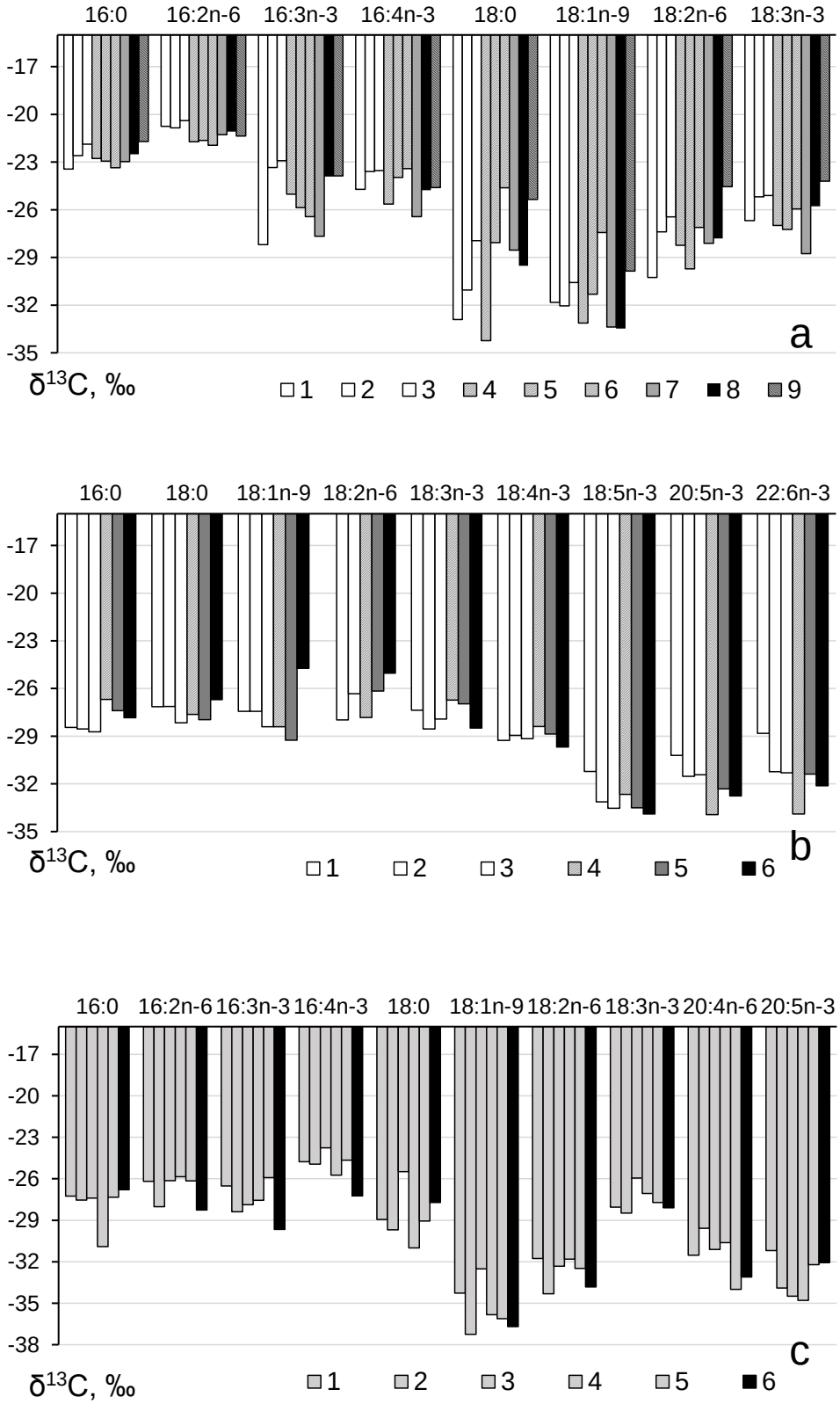
Figure legend

Fig. 1. Stable isotope composition of fatty acids during the experiment: a) Chlorella vulgaris, 1-3 – the end of the first run (10th day), 4-6 – 7th day of the second run, 7 – 8th day of the second run, 8 – 9th day of the second run, 9 – 10th day of the second run; b) *Cryptomonas* sp., 1-3 – 7th day of the second run, 4 – 8th day of the second run, 5 – 9th day of the second run, 6 – 10th day of the second run; c) *Daphnia galeata* (fed *Chlorella*), 1-5 – the end of the first run (10th day), 6 – the end of the second run (10th day).

Fig. 2. 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).

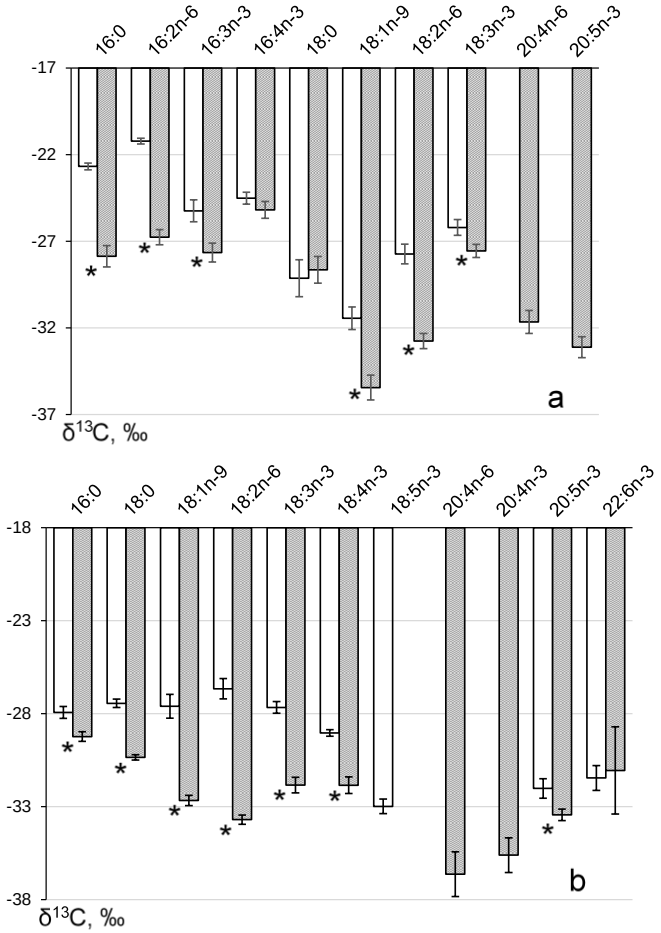
512 Fig. 1

513



514

515 Fig. 2


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

516