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**Variability in amino acid composition of alpine crustacean zooplankton and its relationship with 15-nitrogen fractionation**

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Variability in amino acid composition of alpine crustacean zooplankton and its  
relationship with  $\delta^{15}\text{N}$ -nitrogen fractionation

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RUNNING HEAD: Amino acid composition of zooplankton

**Keywords :** Amino acid composition, homeostasis, food quality, nitrogen fractionation,  
stoichiometry

## Abstract

Amino acids (AAs) are critical biochemical compounds for living organisms. Because of the limited capacity for their *de novo* synthesis in many animals, the nutritional value of food largely depends on its AA composition relative to the animal's requirements. To improve present knowledge on AA variability in freshwater crustacean we studied the inter- and intraspecific variability in three contrasting species from an oligotrophic alpine lake (*Daphnia pulicaria*, cladocera; *Cyclops abyssorum*, cyclopoid copepod and *Diaptomus cyaneus*, calanoid copepod). Inter-species differences were larger than intraspecific variation, confirming a non-strict homeostasis in freshwater crustacean zooplankton. The intraspecific variability differed for each species: in *Daphnia* it was mainly related with ontogenetic changes rather than reproduction; in *Cyclops* both factors were equally important; and reproduction was the most relevant in *Diaptomus*. Reproduction changes were associated with serine and phenylalanine in the three species; while the AAs responsible for ontogenetic changes differed in each species. There were no gender differences in AA composition in either of the two copepod species. Free AAs formed a very low percentage of total AA pool (< 2.7 %). Taking advantage of the fact that *Daphnia* is the main prey for *Cyclops* in the lake studied, we further investigated to what extent the AA composition is related with *Cyclops-Daphnia* nitrogen stable isotope fractionation. Only those AAs that are both essential and are not trans-aminated during protein synthesis had a significant correlation with nitrogen stable isotope fractionation, supporting the hypothesis that an AA imbalance can be responsible for a variable nitrogen stable isotope fractionation.

## Introduction

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9 25 Amino acids (AAs) are critical biochemical compounds for living organisms (i.e. as  
10 building blocks of proteins, as part of coenzymes, and as precursors for the biosynthesis  
11 of some molecules) (Strayer, 1988). Because of the limited capacity for their *de novo*  
12 synthesis in many animals, the AA composition of food becomes very important in  
13 animal nutrition. This has been demonstrated in different types of terrestrial organisms  
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20 30 such as butterflies (O'Brien et al., 2003), birds (Ramsay and Houston, 1998), farm  
21 animals (D'mello, 1994) and humans (Reeds, 2000), and also for aquatic organisms such  
22 as farmed fish (Conceição et al., 2003, Ronnestad et al., 2003), rotifers (Boëchat and  
23 Adrian, 2006), mussels (Kreeger et al., 1996) and marine copepods (Kleppel and  
24 Burkart, 1995, Guisande et al., 1999, Guisande et al., 2000). This knowledge contrasts  
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32 35 with the few ecological studies focussing on the relative importance of AAs for animal  
33 growth and reproduction (Conklin and Provasoli, 1977, Boëchat and Adrian, 2006).  
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44 40 (e.g. Müller-Navarra et al., 2000) have received more attention than AAs. Since most of  
45 animal's nitrogen content is in the form of amino acids, comparison between organisms  
46 and their food AA composition has been suggested as a step forward in using  
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56 45 is unable to synthesise some AAs in significant quantities (i.e., essential AAs) and that  
57 it is homeostatic with respect to the composition in essential AAs. The former  
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60 assumption is supported by numerous studies, including in crustaceans (Claybrook,

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3 1983), whereas the latter has only been partially demonstrated and further studies are  
4  
5 required (Cowgill et al., 1986, Anderson et al., 2004).  
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8           An imbalance between the AA composition of food and those of the consumer  
9  
10 has been proposed as one of the main mechanisms explaining nitrogen stable isotope  
11  
12 fractionation (i.e., change in isotope ratios,  $\Delta^{15}\text{N}$ ) between diet and animal tissues  
13  
14 (Martínez del Rio and Wolf, 2005). Nitrogen stable isotopes together with those of  
15  
16 carbon have become a common tool for describing trophic relationships, although the  
17  
18 physiological mechanism explaining  $\Delta^{15}\text{N}$  (crucial for estimating diet sources) is still  
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20 55  
21 not resolved (Martínez del Rio et al., 2009). The physiological mechanism could be  
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23 caused by the deficiency in one, or several essential AAs leading to greater protein  
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25 intake and eventually to higher catabolism of the AAs in excess for protein synthesis  
26  
27 favouring a preferential body elimination of lighter nitrogen (Martínez del Rio and  
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31 60  
32 Wolf, 2005). Few studies have provided evidence for this hypothesis with the exception  
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34 of Robbins et al. (2005), in a comparative study, and Ventura and Catalan (2008), in a  
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36 field study, that reported a significant negative correlation between  $\Delta^{15}\text{N}$  and the  
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38 protein quality of the diet (e.g. AA composition). An important unresolved question is if  
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40 there are some particular AAs that are more relevant for  $\Delta^{15}\text{N}$ . The essential part of an  
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44 65  
45 AA (and what differentiates them biochemically) is their carbon skeleton. All the  
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47 nitrogen of AAs is in the amino group, which can be trans-aminated by animals in, *a*  
48  
49 *priori*, all AAs. However, it has been observed that some AAs appear to retain  
50  
51 approximately the same nitrogen isotopic composition as their food source (are not  
52  
53 trans-aminated; “source AAs”) whereas others become enriched in  $^{15}\text{N}$  by the animals’  
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56 70  
57 metabolism (undergo trans-amination and obtain a new amino group with enriched  $^{15}\text{N}$ ;  
58  
59 “trophic AAs”) (McClelland and Montoya, 2002, Popp et al., 2007). Different  
60  
proportions of “trophic” and “source” AAs explain why animal tissues differ in  $^{15}\text{N}$

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3 (Martínez del Rio et al., 2009). Since the trans-amination is not related to the AA  
4 carbon skeleton, some trophic / source AAs are essential while others are nonessential,  
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8 75 making a complicated physiological puzzle. What particular AAs or AA group is  
9  
10 directly related to  $\Delta^{15}\text{N}$  has not been described and therefore needs to be explored more  
11  
12 in depth. One may hypothesise that among the essential amino acids, only those that are  
13  
14 not trans-aminated would have a higher relevance than the others.  
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18 Zooplankton has played a central role in stoichiometry studies, particularly  
19  
20 80 *Daphnia*, which has been extensively used as a model organism. Investigations  
21  
22 examining the AA composition are scarce, however. Most past studies of the AA  
23  
24 composition of zooplankton have focused on the variability in the total AA (TAA)  
25  
26 composition of adult marine or freshwater zooplankton compared to their food, the  
27  
28 results showing that adults have a very strict AA composition (Cowey and Corner,  
29  
30  
31 85 1963, Cowgill et al., 1986, Guisande et al., 2000). All those studies are indicative of AA  
32  
33 composition homeostasis in adults of crustaceans. In contrast, there are few studies  
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35 focussing on the TAA intraspecific variability. Significant differences in AA  
36  
37 composition during development have been observed in *Artemia spp.* and lobsters  
38  
39 (Helland et al., 2000, Mente et al., 2001). In cladocerans and copepods Dabrowski and  
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41  
42 90 Rusiecki (1983) found differences in TAA between late developmental stages of  
43  
44 different species, although they did not examine the degree of these TAA changes for  
45  
46 each species. Changes in TAA were reported for two *Calanus* species during  
47  
48 development from egg to the second naupliar stage (Laabir et al., 1999) and between  
49  
50 nauplii of different seasons (Helland et al., 2003a). Ontogenetic variability in species of  
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53 95 cladocerans, calanoid and cyclopoid copepods were studied in different coastal lagoons  
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55 and wetlands by Bruçet et al. (2005). They analysed all the lake-populations  
56  
57 simultaneously, and found a more pronounced variability in the TAA composition  
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3 related with ontogeny in copepods, while *Daphnia* variability was mostly related with  
4 lake type and not with ontogeny. However, the relative importance of ontogeny,  
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8 100 reproduction and gender in AA composition changes has not been evaluated for these  
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10 animal groups.

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12 In this context, with the aim of testing the hypothesis that interspecific  
13 differences among the main freshwater groups of crustacean zooplankton are greater  
14 than their intraspecific variation, we studied the relative proportions of the AA pools  
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20 105 and its variation in crustacean zooplankton species of an alpine lake throughout a whole  
21 seasonal cycle. We selected a lake that had one species of each of the main freshwater  
22 crustacean groups, a cladoceran (*Daphnia pulicaria* Forbes), a cyclopoid copepod  
23 (*Cyclops abyssorum* Sars) and a calanoid copepod (*Diaptomus cyaneus* Gurney). In a  
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32 110 elemental content of carbon and phosphorus during their life cycles associated with  
33 reproduction (Ventura and Catalan 2005). In contrast, nitrogen was the only compound  
34 which did not change significantly. In addition, *Cyclops* was described as being a  
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44 115 consumer amino acid (nitrogen) limitation (Ventura and Catalan, 2008). Therefore these  
45 two species also present an ideal framework for investigating the specific role of AAs in  
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56 120 study were (i) interspecific variability of AA composition is greater than intraspecific  
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60 AA variability (ii) reproduction, gender and ontogenetic changes are important  
intraspecific sources of AA variability; and (iii) essential amino acids that are not trans-



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3 aminated during tissue incorporation are those most closely related with the variability  
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5 in  $\Delta^{15}\text{N}$  between *Cyclops* and *Daphnia*.  
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## 10 Method

### 14 *Study site and sample collection*

17 Lake Redon (formerly lake Redó) is an oligotrophic glacial cirque lake located at 2240  
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19  
20 130 m a.s.l. in the central Pyrenees (42° 38' N, 0° 46' E). It is relatively large (24 ha) and it is  
21  
22 one of the deepest lakes (73 m) in the Pyrenees. It is dimictic, covered by ice during half  
23  
24 of the year, usually from mid December until late May or the beginning of June. The  
25  
26 lake has average microbial biomass ratios of 10 : 2 : 2 : 1 for phytoplankton : bacteria :  
27  
28 heterotrophic nanoflagellates : ciliates during the ice-free period (Felip et al., 1999).  
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31 135 Rotifers are usually less abundant than crustaceans (the ratio between *Daphnia* and  
32  
33 rotifer biomass is approximately 10:1 during the ice-free period; Camarero et al., 1999).  
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36 The lake was sampled on 14 occasions from December 1998, just after the lake  
37  
38 was completely ice covered, to December 1999, after it was ice-covered again. Samples  
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40 were collected at the deepest point of the lake either after drilling the ice-cover or from  
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42  
43 140 a platform anchored throughout the summer at the same point. Zooplankton samples  
44  
45 were collected by vertical hauls from 65 m to the surface with a 200  $\mu\text{m}$  net. Individuals  
46  
47 were kept alive and transported cold (4°C) until they were frozen (-20°C) in the  
48  
49 laboratory a few hours after collection.  
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52 The three crustacean zooplankton species studied had contrasting life histories  
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55 145 although all produced a single cohort per year. A complete description of their life  
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57 cycles during the study period can be found in Ventura and Catalan (2005) and their  
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59 trophic relationships in Ventura and Catalan (2008). *Cyclops* is a predatory copepod  
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3 feeding primarily on *Daphnia* in this lake (Ventura and Catalan, 2008). Its life cycle  
4 was synchronised with that of *Daphnia* being the only two planktonic crustacean  
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8 150 species inhabiting the lake during the whole year. Adult females of *Daphnia* from the  
9  
10 previous summer survived below the ice-cover, reproduced during the end of the ice-  
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12 covered period and the first two months of the ice-free period, and disappeared from the  
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14 lake afterwards, leaving the juveniles growing up during the summer (Fig. 1) (Ventura  
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16 and Catalan 2005). Adult females (termed 'first cohort females' in our study) were  
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19 155 therefore clearly distinguishable from the juveniles born during the ice free period  
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21 (termed 'second cohort females'). We therefore compared the AA composition of the  
22  
23 two cohorts, of the reproducing and non-reproducing first cohort females, and their  
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25 eggs. Similar to *Daphnia*, adult females of *Cyclops* survived below the ice-cover in  
26  
27 order to reproduce during the end of the ice-covered period. Adult males were present in  
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30 160 the lake from December to February below the ice-cover. Nauplii appeared the  
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32 following summer, just after the ice-cover melted (copepods have six naupliar, and five  
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34 copepodite stages before adults develop) and coincided with the spring production  
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36 maximum. Copepodites CI to CIII, dominated *Cyclops* biomass during July and August,  
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38 copepodites CIV and CV were present in the lake from September until November (Fig.  
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40  
41 165 1) (Ventura and Catalan 2005). Therefore, we sampled two generations and the stages  
42  
43 analysed consisted of adult males, adult reproductive and non-reproductive females, and  
44  
45 four copepodites (CII to CV), which were amalgamated in two groups, one with CII and  
46  
47 CIII and the other with CIV and CV. The other copepod, *Diaptomus*, dominated the  
48  
49 plankton in July and August, spending the rest of the year as resting eggs (Fig. 1)  
50  
51  
52 170 (Ventura and Catalan 2005). Due to its fast development, only adult *Diaptomus* were  
53  
54 considered in this study. Unlike *Cyclops*, adult males and females were present in the  
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56 lake during the same period. The July and August samples coincided with the beginning  
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3 and the end of the reproduction period and were therefore expected to cover the  
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5 maximum range of the AA variability for the adults of the species.  
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8 175 *Sample preparation and chemical analysis*  
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10 After thawing the samples, from 10 to several hundreds of individuals, depending on the  
11 weight of each species and stage, were quickly sorted under a dissecting microscope and  
12 were either placed into pre-weighed tin capsules for stable isotope analysis or in Teflon  
13 capsules for analysis of amino acids. From two to six combined sample replicates were  
14 analysed for each species and stage for each sampling date, with the exception of a few  
15 cases where sample material did not suffice. Individuals were kept cold (<4 °C) during  
16 the sorting process, which was always carried out within a few hours of collection. Dry  
17 weights were determined for all samples after drying at 60 °C for 24h and weighing on a  
18 high precision microbalance (Ohaus Analytical Plus, AP250D-0).  
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32 185 For amino acid analysis, between 0.6-0.9 mg of dried sample was vacuum-  
33 sealed and hydrolysed with HCl 6N at 112 °C for 16 h (Fountoulakis and Lahm, 1998).  
34 An internal norleucine standard was included in every sample prior to hydrolysis in  
35 order to minimise among sample variability. Samples were subsequently dried under  
36 vacuum at room temperature. Dried samples were re-dissolved with 0.5 to 1 ml  
37 (depending on the concentration of the sample) of buffer solution (pH 2.2) prior to  
38 analysis and purified by filtration (0.65-µm Durapore filter; Millipore, Bedford,  
39 Massachusetts, USA). The AA analysis was performed on a Biochrom20 (Amersham-  
40 Pharmacia) ion-exchange AA auto analyser following the ninhydrine method of  
41 Spackman et al. (1958). A standard solution of 20 AAs was run for every 10 samples.  
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56 195 The detection limit was 10 pmol for all amino acids and 50 pmol for proline. The  
57 samples injected were always greater than the limit of detection for all amino acids  
58 considered. Analytical precision had a CV of 3.9%. Tryptophan, due to its degradation  
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3 under acid hydrolysis (Fountoulakis and Lahm, 1998), has not been considered in this  
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5 study. Cysteine and methionine are other AAs that usually degrade easily during the  
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8 200 hydrolysis process. Together with taurine they are the three AAs containing sulphur.  
9  
10 Therefore their possible degradation was checked by analysing for elemental sulphur  
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12 (EA 1108 CHNS-O Carlo Erba Instruments elemental analyser). This analysis showed  
13  
14 that the AA sulphur accounted for most of the elemental sulphur indicating a good  
15  
16 recovery of most sulphur-containing AAs. Also due to the acid hydrolysis, asparagine  
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18 and glutamine, were measured together with aspartic acid and glutamic acid,  
19  
20 205 respectively. AAs are expressed as relative mass percentage of TAA through out this  
21  
22 paper. These units were chosen rather than absolute concentrations (e.g. mg AA / mg C  
23  
24 or DW) since a description of the biochemical variability of the present species and  
25  
26 study period showed that there was an important seasonal variability in the lipid and  
27  
28 carbohydrate content (up to 50 % of their DW) (Ventura and Catalan, 2005), which  
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30 210 were much greater than the changes in the AA composition. Using the relative units we  
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32 focus on the variability in the AA composition rather than the factors affecting the gross  
33  
34 biochemical composition. However, we provide the TAA concentration (in % dry  
35  
36 weight) which allows for calculation of the absolute concentration also used in the  
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44 215 literature.

45  
46 In order to determine the relative importance of the free AA (FAA) pool with  
47  
48 respect to proteins, we used the concentration of taurine as an indicator of the FAA  
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50 pool. Taurine is the only AA which does not form proteins, and one of the main FAAs  
51  
52 in crustaceans (Claybrook, 1983). A bibliography search of joint measurements of  
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54 220 taurine and the concentration of FAAs was obtained from Ventura (2006), and these  
55  
56 were found to relate linearly (Fig. 2). The good fit ( $r^2 = 0.90$ ,  $p < 0.001$ ) suggested that  
57  
58 taurine concentration could be used as a predictor of the FAA pool. Therefore, we  
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3 estimated the relative proportion of the FAA by applying the regression formula of  
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6 Figure 2 to the measured taurine concentration.

7  
8 225 Dried samples for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotope analysis were analysed in a Delta  
9  
10 C Finningan MAT mass spectrometer. Results are reported relative to atmospheric  
11  
12 nitrogen and PeeDee belemnite (PDB) carbonate as references. Reproducibility was  
13  
14 better than 0.1 ‰ and 0.3 ‰ for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively. Further methodological  
15  
16 details are provided in Ventura & Catalan (2008).  
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### 21 22 *Statistical analysis* 23

24  
25 To test if the different species or the stages within each species could be distinguished  
26  
27 based on their AA composition, we performed multiple discriminant analyses (MDA).  
28  
29 The MDAs enabled us to determine which AAs were the most significant in  
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31  
32 235 discriminating between two or more groups (species and stages). We performed a first  
33  
34 MDA (MDA1) with the various stages and other sources of intraspecific variation of the  
35  
36 three different species as separate factors in one single analysis in order to find out if the  
37  
38 interspecific variability was greater than the intraspecific variability. The sources of  
39  
40 intraspecific variation of the AA composition evaluated consisted of gender,  
41  
42  
43 240 development stages (ontogenetic changes), differences between reproductive and non-  
44  
45 reproductive females in the three species and also males in *Diaptomus*. Gender  
46  
47 differences were only considered in the two copepod species, since *Daphnia* mainly  
48  
49 reproduced asexually. Ontogenetic changes could only be tested in *Daphnia* and  
50  
51  
52 *Cyclops* due to the short life cycle of *Diaptomus*. Each replicate was entered separately  
53  
54  
55 245 in the analysis. The variable number of samples for each species or stage was due to the  
56  
57 natural variability of their different seasonal occurrences. A different MDA was  
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59 performed for each species (MDA2 for *Daphnia*, MDA3 for *Cyclops* and MDA4 for  
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*Diaptomus*) with the above stages as discriminating groups in the MDA in order to test if they could also be distinguished in terms of their AA composition and to find out which particular amino acids were involved with the different developmental stages of each species. In the specific case of *Diaptomus* due to the lower number of samples (8 cases for 4 variables) present we ran a forward selection procedure to select only the combination of AAs best discriminating among the different groups. MDA analysis is a method previously applied in AA comparisons of zooplankton (Guisande et al., 1999, Guisande et al., 2000, Guisande et al., 2003, Boëchat and Adrian, 2005, Bruçet et al., 2005) and therefore its application facilitates comparison with previous studies.

Average Euclidean distance ( $D_{jk}$ ) was used to test if the interspecific variability of amino acid composition was greater than the intraspecific variability and to estimate the dissimilarity between the amino acid compositions of *Cyclops* and *Daphnia* for comparison with the nitrogen stable isotope composition. We compared *Cyclops* with *Daphnia* since the former was described as being a predatory copepod feeding primarily on *Daphnia* in the same lake and study period (Ventura and Catalan, 2008). The Euclidean distance is a metric distance given by the Pythagorean formula (Legendre and Legendre, 1998):

$$D_{ij} = \sqrt{\sum_{i=1}^n (X_{ij} - X_{ik})^2}$$

where  $X_{ij}$  and  $X_{ik}$  are the percentages of the amino acid  $i$  of species 1 ( $j$ ) or species 2 ( $k$ ), and  $n$  is the number of amino acids considered. The distance was calculated with all the amino acids when evaluating interspecific versus intraspecific amino acid variability.

For comparison of the distance between *Cyclops* and *Daphnia* we calculated the

distance of all amino acids or separately for the essential amino acids (Lysine, Phenylalanine + Tyrosine, Leucine, Valine, Threonine, Isoleucine, Histidine, Cysteine +

1  
2  
3 Methionine), nonessential amino acids (Glutamic acid, Aspartic acid, Alanine,  
4  
5 Arginine, Glycine, Proline and Serine), trophic amino acids (Alanine, Asparagine +  
6  
7 Aspartic acid, Glutamine+Glutamic acid, Isoleucine, Leucine, Proline and Valine) and  
8  
9  
10 275 source amino acids (Arginine, Glycine, Lysine, Serine, Threonine and Phenilalanine  
11  
12 +Tyrosine). The average Euclidean distance between *Cyclops* and *Daphnia* using the  
13  
14 different amino acid groups was compared with the  $\Delta^{15}\text{N}$  of both species with linear  
15  
16 regression analysis.  
17  
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19  
20 In order to determine which particular amino acids could be most imbalanced in  
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22 280 *Daphnia* in comparison with the predator, *Cyclops*, we calculated the amino acid  
23  
24 imbalance as follows (Conceição *et al.*, 2003):  
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$$27 \quad \text{AA}_i = (\text{dietAA}_i - \text{consumerAA}_i) / (\text{consumerAA}_i) * 100$$

28  
29 where  $\text{dietAA}$  and  $\text{consumerAA}$  are the contributions of a given AA (i) to the AA profiles of  
30  
31 the consumer.  
32  
33

34 285 Differences in individual AAs were tested with one-way ANOVA analysis  
35  
36 (Underwood, 1997) with species or stage as independent variable and the percentage of  
37  
38 the AAs as the dependent variable. AA percentage data were previously normalised  
39  
40 with arcsine root square transformation when required, and a test of homogeneity of  
41  
42 variances was performed before ANOVA. To test if Euclidean distances of interspecies  
43  
44 versus intraspecies comparison were significantly different we used one way ANOVA  
45  
46 290 with the Euclidean distance between all the pairs of samples analysed as dependent  
47  
48 variable and if the comparison was from among species comparison (three cases) or  
49  
50 within species (3 more cases) as independent variables. We used Tukey's *post hoc* test  
51  
52 for detecting significant differences among or within species. All statistical analyses  
53  
54 were performed with SPSS V17.  
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## Results

### *Interspecies differences*

The three species had different total AA content. *Daphnia* had the lowest relative concentration, followed by *Cyclops* and *Diaptomus* (27.5 % on dry weight  $\pm$  4.6 SD, 45.4  $\pm$  8.4, 59.7  $\pm$  13.4 respectively) ( $F_{2,22} = 42.3$ ,  $P < 0.001$ ; Tukey post-hoc test  $P < 0.0001$ ) (Table 1). Most of the AAs were forming proteins, the FAA pool being a small proportion of the TAA pool of each species. *Daphnia* FAA relative concentration (2.7 $\pm$ 1.5 % of TAA) was slightly higher than *Diaptomus* (1.2 $\pm$ 0.5 % of TAA,  $F_{2,22} = 4.2$ ,  $P < 0.05$ ; Tukey's  $P = 0.014$ ) but similar to *Cyclops* (1.9 $\pm$ 0.8 % of TAA, Tukey's  $P = 0.162$ ).

The average Euclidean interspecific distance was two times greater than the average intraspecific distance (3.7  $\pm$  1.1 and 1.7  $\pm$  0.6 respectively;  $F_{5,1080} = 706$ ,  $P < 0.001$ ; Tukey's  $P < 0.001$ ; Fig. 3). The two copepods had a more similar composition to each other than to the cladoceran, while the three species had a similar intraspecific distance (Fig. 3).

The AA composition of the three species studied was species specific (Fig. 4a). The most abundant AAs for the two copepods (in order of importance and not taking into account asparagine / aspartic acid and glutamine / glutamic acid which were analysed together), were alanine, leucine, tyrosine, lysine and arginine, which altogether accounted for 37% of the total AA pool. For the cladoceran the most abundant AAs were leucine, arginine, alanine, valine, threonine and lysine, accounting for 40% of the total AA pool (Table 2). The MDA1, including all the stages of the three species was able to classify the stages of the three species based on their AA composition (100% of the samples being correctly classified with the exception of half of the *Diaptomus* non-



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2  
3 320 reproductive females that were identified as males; Fig. 4a and Table 3). The first axis  
4  
5 of the MDA1 explained 47 % of the variance and mainly segregated the two copepods  
6  
7 from the cladoceran. The AAs significantly correlated with this first axis were proline,  
8  
9 serine and threonine, their relative concentrations being higher in *Daphnia*, and glycine  
10  
11 and taurine, with higher relative concentrations in the two copepods (Table 3). The  
12  
13  
14  
15 325 second axis explained 39 % of the variance and segregated *Diaptomus* from the other  
16  
17 two species. *Diaptomus* had a higher alanine and serine relative concentration, while  
18  
19 *Cyclops* and *Daphnia* had higher glycine concentration.  
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#### 25 *Intraspecific variation*

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27  
28 330 Within each species, there were no differences in the relative proportion of TAA,  
29  
30 protein AA (PAA), FAA, essential AA and nonessential AA pools between most of the  
31  
32 different stages, with the exception of *Cyclops* males that had a lower relative  
33  
34 concentration than the females in the lake during the same period ( $2.9 \pm 0.1$  and  $1.6 \pm$   
35  
36  $0.5$  % dry weight of TAA for females and males, respectively;  $F_{1,3} = 15.8$ ,  $P = 0.028$ )  
37  
38 and *Daphnia* eggs that had higher TAA relative concentration than the first cohort  
39  
40 females ( $F_{1,8} = 2.8$ ,  $P = 0.1$  Tukey *post-hoc* test  $P = 0.013$ ) (Table 1). In contrast there  
41  
42 was a decrease from non-reproducing females to reproducing females of FAA and  
43  
44 essential AA pools of *Cyclops* ( $F_{1,9} = 20.5$ ,  $P = 0.001$  and  $F_{1,9} = 7.2$ ,  $P = 0.025$   
45  
46 respectively), a slight decrease in the females of *Daphnia* ( $F_{1,8} = 2.8$ ,  $P = 0.1$  and  $F_{1,8} =$   
47  
48  $3.3$ ,  $P = 0.1$  respectively) and in both males and females of *Diaptomus* ( $F_{1,6} = 3.8$ ,  $P =$   
49  
50  $0.1$  and  $F_{1,6} = 2.8$ ,  $P = 0.1$  respectively).  
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56 The MDA2 correctly classified 100 % of the samples of *Daphnia* (Fig. 4 and  
57  
58 Table 3). The first axes summarised 74.9 % of the variance and separated the three  
59  
60 stages analysed: first cohort females, second cohort females and eggs. The AAs most

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3 345 strongly associated with the axes were proline and arginine, more abundant in the eggs  
4  
5 (positive) side, and glycine and leucine, more abundant in second generation females  
6  
7 (negative) side. The second axis explained the remaining 22.4 % of the variation and  
8  
9 separated the second generation females and eggs (negative) side from the reproductive  
10  
11 and non-reproductive first generation females (positive) side. The AAs most strongly  
12  
13 associated with the axes were positively, threonine and glutamine / glutamic acid and  
14  
15 350 negatively, arginine. Further comparison of individual AAs between reproducing and  
16  
17 non-reproducing first cohort females with one way ANOVAs indicated that reproducing  
18  
19 females had higher histidine, serine and cystine ( $P = 0.003$ ,  $P = 0.003$   $P = 0.007$   
20  
21 respectively) and lower phenylalanine ( $P = 0.021$ ).  
22  
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27 355 For *Cyclops*, results of the MDA3 analysis showed that 100 % of the cases were  
28  
29 correctly classified (Table 3) (Fig. 4b). The first axes explained 78.2 % of the variance  
30  
31 and mainly segregated the CIV-V copepodites, the reproductive females and males from  
32  
33 the non-reproductive females and CII-III copepodites. The AAs most significantly  
34  
35 associated with the first axis were glutamine / glutamic acid and phenylalanine in the  
36  
37 non-reproductive females, males and copepodites CII-III (positive) side and serine at the  
38  
39 360 negative side. The second axis explained 12.9 % of the variance and mainly separated  
40  
41 copepodites CII-III from the rest, leucine and serine being correlated with copepodites  
42  
43 CII-III and valine and glutamine / glutamic acid with the rest (Table 3). Further  
44  
45 comparison of individual AAs between males and females during the months they  
46  
47 365 coexisted in the lake (January and February) with one way ANOVAs confirmed that  
48  
49 there were no statistical differences for any of the 18 AAs analysed and thus no gender  
50  
51 differences in *Cyclops* in terms of their AA composition.  
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58 The MDA4 correctly classified all the samples of *Diaptomus* in one single  
59  
60 significant axis (Fig. 4d and Table 3), showing that almost all the variability was

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3 370 associated with reproduction, while only negligible differences were related to gender.  
4  
5 The AAs that were selected by the forward selection procedure and that significantly  
6  
7 explained this variation were asparagine and phenylalanine that were associated with  
8  
9 the non-reproducing adults and threonine and serine with the reproducing adults (Table  
10  
11 3).  
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14  
15 375 *AAs related with Cyclops – Daphnia  $\Delta^{15}N$*   
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17  
18 The  $\Delta^{15}N$  of the predatory copepod *Cyclops* and those of its predominant prey, *Daphnia*  
19  
20 were calculated for the whole seasonal cycle and compared with the Euclidean distance  
21  
22 (dissimilarity) of the AA composition of the two species. Among the different AA  
23  
24 groups, the dissimilarity calculated only with the essential or source AAs had the  
25  
26  
27 380 highest correlation with the *Cyclops-Daphnia*  $\Delta^{15}N$  ( $r^2 = 0.931$ ,  $P < 0.001$  and  $r^2 =$   
28  
29  $0.835$ ,  $P = 0.01$  for essential and source AAs respectively), while the correlation was  
30  
31 weaker for trophic AAs ( $r^2 = 0.67$ ,  $P = 0.046$ ) or non-significant for the nonessential  
32  
33 AAs ( $r^2 = 0.461$ ,  $P = 0.138$ ) (Fig. 5). To get a closer insight into the relative importance  
34  
35 of both AA groupings we also calculated the dissimilarity of the AAs that were both  
36  
37  
38 385 essential and trophic, essential and source, nonessential and trophic, and nonessential  
39  
40 and source. Among these four groups, only those AAs that were both essential and  
41  
42 source had a significant correlation ( $r^2 = 0.872$ ,  $P = 0.006$ ), suggesting that only this  
43  
44 group of AAs were related with  $\Delta^{15}N$ .  
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49  
50 In order to find out if a particular essential AA might be related to  $\Delta^{15}N$  we  
51  
52 390 calculated the relative imbalance between *Cyclops* and *Daphnia*. Those AAs with a  
53  
54 higher weight in explaining  $\Delta^{15}N_{Cyclops-Daphnia}$  variance were expected to have 1) a  
55  
56 negative imbalance in the consumer with respect to the diet and 2)  $\Delta^{15}N_{Cyclops-Daphnia}$   
57  
58 should increase with decreasing relative concentration of this individual AA in the diet.  
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Among the different essential amino acids, only lysine had an average negative

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3 395 imbalance between both species (Fig. 6). A decrease in  $\Delta^{15}\text{N}_{\text{Cyclops-Daphnia}}$  with the  
4  
5 relative concentration of each individual essential AA in *Daphnia* was analysed with  
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7  
8 Pearson correlation (Table 4). Among the eight essential AAs analysed, only the  
9  
10 essential and source AA lysine had a significant negative correlation.  
11

## 13 Discussion

### 16 400 *Interspecific variability*

18 Our results confirm the hypothesis that interspecific variation in total AA composition  
19 was more pronounced than intraspecific variation. The overall AA interspecific distance  
20 was twice that of intraspecific distance (Fig. 3). In addition, although almost all samples  
21 were successfully classified to their corresponding stages for each species by the  
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28 405 MDA1, the two first axes of the MDA1 basically distinguished among the three species  
29  
30 (Fig. 4a). These results complement a previous extensive study focusing only on  
31 interspecific variation of alpine lake zooplankton in which most (85 %) of the adult  
32  
33 females of 13 species were successfully classified to their respective species (Guisande  
34  
35 et al. 2003). Therefore, it can be concluded that alpine freshwater zooplankton have a  
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40 410 non-strict homeostasis (Sternler and Elser, 2002) in their AA composition. This also  
41  
42 implies that if there are predatory interactions among them, it is likely that predators  
43  
44 may encounter dietary imbalances in their requirements. While phosphorus limitation in  
45  
46 freshwater primary consumers has been documented frequently (Hessen, 1992),  
47  
48 predators have been reported to face frequently nitrogen limitation (White, 1993,  
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50  
51 415 Anderson et al., 2004). We suggest that in these organisms AAs might be a dietary  
52  
53 limiting biochemical more often than is commonly assumed.  
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### 56 *Intraspecific variability*

57  
58 The degree of intraspecific variability was comparable in the three species (average  
59  
60 Euclidean distance within each species of Fig. 3 and dispersal of the points in the

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3 420 MDA1 of Fig. 4a), although the relative importance of ontogeny, reproduction and  
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5 gender was not the same for each species. *Cyclops* had a similar AA composition  
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7 variability related with ontogeny and reproduction, while *Daphnia* had a higher  
8  
9 variability related with ontogeny than reproduction. In the only previous study of  
10  
11 ontogenetic intraspecific AA variability of both copepods and cladocerans, Brucet et al.  
12  
13 (2005) also found an important effect of ontogeny in copepods, although they found  
14  
15 425 (2005) also found an important effect of ontogeny in copepods, although they found  
16  
17 little ontogenetic variability in *Daphnia*. In these latter species all the variability was  
18  
19 associated with differences among populations belonging to different lakes. Our results  
20  
21 show that when studying the intraspecific variability in one particular lake, the  
22  
23 ontogenetic AA variability of copepods and cladocerans can be of comparable  
24  
25 magnitude. Furthermore, we found different AAs responsible for the AA variation in  
26  
27 430 magnitude. Furthermore, we found different AAs responsible for the AA variation in  
28  
29 each species (proline, arginine, glycine and leucine in *Cyclops*; glutamine / glutamic  
30  
31 acid, phenylalanine and serine in *Daphnia*). In other species of copepods, Brucet et al  
32  
33 (2005) found valine, tyrosine, proline and alanine as the AA with a higher weight in the  
34  
35 ontogenetic variability of other copepod species. These results altogether suggest that  
36  
37 435 the AAs involved in the ontogenetic variability of crustaceans are species specific.  
38  
39 Although in this study we have not analysed the AA composition of nauplii, previous  
40  
41 studies have shown they are the stage most similar to adults in terms of their  
42  
43 composition (Brucet et al., 2005), and that they are very restrictive in their AA  
44  
45 composition, not developing further if maternal diet is deficient (Laabir et al., 1999).  
46  
47 440 Therefore, our results of lower intra- versus interspecific AA variability would most  
48  
49 likely have been the same if the naupliar stage of copepods had been included.  
50  
51  
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55 Various studies have described the negative effects of an AA imbalanced diet for  
56  
57 reproductive success in copepods (Cowgill et al., 1986, Guisande et al., 1999, Laabir et  
58  
59 al., 1999, Guisande et al., 2000, Lacoste et al., 2001, Helland et al., 2003b). In this study  
60

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3 445 we show that, in addition, adult female copepods and cladocerans, and males of  
4  
5 *Diaptomus* change their AA composition with reproduction. Contrary to the ontogenetic  
6  
7 effects, two of the AAs involved with the changes related with reproduction were the  
8  
9 same in the three species (the essential AA phenylalanine and the nonessential serine).  
10  
11 Phenylalanine is an aromatic AA which acts as a precursor of tyrosine and other  
12  
13  
14 450 compounds involved in important physiological functions, while serine is involved in  
15  
16 lipid biosynthesis and also a precursor of melanine. The results of this study suggest that  
17  
18 they are relevant during the reproduction of crustaceans. In addition to the changes in  
19  
20 individual AAs we also found a tendency for the FAA content to decrease from non-  
21  
22 reproductive to reproductive females. This agrees with other studies showing that eggs  
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25  
26 455 contain a larger proportion of FAAs and that the AA composition of eggs is decisive for  
27  
28 the successful development of the embryos (Guisande et al., 1999, Laabir et al., 1999,  
29  
30 Helland et al., 2003a, Helland et al., 2003b).  
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34  
35 Contrasting with the ontogenetic and reproduction effects, we found no changes in  
36  
37 the AA composition associated with gender in the two copepod species. The only  
38  
39 460 difference attributed to gender was a higher proportion of the FAA pool in *Cyclops*  
40  
41 females compared to males. This contrasts with the equal proportion between both sexes  
42  
43 in *Diaptomus*, which might reflect the different reproductive strategies described for  
44  
45 freshwater calanoids and cyclopoids. This result also agrees with the findings of a  
46  
47 parallel study (Ventura and Catalan 2005), in which *Diaptomus* males were found to  
48  
49  
50 465 follow similar elemental composition changes with reproduction as females, while the  
51  
52 elemental content of *Cyclops* males did not change with time. This was attributed to the  
53  
54 requirement of *Diaptomus* to mate every egg clutch, while *Cyclops* females can produce  
55  
56 multiple clutches of eggs with only one impregnation. Therefore, the present results on  
57  
58 the changes in the FAA pool also support the hypothesis that the different reproductive  
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3 470 strategies of copepods have a relevant effect on the elemental and biochemical  
4  
5 homeostasis of males.

6  
7  
8 *AAs involved in  $\Delta^{15}N$*

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10 There are different physiological factors that might influence  $\Delta^{15}N$ , including those  
11  
12 related with fasting, growth and diet quality in terms of protein quantity or quality  
13  
14  
15 475 (reviewed by Martínez del Rio and Wolf, 2005). While most of these factors have been  
16  
17 demonstrated either experimentally or through field studies, the effects of varying AA  
18  
19 composition have scarcely been studied (Martínez del Rio et al., 2009). In a previous  
20  
21 study we showed that the carbon isotopic variation in *Cyclops* juveniles could be  
22  
23 explained by fitting an isotopic growth model based on feeding entirely on *Daphnia*.  
24  
25  
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27 480 However this was not the case for nitrogen isotopic variability. *Cyclops* nitrogen  
28  
29 isotopic composition variation and the *Cyclops* to *Daphnia*  $\Delta^{15}N$  were closely correlated  
30  
31 to the dissimilarity in the AA composition between the two species (Ventura and  
32  
33 Catalan, 2008).

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37 In this study we show that among the different AAs, only those that are both  
38  
39 485 essential and source AAs had a high and significant correlation with  $\Delta^{15}N$ . Among  
40  
41 these, only lysine had a negative imbalance in the consumer as it increased its relative  
42  
43 concentration in the diet when the  $\Delta^{15}N$  decreased. This suggests that the imbalance of  
44  
45 only one amino acid can be responsible for a high metabolic turnover, which can  
46  
47 ultimately be responsible for a higher  $\Delta^{15}N$ . Our results therefore are in agreement with  
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50  
51 490 the hypothesis that an AA deficiency can be responsible for a variable  $\Delta^{15}N$  (Martínez  
52  
53 del Rio and Wolf, 2005).

54  
55  
56 Although the recognition of a variable  $\Delta^{15}N$  might seem a difficulty for a  
57  
58 widespread use of  $^{15}N$  as a trophic position estimator, it opens new perspectives for its  
59  
60 interpretation, since it could be used as an indicator of the relative AA imbalance of an

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2  
3 495 animal and as a consequence, an indicator of the relative degree of amino acid limitation  
4  
5 and as a result of the overall nitrogen limitation. Although this might seem difficult to  
6  
7 apply to general field studies, future studies using individual amino acid stable isotope  
8  
9 composition of carbon or nitrogen (e.g. Larsen et al., 2009) might substantially help  
10  
11 interpretations in stable isotope trophic ecology.  
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16 500

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## Figure and table legends

**Table 1.** Relative proportions of the different amino acid (AA) pools in the different species / stages analysed (mean  $\pm$  SD). Total AA (TAA) concentration is expressed as mass percentage of dry weight (DW) and is subdivided in the protein AA pool (PAA) and the free amino pool (FAA) that are expressed as the mass percentage of TAA. The relative proportions of essential and nonessential AA pools (EAA and NEAA respectively) are also calculated. Letters after SDs denote ANOVA statistically significant stages (a, b) or reproducing versus non-reproducing females (c, d) at  $P < 0.05$  or (\*)  $< 0.1$ .

**Table 2.** AA composition (mean  $\pm$  SD weight percentage of total AA) of all AAs of different development stages of the three species of crustacean zooplankton of Lake Redon (*Cyclops abyssorum*, *Daphnia pulex* and *Diaptomus cyaneus*). Those AAs described to be essential (E) / nonessential (N) or trophic (T) / source (S) (the latter category after Popp et al. 2007) are distinguished with letters in parentheses. A question mark designates those amino acids not included into the trophic / source category.

**Table 3.** Standardized canonical coefficients for each AA in the discriminant functions extracted by the multiple discriminant analysis performed to identify AAs (independent variables), which significantly separate species or stages. Missing values for a given AA are those AAs excluded from the analysis of the tolerance test. A graphical representation can be found in Fig. 4.

**Table 4.** Pearson correlation coefficient between the *Cyclops abyssorum* – *Daphnia pulex* nitrogen isotopic fractionation and the relative concentration of each essential amino acid in *D. pulex*.

**Fig. 1.** Seasonal changes in water temperature, chlorophyll *a*, the biomass of the three crustacean planktonic species of Lake Redon (*Cyclops abyssorum*, *Daphnia pulex* and *Diaptomus cyaneus*), the relative contribution of *Cyclops abyssorum* small copepodites (I to III), large copepodites (IV and V) and adults of the total *C. abyssorum* biomass, and the relative contribution of *Daphnia pulex* ovigerous and non-

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3 ovigerous females (first cohort females) and juveniles (second cohort females) to the  
4 total *D. pulcaria* biomass. *Daphnia* juveniles were those not reaching sexual maturity,  
5 which in this year corresponded to a size lower than 2 mm. For temperature and  
6 chlorophyll a, filled circles are the water column average values; dotted lines follow  
7 maximum and minimum values recorded on each sampling occasion.  
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14 **Fig. 2.** Relative concentration of taurine in relation to the free AA pool in eight different  
15 zooplankton species found in the literature (Cowey and Corner 1963a; Suyama et al.  
16 1965; Jeffries 1969; Jeffries and Alzara 1970; Srinivasagam et al. 1971). DW stands for  
17 dry weight.  
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23 **Fig. 3.** Average Euclidean distance (with 10<sup>th</sup> and 25<sup>th</sup> percentiles) of the interspecific  
24 (shaded box plots) and intraspecific (white box plots) distance in the amino acid  
25 composition. Distances were computed among all samples analysed and therefore the  
26 intraspecific distances include both the distances between stages and within stages.  
27 Abbreviations stand for *Cyclops abyssorum* (C.a.), *Daphnia pulicaria* (D.p.) and  
28 *Diaptomus cyaneus* (D.c.). Letters above each box-plot denote significantly different  
29 distances from the one-way ANOVA Tukey post-hoc test ( $P < 0.001$ ).  
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37 **Fig. 4.** Plots of the first two discriminant function scores of the total AA composition of  
38 (a) the various development stages of *Daphnia pulicaria* (open symbols), *Cyclops*  
39 *abyssorum* (shaded symbols) and *Diaptomus cyaneus* (black symbols) altogether, (b)  
40 those of *D. pulicaria* only, (c) *C. abyssorum* only and (d) *D. cyaneus* only. Symbols  
41 correspond to reproducing females (downward triangles), non-reproducing females  
42 (circles), reproducing males (upward triangles), non-reproducing males (diamonds), *C.*  
43 *abyssorum* copepodites CII and CIII (hexagons), *C. abyssorum* copepodites CIV and  
44 CV (stars), *D. pulicaria* young females (squares) and eggs (crossed circles). The AA  
45 scores and details of the analysis are summarised in Table 3.  
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55 **Fig. 5.** Comparison between (a) the *Cyclops abyssorum* – *Daphnia pulicaria* nitrogen  
56 isotopic fractionation ( $\Delta^{15}\text{N}$ ) and the trophic amino acids average Euclidean distance (b)  
57 the source AAs average Euclidean distance, (c) the essential AAs average Euclidean  
58 distance, (d) the nonessential AAs average Euclidean distance, (e) the trophic and  
59 essential AAs average Euclidean distance, (f) the source and essential AAs average  
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3 Euclidean distance, (g) the trophic and non-essential AAs average Euclidean distance  
4 and (h) the source and nonessential AAs average Euclidean distance. The different AAs  
5 belonging to each group are described in Table 2.  
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10 **Fig. 6.** Average relative essential amino acid (AA) imbalance between *Cyclops*  
11 *abyssorum* and *Daphnia pulex* during a seasonal cycle. See text for calculation  
12 procedure. Results are expressed in dry weight percentage of the total AA composition.  
13 Error bars are standard deviations. White and black bars are source and trophic AAs  
14 respectively. Grey bars are those amino acids that have not been described to belong to  
15 any of the two categories. Asterisks denote significant negative correlation between the  
16 *C. abyssorum* – *D. pulex* nitrogen isotopic fractionation and the AA composition of  
17 *D. pulex* as described in Table 4. Amino acid abbreviations are arginine (ARG),  
18 histidine (HIS), taurine (TAU), methionine (MET), isoleucine (ILE), leucine (LEU),  
19 valine (VAL), phenylalanine (PHE), lysine (LYS) and threonine (THR).  
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Table 1

Species	Stage / gender	TAA (% DW)	FAA (% TAA)	PAA (% TAA)	EAA (% TAA)	NEAA (% TAA)
<i>Cyclops abyssorum</i>	CII-III	47.5 ±13.4a	1.7 ±1.0a	98.3 ±1.0a	45.6 ±0.4a	54.4 ±0.4a
	CIV-V	41.9 ±4.8a	2.4 ±0.9a	97.6 ±0.9a	45.9 ±2.4a	54.1 ±2.4a
	non-reproducing females	37.7 ±4.3a;c	2.7 ±0.4a;c	97.3 ±0.4a;c	45.4 ±0.5a;c	54.6 ±0.5a;c
	reproducing females	53.6 ±4.7a;d	1.4 ±0.5a;d	98.6 ±0.5a;d	44.7 ±0.3a;d	55.3 ±0.3a;d
	males	47.6 ±7.3a	1.6 ±0.5a	98.4 ±0.5a	45.2 ±1.6a	54.8 ±1.6a
<i>Daphnia pulicaria</i>	First-cohort non-reproducing females	25.3 ±2.3a;c*	2.6 ±0.7a;c*	97.4 ±0.7a;c	45.5 ±1.2a;c*	54.1 ±0.5a;c*
	First-cohort reproducing females	27.3 ±1.9a;d*	1.9 ±0.4a;d*	98.1 ±0.4a;c	44.6 ±2.0a;d*	55.4 ±2.0a;d*
	Second-cohort females	29.3 ±5.0a,b	3.3 ±2.6a	96.7 ±2.6a	46.2 ±1.0a	53.8 ±1.0a
	eggs	34.1 ±6.6b	2.3 ±0.3a	97.7 ±0.3a	47.7 ±2.2a	52.3 ±2.2a
<i>Diaptomus cyaneus</i>	non-reproducing females	45.8 ±1.3a;c	1.6 ±0.6a;c	98.4 ±0.6a;c	45.1 ±0.0a;c	54.9 ±0.0a;c
	reproducing females	71.4 ±0.9a;d	1.0 ±0.1a;d	99.0 ±0.1a;c	45.3 ±0.4a;c	54.7 ±0.4a;c
	non-reproducing males	49.0 ±0.0a;c	1.3 ±0.1a;c	98.7 ±0.1a;c	45.1 ±0.4a;c	54.9 ±0.4a;c
	reproducing males	72.6 ±4.9a;d	1.0 ±0.1a;d	99.0 ±0.1a;c	45.4 ±0.5a;c	54.6 ±0.5a;c

Table 2

	<i>Cyclops abyssorum</i>					<i>Daphnia pulicaria</i>			<i>Diaptomus cyaneus</i>				
	CII-III	CIV-V	non-reproducing females	reproducing females	males	eggs	juveniles	non-reproducing females	reproducing females	Non-reproducing females	reproducing females	non-reproducing males	reproducing males
Taurine (E, ?)	0.1 ±0.1	0.2 ±0.1	0.3 ±0.1	0.1 ±0.1	0.1 ±0.1	0.2 ±0.0	0.4 ±0.5	0.2 ±0.1	0.1 ±0.1	0.2 ±0.1	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0
Alanine (N, T)	7.6 ±0.0	8.1 ±0.1	8.1 ±0.4	8.2 ±0.2	8.5 ±0.5	6.2 ±0.2	6.9 ±0.1	6.9 ±0.3	6.4 ±0.4	8.6 ±0.3	9.0 ±0.1	8.8 ±0.0	9.2 ±0.2
Arginine (E, S)	7.0 ±0.1	6.9 ±0.6	6.8 ±0.3	6.6 ±0.4	6.5 ±0.3	6.9 ±0.6	6.7 ±0.2	6.8 ±0.2	6.7 ±0.0	6.1 ±0.4	6.1 ±0.1	5.9 ±0.1	5.8 ±0.0
Asparagine + Asparctic ac.(N, T)	9.6 ±0.1	9.0 ±0.7	9.4 ±0.2	9.3 ±0.1	9.4 ±0.7	9.8 ±0.2	9.8 ±0.1	10.3 ±0.1	10.4 ±0.1	9.8 ±0.0	9.1 ±0.1	9.7 ±0.1	9.0 ±0.1
Glutamine + Glutamic ac. (N, T)	13.7 ±0.3	12.5 ±0.6	13.0 ±0.6	13.4 ±0.2	13.5 ±0.8	13.1 ±0.4	12.8 ±0.5	13.1 ±0.5	13.4 ±0.1	13.8 ±0.2	13.1 ±0.2	13.9 ±0.0	13.1 ±0.1
Glycine (N, S)	5.8 ±0.2	6.0 ±0.2	6.1 ±0.1	6.1 ±0.1	6.2 ±0.2	4.5 ±0.9	5.3 ±0.1	5.3 ±0.1	5.2 ±0.1	5.2 ±0.1	5.4 ±0.0	5.2 ±0.0	5.6 ±0.0
Histidine (E, ?)	2.0 ±0.0	2.1 ±0.1	2.0 ±0.1	2.1 ±0.2	2.2 ±0.1	2.3 ±0.1	2.4 ±0.6	2.0 ±0.1	2.2 ±0.0	2.2 ±0.0	2.2 ±0.0	2.1 ±0.0	2.1 ±0.0
Isoleucine (E, T)	4.6 ±0.1	4.5 ±0.2	4.4 ±0.1	4.3 ±0.1	4.4 ±0.3	5.1 ±0.6	4.7 ±0.1	4.8 ±0.1	4.9 ±0.1	4.3 ±0.0	4.1 ±0.1	4.3 ±0.0	4.1 ±0.1
Leucine (E, T)	7.8 ±0.2	7.6 ±0.4	7.5 ±0.4	7.3 ±0.3	7.8 ±0.2	8.4 ±0.5	8.9 ±0.2	8.3 ±0.1	8.3 ±0.1	7.8 ±0.0	7.3 ±0.2	7.9 ±0.1	7.4 ±0.2
Lysine (E, S)	7.2 ±0.2	7.9 ±0.5	6.7 ±0.4	6.7 ±0.1	6.9 ±0.1	6.9 ±0.2	6.4 ±0.5	6.1 ±0.2	6.1 ±0.5	7.5 ±0.4	7.2 ±0.1	7.2 ±0.1	7.4 ±0.3
Phenylalanine (E, S)	4.3 ±0.1	4.2 ±0.2	4.4 ±0.1	4.3 ±0.1	4.3 ±0.2	5.1 ±0.1	4.9 ±0.0	5.1 ±0.1	5.0 ±0.1	4.2 ±0.0	3.9 ±0.0	4.2 ±0.1	3.8 ±0.1
Proline (N, T)	4.8 ±0.1	5.0 ±0.2	5.2 ±0.4	5.5 ±0.1	5.4 ±0.2	4.5 ±1.9	5.2 ±0.3	5.0 ±0.4	5.3 ±0.1	5.2 ±0.2	5.8 ±0.2	5.4 ±0.0	5.8 ±0.2
Serine (N, S)	4.6 ±0.1	4.2 ±0.3	4.4 ±0.2	4.5 ±0.2	4.2 ±0.2	5.9 ±0.4	5.1 ±0.1	5.5 ±0.1	5.7 ±0.1	4.7 ±0.0	4.5 ±0.1	4.8 ±0.0	4.5 ±0.1
Threonine (E, S)	5.3 ±0.2	5.2 ±0.3	5.2 ±0.2	4.9 ±0.2	5.6 ±0.8	6.5 ±0.8	6.2 ±0.2	6.4 ±0.4	6.3 ±0.1	4.9 ±0.2	5.1 ±0.2	4.8 ±0.1	4.8 ±0.1
Tyrosine (N, S)	7.2 ±0.3	7.5 ±0.5	7.6 ±0.2	7.6 ±0.4	7.5 ±0.7	5.0 ±0.5	5.0 ±0.1	4.9 ±0.3	4.8 ±0.0	6.7 ±0.4	7.9 ±0.0	6.7 ±0.4	8.2 ±0.1
Valine (E, T)	5.7 ±0.0	5.9 ±0.2	6.0 ±0.2	6.0 ±0.1	5.9 ±0.2	6.5 ±0.6	6.5 ±0.2	6.4 ±0.1	6.3 ±0.0	5.8 ±0.1	6.0 ±0.0	5.9 ±0.0	5.8 ±0.0
Methionine (E, ?)	1.6 ±0.2	1.8 ±0.1	1.7 ±0.2	1.7 ±0.1	0.8 ±1.0	2.1 ±0.0	1.8 ±0.1	1.9 ±0.2	2.0 ±0.2	1.9 ±0.1	1.8 ±0.1	2.0 ±0.1	1.9 ±0.0
Cystine (N, ?)	1.0 ±0.3	1.3 ±0.1	1.3 ±0.1	1.4 ±0.1	0.7 ±0.8	1.1 ±0.1	0.9 ±0.0	0.9 ±0.1	1.0 ±0.1	1.1 ±0.2	1.4 ±0.1	1.0 ±0.1	1.4 ±0.0

Table 3

	Inter-species classification		<i>Cyclops abyssorum</i>		<i>Daphnia pulicaria</i>		<i>Diaptomus cyaneus</i>
	Function 1	Function 2	Function 1	Function 2	Function 1	Function 2	Function 1
Alanine	1.047	-1.122	10.4	1.9	0.19	3.70	
Arginine	1.08	1.46	9.8	5.6	7.33	-2.20	
Asparagine + Aspartic ac.	1.31	-0.496	10.4	0.0	-1.03	1.16	22.45
Cystine	0.271	-0.38					
Glutamine + Glutamic ac.	0.935	1.36	22.7	10.0	-0.06	6.32	
Glycine	-0.467	2.772	17.2	5.2	-8.97	2.65	
Histidine	0.719	0.639	8.9	2.8	2.87	-0.16	
Isoleucine	0.292	1.156	2.7	0.8	5.50	5.69	
Leucine	1.03	-0.341	8.9	-2.4	-8.28	1.30	
Lysine	0.683	0.73	15.7	8.1	4.86	1.28	
Methionine	0.638	-0.327					
Phenylalanine	0.727	0.71	16.8	5.7	5.32	0.74	22.53
Proline	2.843	-0.373	2.0	5.6	22.49	6.78	
Serine	1.321	-0.537	-6.3	-2.7			-5.55
Taurine	-0.337	0.923	9.2	5.3	-0.13	-0.02	
Threonine	1.141	1.186	15.3	4.3	5.31	9.42	-12.45
Tyrosine	0.863	0.841	13.0	4.1			
Valine	0.945	1.537	5.2	5.6	0.19	3.70	
Eigenvalues	93.0	77.3	214.2	35.4	187.7	56.1	31441
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	0.001
Canonical correlation	0.995	0.994	0.998	0.986	0.997	0.991	1.0
Variance explained	46.5	38.7	78.2	12.9	74.9	22.4	100

Table 4

	Correlation coefficient	<i>P</i> -val
Arginine	0.204	ns
Histidine	-0.274	ns
Methionine	0.407	ns
Taurine	0.138	ns
Isoleucine	0.940	0.003
Leucine	0.725	0.052
Valine	0.455	ns
Lysine	-0.830	0.020
Threonine	0.588	ns
Phenylalanine	0.305	ns

For Peer Review

Figure 1

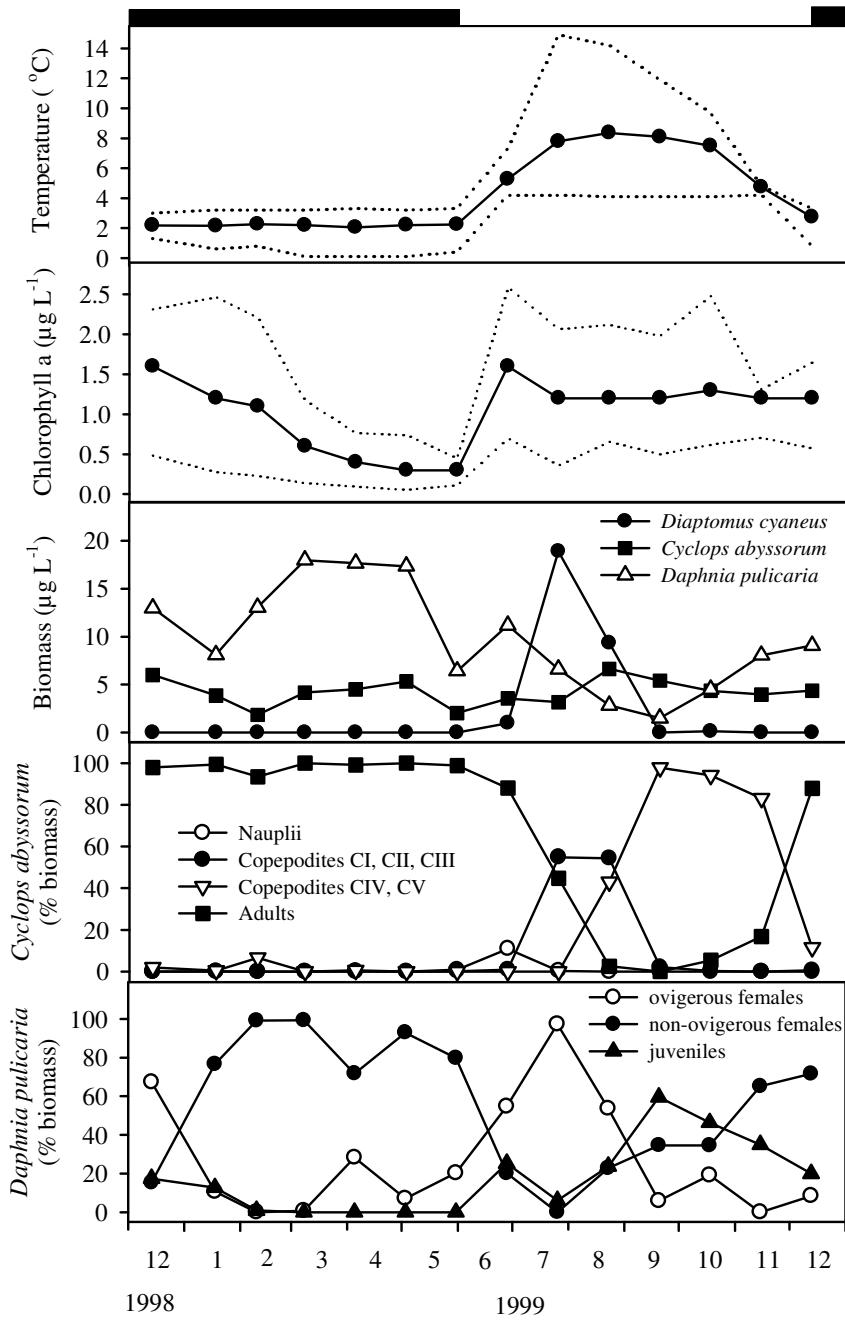
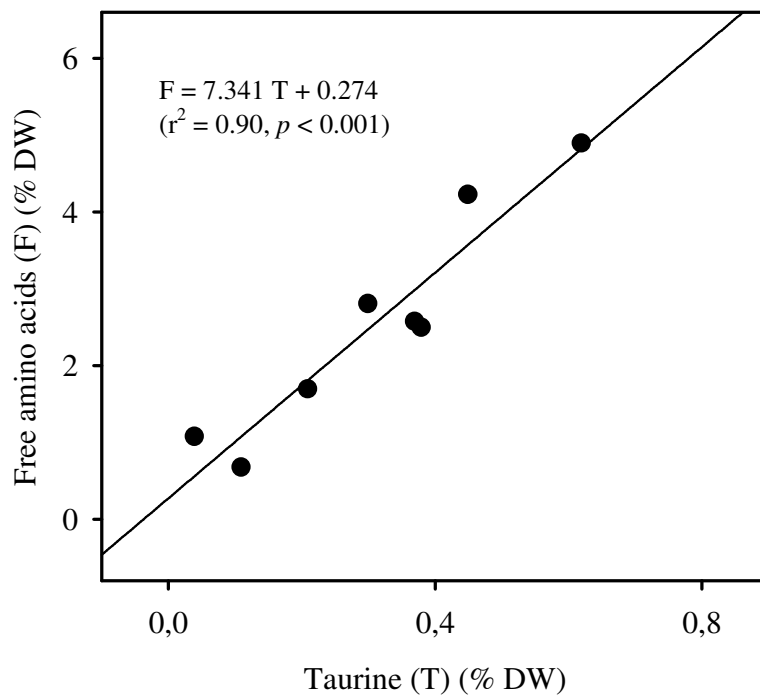


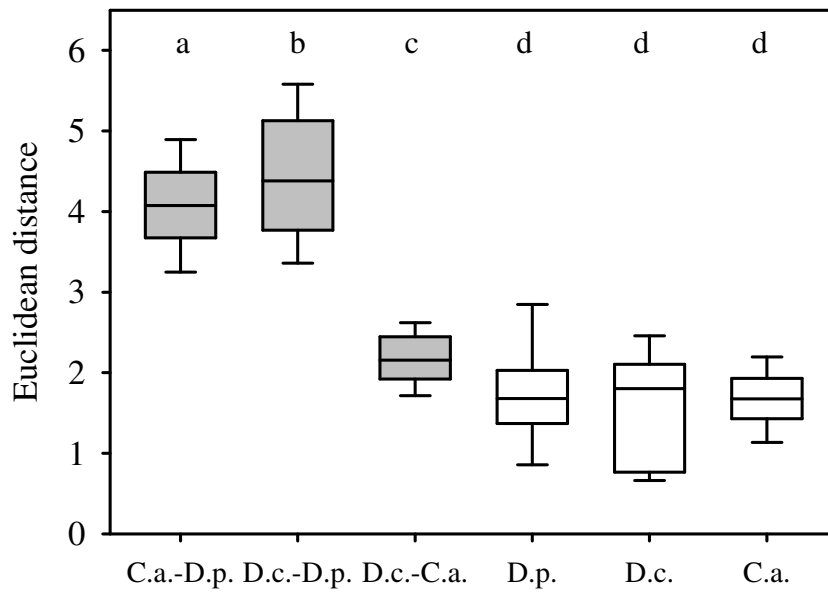
Figure 2



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Figure 3



Peer Review



Figure 4

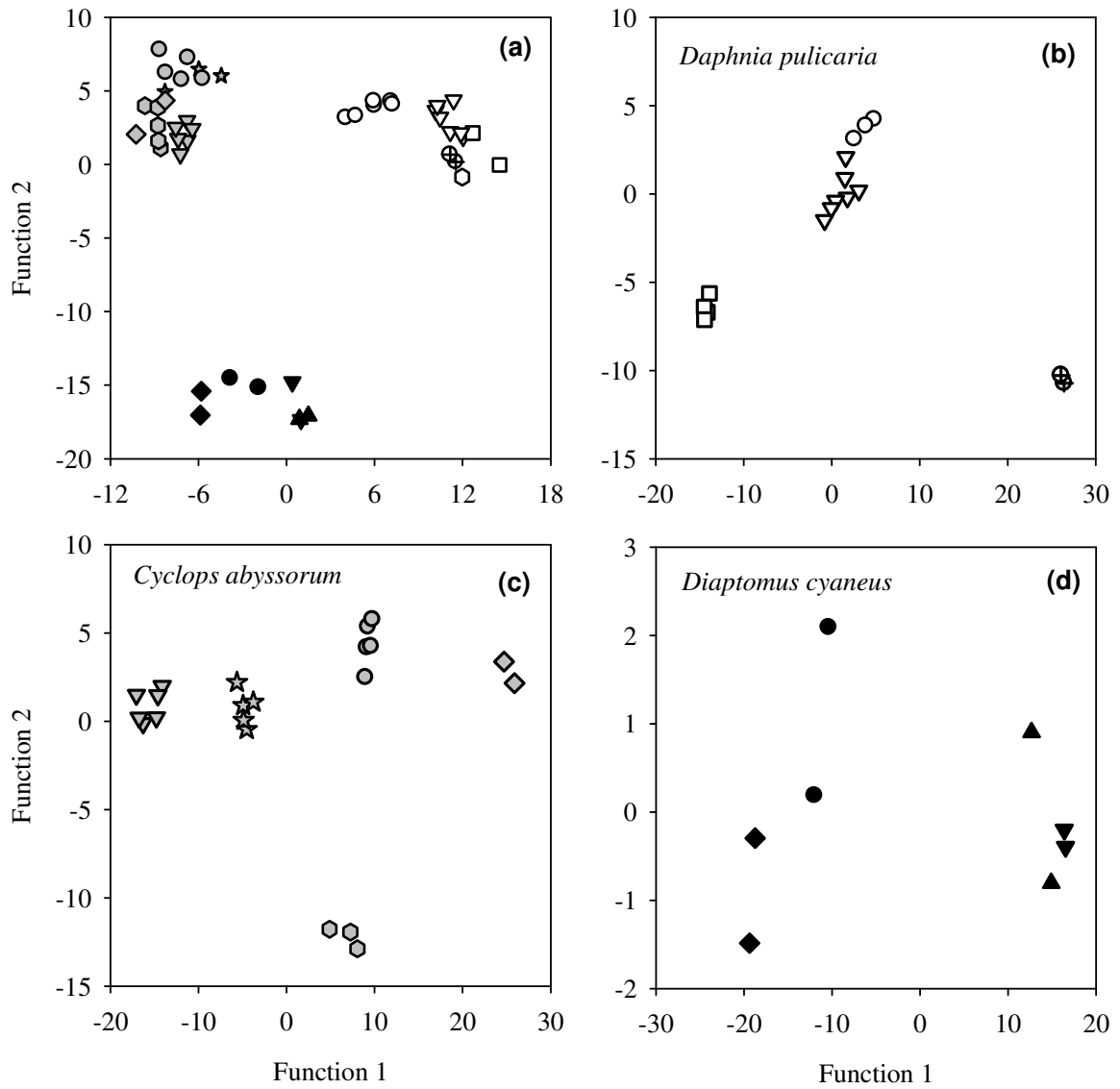


Figure 5

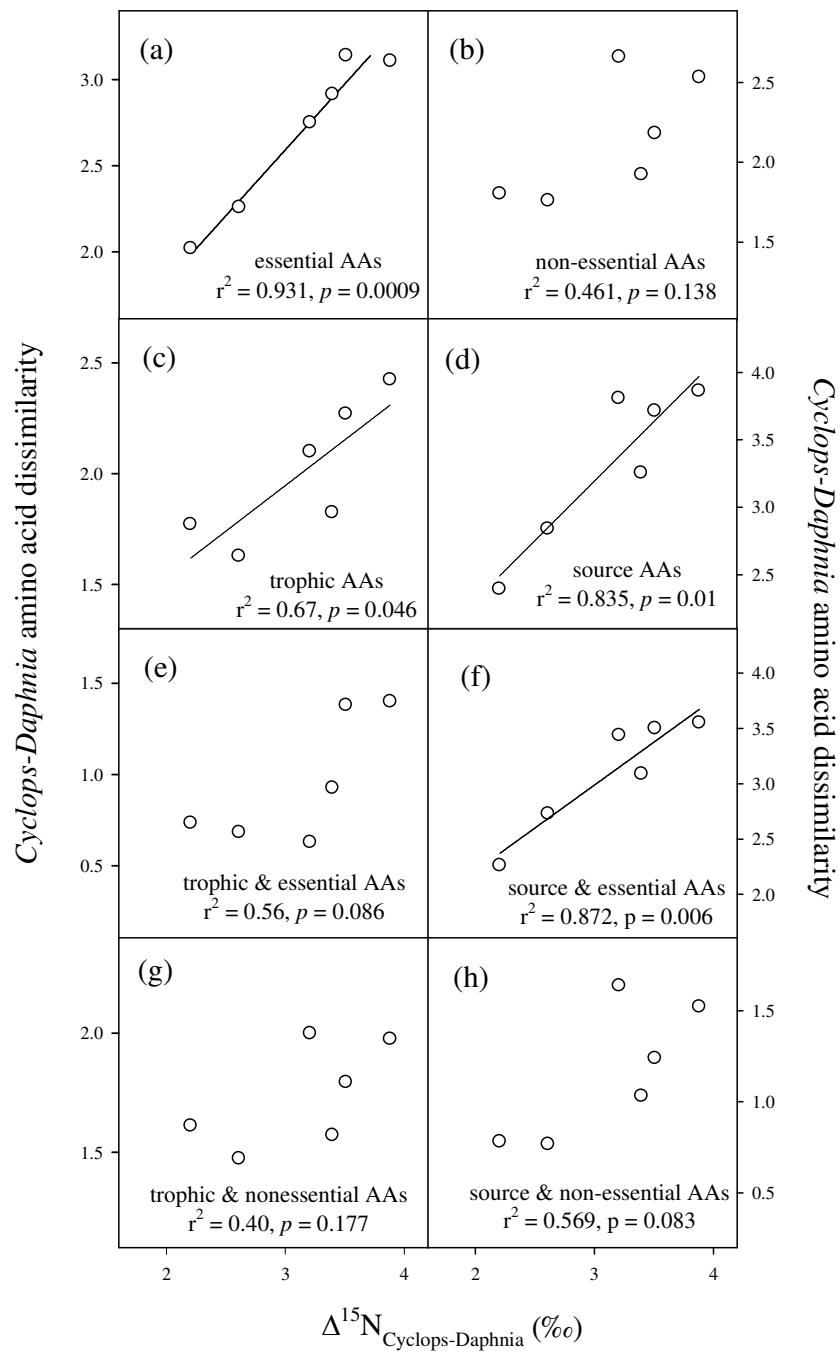
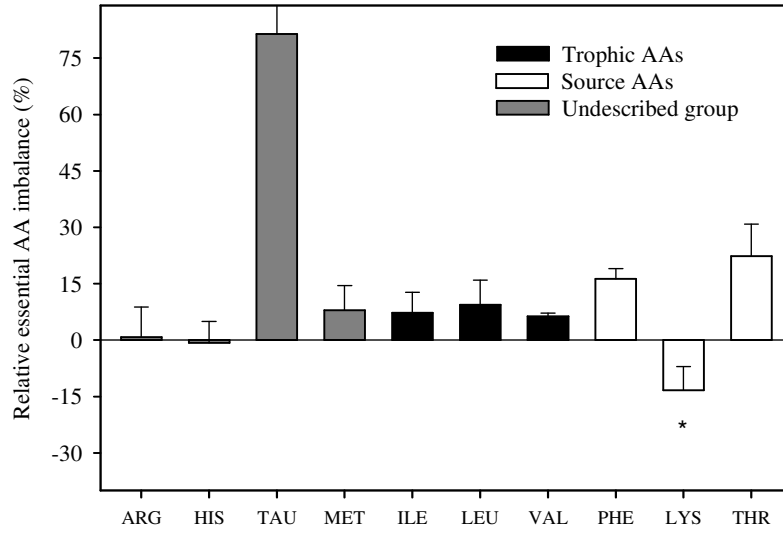


Figure 6



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