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Marc Ventura, Jordi Catalan. Variability in amino acid composition of alpine crustacean zooplankton and its relationship with 15-nitrogen fractionation. *Journal of Plankton Research*, 2010, 10.1093/plankt/FBQ066 . hal-00598965

HAL Id: hal-00598965

<https://hal.science/hal-00598965>

Submitted on 8 Jun 2011

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Journal of
Plankton Research

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

Journal:	<i>Journal of Plankton Research</i>
Manuscript ID:	JPR-2010-045.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	07-Apr-2010
Complete List of Authors:	Ventura, Marc; CEAB-CSIC, Biogeodynamics and Biodiversity group Catalan, Jordi; CEAB-CSIC, Biogeodynamics and Biodiversity Group
Keywords:	Amino acid composition, food quality, nitrogen fractionation, stoichiometry, homeostasis



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

Amino acids (AAs) are critical biochemical compounds for living organisms (i.e. as building blocks of proteins, as part of coenzymes, and as precursors for the biosynthesis of some molecules) (Strayer, 1988). Because of the limited capacity for their *de novo* synthesis in many animals, the AA composition of food becomes very important in animal nutrition. This has been demonstrated in different types of terrestrial organisms such as butterflies (O'Brien et al., 2003), birds (Ramsay and Houston, 1998), farm animals (D'mello, 1994) and humans (Reeds, 2000), and also for aquatic organisms such as farmed fish (Conceição et al., 2003, Ronnestad et al., 2003), rotifers (Boëchat and Adrian, 2006), mussels (Kreeger et al., 1996) and marine copepods (Kleppel and Burkart, 1995, Guisande et al., 1999, Guisande et al., 2000). This knowledge contrasts with the few ecological studies focussing on the relative importance of AAs for animal growth and reproduction (Conklin and Provasoli, 1977, Boëchat and Adrian, 2006). Stoichiometry studies focusing on elements, mainly phosphorus, but also carbon and nitrogen (reviewed in Sterner and Elser, 2002) or fatty acids, mainly highly unsaturated fatty acids (HUFA) such as eicosapentanoic acid (EPA) or docosahexanoic acid (DHA) (e.g. Müller-Navarra et al., 2000) have received more attention than AAs. Since most of animal's nitrogen content is in the form of amino acids, comparison between organisms and their food AA composition has been suggested as a step forward in using stoichiometric arguments for a better understanding of nitrogen limitation (Anderson et al., 2004). This suggestion is based on two main assumptions, namely that the consumer is unable to synthesise some AAs in significant quantities (i.e., essential AAs) and that it is homeostatic with respect to the composition in essential AAs. The former assumption is supported by numerous studies, including in crustaceans (Claybrook,

1983), whereas the latter has only been partially demonstrated and further studies are required (Cowgill et al., 1986, Anderson et al., 2004).

50 An imbalance between the AA composition of food and those of the consumer has been proposed as one of the main mechanisms explaining nitrogen stable isotope fractionation (i.e., change in isotope ratios, $\Delta^{15}\text{N}$) between diet and animal tissues (Martínez del Rio and Wolf, 2005). Nitrogen stable isotopes together with those of carbon have become a common tool for describing trophic relationships, although the physiological mechanism explaining $\Delta^{15}\text{N}$ (crucial for estimating diet sources) is still not resolved (Martínez del Rio et al., 2009). The physiological mechanism could be caused by the deficiency in one, or several essential AAs leading to greater protein intake and eventually to higher catabolism of the AAs in excess for protein synthesis favouring a preferential body elimination of lighter nitrogen (Martínez del Rio and Wolf, 2005). Few studies have provided evidence for this hypothesis with the exception of Robbins et al. (2005), in a comparative study, and Ventura and Catalan (2008), in a field study, that reported a significant negative correlation between $\Delta^{15}\text{N}$ and the protein quality of the diet (e.g. AA composition). An important unresolved question is if there are some particular AAs that are more relevant for $\Delta^{15}\text{N}$. The essential part of an AA (and what differentiates them biochemically) is their carbon skeleton. All the nitrogen of AAs is in the amino group, which can be trans-aminated by animals in, *a priori*, all AAs. However, it has been observed that some AAs appear to retain approximately the same nitrogen isotopic composition as their food source (are not trans-aminated; “source AAs”) whereas others become enriched in ^{15}N by the animals’ metabolism (undergo trans-amination and obtain a new amino group with enriched ^{15}N ; “trophic AAs”) (McClelland and Montoya, 2002, Popp et al., 2007). Different proportions of “trophic” and “source” AAs explain why animal tissues differ in ^{15}N

(Martínez del Río et al., 2009). Since the trans-amination is not related to the AA carbon skeleton, some trophic / source AAs are essential while others are nonessential, making a complicated physiological puzzle. What particular AAs or AA group is directly related to $\Delta^{15}\text{N}$ has not been described and therefore needs to be explored more in depth. One may hypothesise that among the essential amino acids, only those that are not trans-aminated would have a higher relevance than the others.

Zooplankton has played a central role in stoichiometry studies, particularly *Daphnia*, which has been extensively used as a model organism. Investigations examining the AA composition are scarce, however. Most past studies of the AA composition of zooplankton have focused on the variability in the total AA (TAA) composition of adult marine or freshwater zooplankton compared to their food, the results showing that adults have a very strict AA composition (Cowey and Corner, 1963, Cowgill et al., 1986, Guisande et al., 2000). All those studies are indicative of AA composition homeostasis in adults of crustaceans. In contrast, there are few studies focussing on the TAA intraspecific variability. Significant differences in AA composition during development have been observed in *Artemia spp.* and lobsters (Helland et al., 2000, Mente et al., 2001). In cladocerans and copepods Dabrowski and Rusiecki (1983) found differences in TAA between late developmental stages of different species, although they did not examine the degree of these TAA changes for each species. Changes in TAA were reported for two *Calanus* species during development from egg to the second naupliar stage (Laabir et al., 1999) and between nauplii of different seasons (Helland et al., 2003a). Ontogenetic variability in species of cladocerans, calanoid and cyclopoid copepods were studied in different coastal lagoons and wetlands by Brucet et al. (2005). They analysed all the lake-populations simultaneously, and found a more pronounced variability in the TAA composition

related with ontogeny in copepods, while *Daphnia* variability was mostly related with lake type and not with ontogeny. However, the relative importance of ontogeny, reproduction and gender in AA composition changes has not been evaluated for these animal groups.

In this context, with the aim of testing the hypothesis that interspecific differences among the main freshwater groups of crustacean zooplankton are greater than their intraspecific variation, we studied the relative proportions of the AA pools and its variation in crustacean zooplankton species of an alpine lake throughout a whole seasonal cycle. We selected a lake that had one species of each of the main freshwater crustacean groups, a cladoceran (*Daphnia pulicaria* Forbes), a cyclopoid copepod (*Cyclops abyssorum* Sars) and a calanoid copepod (*Diaptomus cyaneus* Gurney). In a previous study these three species were found to have substantial changes in their elemental content of carbon and phosphorus during their life cycles associated with reproduction (Ventura and Catalan 2005). In contrast, nitrogen was the only compound which did not change significantly. In addition, *Cyclops* was described as being a predatory copepod feeding primarily on *Daphnia*, and the $\Delta^{15}\text{N}$ of both species was strongly correlated with their protein quality, which was interpreted as evidence of consumer amino acid (nitrogen) limitation (Ventura and Catalan, 2008). Therefore these two species also present an ideal framework for investigating the specific role of AAs in $\Delta^{15}\text{N}$. Contrasting with *Cyclops*, the other two species, *Diaptomus* and *Daphnia* are more generalistic consumers (an omnivore and herbivore respectively), and therefore they are not suitable for this particular comparison. The specific hypotheses driving the study were (i) interspecific variability of AA composition is greater than intraspecific AA variability (ii) reproduction, gender and ontogenetic changes are important intraspecific sources of AA variability; and (iii) essential amino acids that are not trans-

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

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Method

Study site and sample collection

Lake Redon (formerly lake Redó) is an oligotrophic glacial cirque lake located at 2240 m a.s.l. in the central Pyrenees (42° 38' N, 0° 46' E). It is relatively large (24 ha) and it is one of the deepest lakes (73 m) in the Pyrenees. It is dimictic, covered by ice during half of the year, usually from mid December until late May or the beginning of June. The lake has average microbial biomass ratios of 10 : 2 : 2 : 1 for phytoplankton : bacteria : heterotrophic nanoflagellates : ciliates during the ice-free period (Felip et al., 1999).

135 Rotifers are usually less abundant than crustaceans (the ratio between *Daphnia* and rotifer biomass is approximately 10:1 during the ice-free period; Camarero et al., 1999).

The lake was sampled on 14 occasions from December 1998, just after the lake was completely ice covered, to December 1999, after it was ice-covered again. Samples were collected at the deepest point of the lake either after drilling the ice-cover or from a platform anchored throughout the summer at the same point. Zooplankton samples were collected by vertical hauls from 65 m to the surface with a 200 μm net. Individuals were kept alive and transported cold (4°C) until they were frozen (-20°C) in the laboratory a few hours after collection.

The three crustacean zooplankton species studied had contrasting life histories although all produced a single cohort per year. A complete description of their life cycles during the study period can be found in Ventura and Catalan (2005) and their trophic relationships in Ventura and Catalan (2008). *Cyclops* is a predatory copepod

feeding primarily on *Daphnia* in this lake (Ventura and Catalan, 2008). Its life cycle was synchronised with that of *Daphnia* being the only two planktonic crustacean species inhabiting the lake during the whole year. Adult females of *Daphnia* from the previous summer survived below the ice-cover, reproduced during the end of the ice-covered period and the first two months of the ice-free period, and disappeared from the lake afterwards, leaving the juveniles growing up during the summer (Fig. 1) (Ventura and Catalan 2005). Adult females (termed 'first cohort females' in our study) were therefore clearly distinguishable from the juveniles born during the ice free period (termed 'second cohort females'). We therefore compared the AA composition of the two cohorts, of the reproducing and non-reproducing first cohort females, and their eggs. Similar to *Daphnia*, adult females of *Cyclops* survived below the ice-cover in order to reproduce during the end of the ice-covered period. Adult males were present in the lake from December to February below the ice-cover. Nauplii appeared the following summer, just after the ice-cover melted (copepods have six naupliar, and five copepodite stages before adults develop) and coincided with the spring production maximum. Copepodites CI to CIII, dominated *Cyclops* biomass during July and August, copepodites CIV and CV were present in the lake from September until November (Fig. 1) (Ventura and Catalan 2005). Therefore, we sampled two generations and the stages analysed consisted of adult males, adult reproductive and non-reproductive females, and four copepodites (CII to CV), which were amalgamated in two groups, one with CII and CIII and the other with CIV and CV. The other copepod, *Diaptomus*, dominated the plankton in July and August, spending the rest of the year as resting eggs (Fig. 1) (Ventura and Catalan 2005). Due to its fast development, only adult *Diaptomus* were considered in this study. Unlike *Cyclops*, adult males and females were present in the lake during the same period. The July and August samples coincided with the beginning

and the end of the reproduction period and were therefore expected to cover the maximum range of the AA variability for the adults of the species.

175 *Sample preparation and chemical analysis*

After thawing the samples, from 10 to several hundreds of individuals, depending on the weight of each species and stage, were quickly sorted under a dissecting microscope and were either placed into pre-weighed tin capsules for stable isotope analysis or in Teflon capsules for analysis of amino acids. From two to six combined sample replicates were analysed for each species and stage for each sampling date, with the exception of a few cases where sample material did not suffice. Individuals were kept cold (<4 °C) during the sorting process, which was always carried out within a few hours of collection. Dry weights were determined for all samples after drying at 60 °C for 24h and weighing on a high precision microbalance (Ohaus Analytical Plus, AP250D-0).

185 For amino acid analysis, between 0.6-0.9 mg of dried sample was vacuum-sealed and hydrolysed with HCl 6N at 112 °C for 16 h (Fountoulakis and Lahm, 1998). An internal norleucine standard was included in every sample prior to hydrolysis in order to minimise among sample variability. Samples were subsequently dried under vacuum at room temperature. Dried samples were re-dissolved with 0.5 to 1 ml (depending on the concentration of the sample) of buffer solution (pH 2.2) prior to analysis and purified by filtration (0.65-µm Durapore filter; Millipore, Bedford, Massachusetts, USA). The AA analysis was performed on a Biochrom20 (Amersham-Pharmacia) ion-exchange AA auto analyser following the ninhydrine method of Spackman et al. (1958). A standard solution of 20 AAs was run for every 10 samples.

195 The detection limit was 10 pmol for all amino acids and 50 pmol for proline. The samples injected were always greater than the limit of detection for all amino acids considered. Analytical precision had a CV of 3.9%. Tryptophan, due to its degradation

under acid hydrolysis (Fountoulakis and Lahm, 1998), has not been considered in this study. Cysteine and methionine are other AAs that usually degrade easily during the hydrolysis process. Together with taurine they are the three AAs containing sulphur. Therefore their possible degradation was checked by analysing for elemental sulphur (EA 1108 CHNS-O Carlo Erba Instruments elemental analyser). This analysis showed that the AA sulphur accounted for most of the elemental sulphur indicating a good recovery of most sulphur-containing AAs. Also due to the acid hydrolysis, asparagine and glutamine, were measured together with aspartic acid and glutamic acid, respectively. AAs are expressed as relative mass percentage of TAA through out this paper. These units were chosen rather than absolute concentrations (e.g. mg AA / mg C or DW) since a description of the biochemical variability of the present species and study period showed that there was an important seasonal variability in the lipid and carbohydrate content (up to 50 % of their DW) (Ventura and Catalan, 2005), which were much greater than the changes in the AA composition. Using the relative units we focus on the variability in the AA composition rather than the factors affecting the gross biochemical composition. However, we provide the TAA concentration (in % dry weight) which allows for calculation of the absolute concentration also used in the literature.

In order to determine the relative importance of the free AA (FAA) pool with respect to proteins, we used the concentration of taurine as an indicator of the FAA pool. Taurine is the only AA which does not form proteins, and one of the main FAAs in crustaceans (Claybrook, 1983). A bibliography search of joint measurements of taurine and the concentration of FAAs was obtained from Ventura (2006), and these were found to relate linearly (Fig. 2). The good fit ($r^2 = 0.90$, $p < 0.001$) suggested that taurine concentration could be used as a predictor of the FAA pool. Therefore, we

estimated the relative proportion of the FAA by applying the regression formula of Figure 2 to the measured taurine concentration.

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

Statistical analysis

To test if the different species or the stages within each species could be distinguished based on their AA composition, we performed multiple discriminant analyses (MDA). The MDAs enabled us to determine which AAs were the most significant in discriminating between two or more groups (species and stages). We performed a first MDA (MDA1) with the various stages and other sources of intraspecific variation of the three different species as separate factors in one single analysis in order to find out if the interspecific variability was greater than the intraspecific variability. The sources of intraspecific variation of the AA composition evaluated consisted of gender, development stages (ontogenetic changes), differences between reproductive and non-reproductive females in the three species and also males in *Diaptomus*. Gender differences were only considered in the two copepod species, since *Daphnia* mainly reproduced asexually. Ontogenetic changes could only be tested in *Daphnia* and *Cyclops* due to the short life cycle of *Diaptomus*. Each replicate was entered separately in the analysis. The variable number of samples for each species or stage was due to the natural variability of their different seasonal occurrences. A different MDA was performed for each species (MDA2 for *Daphnia*, MDA3 for *Cyclops* and MDA4 for

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, Brucet 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 +

Methionine), nonessential amino acids (Glutamic acid, Aspartic acid, Alanine, Arginine, Glycine, Proline and Serine), trophic amino acids (Alanine, Asparagine + Aspartic acid, Glutamine+Glutamic acid, Isoleucine, Leucine, Proline and Valine) and source amino acids (Arginine, Glycine, Lysine, Serine, Threonine and Phenilalanine +Tyrosine). The average Euclidean distance between *Cyclops* and *Daphnia* using the different amino acid groups was compared with the $\Delta^{15}\text{N}$ of both species with linear regression analysis.

In order to determine which particular amino acids could be most imbalanced in *Daphnia* in comparison with the predator, *Cyclops*, we calculated the amino acid imbalance as follows (Conceição *et al.*, 2003):

$$AA_i = (\text{diet}AA_i - \text{consumer}AA_i) / (\text{consumer}AA_i) * 100$$

where $\text{diet}AA$ and $\text{consumer}AA$ are the contributions of a given AA (i) to the AA profiles of the consumer.

Differences in individual AAs were tested with one-way ANOVA analysis (Underwood, 1997) with species or stage as independent variable and the percentage of the AAs as the dependent variable. AA percentage data were previously normalised with arcsine root square transformation when required, and a test of homogeneity of variances was performed before ANOVA. To test if Euclidean distances of interspecies versus intraspecies comparison were significantly different we used one way ANOVA with the Euclidean distance between all the pairs of samples analysed as dependent variable and if the comparison was from among species comparison (three cases) or within species (3 more cases) as independent variables. We used Tukey's *post hoc* test for detecting significant differences among or within species. All statistical analyses were performed with SPSS V17.

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-

reproductive females that were identified as males; Fig. 4a and Table 3). The first axis of the MDA1 explained 47 % of the variance and mainly segregated the two copepods from the cladoceran. The AAs significantly correlated with this first axis were proline, serine and threonine, their relative concentrations being higher in *Daphnia*, and glycine and taurine, with higher relative concentrations in the two copepods (Table 3). The second axis explained 39 % of the variance and segregated *Diaptomus* from the other two species. *Diaptomus* had a higher alanine and serine relative concentration, while *Cyclops* and *Daphnia* had higher glycine concentration.

Intraspecific variation

Within each species, there were no differences in the relative proportion of TAA, protein AA (PAA), FAA, essential AA and nonessential AA pools between most of the different stages, with the exception of *Cyclops* males that had a lower relative concentration than the females in the lake during the same period (2.9 ± 0.1 and 1.6 ± 0.5 % dry weight of TAA for females and males, respectively; $F_{1,3} = 15.8$, $P = 0.028$) and *Daphnia* eggs that had higher TAA relative concentration than the first cohort females ($F_{1,8} = 2.8$, $P = 0.1$ Tukey *post-hoc* test $P = 0.013$) (Table 1). In contrast there was a decrease from non-reproducing females to reproducing females of FAA and essential AA pools of *Cyclops* ($F_{1,9} = 20.5$, $P = 0.001$ and $F_{1,9} = 7.2$, $P = 0.025$ respectively), a slight decrease in the females of *Daphnia* ($F_{1,8} = 2.8$, $P = 0.1$ and $F_{1,8} = 3.3$, $P = 0.1$ respectively) and in both males and females of *Diaptomus* ($F_{1,6} = 3.8$, $P = 0.1$ and $F_{1,6} = 2.8$, $P = 0.1$ respectively).

The MDA2 correctly classified 100 % of the samples of *Daphnia* (Fig. 4 and Table 3). The first axes summarised 74.9 % of the variance and separated the three stages analysed: first cohort females, second cohort females and eggs. The AAs most

strongly associated with the axes were proline and arginine, more abundant in the eggs (positive) side, and glycine and leucine, more abundant in second generation females (negative) side. The second axis explained the remaining 22.4 % of the variation and separated the second generation females and eggs (negative) side from the reproductive and non-reproductive first generation females (positive) side. The AAs most strongly associated with the axes were positively, threonine and glutamine / glutamic acid and negatively, arginine. Further comparison of individual AAs between reproducing and non-reproducing first cohort females with one way ANOVAs indicated that reproducing females had higher histidine, serine and cystine ($P = 0.003$, $P = 0.003$ $P = 0.007$ respectively) and lower phenylalanine ($P = 0.021$).

For *Cyclops*, results of the MDA3 analysis showed that 100 % of the cases were correctly classified (Table 3) (Fig. 4b). The first axes explained 78.2 % of the variance and mainly segregated the CIV-V copepodites, the reproductive females and males from the non-reproductive females and CII-III copepodites. The AAs most significantly associated with the first axis were glutamine / glutamic acid and phenylalanine in the non-reproductive females, males and copepodites CII-III (positive) side and serine at the negative side. The second axis explained 12.9 % of the variance and mainly separated copepodites CII-III from the rest, leucine and serine being correlated with copepodites CII-III and valine and glutamine / glutamic acid with the rest (Table 3). Further comparison of individual AAs between males and females during the months they coexisted in the lake (January and February) with one way ANOVAs confirmed that there were no statistical differences for any of the 18 AAs analysed and thus no gender differences in *Cyclops* in terms of their AA composition.

The MDA4 correctly classified all the samples of *Diaptomus* in one single significant axis (Fig. 4d and Table 3), showing that almost all the variability was

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370 associated with reproduction, while only negligible differences were related to gender.
The AAs that were selected by the forward selection procedure and that significantly explained this variation were asparagine and phenylalanine that were associated with the non-reproducing adults and threonine and serine with the reproducing adults (Table 3).

375 AAs related with *Cyclops* – *Daphnia* $\Delta^{15}N$

The $\Delta^{15}N$ of the predatory copepod *Cyclops* and those of its predominant prey, *Daphnia* were calculated for the whole seasonal cycle and compared with the Euclidean distance (dissimilarity) of the AA composition of the two species. Among the different AA groups, the dissimilarity calculated only with the essential or source AAs had the
380 highest correlation with the *Cyclops-Daphnia* $\Delta^{15}N$ ($r^2 = 0.931$, $P < 0.001$ and $r^2 = 0.835$, $P = 0.01$ for essential and source AAs respectively), while the correlation was weaker for trophic AAs ($r^2 = 0.67$, $P = 0.046$) or non-significant for the nonessential AAs ($r^2 = 0.461$, $P = 0.138$) (Fig. 5). To get a closer insight into the relative importance of both AA groupings we also calculated the dissimilarity of the AAs that were both
385 essential and trophic, essential and source, nonessential and trophic, and nonessential and source. Among these four groups, only those AAs that were both essential and source had a significant correlation ($r^2 = 0.872$, $P = 0.006$), suggesting that only this group of AAs were related with $\Delta^{15}N$.

In order to find out if a particular essential AA might be related to $\Delta^{15}N$ we
390 calculated the relative imbalance between *Cyclops* and *Daphnia*. Those AAs with a higher weight in explaining $\Delta^{15}N_{Cyclops-Daphnia}$ variance were expected to have 1) a negative imbalance in the consumer with respect to the diet and 2) $\Delta^{15}N_{Cyclops-Daphnia}$ should increase with decreasing relative concentration of this individual AA in the diet.

Among the different essential amino acids, only lysine had an average negative

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

Discussion

Interspecific variability

Our results confirm the hypothesis that interspecific variation in total AA composition was more pronounced than intraspecific variation. The overall AA interspecific distance was twice that of intraspecific distance (Fig. 3). In addition, although almost all samples were successfully classified to their corresponding stages for each species by the MDA1, the two first axes of the MDA1 basically distinguished among the three species (Fig. 4a). These results complement a previous extensive study focusing only on interspecific variation of alpine lake zooplankton in which most (85 %) of the adult females of 13 species were successfully classified to their respective species (Guisande et al. 2003). Therefore, it can be concluded that alpine freshwater zooplankton have a non-strict homeostasis (Sterner and Elser, 2002) in their AA composition. This also implies that if there are predatory interactions among them, it is likely that predators may encounter dietary imbalances in their requirements. While phosphorus limitation in freshwater primary consumers has been documented frequently (Hessen, 1992), predators have been reported to face frequently nitrogen limitation (White, 1993, Anderson et al., 2004). We suggest that in these organisms AAs might be a dietary limiting biochemical more often than is commonly assumed.

Intraspecific variability

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

MDA1 of Fig. 4a), although the relative importance of ontogeny, reproduction and gender was not the same for each species. *Cyclops* had a similar AA composition variability related with ontogeny and reproduction, while *Daphnia* had a higher variability related with ontogeny than reproduction. In the only previous study of ontogenetic intraspecific AA variability of both copepods and cladocerans, Brucet et al. (2005) also found an important effect of ontogeny in copepods, although they found little ontogenetic variability in *Daphnia*. In these latter species all the variability was associated with differences among populations belonging to different lakes. Our results show that when studying the intraspecific variability in one particular lake, the ontogenetic AA variability of copepods and cladocerans can be of comparable magnitude. Furthermore, we found different AAs responsible for the AA variation in each species (proline, arginine, glycine and leucine in *Cyclops*; glutamine / glutamic acid, phenylalanine and serine in *Daphnia*). In other species of copepods, Brucet et al (2005) found valine, tyrosine, proline and alanine as the AA with a higher weight in the ontogenetic variability of other copepod species. These results altogether suggest that the AAs involved in the ontogenetic variability of crustaceans are species specific. Although in this study we have not analysed the AA composition of nauplii, previous studies have shown they are the stage most similar to adults in terms of their composition (Brucet et al., 2005), and that they are very restrictive in their AA composition, not developing further if maternal diet is deficient (Laabir et al., 1999). Therefore, our results of lower intra- versus interspecific AA variability would most likely have been the same if the naupliar stage of copepods had been included.

Various studies have described the negative effects of an AA imbalanced diet for reproductive success in copepods (Cowgill et al., 1986, Guisande et al., 1999, Laabir et al., 1999, Guisande et al., 2000, Lacoste et al., 2001, Helland et al., 2003b). In this study

we show that, in addition, adult female copepods and cladocerans, and males of *Diaptomus* change their AA composition with reproduction. Contrary to the ontogenetic effects, two of the AAs involved with the changes related with reproduction were the same in the three species (the essential AA phenylalanine and the nonessential serine). Phenylalanine is an aromatic AA which acts as a precursor of tyrosine and other compounds involved in important physiological functions, while serine is involved in lipid biosynthesis and also a precursor of melanine. The results of this study suggest that they are relevant during the reproduction of crustaceans. In addition to the changes in individual AAs we also found a tendency for the FAA content to decrease from non-reproductive to reproductive females. This agrees with other studies showing that eggs contain a larger proportion of FAAs and that the AA composition of eggs is decisive for the successful development of the embryos (Guisande et al., 1999, Laabir et al., 1999, Helland et al., 2003a, Helland et al., 2003b).

Contrasting with the ontogenetic and reproduction effects, we found no changes in the AA composition associated with gender in the two copepod species. The only difference attributed to gender was a higher proportion of the FAA pool in *Cyclops* females compared to males. This contrasts with the equal proportion between both sexes in *Diaptomus*, which might reflect the different reproductive strategies described for freshwater calanoids and cyclopoids. This result also agrees with the findings of a parallel study (Ventura and Catalan 2005), in which *Diaptomus* males were found to follow similar elemental composition changes with reproduction as females, while the elemental content of *Cyclops* males did not change with time. This was attributed to the requirement of *Diaptomus* to mate every egg clutch, while *Cyclops* females can produce multiple clutches of eggs with only one impregnation. Therefore, the present results on 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
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5 homeostasis of males.
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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
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12 related with fasting, growth and diet quality in terms of protein quantity or quality
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15 475 (reviewed by Martínez del Río and Wolf, 2005). While most of these factors have been
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17 demonstrated either experimentally or through field studies, the effects of varying AA
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19 composition have scarcely been studied (Martínez del Río et al., 2009). In a previous
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21 study we showed that the carbon isotopic variation in *Cyclops* juveniles could be
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23 explained by fitting an isotopic growth model based on feeding entirely on *Daphnia*.
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26 480 However this was not the case for nitrogen isotopic variability. *Cyclops* nitrogen
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28 isotopic composition variation and the *Cyclops* to *Daphnia* $\Delta^{15}N$ were closely correlated
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30 to the dissimilarity in the AA composition between the two species (Ventura and
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32 Catalan, 2008).
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37 In this study we show that among the different AAs, only those that are both
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39 485 essential and source AAs had a high and significant correlation with $\Delta^{15}N$. Among
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41 these, only lysine had a negative imbalance in the consumer as it increased its relative
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43 concentration in the diet when the $\Delta^{15}N$ decreased. This suggests that the imbalance of
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45 only one amino acid can be responsible for a high metabolic turnover, which can
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47 ultimately be responsible for a higher $\Delta^{15}N$. Our results therefore are in agreement with
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50 490 the hypothesis that an AA deficiency can be responsible for a variable $\Delta^{15}N$ (Martínez
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52 del Río and Wolf, 2005).
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57 Although the recognition of a variable $\Delta^{15}N$ might seem a difficulty for a
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59 widespread use of ^{15}N as a trophic position estimator, it opens new perspectives for its
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interpretation, since it could be used as an indicator of the relative AA imbalance of an

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3 495 animal and as a consequence, an indicator of the relative degree of amino acid limitation
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5 and as a result of the overall nitrogen limitation. Although this might seem difficult to
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7 apply to general field studies, future studies using individual amino acid stable isotope
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9 composition of carbon or nitrogen (e.g. Larsen et al., 2009) might substantially help
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11 interpretations in stable isotope trophic ecology.
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17 18 19 **Acknowledgements**

20
21 We thank LL. Camarero, B. Claramunt, A. Miró, P. Renom, T. Buchaca and G. Cots for
22
23 their assistance in field, and R. Franco for laboratory assistance. The total AA analyses
24
25 were performed in the Serveis Científics Tècnics of the University of Barcelona. A.M.
26
27
28 505 Poulsen assisted in linguistic/editorial corrections. T. Buchaca, S. Pla, T. Larsen and
29
30 anonymous reviewers are acknowledged for their helpful comments on the manuscript.
31
32
33 This is a study of the Limnology Unit (CSIC-UB) and the group of Ecology of
34
35 Environmental Changes (2005SGR 00510). The research was supported by the research
36
37 project TRAZAS (CGL2004-02989) and the European Commission projects MOLAR
38
39
40 510 (ENV4-CT95-0007) and EUROLIMPACS (GOCE-CT-2003-505540). MV was
41
42 partially supported by a Ramon y Cajal grant.
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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 pulicaria* 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 pulicaria* nitrogen isotopic fractionation and the relative concentration of each essential amino acid in *D. pulicaria*.

Fig. 1. Seasonal changes in water temperature, chlorophyll *a*, the biomass of the three crustacean planktonic species of Lake Redon (*Cyclops abyssorum*, *Daphnia pulicaria* 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 pulicaria* ovigerous and non-

ovigerous females (first cohort females) and juveniles (second cohort females) to the total *D. pulcaria* biomass. *Daphnia* juveniles were those not reaching sexual maturity, which in this year corresponded to a size lower than 2 mm. For temperature and chlorophyll a, filled circles are the water column average values; dotted lines follow maximum and minimum values recorded on each sampling occasion.

Fig. 2. Relative concentration of taurine in relation to the free AA pool in eight different zooplankton species found in the literature (Cowey and Corner 1963a; Suyama et al. 1965; Jeffries 1969; Jeffries and Alzara 1970; Srinivasagam et al. 1971). DW stands for dry weight.

Fig. 3. Average Euclidean distance (with 10th and 25th percentiles) of the interspecific (shaded box plots) and intraspecific (white box plots) distance in the amino acid composition. Distances were computed among all samples analysed and therefore the intraspecific distances include both the distances between stages and within stages. Abbreviations stand for *Cyclops abyssorum* (C.a.), *Daphnia pulicaria* (D.p.) and *Diaptomus cyaneus* (D.c.). Letters above each box-plot denote significantly different distances from the one-way ANOVA Tukey post-hoc test ($P < 0.001$).

Fig. 4. Plots of the first two discriminant function scores of the total AA composition of (a) the various development stages of *Daphnia pulicaria* (open symbols), *Cyclops abyssorum* (shaded symbols) and *Diaptomus cyaneus* (black symbols) altogether, (b) those of *D. pulicaria* only, (c) *C. abyssorum* only and (d) *D. cyaneus* only. Symbols correspond to reproducing females (downward triangles), non-reproducing females (circles), reproducing males (upward triangles), non-reproducing males (diamonds), *C. abyssorum* copepodites CII and CIII (hexagons), *C. abyssorum* copepodites CIV and CV (stars), *D. pulicaria* young females (squares) and eggs (crossed circles). The AA scores and details of the analysis are summarised in Table 3.

Fig. 5. Comparison between (a) the *Cyclops abyssorum* – *Daphnia pulicaria* nitrogen isotopic fractionation ($\Delta^{15}\text{N}$) and the trophic amino acids average Euclidean distance (b) the source AAs average Euclidean distance, (c) the essential AAs average Euclidean distance, (d) the nonessential AAs average Euclidean distance, (e) the trophic and essential AAs average Euclidean distance, (f) the source and essential AAs average

Euclidean distance, (g) the trophic and non-essential AAs average Euclidean distance and (h) the source and nonessential AAs average Euclidean distance. The different AAs belonging to each group are described in Table 2.

Fig. 6. Average relative essential amino acid (AA) imbalance between *Cyclops abyssorum* and *Daphnia pulex* during a seasonal cycle. See text for calculation procedure. Results are expressed in dry weight percentage of the total AA composition. Error bars are standard deviations. White and black bars are source and trophic AAs respectively. Grey bars are those amino acids that have not been described to belong to any of the two categories. Asterisks denote significant negative correlation between the *C. abyssorum* – *D. pulex* nitrogen isotopic fractionation and the AA composition of *D. pulex* as described in Table 4. Amino acid abbreviations are arginine (ARG), histidine (HIS), taurine (TAU), methionine (MET), isoleucine (ILE), leucine (LEU), valine (VAL), phenylalanine (PHE), lysine (LYS) and threonine (THR).

Table 1

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

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

Figure 1

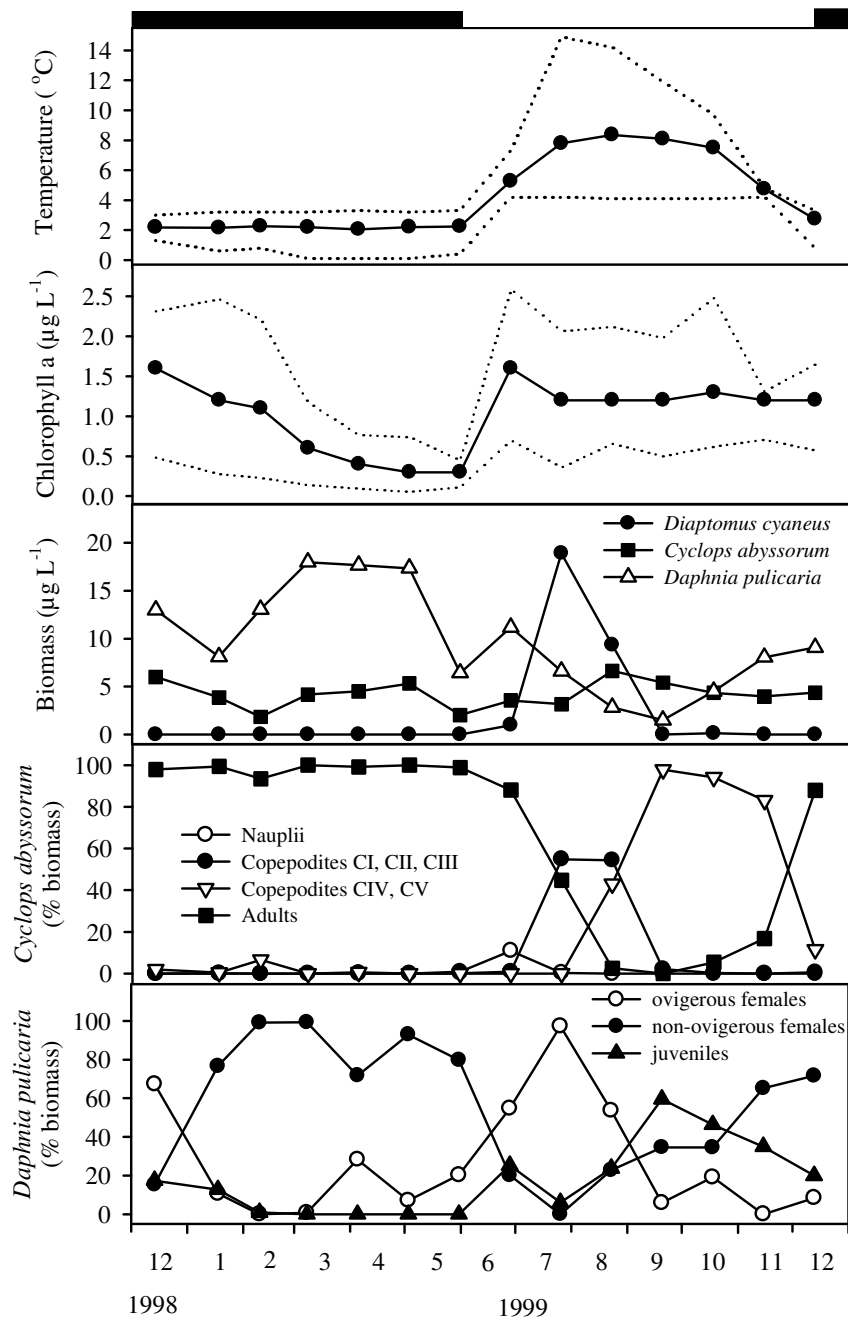


Figure 2

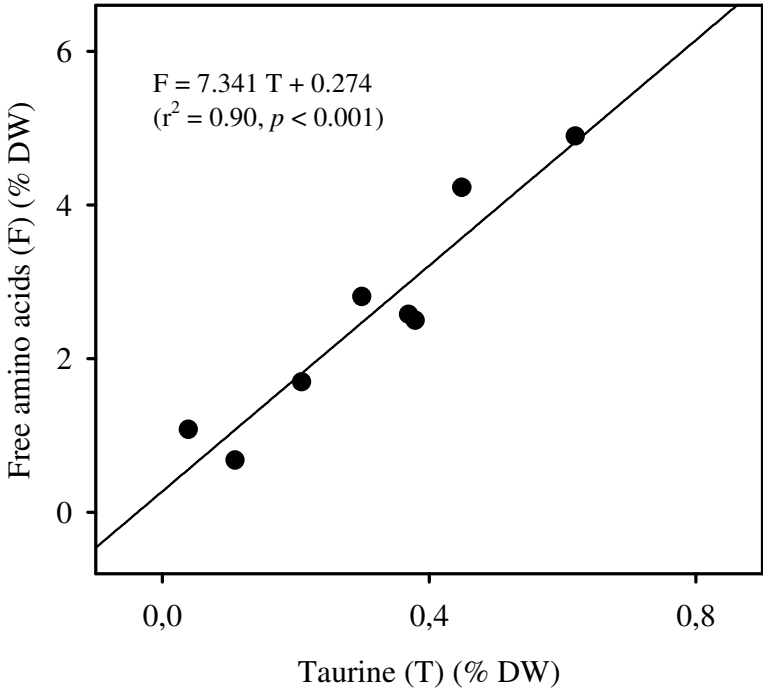


Figure 3

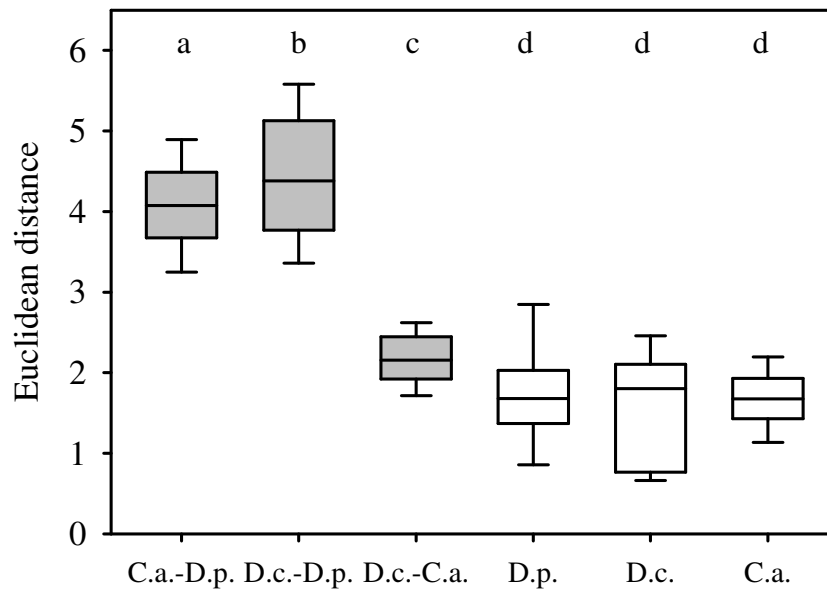


Figure 4

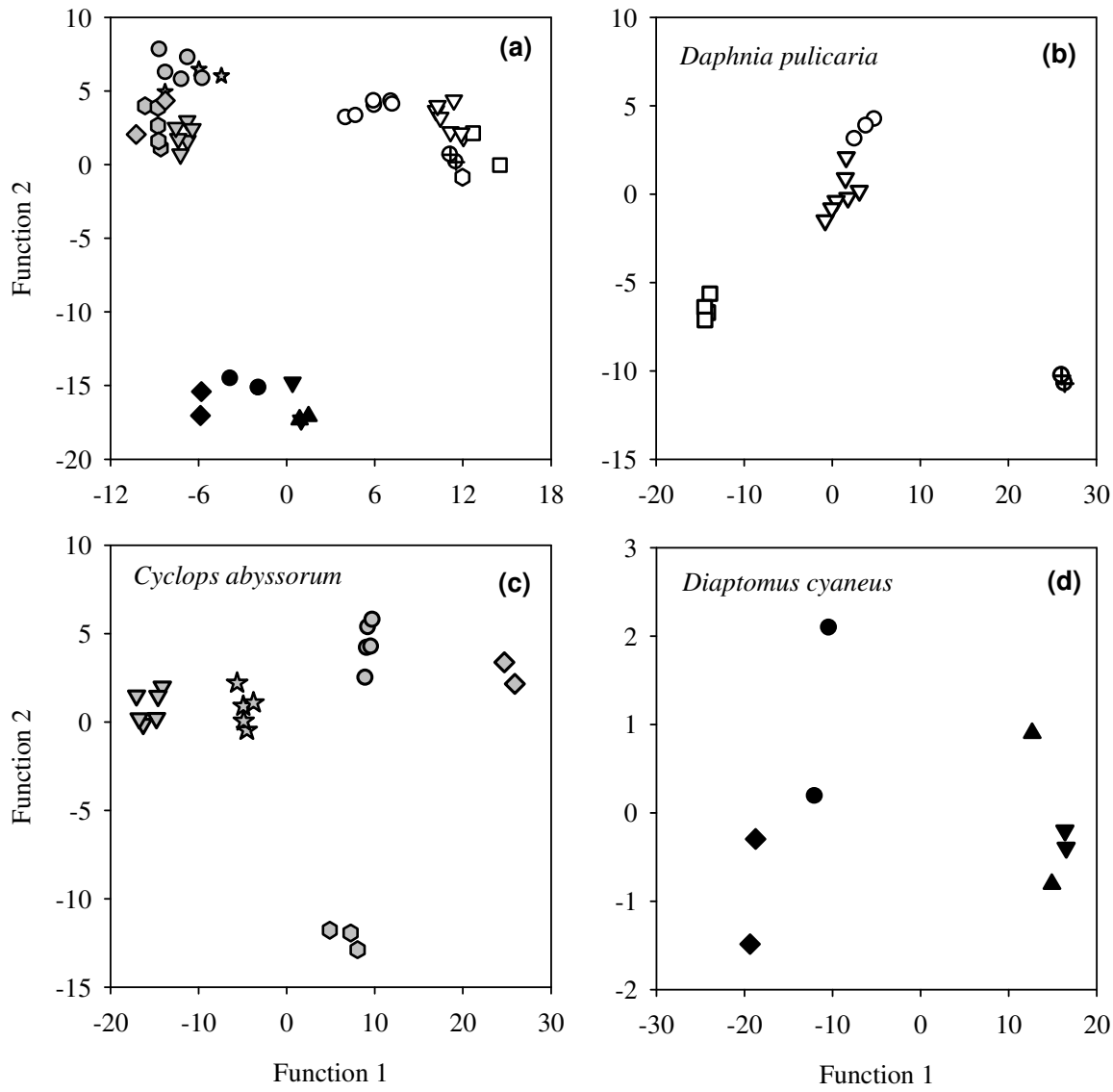


Figure 5

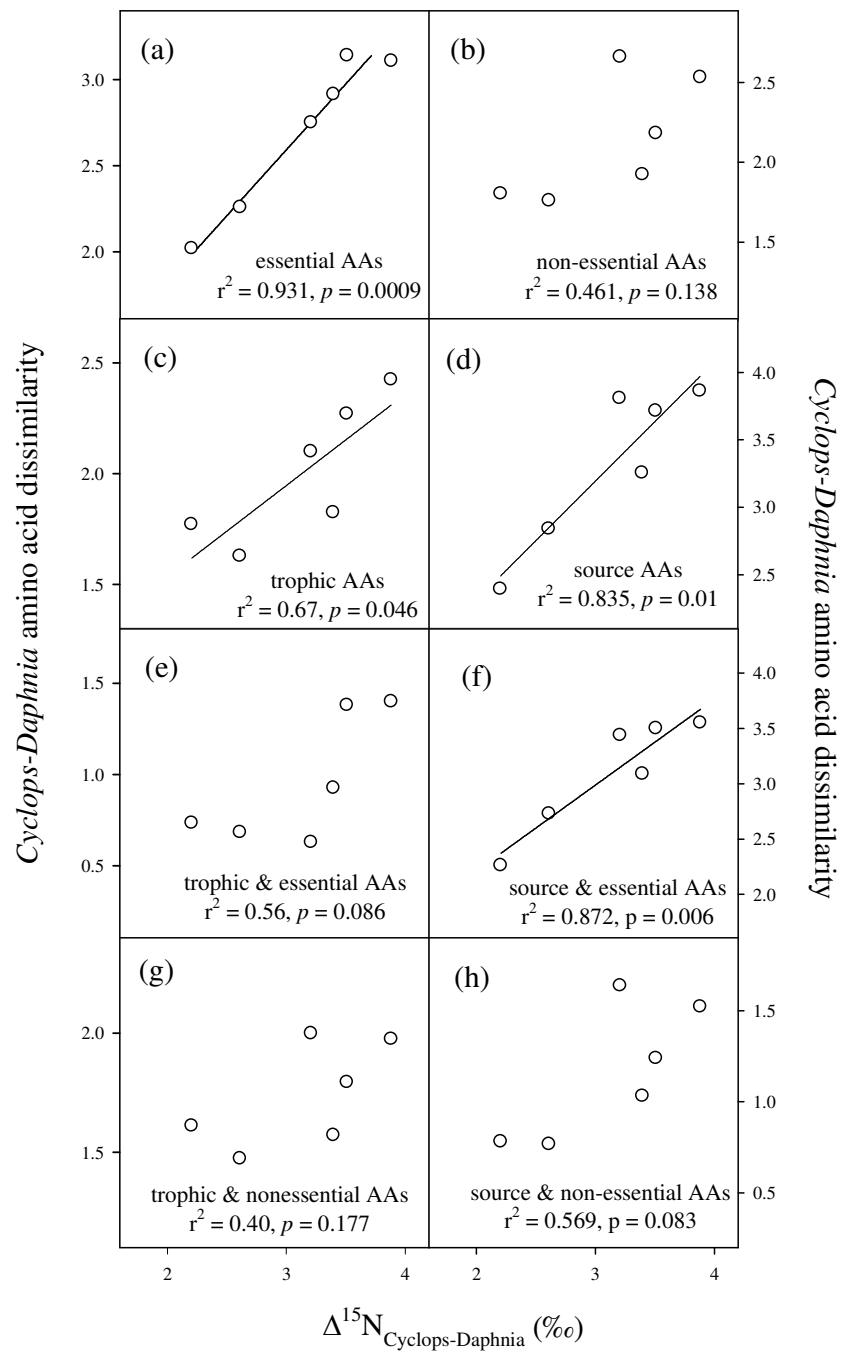


Figure 6

