Aquat Ecol (2010) 44:397–408
DOI 10.1007/s10452-009-9299-7

Effect of fasting on hypogean (*Niphargus stygius*) and epigean (*Gammarus fossarum*) amphipods: a laboratory study

Tadej Mezek · Tatjana Simčič · Michael T. Arts · Anton Brancelj

Received: 20 March 2009 / Accepted: 9 November 2009 / Published online: 28 November 2009
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Abstract Two amphipods, the hypogean *Niphargus stygius* and epigean *Gammarus fossarum*, were analyzed for fatty acid (FA) composition, electron transport system (ETS) activity and respiration (R) during a laboratory fasting experiment. In agreement with ETS and R measurements (and the ETS/R ratio), the hypogean *N. stygius* utilized FA more slowly than the epigean *G. fossarum*. Inter-specific differences in the utilization of certain FA during fasting were also revealed. While *N. stygius* tended to preserve all of its FA during the experimental fasting period, *G. fossarum* showed a tendency to utilize MUFA (monounsaturated FA) and SAFA (saturated FA) and preferentially retain PUFA (polyunsaturated FA). The significant correlations between ETS activity and composition of specific FA during fasting can be linked to R. During the fasting, both ETS activity and respiration rate of *G. fossarum* decreased, however, ETS/R ratio increased. In contrast, *N. stygius* did not show significant changes in these parameters. This is the first report, which connects ETS activity with changes in concentrations of specific FA during fasting. Such evolutionary adaptations of hypogean species enables them to better survive chronically low and/or discontinuous food supplies compared to epigean species, which live in environments where food shortages are much less frequent.

Keywords *Niphargus stygius* · ETS activity · Fasting · Fatty acids · *Gammarus fossarum* · ETS/R

T. Mezek (✉) · T. Simčič · A. Brancelj
Department of Freshwater and Terrestrial Ecosystem Research, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia
e-mail: tadej.mezek@gmail.com

M. T. Arts
National Water Research Institute, Environment Canada, Burlington L7R 4A6, Canada

Introduction

While they share some similarities, there are also many differences between groundwater and surface water ecosystems. Limited food supply, due to the lack of photoautotrophic primary producers, and sporadic allochthonous food inputs are two of the most important elements affecting the survival strategies of hypogean species. Therefore, hypogean species have evolved a number of adaptations, which allow them to successfully exploit the subterranean environment. The ability to sustain a low metabolic activity is one of the most important adaptations of cave animals to low and discontinuous food supplies (Hervant et al. 1997, 1999, 2001; Spicer 1998; Hervant and Renault 2002) and to alternately hypoxic and normoxic waters (Hervant et al. 1995, 1996, 1998; Mejía-Ortiz and López-Mejía 2005).

Oxygen consumption through the process of respiration (R) is a parameter that is commonly measured in metabolic studies including fasting

studies. However, oxygen consumption on its own offers limited insights into the strategies employed by organisms to deal with fasting conditions. Measurements of R can provide information about the intensity of actual metabolism under particular conditions, but the question remains as to what the measured energy demands mean with regard to the details of the organism's metabolic capacity.

Electron transport system (ETS) activity has been measured in order to estimate potential metabolic activity, i.e., the value of respiration rate that would occur if all enzymes involved in this process functioned maximally (Muskó et al. 1995). The ETS activity/respiration rate (ETS/R) ratio is a measure of the exploitation of the metabolic potential that is actually used for respiration (Martinez 1992). Previous studies have shown that this ratio differs between related species inhabiting habitats with different food concentrations (Simčič and Brancelj 1997, 2006; Simčič et al. 2005) and that it changes with changing food quality and quantity (Fanslow et al. 2001) and with fasting (Cammen et al. 1990).

Another estimate of an organism's physiological status and overall condition during fasting can be provided by measuring its lipids. Such data provide insights into the balance between catabolism and synthesis during periods of fasting culminating in starvation. Little is known about the overall rate of utilization of lipids and the effects on fatty acid (FA) composition in hypogean versus epigean species in the context of low and/or discontinuous food conditions; both of which can impose various degrees of fasting stress on an organism. Lipids have the highest energy density (39.4 J mg^{-1}) when compared to proteins (23.6 J mg^{-1}) or carbohydrates (17.2 J mg^{-1}) (Hagen and Auel 2001). Lipids also represent the major carbon reservoirs in most aquatic organisms. For example, triacylglycerols and phospholipids comprised 77 and, 65% of total carbon, respectively, of freshwater crustacean zooplankton (Ventura 2005). However, lipids not only function as energy reserves; but also affect other processes such as thermal isolation, membrane structural integrity and fluidity, chemical signaling and specific density of organisms (e.g. buoyancy control). Quantifying biogenic FA is gaining importance in ecological studies especially those related to determining the nutritional status of organisms and/or exploring food web dynamics (Arts and Wainman 1999; Arts et al. 2001, 2009; Stubling and Hagen 2003; Stubling et al. 2003; Trushenski et al. 2006; Budge et al. 2006; Hebert et al. 2006), including those specifically targeted toward the development of biomarkers (Dalsgaard et al. 2003; Budge et al. 2006). In this study, we measured ETS and R along with FA composition in order to reveal inter- and intra-specific differences between hypogean and epigean amphipods during a laboratory fasting experiment.

Membrane-associated processes contribute significantly to overall metabolism, and it is clear that the acyl composition of the lipids comprising membrane bilayers has a strong influence on metabolic activity (Hulbert 2003; Hulbert et al. 2006). Membrane bilayers in metabolically active systems show more PUFA and less monounsaturated fatty acids (MUFA) than metabolically less-active systems (Hulbert and Else 2000). In the vast majority of cases, animals (but see Tanaka et al. 2007) cannot de novo synthesize α-linoleic acid (ALA; 18:3n-3) and linolenic acid (LIN; 18:2n-6) so that these two FA are termed "essential fatty acids" (EFA); satisfying requirements for these EFA means obtaining them from the diet. In addition, most heterotrophs cannot biosynthesize long chain highly unsaturated fatty acids (HUFA) in sufficient quantities for optimal growth and performance (Arts et al. 2001; Kainz et al. 2004). Therefore, preferential utilization of nonessential FA while retaining EFA in unfavorable conditions (e.g. fasting) is of great importance for maintaining the cell's biochemical competency.

Fatty acids are tightly linked with many key physiological and biochemical processes at the cellular and organism levels (Hillgartner et al. 1995) while ETS activity evaluates the metabolic potential of cells and tissues and is tightly linked to the condition of the cells or tissues at the time of measurement. The condition of cells at the moment ETS activity is measured is, in turn, influenced by the FA composition of the cells (see above). However, very little is known about the changes in FA composition during fasting, especially for species inhabiting starkly contrasting environments (e.g. hypogean and epigean organisms). Simultaneous measurements of ETS, R and FA should offer new insights into the connections between these three variables. All three variables, to varying extents, reveal the current "state of the cell". Thus, simultaneously measuring these variables during an induced stress such as fasting should encourage a novel

understanding of how different biochemical indicators of organism's status interact.

We hypothesized that two organisms, inhabiting environments that differed greatly in food availability, should exhibit different survival strategies, in terms of FA, R and ETS activity, during fasting. Since ETS plays a key role in respiration energy production processes and, since esterified FA is the most common long-term form of energy deposition in aquatic organisms, the interaction between these systems should be well integrated with the survival strategies of organisms in specific environments. In addition, we propose that differential utilization of FA between hypogean and epigean species can be used to further characterize species-specific metabolism (or environment of origin) as well as the responses of different species to stress and food scarcity. For this study, two organisms from same order were selected. *Gammarus fossarum* (*Koch*), 1835 (Amphipoda) is an inhabitant of the well-oxygenated running water where food is not limited and *Niphargus stygius* (*Schiodte*), 1847 (Amphipoda) is a cave-dwelling (hypogean) species frequently faced with food shortages.

Methods

Animal collections and culture conditions

The amphipods *G. fossarum* and *stygius* were collected from two locations in south-western Slovenia; the Iščica River and Velika Pasica Cave, respectively. Sampling occurred on the same day in early spring 2008. The cave is a $\sim$100 m long horizontal gallery, which is divided into an entrance section (higher organic inputs) and an inner section (lower organic inputs). To minimize the possible effect of spatial differences, and thus lipid content of specimens, amphipods were collected from an a single 1 m^2 section of the river for *G. fossarum* and a $\sim$10 m^2 section of the inner part of the cave for *N. stygius*.

In both species, only males were sampled in order to exclude differences in FA composition that might result between males and females and between gravid and nongravid females. Both species were kept at a density of 1 amphipod/20 mL in small, custom made, glass tanks, in the dark and maintained at 10°C until analyzed. The water and the condition of the test animals were replaced/monitored on a weekly basis. The "original", i.e., nonfasting specimens of each species were analyzed 1 day after they were transferred to the laboratory. Amphipods ($n = 3$ for each species and time interval) were analyzed on Day 0, Day 7, Day 14, Day 28 and Day 42. Although *N. stygius* can survive fasting for >42 days, all the specimens of *G. fossarum* had died by Day 49.

Dry mass

Randomly chosen animals from the glass tanks were transferred into preweighed, lipid-free vials followed by 24 h lyophilization. Lyophilized samples were weighed again to obtain dry mass of each individual to the nearest 0.1 mg. The average dry weight of *N. stygius* was 27.1 $\pm$ 13.5 (mean $\pm$ SD) ($n = 15$) mg and for *G. fossarum* 20.4 $\pm$ 7.3 mg ($n = 15$), respectively.

Lipid extraction

Lipids were extracted twice, using a modified Folch procedure (Folch et al. 1956; Parrish 1985; Iverson et al. 2001; Honeycutt et al. 1995; Booij and Van den Berg 1994), by adding a mixture of dichloromethane:methanol (2:1 v/v) to each sample vial. Prior to extraction, 8 μg of methyl nonadecanoate (19:0 methyl ester) was added as an internal standard to each sample vial. After overnight extraction, a 0.9% NaCl solution was added to give a final ratio of dichloromethane:methanol:NaCl solution of 2:1:0.2 (v/v/v). The lower, dichloromethane phase was transferred with a pipette into a clean vial in preparation for methylation of the FA.

Methylation of fatty acids (one step hydrolysis and derivatization)

In order to obtain fatty acid methyl esters (FAME), the lipid extract was dried under a steady flux of nitrogen, re-dissolved in 0.5 mL of 3 M methanolic-HCl and incubated for 15 min at 60°C in a sealed vial (Von Elert and Stampfl 2000; Von Elert 2002). After the sample had cooled, FAME were extracted three times with 1 mL of hexane and pooled in a new vial, dried under a steady flow of nitrogen and re-dissolved in 0.5 mL of isooctane.

GC/MS analysis

FAME were analyzed by gas chromatograph coupled to a mass spectrometer (Agilent Technologies 6890N GC) equipped with a polar capillary column (Agilent Technologies; 60-m × 0.25-mm id × 0.15 μm DB-23 (part. no. 122–2361), an Agilent 7683B injector and a mass selective quadrupole detector (Agilent 5973N). Helium, at a constant pressure and temperature (~180 kPa at 33 cm/s at 50°C), was used as the carrier gas. Injection was done at an oven temperature of 50°C. After 1 min, the oven temperature was raised to 175°C at a rate of 25°C/min, then to 235°C at a rate of 4°C/min and held for 5 min. Transfer line temperature was 180°C. Retention time (RT) locking was used in order to obtain elutions of FA with very little retention time shifting of the peaks (methyl stearate was retention time locked to 14.0 min). Samples were injected in split and/or splitless mode depending on the requirements of individual samples. Samples were analyzed in both SIM and SCAN mode. Individual FAME components were identified by comparing retention times with those obtained for the PUFA standard (Supelco, 37 FAME mix, PUFA-2, catalog# 47015-U). Concentrations of individual FAME were quantified by comparison with 19:0, the internal standard (peak ratio method). Fatty acid composition (SAFA, MUFA, PUFA, ω3 and ω6) was calculated as a sum of each representative FA within each group.

Respiration

Respiration rate was estimated by the closed bottle method (Lampert 1984). Fifty-mL-ground-glass stoppered bottles were filled with synthetic and aerated water from the same, well-mixed, container. The experimental bottles received animals, while three bottles served as controls. All bottles were stoppered and kept at 10°C. After 20 h, the concentration of dissolved oxygen in the experimental and control bottles was measured with a 4-Channal fiber-optic oxygen meter (PreSens OXY-4, Presens GmbH, egensburg, Germany). The difference between the concentrations of dissolved oxygen of each experimental bottle at the start and the end of incubation, minus the mean value of control bottles, was taken as the amount of oxygen consumed by animals.

ETS activity

Electron transport system (ETS) activity was measured using the method originally proposed by Packard (1971) and improved by G.-Tóth (1999). Each preweighed animal was homogenized in 4 mL of homogenization buffer (0.1 M sodium phosphate buffer pH = 8.4; 75 μM $MgSO_4$; 0.15% (w/v) polyvinyl pyrrolidone; 0.2% (v/v) Triton-X-100) for 3 min using a glass potter (IKA). The homogenate was then sonicated for 20 sec (4710; Cole-Parmer) and centrifuged for 4 min at 0°C at 8,500×g (Sigma). Three 0.5 mL samples from each homogenate were incubated in 1.5 mL substrate solution (0.1 M sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% (v/v) Triton-X-100) with 0.5 mL 2.5 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution for 40 min at 10°C. Formazan production was determined spectrophotometrically from the absorbance of the sample at 490 nm wavelength against the blank within 10 min of stopping the reaction (WTW photoLabSpektral). ETS activity was calculated according to Kenner and Ahmed (1975).

Statistical analyses

Intra- and inter-specific differences between experimental intervals were obtained by using ANOVA and Tukey–Kramer HSD (Honestly Significantly Different) test. All statistical analyses were performed using JMP—SAS (ver. 7.0.1) and Sigmastat (ver. 3.5).

Results

Inter-specific differences in total FAME concentrations (Fig. 1a) between the two amphipod species were not statistically significant on either the first or the last day of the experiment (Day 0; $F = 1.67$, $df = 5$, $P = 0.266$; Day 42; $F = 2.96$, $df = 5$, $P = 0.160$). Similarly, total SAFA, MUFA and PUFA concentrations were also not significantly different between the two species on these 2 days. However, statistically significant differences between the species were detected in the amounts of ω3 and ω6 FA. While *G. fossarum* contained ~3 × higher amounts of ω3 FA compared to *N. stygius* on Day 0

($F = 24.40$, $df = 5$, $P = 0.007$) and Day 7 ($F = 30.85$, $df = 5$, $P = 0.005$), the difference narrowed to less than 2× on Day 42 ($F = 19.18$, $df = 5$, $P = 0.011$). The concentrations of ω6 FA in both species on Day 0 were not significantly different (ANOVA, $F = 0.091$, $df = 5$, $P = 0.777$); however, this changed as the fasting experiment progressed so that, on Day 42 the amount of ω6 FA of *N. stygius* was greater than that of to *G. Fossarum* (ANOVA, $F = 22.97$, $df = 5$, $P = 0.008$) (Fig. 1f).

The main inter-specific differences that we detected were with respect to utilization of certain FA groups during fasting. We were unable to reveal statistically significant change in the composition of groups of FA of *N. stygius* during the 42 days long fasting period (Fig. 1b, c, d, e, f). In contrast, *G. fossarum* showed statistically significant utilization of certain groups of FA from Day 7 onward (Fig. 1b, c, d, e, f).

The epigean *G. fossarum* starts preferentially utilizing MUFA (Fig. 1c) during fasting (significant differences detected from Day 7 onward) (Table 2b) followed by SAFA (significant difference from Day 14 onward) and ω3 (significant difference from Day 14 onward) and, finally, PUFA (significant changes detected only on Day 28).

During fasting, ETS activity (on a wet weight basis) ranged from 0.39 to 0.49 μL O_2 mg^{-1} h^{-1} for *N. stygius* and from 0.36 to 0.58 μL O_2 mg^{-1} h^{-1} for *G. fossarum* (Fig. 2a). ETS activity of *N. stygius* did not differ significantly from that of *G. fossarum* on any single experimental day ($P > 0.05$). The ETS activity differed significantly during the fasting period for *G. fossarum* (ANOVA, $P < 0.05$); the Tukey–Kramer *post hoc* test showed that lower ETS activities were observed after 28 days of fasting. The ETS activity of *N. stygius* did not change significantly during the 42 days of fasting (ANOVA, $P > 0.05$).

Respiration rates of *G. fossarum* differed significantly during 42 days of fasting (ANOVA, $P = 0.001$) with the values ranging from 0.07 μL O_2 mg^{-1} h^{-1} on Day 42 to 0.25 μL O_2 mg^{-1} h^{-1} on Day 7 (Fig. 2b). *N. stygius* had significantly lower respiration rates than *G. fossarum* on Day 0 ($F = 9.18$, $df = 5$, $P = 0.039$) and Day 7 ($F = 32.18$, $df = 5$, $P = 0.005$). Significantly decreased respiration rates were observed after 28 days of fasting in *G. fossarum*, while *N. stygius* did not show significant changes in respiration rates during 42 days of fasting (ANOVA, $P > 0.05$).

The ETS/R ratios of *G. fossarum* ranged from 1.8 to 6.0 and differed significantly during the fasting period (ANOVA, $P < 0.01$) (Fig. 2c). Higher ETS/R ratios for *G. fossarum* than *N. stygius* were measured on Day 7 ($F = 59.16$, $df = 5$, $P = 0.001$). ETS/R ratios of *N. stygius* did not differ significantly during the fasting period (ANOVA, $P > 0.05$).

Although a common molecular substrate does not exist between the FA examined here and ETS activity and respiration rate, correlation analyses were performed in an effort to gauge the potential interdependences among these variables. ETS activity and respiration rate of *G. fossarum* correlated positively with total FA and all individual FA, while the ETS/R ratio correlated negatively with SAFA, ω6 and ω3 FA (Table 1). In contrast, the ETS/R ratio of *N. stygius* correlated positively to all FA ($P < 0.05$), but ETS activity and respiration rate did not correlate significantly with any of the FA ($P > 0.05$), except SAFA.

Calculation of the relationship between pooled data (i.e. ETS and R for *N. stygius* and *G. fossarum* combined) and the FA composition (i.e. ω3, ω6, SAFA, MUFA, PUFA, Total FAME) showed a positive correlation between R and ω3 FA ($N = 30$, $P < 0.001$), and a negative correlation between ETS/R ratio and ω3 FA ($N = 30$, $P < 0.05$).

Discussion

Studies comparing the physiological responses of two phylogenetically related species under fasting conditions are rare. The two amphipod species examined here inhabit very different environments to which they have adapted by adopting markedly different physiological strategies to deal with uncertain food availability.

While both amphipods share similar levels of the major FA groups (SAFA, MUFA, PUFA), it is also clear that patterns of FA utilization are different between the two species and most probably related to specific adaptations to the local environment (Table 1a, b). We demonstrated that total FAME concentrations were not significantly different on Day 0 and by Day 42 for both species despite the fact that significant differences in the utilization of certain FA were recorded in the epigean amphipod *G. fossarum* (Fig. 1a). The overall loss of total FAME was greater

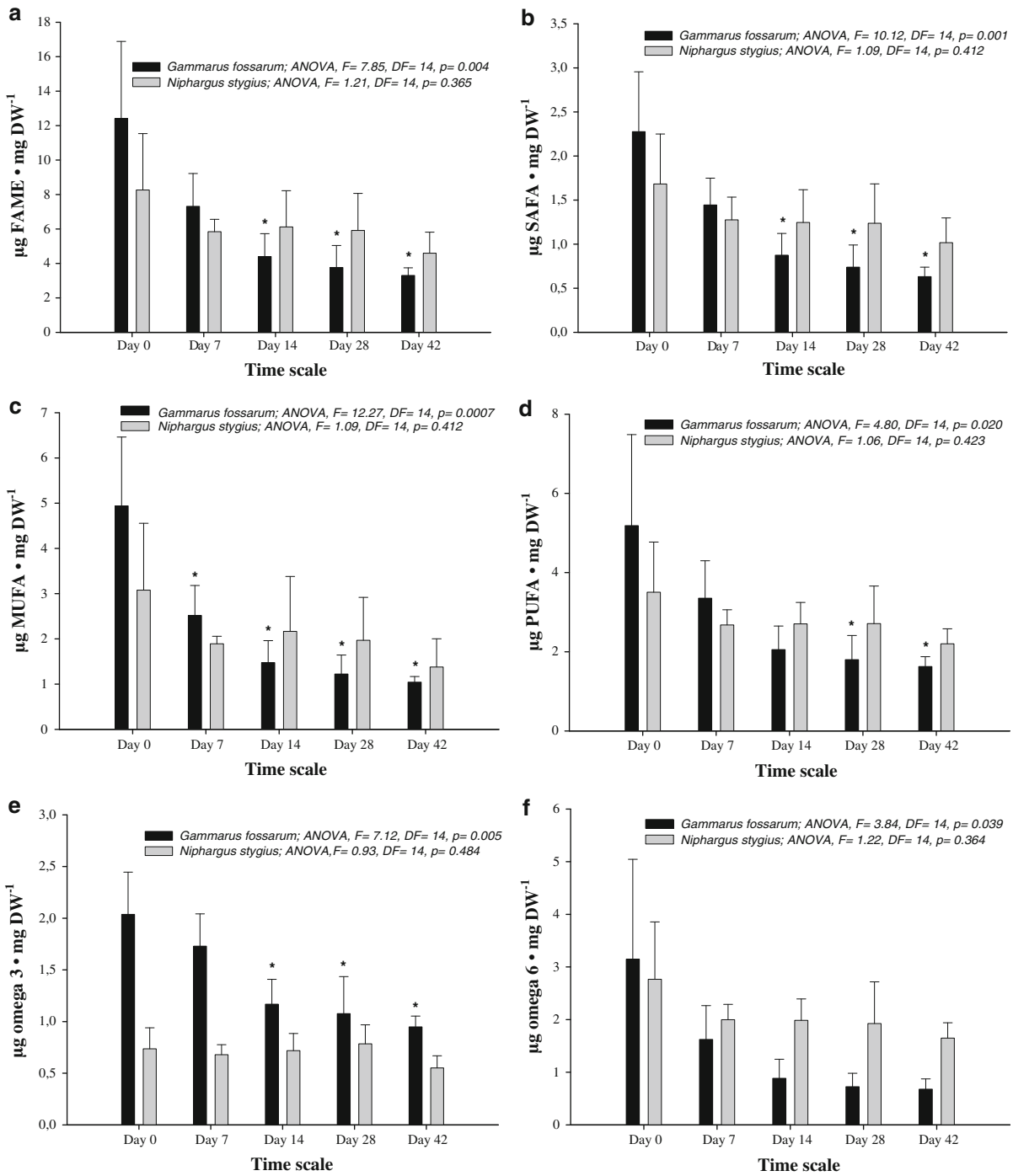

Fig. 1 Fatty acid distribution of *Niphargus stygius* and *Gammarus fossarum* during fasting experiment; Total fatty acid (**a**), Saturated fatty acid (SAFA) (**b**), Monounsaturated fatty acid (MUFA) (**c**), Polyunsaturated fatty acid (PUFA) (**d**), omega 3 fatty acids (**e**) and omega 6 fatty acids (**f**). *Vertical lines* represent standard deviations. Results of Tukey–Kramer HSD test are presented with (*) above *vertical lines* if $P < 0.05$

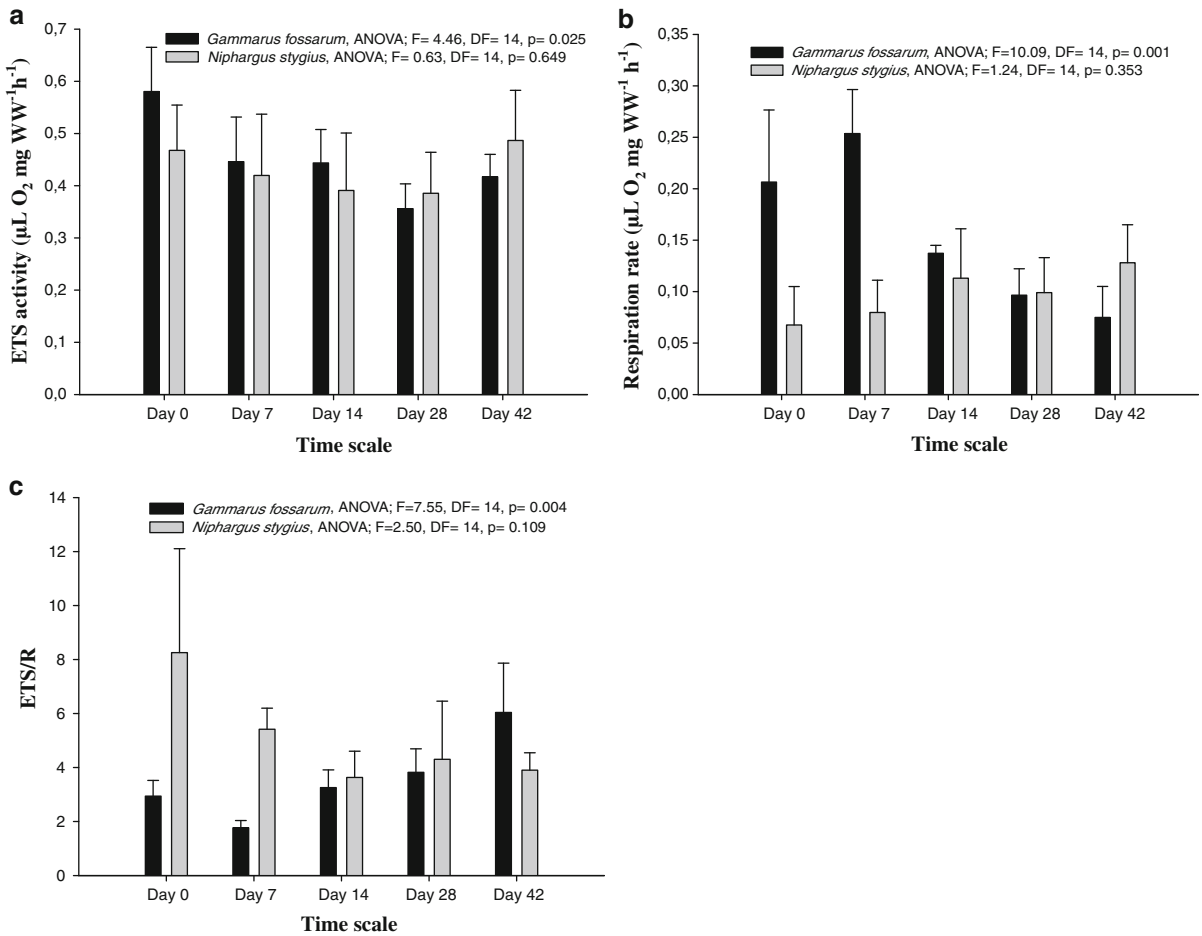

Fig. 2 Electron transport system (ETS) activities, respiration rates and ETS/R ratios of *Niphargus stygius* and *Gammarus fossarum* during fasting experiment; **a** ETS activity, **b** Respiration measurements, **c** ETS/R ratio. *Vertical lines* represent standard deviations. Results of Tukey–Kramer HSD test are presented with (*) above *vertical lines* if $P < 0.05$

Table 1 Correlations between ETS activity, respiration rate and ETS/R ratio, and saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids PUFA, Total FA, $\omega 6$ and $\omega 3$ FA of *Niphargus stygius* and *Gammarus fossarum* during fasting experiment

	ETS activity		Respiration rate		ETS/R ratio	
	Niphargus	*Gammarus*	*Niphargus*	*Gammarus*	*Niphargus*	*Gammarus*
SAFA	NS	+	−	+	+	−
MUFA	NS	+	NS	+	+	NS
PUFA	NS	+	NS	+	+	NS
Total	NS	+	NS	+	+	NS
$\omega 6$	NS	+	NS	+	+	−
$\omega 3$	NS	+	NS	+	+	−

Symbol + indicates positive correlation, $P < 0.05$; − negative correlation, $P < 0.05$; NS- no significance, $P > 0.05$; $N = 15$

in *G. fossarum* compared to *N. stygius* (Table 1a, b). Inter-specific differences in the amounts of SAFA, MUFA and PUFA were also not significantly different between the two species in any of the time intervals. This is partly due to the relatively lower total FA content in *N. stygius* compared to *G. fossarum* on Day 0. As *G. fossarum* utilized certain FA by Day 42, its total FA content approached the FA content of *N. stygius*. Also, test animals were taken from the field and immediately analyzed—the experiment started within 24 h of animal collection. We have no data on gut contents, nor on when specific individuals fed for the last time before being caught, which might explain the relatively high standard deviation in total FA on Day 0 (Fig. 1a). A higher number of replicates would probably have lowered intra-specific variability within the experimental interval; however, the abundance and density of *N. stygius* were rather low preventing us from obtaining a higher number of replicates. This was not the case for the epigean amphipod; however, we opted to maintain a balanced experimental design and therefore collected similar numbers of animals of each species.

Inter-specific differences were observed in the relative concentrations of ω3 and ω6 FA on Day 0 between the two species suggesting that the two amphipods access different food items in their natural environment. While *G. fossarum* feeds on organic detritus and decaying leaves in an environment where primary producers are present (i.e. where the majority of essential ω3 FA originate; Gladyshev et al. 2009), *N. stygius* feeds, only occasionally, mainly by preying and scavenging material brought to their habitat most often after floods and in an environment where algal primary producers are generally absent and thus where overall ω3 FA availability is likely lower. Since our fasting experiment started early in the spring, it is also possible that higher ω3 FA values in *G. fossarum* arise because of the connection between cold water temperatures and higher membrane PUFA concentrations as a result of the general need of poikilothermic organisms to maintain sufficient fluidity in their cell membranes (Arts and Kohler 2009). Conversely, the overall lower ω3 FA concentrations in *N. stygius* may have been the result of lower water flow through the cave at this time. Overall, our results confirm that the epigean amphipod *G. fossarum* utilizes FA at a higher rate than its hypogean relative *N. stygius* and that PUFA are the most retained class of FA in agreement with findings from other studies (Kainz et al. 2004; Schlechtriem et al. 2006; Arts et al., 2009). This finding is supported by the respiration rate measurements, which were found to be lower in *N. stygius* compared to *G. fossarum* (Fig. 2b) as well as the ETS/R ratio (Fig. 2c), which was higher in *N. stygius*.

The relatively constant food supply with which *G. fossarum* is faced with, when compared to *N. stygius* for which food shortages are common, could lead us to hypothesize that *N. stygius* might employ a strategy of storing a higher amount of lipids in order to survive periods of food shortage. Our results, however, did not support this hypothesis but suggest instead that the main difference between the two species lies in the utilization of different FA composition and not in the total amount of FA (which is correlated to energy reserve levels). Thus, while both species share similar amounts of total energy reserves (total FAME) (Fig. 1a) on Day 0, a significant drop of these energy reserves during fasting was recorded in *G. fossarum* but not in *N. stygius.*

Although it has been reported that subterranean crustaceans can survive fasting for periods well in excess of a year (Gibert and Mathieu 1980; Mathieu and Gibert 1980), our experiment with *N. stygius* and *G. fossarum* lasted only for 42 days. Although we have no data on the ultimate ability of *N. stygius* to survive when exposed to fasting (all of the specimens in our experiments survived to Day 42), none of the specimens of *G. fossarum* survived past Day 49.

Invertebrates utilize certain classes of biomolecules first when faced with fasting stress, for example, preserving PUFA as long as possible (Schlechtriem et al. 2006, 2008). This general observation is in agreement with our finding that *G. fossarum* starts utilizing SAFA and MUFA first to fuel metabolism (Fig. 1b, c; Table 2a, b) and preserves PUFA (Fig. 1d); a significant drop of PUFA was only recorded from Day 28 onward. Because of their key role in maintaining membrane and immune system competency, conserving PUFA during periods of stress or/and fluctuating food supply is of great ecological importance for an organism's survival. In addition, it has been shown that PUFA (and especially DHA) are retained during fasting with different temperature-dependant retention efficiencies (Schlechtriem et al. 2006). It has been demonstrated

Table 2 Mean values of the individual FA class with the corresponding calculated losses

	Day 0 Mean ± SD	Overall loss from Day 0			
		Day 7	Day 14	Day 28	Day 42
(a) *Niphargus stygius* (μg mg DW)					
ω3	0.73 ± 0.20	−0.05 ± 0.10	−0.01 ± 0.03	0.05 ± 0.02	−0.18 ± 0.08
		(7 ± 13%)	(1 ± 4%)	(7 ± 3%)	(24 ± 11%)
ω6	2.7 ± 1.09	−0.77 ± 0.79	−0.78 ± 0.68	−0.84 ± 0.29	−1.11 ± 0.79
		(28 ± 29%)	(28 ± 25%)	(31 ± 10%)	(41 ± 29%)
SAFA	1.68 ± 0.56	−0.40 ± 0.30	−0.43 ± 0.19	−0.44 ± 0.12	−0.66 ± 0.28
		(23 ± 18%)	(25 ± 11%)	(26 ± 6%)	(39 ± 16%)
MUFA	3.07 ± 1.47	−1.18 ± 1.31	−0.91 ± 0.26	−1.10 ± 0.53	−1.70 ± 0.85
		(38 ± 42%)	(29 ± 8%)	(36 ± 17%)	(55 ± 27%)
PUFA	3.50 ± 1.26	−0.82 ± 0.88	−0.79 ± 0.72	−0.79 ± 0.31	−1.30 ± 0.88
		(23 ± 25%)	(22 ± 20%)	(22 ± 9%)	(37 ± 25%)
Total FAME	8.26 ± 3.27	−2.41 ± 2.55	−2.14 ± 1.16	−2.34 ± 1.12	−3.66 ± 2.05
		(29 ± 30%)	(25 ± 14%)	(28 ± 13%)	(44 ± 24%)
(b) *Gammarus fossarum* (μg mg DW)					
ω3	2.03 ± 0.41	−0.31 ± 0.09	−0.87 ± 0.16	0.96 ± 0.05	−1.08 ± 0.09
		(15 ± 4%)	(42 ± 8%)	(47 ± 2%)	(53 ± 2%)
ω6	3.15 ± 1.89	−1.52 ± 1.25	−2.26 ± 1.53	−2.42 ± 1.64	−2.46 ± 1.70
		(48 ± 40%)	(71 ± 48%)	(76 ± 52%)	(78 ± 54%)
SAFA	2.27 ± 0.67	−0.83 ± 0.37	−1.40 ± 0.43	−1.53 ± 0.42	−1.64 ± 0.57
		(36 ± 16%)	(61 ± 19%)	(67 ± 18%)	(72 ± 25%)
MUFA	4.94 ± 1.52	−2.42 ± 0.86	−3.46 ± 1.03	−3.71 ± 1.10	−3.89 ± 1.39
		(48 ± 17%)	(70 ± 21%)	(75 ± 22%)	(78 ± 28%)
PUFA	5.18 ± 2.29	−1.83 ± 1.35	−3.13 ± 1.70	−3.38 ± 1.69	−3.55 ± 2.05
		(35 ± 26%)	(60 ± 33%)	(65 ± 32%)	(68 ± 40%)
Total FAME	12.40 ± 4.48	−5.08 ± 2.58	−7.99 ± 3.16	−8.63 ± 3.20	−9.09 ± 4.04
		(40 ± 21%)	(64 ± 25%)	(69 ± 25%)	(73 ± 32%)

that DHA retention efficiency is higher at lower temperatures (Olsen and Skjervold 1991); however, we were unable to reveal that with the current survey as we were comparing two contrasting habitats and not the same species adapted to varying seasonal temperatures.

The lower respiration rate in *N. stygius* compared to *G. fossarum* is in accordance with published reports that hypogean animals have lower metabolic activity than epigean ones (Hervant et al. 1997; Hervant et al. 1998; Spicer 1998; Hervant and Renault 2002; Simčič et al. 2005). Lower metabolic activity is one of the most important adaptations in subterranean environments to low and discontinuous food supplies and to alternately hypoxic and normoxic waters. Moreover, Hervant et al. (1999) reported that the metabolic response to food deprivation was monophasic in *G. fossarum,* showing an immediate, linear and large decline in all of the energy reserves including lipids. In contrast, two hypogean species—*Niphargus rhenorhodanensis* and *N. virei*—displayed successive periods of glucidic, proteo-glucidic and lipidic-dominant catabolism during food deprivation.

Measurements of ETS activity showed that *N. stygius* and *G. fossarum* had similar metabolic potential (Fig. 2a). This finding is in agreement with that of Simčič et al. (2005) who reported that hypogean animals possess a relatively high metabolic potential, which can be exploited for energy production immediately

following a pulse in the food supply. When food is available after prolonged fasting, it is ecologically very advantageous for organisms to quickly restore energy reserves that were depleted during previous food shortages (Hervant and Renault 2002). Previous comparative studies on the metabolic responses of hypogean and epigean animals to long-term starvation and the subsequent resumption of feeding (Hervant et al. 1999, 2001; Hervant and Renault 2002), and to hypoxia and subsequent recovery (Hervant et al. 1995, 1996), reveal that hypogean species possess faster and more efficient assimilation mechanisms for available nutrients during recovery from starvation and, in the case of hypoxia, they replenish their ATP levels faster. The high ETS/R ratio of *N. stygius* on Day 0 and Day 7 indicates low exploitation of metabolic potential and is one of the characteristics of hypogean animals (Simčič et al. 2005). In organisms with high ETS/R ratios, the capacity to rapidly increase metabolism is maintained (Fanslow et al. 2001).

The significant decrease in ETS activity of *G. fossarum* during starvation is in agreement with the findings of García-Esquivel et al. (2002) who reported that starvation resulted in a significant decrease in enzymatic activity. Lower respiration rate in *G. fossarum* after 28 days of fasting, observed in the present study, was also reported by Hervant et al. (1997). Contrary to *G. fossarum,* metabolic potential and respiration rate of *N. stygius* did not change significantly during the fasting experiment. The lower utilization rate of FA per unit time in *N. stygius* compared with *G. fossarum* during fasting is in agreement with the ETS and R measurements further confirming the lower exploitation of energy reserves of *N. stygius*.

Total FA as well as the FA composition correlates significantly with ETS activity and R in *G. fossarum* (Table 1), although the connection between the two variables is not straightforward because there is no common molecular substrate between ETS and FA synthesis. However, the significant decrease in ETS activity and respiration rate with decreasing amount of FA during fasting in *G. fossarum* is in agreement with the key role that FA play in membrane-associated processes, such as ETS activity and R. However, with the exception of R and SAFA, ETS activity and R of *N. stygius* did not correlate significantly with total FA or FA composition, except respiration rate and SAFA.

Although both respiration rate and ETS activity of *G. fossarum* decreased during fasting, the ETS/R ratio increased (Fig. 2c). The reason is a different response of both parameters to fasting. García-Esquivel et al. (2002) found an exponential reduction in postlarval respiration rate and a liner decrease in enzyme activity during fasting of *Crassostrea gigas* postlarvae. Cammen et al. (1990) also reported that R of *Nereis virens* (Sars) and *Corophium volutator* (Pallas) declined during fasting, but ETS activity remained relatively constant. Therefore, the increased ETS/R ratio of *G. fossarum* means a faster decrease in R compared to ETS activity during the 42 days fasting period. ETS activity is, however, a direct enzymatic process, depending on the concentration (Bamstedt 1980) and characteristics (Packard 1971) of the enzymes, whereas R is a complex physiological process. It means that R is also influenced by the intact intracellular environment, substrate concentrations and structure and properties of intact lipid membranes (Withers 1992). Thus, the negative correlation between ETS/R ratio and some FA composition (i.e. SAFA, ω6 FA, ω3 FA) could indicate their greater influence on R than on ETS activity. Moreover, the positive correlation between pooled R data of both species and ω3 FA likely reflected a key role of ω3 FA on the respiration intensity of amphipods. This agrees with observations made by Hulbert (2003) and Hulbert and Else (2000) that high PUFA and especially DHA contents are normally associated with increased activity of membrane-associated processes. Moreover, Spicer (1998) reported that both a genetic and an environmental component contribute to the reduced metabolism characteristics of hypogean animals. Therefore, it seems that the ω3 FA content could be one of the factors, which define different standards of metabolic activity in both species.

Contrary to *G. fossarum*, the ETS/R ratio of *N. stygius* correlated positively with total FA and FA composition. This means that the temporal change in FA content during fasting had a greater influence on metabolic potential than on actual respiration rate. This trend leads us to assume that during long-term fasting (>50 days) the utilization of lipids as energy reserves would eventually cause a decrease in both ETS activity and respiration rate in *N. stygius*, but ETS/R ratio would still be significantly lower at the end of a more sustained period of fasting compared to

the ratio of *N. stygius* in their natural habitat (Simčič et al. 2005). Under long-term fasting conditions, we would expect animals to eventually decrease their metabolic potential to a large degree to minimize energy demands for maintaining basal metabolism. Efficient use of metabolic potential would be an advantage in such extremely unfavorable conditions because comparatively higher amounts of ATP should be produced with the existing enzyme complexes by hypogean compared with epigean animals. Nevertheless, long-term fasting experiments (>200 days) should be preformed to test this assumption.

We concluded that both species show different responses to fasting. Although *N. stygius* and *G. fossarum* have similar metabolic potentials, the former species has a lower respiration rate, and it utilizes FA at a much slower rate. Moreover, the results of the present study reveal a lower ω3 FA content in *N. stygius* than in *G. fossarum* at the beginning of the fasting experiment. This could be one of the explanations for the relatively low standard metabolic rate in the hypogean amphipod too. Such adaptations enable *N. stygius* to survive in low and discontinuous food supplies on a longer term basis than *G. fossarum,* which live in food-rich environments or whereas food shortages are less likely to occur.

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