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Famine and feast in a common freshwater calanoid: Effects of diet and temperature on fatty acid dynamics of *Eudiaptomus gracilis*

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Abstract

We investigated the effects of temperature (4°C, 8°C, and 12°C) on structural and storage dynamics, as measured by changes in fatty acids (FA) associated with cell membrane phospholipids (PL) and triacylglycerols (TAG), respectively, as well as on body weight and survival of a freshwater calanoid copepod (*Eudiaptomus gracilis*) during fasting (10 d) and refeeding (10 d) with two algae of differing nutritional quality (*Cryptomonas ozolinii* and *Scenedesmus obliquus*). Fasting led to 50% loss in body weight, a near total depletion of TAG, and a drastic decrease of the polyunsaturated FA (PUFA) in TAG and PL, indicating their preferential utilization and alterations in membrane function, respectively. Higher temperatures accelerated the decrease of body weight and of PUFA in PL and TAG, and decreased survival. After 10 d of refeeding, copepods partially recovered their initial lipid stores and cell membrane composition. The effects of food quality were temperature dependent: *Cryptomonas* promoted better recovery (i.e., return to or close to the levels at the beginning of the experiment) of both body weight and TAG at only the two higher temperatures (8°C and 12°C), whereas no recovery was observed at 4°C. Higher temperatures and refeeding on *Cryptomonas* also had a positive, but minor, influence on the recovery of membrane FA composition. Survival differed among treatments but was lowest at the intermediate temperature (8°C) for both diets. We conclude that temperature changes on the order of 4–8°C significantly influence TAG and PL during fasting periods and interact with food quality to determine the extent of recovery in copepod lipids.

Thermal and food spatiotemporal gradients are among the main factors that shape—often in concert—zooplankton behavior, life history traits, and species assemblages (Threlkeld 1976; Kirk 1997; Lampert et al. 2003). Zooplankton are often food limited (quantity and/or quality) and sometimes experience periods of fasting (Tessier et al. 1983; Lampert and Muck 1985). Fasting in zooplankton may arise in periods of low primary productivity and/or overgrazing. Zooplankton may also be food limited in highly productive systems, e.g., when food particles are inedible or digestion resistant (Gliwicz and Siedlar 1980; DeMott et al. 2001; Ortega-Mayagoitia et al. 2011). In addition to fluctuations in food availability and nutritional quality, zooplankton must deal with spatially and temporally variable thermal environments. For ectotherms such as zooplankton, temperature strongly affects metabolic rates (Arts et al. 1993; Gillooly et al. 2001) and can therefore influence the rate at which energy stores are used (e.g., during a period of resource limitation or fasting) or restored (during refeeding).

Nutritional stress and temperature have a direct bearing on lipid dynamics in copepods, as for many animals. During periods of sufficient food, copepods store fatty acids (FA) as triacylglycerols (TAG) and/or wax esters and use them as primary energy sources during periods of food deprivation (Adams 1998; Lee et al. 2006). The current knowledge on how temperature, fasting, and food quality affect lipid metabolism in freshwater zooplankton is limited and mostly focused on cladocerans (Bychek et al. 2005; Schlechtriem et al. 2006). Copepods often comprise much of the entire zooplankton biomass in lakes (Arts et al. 1993; Kainz et al. 2004; Persson and Vrede 2006), but basic knowledge concerning how quickly and which TAGFA are preferentially utilized during fasting, how temperature influences TAGFA utilization, or how diet quality and temperature influence TAGFA deposition when feeding resumes is missing. Preferential utilization of specific FA has been observed in mammals, fish, and crustaceans, indicating that, in addition to mass fraction of TAG, their FA composition might also determine their quality as metabolic fuel (Raclot 2003; Hansen et al. 2008; Pasquevich et al. 2011). Also understudied in copepods, temperature seems to influence the composition of TAG of certain invertebrates (Van Dooremalen and Ellers 2010) and could consequently influence which FA are preferentially used during fasting and preferentially incorporated when food is abundant.

FA, as components of complex lipids, serve not only as metabolic fuel, but also as structural components of cell membranes (Arts and Kohler 2009) and as precursors of signaling molecules (Sardesai 1992). There is mounting evidence that the FA composition of cell membrane

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phospholipids (PL), which are important building blocks of cell membranes, has a major influence on the activity of many membrane-bound proteins, for example, by altering the leakiness of membranes, which ultimately affects metabolism (Hulbert and Else 2000). Hence, organisms modulate cell membrane FA composition to adjust metabolic rates to ambient conditions. Although the membrane FA compositional adaptation to temperature has been described in copepods (i.e., homeoviscous adaptation; Farkas 1979), the implications of nutritional stress for cell membrane lipids remain unknown for zooplankton and poorly studied in animals in general.

In this study, we examined the influence of water temperature and food quality on TAG, PL, and their FA (TAGFA and membrane phospholipid FA (PLFA), respectively) trajectories during 10 d of fasting and 10 d of subsequent refeeding in the copepod *Eudiaptomus gracilis*, the most common calanoid copepod species in European lakes (Kiefer 1978). We tested the null hypotheses that during fasting, all TAGFA are preferentially and equally used by *Eudiaptomus* and that cell membrane competency is maintained so that the compositions of TAGFA and PLFA remain unaffected (hypothesis 1). Moreover, we tested the null hypothesis (hypothesis 2) that, during fasting, the mobilization of TAGFA, as well as the TAGFA and PLFA compositions after 10 d, is not affected by temperature. For the subsequent refeeding period we used either *Cryptomonas ozolinii* Skuja, 1939 (high nutritional quality; Santer 1994; Von Elert and Stampfl 2000) or *Scenedesmus obliquus* Turpin Kützing, 1833 (low nutritional quality; Lampert and Muck 1985; Santer 1994). Although the importance of nutrition for the somatic development and reproduction of freshwater zooplankton is beginning to be better understood (Sterner and Elser 2002; Martin-Creuzburg et al. 2009), the extent to which the nutritional quality of ingested phytoplankton affects recovery from fasting (i.e., the return to pre-fasting TAG and PLFA levels and composition) as a function of temperature still needs to be elucidated (but *see* Bourdier and Amblard 1989). We therefore tested the null hypothesis (hypothesis 3) that the extent of recovery (i.e., TAGFA and PLFA mass fraction and composition) after 10 d is independent from food and temperature. Finally, we related changes in the above biochemical parameters to the physiological condition of the copepods by quantifying their body weight and survival during the experiment.

To our knowledge, this is the first study documenting how temperature, fasting, and the quality of food resources interact and influence freshwater copepod lipid biochemistry and survival. We do not claim that our experimental setting exactly replicates natural conditions, and we acknowledge that it is unknown whether copepods like *Eudiaptomus* ever face zero-food conditions. However, our carefully controlled experiments provide an indication on how *Eudiaptomus* responds to and recovers from nutritional stress in particular thermal habitats and in the presence of different dietary quality. Such knowledge is important to the mechanistic understanding of habitat selection behavior of *Eudiaptomus* (and perhaps freshwater calanoids in general) in the field and to the assessment of the potential effects of natural or anthropogenic temperature and algal assemblage changes on their physiology.

Methods

Animal collection and experimental setup—Copepods (*E. gracilis*) were collected using 6 m long vertical net hauls (100 μm mesh size) in fall 2011 from Lake Lunzer Obersee, a pre-alpine (1116 m above sea level), small (0.144 km^2), and shallow (Z_{max} = 15 m) lake in Lower Austria (47°48′N, 15°04′E; Rauch et al 2006). The temperatures at the lake surface and at 6 m were 11.8°C and 6°C, respectively. Within a few hours of collection, depth-integrated seston samples (< 30 μm) were filtered onto pre-weighed, precombusted GF/C filters (n = 3, mesh size 1.2 μm), freeze-dried, weighed, and stored at −80°C until lipid analysis. Adult non-ovigerous copepods were separated in the lab under a dissecting microscope. Three replicates of 50 individuals (ind.) each were placed in pre-weighed tin cups and stored at −80°C. The rest of the copepods were evenly distributed among 54 beakers (100 ind. beaker^{-1}) filled with 500 mL of 0.2 μm filtered lake water. The copepod-containing beakers were separated into three groups and kept at 4°C, 8°C, and 12°C without food in temperature-controlled chambers with a 12 : 12 h light : dark cycle for 10 d. The three experimental temperatures were chosen as representative of the temperature gradient that *Eudiaptomus* was experiencing at the moment of capture (*see* above). After 10 d, copepods were fed for 10 more days with either *C. ozolinii* or *S. obliquus*. In every beaker, 1 mg C of algae per liter of water was supplied on days 11, 14, and 18 in order to keep the food concentration above limiting levels (Lampert and Muck 1985).

The two algae were chosen because of their different nutritional quality for *Eudiaptomus. Cryptomonas* sp. are generally considered to support higher development, growth, reproduction, and survivorship rates than *Scenedesmus* sp. (Lampert and Muck 1985; Santer 1994; Von Elert and Stampfl 2000). The pertinent aspect of these two algae (regarding their putative influence on *Eudiaptomus* lipid physiology) is that they differ in their FA composition (Ahlgren et al. 1992; Masclaux et al. 2009; Koussoroplis et al. 2012). *Scenedesmus* is rich in 18:3n-3 (alpha-linolenic acid), 18:2n-6 (linoleic acid), and 18:4n-3 (stearidonic acid), but is devoid of physiologically important long-chain (i.e., > 20 carbon atoms) polyunsaturated FA (LC-PUFA). *Cryptomonas* contains 18-carbon PUFA, but is also rich in 20:5n-3 (eicosapentaenoic acid). Moreover, *Cryptomonas* also contains 22:5n-6 (docosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid; Masclaux et al. 2009; Martin-Creuzburg et al. 2011). The two algae were grown semi-continuously in Wright's Chu medium (Guillard and Lorenzen 1972) at 20°C. C : P ratios of algal cultures were kept low by renewing 10% of the media every 2 d. At the onset of feeding, samples of the algal cultures were filtered on pre-weighed GF/C filters, freeze-dried, weighed, and stored at −80°C until lipid analysis.

Every day, beakers were examined and dead animals removed and counted. Mortality was recorded daily until day 14, and thereafter only at days 17 and 20. On days 1, 7,

10, 11, 17, and 20, triplicates (20–40 ind. sample^{-1}) of live individuals were collected from each replicate temperature treatment, placed in pre-weighed tin cups, and stored at −80°C until lipid analysis.

Lipid analysis—Lipids of freeze-dried and homogenized algae and copepods were extracted according to Heissenberger et al. (2010). For copepods, TAG were separated from PL by one-dimensional thin-layer chromatography on 10 × 10 cm silica gel plates (Supelco™ SP-2560) by developing to full height with 90 : 10 : 3 : 2 (by volume) hexane : diethyl ether : methanol : formic acid (Desvilettes et al. 1994). Plates were sprayed with 0.05% (w : v) 8-anilino-4-naphthosulfonic acid (Bychek et al. 2005) and viewed under ultraviolet light to identify spots of TAG and PL, which were then scraped and placed in glass vials to which toluene (1 mL) was added. FA were derivatized to methyl esters (FAME) using sulfuric acid in methanol (1% v : v) at 50°C for 15 h (Heissenberger et al. 2010). To obtain sufficient amounts for analysis, FAME were concentrated to 50 μL in GC vials with glass inserts, from which they were injected and analysed by a gas chromatograph (TRACE GC Thermo; detector = flame ionization detector at 260°C; carrier gas = He at 1 mL min^{-1}; detector gases = H_2 at 40 mL min^{-1}, N_2 at 45 mL min^{-1}, air = 450 mL min^{-1}; temperature ramp = 140°C [5 min] then 4°C min^{-1} to 240°C [20 min]) equipped with a temperature-programmable injector and an autosampler. A Supelco™ SP-2560 column (100 m, 25 mm internal diameter, 0.2 μm film thickness) was used to separate FAME. FAME were identified by comparison of their retention times with known standards (37-component FAME mix, Supelco™ 47885-U; bacterial FA, Supelco™ 47080-U; and the following individual FAME standards: stearidonic acid, O5130 SIGMA™, and n-3 docosapentaenoic acid, Supelco™ 47563-U) and quantified with reference to seven-point calibration curves derived from 2.5, 50, 100, 250, 500, 1000, and 2000 ng μL^{-1} solutions of the FAME standard. The FA were expressed as total PLFA or TAGFA mass fractions (μg FA mg dry weight [dry wt]$^{-1}$) and as individual FA relative proportions (% of total identified FA).

Data analysis—Before the statistical analysis, relative (%) FA data were arcsine square root-transformed and PLFA and TAGFA total mass fractions (mg wt^{-1}) and, when necessary, individual dry wts were log transformed to meet assumptions for normality and homogeneity of variance for principal component analysis (PCA) and ANOVA. Only FA with less than 20% of zero values were used for the PCA.

PCA allowed us to reduce the number of response variables without losing information on temporal patterns of the main FA (Van Dooremalen and Ellers 2010). This allowed us to focus on the most important FA compositional changes in TAG and PL fractions separately. The two PCAs were run for fasting and feeding treatments together. The data transformation reduced the scale differences among FA below one order of magnitude; hence, the PCA was run on a covariance matrix. The comparison of the eigenvalues with the broken stick model (Jackson 1993) indicated that, for both PL and TAG, only the first principal component (PC1) was significant (eigenvalue variation explained by PC1: 67% and 71% for PL and TAG, respectively). After verifying that they met assumptions for normality and homogeneity of variance, the sample scores on the PC1 were further used for statistical analysis as the new variable "$PC1_{score}$" representing the major trends in FA composition. To relate the PC scores to single FA, a Pearson correlation coefficient between $PC1_{score}$ and each single FA proportion was calculated. The validity of this approach was previously tested (not shown) by submitting the most strongly correlated FA to the same statistical tests as $PC1_{score}$.

Based on the assumption that treatment effects need time to be fully expressed, we focused our statistical analysis on the endpoints of our data set (i.e., days 0, 10, and 20). To determine the effects of fasting at the three different experimental temperatures on FA and biomass changes in *Eudiaptomus*, we used ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test to compare FA profiles ($PC1_{score}$) and the TAGFA and PLFA mass fractions (hypotheses 1 and 2) as well as the copepods' individual dry wt between days 0 and 10. For the feeding period, the effects of food, temperature, and their interaction on the different physiological parameters at the end of the feeding period (day 20) were assessed by two-way ANOVA followed by Tukey's HSD post hoc tests (hypothesis 3). We also assessed the significance of changes in FA and copepod dry wt induced by the two feeding treatments at the different temperatures by pairwise comparisons (Student's *t*-test) at the ends of fasting (day 10) and feeding (day 20). In a few cases, because of the nonlinearity of the time-resolved responses of the different parameters and in order to correctly interpret the results, we also applied the above analyses (ANOVA and Student's *t*-test) to intermediate time steps.

The Kaplan-Meier procedure was used to obtain estimates of the survival functions. The analysis was performed separately for feeding by considering the individuals still alive at day 10 as the starting point. Differences between survival functions in treatments were tested using nonparametric log-rank tests for homogeneity of survival functions. Mean survival time (mean time from the onset of fasting to death) was calculated from survival functions.

R (R Development Core Team 2008) was used for PCA and ANOVA, and Medcalc Software was used for survival analysis.

Results

FA composition and lipid content of the algae—The mass fraction of total lipid was considerably less in *Scenedesmus* than in *Cryptomonas* (Table 1). As expected, *Scenedesmus*'s FA composition was dominated by 18:3n-3, 16:4n-3, 16:0, and, to a lesser extent, 18:1n-9, 18:2n-6, and 18:4n-3, and was devoid of LC-PUFA (Table 1). The FA composition of *Cryptomonas* was dominated by 18:4n-3, 18:3n-3, and 20:5n-3, and, to a lesser extent, by 16:0 as well as 18:2n-6, 22:6n-3, and 20:4n-3 (Table 1).

Table 1. FA composition (mean ± SE, $n = 3$) of total lipids (% of total FA weight) and total lipid content (μg lipids mg dry wt^{-1}) of natural seston from Lunzer Obersee the day the copepods were collected, of *Cryptomonas ozolinii* and of *Scenedesmus obliquus*. The total lipid content was determined gravimetrically as described in Heissenberger et al. (2010). tr, traces (< 1%); —, not detected; na, not available.

	% of total FA weight		
	Seston	*S. obliquus*	*C. ozolinii*
14:0	2.1±0.2	tr	2.5±0.1
16:0	21.8±0.7	15.8±1.1	11.1±0.4
18:0	9.5±0.6	1.1±0.3	1.4±0.2
20:0	tr	tr	—
22:0	tr	tr	—
24:0	tr	—	—
15:0+17:0	1.5±0.1	1.9±0.4	1.8±0.2
16:1n-9	1.6±0.2	1.3±0.3	tr
16:1n-7	2.2±0.1	tr	tr
18:1n-9	5.2±0.1	9.6±0.5	1.2±0.1
18:1n-7	2.0±0.1	2.0±0.1	—
22:1n-9	4.7±0.2	tr	—
16:4n-3		22.3±1.5	
18:2n-6	4.8±0.1	9.0±0.4	6.6±0.1
18:3n-6	tr	tr	tr
18:3n-3	15.4±0.5	30.7±1.3	26.7±0.2
18:4n-3	18.3±0.8	5.3±0.6	27.4±0.3
20:3n-3	—	—	—
20:4n-6	—	tr	—
20:4n-3	—	tr	1.2±0.2
20:5n-3	4.9±0.3	—	16.6±0.4
22:6n-3	4.0±0.1	—	1.6±0.1
Total lipids (μg mg dry wt^{-1})	na	128.0±8.9	198.5±13.4

PLFA and TAGFA mass fraction—At the onset of the experiment (day 0), copepods contained 29.5 ± 2.8 μg PLFA per mg dry wt (Fig. 1a). PLFA consisted mainly of LC-PUFA (15.7 ± 1.1 μg mg dry wt^{-1}) and saturated FA (SAFA; 7.7 ± 0.5 μg mg dry wt^{-1}) with only 3.5 ± 0.3 and 2.5 ± 0.3 μg mg dry wt^{-1} of C_{18}PUFA and monounsaturated FA (MUFA), respectively. During fasting, and despite a slight increase (significant only at 4°C on day 7, ANOVA, $p < 0.05$, not shown), the total PFLA mass fractions at day 10 did not differ across temperatures and were not significantly different from the initial level on day 0 (Fig. 1a; Table 2). Furthermore, no differences among treatments were detected at day 20, and PLFA mass fractions did not differ from those at the end of fasting (Fig. 1a; Tables 2 and 3).

At the onset of the experiment (day 0), the copepods contained 40.9 ± 3.9 μg TAGFA mg dry wt^{-1} (Fig. 1b), mostly C_{18}PUFA (16.9 ± 1.3 μg mg dry wt^{-1}) and SAFA (11.3 ± 1.3 μg mg dry wt^{-1}), with only 6.1 ± 0.5 and 5.6 ± 0.7 μg mg dry wt^{-1} of LC-PUFA and MUFA, respectively.

After 10 d of fasting, TAGFA mass fractions significantly decreased over time to nearly full depletion for all temperatures, but no differences between temperatures were found (also when comparing day 0 to days 1 and 7, one-way ANOVA, $p > 0.05$, not shown; Fig. 1b; Table 2). The effects of refeeding and temperature on TAGFA mass fraction were not apparent up to day 17 (two-way ANOVA, $p > 0.05$ and t-test, $p > 0.05$, not shown). At the end of the feeding period, however, food and temperature interacted to determine total TAGFA levels. Interestingly, TAGFA levels were highest in *Eudiaptomus* fed on *Cryptomonas,* but only at 8°C and 12°C (Fig. 1b; Table 3). The rest of the treatments showed little or no difference from each other as well as from their respective levels at the end of fasting (Fig. 1b; Table 3).

Body weight—Temperature had a significant effect on the decrease in dry wt following fasting, and the lower values were observed at 8°C and 12°C (dry wt; μg ind.$^{-1}$; Fig. 1c; Table 2). Food and temperature interacted to determine total TAGFA levels at the end of the refeeding period. More specifically, feeding on *Cryptomonas* resulted in a significantly higher dry wt, but only at 8°C and 12°C (Fig. 1c; Table 4). Whereas in all *Cryptomonas* treatments dry wt significantly increased as compared to day 10, in the *Scenedesmus* treatments it was only the case for the copepods fed at 8°C (Table 3).

Survival—Death of copepods started as of day 4. A high-mortality event occurred between days 4 and 5 in the 12°C treatment, whereas in the other treatments mortality was relatively equal over time (Fig. 1d). The starvation survival functions for the three experimental temperatures differed significantly from each other, and copepods fasting at lower temperatures (4°C and 8°C) had longer mean survival times (log-rank test, $\chi^2 = 209, 37$; $p < 0.0001$; 9.04 ± 0.05, 9.27 ± 0.04, and 8.4 ± 0.06 d for 4°C, 8°C, and 12°C, respectively). In the refeeding phase, mortality remained low until day 14, but peaked between days 14 and 20 (Fig. 1d). Significant differences were also found between the survival functions of refeeding copepods (log-rank test, $\chi^2 = 209, 37$, $p < 0.0001$). These differences appeared to be mainly linked to temperature rather than diet: 8.4 ± 0.1, 8.2 ± 0.1, and 8.5 ± 0.1 d for *Scenedesmus* and 8.4 ± 0.1, 8.3 ± 0.1, and 8.7 ± 0.1 d for *Cryptomonas* at 4°C, 8°C, and 12°C, respectively. Unlike fasting, however, survival was not linearly related to the temperature gradient, and the lowest mean survival was observed at 8°C (Fig. 1d).

Correlations between FA composition and $PC1_{score}$—The FA used in the PCA are given in Table 5. PC1 explained 67.7% and 71.7% of the variability in eigenvalues for PLFA and TAGFA, respectively. For PLFA, the proportions (%) of PUFA and 16:1n-9 were positively correlated, and the proportions of 14:0, 16:0, 18:0, 20:0, 16:1n-7, and 18:1n-7 were negatively correlated to the $PC1_{score}$ (Fig. 2a). For TAGFA, the proportions (%) of 14:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, and LC-PUFA were positively correlated, and 16:0, 18:0, and 18:1n-7 were negatively correlated to the $PC1_{score}$ (Fig. 2b).

PLFA composition ($PC1_{score}$)—Fasting significantly decreased the copepods' PLFA $PC1_{score}$ in all treatments with the extent of the decrease following 12°C > 8°C > 4°C (Fig. 2a; Table 2). This indicates that fasting copepods

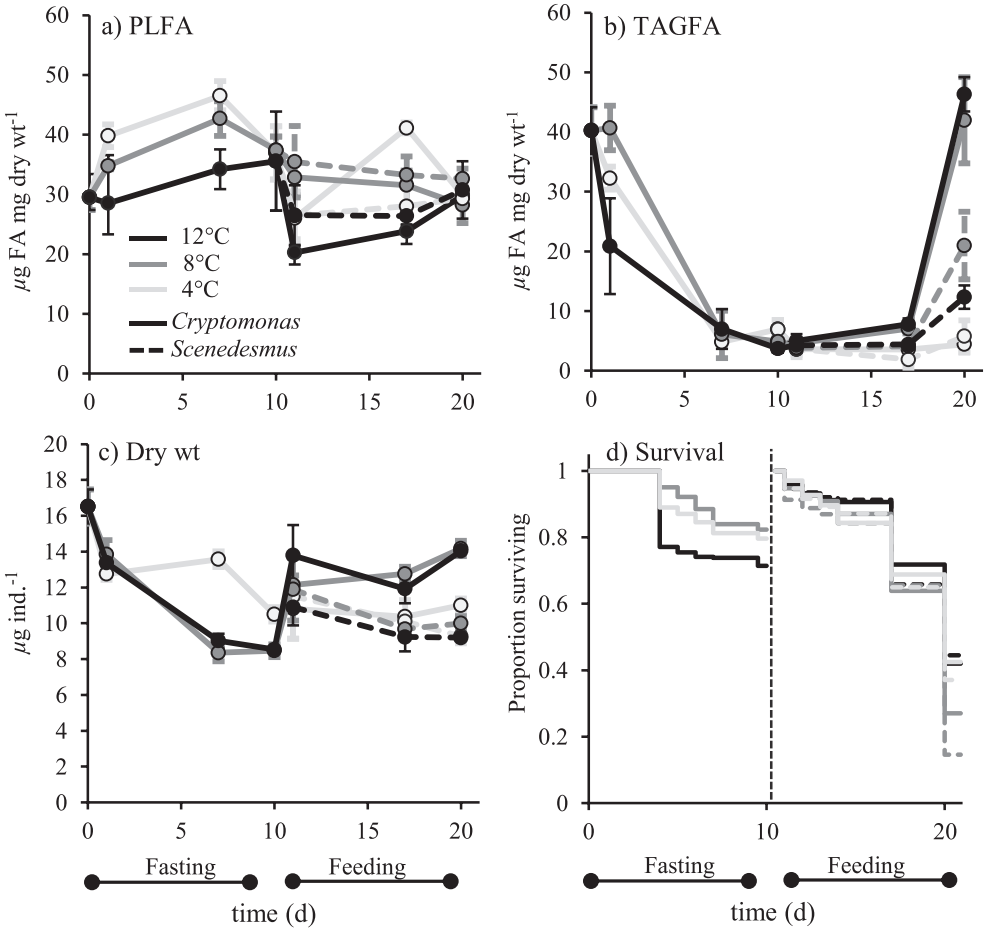

Fig. 1. FA of membrane phospholipids and triacylglycerols, body weight, and survival parameters of adult *Eudiaptomus gracilis* during 10 d of fasting and 10 d of subsequent feeding at 4°C, 8°C, and 12°C. (a) Total PLFA and (b) the total TAGFA mass fractions (μg mg dry wt $^{-1}$, mean ± standard error [SE], $n = 3$), (c) dry wt (μg copepod^{-1}, mean ± SE, $n = 3$), and (d) Kaplan-Meier product limit estimates of the proportion of individuals surviving at each time step. Dashed vertical line shows the onset of refeeding.

decrease the proportion of PUFA while increasing that of SAFA in their membranes, particularly at higher temperatures (Table 5).

All copepods, except those fed on *Scenedesmus* at 4°C, significantly increased their PLFA $PC1_{score}$ between day 10 and day 20 (Fig. 2a; Table 3), indicating that copepods that resumed feeding increased the proportion of PUFA in their membranes (Table 5). The PLFA $PC1_{score}$ at day 20 was significantly influenced by food, with *Cryptomonas* treatments having, on average, higher values than *Scenedesmus* treatments (mostly because of the low *Scenedesmus* $PC1_{score}$ values at 4°C; Fig. 2a; Table 4). However, no effect of temperature on PLFA $PC1_{score}$ or interaction between the two (food and temperature) was observed (Table 4). An examination of the individual FA that were strongly correlated to $PC1_{score}$ confirmed that food was the main factor influencing PUFA proportion (Table 5).

Finally, we note that, despite the absence of 20:5n-3 and 22:6n-3 in *Scenedesmus*, the proportions of these two LC-PUFA increased in PLFA in copepods fed on this alga,

Table 2. One-way ANOVA and Tukey's HSD of the different physiological parameters of adult *Eudiaptomus gracilis* at the onset (day 0) and at the end of fasting at 4°C, 8°C, and 12°C (day 10).

Variable	df	*F*	*p*	Tukey's HSD
Dry wt	3	101.80	<0.0001	$Day0 > Day10_{4°C} > Day10_{8°C} = Day10_{12°C}$
Total PLFA	3	0.55	0.663	—
Total TAGFA	3	34.50	<0.0001	$Day0 > Day10_{4°C} = Day10_{8°C} = Day_{12°C}$
$PC1_{score}$ PLFA	3	90.10	<0.0001	$Day0 > Day10_{4°C} > Day10_{8°C} > Day10_{12°C}$
$PC1_{score}$ TAGFA	3	49.1	<0.00001	$Day0 > Day10_{4°C} > Day10_{8°C} = Day10_{12°C}$

Table 3. Pairwise comparison (Student's *t*-test) of the different physiological parameter values of adult *Eudiaptomus gracilis* fed on *Cryptomonas* (Cry) or *Scenedesmus* (Sce) at three different temperatures (4°C, 8°C, and 12°C) at end of feeding period (day 20) and their respective values at the onset of feeding (day 10). Dry wt, dry weight; PLFA, membrane phospholipid fatty acids; TAGFA, triacylglycerol fatty acids; PC1score, scores of fatty acid composition (%) samples on the first principal component. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Day 10 vs. day 20	Dry wt	Total PLFA	Total TAGFA	$PC1_{score}$ PLFA	$PC1_{score}$ TAGFA
Cry 4°C	−6.94**	1.49, ns	1.20, ns	−11.84***	−0.22, ns
Cry 8°C	4.82**	2.44, ns	−5.09*	−13.49***	−20.15***
Cry 12°C	−7.54**	0.68, ns	−24.34***	−9.67***	−21.40***
Sce 4°C	−1.90, ns	1.70, ns	−0.38, ns	−1.74, ns	−1.13, ns
Sce 8°C	−5.47**	1.75, ns	−1.78, ns	−10.75***	−2.91*
Sce 12°C	−1.66, ns	−0.50, ns	−4.12*	−7.06**	−14.10***

ultimately reaching similar proportions to those found in the copepods fed *Cryptomonas* (Table 5).

TAGFA composition ($PC1_{score}$)—Fasting significantly decreased the copepods' TAGFA $PC1_{score}$ in all treatments, with the more important decreases at 8°C and 12°C (Fig. 2b; Table 2), indicating that fasting copepods tend to preferentially mobilize PUFA from their lipid stores, particularly at higher temperatures (Table 5).

As was the case for PLFA, all copepods, except those fed at 4°C, significantly increased their TAGFA $PC1_{score}$ between days 10 and 20 (refeeding period; Fig. 2b; Table 4), indicating that when they resumed feeding they increased again the proportion of the PUFA in their lipid stores (Table 5). However, when considering that in all treatments the lowest TAGFA $PC1_{score}$ was observed at day 11, a significant increase between days 11 and 20 was also observed for *Scenedesmus* treatments at 4°C (*t*-test, $t = -3{,}17$, df = 1, $p = 0.033$). At day 20, the TAGFA $PC1_{score}$ was interactively dependent on food and temperature, as the copepods fed with *Cryptomonas* had higher $PC1_{score}$, but only at 8°C and 12°C (Fig. 2b; Table 4). At 4°C, the lowest $PC1_{scores}$ were observed without any differences between diets.

Discussion

Fasting—As expected, *Eudiaptomus* used TAGFA during fasting, thus confirming the role of TAGFA as energy source during periods of food shortage (Arts and Evans 1991). In copepods, the amounts of stored lipids may be indicative of how fast they respond to resource fluctuations (Arts 1999). Although the maximum lipid storage capacity of *Eudiaptomus* is unknown, similar total FA levels to those at the onset of our study were reported in lab-reared *Eudiaptomus* fed ad libitum on a high-quality lipid-rich diet (Von Elert and Stampfl 2000), suggesting relatively limited storage abilities. Based on observations of marine calanoids with comparable lipid stores (3–19% total lipid content), it could be expected that *Eudiaptomus* (~ 4% total FA content on day 0) respond to short fasting periods (i.e., few days of short fasting spells) by decreases in reproduction and might not survive more than 4–7 d of fasting (Dagg 1977; Lee et al. 2006).

In our experiment, *Eudiaptomus* nearly depleted their TAGFA within 7 d, confirming that TAGFA stores could sustain energetic demands only for relatively short periods. However, more than 70% of the copepods were still alive after 10 d of fasting (mean estimated survival time > 8 d).

Table 4. Two-way ANOVA and Tukey's HSD of the different physiological parameters of adult *Eudiaptomus gracilis* fed on *Cryptomonas* or *Scenedesmus* at three different temperatures (4°C, 8°C, and 12°C) at day 20. Temp, temperature; Dry wt, dry weight; PLFA, membrane phospholipid fatty acids; TAGFA, triacylglycerol fatty acids; PC1score, scores of fatty acid composition (%) samples on the first principal component.

Variable	Source	df	*F*	*p*	Tukey's HSD
Dry wt	Food	1	118.02	<0.0001	*Scenedesmus*<*Cryptomonas*
	Temp	2	13.20	0.001	4°C<8°C=12°C
	Food×Temp	2	7.93	0.006	$4°C_{Sce}=8°C_{Sce}=12°C_{Sce}=4°C_{Cry}<8°C_{Cry}<12°C_{Cry}$
$PLFA_{total}$	Food	1	0.528	0.481	—
	Temp	2	0.055	0.947	—
	Food×Temp	2	0.473	0.634	—
$TAGFA_{total}$	Food	1	23.80	<0.001	*Scenedesmus*<*Cryptomonas*
	Temp	2	18.71	<0.001	4°C<8°C=12°C
	Food×Temp	2	7.56	0.008	$4°C_{Sce}=4°C_{Cry}<8°C_{Cry}=12°C_{cry}$
$PC1_{score}$ PLFA	Food	1	37.86	<0.0001	Scenedesmus<Cryptomonas
	Temp	2	3.42	0.068	—
	Food×Temp	2	2.67	0.114	—
$PC1_{score}$ TAGFA	Food	1	18.86	<0.0001	*Scenedesmus*<*Cryptomonas*
	Temp	2	22.42	<0.0001	4°C<8°C=12°C
	Food×Temp	2	2.67	0.049	—
$PC1_{score}$ TAGFA without outliers	Food	1	75.91	<0.0001	*Scenedesmus*<*Cryptomonas*
	Temp	2	258.82	<0.0001	4°C<8°C=12°C
	Food×Temp	2	34.07	<0.0001	$4°C_{Sce}=4°C_{Cry}<8°C_{Sce}=12°C_{Sce}<8°C_{Cry}=12°C_{Cry}$

Table 5. Relative proportions (% of total FA weight) of the FA (mean ± SE, $n = 3$) of *Eudiaptomus gracilis* used for the PCA (see Methods) at the onset of fasting (day 0), after the fasting period (day 10), and at the end of the refeeding period (day 20) with *Cryptomonas ozolinii* and of *Scenedesmus obliquus*. tr, traces (< 1%); PLFA, membrane phospholipid fatty acids; TAGFA, triacylglycerol fatty acids; LC-PUFA, sum of LC-PUFA.

		Day 10			Day 20			Day 20		
		Fasting			*Cryptomonas*			*Scenedesmus*		
	Day 0	4°C	8°C	12°C	4°C	8°C	12°C	4°C	8°C	12°C
a) PLFA (%)										
14:0	tr	1.6±0.1	2.5±0.2	1.4±0.1	2.2±0.3	1.0±0.2	tr	1.6±0.2	tr	1.0±0.4
16:0	15.9±0.2	27.6±0.1	28.1±0.6	33.3±0.3	21.1±3.3	20.3±0.6	17.3±0.5	23.5±1.4	20.3±5.7	21±2.2
18:0	7.1±0.2	22.5±0.4	21.0±0.8	27±0.5	9.1±2.3	13±0.5	11.5±0.3	16.5±1	18.1±3.7	14.4±0.2
20:0	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
15:0+17:0	1.4±0.1	1.2±0.1	1.5±0.1	1.2±0.1	1.7±0.2	1.9±0.1	2.0±0.1	1.7±0.1	1.0±0.3	1.3±0.1
16:1n-7	tr	2.7±0.1	5.7±0.2	6.8±0.2	4.4±0.2	1.0±0.2	1.0±0.1	5.6±0.4	tr	1.7±0.3
18:1n-9	2.9±0.1	2.2±0.1	3.4±0.2	2.7±0.1	3.8±0.1	2.6±0.1	2.4±0	4.2±0.2	1.9±0.6	3.3±0.2
18:1n-7	2.4±0.1	2.4±0.1	4.2±0.2	5.8±0.1	2.7±0.1	2.0±0.1	1.8±0	3.1±0.1	7.5±2.3	3.5±0.1
18:2n-6	2.6±0.1	1.5±0.1	2.0±0.1	1.3±0.1	2.9±0.2	3.0±0.1	2.4±0.1	2.4±0.1	3.3±1.1	2.7±0.1
18:3n-3	4.5±0.1	3.1±0.1	2.1±0.3	1.2±0.1	5.2±0.4	6.5±0.4	6.7±0.1	4.6±0.2	7.2±2.1	10.5±0.6
18:4n-3	4.7±0.1	1.6±0.1	1.9±0.4	1.1±0.1	4.8±0.3	9.2±0.5	5.9±0.2	3.3±0.1	2.5±0.8	2.4±0.1
20:4n-6	1.6±0.1	1.0±0.1	1.0±0.1	tr	1.0±0.2	tr	1.1±0	1.1±0.1	1.1±0.3	1.4±0.1
20:5n-3	17.0±0.1	9.1±0.1	6.0±0.1	4.2±0.1	13.5±2.4	15.4±0.9	21.9±0.5	10.5±0.8	9.8±2.4	9.6±0.4
22:6n-3	33.6±0.4	19.8±0.1	13.3±0.2	8.6±0.2	22.5±4.3	20.8±1.3	22.7±0.8	19.2±2.0	23.2±5.6	24.1±2.0
b) TAGFA (%)										
14:0	3.4±0.2	4.0±0.6	2.9±0.2	1.6±0.2	2.7±1.0	2.6±0.6	2.6±0.3	4.0±0.7	2.3±0.8	1.0±0.2
16:0	17.2±0.1	24.5±0.8	27.2±1.0	30.6±0.7	30.8±2.4	14.6±1.4	14.2±0.9	40.3±10.1	24.3±2.6	19.7±0.2
18:0	3.9±0.1	20.2±3.7	27.7±0.9	35.6±2.5	22.7±4.8	4.1±0.1	4.2±0.2	19±5.5	25.3±5.6	13.4±0.3
16:1n-9	tr	3.8±0.4	1.2±0.1	tr	2.9±0.5	tr	tr	4.7±0.9	5.5±2.1	3.5±0.6
16:1n-7	2.8±0.1	2.1±0.3	1.7±0.1	2.1±0.2	2.3±0.2	1.9±0.3	2.2±0.2	1.1±0.6	1.3±0.2	1.8±0.2
18:1n-9	5.5±0.1	10.2±0.7	13.0±0.1	11.6±1.1	8.7±0.3	2.3±0.3	3.8±0.3	6.7±3.4	18.1±1.1	12.4±0.5
18:1n-7	2.3±0.1	2.2±0.3	2.2±0.1	1.2±0.1	1.9±0.2	1.6±0.1	1.7±0.1	2.5±0.1	1.7±0.1	2.9±0.2
18:2n-6	6.2±0.1	4.6±0.2	2.9±0.1	4.3±1.1	5.2±0.4	5.8±0.4	5.5±0.2	3.0±1.5	5.2±0.3	6.1±0.1
18:3n-3	16.2±0.2	5.3±0.7	1.4±0.1	tr	5.6±1.7	18.5±0.3	21.2±0.3	2.4±1.2	7.0±3.7	20.1±3.7
18:4n-3	18.1±0.5	3.8±0.6	1.2±0.2	tr	4.7±1.1	30.9±2.8	20.7±1.4	5.0±1.5	2.9±1.9	5.6±0.7
LC- PUFA	16.3±0.3	8.3±1.0	5.8±0.4	2.5±0.2	6.8±2.3	13.1±1.0	18.0±1.0	5.9±1.2	6.1±4.4	9.7±1.8

One of the possible explanations for such a high survival is that the relatively low temperatures (as compared to other studies on copepod starvation) at which the experiment was conducted prolonged survival by keeping copepod energy use (maintenance cost) rate low. Interestingly, within the tested temperature gradient, *Eudiaptomus* survival times were longer at the two lower temperatures (4°C and 8°C). Nevertheless, longer survival was not related to slower depletion of lipid stores, as our null hypothesis on equal utilization of TAGFA across temperatures could not be rejected based on the ANOVA results. The catabolized TAGFA represented only a small proportion (≤ 10%) of the copepod dry wt loss, indicating that fasting *Eudiaptomus* utilized mostly other body compounds (proteins, carbohydrates) to supply their energetic requirements. Extensive protein catabolism, often after lipid stores have been depleted, has been observed in certain copepods and many other crustacean taxa (Evjemo et al. 2001; Sanchez-Paz et al. 2006; Mayor et al. 2011). Body mass loss was in better agreement with an earlier depletion of energy stores at higher temperature and survivorship. Copepods fasting at 8°C and 12°C lost significantly more body mass (already visible at day 7, ANOVA $p < 0.0001$, not shown) than those at 4°C. Hence, although detected by the ANOVA at the sampled time steps, increased temperatures probably accelerated the depletion of TAGFA, resulting in an earlier and more extensive utilization for other body components (i.e., earlier decrease in body weight). Higher observation frequency (e.g., between days 1 and 7) may have provided better information about the time at which TAGFA mass fractions differed among temperatures before depletion.

Contrary to our null hypothesis on unselective TAGFA use, the composition of the remaining TAGFA in fasted *Eudiaptomus* strongly differed from that at the onset of fasting, indicating selective mobilization of particular FA from stored TAG. As observed in some marine copepods, notothenioid fish, and mammals (Raclot 2003; Hazel and Sidell 2004; Pond 2012), we found a relative retention of SAFA at the expense of PUFA and certain MUFA. Furthermore, the extent to which the TAGFA composition changed during fasting was affected by temperature. TAGFA of copepods fasting at 4°C were richer in PUFA than those at 8°C and 12°C, thus rejecting our null hypothesis that mobilization of specific TAGFA is independent of temperature. The mechanism behind preferential utilization of PUFA-containing TAG in copepods is unknown, but it could be related to higher affinity of lipases to these particular substrates (Raclot 2003). Never studied so far in freshwater copepods, selective accumulation and catabolism of given FA in lipid

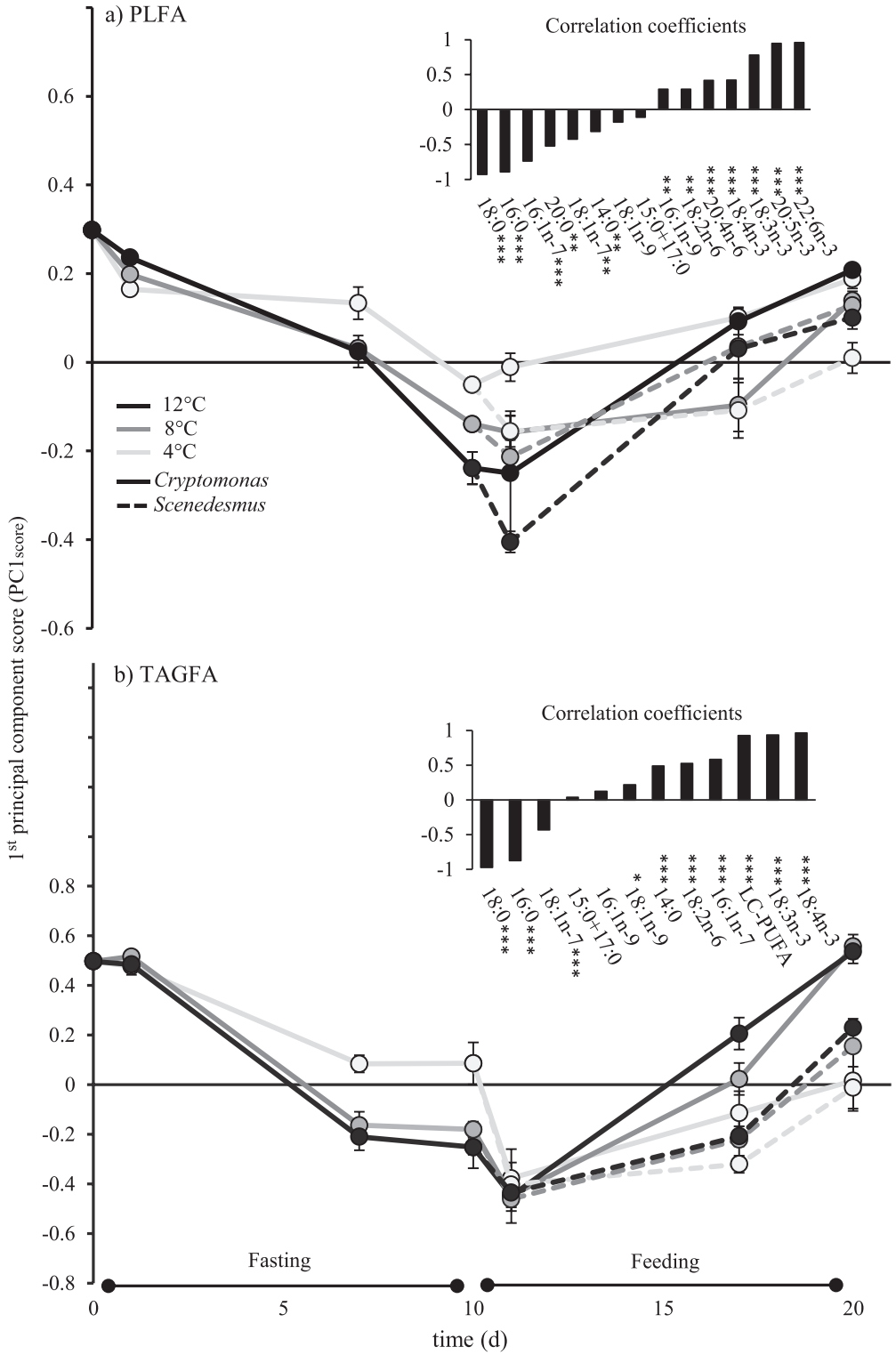

Fig. 2. FA composition as reflected in $PC1_{score}$ (mean ± SE, $n = 3$) of adult *Eudiaptomus gracilis* during 10 d of fasting and 10 d of subsequent feeding at 4°C, 8°C, and 12°C. (a) membrane phospholipid fatty acids (PLFA) and (b) triacylglycerol fatty acids (TAGFA). Nested in the figures are the coefficient of correlation (R^2) between the proportions (% of total FA weight) of the FA used for the PCA and the scores of copepod samples on $PC1_{score}$ for PLFA and TAGFA; Correlation significance levels: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

stores have been, at least in some wax ester–storing marine species, proposed as possible energy storage optimization or buoyancy control strategies (Pond 2012). Finally, even less is known on the response of copepod TAGFA composition to temperature changes, but some studies on insects suggest that the degree of unsaturation in TAGFA increases with decreasing temperature (Haubert et al. 2008; Van Dooremalen and Ellers 2010). Although not yet experimentally tested, it has been proposed that such changes may prevent the solidification of lipid stores that could interfere with lipolysis, leading to decreased accessibility to stored energy (Van Dooremalen and Ellers 2010).

As predicted, total PLFA mass fraction showed only moderate changes during fasting. Contrary to the null hypotheses, PLFA composition was strongly affected by fasting as well as by temperature during fasting. These compositional changes were characterized by decreases in the proportion of PUFA and an increase in SAFA and certain MUFA. The changes were very moderate until day 7, indicating that copepods maintained their membrane FA composition for at least some time during fasting. However, after day 7, the proportion of PUFA in PLFA dramatically decreased. Usually cell membrane lipids are rich in LC-PUFA (e.g., 22:6n-3 and 20:5n-3) that are involved in the maintenance of cell membrane fluidity, elasticity, and permeability at colder temperatures (Arts and Kohler 2009). In addition, LC-PUFA such as 20:5n-3 and 20:4n-6 are involved in chemical signaling related to immunity, inflammation, mineral balance, and reproductive processes (Sardesai 1992). Therefore, given the physiological importance of PUFA and that they are mostly obtained from diet (so that such losses in PUFA are irreversible as long as feeding is not resumed), our observations on fasting *Eudiaptomus* PLFA seem counterintuitive. Preferential PUFA retention during fasting has been shown in other taxa, such as cladocerans (Schlechtriem et al. 2006) and amphipods (Mezek et al. 2010). The mass fractions of several PUFA also decreased more quickly than those of SAFA and MUFA in a fasting experiment with *Calanus* (Mayor et al. 2011). Moreover, Stuart et al. (1998) observed important decreases and increases of MUFA and PUFA, respectively, in the mitochondrial membranes of estivating snails and correlated them to adaptive metabolic depression. Such changes in PLFA composition, along with PL species shifts, could contribute to reducing the high energetic cost of maintaining the mitochondrial transmembrane gradients through alterations of membrane permeability and transmembrane protein functioning (Stuart et al. 1998; Hulbert and Else 2000). It can thus be speculated that when exposed to fasting, certain marine and freshwater calanoid copepod species (e.g., *E. gracilis* and *Calanus* spp.) might be able to modulate the FA composition of their cell membranes to slow down metabolism and to prolong survival as energy stores progressively decrease. The fact that these compositional changes in ectotherms are temperature sensitive and in agreement with homeoviscous adaptation (i.e., higher unsaturation at lower temperatures) further suggests that this putative, membrane-driven, metabolic depression is finely tuned to ambient temperatures.

Our results contribute to our understanding on how *Eudiaptomus* might choose their location in the water column. The considerable effects of relatively moderate temperature changes on body physiological condition and survival suggest that during food-limiting periods *Eudiaptomus* might gain a key advantage by seeking energy-sparing colder waters. Finally, our results suggest that this particular species is able to massively catabolize other body compounds in addition to their limited lipid stores to survive at least 10 days of fasting. This ability, as well as the indications that they might be able to finely tune their cell membranes to ambient food and temperature levels, could contribute to their unique capacity to actively overwinter and also to their dominance in crustacean communities of oligotrophic lakes (Straile and Geller 1998; Santer et al. 2000; Riccardi and Giussani 2007). To investigate this putative membrane-mediated mechanism, future studies should compare *E. gracilis* to the often co-occurring and closely related but not overwintering *Eudiaptomus graciloides*.

Feeding—Contrary to our null hypothesis, within the 10 d long period of refeeding, higher temperatures combined with a high-quality diet (*Cryptomonas*) favored both TAGFA mass fraction and PLFA composition recovery. Only copepods fed on *Cryptomonas* at 8°C and 12°C were able to fully recover their TAGFA; those fed on *Scenedesmus* only partially recovered. Similar to TAGFA, body weight recovery was higher for copepods fed at 8°C and 12°C. At 4°C, no significant increase of body weight or TAGFA was found for copepods fed on either of the algae, indicating that, within the observed timescale, the food quality effect on storage lipid deposition rates is temperature dependent. Although *Cryptomonas* promoted a better membrane FA composition recovery, the differences between the PLFA composition in the different treatments were limited compared to those in TAGFA. This clearly indicates that refeeding copepods are able to efficiently regulate their cell membrane FA composition with relatively little influence from diet and temperature. Furthermore, we found that *Eudiaptomus* was able to convert n-3 C_{18}PUFA from *Scenedesmus* to 20:5n-3 and 22:6n-3, as previously hypothesized by Von Elert and Stampfl (2000), to restore anterior LC-PUFA levels in its membranes. Hence, although our results confirm the nutritional advantage of *Cryptomonas*, it is difficult to attribute its positive benefits solely to its PUFA composition as we had implicitly hypothesized. Although PUFA conversion costs cannot be excluded, dietary supply of $C_{20–22}$ PUFA likely has little influence on the TAGFA deposition rates in *Eudiaptomus* during feeding. The higher total lipid content of *Cryptomonas* seems more likely to be the main reason for the higher TAGFA contents in copepods fed this diet. The composition in other required nutrients such as sterols and amino acids (Martin-Creuzburg and Von Elert 2009; Wacker and Martin-Creuzburg 2012) and/or poor digestibility of *Scenedesmus* by *Eudiaptomus* (Santer 1994) might also partially explain the lower TGFA deposition when feeding on *Scenedesmus*.

With both algae, TAGFA started to increase again only after 7 d, indicating that these copepods had no energy

surplus to store despite the fact that food concentration was always above the limiting levels for somatic growth and reproduction (0.1 and 1 mg C L^{-1}, respectively; Lampert and Muck 1985; Santer 1994). These results strongly suggest that recovery from fasting requires dietary lipids first being allocated to readjusting membrane lipids, as compositional changes occurred in PLFA before any changes were observed in TAGFA. As hypothesized, higher temperatures accelerated FA recovery, leading to the observed differences in TAGFA replenishment. A longer observational timescale would likely have revealed food quality effects also at 4°C. However, by increasing metabolic rates, temperature also increases energetic demands, and therefore more food might be necessary to achieve a positive energetic balance at higher temperatures. Consequently, under equal yet limiting food conditions, higher temperatures may compromise recovery if the temperature-driven energetic demand rises faster than energy acquisition rate. Thus, the positive effect of temperature on the TAGFA recovery rate does not necessarily imply that *Eudiaptomus* living at higher temperatures will have higher lipid reserves. Given relatively constant food conditions, within a certain range of temperatures and food availabilities, lower temperatures have been observed to promote higher lipid deposition in freshwater copepods (Vanderploeg et al. 1992; Arts et al. 1993).

Contrary to the fasting period, survival was not directly linked to final body weight or TAGFA mass fraction of the copepods surviving at day 20. This could indicate high interindividual variability linked to the fact we used individuals from a natural population. Future studies should consider biochemical data of survivors and dying individuals in parallel to gain better insight on how body weight or TAGFA mass fractions are related to mortality. Oddly, the influence of temperature on survival during the feeding period was not linear, and mortality peaked at 8°C. The energy acquisition and maintenance costs do not necessarily scale up with temperature at the same rate (Rinke and Vijverberg 2005) and might differ among individuals. It is therefore possible that a net energy gain at low temperatures (4°C and 8°C) was not possible for all copepods, especially if possible extra energetic costs linked to cell membrane recovery are considered. At 4°C, however, the energy loss rate might be so slow that the high mortality observed for 8°C occurs later than day 20.

In conclusion, we provide experimental evidence that fasting results in a significant decrease in body weight, TAGFA, and the proportion of PUFA in both storage and membrane lipids of *Eudiaptomus*. Higher temperatures accelerated the decrease of body weight and total TAGFA as well as the decrease of PUFA in storage and membrane lipids. We suggest that predicted temperature increases due to climate warming, particularly at high latitudes (IPCC 2007), may interact with habitat preferences and strategies to balance lipid acquisition and metabolism, especially in fasting copepods, through a more rapid depletion of storage lipids as well as the loss of lipids containing LC-PUFA of known beneficial physiological properties. The consequence of such decreases may weaken the survival or recovery potential of fasting individuals and also the quality of their offspring, as lipids and particularly PUFA are heavily invested in reproduction (Arts 1999; Lee et al. 2006). Finally, the quality of resources consumed by copepods affects their response to fasting episodes in various ways. Besides the role of food quality in promoting accumulation of lipid stores, the selective TAGFA mobilization suggests that TAGFA composition might also affect the quality of TAGFA as energy source in fasting *Eudiaptomus*. Consequently, as TAGFA composition is strongly influenced by diet (Brett et al. 2009), decreases in algal PUFA due to climate warming and cultural eutrophication (Müller-Navarra et al. 2004; Fuschino et al. 2011) may alter the responses of copepods exposed to fasting. Similarly, changes in algae species may affect the rate at which fasted copepods recover. Taken together, these results allow more detailed understanding of how anticipated changes in temperature and dietary resources affect the ecophysiology of widespread *Eudiaptomus* in nature and their ability to cope with natural challenges such as fasting.

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