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Differential effects of phosphorus and fatty acids on *Daphnia magna* growth and reproduction

Claes Becker [1]
Max-Planck-Institut für Limnologie, Postfach 165, 24302 Plön, Germany

Maarten Boersma
Alfred-Wegener-Institut für Polar und Meeresforschung, Biologische Anstalt Helgoland, Postfach 180, 27483 Helgoland, Germany

Abstract

We investigated the effects of various mineral and biochemical limitations on *Daphnia magna.* These daphniids have much lower saturation thresholds for growth for the polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA), and arachidonic acid (ARA) than has been previously described for other *Daphnia* species. Daphniids take up large amount of fatty acids from food, and different fatty acids are handled differently by *D. magna.* The saturated fatty acid (20:0; EPA) was not retained, and metabolized, the PUFAs were preferably stored. There were also differences among the PUFAs: EPA was found in higher concentrations in the eggs than ARA. In contrast, although there were some variations in *D. magna* phosphorus levels with varying levels of phosphorus in the food, these differences were small compared with the changes in *D. magna* fatty-acid concentrations. Independent of these small changes, the P content of eggs was constant at 14 mg P (g dry wt)$^{-1}$. Storage of EPA, but not P, fully compensated *D. magna* growth during periods of bad food quality. Egg production was a major drain of fatty acids from female *D. magna.*

All consumers require essential nutrients in their food, and limitations of these nutrients induce changes in their life histories. In freshwater, phosphorus (P) and fatty acids are particularly important in zooplankton nutrition. In zooplankton, most of the P is found in DNA, RNA, and phospholipids (Vrede et al. 1999). Essential fatty acids are necessary in cell membranes and probably also as precursors involved in immune responses (Vance and Vance 1985). The location of the double bonds from the methyl end of the fatty-acid molecule determines the essentiality of the fatty acid, because most animals lack the specific desaturases to insert double bonds at the ω3 and ω6 positions of the fatty-acid molecule (Vance and Vance 1985). Another (nonessential) trait of fatty acids is that they are an effective energy storage that can be utilized when food conditions are poor. Zooplankton can accumulate considerable amounts of lipids (up to 40% of dry weight; Goulden and Place 1990). Zooplankters derive most (98%) of their lipids from food; only a small fraction is synthesized de novo (Goulden and Place 1993). The lipid accumulation process is tightly coupled to egg production, where each cycle is preceded by a lipid increase (Tessier and Goulden 1982).

Compared with lipids, zooplankton storage and utilization of P is less well understood. Typically, daphniids have high requirements for phosphorus and are considered to be homeostatic consumers (Sterner 1990). To maintain constant C : P ratios, daphniids make physiological adjustments when the food quality (P content) changes—for example, they increase their excretion and respiration rates when food becomes P deficient (Darchambeau et al. 2003). This increase in respiration rate could be the result of increased *Daphnia* feeding activity (Plath and Boersma 2001). Homeostasis implies that daphniids should not have luxury consumption rate of P, because a stored pool of P would alter the somatic C : P ratio. In daphniids, the largest compartments of P are nucleic acids (40–60%) and phospholipids (20%; Vrede et al. 1999). Moreover, daphniids with low amounts of RNA enhance the P content of ''other'' P compounds; Vrede et al. (1999) suggested that these compounds were free nucleotides that enable them to maintain homeostasis. However, several studies have shown that homeostasis for P is not as strict as was previously believed (DeMott et al. 1998; Plath and Boersma 2001; DeMott 2003). Furthermore, Sterner and Schwalbach (2001) showed that daphniids could utilize stored P during short periods of P limitation.

The interaction between zooplankton and phytoplankton is very well suited to investigate questions of nutritional quality and essential components of food; both the consumers and their food are easy to culture; and, because cladoceran zooplankters, such as *Daphnia,* are cyclical parthenogens, using clones reduces variation in experiments. Although some studies have focused on the effects of amino acids and vitamins (and, recently, also sterols) in the food of zooplankters, the main interest has been minerals (especially P, but also nitrogen and selenium) and fatty acids. Algae with low concentrations of P are poor food for daph-

[1] Corresponding author. Present address: Michigan State University, WK Kellogg Biological Station, Hickory Corners, Michigan 49060 (beckerc@kbs.msu.edu).

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niids (Sterner et al. 1993; Urabe et al. 1997; DeMott et al. 1998), and the quantities of polyunsaturated fatty acids (PUFAs) are important for *Daphnia* growth both in field (Müller-Navarra 1995*b;* Wacker and von Elert 2001; Becker et al. in press) and laboratory (DeMott and Müller-Navarra 1997; Park et al. 2002; von Elert 2002) studies. However, even though the importance of PUFAs is known, our understanding of the concentration ranges these resources are required in is still meager. This knowledge is important because it allows the assessment of the probability of PUFA limitation in the field. On the basis of correlations between *D. galeata* growth rates and seston eicosapentaenoic acid (EPA) concentrations in a mesotrophic lake over an entire season, Müller-Navarra (1995*b*) established the saturation threshold to be 0.8 μg EPA L^{-1}. Recently, fatty-acid enrichment techniques have been developed, and this allows additions of single fatty acids to algal cells; mixing enriched and unenriched algae enables the establishment of a gradient of fatty-acid concentrations and, hence, the determination of saturation thresholds.

In this study, we set out to investigate the effect of different nutritional limitations on the growth and reproduction of *D. magna*, contrasting the narrow range of P within the animals with PUFAs, which are stored in substantial amounts. We addressed the following questions. (1) How do daphniids cope with excess amounts of essential components of the food? Are they stored, and, if so, are they reallocated during periods of bad food quality? (2) How do daphniids divide up essential nutrients between somatic growth and reproduction? Are there differences between P and PUFAs?

To answer these questions, we performed several experiments that studied (1) fatty-acid saturation thresholds for *D. magna* growth using gradients of fatty-acid additions; (2) storage of EPA by *D. magna;* (3) how the specific P content of females and eggs change when they are fed a food ranging in molar C : P ratios; (4) how different fatty acids are stored and allocated for reproduction; (5) whether EPA and P can be reallocated from storage to balance periods of poor food quality; and (6) how EPA is allocated to reproduction.

Material and methods

Algae and animals—We used a clone of *D. magna* from a pond in Frankfurt and kept at the Max Planck Institute for Limnology for many years. All experiments were conducted at 20°C in constant, dim light. In all experiments, neonates <24 h old were collected from third brood females fed P-sufficient *Scenedesmus obliquus* in ample supply (1 mg C L^{-1}) during their whole life. The neonates were pooled and randomly divided over the different treatments. To reduce the effect of uncontrolled P addition, daphniids in all experiments were kept in the P-free growth medium Aachener Daphnien medium (ADaM; Klüttgen et al. 1994). We used two different food sources: P-sufficient *S. obliquus,* from a continuous culture, and semicontinuously cultured P-limited *S. obliquus.* In the cultures, we used Z/4 medium (Zehnder and Gorham 1960) with two different P concentrations (1.39 mg P L^{-1} and 83.5 μg P L^{-1}). All algae were obtained from stock cultures of the Max Planck Institute for Limnology, Germany. During all experiments, daphniids were transferred daily into fresh food suspensions or kept in a flow-through system.

General experimental procedure—To investigate how different essential compounds affect the fitness and the stoichiometry of *D. magna* we performed a range of experiments. We first investigated how *D. magna* responded to different food conditions in flow-through experiments. Six pooled newborn neonates were placed in flow-through vessels filled with 120 ml of the different experimental foods added at a rate of 1 L d^{-1} to ensure constant food concentrations. The initial dry weight of daphniids was determined from a subsample of pooled individuals. At each sampling, three to six neonates were transferred to preweighed aluminum boats, dried overnight at 60°C, and weighed to the nearest 0.1 μg on a Sartorius microbalance. Growth rates were computed from the dry-weight increments. We investigated how nutrients are incorporated into *D. magna* tissue and thus affect the stoichiometry of the animals in experiments using 1-liter jars. All daphniids were pooled before the experiments and randomly divided over the different treatments. In the studies where the fatty-acid stoichiometry was investigated, triplicates or quadruplicates with 20 individuals each were used; when P stoichiometry was considered, quintuplicates were stocked with 10 individuals each. Before sampling, the daphniids were transferred to a suspension of unenriched control algae for 45 min, to minimize the effect on ingested but not yet incorporated nutrients.

Nutrient enrichment techniques—P-sufficient *S. obliquus* were enriched with three different fatty acids, eicosanoic acid (20:0, ESA), 5,8,11,14,17-eicosapentaenoic acid (20:5ω3, EPA), and 5,8,11,14-eicosatetraenoic acid (20:4ω6, arachidonic acid [ARA]). We also had a control treatment that was incubated like the other treatments, only differing in the fatty-acid addition. In the experiment where the food quality was alternated, P-limited *S. obliquus* was enriched with EPA. The fatty-acid enrichments were conducted according to the method of von Elert (2002), by incubating *S. obliquus* in a suspension of single fatty acids and bovine serum albumin (BSA) for 13 h, which is the period that algae need to reach saturation (von Elert 2002). The food suspensions were diluted with ADaM to reach food concentrations of 1 mg C L^{-1}. The different P treatments were obtained according to the method of Plath and Boersma (2001) by enriching P-limited *S. obliquus* diluted in ADaM with various pulses of K_2HPO_4.

Analyses—At three dates during the experiments, algal samples for carbon, P, and fatty-acid analysis were sampled by filtration over GF/C filters (Whatman) directly after the new food suspensions were mixed. The samples for carbon and nitrogen were dried at 60°C overnight and stored in a desiccator until analysis with a FISONS NA2000 elemental analyzer. The particulate P samples were analyzed directly, by oxidation, and subsequently by the ammonium-molybdate method on a spectrophotometer at 720 nm. Samples for fatty-acid analysis were stored at −18°C in Eppendorf vials under N_2 gas until analysis. The fatty acids were extracted

and esterified according to the method of Wiltshire et al. (2000) and quantified in a gas chromatograph using the temperature configuration of von Elert (2002). The fatty-acid methyl esters were identified by comparison of retention times of known reference compounds. We used heptadecanoic acid methyl-ester (17:0) and tricosanoic acid methyl-ester (23:0) as internal standards.

Fatty-acid demand and stoichiometry—To investigate in which amounts fatty acids are important for *D. magna* growth, we performed an experiment with variable fatty-acid concentrations. An array of EPA and ARA concentrations in the food were obtained through mixing control algae with enriched algae in different proportions: 1.0, 0.5, 0.25, 0.125, and 0 of enriched algae; we used a logarithmic gradient because we expected the largest differences in the lower range of the fatty-acid gradient. The juvenile growth rate was determined over 3 d. This experiment was followed up to confirm growth-rates results and to determine how the daphniids take up and incorporate fatty acids when they are above the fatty-acid saturation threshold. These experiments used a concentration gradient of EPA obtained with the same dilution technique and the gradient as described above, and neonates were cultivated in 1-liter jars on daily renewed food suspensions in quadruplicate. Experiments were terminated after 6 d, followed by dry-weight determinations and fatty-acid analysis.

Female and eggs—We studied how *D. magna* allocates essential nutrients between females and eggs by alternating both P conditions and availability of fatty acids in the food. Several of the food sources used in this study do not support juvenile growth, and, hence, did not allow us to study effects on reproduction. Therefore, to study allocations between eggs and adults, we worked with adult daphniids. These females were obtained by cultivating <24-h-old neonates on P-sufficient *S. obliquus.* At the age of 10 d, most of these individuals were carrying their first brood of eggs, and the females were pooled and randomly divided over the experimental treatments. To study the effects of P, we performed two consecutive experiments alternating C : P molar ratios of P-limited *S. obliquus* enriched with P using the method from Plath and Boersma (2001). In the first experiment, the C : P ranged between 433 and 93 and in the second between 848 and 477. For the fatty acids, we studied how the daphniids incorporated different fatty acids when fed P-sufficient *S. obliquus* enriched with ESA, EPA, ARA, and an unenriched control. After 7 d of feeding, the experiment was terminated. For the sampling, we selected females with eggs in their spherical phase, to ensure that the eggs were in similar stages, which, during favorable food conditions corresponds to eggs ~30 h old (Sobral et al. 2001). Eggs from the brood pouch of these females were removed with a gentle current of a small syringe. From each replicate, we concentrated the eggs in Eppendorf tubes. The samples for P analysis were transferred to preweighed silver capsules in a drop of ADaM and dried at 60°C overnight; samples were ashed at 550°C and analyzed for P content, calculated as mg P (g dry wt)$^{-1}$.

Table 1. Schedule of food regimes describing timing and the proportion (in percent) of the enrichments the daphniids received each day. The six different treatment identifications denote timing in days (numbers) and availability (letters) of enriched (E) or control (C) food. Over the 6-d period, all treatments received an equal total amount of enrichments (except treatment 6C). All food suspensions had a concentration of 1 mg C L^{-1} of P-limited *S. obliquus*.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1E : 1C	33	0	33	0	33	0
1E : 2C	50	0	0	50	0	0
3E : 3C	33	33	33	0	0	0
3C : 3E	0	0	0	33	33	33
6E	17	17	17	17	17	17
6C	0	0	0	0	0	0

Alternating food conditions—To determine whether daphniids during bad food conditions can use stored fatty acids and P for growth, we performed experiments in which the availability of P and EPA was alternated over a 6-d period. In both experiments, we used P-limited *S. obliquus* (1 mg C L^{-1}) enriched with P and EPA using the methods of Plath and Boersma (2001) and von Elert (2002). We used P-limited *S. obliquus* as well in the EPA experiment, because they have low content of EPA (Müller-Navarra 1995*a;* Boersma 2000), which limits *D. magna* growth (Becker and Boersma 2003). To ensure that EPA-enriched *S. obliquus* were not P limited, a pulse of P was added before replacing the food suspensions. In total, we had six different treatments in quintuplicate, for a total of 30 vessels per enrichment (EPA and P), with six neonates each. During the growth phase, the neonate daphniids were fed enriched and unenriched food at various times. To ensure the same availability of each addition over the total experimental period, the enriched food was given in different concentrations (Table 1). Growth rates were determined over 6 d. Initially, we performed experiments with EPA and P additions in flow through, to ensure stable food concentrations. Consequently, in the flow-through vessel (120 ml), there will be a time lag before a suspension is fully replaced. Unfortunately, this fact, in combination with the very low EPA concentration threshold for *D. magna* (*see Fig. 1*), only induced gradients of EPA limitations and made our results impossible to interpret from a storage utilization perspective. Therefore, it was necessary to redo the experiment in a batch setup where dilution gradients could be avoided by daily transferring the daphniids into new food suspensions.

EPA and egg production—To determine how important EPA is for egg production, we enriched adult females with EPA, as described above, for 7 d. Thereafter, the females were transferred to control *S. obliquus* and cultured on this food for 6 d. Samples for fatty-acid analysis of females and their eggs were collected after 45 min, 3 d, and 6 d of feeding. The interval between samplings corresponds roughly to one egg production cycle of *D. magna*. Furthermore, to estimate how important egg production is for EPA losses in the daphniids, we calculated the predicted total EPA content for the females and the released broods (e.g., female day 3

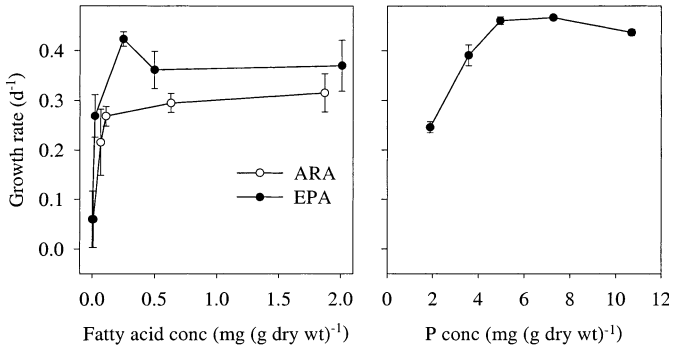

Fig. 1. *D. magna* juvenile growth rates for on P-sufficient *S. obliquus* enriched with various concentrations of ARA and EPA. For the P gradient, P-limited algae were spiked with various concentrations of P (Plath and Boersma 2001). The food concentration was 1 mg C L^{-1}. Fatty-acid concentrations are given in mg (g dry wt)$^{-1}$. In brackets are ±SE for quintuplicate studies.

+ brood day 0) and compared these with the initial amounts in the females.

Results

Enrichment of algae—We enriched P-sufficient *S. obliquus* with three different fatty acids. This enrichment technique allowed the addition of a single fatty acid to the algae without affecting the others (Table 2). Nevertheless, in our study, we found a slight increase of the closest similar fatty acid—for example, enrichment with 20:4ω6 also affected the content of 20:3ω6, and enrichment with 20:5ω3 also enhanced 20:4ω3 (Table 2).

Fatty-acid demand and stoichiometry—We found that even the lowest addition of PUFAs, 0.06 and 0.02 mg (g dry wt)$^{-1}$ for ARA and EPA, respectively, increased the growth rates of *D. magna* significantly compared with the controls (analysis of variance [ANOVA]; $p < 0.001$) (Fig. 1). Enhancing the ARA and EPA contents furthermore had no effect on growth (Newman-Keuls' test). Because of unexpectedly low growth on the control *S. obliquus*, we repeated the EPA dilution gradient. In this setup, we also determined how fatty acids are incorporated into the *D. magna* biomass. The results were the same concerning growth and EPA concentrations; the lowest EPA concentration (0.27 mg EPA g dry wt^{-1}) supported higher growth than the control (Fig. 2) (ANOVA; $p < 0.001$), and the higher EPA concentrations did not have any effect on growth when the control was omitted (ANOVA; $p = 0.68$). Daphniids increased their somatic EPA content with increasing EPA content in the food (ANOVA; $p < 0.001$) and retained a higher proportion of the EPA about the double concentration of EPA in the daphniids than the algae (Fig. 2; $y = 2.08\times-4\times10^{-11}$; $r^2 = 0.84$; $p < 0.001$).

Stoichiometry of female tissues and eggs—The C : P molar ratio of food had a strong effect on the P content of *D. magna.* There was a decrease in P content of the daphniids with elevated molar C : P ratios (Table 3; Fig 2); the P content of eggs was stable, despite being variable in females (Table 3; Fig. 3). Overall, the P content of females decreased from ~17 to 9.0 mg P (g dry wt)$^{-1}$ (ANOVA; $p < 0.001$); eggs, on the other hand, remained stable at ~14 mg P (g dry wt)$^{-1}$ over all treatments (Fig. 3) (ANOVA; $p = 0.75$). When grown in P-sufficient conditions, below a C : P of ~350 (Brett et al. 2000; Becker and Boersma 2003), P was equally distributed between eggs and females (two-way ANOVA between stage and C : P (93–328); $p = 0.45$). However, P decreased in females with elevated C : P molar ratios, and this decrease was already considerable in the nonlimiting C : P range (93–328, Fig. 3; ANOVA; $p < 0.01$). At the higher C : P ratios in experiment 2, the eggs had a considerably higher P than the females (Newman-Keuls' test; Table 3, Fig. 3).

Different fatty acids enriched in the food were incorporated in adult *D. magna* with different efficiencies. In these analyses, two ''neutral fatty acids,'' 18:3ω4 and 20:1ω9, that were not enriched, were included for comparison. These fatty acids belong to other ω-families than ω3-EPA and should therefore not be affected by our experimental additions. Overall, there was a tendency for fatty acids to be in higher concentrations, per dry weight, in eggs than the females (Table 4). Neither ARA nor 20:1ω9 was preferably allocated in the eggs (Table 4). All fatty-acid additions had a significant effect on the somatic fatty-acid content of daphniids (Table 4; Fig. 4). Only the EPA enrichment of the algae increased the concentration within the eggs compared with the females (post hoc comparisons, Newman-Keuls' test; $p < 0.001$).

Alternating food conditions—We determined whether daphniids could reallocate stored resources of P or EPA during periods of lower food quality. In these experiments, P additions altered the C : P ratios between 70 and 460, and the enrichments of EPA ranged 0–1.6 mg EPA (g C)$^{-1}$. When EPA storage reallocation was considered in batch experiments, we found a positive effect of the EPA additions (Fig. 5A, ANOVA; $p < 0.001$). Post hoc comparison showed that all treatments had higher growth rates than treatment 6C and that there were no differences between the other treatments (Newman-Keuls' test). When the P content was alternated, we found an overall significant difference between the treatments (Fig. 5B, ANOVA; $p < 0.001$). All treatments with alternating food conditions affected *D. magna* growth rates negatively, and these treatments supported intermediate growth levels between the two extremes, treatments 6E and 6C (Newman-Keuls' test).

EPA and egg production—To compare the effect on EPA, we again used 18:3ω4 and 20:1ω9, which, in contrast to EPA, were continuously available in the food. At the start of the experiment, females had a higher absolute EPA content than the broods (Fig. 6C). During the initial 3-d period, EPA decreased in absolute amount over time in both eggs and adults (Table 5, Fig. 6C) but the decrease was larger in females (39% vs. 23% for the brood). Over the whole 6-d period, there was a similar decrease between the females and the broods (60% vs. 67%). Conversely, the contents of 18:3ω4 and 20:1ω9 that were available in food increased both in females and eggs over time (Table 5, Fig. 6A,B). To es-

Table 2. Fatty-acid concentrations of P-sufficient *S. obliquus* [mg (g C)$^{-1}$] enriched with three different fatty acids used as food source (1 mg C L^{-1}) for *D. magna*. The values are mean from triplicate samples (SE). Fatty acids marked with letters in superscript represent an overall significant difference betweeen the additions (ANOVA; $F_{3,8} > 5.7$; $p < 0.05$) where treatments marked with identical letters are not significantly different (Newman-Keuls' test).

Parameter	Control	Saturated	EPA	ARA
14:0	0.54 (0.02)	0.55 (0.04)	0.45 (0.01)	0.47 (0.06)
16:0	49.23 (3.01)	47.94 (5.30)	38.75 (3.53)	39.76 (3.79)
15:1ω7	0.36 (0.04)	0.35 (0.02)	0.27 (0.02)	0.27 (0.01)
18:0	7.95 (0.45)^{A}	8.11 (0.48)^{AC}	6.75 (0.12)^{BC}	6.32 (0.28)^{B}
18:1ω12/ω9/ω7	87.29 (11.49)	72.02 (8.31)	73.54 (5.26)	69.21 (2.48)
18:2ω6	7.11 (1.35)	5.93 (2.27)	6.73 (0.57)	6.07 (1.95)
18:3ω6	0.84 (0.15)	0.89 (0.13)	0.54 (0.02)	0.67 (0.06)
18:3ω4	0.60 (0.24)	0.54 (0.17)	0.48 (0.19)	0.52 (0.17)
18:3ω3	4.97 (1.15)	4.37 (2.00)	5.18 (0.66)	4.65 (1.76)
18:4ω3	0.94 (0.25)	0.84 (0.41)	1.51 (0.21)	0.90 (0.36)
20:0	0.39 (0.05)^{A}	14.11 (0.92)^{B}	0.47 (0.04)^{A}	0.55 (0.01)^{A}
20:1ω9	0.62 (0.11)	0.49 (0.19)	0.72 (0.05)	0.97 (0.07)
20:2ω6	0.13 (0.02)	0.04 (0.04)	0.04 (0.03)	0.10 (0.00)
20:3ω6	0.01 (0.01)^{A}	0.05 (0.01)^{A}	0.02 (0.02)^{A}	0.28 (0.10)^{B}
20:4ω6	0.01 (0.01)^{A}	0.04 (0.03)^{A}	0.02 (0.01)^{A}	3.85 (1.33)^{B}
20:3ω3	0.03 (0.03)	0.04 (0.04)	0.06 (0.03)	0.11 (0.07)
20:4ω3	0.00 (0.00)^{A}	0.00 (0.00)^{A}	0.45 (0.01)^{B}	0.01 (0.01)^{A}
20:5ω3	0.00 (0.00)^{A}	0.00 (0.00)^{A}	4.47 (0.68)^{B}	0.00 (0.00)^{A}
22:0	0.77 (0.03)	0.79 (0.06)	0.43 (0.22)	0.65 (0.02)
22:5ω3	0.15 (0.07)	0.16 (0.02)	0.10 (0.05)	0.14 (0.01)
22:6ω3	0.14 (0.01)^{A}	0.11 (0.02)^{AB}	0.06 (0.01)^{B}	0.10 (0.01)^{AB}
24:0	0.29 (0.12)	0.34 (0.05)	0.31 (0.02)	0.30 (0.05)
24:1ω9	0.00 (0.00)	0.02 (0.02)	0.01 (0.01)	0.03 (0.01)
SAFA	59.17 (3.24)^{AB}	71.84 (5.37)^{A}	47.15 (3.42)^{B}	48.06 (4.01)^{B}
MUFA	88.27 (11.55)	72.88 (8.53)	74.54 (5.26)	70.48 (2.43)
PUFA	14.95 (3.04)	13.02 (4.76)	19.67 (2.18)	17.40 (5.55)
HUFA	1.24 (0.30)^{A}	1.16 (0.44)^{AB}	6.61 (0.92)^{C}	4.99 (1.67)^{BC}
ω3	6.24 (1.49)	5.53 (2.42)	11.83 (1.56)	5.91 (2.09)
ω6	8.11 (1.31)	6.95 (2.17)	7.37 (0.61)	10.97 (3.29)
ω3:ω6	0.75 (0.05)^{A}	0.74 (0.09)^{A}	1.59 (0.09)^{B}	0.51 (0.05)^{A}

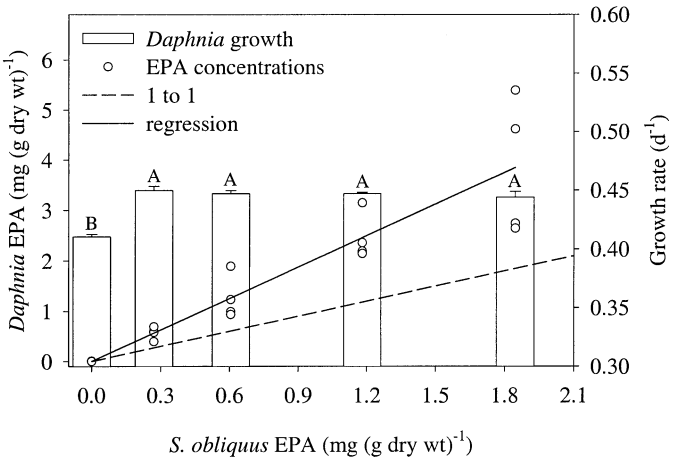

Fig. 2. *D. magna* juvenile growth rates and the incorporation of EPA over 6 d when fed P-sufficient *S. obliquus* enriched with EPA and diluted with control algae to achieve five different concentrations of EPA. The regression equation is $y = 2.08x - 4 \times 10^{-11}$; $r^2 = 0.84$; $p < 0.001$. Error bars are ±SE from quintuplicate studies. Bars marked with identical letters are not significantly different (Newman-Keuls' test).

timate how egg production affected the EPA content of females, we computed the total EPA content of females plus the previous brood or broods. The sum of EPA content from days 3 and 6 was higher than the content of the initial females (Fig. 6C). This indicates an overall accumulation of EPA when the egg production was neglected. When only the concentrations of EPA in eggs and females were considered, the eggs had higher concentrations of EPA (mg g C^{-1}) over the whole experiment period than the adults (Fig. 6D).

Discussion

Nutritional requirements—Many studies have used phytoplankton of various nutritional states—that is, with different fatty-acid contents—to study which factors that determine *Daphnia* growth and development in the field (Müller-Navarra 1995*b;* Brett and Müller-Navarra 1997; Wacker and von Elert 2001) and in laboratory studies (Müller-Navarra 1995*a;* Park et al. 2002). Recent studies have also used various techniques to enrich the *Daphnia* diet with different fatty acids, be it fatty acid emulsions, microencapsulated lipids, or PUFA-rich algae (Sundbom and Vrede 1997; Weers and Gulati 1997; Plath and Boersma 2001). The disadvantage of these techniques is that mixtures of fatty

Table 3. Summary table of 2-way ANOVAs testing for differences in specific P content of *D. magna* females and their eggs (stage), *p* values of two-way ANOVAs. Experiment 1 was conducted with *S. obliquus* with C : P ratios ranging from 93 to 433 and experiment 2 from 477 to 848.

Experiment	C : P ratio	Stage	Interaction
1	<0.001	0.68	<0.001
2	0.99	<0.001	0.39
1 and 2	<0.001	<0.001	<0.001

acids were used, which made it difficult to attribute effects to a single fatty acid. Recently however, various techniques to enrich *Daphnia* food with single fatty acids were developed (von Elert 2002; Ravet et al. 2003). Overall, there is now a substantial amount of evidence that indicates that various ω3 fatty acids in general and EPA in particular are important determinants for the food quality of daphniids (*but see* von Elert and Wolffrom 2001; von Elert et al. 2003). However, most studies have only described the effect on growth by switching the availability of fatty acids between present or not present, although recently Ravet et al. (2003) investigated gradients of fatty-acid mixtures; to our knowledge, no one has investigated gradients of single fatty acids. Hence, it was not possible to assess the quantities of single compounds necessary for growth. This is a very important question, because establishing saturation thresholds would help assess the likelihood of fatty-acid limitations in the field. Furthermore, it is important to determine whether the amounts used in previously mentioned studies were in this range, especially because some reports have indicated that free fatty acids can actually be deleterious (D'abramo 1979; Reinikainen et al. 2001). Moreover, it is possible that fatty

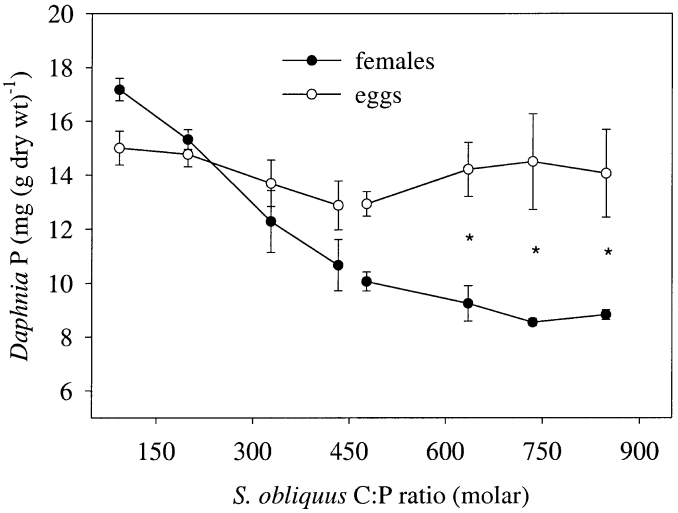

Fig. 3. *D. magna* P content (mg g dry wt^{-1}) of females and eggs after 7 d of feeding on *S. obliquus* of various C : P molar ratios. Data are from two consecutive experiments—experiment 1 was conducted with lower C : P ratios and experiment 2 with higher C : P ratios. Asterisks denote significant differences between female and eggs (Newman-Keuls' test). Error bars are ±SE for quintuplicate studies.

Table 4. Summary table of two-way-ANOVAs testing for differences in the somatic fatty-acid composition of *D. magna* dependent of various fatty-acid additions (EPA, ARA, and ESA) and the allocation of various fatty acids between females and their eggs (stage), *p* values of two-way ANOVAs, NS = nonsignificance. The females were fed P-sufficient *S. obliquus* (1 mg C L^{-1}) enriched with fatty acids.

Parameter	Stage	FA addition	Interaction
18:3ω4	<0.001	NS	NS
20:1ω9	NS	NS	NS
ESA, 20:0	<0.01	<0.05	NS
ARA, 20:4ω6	NS	<0.001	NS
EPA, 20:5ω3	<0.001	<0.001	<0.01

acids in great surplus could be used differently than those administrated in the ''natural'' range. To investigate this, we determined *D. magna* growth on concentration gradients of fatty acids.

We used P-sufficient *S. obliquus* enriched with different fatty acids in a logarithmic dilution gradient of natural concentrations (Müller-Navarra 1995*b;* Ahlgren et al. 1997), concentrating on two polyunsaturated fatty acids with the same length but from different ω-families (ω6-ARA and ω3-EPA). These fatty acids might be used similarly within animals (e.g., in membranes and prostaglandins), although the differences between the fatty acids might also induce dissimilar effects. ARA and EPA were supplemented in equal amounts with *S. obliquus* (Table 2, Fig. 1). The highest concentrations for both EPA and ARA were ~2 mg fatty acids (g dry wt)$^{-1}$ (~4 μg fatty acids L^{-1}). Even in the lowest EPA additions in both experiments (0.02 and 0.27 mg EPA g dry wt^{-1}) the growth rates were higher than the unenriched controls. Above these concentrations, increased enrichments did not affect the growth rates (Figs. 1, 2). This pattern is contradictory to the findings of: Müller-Navarra (1995*b*), where *D. galeata* growth rates leveled off at >0.8 μg EPA L^{-1}. It is possible that the fatty-acid requirements differ be-

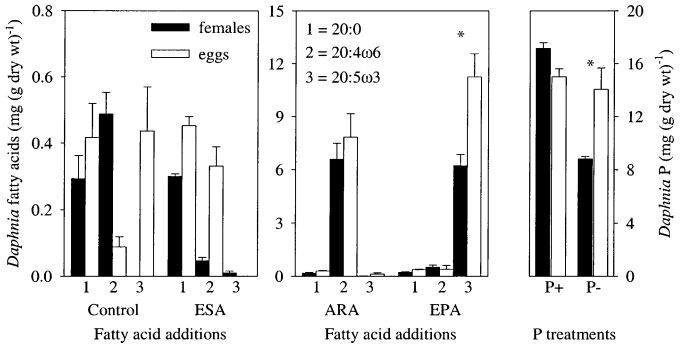

Fig. 4. Fatty-acid (FA) and P content (mg g dry wt^{-1}) of *D. magna* females and their eggs. For 7 d, the daphniids were fed either P-sufficient *S. obliquus* enriched with FAs or P-limited *S. obliquus* spiked with a pulse of P to achieve C : P of 93 (P+) or 848 (P−). The FA enrichments were control, ESA (20:0), ARA (20:4ω6), and EPA (20:5ω3). The number 1–3 describes the content of each FA. Asterisks denote significant differences between female and eggs (Newman-Keuls' test). Error bars are ±SE for four (FA) respective quintuplicate studies.

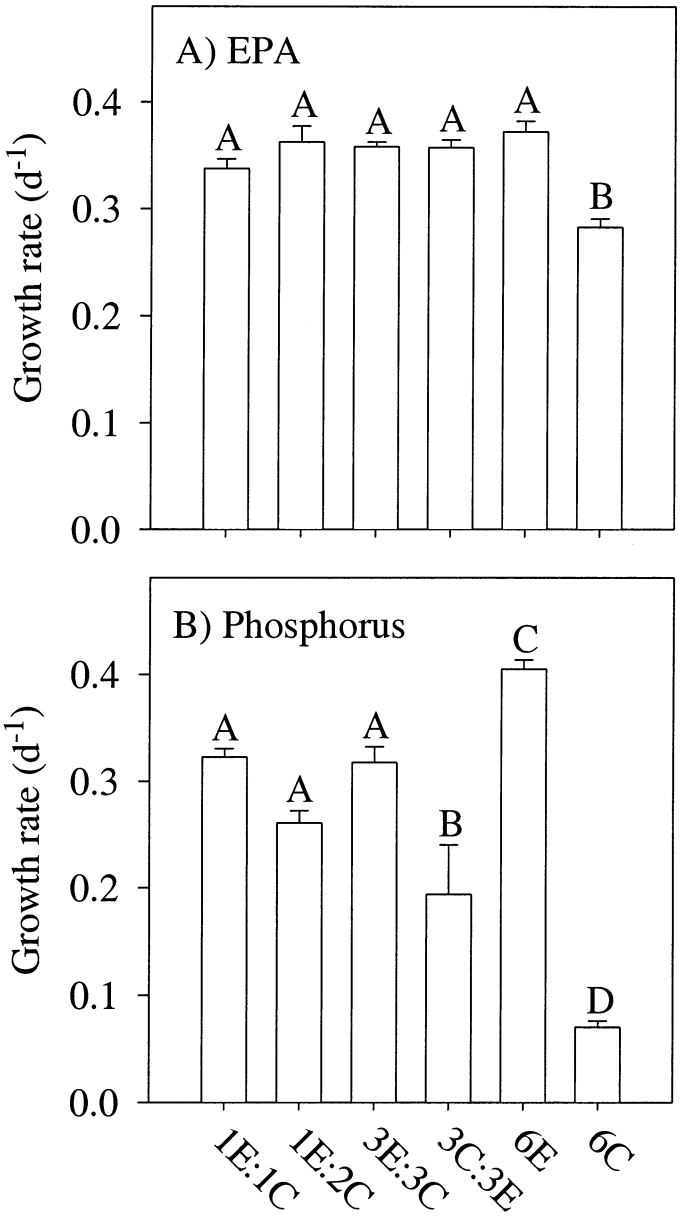

Fig. 5. Effects on *D. magna* juvenile growth rate over 6 d on variable food conditions in regards to EPA and P content. The *X*-axes indicate the timing of food regimes where numbers indicate time (d) and letter enriched (E) or control (C) food availability. For details, see Table 1. All juveniles were fed P-limited *S. obliquus* that were enriched with EPA and P according to Table 1. After 6 d, the experiment was terminated. Error bars denote ±SE for quintuplicate studies. Bars marked with identical letters are not significantly different (Newman-Keuls' test).

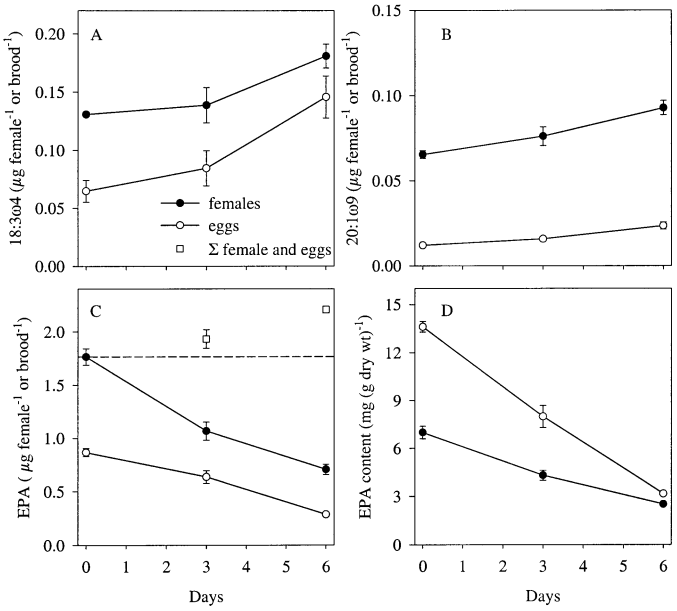

Fig. 6. Development of the fatty acid pool within adult *D. magna* and their eggs over 6 d. The daphniids were fed P-sufficient *S. obliquus* enriched with EPA for 6 d, after which all daphniids were fed unenriched, P-sufficient *S. obliquus*. (A) The total content of 18:3ω4 female^{-1} or brood^{-1}. (B) The total content of 20:1ω9 female^{-1} or brood^{-1}. (C) The total content of EPA female^{-1} and brood^{-1}. Squares describe the sum fatty acid of females plus the previous brood or broods. (D) The concentration of EPA (mg g dry wt^{-1}). Error bars are ±SE from triplicate samples.

tween *Daphnia* species, because food concentration thresholds typically decline with increasing body size (Gliwicz 1990). Hence, if this pattern is also applicable to EPA concentrations, large *D. magna* should have a lower EPA threshold than *D. galeata*. Nevertheless, our findings suggest that the EPA requirement for *D. magna* growth is far less than their requirements for P (2–4 mg P g dry wt^{-1}; Fig. 1; Plath and Boersma 2001). Moreover, 0.8 μg EPA L^{-1} (*D. galeata*, Müller-Navarra 1995*b*) and 0.04 μg EPA L^{-1} (*D. magna*, this study) are considerably lower than the enrichments used in recent studies; ~8 (Becker and Boersma 2003), 105 (von Elert and Wolffrom 2001), and 146 (von Elert 2002) μg EPA L^{-1}. We also found elevated growth with the addition of ARA, although this caused lower growth than EPA (Fig. 1; two-way ANOVA, $p < 0.05$). The elevated growth with ARA additions was not consistent with the findings of von Elert (2002), even though he also observed a slight but nonsignificant growth increase with this particular fatty acid compared with the control. Only the additions of three different ω3 fatty acids had a positive effect on *D. galeata* growth in his study. From these experiments, we conclude

Table 5. Summary table of differences in the total somatic fatty-acid distribution of *D. magna* per female and brood for three repeated measurements from Fig. 5A–C. The females were fed P-sufficient *S. obilquus* enriched with EPA for 6 d before the first sampling, after which all daphniids were fed unenriched P-sufficient *S. obliquus* until the experiment was terminated after 6 d. *F* and *p* (values of time (0, 3, and 6 d) vs. stage (female and brood), repeated measurement analysis; NS = nonsignificance.

	Female vs. brood		Time		Interaction	
Parameter	$F_{1,4}$	p	$F_{2,8}$	p	$F_{2,8}$	p
18:3ω4	22.0	<0.01	15.6	<0.01	0.80	NS
20:1ω9	1233	<0.001	15.7	<0.01	2.5	NS
EPA, 20:5ω3	149	<0.001	99.2	<0.001	10.94	0.001

that PUFAs and EPA are important food quality determinants that enhance the growth of daphniids. However, the fatty-acid requirements for growth were fulfilled even with minute additions—that is, larger additions did not improve growth further.

Nutrient storage—In general, daphniids can allocate resources for different purposes: reproduction, storage, somatic growth, and maintenance. Of these, investments in egg production seem to be the most sensitive to starvation. Daphniids store lipids in a cyclic process that is tightly coupled to egg production (Tessier and Goulden 1982). Each egg-production cycle is preceded by visible increases in lipids, which decrease when eggs are produced. Egg production is determined by the energy availability during the first part of each instar (Bradley et al. 1991). Thus, females enrich their eggs with a pool of fat, which improves neonates' resistance to starvation.

The increased concentration of EPA, ARA, and ESA fatty acids in food resulted in higher contents of these compounds in *D. magna* (Table 4). As expected, these fatty-acid additions did not affect the content of 18:3ω4 or 18:1ω9 (Table 4). Although significant, the net incorporation of ESA was much lower than that of ARA and EPA (Fig. 4). These two compounds were preferably accumulated in females and eggs. Concentrations of EPA were considerably higher in the eggs than those of ARA (Table 4), which suggests the selective storage of PUFAs and that the ESA is metabolized either for energy or into other compounds (Fig. 4). The higher concentration of the EPA in the eggs of enriched daphniids also suggests a potentially higher need for this fatty acid for neonatal development.

The EPA saturation threshold for *D. magna* was very low. If this is a general pattern for daphniids, this suggests that these zooplankters often face an environment above this threshold. We investigated how the *D. magna* handle this surplus and studied whether they incorporate EPA under different levels of availability. To our knowledge, only one study has investigated *D. galeata* incorporation of fatty acids from a food source with variable fatty-acid concentrations (Weers et al. 1997), where the daphniids were fed a diet of *Chlamydomonas* with emulsions differing mainly (but not solely) in docosahexanoic acid (DHA):EPA ratios. Weers et al. (1997) found that when the EPA content varied in the food, it was retained in the same proportions in the daphniids; on the other hand, when the concentration of DHA was altered in food, the *Daphnia* DHA concentration remained low. Olsen (1999) also described this relationship between food and rotifers (*Brachionus plicatilis*), which initially incorporated similar proportions ($\sim$1:1) of ω3 and ω6 fatty acids as in the food. This relationship reached a plateau when the amount of ω3 fatty acids in the food increased to $>$60% of total fatty acids. We found quite another pattern when the EPA availability was altered: *D. magna* incorporated twice as much EPA than the concentrations in the food (mg g dry wt^{-1}; Fig. 2). Evidently, these daphniids can retain considerable amounts of EPA. However, it is unclear how and for what the storage can be reallocated.

Daphniids are more or less homeostatic consumers (Sterner 1990) and should therefore have limited possibilities to store P. The fact that up to 40% of the zooplankton dry weight consist of carbon-rich lipids (Goulden and Place 1990), which fluctuate considerably during each egg-production cycle (Tessier and Goulden 1982), makes this difficult to understand. During the lipid accumulation phase, the carbon content of the female increases. Conversely, the carbon content of females decreases when the eggs are transferred to the brood pouch. However, such changes will, to some extent, be hidden when whole animals are sampled (female + eggs). The carbon content will probably show a larger variation when only the females are analyzed. Nevertheless, in order for the females to maintain a constant C:P ratio over an egg-production cycle, P would also have to be incorporated by the female. Several recent studies have shown that this homeostasis is not as strict as was previously assumed, and there are some variations in somatic C:P and specific P (%P dry wt^{-1}) with C:P changes of the food (Plath and Boersma 2001; DeMott 2003). DeMott et al. (1998) found a positive relationship between the specific P content of the daphniids and their growth rate. However, this relationship could not be reproduced by Sterner and Schwalbach (2001), who showed that daphniids during alternating P food quality conditions could compensate for periods of low P. For example, daphniids that spent $\sim$50% of the day in P-deficient food and the remaining time in P-sufficient food had similar growth as those fed a constant 50% mixture of these foods. This compensation suggests that the daphniids can reallocate stored compounds for growth, at least over short intervals.

We found that the *D. magna* females show a similar pattern of P allocation as for EPA—that is, if there was more P in the diet the P content in the females increased and varied between 9.0 and 17 mg P (g dry wt)$^{-1}$. Under severe P limitation females stabilized at $\sim$9.0 mg P (g dry wt)$^{-1}$, which could indicate the lowest possible P content for survival. This level was in a similar range as to that reported by DeMott (2003) and previously described for *D. magna* fed P-limited algae. In our study, *D. magna* reached this level when fed a food with a C:P ratio (molar) of $>$450 (Fig. 3). However, females showed a considerable decrease in P content when feeding on algae with low (nonlimiting) C:P ratios. This pattern could indicate storage, where females reallocated P to reproduction and molting. Furthermore, *D. magna* always allocated P in same concentration to eggs independent of algal P content (Fig. 3). This is not in agreement with the results of Boersma and Kreutzer (2002), who found that females feeding on P-sufficient algae gave birth to neonates with a higher P content than females fed P-limited algae. This can possibly be explained by the fact that neonates in Boersma and Kreutzer's (2002) study were allowed to feed on the maternal food sources for $<$24 h, which altered the neonates' specific P content. Nevertheless, the stable P content in *D. magna* eggs is consistent with the results of a recent study (Faerovig and Hessen 2003) and suggests a constant allocation of P to egg production (Fig. 3). The consequence of this is that egg production under P limitation will drain P from females. On the other hand, under excess P, females cannot load their eggs with P to improve the neonates' fitness. Comparing the distributions of P with fatty acids, a different allocation pattern was found.

Adult females accumulated both ARA and EPA from the food, but although the ARA content was allocated in similar proportions as in the females, EPA concentration was considerably higher in eggs (Fig. 4, Table 4).

Reallocation of storage—We investigated whether daphniids can reallocate stored fatty acids and P to maintain growth during periods of poor food quality by switching the availability of enriched and nonenriched foods over various periods in flow-through experiments. When the EPA availability was altered, we did not find any overall changes (Fig. 5A). This was probably due to an insufficient exchange rate in the flow-through in combination with the low EPA saturation threshold for *D. magna* growth (<0.02 mg EPA g dry wt^{-1}). Thus, in this experiment, daphniids might never have to have utilized any storage (Fig. 5A). Of course, the same reasoning could be used for the P experiment. However, the P saturation threshold is much higher than for EPA (>2 mg P g dry wt^{-1}; Plath and Boersma 2001), and we argue that, with regard to P, the exchange rate was high enough to induce specific P-limitation pressures in the flow-through experiments. Hence, we also found strong effects on *D. magna* growth of our alternating P treatments (Fig. 5B).

Clearly, there are differences in how P and EPA can be reallocated for growth. As expected, EPA, which we had previously found in variable concentrations within the daphniids (Fig. 2), can be reallocated for growth to a much larger extent than P (Fig. 5). Alternating the EPA availability in the food did not have a negative effect on *D. magna* growth, and growth rates did not even decline when EPA-enriched food was available only every 3 d (Fig. 5). In contrast, when the availability of P was decreased to every second day the growth decreased considerably compared with the daphniids continuously fed enriched algae. Increasing this period lowered the growth slightly more. We also found differences depending on when the enrichment occurred—that is, over the initial 3 d or the final 3 d. This timing did not have a negative effect on the EPA-enriched daphniids. Apparently, the initially EPA-starved daphniids could recover over the final 3-d period and the initially EPA fed daphniids could reallocate their storage. On the other hand, in P treatments, the timing of enrichments had clear effects: initially, P-enriched daphniids (3E:3C) had a considerably higher growth rate than the initially P-starved daphniids (3C:3E), and differences between these treatments demonstrate that some of the stored P for growth can be reallocated, which supports the findings of Sterner and Schwalbach (2001). In our study, the previously P-enriched daphniids grew faster because of their internal P storage. The initially P-starved daphniids did not have stored P to use and could therefore only use the enriched food during the final period, which resulted in a lower total growth rate. Another explanation could be that, after 3 d, the initially P-starved daphniids were in too bad a state and could not recover. This could indicate that the various requirements are different throughout ontogenesis. However, these daphniids (3C:3E) grew faster than the control daphniids (6C), which indicates that the daphniids were not in too poor a condition. From these findings, we conclude that the reallocation of P is far less efficient than the reallocation of EPA (Fig. 5).

EPA and egg production—We studied how daphniids enriched with EPA allocate this resource when transferred to unenriched food. After 6 d of feeding daphniids enriched food, the eggs were rich in EPA, and the females had approximately twice as much EPA in absolute amounts than the brood (1.7 μg $female^{-1}$ vs. 0.8 μg $brood^{-1}$) (Fig. 6C). A similar pattern was also found with the absolute concentration of 18:3ω4 and 20:1ω9. However, when concentrations are considered, eggs had a considerably higher EPA content (mg EPA g dry wt^{-1}) than females (Fig. 6D), which could indicate the greater importance of this fatty acid on earlier life stages. After the first day, the daphniids were cultivated on control P-enriched *S. obliquus*, and we monitored the fatty acid content every 3 d over two intervals. The fatty acids available in the food (18:3ω4 and 20:1ω9) increased slightly. EPA, on the contrary, decreased drastically in the females and also in the broods (Table 5). To determine in what range egg production is responsible for the maternal decrease, we computed the expected total EPA content. This is the EPA in the female plus that in previously released broods (Fig. 6C), which is possible to calculate because the interval of 3 d is about one egg production cycle for *D. magna.* EPA was enriched in the system over time even when EPA was not available in the food (Fig. 6C), a pattern that was earlier described by von Elert (2002) when *D. galeata* transformed other ω3 fatty acids to EPA. By using the expected EPA content after 6 d a rough bioconversion rate, measured as the EPA increase, was calculated at 0.037 d^{-1}. At the same time, the overall decrease of EPA was much larger (-0.16 d^{-1}). Egg production and release of eggs is thus the major drain of EPA from the females.

We conclude that *D. magna* has a low saturation threshold for fatty acids but a high storage capacity for these nutrients. In contrast, P requirements of *D. magna* are high, and they have only limited possibilities to utilize storage of this nutrient. Fatty-acid storage can be reallocated to support growth and is also very important for reproduction. Egg production is a major drain of EPA from the females. These results strongly suggest that daphniids are much more vulnerable to periods of P limitation than to essential fatty-acid limitation, and it is difficult to understand those studies that found strong correlations between EPA content and *Daphnia* growth above the very low concentrations for saturation that we observed.

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