

Biochemical limitation of resting egg production in *Daphnia*

Abstract—Many planktonic invertebrates can reproduce by immediately developing subitaneous eggs, which allow fast reproduction, and by resting eggs, which enable the survival of eggs in unfavorable environmental conditions. Since resting eggs can stay viable for decades or even centuries, they are likely to be richer in essential, easily degrading biochemicals than subitaneous eggs. Using the freshwater cladoceran *Daphnia pulicaria* we test whether the switch between these two qualitatively different reproductive modes is influenced by the availability of essential polyunsaturated fatty acids (PUFAs) in the food. We show that (1) when raised on a PUFA-poor diet, resting eggs of *D. pulicaria* contain much more PUFAs than subitaneous eggs, and unlike subitaneous eggs, contain considerable amounts of eicosapentaenoic acid (EPA), which was not present in their diet; (2) supplementation with EPA or using EPA-rich algae as food results in dramatically increased resting egg production; (3) when resting eggs are induced by starvation, their increased frequency in the presence of EPA-rich food can be entirely explained by maternal effects. Since dormancy is a ubiquitous phenomenon among invertebrates, our result that its onset can be severely limited by the availability of essential biochemicals may hold for a variety of taxa.

In the last decade, considerable research has been carried out to identify the key biochemical factors that determine the quality of seston for aquatic suspension feeders. Besides particle toxicity, which was studied for decades, three new factors have been identified, which can limit zooplankton growth: polyunsaturated fatty acids (PUFAs), the C:P ratio of seston, and its sterol content. Ahlgren et al. (1990) suggested that PUFAs, which cannot be synthesized by zooplankton, particularly eicosapentaenoic acid (EPA), can limit their growth; hence the differences in their PUFA content determine the quality of algae as food. Subsequent studies have found clear correlations between the amount of EPA in lake seston and the growth of *Daphnia* species (e.g., Müller-Navarra 1995; Müller-Navarra et al. 2000), and experiments with PUFA and EPA supplementation have confirmed their importance in determining herbivore growth rate (DeMott and Müller-Navarra 1997; Sundbom and Vrede 1997; Weers and Gulati 1997). However, supplementation with fatty acids does not improve the food quality of cyanobacteria for *Daphnia* (von Elert and Wolfrum 2001), and more recently it has been shown that the key compounds missing from cyanobacteria are sterols (von Elert et al. 2003). Besides essential lipids and sterols, the mineral composition of food influences the quality of algae as well; algae with low phosphorus content are poor food for zooplankton (Urabe et al. 1997; DeMott 1998).

Daphnia are cyclic parthenogens that lay resting eggs (enclosed in an ephippium) in conditions of stress, usually in spring and autumn (e.g., Spaak 1995), but otherwise

reproduce by subitaneous eggs. The factors that can induce ephippium production are high population density and photoperiod (Carvalho and Hughes 1983), rapid decline in food concentration (Hobaek and Larsson 1990), or the presence of predators (Ślusarczyk 2001). In addition to the environmental conditions at the time of the stimulation, the environmental condition of the mothers has significant influence on the frequency of resting egg production of their offspring (Alekseev and Lampert 2001; LaMontagne and McCauley 2001). As the function of resting eggs is to guarantee survival during unfavorable environmental conditions, which may last years or even decades (Hairston et al. 1995; Weider et al. 1997), one would expect them to contain more “precious biochemicals” than subitaneous eggs. This expectation is consistent with the findings of Arbaciauskas and Lampert (2003), showing that *Daphnia* hatched from resting eggs have higher metabolic, growth, and reproduction rates than individuals hatched from subitaneous eggs.

So far food quality has only been shown to influence quantitative (continuous) traits of zooplankton life histories, like growth rate, the timing of reproduction (Becker and Boersma 2003), and the growth rate of the offspring (Brett 1993; Becker and Boersma 2003). In this paper we test the hypothesis that food quality can induce qualitative changes in the reproductive mode of freshwater cladocerans. More specifically, we test whether (1) resting eggs of *Daphnia pulicaria* contain more essential fatty acids than subitaneous eggs; (2) the availability of fatty acids in the food, particularly of EPA, has significant influence on the frequency of resting egg production.

Methods—To determine the fatty acid profiles of subitaneous and resting eggs of *Daphnia*, we cultured *D. pulicaria* originating from a shallow lake in northern Germany (Grosser Binnensee) in 1.5-liter jars at room temperature, fed ad libitum with the EPA-free green alga *Scenedesmus obliquus* (strain SAG-276-3a) as the sole food. The culturing medium for *Daphnia* was membrane-filtered (0.45 μm) lake water; *S. obliquus* originated from chemostats (Chu-12 medium, 20°C, dilution rate 0.5 d⁻¹). The subitaneous eggs were removed directly from the brood chamber and were transferred to Eppendorf caps. Ephippia were collected from the bottom of culture jars, opened with needles, and the resting eggs were removed. All eggs were stored at -80°C. The fatty acid content of the eggs was determined with the method described by Becker and Boersma (2005). In short, eggs were freeze-dried and the lipids were extracted from batches of approximately 0.5 mg dry weight. The lipid extract was esterified, and the lipids were subsequently quantified in a HP 5890 gas chromatograph. The measurements were carried out in triplicate.

To investigate whether PUFA supplementation influences resting egg production we performed experiments with three different food sources: *S. obliquus*, EPA-

Table 1. Fatty acid profiles (means [\pm SD]) of subitaneous eggs, resting eggs, and the algae used in the experiments. For the resting and subitaneous eggs the concentrations of fatty acids are given in $\mu\text{g mg}^{-1}$ dry weight, and in $\mu\text{g mg}^{-1}$ C for the algae. Significant differences between the two egg types are highlighted with boldface (Wilcoxon rank sum test, $n = 3$).

FA	Eggs		Algae		
	Resting	Subitaneous	<i>Scenedesmus</i>	<i>Scen+EPA</i>	<i>Cryptomonas</i>
C14:0	7.44 (2.04)	3.48 (0.57)			4.55 (1.38)
C16:0	31.54 (7.88)	31.49 (7.49)	23.05 (2.46)	23.39 (2.58)	35.79 (3.35)
C16:1 ω 9	13.22 (3.36)	11.44 (1.40)			
C16:2 ω 4	2.65 (0.44)	0.06 (0.05)			
C18:0	8.56 (4.21)	1.35 (0.26)	0.96 (0.55)	1.08 (0.12)	1.36 (0.318)
C18:1 ω 9	87.67 (30.58)	21.98 (6.21)	14.78 (2.67)	11.67 (1.37)	0.98 (0.91)
C18:1 ω 7	9.03 (3.47)	1.30 (0.60)	0.29 (0.4)	0.345 (0.30)	0.7 (0.655)
C18:2 ω 6	67.98 (10.99)	12.26 (2.00)	18.97 (2.81)	16.73 (1.49)	14.26 (5.39)
C18:3 ω 6	6.06 (1.62)	0.96 (0.12)	0.97 (0.15)	0.86 (0.08)	nd
C18:3 ω 3	82.33 (22.13)	10.70 (2.13)	29.27 (8.06)	30.05 (2.37)	54.98 (6.18)
C18:4 ω 3	18.93 (4.76)	2.20 (0.46)			
C20:0	1.12 (1.94)	0.68 (0.98)			
C20:1 ω 9	1.02 (1.77)	0.05 (0.04)			
C20:5 ω 3 (EPA)	2.40 (1.63)	0.01 (0.02)	nd	7.44 (0.55)	31.37 (4.74)
Σ FA	341.53 (68.68)	98.31 (7.41)	88.33 (14.35)	90.93 (8.38)	151.87 (19.08)
Σ (ω 3)	103.66 (26.21)	12.94 (2.42)	29.27 (8.06)	29.27 (8.06)	91.87 (10.92)
Σ (ω 6)	74.04 (12.24)	13.34 (2.15)			
Σ PUFA	177.70 (37.36)	26.28 (4.57)	49.24 (10.76)	54.43 (4.20)	106.13 (15.91)

enriched *S. obliquus*, and *Cryptomonas erosa*. An obligately parthenogenetic clone of *D. pulicaria* that has been kept in the laboratory more than 20 yr was used in the experiment (the same clone was used by Alekseev and Lampert [2001]). *S. obliquus* was enriched with EPA using the method of von Elert (2002) with slight modifications: 25 mg of bovine serum albumin was dissolved in 6.25 mL of ultrapure water, and 1.25 mg of EPA was added in 250 μL of ethanolic solution. The EPA solution was added to 50 mL of *S. obliquus* suspension containing a total of 6 mg of particulate organic carbon. The algae were incubated on a shaker for 5 h, and were subsequently separated from their medium by centrifugation, and washed twice with filtered lake water. The resulting *S. obliquus* suspension was used as food in the experiments. A similar threefold washing procedure was applied for the *S. obliquus* used in the treatments without EPA enrichment. *C. erosa* was grown on WC medium in a semicontinuous culture. The fatty acid content of the experimental food was determined in a procedure similar to the one described above, with the difference that the algal biomass, equivalent to 0.5 mg, was collected on GF/C filters.

The experimental procedure was as follows: In the first generation, 25 neonates originating from cultures fed only *S. obliquus* were put into 1 liter of filtered lake water, containing 0.6 mg of one of the experimental algal suspensions: *S. obliquus*, *S. obliquus* enriched with EPA, or *C. erosa*. Every treatment was run in five replicates, at 22°C, with 10 h day⁻¹ illumination. The animals were transferred every day to freshly prepared media, and during the first 3 d the number of animals in each jar was gradually reduced to 20. When animals reached maturity, the number of individuals with ephippia was counted, and the experiment was continued with the newborns (25 individuals per jar, reduced

to 20). When the second generation reached maturity, individuals with ephippia were counted, and every treatment was split into two: one with 0.1 mg L⁻¹ C of the original food and one with 0.1 mg L⁻¹ C of *S. obliquus*. This allows the separation of the effect of the food quality from maternal effects in the resting egg induction. Only adults carrying subitaneous eggs were transferred; therefore the neonates of the third generation were already born in the low-food-concentration treatments. Similarly to the previous treatments the number of juveniles was gradually reduced to 20. Individuals were kept until they produced ephippia or subitaneous eggs. Statistical analyses were made on arcsine-transformed frequencies.

Results—Fatty acid profiles: When fed *S. obliquus*, the fatty acid profiles of subitaneous and resting eggs are very different (Table 1). In subitaneous eggs, the amount and the composition of fatty acids are qualitatively similar to the fatty acid composition of their food, *S. obliquus*. In contrast, the total fatty acid content of resting eggs is approximately 3.5 times higher than that of the subitaneous eggs, and the difference in PUFAs is even larger, almost sevenfold. The differences are most pronounced in the concentrations of C:18 fatty acids, which are all present in significantly higher amounts in the resting eggs than in subitaneous eggs, particularly linoleic (C18:2 ω 6), α -linolenic (C18:3 ω 3), and stearidonic (C18:4 ω 3) acids. Resting eggs contain not just more but also different PUFAs: EPA (C20:5 ω 3) is present in well-measurable amounts in the resting eggs, but only in trace amounts in subitaneous eggs (Table 1). The fatty acid profiles of *S. obliquus*, EPA-enriched *S. obliquus*, and *C. erosa* were qualitatively similar to the profiles described in the literature (e.g., Ahlgren et al. 1990; von Elert 2002, Table 1).

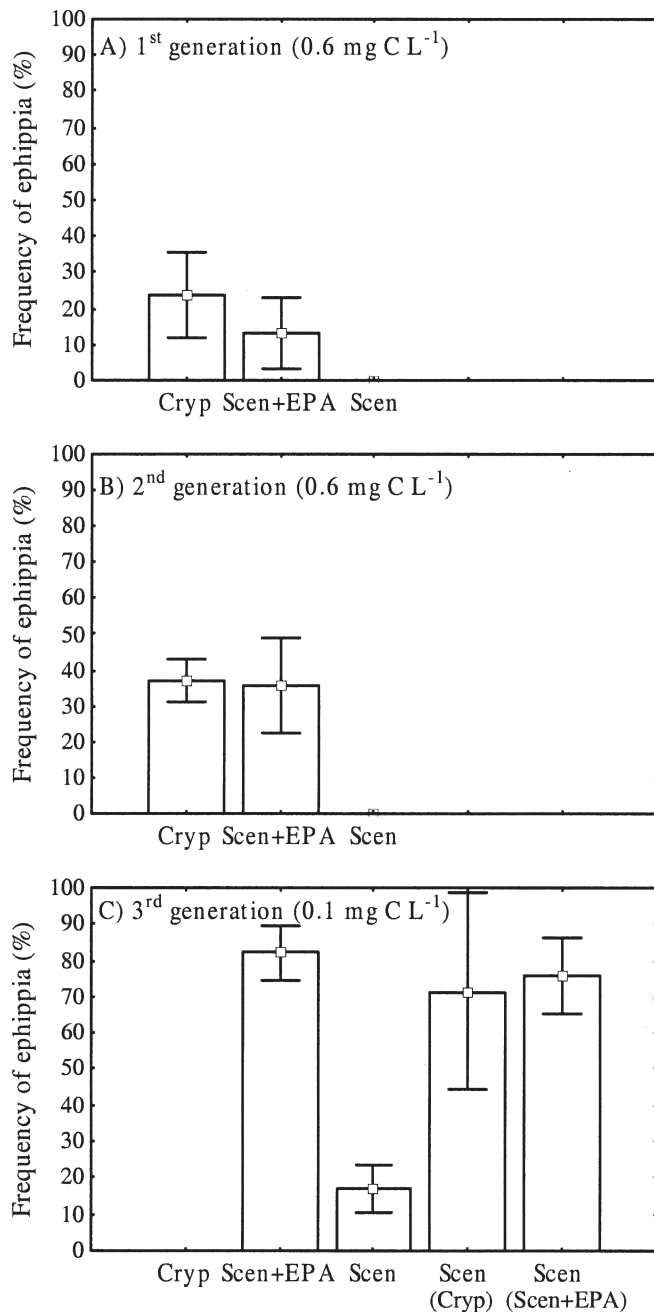


Fig. 1. Frequency of individuals with ephippia (\pm SD) in the three *Daphnia* generations of the experiment. The algal suspension used in the treatments is indicated below the x-axes. In two treatments of the third generation, where maternal food quality was different from the food of their offspring, maternal food is indicated in brackets. (A) First generation ($0.6 \text{ mg L}^{-1} \text{ C}$); (B) second generation ($0.6 \text{ mg L}^{-1} \text{ C}$); (C) third generation ($0.1 \text{ mg L}^{-1} \text{ C}$).

Life history experiment: In the first generation, ephippium production was observed in the *C. erosa* and EPA treatments (Fig. 1) but not in the treatment with only *Scenedesmus*. There was no significant difference between the frequency of resting eggs between the *C. erosa* and EPA treatments (t -test, $p = 0.136$). In the second generation,

ephippium production was observed in the same treatments, and similarly to the first generation there was no difference between the two treatments (t -test, $p = 0.789$). In the EPA treatment, the frequency of ephippia increased significantly compared with the first generation (t -test, $p = 0.013$), but not in the *Cryptomonas* treatment (t -test, $p = 0.0502$). In the third generation individuals with ephippia were present in all treatments. We observed very high mortality in the *Cryptomonas* treatment (73%); therefore the correct rates of resting egg production could not be estimated there. The high mortality indicates that despite its high PUFA content, in the long run this species is not suitable as the sole food source for *Daphnia*. No comparable mortality was observed in the other treatments or previous generations. The frequency of individuals with resting eggs was much higher (t -test, $p < 0.001$) in the third generation of the *Scenedesmus* + EPA treatment than in the second generation. The lowest frequency of ephippia was observed in the individuals that were fed only *Scenedesmus* in the first and second generations, which was significantly different from all other treatments (analysis of variance, multiple comparisons with Tukey HSD tests, $p < 0.001$ for all comparisons). There is no significant difference between the three treatments in which the *Daphnia*'s mothers were raised on PUFA-rich diet ($p > 0.7$ for all comparisons), regardless of whether it was *C. erosa* or EPA-enriched *S. obliquus* (Fig. 1).

Discussion—PUFAs are essential in maintaining the integrity of cell membranes. However, the synthesis of ω -3 PUFAs is largely restricted to the plant kingdom, as most animals lack the desaturase enzymes necessary for their synthesis. In consequence, the PUFA content of animals, including *Daphnia*, is determined mainly by its availability in the food. Most animals can synthesize oleic acid (C18:1 ω 9), which can be converted (at least by some insects) to linoleic (C18:2 ω 6) and arachidonic acids (C20:4 ω 6) (Weers et al. 1997). However, further conversion of these FAs to α -linolenic acid (C18:3 ω 3) and EPA (C20:5 ω 3) in *Daphnia* is very inefficient (Weers et al. 1997; Von Elert 2002), and in consequence the amount of EPA in *Daphnia* tissues is primarily determined by its concentration in the food. This is supported by the findings of Becker and Boersma (2005) that show that the EPA content of *Daphnia* increases linearly with the EPA content of their food. The concentration of EPA in the resting eggs ($2.4 \mu\text{g mg}^{-1}$ dry weight) should be considered as high, since EPA concentrations higher than $0.3 \mu\text{g mg}^{-1} \text{ C}$ in the food and $\sim 0.6 \mu\text{g mg}^{-1}$ dry weight in *Daphnia* tissues have no further effect on the growth rate of *Daphnia* (Becker and Boersma 2005).

The result that resting eggs contain much more PUFAs (both ω 3 and ω 6 types) and EPA than subitaneous eggs (at least when fed with *S. obliquus*) suggests that they are necessary to produce viable resting eggs or to maintain their long-term viability. It shows that the production of ephippia is more costly than the production of subitaneous eggs, and suggests that the availability of PUFAs/EPA may limit the frequency of resting egg production in nature. (Strictly speaking, the word limitation is inappropriate, as

Daphnia individuals have to choose between two alternative reproductive strategies, and they can make only a single ephippium.) The prediction of this hypothesis is that in the presence of cues inducing resting egg formation, if PUFAs/EPA are present in insufficient amounts, most animals will keep reproducing by subitaneous eggs to avoid producing very-low-quality ephippia. Our experimental findings confirmed this prediction (Fig. 1). In the first two generations, despite the fact that no special treatments were applied to induce resting egg production, individuals with resting eggs were present in the PUFA/EPA-rich treatments in intermediate frequencies, but were absent in the treatment where *S. obliquus* was the sole food. We used animal densities that we previously found to be below the threshold that induces resting egg production when *S. obliquus* is the only food source. However, the results show that this threshold density depends largely on the availability of EPA. The lack of a significant difference between the *C. erosa* and EPA-supplemented *S. obliquus* treatments suggests that the high EPA content of *C. erosa* is sufficient to explain the elevated resting egg production. In the third generation, *Daphnia* produced resting eggs in every treatment (Fig. 1), and the influence of high PUFA content of *C. erosa* and EPA supplementation on the frequency of ephippium production was overwhelming. A surprising result is that maternal effects explain all the difference between the “pure” *S. obliquus* and PUFA-rich treatments, and once mothers were grown on an EPA-rich diet, its further availability during their daughters’ lifetime has no significant effect on the frequency of their resting egg production. One possible explanation is that mothers can supply their offspring with all the necessary PUFAs, when available. This could explain also the presence of EPA in the ephippia of animals that were fed only with *S. obliquus* (although *Daphnia* fed only with *S. obliquus* produced ephippia even in the third generation of the experiment; therefore the synthesis of EPA cannot be excluded as well). However, recent studies show that PUFAs, at least in mammals, are involved in the regulation of expression of many genes (Wahle et al. 2003). Since the switch from the production of subitaneous eggs to diapausing eggs certainly involves changes in gene expression, this offers the intriguing possibility that PUFAs may influence the production of diapausing eggs not just through limitation, but also through direct alteration of the expression of genes involved.

The results offer an alternative explanation for the timing of resting egg production of *Daphnia*. In stratified lakes, resting eggs are frequently produced after the clear water phase. Since before and during the clear water phase PUFA-rich algae like diatoms and cryptophytes dominate the phytoplankton, the availability of EPA could be one of the main factors responsible for the spring peak of ephippia and sexual reproduction.

Resting egg production is ubiquitous among all three major groups of zooplankton: rotifers (Gilbert and Schroder 2004), copepods (Hairston et al. 1995), and daphniids, and it is also common in terrestrial invertebrates (Caceres 1997). We believe essential biochemicals may limit resting-egg formation in many other taxo-

nomic groups where resting eggs remain viable for long periods.

György Abrusán¹
Patrick Fink²
Winfried Lampert

Department of Ecophysiology
Max Planck Institute of Limnology
August Thienemann Str.2, 24306 Plön, Germany

References

- AHLGREN, G., L. LUNDSTEDT, M. BRETT, AND C. FORSBERG. 1990. Lipid composition and food quality of some fresh-water phytoplankton for cladoceran zooplankters. *J. Plankton Res.* **12**: 809–818.
- ALEKSEEV, V., AND W. LAMPERT. 2001. Maternal control of resting-egg production in *Daphnia*. *Nature* **414**: 899–901.
- ARBACIAUSKAS, K., AND W. LAMPERT. 2003. Seasonal adaptation of ex-ephippium and parthenogenetic offspring of *Daphnia magna*: Differences in life history and physiology. *Funct. Ecol.* **17**: 431–437.
- BECKER, C., AND M. BOERSMA. 2003. Resource quality effects on life histories of *Daphnia*. *Limnol. Oceanogr.* **48**: 700–706.
- , AND ———. 2005. Differential effects of phosphorus and fatty acids on *Daphnia magna* growth and reproduction. *Limnol. Oceanogr.* **50**: 388–397.
- BRETT, M. T. 1993. Resource quality effects on *Daphnia longispina* offspring fitness. *J. Plankton Res.* **15**: 403–412.
- CACERES, C. E. 1997. Dormancy in invertebrates. *Invertebr. Biol.* **116**: 371–383.
- CARVALHO, G. R., AND R. N. HUGHES. 1983. The effect of food availability, female culture-density and photoperiod on ephippium production in *Daphnia magna* Straus (Crustacea, Cladocera). *Freshw. Biol.* **13**: 37–46.
- DEMOTT, W. R. 1998. Utilization of a cyanobacterium and a phosphorus-deficient green alga as complementary resources by daphnids. *Ecology* **79**: 2463–2481.
- , AND D. C. MÜLLER-NAVARRA. 1997. The importance of highly unsaturated fatty acids in zooplankton nutrition: Evidence from experiments with *Daphnia*, a cyanobacterium and lipid emulsions. *Freshw. Biol.* **38**: 649–664.
- GILBERT, J. J., AND T. SCHRODER. 2004. Rotifers from diapausing, fertilized eggs: Unique features and emergence. *Limnol. Oceanogr.* **49**: 1341–1354.
- HAIRSTON, N. G., R. A. VANBRUNT, C. M. KEARNS, AND D. R. ENGSTROM. 1995. Age and survivorship of diapausing eggs in a sediment egg bank. *Ecology* **76**: 1706–1711.
- HOBBAEK, A., AND P. LARSSON. 1990. Sex determination in *Daphnia magna*. *Ecology* **71**: 2255–2268.

¹ To whom correspondence should be addressed. Present address: Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Charles Deberiotstraat 32, B-3000 Leuven Belgium (Gyorgy.Abrusan@bio.kuleuven.be, Gyorgy.Abrusan@mssm.edu).

² Present address: University of Cologne, Zoological Institute, Weyertal 119, 50923 Cologne, Germany.

Acknowledgments

We thank Claes Becker and Heinke Buhtz for the analysis of lipids of the eggs, and Maren Volquardsen, Ines Schultz, and Heike Paul-Wardenga for help with *Daphnia* transfer and labeling of algae. We thank both referees for their constructive reviews. György Abrusán received a fellowship from the Alexander von Humboldt Foundation and the Max Planck Society, Patrick Fink from the Max Planck Society.

- LAMONTAGNE, J. M., AND E. McCAULEY. 2001. Maternal effects in *Daphnia*: What mothers are telling their offspring and do they listen? *Ecol. Lett.* **4**: 64–71.
- MÜLLER-NAVARRA, D. 1995. Evidence that a highly unsaturated fatty-acid limits *Daphnia* growth in nature. *Arch. Hydrobiol.* **132**: 297–307.
- MÜLLER-NAVARRA, D. C., M. T. BRETT, A. M. LISTON, AND C. R. GOLDMAN. 2000. A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature* **403**: 74–77.
- ŚLUSARCZYK, M. 2001. Food threshold for diapause in *Daphnia* under the threat of fish predation. *Ecology* **82**: 1089–1096.
- SPAAK, P. 1995. Sexual reproduction in *Daphnia*—interspecific differences in a hybrid species complex. *Oecologia* **104**: 501–507.
- SUNDBOM, M., AND T. VREDE. 1997. Effects of fatty acid and phosphorus content of food on the growth, survival and reproduction of *Daphnia*. *Freshw. Biol.* **38**: 665–674.
- URABE, J., J. CLASEN, AND R. W. STERNER. 1997. Phosphorus limitation of *Daphnia* growth: Is it real? *Limnol. Oceanogr.* **42**: 1436–1443.
- VON ELERT, E. 2002. Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnol. Oceanogr.* **47**: 1764–1773.
- , D. MARTIN-CREUZBURG, AND J. R. LE COZ. 2003. Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 1209–1214.
- , AND T. WOLFFROM. 2001. Supplementation of cyanobacterial food with polyunsaturated fatty acids does not improve growth of *Daphnia*. *Limnol. Oceanogr.* **46**: 1552–1558.
- WAHLE, K. W. J., D. ROTONDO, AND S. D. HEYS. 2003. Polyunsaturated fatty acids and gene expression in mammalian systems. *Proc. Nutr. Soc.* **62**: 349–360.
- WEERS, P. M. M., AND R. D. GULATI. 1997. Effect of the addition of polyunsaturated fatty acids to the diet on the growth and fecundity of *Daphnia galeata*. *Freshw. Biol.* **38**: 721–729.
- , K. SIEWERTSEN, AND R. D. GULATI. 1997. Is the fatty acid composition of *Daphnia galeata* determined by the fatty acid composition of the ingested diet? *Freshw. Biol.* **38**: 731–738.
- WEIDER, L. J., W. LAMPERT, M. WESSELS, J. K. COLBOURNE, AND P. LIMBURG. 1997. Long-term genetic shifts in a microcrustacean egg bank associated with anthropogenic changes in the Lake Constance ecosystem. *Proc. R. Soc. Lond. B Biol. Sci.* **264**: 1613–1618.

Received: 19 October 2006
Accepted: 13 February 2007
Amended: 12 March 2007