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Effects of food quantity, dietary fatty acids and temperature on fitness of *Daphnia magna*

A factorial experiment with *Scenedesmus acutus* and EPA amendments

Bernadette Pree

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Abstract

The performance of zooplankton has a major impact on the efficiency in trophic transfer in pelagic food webs and is therefore examined in this study. I investigated the effect of different food quantity and food quality, as measured by omega-3 fatty acid (ω3-FA) content on survival, somatic growth and reproduction of *Daphnia magna* at high and low temperatures in laboratory growth experiments.

I first investigated the response across a range from low to high food quantity (0.02, 0.07, 0.2, 0.7, 2.0 mg C l^{-1}) of the green alga *Scenedesmus acutus* at 12.0˚C and 20.6°C. Food quantity constraints on somatic growth of *Daphnia* and increasing growth rate were found with increasing food concentration. An interaction between quantity and temperature showed a higher maximal growth at higher temperature and high food levels. Furthermore, the starvation point shifted to a lower food concentration at low temperatures.

Subsequently I tested the response in survival, somatic and reproductive growth of *D. magna* to different quality of food in terms of FA content, temperature and food quantity. It was a 2x2x3 factorial design performed at two food levels (maximum growth at 2.0 mg C l^{-1} and close to the reproduction threshold concentration at 0.2 mg C l^{-1}) and two temperatures (14.1˚C and 21.7°C). To address the question if ω3-FA enrichment enhances fitness of *D. magna*, algal food suspensions were amended with eicosapentaenoic acid (EPA) or oleic acid (non-essential control treatment). The results show, besides the expected effects of food quantity and the interaction of temperature and food quantity, a significant effect of EPA enrichment on somatic growth rate and reproduction. EPA amendment improved somatic growth and egg production at both temperatures. The strongest effect of EPA enrichment was manifested on somatic and reproductive growth at low temperature and at high food concentration (2.0 mg C l^{-1}). These results indicate that food quality is of greater ecological importance in cold freshwater systems, like at high latitudes and high altitudes. In temperate lakes, the effect of interaction between food quantity, quality and temperature is manifested in the seasonal as well as the vertical variation of these factors. When EPA content is high in surface waters of stratified lakes and zooplankton migrates vertically during night to colder deeper layers during night the combined effect of FA content and temperature can be expected to result in improved somatic growth.

Abbreviations

ALA	alpha-linolenic acid
DHA	docosahexaeonic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
ILL	Incipient limiting level
LIN	linoleic acid
MUFA	monounsaturated fatty acids
OA	Oleic acid
POC	Particulate organic carbon
PUFA	Polyunsaturated fatty acid
SAFA	saturated fatty acids

Contents

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Introduction

In aquatic food webs, herbivorous zooplankton links primary producers with higher trophic levels. Zooplankton has a paramount role in the process of energy, carbon and nutrient transfer among different trophic levels (Cebrian 2004), therefore it is ecologically important to investigate the factors influencing their survival, reproduction and somatic growth. Several studies have shown that the efficiency of the trophic transfer is determined by food quality and quantity (Sterner and Hessen 1994, Brett and Mueller-Navarra 1997) as well as temperature (Giebelhausen and Lampert 2001). Interactions of these factors are important for accounting patterns of seasonal succession in temperate lakes (Sommer et al. 1986). In spring low temperature is combined with high levels of edible algal concentrations. In summer and autumn the food supply decreases due to grazing and water temperatures increase (Giebelhausen and Lampert 2001). When lakes are stratified and have low external nutrient inputs, nutrient limitation can be strong and hence primary production low. Later in the season, the cooling of the water in autumn causes a circulation and nutrients are mixed into the photic zone. Furthermore, food quality is varying during the year, since the composition of phytoplankton community is changing seasonally (Mueller-Navarra 1995).

However, interactions between temperature, food quality and quantity on survival, somatic and reproductive growth of zooplankton are still poorly investigated. This study aims to improve the understanding of the interactions of these factors and the ecological relevance they have for aquatic food webs. To elaborate this, *Daphnia magna*, a geographically widespread and well investigated Cladocera, was used as a model system. As diet *Scenedesmus acutus*, a green alga of mediocre food quality, was used.

Food quantity

The overall availability of food is the most basic factor affecting somatic growth of *Daphnia*. Even when a resource is very abundant the rate at which organisms can consume it is limited since they need a certain amount of time to handle and ingest it. This relationship between feeding rate and density of a resource is called "the functional response". Holling (1959) described three different types of functional responses, of which *D. magna* typically exhibit a Holling Type II functional response with an asymptotic increase of ingestion rate with increasing food concentration (Fig. 1) (Porter et al. 1983). The initial slope of the functional response curve defines the ability to use a limited resource (Lampert and Sommer 2007). In case of *D. magna*, which is a non-selective filter feeder, the curve is asymptotically increasing and exhibits a plateau. The food concentration where the plateau starts is called the "incipient limiting level" (ILL), beyond which ingestion remains constant (Porter et al. 1983). There are no clear defined concentrations of ILL for *D. magna* since they are depending on several conditions. However, Porter et al. (1983) note 0.2 mg C l^{-1} as the ILL and a concentration of 2.0 mg C l^{-1} where most parameters of fitness (somatic growth, reproduction and survival) were maximal.

Somatic growth rate can be directly related to the available food concentration (Lampert and Sommer 2007). The concentration where the growth curve intercepts with the x- axis is the starvation point, where the availability of the resource is so low that no biomass can be accumulated. Somatic growth as well as reproduction exhibit to a saturating level from which on growth and reproduction are not increasing anymore considerably with higher food concentration. For long term persistence of populations it is crucially important at what food concentration reproduction is initiated. Therefore allocation of the reproduction threshold concentration is topic of interest.

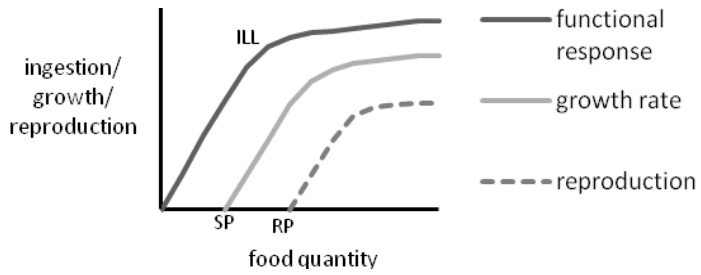


Fig. 1: Models illustrating the relationship between food concentration and functional response, growth rate and reproduction curves. ILL: Incipient limiting level; SP: starvation point; RP: reproduction point (modified after Holling (1959)).

Food quality

Besides food quantity, nutritional composition is essential for development and population growth of zooplankton and will have cascading effects on community level in aquatic ecosystems due to their intermediate role in the pelagic food web (Sundbom and Vrede 1997). For the assessment of food quality for zooplankton, research has developed two different approaches. The first one focuses on elemental carbon, nitrogen and phosphorus in phytoplankton as main determinant of food quality (Sterner and Hessen 1994). The second one emphasizes the role of specific biochemical such as amino acids, sterols and fatty acids (FA) (Brett and Mueller-Navarra 1997). Among FAs, some polyunsaturated fatty acids (PUFAs) are essential for organisms (Kainz et al. 2004), since they have a key regulatory role for maintaining cell membrane fluidity (Pruitt 1990) and some are precursors to many animal hormones (Bell et al. 1991). In aquatic food webs eicosapentaenoic acid (EPA) and docosahexaeonic acid (DHA) have been shown to be important for the somatic growth of herbivorous zooplankton (Mueller-Navarra et al. 2000, Mueller-Navarra et al. 2004) and a high content of EPA can be used as a predictor for good food quality for Cladocera as *Daphnia* (Ahlgren et al. 1990, Mueller-Navarra 1995). EPA and DHA can only be obtained from zooplankton directly from phytoplankton or by converting (via elongation and desaturation) linoleic (LIN) and alpha-linolenic acid (ALA) which are only synthesized by plants (Brett and Mueller-Navarra 1997). In fact, LIN and ALA are the two truly essential FA since they are the first ones in which a ω3 and ω6 double bond is introduced (Cunnane 2000, 2003).

Previous experiments show that dietary EPA enrichment improves reproduction of zooplankton, since dietary EPA is *preferentially allocated into the eggs* (Becker and Boersma 2005, Wacker and Martin-Creuzburg 2007). Therefore a high content of EPA can be used as a predictor for good food quality for zooplankton (Ahlgren et al. 1990). Different groups of algae differ in their FA composition, hence the growth and reproduction is not only depending on algal quantity but also on the algal community composition (Brett et al. 2000). Since phytoplankton species composition follows predictable patterns in temperate lakes throughout the season (Sommer et al. 1986), predictions in regard the available dietary EFA are possible. During spring, a bloom in PUFA rich diatoms und cryptomonad flagellates is typical and consequently a rapid growth of zooplankton populations is occurring. Due to intense grazing the phytoplankton populations undergo a crash and a new phytoplankton community with a lower PUFA content establishes (Mueller-Navarra and Lampert 1996).

Temperature

Temperature is one of the major factors affecting metabolism of ectothermic species as *D. magna* (Dawidowicz and Loose 1992). In addition to a decreased metabolic rate at lower temperatures, the increased viscosity at low temperature causes a lowering of the ingestion rate (Loiterton et al. 2004). Results from previous experiments show that daphnids grow and reproduce faster at higher temperatures (Giebelhausen and Lampert 2001, Rinke and Petzoldt 2003).

Interactions between food quantity, food quality and temperature

Although the effects of food quantity, food quality and temperature on survival, somatic and reproductive growth of *Daphnia* have been the issue of many studies, their interactions are yet poorly investigated.

Giebelhausen and Lampert (2001) performed feeding experiments testing the effect of temperature and food concentration on the fitness of *D. magna.* Their results show a significant interaction of temperature and food concentration as the temperature response was most pronounced at saturating food levels (Giebelhausen and Lampert 2001). Several other studies also show similar effects e.g. (Orcutt and Porter 1984), (Foran 1986) and (Mitchell 1997). Giebelhausen and Lampert (2001) found that daphnids mature at a smaller size at limiting food concentrations when temperature is high. At the same conditions, also the number of eggs was dropping (Giebelhausen and Lampert 2001).

Masclaux et al. (2009) investigated the effect of food quality and temperature and their interplay on somatic and reproductive growth of zooplankton. The study suggests that food quality effects on somatic growth and reproduction of cladocerans are less pronounced at increasing temperature (Masclaux et al. 2009). Earlier studies describe homeoviscous adaptation as a widespread strategy among ectotherms to regulate membrane fluidity by enriching PUFAs of membrane phospholipids at lower temperatures (Sinensky 1974, Pruitt 1990). Hessen and Leu (2006) could find high fractions of PUFAs in daphnids of arctic lakes compared to other freshwater studies, possibly due to the low temperatures.

Boersma and Kreutzer (2002) performed growth experiments to investigate the effect on zooplankton production of food quality in terms of phosphorus with various levels of C (from 30µg to 150 µg C l^{1}). They hypothesized that under scarce food supply the quality is of less importance since it is mainly the C content that determines growth of *D. magna*. Anyhow, results from this research show that at low food concentrations, the mineral content of nutrition is important and there is a quality dependent shift in threshold food concentrations (Boersma and Kreutzer 2002).

Persson et al. (2007) performed a study with the aim to create a model which is able to address the effect of food quantity and quality in relation to the trophic state of lakes (total phosphorus, TP). When TP was low, food quantity was the main determining factor for Daphnia growth, whereas at high TP values EPA content is the main constraint (Persson et al. 2007).

Sperfeld and Wacker (2011) examined how the EPA growth saturation thresholds of *D. magna* is affected by the availability of another essential nutrient (cholesterol), which is an indispensable cell membrane component. They further tested how temperature affects the EPA growth saturation thresholds of *D. magna* at 15°C and 20°C. When cholesterol content was low a higher EPA threshold was estimated. At lower temperatures the estimated EPA thresholds were lower too. Generally, their study found a stronger effect on EPA-dependent growth responses by temperature than by cholesterol availability (Sperfeld and Wacker 2011).

To the best of my knowledge, there is at present no study that address the interplay of food quality and food quantity and temperature on survival, somatic and reproductive growth of *D. magna*, even though they can be expected to be present and definitely ecologically relevant.

Aim and hypothesis

Improving the understanding of the combined effects of food quantity, food quality, temperature and their interactions on survival, somatic and reproductive growth of *D. magna* is the main objective of this thesis. The main research question is: How does dietary PUFA content effect the survival, somatic and reproductive growth of *Daphnia* at high versus low food concentrations and at high versus low temperatures? In order to address this question, two laboratory experiments were conducted.

The first experiment explores the response in survival, somatic and reproductive growth of *D. magna* when exposed to different food concentrations at 12.0 °C and 20.6 °C. I expect that at low food levels growth and reproduction is lower compared to higher food levels. Additionally, the first experiment aimed to identify the allocation of the point of starvation, the reproduction threshold concentration and the concentration where somatic and reproductive growth were maximal at both temperatures.

The second experiment is carried out at the sufficient food concentration (2.0 mg C l^{-1}) and at the reproduction threshold concentration (0.2 mg C l^{-1}) and at two temperatures (14.1°C and 21.7°C). Experiment 2 assesses the effect of EPA enrichment on daphnid performance, and to what extent food quality interacts with temperature at high and low food concentration. Zooplankton will therefore be fed with green algae with well-defined quantities of microencapsulated FA. This approach has the advantage in comparison to previous studies that were either focusing on temperature and FA (Schlechtriem et al. 2006) or food quality in terms of P content and quantity (Boersma and Kreutzer 2002) that it elaborates temperature, FA and concentrations effects and their interactions at once.

I hypothesize that:

- somatic growth will be higher at increasing food quantity and at higher temperature. I expect a more pronounced effect of temperature at high food quantity.
- dietary addition of EPA will increase the somatic and reproductive growth of *D. magna* at low and high temperatures.
- there is an interaction between food quality × food quantity × temperature. I expect a more pronounced effect of dietary EPA enrichment at low temperatures and high food concentrations.

Material and Methods

Culturing algae and daphnids

Prior to the experiments a stock culture of a clone of *Daphnia magna* (Straus 1820) was maintained in M4 medium (Appendix 1) and kept under stable conditions of 16:8 hours light:dark cycle at 12.0±1.0˚C (mean ± SD) and 20.6±0.2°C. Algae were grown under the same conditions as the stock culture of daphnids in a standardized medium (Appendix 2). The animals were fed ad libitum with green algae *Scenedesmus acutus* (no dietary EPA, DHA content). Since FA of *S. acutus* were not analyzed data on its FA were taken from the literature (Ahlgren et al. 1992) (Tab. 1), and should provide an approximation of the FA content. However, variation is possible due to variations among clones and analytical methods.

Table 1. Fatty acid content (FA) of *S. acutus (Ahlgren et al. 1992).*

FA		mg g^{-1} dry weight	% of total FA
∑ SAFA		33,98	29
∑ MUFA		50,36	43
C18:2ω6	linoleic (LIN)	8,49	7
C18:3ω3	alpha-linolenic (ALA)	14,89	13
C18:3ω6	gamma-linolenic (GLA)	0,46	0
C18:4ω3	stearidonic acid (SDA)	3,57	3
C20:4ω6	arachidonic acid (ARA)	-	-
C20:5ω3	eicosapentaenoic (EPA)	0,07	0
C22:6ω3	docosahexaenoic acid (DHA)	-	-
∑ PUFA		27,48	23
∑ ω3		18,53	16
∑ ω6		8,95	8
∑ ω3 / ∑ ω6		2,10	
∑ FA (% of DW)		11,79	
∑ PUFA / ∑ FA		0,23	
∑ unidentified FA		6,08	5
∑		117,90	100

Note: SAFA, saturated FA; MUFA, monounsaturated FA.

In the experiments, animals from the first clutch of the third or later generation were used to assure that maternal size and weight effects are eliminated (Lampert 1993).

To enable dilutions of algal food suspensions to desired food quantities, a relationship between absorbance and particulate carbon (POC) concentration was established (Fig. 2). The algal suspensions were measured photometrically at 630 nm and at the same time filtered on a precombusted GF/C filter (Whatman; 25

mm). Filters for POC analysis were measured using a Carlo-Erba elemental analyzer with acetanilide as standard.

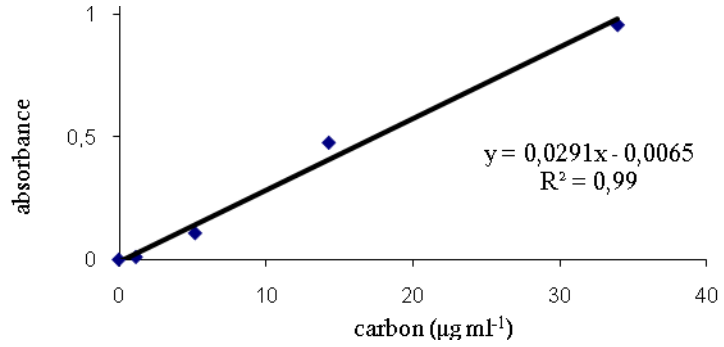


Fig. 2: Relationship between absorbance at 630nm (cuvette length 5.0 cm) and particulate carbon content of algal food suspensions.

To ensure that algae have the same density, *S. acutus* were kept in 1 l semi-continuous cultures. Every day 0.5 l of algae suspension was removed and the same amount of new algal medium was added. This resulted in a carbon concentration ranging from 11.84 to 8.35 mg C l^{-1} and gave reasonable ratios of algae to zooplankton medium when preparing the food concentrations. This procedure of algae culturing most likely produced algae which are non-nutrient limited and hence no P (or N) limitation should therefore be expected to occur in the experiments.

Experimental set-up 1st experiment

The first experiment was designed to estimate the response in survival, reproduction and somatic growth rate of *D. magna* of different food quantities and at two temperatures (12.0±1.0˚C and 20.6±0.2°C). Concentrations that needed to be established were the point of starvation, the reproduction threshold concentration and the incipient limiting concentration. Therefore a gradient design with 2 temperatures and 5 food levels was chosen with 4 replicates and 5 individuals of *D. magna* in each 250 ml flask.

Different food concentrations that are approximately equally spaced on a log scale, and expected to cover a concentration range from close to the starvation point to above the ILL were chosen:

- 2.0 mg C l^{-1}
- 0.7 mg C l^{-1}
- 0.2 mg C l^{-1}
- 0.07 mg C l^{-1}
- 0.02 mg C l^{-1}

Fresh food suspensions were prepared daily by using the results of the pre-established Abs_{630}–POC relationship (Fig. 2) and adding an appropriate amount of algae solution to zooplankton medium for each 250 ml flask (Nunc™ Cell Culture flasks). The animals were transferred with a wide mouthed 5ml pipette into the new flasks. Survival and number of neonates was recorded daily.

The experiment in the 20.6±0.2°C and at 2.0 mg C l^{-1} treatment was terminated after 6 days when reproduction started and repeated for 0.07 and 0.2 mg C l^{-1} for 13 days. At 12.1±1.0°C the experiment was terminated after 13 days even though no reproduction occurred.

Experimental set-up 2nd experiment

In this experiment the response in survival, somatic and reproductive growth of *D. magna* to different quantity and quality of food (FA content) and temperature was assessed. Food suspensions were manipulated by adding 10 mg EPA (g C)$^{-1}$ to the algae suspension. As a control treatment, 10 mg (g C)$^{-1}$ oleic acid (OA; C18:1ω9) was added to algae. Oleic acid is a monounsaturated FA and is therefore from its energy content comparable to EPA (C20:5ω3) but is not an essential FA. The OA control treatment enables conclusions whether variation of survival, somatic growth and reproduction are resulting from the higher energy supply (as would be equally expected for additional dietary OA and EPA) or from the enrichment of the highly unsaturated EPA only (i.e., effect of high degree of double bond). To test this, a growth experiment at two food levels, two temperatures and three different food qualities was conducted (Tab. 2). Food quantity was chosen at sufficient food concentration (2.0 mg C L^{-1}) and the reproduction threshold concentration of 0.2 mg C L^{-1}. Temperatures were slightly increased compared to experiment 1 and resulted in 14.1±2.3 ˚C and 21.7±1.2 °C. The 2 x 2 x 3 factorial design was carried out with 4 replicates and 5 individuals in each flask (in total 240 individuals).

Table 2. Experimental set-up to test the effect of temperature, food quantity and FA enrichment and their interactions on survival, somatic and reproductive growth of *D. magna*.

Quantity	Temperature	Quality		
0.2 mg C l^{-1}	21.7 °C	algae	algae+EPA	algae+OA
	14.1 °C	algae	algae+EPA	algae+OA
2.0 mg C l^{-1}	21.7 °C	algae	algae+EPA	algae+OA
	14.1 °C	algae	algae+EPA	algae+OA

Microcapsule production

For the enrichment of the algal suspensions with FA the microcapsule method of Cary et al. (1992), was employed as adapted by Sundbom and Vrede (1997). A gel of sodium alginate (1.6% w/v) and gelatine (0.5% w/v) was prepared and pH was set to 12.0 with 1.0 m NaOH. The suspension was stirred at 50 °C until it fully dissolved. A syringe (3 ml) was placed on a balance and the gel and the FA (EPA or oleic acid) were added into the syringe until the lipid content was 1.5% w/w. According to Sundbom and Vrede (1997) this proportion results in stable capsules with a density similar to algal cells. To ensure a homogenous suspension the syringe was shaken and thereafter stored for 5 min upside down, so that the air could be removed with only loosing little of the suspension.

A glass capillary (0.3 x 13 mm) with a cut tip was fitted to the syringe. A Pasteur pipette was prepared by melting the tip with a Bunsen burner and stretching the glass till the outer diameter was approximately 0.7 mm. The syringe was placed horizontally and its capillary was pointing perpendicularly, tip to tip, to the Pasteur pipette, which was blowing out compressed N_2. By pressing the emulsion steadily and slowly out of the syringe into the N_2 stream, an aerosol fan was created, which was collected into a beaker with 20 ml ice-cold $CaCl_2$ (20% w/v) and stirred to avoid aggregation. After spraying 1ml of emulsion the $CaCl_2$ solution was put into the fridge to allow the capsules to harden. The solution was later sieved through a 30-µm mesh. The fraction <30 µm was used for the 2nd experiment. Subsamples were taken, and capsule density, shape and size distribution were assessed in a microscope by using a counting chamber (Buerker). The average amount of FA/ml was calculated and the required concentration of FA (10 mg FA (g C)$^{-1}$) was calculated. The microcapsules were stored in Eppendorf vials in the fridge and thoroughly shaken before usage.

When an experiment started a random subsample of neonates from each temperature was collected. The animals were dried and frozen in pre-weighed aluminum capsules for 12 h in a freeze dryer (Edwards 4K Modulyo). The daphnids dry mass was then measured with the electro balance (Cahn microbalance; ±1 µg) to provide an initial biomass estimate. When terminating the experiments, daphnids and neonates born during the experimental period followed the same procedure to determine dry mass. Somatic growth rate was then calculated as:

$$r = (\ln C_t - \ln[C_0])\, t^{-1}$$

where C_0 is the initial animal dry weight and C_t the final animal dry weight, t is the duration of the experiment in days.

After weighing the animals were pooled treatment wise and transferred into cryogenic vials, filled up with nitrogen gas and stored in the freezer until later FA analysis.

During the experiment mortality was recorded daily. As soon as egg production was possible to detect the day was noted.

Statistics

Statistical analyses were run using JMP software (version 8) with α set at 0.05.

For survival individuals in each flask was averaged and a Wilcoxon/ Kruskal Wallis analysis was performed.

Data for somatic growth rates and reproduction (average clutch size individual $^{-1}$, average number of neonates individual $^{-1}$ and average weight of neonates) were analyzed with factorial ANOVA after Box Cox transformation of x+1 (except data for average dry weight neonate^{-1}).

Results

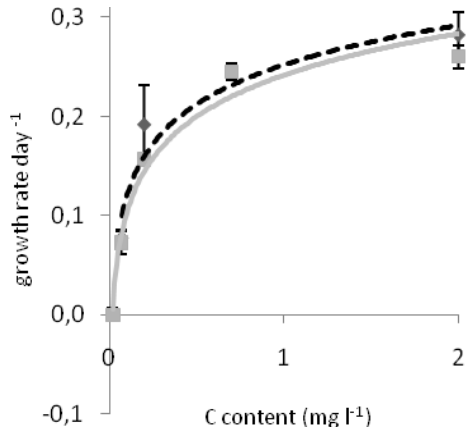
1st Experiment: effect of food concentration and temperature on somatic growth

Initial dry mass of *D. magna* at 12.0 °C was 7.9 ±1.0 µg and increased to 196.8 ±31.2 µg when exposed to 2.0 mg C l^{-1} of food. In comparison, initial weight at 20.6°C was lower with 6.7 ±1.2 µg but showed a higher increase at highest food concentration to 238.9 ±77.1 µg. At both temperatures *Daphnias* growth rate increased with increasing availability of algal carbon and showed maximum growth rates at 2.0 mg C l^{-1} (12.0°C: 0.260 day^{-1}, 20.6°C: 0.282 day^{-1}) (Fig. 3a).

The starvation point was 0.02 mg C L^{-1} at 12.0 °C, and the average weight of these individuals was 7±1 µg after 13 days of exposure to this conditions. *D. magna* rapidly exhibited increased growth with increasing carbon availability. When diet supply was >0.5 mg C l^{-1} the rate of increase in growth rate was declining. *Daphnia* at 20.6°C showed a similar pattern in growth when the experiment was run for 6 days, with a negative growth rate at 0.02 mg C L^{-1}. Data for the 20.6 °C are not complete for 0.02, 0.7 mg C l^{-1} for the same experimental period (13 days) as at 12.0°C therefore no precise localization of the point of starvation and the ILL is possible. However, these data allow a more direct comparison of growth rates at different temperatures (Fig. 3 a and b). When plotting logarithmic trend lines of both growth rates (Fig. 3 a) a similar response in growth to different food concentrations at 20.6°C and 12.0 °C can be seen. When plotting linear regression lines of growth rates on a logarithmic x- axis (Fig. 3b), the 20.6 °C treatment shows a higher starvation point (at 0.03 mg C l^{-1}) than the 12.0 °C (at 0.02 mg C l^{-1}) treatment. For calculating the starvation point, data of growth rate at high food concentration (2.0 mg C l^{-1}) were not included.

At 20.6 °C and at a food concentration of 2.0 mg C l^{-1} *D. magna* produced eggs from day 6 on, when exposed to 0.2 mg C l^{-1} eggs were detected from day 8 on. In contrast, within 13 days no reproductive growth occurred at 12.0°C.

a)



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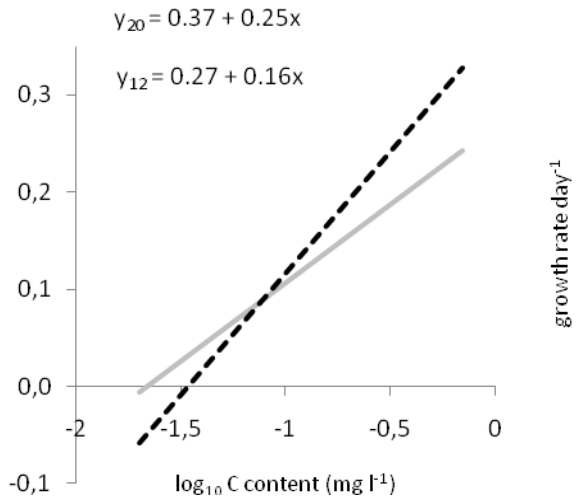

Fig. 3 Somatic growth of *D. magna* in response to different algal carbon concentration levels at 12.0 °C and 20.6 °C. Experimental period was 13 days. Dashed, blacked lines display the results at 20.6 °C, grey and solid lines the results at 12.0 °C. a) Markers and logarithmic trend lines display somatic growth rates (±SD). 20.6 °C (R^2 = 0.92; y (20.6°C) = 0.06ln(x) + 0.25), 12.0 °C (R^2 = 0.97, y (12.0°C) = 0.06ln(x) + 0.24). b) Data points (±SD) and linear regression line of somatic growth plotted on a logarithmic x scale.

2nd Experiment: effect of EPA enrichment, food concentration and temperature on survival

Temperature and food concentration both affect survival of *D. magna*, whereas food quality does not explain the variation in survival of the daphnids (Tab.3). Survival ranged between 66% and 100% and was higher at 14.1°C than at 21.7 °C (Fig. 4). At 14.1°C and 0.2 mg C l^{-1}, individuals showed a survival of 98.3% (±2.9), whereas the same food level enabled only a survival of 65.6 % (±5.1) in the 21.7°C treatment and only 2.8 daphnids survived under these conditions. At 2.0 mg C l^{-1} and 21.7°C on average 4.9 individuals were alive on day 13 (Fig. 4). Data of survival of *Daphnia* suggest that there is an interaction between food quantity and temperature with lower survival at high temperature when food is scarce. Even if the assumption of homogeneity of variance of survival data is not met, factorial ANOVA reveals that there is a combined effect of temperature and food quantity (Tab. 4).

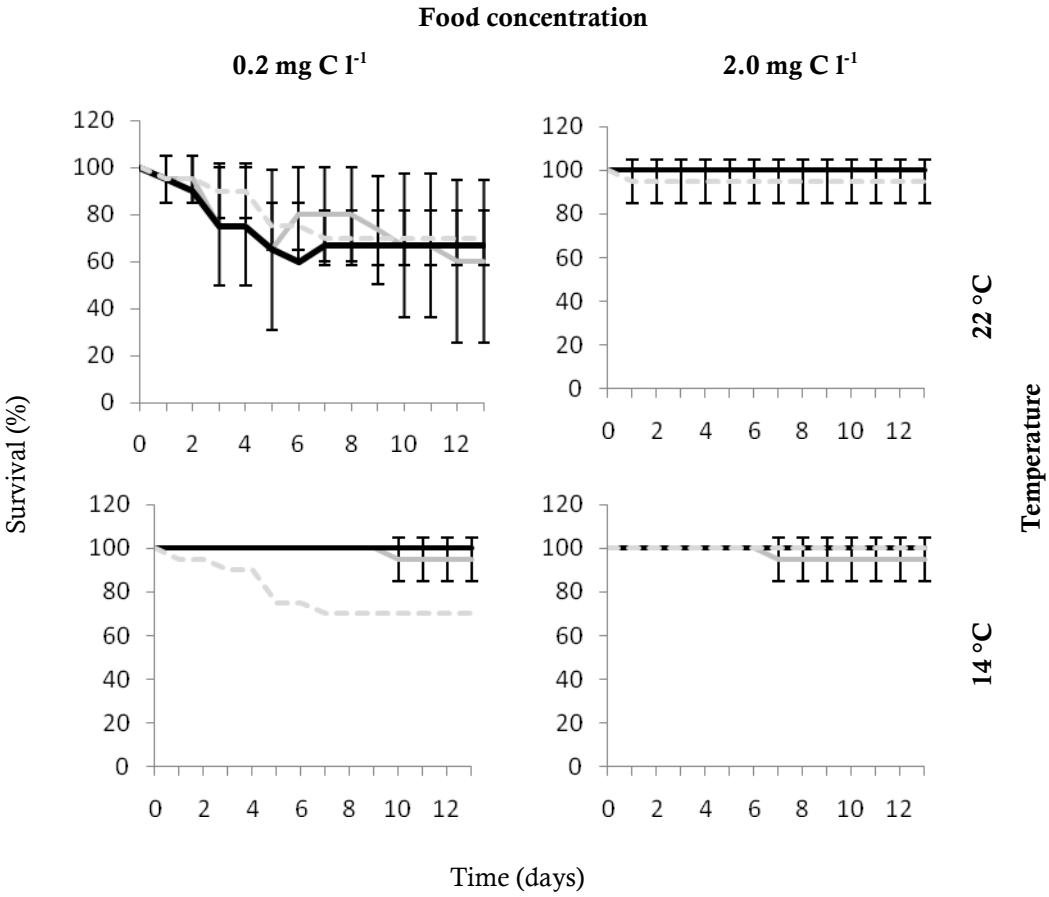

Fig. 4 Average survival (%) of *D. magna* (±STDEV) in response to different algal carbon concentration levels at 14.1 °C and 21.7°C.The increase in survival in the 0.2 mg C l $^{-1}$, 21.7 °C is explained by the loss of replicates (grey: algae; black: algae+EPA, grey dashed: algae+OA). Number of replicates was 4 for all treatments, except for 2.0 mg C l^{-1} EPA and algae at 21.7 °C where n was 3 from day 6 and 7 on due to high mortality.

Table 3. Wilcoxon / Kruskal Wallis analysis of the effects of temperature (12.0 °C and 20.6 °C), food quantity (0.2 mg C l^{-1} and 2.0 mg C l^{-1}) and food quality (algae, algae+EPA, algae+OA) on survival of *D. magna*.

	DF	Chi²	p
temperature	1	8.812	0.003
food quantity	1	8.812	0.003
food quality	2	0.386	0.824

Table 4. Results of full factorial ANOVA of the effects of temperature, food quantity, quality and their interactions on survival of *D. magna*. Data were Box Cox transformed (x+1).

survival		DF	∑ Squares	F Ratio	p	R^2
analysis of variance	Model	11	16.017	7.1	< 0.0001	0.696
	error	34	6.999			
effect tests	temp.	1	5.695	27.7	< 0.0001	
	quantity	1	5.695	27.7	< 0.0001	
	temp × quantity	1	5.695	27.7	< 0.0001	
	quality	2	0.150	0.4	0.698	
	temp. × quality	2	0.182	0.4	0.646	
	quantity × quality	2	0.105	0.3	0.776	
	temp. × quantity × quality	2	0.105	0.3	0.776	

2nd Experiment: effect of EPA enrichment, food concentration and temperature on somatic growth

Food quantity, quality, temperature and their interactions explained 96 % of the variation in growth rate (Tab. 5). The strongest effects were food quantity (positive), quality (EPA>OA>algae) and the positive temperature × quantity interaction (Tab. 5, Fig. 5).

Growth was highest at 14.1°C at both food concentrations (0.2 mg C l^{-1}: 0.203; 2.0 mg C l^{-1}: 0.322). The effect of EPA enrichment is most pronounced at 14.1°C and 2.0 mg C l^{-1} whereas at 21.7 °C the effect of food quality is less conspicuous.

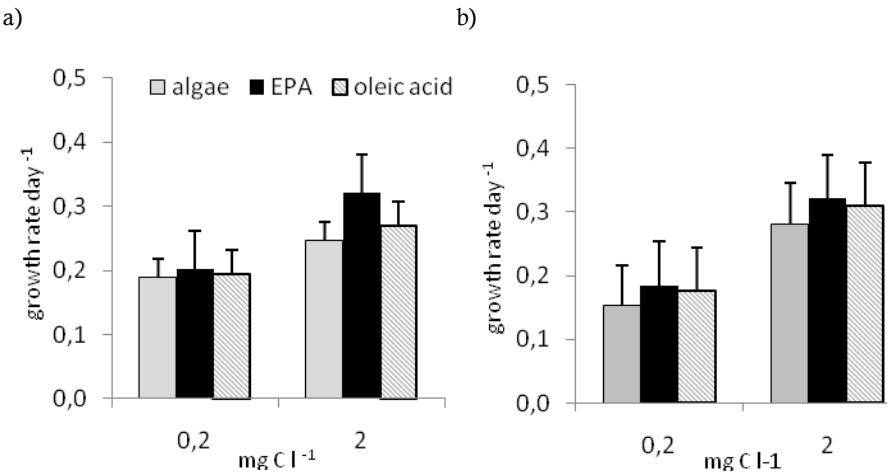

Fig. 5. Growth rate of *D. magna* (mean +SE) in response to different algal carbon concentration levels (0.2 mg C l^{-1} and 2.0 mg C l^{-1}) and different dietary FA at 14.1 °C (a) and 21.7°C (b).

Table 5. Results of full factorial ANOVA of the effects of temperature, food quantity, quality and their interactions on somatic growth of *D. magna*. Data were Box Cox transformed (x+1).

Somatic growth rate						
analysis	Model	11	0.157	65.2	< 0.0001	0.955
of variance	error	34	0.007			
	temp.	1	0.000	0.3	0.590	
	quantity	1	0.132	606.5	< 0.0001	
	temp. × quantity	1	0.007	32.1	< 0.0001	
effect tests	quality	2	0.012	28.5	< 0.0001	
	temp. × quality	2	0.001	2.2	0.127	
	quantity × quality	2	0.003	6.6	0.004	
	temp. × quantity × quality	2	0.002	3.8	0.033	

2nd Experiment: effect of EPA enrichment, food concentration and temperature on reproduction (clutch size, number off neonates, average weight of neonates)

Food quantity, quality, temperature and their interactions explained 91 % of the variation in clutch size on the day of termination of the experiment (Tab. 6). Food concentration is explaining most of the variance of reproduction in terms of clutch size on day 13, followed by temperature and the interaction quantity × temperature.

At 21.7 °C individuals showed reproduction at 2.0 mg C l^{-1} as well as at 0.2 mg C l^{-1} in algae, EPA and OA treatments. The average clutch size (per treatment, n=4) on day 13 was highest for animals exposed to 2.0 mg C l^{-1} and EPA enrichment at 21.7 °C with on average 20 eggs per individual (Fig. 6).

At 14.1 °C the average clutch size (per treatment, n=4) was 11 eggs per individual, when exposed to 2.0 mg C l^{-1} and EPA enrichment. At a food concentration of 0.2 mg C l^{-1} only animals in the EPA treatment showed reproduction, no eggs were produced in algae and OA treatments. The effect of FA content of diet marginally significantly explains variances of clutch size whereas the interaction between quality, temperature and/or food quantity are clearly insignificant.

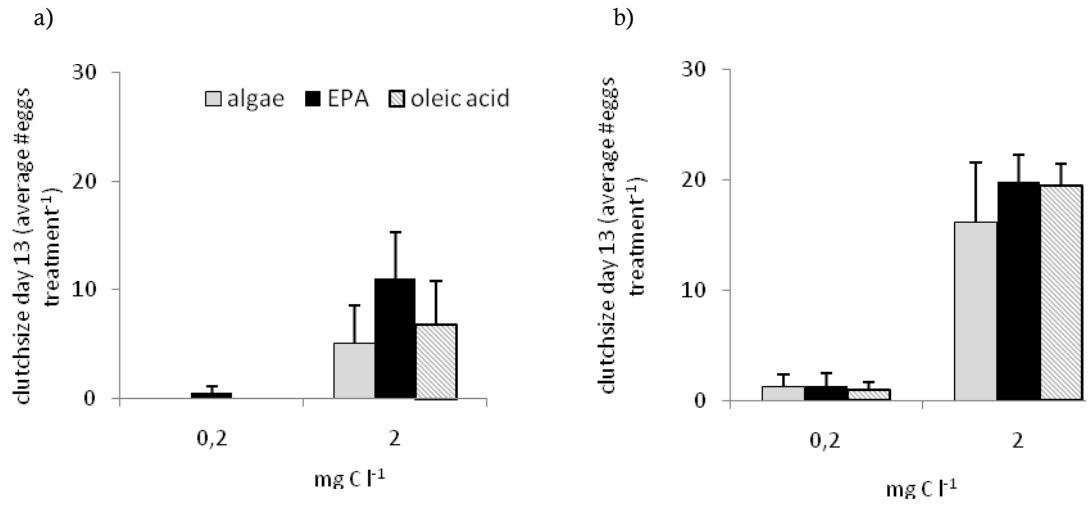

Fig. 6. Effect of food quality on clutch size on day 13 (termination of experiments) of *D. magna* in response to different algal carbon concentration levels at 14.1°C (a) and 21.7°C (b). Data are means +SE.

At 21.7°C but not at 14.1°C neonates hatched during the experiment. The age of first reproduction at 21.7°C ranged between 8 and 12 days and was lower at 2.0 mg C l^{-1} than at 0.2 mg C l^{-1}. At high food concentration age of first reproduction was the same for all food qualities (8d), where at the low food concentration there was an effect of FA content on age of first reproduction. Neonates hatched in OA treatment on the 9th day, followed by EPA (10th) and algae (12th). Mean values of neonates (day female)$^{-1}$ indicate, that even if there is a delay in reproduction at lower food quantities, the average number of neonates (day female)$^{-1}$ is still much lower at this low food concentration (Fig. 7 a).

The variance in number of neonates per female (born during the 13 days of experimental period) is to 96% explained by food quantity, quality and temperature (Tab. 6). The strongest effect was food concentration, at 2.0 mg C l^{-1} the average number of neonates was 36, whereas at 0.2 mg C l^{-1} on average only 11 neonates were born during the experiment. The effect of the FA enrichment was significant and the number of neonates was highest when animals were exposed to FA enriched food. The difference of neonates between EPA and OA enrichments (37 and 36, respectively) was negligible, but differed significantly to the average number of neonates of the treatment without dietary FA enrichment (22, Fig. 7 a).

Average body mass of neonates was lower (mean 6.5 µg) when fed with 2.0 mg C l^{-1} compared to 0.2 mg C l^{-1} where neonates weighed 8.4 µg (Fig. 7 b). The effect of quantity is greater than of FA enrichment, their interaction is not significant (Tab. 6). The average body mass was highest for the animals exposed to EPA enriched nutrition at 2.0 mg C l^{-1} (8.3 µg) and 0.2 mg C l^{-1} (8.5 µg).

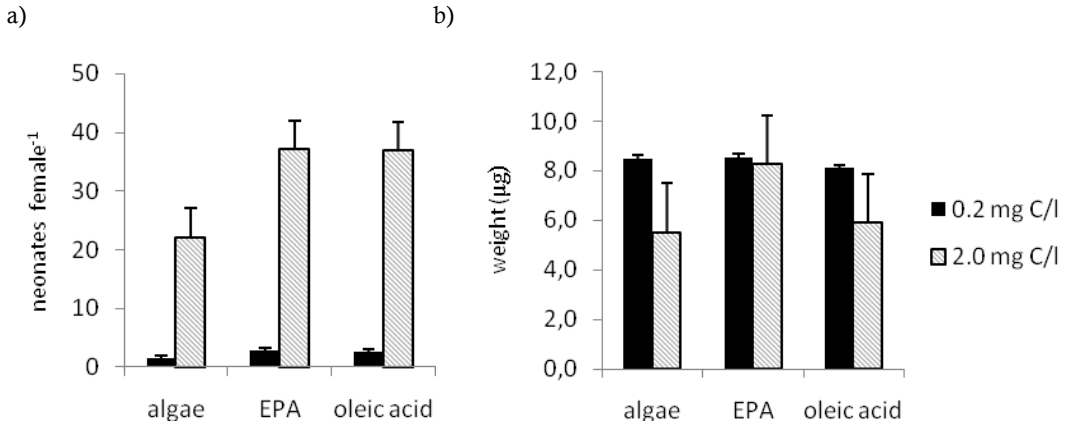

Fig. 7. a) Number of neonates (mean + SE) of *D. magna* born under different algal carbon concentrations (0.2 mg C l^{-1} and 2.0 mg C l^{-1}) and different FA content at 21.7°C during experimental period of 13 days. b) Weight of neonates (mean + SE) born under different algal carbon concentrations (0.2 mg C l^{-1} and 2.0 mg C l^{-1}) and different FA content at 21.7°C.

Table 6. Summary of the results of full factorial ANOVA of the effects of temperature, food quantity, quality and their interactions on reproduction measures of *D. magna*. Data, except of average weight of neonates, were Box Cox transformed (x+1).

clutch size day 13						
analysis of variance	Model	11	1185.6	29.742	< 0.0001	0.906
	error	34	123.2			
effect tests	temp.	1	143.0	39.5	< 0.0001	
	quantity	1	938.9	259.1	< 0.0001	
	temp. × quantity	1	23.0	6.3	0.017	
	quality	2	23.1	3.2	0.054	
	temp. × quality	2	6.9	1.0	0.396	
	quantity × quality	2	7.7	1.1	0.356	
	temp. × quantity × quality	2	1.6	0.2	0.798	
neonates/individual						
analysis of variance	Model	5	3717.6	70.0	< 0.0001	0.956
	error	16	170.1			
effect tests	quantity	1	3451.2	324.7	< 0.0001	
	quality	2	308.0	14.5	0.000	
	quantity × quality	2	36.4	1.2	0.315	
average weight neonates						
analysis of variance	Model	5	0.0000349	8.9	0.001	0.762
	error	14	0.0000109			
effect tests	quantity	1	0.0000134	17.1	0.001	
	quality	2	0.0000072	4.6	0.029	
	quantity × quality	2	0.0000054	3.4	0.061	

Discussion

As hypothesized, the results clearly show that food quantity is the major factor influencing somatic and reproductive growth of *D. magna*. Surprisingly somatic growth was fairly similar at low and high temperatures with at the same time a high mortality when food quantity is low and temperature is high. Reproduction was mostly determined by temperature, since no offspring was released during the experiment at low temperatures. EPA amendment resulted in the hypothesized positive response, since it improved both somatic growth and reproduction in all treatments but had a more pronounced effect at lower temperatures and high food concentration.

Effect of food concentration, food quality and temperature on survival

Survival of *D. magna* is affected by temperature and food concentration, but not by food quality. Boersma and Kreutzer (2002) assumed in their study that basic metabolic costs, like the basal respiration rates (Bohrer and Lampert 1988), are not effected by food quality. It can be speculated that survival is not improved by higher FA content because it is mainly the carbon content and the digestibility of the food that

sustains survival. To ensure high survival at high temperature, a high quantity of food is required. This might cause a limitation of *D. magna´*s survival in nature within the typical pattern of seasonal succession in temperate lakes during summer (Sommer et al. 1986). Lampert and Summer (2007) report that *Daphnia* follows a bimodal population pattern in temperate lakes. They exhibit the highest peak in May and a second but smaller one in September/August as a consequence of temperature increase and less food availability during summer (Giebelhausen and Lampert 2001). Consequently, high mortality when temperature is high and food quantity low has implications on the entire food web. A decrease in fish production and accumulation of algae during warm periods can result.

In contrast, higher survival at lower temperatures can have implications on higher trophic levels in lakes that are not strongly warming up during summer, like high latitude or high altitude lakes. Survival of zooplankton and therefore also food supply for higher trophic levels can then be expected to be stable during summer months.

Effect of food concentration, food quality and temperature on somatic growth

Individual somatic growth rate is highly dependent on food quantity and is not significantly affected by temperature. Still, *Daphnia* grew best at higher temperature and at high food level. The interaction of temperature and food concentration for somatic growth resulted from the increased starvation point and steeper slope in growth curve at high temperature. In contrast to Giebelhausen and Lampert (2001) who found a more pronounced effect of temperature (looking at a range of 15-30 °C) at high food concentrations (0.1-1.0 mg C l^{-1}) on growth, this study shows a higher influence of temperature at low food levels (0.02 mg C l -1). However, it has to be considered that Giebelhausen and Lampert (2001) did not aim to identify the effect of temperature at starvation concentration and that the present study did not cover a broad temperature range.

Lower temperature is reported to lead to reduced metabolic rate, furthermore in a decrease in locomotion and ingestion rate (Loiterton et al. 2004). Lower metabolic rate might be beneficial when food concentration is poor and temperature low as in temperate lakes in winter. Besides the seasonal variation in temperature and food availability also a vertical gradient of these two factors can be found in temperate lakes. During summer temperature and food conditions are decreasing with depth and zooplankton. Lower metabolic rate is then also beneficial when zooplankton migrates to colder deep-water layers to avoid fish predation during daytime and swims up to surface waters to feed on more abundant food there (Lampert and Sommer 2007). Contrary to decrease in metabolic rate, lower ingestion rate and therefore decrease in energy uptake at lower temperatures is affecting the energy budget of a filter-feeder negatively and influencing their fitness (Loiterton et al. 2004). The present results suggest that somatic growth of *D. magna* is not affected negatively by low temperature. However, the effect on population growth was not tested.

D. magna fed on EPA amended diet showed the highest growth at both food levels and both temperatures. There is a positive combined effect of EPA at low temperature and high food concentration. This present study suggests that *D. magna* has the ability to improve growth at low temperature conditions when food quality is high in EPA content. Arctic freshwater ecosystems are of special interest in this context, since

they are characterized by cold temperatures and food webs that are rich in lipids (Hessen and Leu 2006). Furthermore arctic lakes follow a predictable seasonal pattern with a short growing season for phytoplankton just after ice thaw and a consequently high food supply for a short period. When phytoplankton is rich in EPA, *Daphnids* somatic growth can be improved under these conditions and have cascading effects on higher trophic levels. Previous studies describe the greater importance of dietary PUFA at lower temperatures as an important mechanism for sustaining cell membrane fluidity (i.e. the principle of `homeoviscous membrane adaption´ (Sinensky 1974)). *Daphnia* in arctic lakes has been shown to accumulate considerably higher fractions of PUFA (Hessen and Leu 2006) than recorded in studies of temperate lakes (Kainz et al. 2004). At low temperatures and low food quantity, the quality of food seems to be less important to somatic growth of *Daphnia*, since it is mostly affected by the available quantity.

In temperate and stratified lakes the interactive effect of EPA at low temperature and high food concentration is mostly manifested in a vertical variation of these factors. During summer food quantity and temperature are declining with depth. Additionally, sestonic EPA concentrations is reported to vary diurnal and vertical (Park et al. 2004). The traditional concept of diel vertical migration of zooplankton assumes that food conditions are better in the surface waters and zooplankton remains there during night for feeding and migrates to colder deep-water layers to avoid fish predation during daytime (Lampert and Sommer 2007). When EPA content is high in surface waters of stratified lakes and zooplankton migrates vertically during night to colder deeper layers during night the combined effect of FA content and temperature can be expected to result in improved somatic growth.

Effect of food concentration, food quality and temperature on reproduction

Clutch size at the end of the experiment was mostly determined by food concentration followed by temperature and quality. The interaction shows a positive effect of high food concentration and high temperatures. In this treatment highest number of eggs was observed. Present results display the trend that EPA enriched diet leads to higher number of eggs in the brood pouches. Recent studies found a similar effect of EPA enrichment at low temperatures that favored higher clutch sizes of Cladocerans (Masclaux et al. 2009). Oleic acid affected the clutch size too but to a lower extent than EPA did. The high proportion of egg-bearing females when fed microencapsulated EPA is interpreted as a shorter time to first reproduction, which is an important feature for increasing the fitness of animals (Sundbom and Vrede 1997). This is of great ecological importance in lakes in the high Arctic which are characterized by low temperatures and a brief spring bloom when the bulk phytoplankton is produced (Leu et al. 2006). The highest amount of PUFA is then found in the early phase of this bloom and hence the success of a species is mainly driven by the ability to utilize the resources and reproduce.

The time span until neonates hatched increased with decreasing temperature, which is in line with previous findings (Rinke and Petzoldt 2003). *Daphnia* at low temperatures did not show any reproduction after 13 days but showed higher growth rates compared to the animals at high temperatures. This is in accordance with the temperature-size rule (Atkinson 1995), which states that low temperature causes an increase in

size at maturity of many ectotherms. In lakes with low temperatures and large population of planktivorous fish an increase of body size at maturity might cause limitation in zooplanktons success because of fish preference to feed on large zooplankton.
The age at first reproduction at high temperatures was improved by the amendment of FA. An earlier maturation when exposure to higher dietary FA might be beneficial for *Daphnids* long term success in aquatic ecosystems.

The number of neonates was strongly determined by temperature since only females at high temperature had viable offspring. At high temperature food quantity was the major factor affecting number of neonates as well as their body weight. Neonates hatched at high food concentration were greater in their number but had less weight. Additionally, food quality influenced number and weight of neonates, EPA enriched food showed a higher number of viable offspring and slightly higher biomass of neonates than dietary *S. acutus* or oleic acid enrichment. In contrast to this results Martin-Creuzburg et al. (2005) found that daphnids tend to produce larger but fewer eggs and consequently larger and fewer neonates when dietary EPA is missing. However, both studies underline the potential of EPA to improve reproduction of zooplankton.

Concluding remarks

There are still gaps in our knowledge about the relevance of PUFA in aquatic food webs at low versus high temperatures and at low versus high food concentrations. It should be recognized that in addition to growth rate, survival and reproduction other measures of fitness, behavior or other traits may be relevant. Swimming ability, stress tolerance and/or occurrence of deformities – just to name a few – might be complementary assessments of the impact of food quality on zooplankton performance (Ahlgren et al. 2009). Previous studies also suggest that there are interspecific as well as intraspecific differences of *Daphnia* to varying food and environmental conditions (Boersma et al. 1999). Furthermore, the fitness of zooplankton *in situ* is influenced by various factors besides food quantity, quality and temperature, i.e. by predation (Gliwicz and Pijanowska 1989) and UV radiation (Hessen et al. 1997, Hessen et al. 2002).

Although there admittedly are gaps that are not filled in by this study, it contributes to an increased understanding of when and where food quality limitation can be predicted to be more important in nature. At low temperatures less food quantity is necessary to maintain survival of Daphnia. When dietary EPA is amended, zooplankton growth and reproduction were improved to a more pronounced extend at low temperatures. The strongest effect of dietary EPA enrichment was found in the increase of reproduction at low temperature. One might speculate that especially in cold freshwater systems (high altitude and high latitude lakes) dietary intake of essential FA is crucial for the success of zooplankton, whereas the FA content of phytoplankton is less important in nutrient rich and temperate lakes. In these lakes, the effect of interaction between food quantity, quality and temperature is mostly manifested in the vertical variation of these factors. When EPA content is high in surface waters of stratified lakes and zooplankton migrates vertically during night to colder deeper layers during night the combined effect of FA content and temperature can be expected to result in improved somatic growth.

This study suggests that dietary EPA content has the potential to improve somatic and reproductive growth of *D. magna*. Especially at low temperatures and high food concentrations an increase in this PUFA has a pronounced positive effect and reproduction and somatic growth of zooplankton were improved. The findings of this study help to understand the factors and the interactions that affect the efficiency of energy transfer in aquatic food webs and point out that food quality is of greater ecological importance in cold freshwater systems, like they can be found in high latitudes.

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Appendix 1

Preparation (OECD/OCDE 2004)

Trace elements: Separate stock solutions (I) of individual elements are first prepared in water of suitable purity, e.g. deionized, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements, i.e.:

Stock solution(s) I (single substance)	Amount added to water (mg l^{-1})	Concentration (related to Medium M4)	To prepare the combined stocksolution II add the following amount of stock solution I to water (ml l^{-1})
H_3BO_3	57 190	20 000-fold	1.0
$MnCl_2 \cdot 4H_2O$	7 210	20 000-fold	1.0
LiCl	6 120	20 000-fold	1.0
RbCl	1 420	20 000-fold	1.0
$SrCl_2 \cdot 6\ H_2O$	3 040	20 000-fold	1.0
NaBr	320	20 000-fold	1.0
$Na_2MoO_4 \cdot 2\ H_2O$	1 260	20 000-fold	1.0
$CuCl_2 \cdot 6\ H_2O$	335	20 000-fold	1.0
$ZnCl_2$	260	20 000-fold	1.0
$CoCl_2 \cdot 6\ H_2O$	200	20 000-fold	1.0
KI	65	20 000-fold	1.0
Na_2SeO_3	43.8	20 000-fold	1.0
NH_4VO_3	11.5	20 000-fold	1.0
$Na_2EDTA \cdot 2\ H_2O$	5 000	2 000-fold	-
$FeSo_4 \cdot 7\ H_2O$	1 991	2 000-fold	-
Both Na_2EDTA and $FeSo_4$ solutions are prepared singly, poured together and autoclaved immediately. This gives:			
21 Fe-EDTA solution		1 000-fold	20.0

M4 media is prepared using stock solution II, the macro-nutrients and vitamins as follows:

	Amount added to water (mg l^{-1})	Concentration (related to Medium M4)	Amount of stock Solution added to prepare medium (ml l^{-1})
Stock solution II (combined trace elements)		20-fold	50
Macro nutrient stock solutions (single substances)			
$CaCl_2 \cdot 2\ H_2O$	293 800	1 000-fold	1.0
$MgSO_4 \cdot 7\ H_2O$	246 600	2 000-fold	0.5
KCL	58 000	10 000-fold	0.1
$NaHCO^3$	64 800	1 000-fold	1.0
$Na_2SiO_3 \cdot 9\ H_2O$	50 000	5 000-fold	0.2
$NaNO_3$	2 740	10 000-fold	0.1
KH_2PO_4	1 430	10 000-fold	0.1
K_2HPO_4	1 840	10 000-fold	0.1
Combined vitamin stock	-	10 000-fold	0.1
The prepared vitamin stock solution is prepared by adding the 3 vitamins to 1 litre water, as shown below:			
Thiamine hydrochloride	750	10 000-fold	
Cyanocobalamine (B_{12})	10	10 000-fold	
Biotine	7.5	10 000-fold	

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

To avoid precipitation of salts when preparing complete media, add the aliquots of stock solutions to about 500 – 800 ml deionized water and then fill it up to 1 litre.

The first publication of the M4 medium can be found in (Elendt 1990).

Appendix 2

Recipe for algal medium (SIS 1993):

	Nutrient	concentration in stock solution	final concentration in testsolution
Stock solution 1:	NH_4Cl	1.5 g l^{-1}	15 mg l^{-1}
macronutrients	$MgCl_2$ $6H_2O$	1.2 g l^{-1}	12 mg l^{-1}
	$CaCl_2$ $2H_2O$	1.8 g l^{-1}	18 mg l^{-1}
	$MgSO_4$ $7H_2O$	1.5 g l^{-1}	15 mg l^{-1}
	KH_2PO_4	0.16 g l^{-1}	1.6 mg l^{-1}
Stock solution 2:	$FeCl_3$ $6H_2O$	80 mg l^{-1}	80 µg l^{-1}
Fe-EDTA	Na_2EDTA $2H_2O$	100 mg l^{-1}	100 µg l^{-1}
Stock solution 3:	H_3BO_3	185 mg l^{-1}	185 µg l^{-1}
Trace elements	$MnCl_2 \cdot 4H_2O$	415 mg l^{-1}	415 µg l^{-1}
	$ZnCl_2 \cdot 6H_2O$	3 mg l^{-1}	3 µg l^{-1}
	$CoCl_2 \cdot 6H_2O$	1.5 mg l^{-1}	1.5 µg l^{-1}
	$CuCl_2 \cdot 2H_2O$	0.01 mg l^{-1}	0.01 µg l^{-1}
	$Na_2MoO_4 \cdot 2H_2O$	7 mg l^{-1}	7 µg l^{-1}
Stock solution 4:	$NaHCO_3$	50 g l^{-1}	50 mg l^{-1}

1000 ml of a solution are prepared by adding

- 100 ml of stock solution 1
- 10 ml of stock solution 2
- 10 ml of stock solution 3
- 10 ml of stock solution 4

and filling up till 1000 ml with mQ water. The resulting solution is diluted by a factor of 10.