A dietary polyunsaturated fatty acid improves consumer performance during challenge with an opportunistic bacterial pathogen

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Keywords
food quality; gut pathogen; host resistance; intestinal microbiology.

Abstract
A dietary deficiency in polyunsaturated fatty acids (PUFAs) and/or sterols can severely constrain growth and reproduction of invertebrate consumers. Single nutrients are potentially assigned to different physiological processes, for example to support defence mechanisms; therefore, lipid requirements of healthy and pathogen challenged consumers might differ. In an oral exposure experiment, we explored the effects of dietary PUFAs and cholesterol on growth, reproduction and survival of an aquatic key herbivore (Daphnia magna) exposed to an opportunistic pathogen (Pseudomonas sp.). We show that healthy and pathogen challenged D. magna are strongly albeit differentially affected by the biochemical composition of their food sources. Supplementation of a C20 PUFA deficient diet with arachidonic acid (ARA) resulted in increased survival and reproduction of pathogen challenged D. magna. We propose that the observed benefit of consuming an ARA rich diet during pathogen challenge is conveyed partially via ARA derived eicosanoids. This study is one of the first to consider the importance of dietary PUFAs in modifying fitness parameters of pathogen challenged invertebrate hosts. Our results suggest that dietary PUFA supply should receive increased attention in host microorganism interactions and invertebrate disease models to better understand and predict disease dynamics in natural populations.

Introduction

The natural diet of the freshwater keystone herbivore Daphnia consists of various microorganisms, including both eukaryotic microalgae and prokaryotes (Lampert, 1987). Depending on the environmental conditions, cyanobacteria and heterotrophic bacteria can constitute a substantial share of lake seston (Simon et al., 1992; Paerl & Huisman, 2008; Hartwich et al., 2012). The different blends of food organisms can crucially influence the performance of the filter feeder Daphnia, which is unable to discriminate between food particles of different nutritional quality (DeMott, 1986).

Cyanobacteria and heterotrophic bacteria are of low food quality for Daphnia and other aquatic consumers (Martin Creuzburg et al., 2008, 2011; Basen et al., 2012; Taipale et al., 2012; Wenzel et al., 2012) because they lack sterols and are characterized by a deficiency in long chain polyunsaturated fatty acids (PUFAs) (Napolitano, 1998; Volkman, 2003). Both lipid classes, sterols and PUFAs, are indispensable structural components of cell membranes (Clandinin et al., 1991; van Meer et al., 2008) and serve as precursors for a large number of bioactive molecules. For instance, sterols serve as precursors for the moult inducing ecdysteroids in arthropods (Mykles, 2011) and certain C20 PUFAs including arachidonic acid (ARA, 20:4n 6) and eicosapentaenoic acid (EPA, 20:5n 3) are required as precursors for prostaglandins and other eicosanoids. Eicosanoids are involved in reproduction, ion transport physiology and an array of defence mechanisms in vertebrates and invertebrates (Stanley, 2000). In vertebrates, n 6 long chain PUFAs are considered to predominantly...
mediate pro inflammatory processes and n 3 PUFAs are
deed to be their anti inflammatory counterparts (Cal
der, 2007; Schmitz & Ecker, 2008; Alcock et al., 2012).
Whether n 6 and n 3 PUFAs can be assigned similarly to
differential functions with opposing outcome in inverte
brates has not yet been conclusively investigated. Neverthe
less, long chain PUFAs of both families, like ARA and EPA,
greatly impact growth and reproduction of Daphnia (von
Elert, 2002; Becker & Boersma, 2003; Martin Creuzburg
et al., 2010).

Besides being deficient in essential biochemicals, bacteria
may be associated with consumers as members of the natu
ral gut microbiota or may act as pathogenic agents within
their consumers (Carmichael, 1994; Deines et al., 2009;
Freese & Schink, 2011). Representatives of the genus Pseu
domonas, for instance, are ubiquitous and belong to the
most common bacteria in aquatic habitats (Pearce et al.,
2005). While many Pseudomonas species are benign or even
beneficial, for example for plants (Mercado Blanco & Bak
ker, 2007), some are notorious pathogens of vertebrates
and invertebrates, for example P. aeruginosa or P. entomo
phila (Ziprin & Hartman, 1971; Hardalo & Edberg, 1997;
Tan et al., 1999; Ramos, 2004a; Vodovar et al., 2005). The
mechanisms of pathogenicity are manifold and can involve
toxin production as well as detrimental bacteraemia, that is
the presence and proliferation of bacteria in the blood
(Tan et al., 1999; Ramos, 2004b; a, Limmer et al., 2011; Le
Coadic et al., 2012).

In Daphnia, Pseudomonas species have been identified as
members of the intrinsic microbiota (Qi et al., 2009; Freese
& Schink, 2011). In its natural state, the gut microbiota of
invertebrates may benefit the host by, for example aiding
digestion, producing vitamins or providing protection
from pathogens (e.g. Dillon et al., 2005; Pester & Brune,
2007; Koch & Schmid Hempel, 2011). Disturbance of this
natural balance between host and gut microbiota, however,
may facilitate growth and establishment of opportunistic
pathogens (Stecher & Hardt, 2008). Differences in lipid
mediated food quality can strongly influence the fitness of
consumers and affect the gut microbiota (Scott et al.,
2013), may affect the outcome of host parasite interactions
(Schlotz et al., 2013), and even provide signals for the host
inflammatory machinery (Alcock et al., 2012). At the same
time, there is evidence that some Pseudomonas species have
the potential to modulate eicosanoid production of its
host, thereby interfering with the host’s defence mecha
nisms (Vance et al., 2004).

Therefore, we raised the question whether supplemen
tation of dietary lipids can ameliorate fitness costs
imposed by opportunistic pathogenic bacteria. To test
this hypothesis, we orally exposed Daphnia magna to a
Pseudomonas strain, which was previously isolated from
the gut of the same clone of D. magna. This strain has
been shown to be acutely detrimental for D. magna
regarding somatic growth and survival both when pro
vided as the sole food source and in combination with algae (Martin Creuzburg et al., 2011; Freese & Martin
Creuzburg, 2013).

In an oral exposure experiment, in which the total die
tary carbon provided was partially substituted by Pseudo
monas sp., we investigated if the ability of D. magna to
cope with this pathogenic threat is affected by the dietary
sterol or PUFA supply. To disentangle general nutritional
constraints imposed by feeding on bacterial food sources
(i.e. a sterol and PUFA deficiency) from pathogenic
effects, we additionally used the picocyanobacterium Syn
echococcus elongatus as a nonpathogenic reference food. S.
elongatus is nontoxic and well assimilated by Daphnia
(Lampert, 1981), but, like the Pseudomonas strain, does
not contain sterols and PUFAs (Martin Creuzburg et al.,
2008, 2011). Both prokaryotes were provided in conjunc
tion with the eukaryotic green alga Scenedesmus obliquus,
which is of moderate food quality for Daphnia primarily
because it is deficient in PUFAs with more than 18 car
bon atoms (von Elert, 2002; Martin Creuzburg et al.,
2012). To unequivocally attribute food quality effects to
certain lipids, the PUFAs ARA and EPA as well as choles
terol were supplemented to the algal bacterial food mix
tures via liposomes. Another alga (Nannochloropsis
limnetica), rich in all of the examined lipids and thus of
superior food quality for Daphnia (Martin Creuzburg
et al., 2010), completed the set of examined microorgan
isms. Bacterial effects on survival, somatic growth and
reproduction of the consumer were recorded.

Materials and methods

Cultivation of food organisms and preparation of
food suspensions

The green alga S. obliquus (culture collection of the Univer
sity of Göttingen, Germany, SAG 276 3a) and the eustigmatophyte N. limnetica (SAG 18.99) were cultured
semi continuously in modified Woods Hole (WC) medium
(Guillard, 1975) with vitamins in aerated 5 L vessels (20 °C;
dilution rate: 0.2 d−1; illumination: 100 μmol quanta
om−2 s−1); S. elongatus was cultured in Cyano medium
(Jüttner et al., 1983) under the same conditions. The oppor
tunistic pathogen Pseudomonas sp. (strain DD1; 99.9%
similarity to P. gessardii CIP 105469; NCBI accession
number NR 024928) was cultivated in a mineral medium
using glucose as carbon source (Martin Creuzburg et al.,
2011).

Food suspensions were prepared by centrifugation of the
harvested cells and resuspension in < 0.2 μm filtrated
Lake Constance water. Carbon concentrations of the food
Table 1. Lipid composition (PUFAs and sterols) of Scenedesmus obliquus, of the sterol or PUFA containing liposomes used for supplementation (cholesterol, ARA, EPA) and of Nannochloropsis limnetica. Data are means of three replicates ± SD in μg mg C⁻¹ (n.d. not detectable). No PUFAs or sterols were detected in Synechococcus elongatus, Pseudomonas sp. or control liposomes.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>S. obliquus</th>
<th>+ Cholesterol</th>
<th>+ ARA</th>
<th>+ EPA</th>
<th>N. limnetica</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n 6 (LIN)</td>
<td>14.77 ± 0.75</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>14.68 ± 0.13</td>
</tr>
<tr>
<td>18:3n 6 (GLA)</td>
<td>1.81 ± 0.07</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.84 ± 0.01</td>
</tr>
<tr>
<td>18:3n 3 (ALA)</td>
<td>110.07 ± 5.85</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.13 ± 0.04</td>
</tr>
<tr>
<td>18:2n 6 (LIN)</td>
<td>7.73 ± 0.39</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>20:4n 6 (ARA)</td>
<td>n.d.</td>
<td>29.20 ± 0.34</td>
<td>n.d.</td>
<td>n.d.</td>
<td>28.05 ± 2.35</td>
</tr>
<tr>
<td>20:5n 3 (EPA)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>29.33 ± 0.45</td>
</tr>
<tr>
<td>Fungisterol</td>
<td>4.67 ± 0.58</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Schottenol</td>
<td>0.93 ± 0.33</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15.82 ± 1.33</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>11.64 ± 0.31</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.53 ± 0.03</td>
</tr>
<tr>
<td>Isofucosterol</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.04 ± 0.26</td>
</tr>
</tbody>
</table>

Suspensions were estimated from photometric light extinctions and from previously determined carbon extinction equations. The carbon light extinction regressions were confirmed by subsequent carbon analysis of the food suspensions.

Liposomes were produced and prepared as described in Martin Creuzburg et al., 2009. The amount of daily supplied ARA containing liposomes was adjusted to an amount of ARA comparable to what is found in the daily supplied N. limnetica food suspension (Table 1). To be able to directly compare effects of dietary ARA to those of EPA, we did not provide the exceptionally high amounts of EPA contained in N. limnetica (Table 1), but instead supplemented equal amounts of ARA and EPA.

Chemical analyses of food organisms

Fatty acids and sterols

For the analysis of dietary fatty acids and sterols, ~1 mg particulate organic carbon (POC) was filtered separately onto precombusted GF/F filters (Whatman, 25 mm). Filters were placed in 5 mL of dichloromethane : methanol (2:1, v:v) and stored at −20 °C. Total lipids were extracted three times from sonicated filters with dichloromethane : methanol : diethyl ether (9:1:1, v:v). The lipid containing fraction was evaporated to dryness under N₂ and resuspended in iso hexane (10 μL). Lipids were analyzed by gas chromatography (GC; Hewlett Packard 6890TM) equipped with a flame ionization detector (FID) and a DB 225 (J&W Scientific, 30 m x 0.25 mm inner diameter (i.d.) x 0.25 μm film) capillary column for FAME analysis and with a HP 5 (Agilent, 30 m x 0.25 mm i.d. x 0.25 μm film) capillary column for sterol analysis. Details of GC configurations are given elsewhere (Martin Creuzburg et al., 2009, 2010). Lipids were quantified by comparison to internal standards (C23:0 ME; 5α cholestane) of known concentrations using multipoint standard calibration curves. Lipids were identified by their retention times and their mass spectra, which were recorded with a GC mass spectrometer (7890A GC system, 5975C inert MSD, Agilent Technologies) equipped with a fused silica capillary column (DB 225MS, J&W for FAMEs; DB 5MS, Agilent for sterols; GC configurations as described for FID). Sterols were analyzed in their free form and as their trimethylsilyl derivatives, which were prepared by incubating 20 μL of iso hexane sterol extract with 10 μL of N,O bis(trimethylsilyl)trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) for 1 h at room temperature. Spectra were recorded between 50 and 600 amu in the electron impact (EI) ionization mode. The limit for quantitation of fatty acids and sterols was 20 ng. The absolute amount of each lipid was related to POC.

Elemental composition

Aliquots of food suspensions were filtered onto precombusted glass fibre filters (Whatman GF/F, 25 mm diameter) and analyzed for POC and nitrogen using an elemental analyser (EuroEA3000, HEKAtech GmbH, Wegberg, Germany). For the determination of particulate phosphorus, aliquots were collected on acid rinsed polysulphone filters.
In juvenile somatic growth rates (increase in dry mass from day 0) saturating amounts of EPA or cholesterol and ‘bacteria’ (ANOVA1: none, S. obliquus or assigned to one of the following food regimes: (1) 100% were kept individually in 80 mL of 0.2 mental animals (third clutch neonates born within 12 h) water (20 sp.; (3) The domonas bacterial carbon represented either by of which 30% of the provided carbon was exchanged by quus Pseudomonas individuals per treatment for the determination of cumula experiment to determine somatic growth rates leaving 24 um and freshly prepared food suspensions. The experi ment started with 30 individuals per treatment; six individ uals of each treatment were subsampled at day 6 of the ment. Animals were transferred daily to fresh med the experiment to determine somatic growth rates leaving 24 individuals per treatment for the determination of cumula tive numbers of viable offspring.

Data analyses
To determine somatic growth rates, subsamples of the experimental animals were taken at the beginning and at day 6 of the experiment, dried for 24 h, and weighed on an electronic balance (Mettler Toledo XP2U; ± 0.1 µg). Juvenile somatic growth rates (g) were calculated as the increase in dry mass from day 0 (M0) to day 6 (M6) using the equation:

\[ g = \frac{\ln M_6 - \ln M_0}{t} \]

Somatic growth rates and cumulative numbers of off spring of D. magna were analyzed using factorial analyses of variance (two way ANOVA). The experimental factors were ‘algae’ (ANOVA1: S. obliquus, N. limnetica; ANOVA2: S. obliquus, N. limnetica, S. obliquus + liposomes, ARA, EPA or cholesterol) and ‘bacteria’ (ANOVA1: none, S. elonga tus, Pseudomonas sp.; ANOVA2: S. elongatus, Pseudomonas sp.). In the first ANOVA, we analyzed the effects associated with the exchange of 30% of total provided carbon by the bacteria by comparing growth rates or offspring numbers obtained on the pure algal diets S. obliquus and N. limnetica with those obtained on the mixtures S. obliquus S. elongatus, S. obliquus Pseudomonas sp., N. limnetica N. limnetica Pseudomonas sp. (ANOVA1; Table 3). In the second ANOVA, we analyzed the effects of supple mentation by comparing the results obtained on the mix tures S. obliquus S. elongatus and S. obliquus Pseudomonas sp. with those obtained on the mixed diets supplemented with control liposomes, cholesterol, ARA or EPA containing liposomes. In addition, to evaluate the obtained effects of supplementation, the mixed diets N. limnetica S. elongatus and N. limnetica Pseudomonas sp. were included in the second analysis (ANOVA2; Table 3 and Fig. 3). Somatic growth rates were log transformed, and off spring numbers were square root transformed to meet the assumptions of ANOVA. In ANOVA2, homogeneity of variances could not be accomplished by data transformation. How ever, in large experiments with balanced data, the ANOVA is robust to departures from its assumptions (Underwood, 2006) and thus the nonheterogeneity of variances was ignored. Treatment effects were tested by Tukey’s honestly significant difference (HSD) post hoc tests. Effects of ‘algae’ and ‘bacteria’ on the survival of D. magna were analyzed using a generalized linear model (GLM) and the logit link function for binominal distribution. All analyses were car ried out using the statistical software package R (v.2.12.0).

Results
Element ratios and lipid profiles of food organisms
The algal and especially bacterial food sources were charac terized by high nitrogen and phosphorus contents, result ing in low carbon to nitrogen (C:N) and carbon to phosphorus (C:P) ratios (means ± SD; S. obliquus: C:N 5.9 ± 0.0, C:P 103.4 ± 2.1; N. limnetica: C:N 7.9 ± 0.1, C:P 151.0 ± 1.2; S. elongatus: C:N 5.0 ± 0.1, C:P 72.7 ± 0.3; Pseudomonas sp.: C:N 4.4 ± 0.0, C:P 36.6 ± 2.1). As saturating amounts of food (2 mg C L⁻¹) were provided daily and as the C:N and C:P ratios of the food sources were clearly below any published thresholds (C:N > 20; C: P > 200) for N or P limited growth (Sterner & Elser, 2002), a limitation of D. magna by C, N or P in our experi ment is very unlikely.

PUFA and sterol profiles of the two algae differed con siderably (Table 1). S. obliquus contained linoleic acid (LIN, 18:2n 6), γ linolenic acid (GLA, 18:3n 6), stearidonic acid (STA, 18:4n 3) and high concentrations of α linolenic acid (ALA, 18:3n 3), but no PUFA with more
Performance of *D. magna*

Survival of *D. magna* was strongly affected by *Pseudomonas* sp. When raised on *S. obliquus* *Pseudomonas* sp., only 29% of the animals survived until the end of the experiment (Fig. 1a). In contrast, when raised on *N. limnetica* *Pseudomonas* sp., mortality was absent (Fig. 1b). Exchanging 30% of the provided carbon by *S. elongatus* in *S. obliquus* or *N. limnetica* based diets did not affect survival. Supplementation of *S. obliquus* *Pseudomonas* sp. with ARA significantly increased survival (71%; Fig. 1c, Table 2). In contrast, neither cholesterol nor EPA supplementation increased survival in the *S. obliquus* *Pseudomonas* sp. treatment. Survival rates of animals fed the *S. obliquus* *S. elongatus* mixtures were not affected by lipid supplementation (Table 2b; data not shown). Additional pairwise comparisons revealed that survival rates did not differ between *S. obliquus* and *N. limnetica* ($P = 0.899$), between supplemented and with control liposome supplemented *S. obliquus* *S. elongatus* mixtures ($P = 0.476$), between unsupplemented and with control liposome supplemented *S. obliquus* *S. elongatus* mixtures ($P = 0.476$), between unsupplemented and with control liposome supplemented *S. obliquus* *Pseudomonas* sp. mixtures ($P = 0.247$), and also not between the *N. limnetica* liposomes mixtures and the ARA supplemented *S. obliquus* *Pseudomonas* sp. mixtures ($P = 0.033$; not significant after Bonferroni adjustment).

Juvenile somatic growth rates of animals raised on *N. limnetica* were significantly higher than those of animals raised on *S. obliquus* (Fig. 2). Growth rates obtained on the algal mixtures with *S. elongatus* did not differ from those obtained on the respective alga alone (Table 3). When fed the *S. obliquus* *Pseudomonas* sp. mixture, somatic growth rates were significantly reduced by 64%, when fed *N. limnetica* *Pseudomonas* sp. by 49% (compared to the respective alga *S. elongatus* control; Fig. 2). Supplementation of *S. obliquus* *S. elongatus* with ARA and EPA significantly
increased somatic growth rates. In contrast to the effect observed on survival, we could not find a significant effect of any of the supplemented lipids on somatic growth of *Pseudomonas* sp. exposed animals (Fig. 2).

The cumulative numbers of viable offspring produced by *D. magna* during the experiment were significantly higher when fed *N. limnetica* than when fed *S. obliquus* (Table 3, Fig. 3). The presence of 30% *S. elongatus* in both algal food mixtures did not impact reproduction (Table 3). Supplementation of the *S. obliquus* *S. elongatus* mixture with cholesterol significantly increased offspring production, but offspring numbers increased even more upon supplementation with ARA and EPA. The cumulative numbers of viable offspring produced on cholesterol, ARA and EPA supplemented *S. obliquus* *S. elongatus* mixtures did not significantly differ from those produced on *N. limnetica* (Fig. 3). In the presence of *Pseudomonas* sp., offspring production was drastically reduced, but animals fed *N. limnetica* produced significantly more offspring than animals fed *S. obliquus*. We did not find beneficial effects of cholesterol or EPA on reproduction when given as supplement along with the *S. obliquus* *Pseudomonas* sp. mixture. However, supplementation with ARA significantly increased offspring production in *Pseudomonas* sp. exposed animals. The numbers of viable offspring produced on the ARA supplemented *S. obliquus* *Pseudomonas* sp. mixture did not differ significantly from those produced on the *N. limnetica* *Pseudomonas* sp. mixture (Fig. 3).

### Discussion

Pathogens immensely impair growth, fecundity and survival of their hosts and thus can crucially affect population dynamics. Members of the genus *Daphnia* are key species in freshwater food webs, where they play a major role in the transfer of biomass from primary producers to higher trophic levels. Moreover, *Daphnia* species have become important model organisms to study evolutionary phenomena, such as co evolution in host-pathogen interactions. Consequently, assessing the potential of pathogens to impair *Daphnia* life history traits and to understand the underlying physiological mechanisms is of great ecological and evolutionary interest. Host nutrition presumably impacts both the host and its pathogens, implying complex resource consumer-microbial interactions (Cory & Hoover, 2006).

It has been demonstrated that bacterial food sources, that is cyanobacteria and heterotrophic bacteria, are of poor quality for *Daphnia* due to the absence of essential
Table 3. Results of factorial analysis of variance (ANOVA) of somatic growth rates and cumulative numbers of offspring of Daphnia magna raised on different food regimes. *ANOVA1*: effects associated with the exchange of 30% of total provided carbon by bacterial carbon; comprised the two algae Scenedesmus obliquus and Nannochloropsis limnetica (algae), as sole food source or as mixtures of each alga with either Synechococcus elongatus or Pseudomonas sp. (*bacteria*) as categorical variables. *ANOVA2*: effects of lipid supplementation; comprised Synechococcus, N. limnetica, S. obliquus supplemented with control liposomes, cholesterol, ARA, and EPA containing liposomes (*algae*) as mixtures of each food treatment with either S. elongatus or Pseudomonas sp. (*bacteria*) as categorical variables.

<table>
<thead>
<tr>
<th></th>
<th>Somatic growth rate</th>
<th>Cumulative number of offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>SS</td>
</tr>
<tr>
<td>Algae only (<em>ANOVA1</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Algae</td>
<td>1</td>
<td>0.252</td>
</tr>
<tr>
<td>Bacteria</td>
<td>2</td>
<td>0.056</td>
</tr>
<tr>
<td>Algae × bacteria</td>
<td>2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Residuals</td>
<td>30</td>
<td>0.002</td>
</tr>
<tr>
<td>Algae supplemented (<em>ANOVA2</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Algae</td>
<td>5</td>
<td>0.035</td>
</tr>
<tr>
<td>Bacteria</td>
<td>1</td>
<td>0.188</td>
</tr>
<tr>
<td>Algae × bacteria</td>
<td>5</td>
<td>0.004</td>
</tr>
<tr>
<td>Residuals</td>
<td>60</td>
<td>0.013</td>
</tr>
</tbody>
</table>

![Graph](image)

Fig. 3. Cumulative numbers of viable offspring produced by Daphnia magna within the experimental period (21 days) on Scenedesmus obliquus (Scen) or Nannochloropsis limnetica (Nanno) (black bars) or on 70:30% mixtures (with respect to total provided carbon) with Synechococcus elongatus (grey bars) or Pseudomonas sp. (hatched bars). Labels on the x-axis indicate food treatments. *S. obliquus* *S. elongatus* and *S. obliquus* *Pseudomonas* sp. mixtures were supplemented using liposomes (no without lipid supplementation; + lipo control liposomes; + chol/ARA/EPA; supplementation with cholesterol, ARA or EPA, respectively). Data are means of 24 replicates ± SD. Bars labelled with the same letters are not significantly different (Tukey's HSD test, *P* < 0.05 following *ANOVA*; lower case letters: *ANOVA*, upper case letters: *ANOVA2*; cf. Table 3).

Lipids and hence do not sustain growth and reproduction to the same extent as eukaryotic food sources (Martin Creuzburg *et al.*, 2008, 2011). To disentangle these nutritional constraints from pathogenic effects, we compared life history traits of *D. magna* exposed to the opportunistic bacterial pathogen *Pseudomonas* sp. with life history traits of animals exposed to the nontoxic cyanobacterium *S. elongatus*. As the partial substitution of algal for cyanobacterial carbon did not provoke any fitness impairment, we concluded that adverse effects seen in the presence of *Pseudomonas* sp. can specifically be attributed to its pathogenicity and not generally to a lack of essential nutrients.

When exposed to *Pseudomonas* sp., the probability of survival, somatic growth rates and offspring production of *D. magna* were all drastically reduced. The extent of this reduction was clearly affected by the food treatment. The ability to resist the adverse effects of *Pseudomonas* sp. exposure was most pronounced in the presence of *N. limnetica*, in particular with respect to survival. This provides strong evidence for a diet induced resistance to an opportunistic pathogen. A similar dietary impact on the outcome of infection has been reported recently in a study using *Pasteuria ramosa*, a bacterial parasite of *D. magna* (Schlotz *et al.*, 2013). The effect of PUFA supplementation in the present study suggests that the increased resistance to *Pseudomonas* sp. on a *N. limnetica* containing diet is at least partially due to the availability of ARA in *N. limnetica*, because the enrichment of *S. obliquus* with ARA significantly reduced the pathogen induced mortality and fecundity loss. However, as effects obtained by ARA supplementation never completely matched those on a *N. limnetica* based diet other factors must contribute to the protective effect. For example, EPA was not provided in the same amounts as present in *N. limnetica*; likewise, additive or synergistic effects of combined ARA and EPA provision cannot be excluded. Furthermore, the n 6 to n 3 ratio might be important and the optimal ratio may vary with the presence and kind of pathogenic agents. Therefore, future investigations should consider potential effects of dietary nutrient mixing on the performance of *Daphnia* under pathogen challenge.

Concerning somatic growth and reproduction, *N. limnetica* was the superior food source to *S. obliquus*,
irrespective of the bacterial food source present. In animals not exposed to Pseudomonas sp., supplementation of both ARA and EPA increased somatic growth rates and offspring numbers at least up to the level obtained on a N. limnetica diet, indicating that the moderate food quality of S. obliquus is due to the absence of C20 PUFA, which corroborates previous studies on the effects of PUFA on Daphnia life history traits (von Elert, 2002; Martin Creuzburg et al., 2012). Although to a lower extent than upon PUFA supplementation, offspring numbers also increased upon cholesterol supplementation, suggesting that animals raised on the S. obliquus S. elongatus mixtures were simultaneously limited by C20 PUFA and sterols, as has been reported previously for diets consisting solely of the cyanobacterium S. elongatus (Martin Creuzburg et al., 2009; Sperfeld et al., 2012).

In pathogen exposed animals, a distinct pattern diverging from this general beneficial lipid supplementation effect was observed. When exposed to Pseudomonas sp., offspring production on a S. obliquus based diet increased upon ARA supplementation up to the level obtained on a N. limnetica based diet. However, the addition of EPA or cholesterol to the S. obliquus Pseudomonas sp. mixture did not improve reproduction. Together with the reduced mortality observed on the ARA supplemented diets, this suggests that a dietary source of ARA is crucial not only for reproduction but also for sustaining resistance to pathogenic threats. As ARA gives rise to eicosanoids mediating important reproductive and immunological functions (Stanley, 2000; Machado et al., 2007; Hayashi et al., 2008; Tootle & Spradling, 2008; Zhao et al., 2009; Wimuttisuk et al., 2013), we hypothesize that the increased resistance of D. magna to Pseudomonas sp. on ARA containing diets is related to the host’s eicosanoid repertoire. A possible involvement of eicosanoids in host pathogen interactions — either through modulation by the pathogen or through mediation of host defence mechanisms — is especially intriguing, because this could assign a major role to dietary PUFA as eicosanoid precursors in influencing the outcome of a bacterial challenge. In vertebrates, eicosanoids synthesized from ARA and EPA have different functions and partially even opposing effects, best described regarding their pro vs. anti inflammatory activity (Schmitz & Ecker, 2008; Alcock et al., 2012). Assuming similar processes in Daphnia, this may explain why EPA supplementation failed to induce the same effect as seen upon ARA supplementation.

The potential of algal food sources differing in their C20 PUFA content to modulate gene expression related to eicosanoid synthesis has been demonstrated recently (Schlotz et al., 2012). In contrast to earlier research employing eicosanoid biosynthesis inhibitors (e.g. Carton et al., 2002; Garcia et al., 2004; Heckmann et al., 2008; Merchant et al., 2008; Zhao et al., 2009), we here varied the dietary supply of eicosanoid precursors and thus show that dietary ARA can crucially influence consumer performance under pathogen challenge. The exact mechanism of pathogenesis of Pseudomonas sp. (strain DD1) in D. magna remains to be elucidated. However, additional experiments suggest that viable bacterial cells are required to mediate the observed pathogenicity during an infection process, because the pathogenicity of Pseudomonas strain DD1 can be abrogated by heat, inactivating the bacterial cells prior to exposure (Appendix S1). Hence, the involvement of toxic secondary metabolites seems unlikely although we cannot exclude that the observed harmful effects on D. magna are mediated by heat sensitive toxins. The mechanism may resemble that observed in Drosophila melanogaster after ingestion of another pathogenic Pseudomonas species, P. aeruginosa. Here, bacteria crossed the gut barrier, proliferated in the haemolymph and caused severe bacteraemia (Limmer et al., 2011). In this case, innate immune functions would become effective in an attempt to control intestinal damage and systemic infection. Many invertebrate defence mechanisms, that is nodulation response, prophenoloxidase cascade, encapsulation reaction, phagocytosis and hemocyte migration, have been shown to rely on eicosanoid action (Stanley Samuelsen et al., 1991, 1997; Mandato et al., 1997; Carton et al., 2002; Garcia et al., 2004; Merchant et al., 2008; Zhao et al., 2009; Shrestha et al., 2010) and thus can potentially be modulated by the availability of dietary precursor PUFA.

An alternative explanation for the increased resistance to the bacterial pathogen on ARA containing diets could be the bactericidal activity of PUFA per se. In their free form, PUFA can impair important cell membrane properties, inhibit the activity of enzymes and damage bacterial cells via peroxidation or auto oxidation products (Desbois & Smith, 2010). While Gram positive bacteria seem to be particularly susceptible to PUFA induced mortality, reports on the susceptibility of Gram negative bacteria, such as Pseudomonas sp., are conflicting, as are reports on the effectiveness of different PUFA in acting as antibacterial agents (Kabara et al., 1972; Knapp & Melly, 1986; Giamarellos Bourboulis et al., 1998). In general, PUFA with a higher degree of desaturation tend to be more effective (Kabara et al., 1972). Thus, assuming that the effects observed in our study can be attributed to a general bactericidal activity of free PUFA, one would expect equal responses on both ARA and EPA containing diets. However, EPA supplementation did not improve the resistance of D. magna, at least not at the amount provided in our experiment via supplementation. Pure culture growth experiments in which Pseudomonas sp. (strain DD1) was challenged with PUFA containing liposomes did not reveal any inhibitory effects and thus clearly argue against
a direct bactericidal activity of the PUFA containing liposomes (Appendix S2). Qualitative plate diffusion tests also did not reveal any bactericidal activity of the PUFA containing liposomes or of free PUFA on this *Pseudomonas* strain (Appendix S3). Moreover, a grazing induced release of allelochemicals out of *N. limnetica* is very unlikely: we did neither find evidence for growth inhibitory effects of intact or disrupted algal cells on *Pseudomonas* strain DD1 (Appendix S3) nor for the release of free PUFA out of *N. limnetica* after cell damage (G. Pohnert, unpublished data).

It has been recognized that interactions between hosts and their parasites are affected by food quality constraints both in model systems and natural populations (Frost et al., 2008; Hall et al., 2009; Schlotz et al., 2013). Here, we provide a potential link between nutritional constraints for the host and the outcome of pathogenic infections by showing that the availability of a single dietary PUFA affects the ability of *Daphnia* to resist pathogenic challenges. Considering the well established positive effects of dietary PUFA on growth and in particular reproduction of *Daphnia*, this implies that dietary PUFA deficiencies severely affect the consumer, its associated symbionts and pathogens, and in consequence population dynamics and thus food web processes.

**Acknowledgements**

We thank Alexander Wacker and Dieter Ebert for much appreciated advice, fruitful discussions and valuable comments on the manuscript. Georg Pohnert generously tested the potential release of allelochemicals from *N. limnetica* following cell disruption. Bernd Kress, Petra Merkel, Sylke Wiechmann, Antje Wiese and Susanne Wörner provided excellent technical assistance. This work was supported financially by the German Research Foundation (DFG, MA 5005/1 1, PE 2147/1 1) and the Zukunftskolleg of the University of Konstanz.

**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Information 1 – Pathogenicity of *Pseudomonas* sp. (DD1)

To test whether the pathogenicity of the *Pseudomonas* strain DD1 is connected to viable pathogenic bacteria or simply to one of their cell constituents, *D. magna* were reared either on the green alga *S. obliquus* alone or on dietary mixtures containing intact or heat-inactivated bacteria.

**Figure S2:** Survival (A) and offspring production (B) of *D. magna* reared on *S. obliquus* (Scen), a mixture of *S. obliquus* and *Pseudomonas* sp. (DD1) (Scen + Pseudo, 70% + 30% in terms of carbon), or on a mixture of *S. obliquus* and heat-inactivated *Pseudomonas* sp. (DD1) (Scen + Pseudo HI, 70% + 30%). Offspring production is expressed as the cumulative number of offspring that were produced in the first and the second clutch. Heat inactivation was achieved by incubating the bacterial suspension in a water bath at 80°C for 30 minutes. Preliminary experiments at lower temperatures and shorter heat-inactivation times revealed that these conditions are required to fully inactivate the growth of *Pseudomonas* sp. (DD1) (data not shown).

The results of this experiment show that the pathogenicity of *Pseudomonas* sp. (DD1) can be abrogated by heat-inactivating the bacterial cells. Based on this experiment one may conclude that active cells are required to mediate the observed pathogenicity during an infection process. However, it cannot be excluded that the pathogenicity is mediated by heat-labile harmful secondary metabolites.
Supplementary Information 2 – Growth response of *Pseudomonas* sp. (DD1) to PUFA-containing liposomes

The *Pseudomonas* strain DD1 was cultured either in the absence or presence of different PUFA-containing liposomes in order to assess potential bactericidal effects of the PUFA-containing liposomes used in the *Daphnia* infection experiment.

![Growth response curves](image)

**Figure S1:** Growth response curves of *Pseudomonas* sp. (DD1) recorded for a period of 26 h (22°C, pH 7.2). *Pseudomonas* sp. (DD1) was cultured in mineral medium containing 20 mM glucose in the absence (control) or in the presence of liposomes. Liposomes were either free of PUFAs (lipo) or were enriched with arachidonic acid (ARA) or eicosapentaenoic acid (EPA). Liposomes were tested in two different concentrations: low = concentration as in the experimental beakers in which *Daphnia* were exposed to *Pseudomonas* sp. (DD1); high = concentration ten times higher than in the experimental beakers. The experiment was conducted in test tubes containing 7 ml of medium (four replicates) placed on a shaker; the optical density (OD) was recorded at 600 nm.

The addition of liposomes to the bacterial cultures did not affect the growth of *Pseudomonas* sp. (DD1), irrespective of the concentration and of whether they contained PUFAs. This suggests that the presence of PUFA-containing liposomes did neither impair nor improve the growth of *Pseudomonas* sp. (DD1) in our infection experiments, which supports the hypothesis that the increased resistance of the host is due to the dietary PUFA supply rather than to a direct bactericidal activity of the supplemented PUFAs. Liposomes were used as a vehicle to provide experimental animals with dietary PUFAs and to avoid potential harmful effects of free PUFAs on *Daphnia*. It should be noted that the use of liposomes may have prevented direct contact between bacterial cells and PUFAs and thus a potential bactericidal activity on *Pseudomonas* sp. (DD1).
Supplementary Information 3 – Agar diffusion assay

To investigate whether *Pseudomonas* strain DD1 is inhibited in the presence of the two algae *S. obliquus* and *N. limnetica*, the PUFA-containing liposomes, or free PUFAs, qualitative agar diffusion assays were conducted.

Figure S3: Agar diffusion assay to test for a potential inhibition of *Pseudomonas* sp. (DD1) by free PUFAs, PUFA-containing liposomes, or the two algae *S. obliquus* (Scen) and *N. limnetica* (Nanno). Aliquots (50 μl) of a liquid overnight culture of *Pseudomonas* sp. (DD1) were spread evenly on plates (5 replicates; M1 medium: 8 g nutrient broth + 15 g agar L⁻¹). Each well was loaded with 50 μl of the respective stock solutions and the formation of inhibition zones was recorded after 48 h of incubation. 2 + 3 = free PUFAs (both from stock solutions dissolved in ethanol, 2.5 mg ml⁻¹), 5 + 6 = PUFA-containing liposomes (from the stock solutions used in the *Daphnia* experiment), 9 + 10 = algae. Ethanol (1) and PUFA-free liposomes (4) served as reference treatments.

The results show that free PUFAs dissolved in ethanol inhibit the growth of *Pseudomonas* sp. (DD1). However, the zones of inhibition did not differ from the ethanol control treatment, suggesting that inhibition is mediated by ethanol rather than by the dissolved free PUFAs. The PUFA-free as well as the PUFA-containing liposomes led to an increased growth of *Pseudomonas* sp. (DD1) at the contact zones, suggesting that the bacteria metabolized the phospholipids that were used to prepare the liposomes. *S. obliquus* and *N. limnetica* (in 1× and 10× concentrations) did not influence the growth of *Pseudomonas* sp. (DD1), suggesting that both algae did not impose allelopathic effects on *Pseudomonas* sp. (DD1). Additional agar diffusion assays, in which intact and partially disrupted (sonicated) algal cells were tested, also did not reveal allelopathic effects on *Pseudomonas* sp. (DD1) (data not shown), suggesting that wound-activated allelopathic chemicals are also not produced by these algae (cf. Pohnert 2005, *Chem Bio Chem* 6: 946-959).