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

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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 exam ple to support defence mechanisms; therefore, lipid requirements of healthy and pathogen challenged consumers might differ. In an oral exposure experi ment, 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. Supplementa tion 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 path ogen challenge is conveyed partially via ARA derived eicosanoids. This study is one of the first to consider the importance of dietary PUFAs in modifying fit ness parameters of pathogen challenged invertebrate hosts. Our results suggest that dietary PUFA supply should receive increased attention in host microor ganisms 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, cyano bacteria and heterotrophic bacteria can constitute a sub stantial 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 perfor mance of the filter feeder *Daphnia*, which is unable to dis criminate 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 verte brates and invertebrates (Stanley, 2000). In vertebrates, n 6 long chain PUFAs are considered to predominantly

mediate pro inflammatory processes and n 3 PUFAs are deemed 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

	S. obliquus	+ Cholesterol	+ ARA	+ EPA	*N. limnetica*
18:2n 6 (LIN)	14.77 ± 0.75	n.d	n.d	n.d	14.68 ± 0.13
18:3n 6 (GLA)	1.81 ± 0.07	n.d	n.d	n.d	1.84 ± 0.01
18:3n 3 (ALA)	110.07 ± 5.85	n.d	n.d	n.d	2.13 ± 0.04
18:4n 3 (STA)	7.73 ± 0.39	n.d	n.d	n.d	n.d.
20:3n 6 (DGLA)	n.d.	n.d	n.d	n.d	2.17 ± 0.01
20:4n 6 (ARA)	n.d.	n.d	29.20 ± 0.34	n.d	24.33 ± 0.45
20:5n 3 (EPA)	n.d.	n.d	n.d	28.05 ± 2.35	186.08 ± 3.68
Fungisterol	4.67 ± 0.58	n.d	n.d	n.d	n.d.
Chondrillasterol	8.71 ± 0.84	n.d	n.d	n.d	n.d.
Schottenol	0.93 ± 0.33	n.d	n.d	n.d	n.d.
Cholesterol	n.d.	15.82 ± 1.33	n.d	n.d	11.64 ± 0.31
Sitosterol	n.d.	n.d	n.d	n.d	2.53 ± 0.03
Isofucosterol	n.d.	n.d	n.d	n.d	3.04 ± 0.26

suspensions were estimated from photometric light extinctions and from previously determined carbon extinction equations. The carbon light extinction regres sions 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 sup plied ARA containing liposomes was adjusted to an amount of ARA comparable to what is found in the daily supplied *N. limnetica* food suspension to create similar conditions with respect to this PUFA (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). Fil ters 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 dichlo romethane : methanol (2 : 1, v:v). Pooled cell free extracts were evaporated to dryness under a N_2 atmosphere. The lipid extracts were transesterified with methanolic HCl (3 M, 60 °C, 15 min) for fatty acid analysis or saponified with methanolic KOH (0.2 M, 70 °C, 1 h) for sterol analy sis. Subsequently, fatty acid methyl esters (FAME) were extracted three times with *iso* hexane (2 mL); the neutral lipids were partitioned into *iso* hexane : diethyl ether (9 : 1, v:v). The lipid containing fraction was evaporated to dryness under N_2 and resuspended in *iso* hexane (10 20 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 × 0.25 mm inner diameter (i.d.) × 0.25 μm film) capillary column for FAME analysis and with a HP 5 (Agilent, 30 m × 0.25 mm i.d. × 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 identi fied 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 precombu sted glass fibre filters (Whatman GF/F, 25 mm diameter) and analyzed for POC and nitrogen using an elemental analyser (EuroEA3000, HEKAtech GmbH, Wegberg, Ger many). For the determination of particulate phosphorus, aliquots were collected on acid rinsed polysulphone filters

(HT 200; Pall, Ann Arbor, MI) and digested with a solution of 10% potassium peroxodisulphate and 1.5% sodium hydroxide for 60 min at 121 °C. Soluble reactive phosphorus was determined using the molybdate ascorbic acid method (Greenberg *et al.* 1985).

Experimental design

The life history experiment was conducted with a clone of *D. magna* originally isolated from Großer Binnensee, Germany (Lampert, 1991). Stock cultures were cultivated in filtrated Lake Constance water (< 0.2 μm) containing saturating amounts of *S. obliquus* (2 mg C L^{-1}). Experimental animals (third clutch neonates born within 12 h) were kept individually in 80 mL of 0.2 μm filtrated lake water (20 °C, 16:8 h light:dark cycle). They were randomly assigned to one of the following food regimes: (1) 100% *S. obliquus* or *N. limnetica*; (2) *S. obliquus* or *N. limnetica* of which 30% of the provided carbon was exchanged by bacterial carbon represented either by *S. elongatus* or *Pseudomonas* sp.; (3) The *S. obliquus S. elongatus* or *S. obliquus Pseudomonas* sp. mixtures supplemented with single lipids (cholesterol, ARA or EPA) via liposomes. Liposomes not containing any C20 PUFAs or sterols served as control supplements. Animals were transferred daily to fresh medium and freshly prepared food suspensions. The experiment lasted for 21 days during which mortality and reproduction (viable offspring) were recorded. The experiment started with 30 individuals per treatment; six individuals of each treatment were subsampled at day 6 of the experiment to determine somatic growth rates leaving 24 individuals per treatment for the determination of cumulative 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 (M_0) to day 6 (M_t) using the equation:

$$g = \frac{\mathrm{In}M_t - \mathrm{In}M_o}{t}$$

Somatic growth rates and cumulative numbers of offspring 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. elongatus*, *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 S. elongatus, N. limnetica Pseudomonas* sp. (ANOVA1; Table 3). In the second ANOVA, we analyzed the effects of supplementation by comparing the results obtained on the mixtures *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 offspring numbers were square root transformed to meet the assumptions of ANOVA. In ANOVA2, homogeneity of variances could not be accomplished by data transformation. However, 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 carried 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 characterized by high nitrogen and phosphorus contents, resulting 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 experiment is very unlikely.

PUFA and sterol profiles of the two algae differed considerably (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

than 18 C atoms. In contrast, the PUFA composition of *N. limnetica* was characterized by moderate concentra tions of ARA and exceptionally high concentrations of EPA. The concentrations of LIN and GLA in *N. limnetica* were comparable to those of *S. obliquus*, but the concen tration of ALA was notably lower (Table 1).

Principal sterols found in *S. obliquus* were fungisterol (5α ergost 7 en 3β ol) and chondrillasterol ((22E) 5α po riferasta 7,22 dien 3β ol), together with lower amounts of schottenol (5α stigmast 7 en 3β ol). *N. limnetica* con tained, in addition to the two phytosterols sitosterol (stig mast 5 en 3β ol) and isofucosterol ((24Z) stigmasta 5,24 (28) dien 3β ol), notable amounts of cholesterol (cholest 5 en 3β ol), the main sterol found in animals (Table 1). In *S. elongatus* and *Pseudomonas* sp., PUFA or sterols could not be detected. Liposomes did not contain any lipids apart from phospholipid derived fatty acids (16:0 and 18:1n 9) and the respective added PUFA or choles terol. In relation to carbon, the amounts of ARA and cholesterol supplied via liposomes were comparable to those provided with *N. limnetica* (Table 1). As intended, the amount of EPA supplied via liposomes equalled the amount of ARA.

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 Pseudo monas* 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. Supple mentation of *S. obliquus Pseudomonas* sp. with ARA signif icantly increased survival (71%; Fig. 1c, Table 2). In contrast, neither cholesterol nor EPA supplementation increased survival in the *S. obliquus Pseudomonas* sp. treat ment. Survival rates of animals fed the *S. obliquus S. elong atus* mixtures were not affected by lipid supplementation (Table 2b; data not shown). Additional pairwise compari sons revealed that survival rates did not differ between *S. obliquus* and *N. limnetica* ($P = 0.899$), between unsup plemented and with control liposome supplemented *S. obliquus S. elongatus* mixtures ($P = 0.476$), between un supplemented and with control liposome supplemented *S. obliquus Pseudomonas* sp. mixtures ($P = 0.247$), and also not between the *N. limnetica Pseudomonas* sp. 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. li mnetica* 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

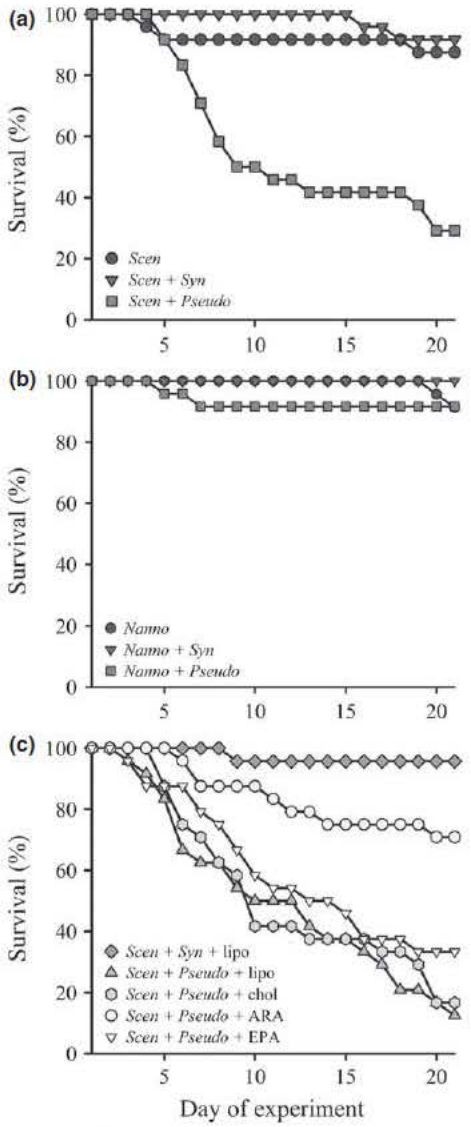

Fig. 1. Survival of *Daphnia magna* raised on (a) *Scenedesmus obliquus* (*Scen*) or (b) *Nannochloropsis limnetica* (*Nanno*) and on 70:30% mixtures (with respect to total provided carbon) with *Synechococcus elongatus* (*Syn*) or *Pseudomonas* sp. (*Pseudo*) and on (c) the *S. obliquus Pseudomonas* sp. mixture supplemented with control liposomes (+ lipo), and cholesterol , ARA or EPA containing liposomes (+ chol/ARA/EPA). Mortality on the supplemented *S. obliquus S. elongatus* mixtures did not differ from the unsupplemented *S. obliquus S. elongatus* treatment (data not shown). Note, all treatments were performed together in one experiment, but are shown here in three graphs for better presentation.

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. li mnetica Pseudomonas* sp. by 49% (compared to the respec tive alga *S. elongatus* control; Fig. 2). Supplementation of *S. obliquus S. elongatus* with ARA and EPA significantly

Table 2. Comparison of survival rates of *Daphnia magna* raised on different food sources using a generalized linear model. (a) Survival of *D. magna* fed the eukaryotic algae *Scenedesmus obliquus* (*Scen*) or *Nannochloropsis limnetica* (*Nanno*) (references) in comparison to the survival on algal based diets containing 30% of prokaryotic carbon, that is either *Synechococcus elongatus* (*Syn*) or *Pseudomonas* sp. (*Pseudo*). (b) Survival of *D. magna* fed *S. obliquus* (*Scen*) in mixtures with either *S. elongatus* (*Syn*) or *Pseudomonas* sp. (*Pseudo*) supplemented with control liposomes (reference) in comparison to survival obtained by supplementing either cholesterol , ARA , or EPA containing liposomes (* significant after Bonferroni adjustment)

	Scen + bacteria (*Reference: Scen*)		*Nanno* + bacteria (*Reference: Nanno*)	
(a)	*z* value	*P* value	*z* value	*P* value
(Intercept)	8.233	< 0.001	7.271	< 0.001
Time	1.773	0.076	0.996	0.319
+ Syn	2.182	0.029	0.463	0.643
+ Pseudo	0.687	0.492	1.172	0.241
Time × Syn	0.924	0.355	0.686	0.493
Time × Pseudo	4.416	< 0.001*	0.254	0.800
	Scen + *Syn* supplemented (*Reference: Scen* + *Syn* + *lipo*)		*Scen* + *Pseudo* supplemented (*Reference: Scen* + *Pseudo* + *lipo*)	
(b)	*z* value	*P* value	*z* value	*P* value
(Intercept)	7.769	< 0.001	9.937	< 0.001
Time	1.512	0.130	10.578	< 0.001
+ Cholesterol	0.449	0.654	0.257	0.797
+ ARA	0.031	0.976	1.582	0.114
+ EPA	0.104	0.917	0.328	0.743
Time × cholesterol	0.177	0.860	0.041	0.967
Time × ARA	0.955	0.340	2.975	0.003*
Time × EPA	0.954	0.340	1.057	0.290

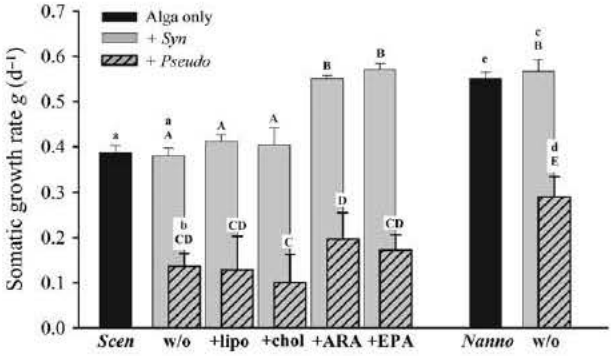

Fig. 2. Somatic growth rates of *Daphnia magna* raised on *Scenedesmus obliquus* (*Scen*) or *Nannochloropsis limnetica* (*Nanno*) (black bars) or on 70:30% mixtures (with respect to total provided carbon) with *Synechococcus elongatus* (grey bars) or *Pseudomonas* sp. (hatched bars). Labels on the *x* axis indicate food treatments. *S. obliquus S. elongatus* and *S. obliquus Pseudomonas* sp. mixtures were supplemented using liposomes (w/o without liposome supplementation; + ipo control liposomes; + chol/ARA/EPA supplementation with cholesterol, ARA or EPA, respectively). Data are means of 6 replicates ± SD. Bars labelled with the same letters are not significantly different (Tukey's HSD test, $P < 0.05$ following ANOVA; lower case letters: ANOVA1, upper case letters: ANOVA2; cf. Table 3).

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 keystone 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 microorganisms 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 *S. obliquus*, *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

	Somatic growth rate				Cumulative number of offspring			
	d.f.	SS	*F*	*P*	d.f.	SS	*F*	*P*
Algae only (ANOVA1)								
Algae	1	0.252	331.4	< 0.001	1	198.40	199.7	< 0.001
Bacteria	2	0.056	368.8	< 0.001	2	1526.3	768.0	< 0.001
Algae × bacteria	2	0.0001	0.5	0.592	2	7.68	3.9	0.023
Residuals	30	0.002			133	132.16		
Supplemented (ANOVA2)								
Algae	5	0.035	32.1	< 0.001	5	326.4	46.8	< 0.001
Bacteria	1	0.188	850.3	< 0.001	1	4740.5	3401.8	< 0.001
Algae × bacteria	5	0.004	3.8	0.005	5	116.5	16.7	< 0.001
Residuals	60	0.013			271	377.6		

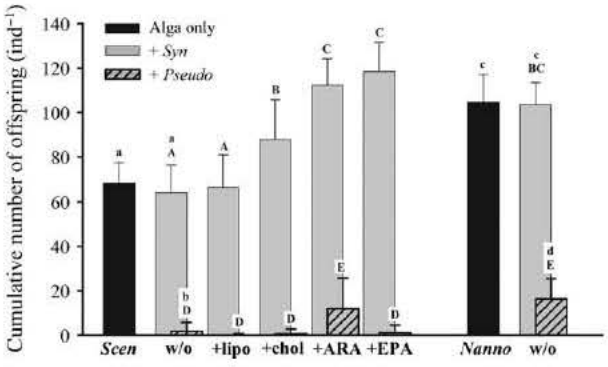

Fig. 3. Cumulative numbers of viable offspring produced by *Daphnia magna* within the experimental period (21 days) on *Scenedesmus obliquus* (*Scen*) or *Nannochloropsis limnetica* (*Nanno*) (black bars) or on 70:30% mixtures (with respect to total provided carbon) with *Synechococcus elongatus* (grey 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 (w/o without liposome 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: ANOVA1, 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 nutri tional 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 cyano bacterial 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 patho genicity 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. expo sure 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 *Paste uria 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 *Pseu domonas* 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 signifi cantly reduced the pathogen induced mortality and fecun dity 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. li mnetica* 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 PUFAs 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 diet, 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 PUFAs 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 Samuelson *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 PUFAs.

An alternative explanation for the increased resistance to the bacterial pathogen on ARA containing diets could be the bactericidal activity of PUFAs *per se*. In their free form, PUFAs 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 PUFAs in acting as antibacterial agents (Kabara *et al.*, 1972; Knapp & Melly, 1986; Giamarellos-Bourboulis *et al.*, 1998). In general, PUFAs 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 PUFAs, 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 lipo somes (Appendix S2). Qualitative plate diffusion tests also did not reveal any bactericidal activity of the PUFA con taining liposomes or of free PUFAs 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 PUFAs 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 con straints for the host and the outcome of pathogenic infec tions 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 PUFAs 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.

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Supporting Information

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

Appendix S1. Pathogenicity of *Pseudomonas* sp. (DD1).
Appendix S2. Growth response of *Pseudomonas* sp. (DD1) to PUFA containing liposomes.
Appendix S3. Agar diffusion assay.

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.

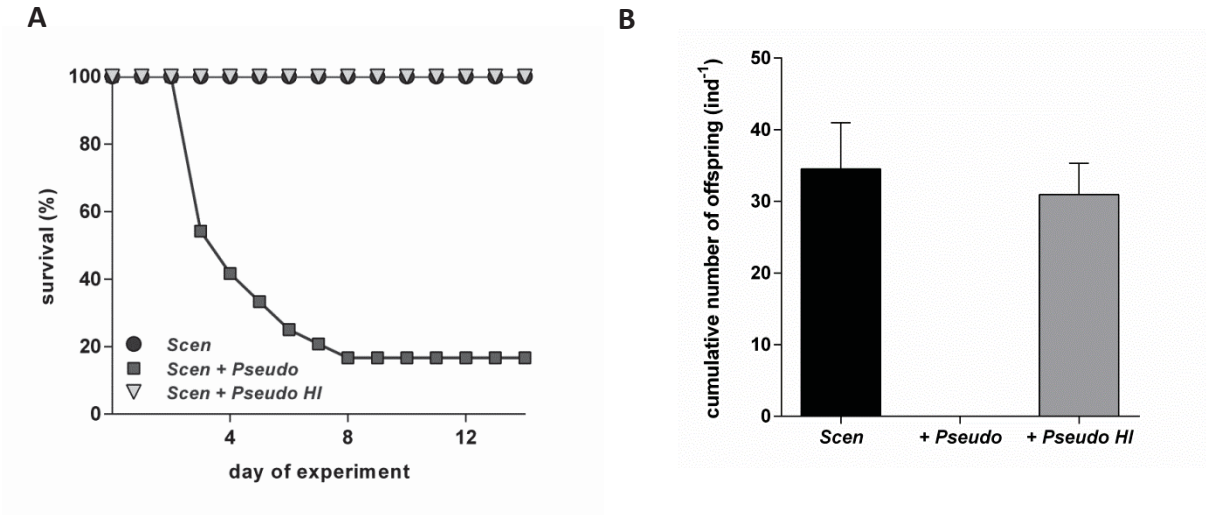


Figure S2: Survival (A) and offspring production (B) of *D. magna* reared on *S. obliquus* (Scen), a mixture of *S. obliquus* and *Pseudomonas* sp. (DD1) (*Scen + Pseudo*, 70% + 30% in terms of carbon), or on a mixture of *S. obliquus* and heat-inactivated *Pseudomonas* sp. (DD1) (*Scen + Pseudo HI*, 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.

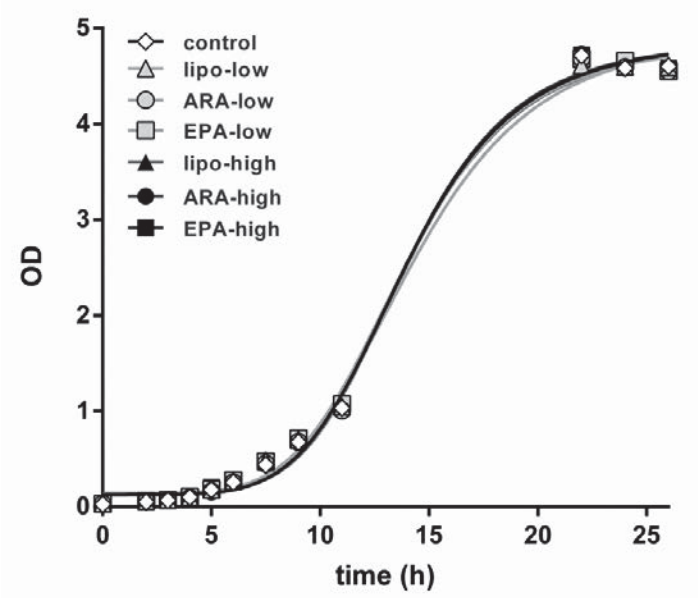
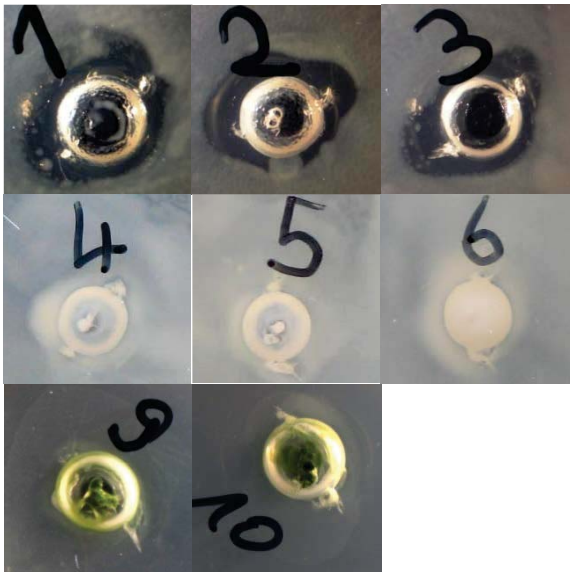


Figure S1: Growth response curves of *Pseudomonas* sp. (DD1) recorded for a period of 26 h (22°C, pH 7.2). *Pseudomonas* sp. (DD1) was cultured in mineral medium containing 20 mM glucose in the absence (control) or in the presence of liposomes. Liposomes were either free of PUFAs (lipo) or were enriched 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.



1: EtOH

2: Arachidonic acid (ARA; in EtOH)

3: Eicosapentaenoic acid (EPA; in EtOH)

4: Liposomes (control)

5: ARA (liposomes)

6: EPA (liposomes)

7: *S. obliquus* (*Scen*; concentration as in main experiment; not shown)

8: *N. limnetica* (*Nanno*; concentration as in main experiment; not shown)

9: *Scen* (10x higher concentrated)

10: *Nanno* (10x higher concentrated)

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 inhibitions 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).