Equal relevance of omega-3 and omega-6 polyunsaturated fatty acids for the fitness of Daphnia spp.

Maja Ilić, Christian Werner, Patrick Fink

Institute for Zoology, University of Cologne, Koeln, Germany
School of Biological Sciences, Queen’s University Belfast, Belfast, United Kingdom
Department of River Ecology, Helmholtz Centre of Environmental Research—UFZ, Magdeburg, Germany
Department of Aquatic Ecosystem Analysis, Helmholtz Centre of Environmental Research—UFZ, Magdeburg, Germany

Abstract

Essential polyunsaturated fatty acids (PUFAs) have been recognized as a crucial factor that determines the trophic transfer efficiency in plankton communities. As many animals cannot synthesize the classes of ω3- and ω6-PUFAs, the dietary availability of these PUFAs can constrain the fitness of freshwater zooplankton such as Daphnia spp. In particular, eicosapentaenoic acid (EPA, 20:5ω3) is considered to be a crucial determinant of the transfer of biomass at the freshwater plant-herbivore interface. In contrast to ω3-PUFAs, the group of ω6-PUFAs has previously been considered to be of less ecological relevance, although the potential role of the ω6-PUFA arachidonic acid (ARA, 20:4ω6) remains controversial. To investigate its potential role, we conducted dose-response growth experiments with two Daphnia species, D. pulex and D. magna, supplemented with EPA or ARA, which allowed us to calculate EPA and ARA saturation thresholds for growth and reproduction of both Daphnia species. Our results provide evidence that not only the availability of ω3-PUFAs, but also the availability of a ω6-PUFA, namely ARA, can limit both the growth and reproduction of Daphnia spp. to an equal extent. The saturation thresholds for growth and reproduction were consistently, but not significantly, higher for EPA than for ARA in both Daphnia species. As shifts in phytoplankton community composition might result in environmental fluctuations in the dietary availability of ω3- and ω6-PUFAs, our findings present a significant step in understanding the consequences of the ongoing global biodiversity loss for trophic transfer efficiency at the phytoplankton-zooplankton interface.

The freshwater crustacean Daphnia is a keystone genus for lake ecosystems, as it represents the major link between primary producers, the phytoplankton, and secondary consumers, like planktivorous fish (Gaedke and Straile 1998). As such, Daphnia play an important role in the trophic transfer of energy within the aquatic food web.

Two major factors that determine the nutritional value and thus the food quality of Daphnia’s diet are the elemental and biochemical composition of the phytoplankton (Ahlgren et al. 1990; Müller-Navarra 1995a; Park et al. 2002; Becker and Boersma 2003). Besides dietary carbon to nutrient ratios (Sterner et al. 1993; Urabe et al. 1997; Ravet and Brett 2006), Daphnia’s performance has often been associated with the availability of polyunsaturated fatty acids (PUFAs) in their diet (Müller-Navarra 1995b; Wacker and von Elert 2001; von Elert 2002), i.e., fatty acids with two or more double bonds in their carbon chain.

Arthropod animals, including Daphnia, are incapable of synthesizing ω3- and ω6-PUFAs de novo (Stanley-Samuelson et al. 1987; Harrison 1990; Leonard et al. 2004). Although previous studies suggest that at least some Daphnia species are able to convert ω3- and ω6-PUFAs within the respective PUFA family, these PUFA families must be considered essential for Daphnia and have to be derived from the diet (Weers et al. 1997; von Elert 2002; Schlechtriem et al. 2006). The presence and the amount of PUFA in the diet, however, vary drastically among different phytoplankton groups (Ahlgren et al. 1990; Lang et al. 2011).

In several field studies, the juvenile somatic growth of Daphnia spp. was shown to correlate with the content of ω3-PUFAs in the seston, in particular α-linolenic acid (α-LA, 18:3ω3; Wacker and von Elert 2001) and eicosapentaenoic acid (EPA, 20:5ω3; Müller-Navarra 1995b). As juvenile somatic growth rate is a good proxy for Daphnia fitness (Lampert and Trubetskova 1996), this suggests that the dietary availability of

*Correspondence: maja.ilic.bio@gmail.com

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Additional Supporting Information may be found in the online version of this article.
ω3-PUFAs can limit the fitness of *Daphnia* in nature. This view is supported by multiple laboratory studies: *Daphnia galeata* fed with algal food supplemented with single ω3-PUFAs showed increased somatic growth rates when EPA, α-LA, and docosahexaenoic acid (22:6ω3) were supplemented (von Elert 2002). In particular, the dietary EPA availability was shown to limit not only the somatic growth rate of *Daphnia* (Becker and Boersma 2003; Sperfeld and Wacker 2012), but also their reproduction (Ravet et al. 2003; Martin-Creuzburg et al. 2008, 2010) and population growth (Martin-Creuzburg et al. 2010). Accordingly, EPA has been strongly recognized as a dietary PUFA limiting the fitness of various *Daphnia* species. Additionally, the dietary EPA concentrations below which the fitness of *Daphnia* is limited were found to differ between *Daphnia* species and to increase with body size (Sikora et al. 2016). This can be explained by the finding that larger species show higher growth rates and thus have higher PUFA demands (Goulden and Place 1990; Sikora et al. 2016).

Besides EPA, the C20-PUFA arachidonic acid (ARA, 20:4ω6) is assumed to play an important role for *Daphnia*’s fitness and physiology (Kainz et al. 2004; Brett et al. 2006; Schlotz et al. 2014). The main structural difference between these two PUFAs is the position of the first double-bond relative to the ω-end (methyl-end) of the carbon chain (ω3 vs. ω6). Both ARA and EPA serve as precursors for eicosanoids, a family of hormone-like substances such as prostaglandins, which are known to affect the reproduction, the immune system, and the ion transport physiology of both vertebrates and invertebrates (Stanley-Samuelson 1994; Stanley 2000). Nevertheless, studies investigating the eicosanoid pathway in *Daphnia* were mostly focused on the role of ARA (Heckmann et al. 2008b; Schlotz et al. 2012), while the relevance of EPA for the eicosanoid metabolisms was poorly understood until recently (Schlotz et al. 2016; Fink and Windisch 2018). The relevance of ARA for *Daphnia* is further supported by the finding that daphnids accumulate significant amounts of ARA, both during starvation and feeding, either by direct uptake from the diet or by bioconversion of other available ω6-PUFAs (Kainz et al. 2004; Schlechtriem et al. 2006; Smyntek et al. 2008; Burns et al. 2011; Taipale et al. 2011).

However, in contrast to EPA, the reports on potential constraints of *Daphnia*’s fitness through ARA availability are rather inconsistent. In a field study, Wacker and von Elert (2001) applied a modified Monod model to describe the relationship between the somatic growth rate of *D. galeata*, which was raised on natural seston of Lake Constance, and the sestonic content of different ω3- and ω6-PUFAs. They found equal proportions of variance explained by the Monod model when the sum of the sestonic content of all ω3-PUFAs was considered compared to the sum of ω6-PUFAs ($R^2 = 0.86$ for both ω-families). Furthermore, their Monod model describing the relationship between the growth of daphnids and the sestonic concentration of ARA explained 70% of the variance. This provided the first evidence that in the field, ω6-PUFAs might play a role for the fitness of *Daphnia*. The first controlled laboratory study on the role of PUFAs for *Daphnia* fitness by von Elert (2002) found *D. galeata* to be limited only by the availability of ω3-PUFA, but not of ω6-PUFA (namely ARA) availability. These findings were supported by a later study by Ravet et al. (2012), where an ARA-supplemented diet did (in contrast to EPA supplementation) not increase the somatic growth rate or reproduction of *Daphnia pulex*, although equal amounts of EPA and ARA were used in the respective diet treatments. This leads to the assumption that the effect size of ARA-supplementation in terms of increased growth or reproduction is smaller than the effect size of EPA-supplementation. On the other hand, Martin-Creuzburg et al. (2010) reported an increase in reproduction (determined as cumulative number of offspring), but not of somatic growth of *Daphnia magna* when fed a cholesterol- and ARA-supplemented cyanobacterium diet. Furthermore, the survival and reproduction of *D. magna* when exposed to an opportunistic bacterial pathogen (*Pseudomonas* sp.) were increased when ARA was supplemented to a C20-PUFA-deficient diet (Schlotz et al. 2014). Additionally, Schlotz et al. (2014) also observed an increased growth of *D. magna* when a C20-PUFA-free diet mixture of *Acutodesmus obliquus* (formerly *Scenedesmus*) and *Synechococcus elongatus* was supplemented with ARA, which is in contrast to the previous findings from von Elert (2002), Martin-Creuzburg et al. (2010), and Ravet et al. (2012).

Due to the highly inconsistent findings, the limitation of *Daphnia*’s fitness and in particular its growth and reproduction by the availability of this ω6-PUFA cannot be resolved without the determination of threshold saturation concentrations, as previously established for ω3-PUFAs (Sperfeld and Wacker 2011). Such saturation thresholds are defined as the minimum dietary PUFA concentrations that are necessary for a saturated (i.e., unlimited) growth or reproduction (Sperfeld and Wacker 2011). Along a nutrient concentration gradient, starting with an infinite availability of a particular PUFA, a higher saturation threshold indicates a stronger and earlier occurring limitation of *Daphnia*’s fitness compared to lower saturation thresholds. The determination of threshold saturation concentrations of dietary PUFAs may therefore not only allow for identification of PUFAs that limit the fitness of *Daphnia*, but also may provide an indication about PUFA-limiting conditions for *Daphnia* in nature (Becker and Boersma 2005).
found to increase with increasing body size (Goulden and Place 1993). Therefore, it is reasonable to assume that larger sized Daphnia species (e.g., D. magna) might have higher rates of assimilation and accumulation of ARA and EPA compared to smaller sized Daphnia (e.g., D. pulex, D. pulicaria, and D. longispina). Furthermore, it is not yet clear, if and at which rates ARA and EPA can be synthesized due to (retro-)conversion of other available PUFAs by different Daphnia species (Weers et al. 1997; von Elert 2002; Schlechtriem et al. 2006). While specific EPA demands by different Daphnia species have been previously reported by Sikora et al. (2016), intraspecific and interspecific differences in ARA requirements within the genus Daphnia have, to our knowledge, not yet been studied. Although addressing such intraspecific and interspecific differences would require investigations with more genotypes from multiple species, single clone studies can nevertheless provide first evidence on possible species-specific ARA requirements.

We specifically hypothesize that: (1) insufficient ARA availability limits the fitness of Daphnia species; (2) ARA threshold concentrations that are necessary to allow for saturated (i.e., ARA-unlimited) growth and reproduction of Daphnia are similar to the previously described EPA saturation thresholds. To address these hypotheses, we conducted dose-response growth experiments with two Daphnia species of different body size fed with C_{20}-PUFA-free diet supplemented with either EPA or ARA.

**Materials and methods**

**Study organisms and cultivation**

Two Daphnia clones, one from the medium-sized species D. pulex (clone Gerstel, Koch et al. 2009) and one from the large species D. magna (clone B, Lampert and Rothhaupt 1991), were cultured in clonal lines in aged, aerated, and sterile-filtered (0.45 μm) tap water at 20°C and a 16:8 h light:dark cycle. During the preculture phase (at least three generations), the daphnids were fed with the green alga A. obliquus (strain SAG 276-3a from the Göttingen Algal Culture Collection SAG, Germany), which is rich in C_{18}-PUFAs, but lacks C_{20}-PUFAs such as ARA (C20:4 n-6) and EPA (C20:5 n-3, Windisch and Fink 2018), at a concentration of 2 mg particulate organic carbon (POC) L^{-1} every other day. 

*Acutodesmus obliquus* was cultured in Z/4 medium (Zehnder and Gorham 1960) in semi-continuous (dilution rate 0.1 d^{-1}) 5 L batch cultures at 100 μPAR. To estimate the volume of the liposome suspensions that had to be added to the treatments in order to achieve a certain concentration gradient, we analyzed the fatty acid content of the respective liposomes via gas chromatography (GC). We first extracted lipids with 5 mL of dichloromethane/methanol (2:1, v/v) from 100 μL of each liposome suspension (control liposomes, ARA- and EPA-containing liposomes; in triplicates). For subsequent quantification of fatty acids, we added two internal standards to the samples, 10 μg heptadecanoic acid methyl ester (C17:0 ME) and 5 μg tricosanoic acid methyl ester (C23:0 ME) and sonicated for 1 min. The samples were evaporated to dryness at 40°C under a stream of nitrogen gas and fatty acids were transterified at 70°C for 20 min in 5 mL of 10% methanic HCl. The methanic HCl was prepared by addition of acetyl chloride (> 99%, Acros Organics, Geel, Belgium) to ice-cooled methanol. The resulting fatty acid methyl esters (FAMES) were extracted twice with approximately 2 mL of isohexane. The isohexane phases were joined

where \( W_0 \) is the dry mass at the beginning of the experiment, \( W_f \) is the dry mass at the end of the experiment, and \( t \) is the duration of the experiment in days.

Both growth experiments (with *D. pulex* and *D. magna*) were performed in January of 2018.

**Fatty acid analyses**

We placed six randomly assigned neonates of the 3rd clutch (hatched within 20 h) per species into 200 mL (D. pulex) or 300 mL (D. magna) aged and aerated tap water. The neonates were fed with *A. obliquus* (2 mg POC L^{-1}). ARA and EPA were supplemented via liposome carriers (Martin-Creuzburg et al. 2008) loaded with either ARA (122 ng \( \mu \)L^{-1}) or EPA (142 ng \( \mu \)L^{-1}). Different volumes of the ARA or EPA containing liposome suspensions were used, which resulted in a dietary gradient ranging from 0.5 to 10 μg ARA or EPA mg POC^{-1}. In order to exclude a possible effect of lipidic carriers on the growth of the daphnids, we maintained equal concentration of liposomes in all food treatments by adding appropriate amounts of PUFAFree control liposomes to all treatments with < 10 μg PUFAs mg POC^{-1}. Furthermore, in the control treatment, we supplied the same volume of PUFAs-free control liposomes as was necessary to achieve the highest (10 μg mg POC^{-1}) PUFAs concentrations. In total, we had one control treatment (A. obliquus control liposomes = 0 μg PUFAs mg POC^{-1}), which served as the C_{20}-PUFA-free treatment for both experimental lines and nine PUFAs-treatments (0.5, 1, 1.5, 2, 2.5, 3, 5, 7, and 10 μg PUFAs mg POC^{-1}) supplemented with either EPA or ARA, resulting in a total of 19 treatments in triplicates.

Juveniles were transferred into fresh food + liposome suspensions daily. At the start of the experiments, we placed 3 × 10 neonates into preweighed aluminum boats and dried them at 60°C in the drying oven for at least 24 h. After 6 d (D. pulex) or 7 d (D. magna), we sampled half of the daphnids (all not egg-bearing) and dried them in aluminum boats. The second half (egg-bearing individuals; all eggs were counted to determine the clutch size) was sampled 1 d later, i.e., after 7 d (D. pulex) or 8 d (D. magna) and dried in aluminum boats. After drying, aluminium boats with daphnids were weighed on a Sartorius microbalance type CP2 P (accuracy 1 μg). The somatic growth rate \( g \) (d^{-1}) of the daphnids was calculated as:

\[
g = \frac{\ln(W_f) - \ln(W_0)}{t}
\]
and subsequently evaporated at 40°C under a stream of nitrogen gas and the remaining FAMEs were redissolved in 50 μL isohexane per sample.

One microliter of each sample was injected splitless into a 6890-N GC System (Agilent Technologies, Waldbronn, Germany) and analyzed using the same method as described by Windisch and Fink (2018). We found 122 ng μL−1 of ARA and ~ 142 ng μL−1 of EPA in the respective liposomes, while we confirmed the control liposomes to be PUFA-free.

Growth and reproduction saturation thresholds

Using the saturation curve procedure described in Sperfeld and Wacker (2011), we determined growth and reproduction saturation thresholds, i.e., PUFA-concentrations at which the growth and reproduction of juvenile daphnids reaches saturation. This procedure has two main advantages compared to the conventional statistical method (ANOVA) used to determine growth saturation thresholds: (1) it allows for a calculation of thresholds for different saturation levels; (2) combined with a bootstrapping method, it allows for an uncertainty (variance) of the estimated thresholds, which is necessary for statistical comparisons of thresholds between, e.g., species or PUFAs. Furthermore, the threshold concentration estimated with ANOVA depends on the distances between the PUFA concentrations within the chosen concentration gradient (for further discussion, see Sperfeld and Wacker 2011).

To estimate the EPA and ARA threshold concentrations for the saturated growth of daphnids, we applied a modified Monod function (Monod 1950) to describe the growth rate g (d−1) of the daphnids along the EPA or ARA concentration gradient as:

\[
g = g_0 + \frac{(g_\infty - g_0) \times S}{(S + K_S)}
\]  

(2)

where \(g_0\) is the growth rate observed in the PUFA-free treatment (0 μg mg POC−1 of EPA and ARA), \(g_\infty\) the asymptotic growth rate (d−1), S the amount of EPA or ARA supplemented to the diet (μg mg POC−1), and \(K_S\) the half-saturation constant (μg mg POC−1; threshold for the 50% growth saturation level \(g_{50\%}\)). We separately analyzed the growth rates of not egg-bearing vs. egg-bearing mothers. \(g_0\) was calculated as the mean growth rate of the daphnids raised in the C20-PUFA-free treatment (0 μg PUFAmg POC−1) of D. pulex and not egg-bearing D. magna and \(n = 2\) for egg-bearing D. magna, while \(g_\infty\) and \(K_S\) were predicted from the Monod model. Additionally, we calculated the adjusted \(R^2\) as a measure for the proportion of variance explained by the fitted Monod model.

As asymptotic growth rates can only be reached at an infinite amount of EPA and ARA, we used the predicted parameters for the saturated growth of the two Daphnia species from the fitted curves to additionally calculate the EPA and ARA thresholds \(S_{75\%}\) (in μg mg POC−1) for the 75% growth saturation level \(g_{75\%}\) which corresponds to the reduction of \(g_\infty\) by 25% relative to the baseline \(g_0\). This saturation level was used for two main reasons, which are both further discussed in Sperfeld and Wacker (2011): (1) although higher saturation levels (e.g., 90% or 95%) are much closer to the asymptotic growth rate \(g_\infty\), calculation of PUFA threshold concentrations for \(g_{90\%}\) and \(g_{95\%}\) is not recommended as variability in threshold concentrations increases with higher saturation levels and can lead to biased threshold estimates; (2) to avoid biased PUFA threshold estimation by neglecting bootstrapped data sets for which regression curves do not intersect with the line of the growth level, which is likely to occur for growth saturation levels ≥ 90%. Using the same model (Eq. 2), we predicted the asymptotic clutch size (eggs\(\infty\)), the half-saturation constant \(K_S\) for the saturated reproduction and the EPA and ARA threshold concentrations \(S_{75\%}\) for the 75% reproduction saturation level eggs\(75\%\) (equivalent to a reduction of eggs\(\infty\) by 25% relative to the baseline eggs\(0\)). The initial clutch size (eggs\(0\)) was calculated as the mean number of eggs produced by daphnids raised in the C20-PUFA-free treatment (\(n = 3\) for D. pulex and \(n = 2\) for D. magna).

Bootstrapping

To be able to statistically compare the estimated growth saturation thresholds between PUFAs and species, we applied the bootstrapping procedure described in Sperfeld and Wacker (2011); we allowed for uncertainty in the calculated thresholds by randomly leaving out one replicate per concentration level > 0 μg PUFAmg POC−1 (separately for the EPA- and ARA-limited growth) and thus generating \(N = 1000\) new data sets per species and PUFA-specific growth response with a sample size of \(n = 9\) (as we used nine different EPA and ARA concentration levels). Those new data sets were used again for curve fitting (using the Eq. 2) resulting in \(N\) values for each parameter (\(g_\infty\), \(K_S\), \(S_{75\%}\), for eggs\(75\%\), and adjusted \(R^2\)) per species and PUFA. \(g_0\) was calculated as the mean growth rate of the daphnids raised in the C20-PUFA-free treatment and was set as a fixed starting point for every curve; thus, there was no variance in \(g_0\) within one species, as we used the same C20-PUFA-free control treatment for both PUFA experimental lines. In some cases, it was not possible to fit the curves through the bootstrapped growth rate data; therefore, these bootstrapped data sets were omitted from further analyses. Additionally, we applied a second data cleaning step by leaving out all data sets where curve fitting resulted in a negative \(K_S\) (i.e., \(K_S < 0\) μg PUFAmg POC−1), as such results were biological meaningless. The same bootstrapping procedure and the subsequent data cleaning were applied for the reproduction data. The final counts \(N\) (maximum 1000) of biologically meaningful growth and reproduction saturation curves for both species and both tested PUFAs (and thus the number of bootstrapped values for each parameter) are given in Table 2. Additionally, using the bootstrapped data, we calculated intersection points between 1000 randomly chosen EPA- and ARA-saturation curves for the growth and reproduction of the two Daphnia species. We used the function optimise() in R, with a fixed maximum of
1 mg PUFA mg POC\(^{-1}\). Although this upper limit is biologically not meaningful and technically not reachable (e.g., via liposome supplementation), it allows for the maximal possible variation within the potential intersection points. All intersection points ≤ 0 μg PUFA mg POC\(^{-1}\) and exactly equal to 1 mg PUFA mg POC\(^{-1}\) were excluded from the further analysis for following reasons: (1) values below zero are not biologically significant; (2) values that are equal to zero indicate no intersection of the curves after the initial starting point \(g_0\) which was equal for EPA- and ARA-curves within one species; and (3) values equal to 1 mg (fixed maximum) indicate that the intersection point is equal to or above 1 mg PUFA mg POC\(^{-1}\), which is biologically not meaningful.

**Statistical analyses**

Bootstrapping, fitting of the modified Monod function, and subsequent statistical analyses were performed in R (version 3.3.2, R Core Team 2016) and RStudio (version 1.1.383, RStudio Team 2016). We tested the effect of the factors PUFA (EPA vs. ARA), Species (\(D. \) pulex vs. \(D. \) magna), and their interaction PUFA \(\times\) Species on the predicted PUFA threshold concentrations \(S_{75\%}\) for 75% growth and reproduction saturation levels as well as the asymptotic growth rate \(g_{\infty}\) and asymptotic clutch size \(e_{\infty}\) via two-way ANOVA, using the statistical procedure described in Martin-Creuzburg et al. (2014). For each of the predicted parameters, we randomly chose the same number of replicates as in the growth experiments \((n = 3\) for both species and both PUFAs) from the distribution of N bootstrapped parameters. These were compared via two-way ANOVA. The procedure was repeated 400 times and the \(p\) values for the two main factors and the interaction were recorded. As suggested by Martin-Creuzburg et al. (2014), we searched for a critical \(p\) value necessary to hold for a statistical power of 0.8 (equivalent to a type II error of 0.2). Homogeneity of variances was checked with Levene’s test. Finally, we compared the estimated intersection points of EPA- and ARA-curves for growth and reproduction between the two \(Daphnia\) species: we randomly chose six replicates (accounting for three replicates per EPA and ARA experimental line in the original growth experiments) from the distribution of intersection points and compared these via the nonparametric Wilcoxon–Mann–Whitney test (due to the lack of residuals normal distribution, checked via Shapiro–Wilk’s test). This procedure was repeated 400 times (separately for growth and reproduction) to assess the critical \(p\) value necessary to hold for a statistical power of 0.8.

**Results**

**\(C_{20}\)-PUFA-limited growth of \(Daphnia\) spp.**

Growth rates of egg-bearing \(D. \) pulex and \(D. \) magna increased with increasing amounts of ARA supplemented to \(A. \) obliquus, following a typical saturation curve (Fig. 1). We observed a similar pattern for the EPA-limited growth of \(D. \) magna (Fig. 1b). Accordingly, 58.34% and 47.47% of the variances of \(D. \) magna growth were explained by the saturation curves fitted along the EPA and ARA concentration gradient, respectively. For \(D. \) pulex, however, the modified Monod model explained only 22.10% of the variance of the ARA-limited growth of \(D. \) pulex. Growth rates of \(D. \) pulex clearly increased at EPA concentrations ≥ 5 μg EPA mg POC\(^{-1}\), but did not show a clear pattern along the experimental EPA concentration gradient (Fig. 1a). As a consequence, the modified Monod model explained only 18.74% of the variance of EPA-limited growth of \(D. \) pulex (Table 1). In \(D. \) pulex, the mean growth rate \(g_0\) in the \(C_{20}\)-PUFA-free control treatment was 0.370 d\(^{-1}\), while the predicted asymptotic growth rate \(g_{\infty}\) with EPA supply was 0.407 and 0.401 d\(^{-1}\) when ARA was supplemented. This accounted for a 9% and 8% growth increase (gain in growth from \(g_0\) in absence of \(C_{20}\)-PUFAs to \(g_{\infty}\) by EPA and ARA supply, respectively (Fig. 1a; Table 1). In \(D. \) magna, the growth rate increased from 0.391 d\(^{-1}\) \((g_0\) to a predicted asymptotic growth rate of 0.461 d\(^{-1}\) (~15% increase) when EPA was supplemented and to 0.449 d\(^{-1}\) when ARA was supplemented (~13% increase, Fig. 1b, Table 1). We further used the predicted parameters \(g_{\infty}\) and \(K_S\) (Table 1) from the fitted saturation curves (Fig. 1) to calculate EPA and ARA threshold concentration \(S_{75\%}\) (given in μg mg POC\(^{-1}\)) for the 75% growth saturation level \(S_{75\%}\) (equivalent to a reduction of \(g_{\infty}\) by 25% relative to the baseline \(g_0\) for both \(Daphnia\) species. The estimated PUFA threshold concentration \(S_{75\%}\) for the 75% growth saturation level \(S_{75\%}\) was below 5 μg PUFA mg POC\(^{-1}\) for \(D. \) magna \((S_{75\%} \ (\text{EPA}) = 4.183 \ μg \ mg \ POC^{-1}, \ S_{75\%} \ (\text{ARA}) = 2.230 \ μg \ mg \ POC^{-1}, \ Table 1)\). For \(D. \) pulex, we found similar \(S_{75\%}\) for ARA-limited growth \((S_{75\%} \ (\text{ARA}) = 2.625 \ μg \ mg \ POC^{-1})\) and a threefold higher \(S_{75\%}\) for EPA-limited growth \((K_{S\text{EPA}} = 13.850 \ μg \ mg \ POC^{-1}, \ Table 1)\) compared to \(D. \) magna. Note that these findings refer to the saturation curves fitted through the raw growth rate data. Interestingly, the growth rate of not egg-bearing \(D. \) pulex (sampled after 6 d) did not show any clear patterns along the PUFA concentration gradient (Supporting Information Fig. S1a), while the EPA- and ARA-limited growth of \(D. \) magna followed the saturation curve (Supporting Information Fig. S1b). Similar to egg-bearing \(D. \) magna, the saturation curves fitted through the growth rate data of not egg-bearing \(D. \) magna explained 58.93% and 50.12% of variance (adjusted \(R^2\)) for the EPA- and ARA-limited growth, respectively.

The bootstrapping procedure and the subsequent statistical analyses revealed significant differences in the predicted asymptotic growth rates \(g_{\infty}\) among egg-bearing \(Daphnia\): we found significantly higher \(g_{\infty}\) for \(D. \) magna compared to \(D. \) pulex (two-way ANOVA; \(p[\text{Species}] < 0.001, \ Fig. \ 2a, \ Tables 2 (a), 3\)). The factors PUFA (EPA vs. ARA) and the interaction PUFA \(\times\) Species did not have any significant effect on the asymptotic growth rate \(g_{\infty}\) (two-way ANOVA; \(p[\text{PUFA}] = 0.298, \ p[\text{PUFA} \times \text{Species}] = 0.809, \ Table 3\). We did not find any significant effects of the two main factors and their interaction on the threshold concentrations \(S_{75\%}\) for the 75% growth
Fig. 1. Somatic growth rate $g$ (d$^{-1}$) and reproduction (clutch size, i.e., number of eggs per individual) of $D. pulex$ (egg-bearing, sampled after 7 d; panels a and c, respectively) and $D. magna$ (egg-bearing, sampled after 8 d; panels b and d, respectively) grown on $A. obliquus$ supplemented with different amounts ($\mu g$ mg POC$^{-1}$) of EPA (green circles) or ARA (blue triangles). Solid (EPA) and dashed (ARA) saturation curves are based on modified Monod functions (nonlinear least-square fits, Eq. 2). Vertical lines (green solid: EPA, blue dashed: ARA) indicate half-saturation constants $K_S$ ($\mu g$ PUFA mg POC$^{-1}$) of growth and reproduction saturation curves. Summary of the nonlinear least-square fits can be found in Table 1.

Table 1. Somatic growth rate $g_0$ (d$^{-1}$) and clutch size eggs$_0$ (Ind$^{-1}$) for $D. pulex$ (egg-bearing, sampled after 7 d) and $D. magna$ (egg-bearing, sampled after 8 d) grown on $A. obliquus$ without C$_{20}$-PUFA supplementation; asymptotic growth rate $g_\infty$ (d$^{-1}$), asymptotic clutch size eggs$_\infty$ (Ind$^{-1}$); half-saturation constant $K_S$ (in $\mu g$ PUFA mg POC$^{-1}$) for the EPA- and ARA-limited growth and reproduction of the two $Daphnia$ species; PUFA threshold concentration $S_{75\%}$ (in $\mu g$ PUFA mg POC$^{-1}$) for growth and reproduction saturation levels $g_{75\%}$ and eggs$_{75\%}$ (corresponding to reduction of $g_\infty$ and eggs$_\infty$ by 25% relative to the baseline $g_0$ and eggs$_0$, respectively).

|                   | Somatic growth | Reproduction |
|-------------------|---------------|--------------|
|                   | $D. pulex$    | $D. magna$   | $D. pulex$    | $D. magna$   |
|                   | ARA | EPA | ARA | EPA | ARA | EPA | ARA | EPA | ARA | EPA |
| $g_0$ (d$^{-1}$)  | 0.370 | 0.370 | 0.391 | 0.391 | 4.556 | 4.556 | 9.250 | 9.250 |
| $g_\infty$ (d$^{-1}$) | 0.401 | 0.407 | 0.449 | 0.461 | 6.442 | 6.456 | 14.284 | 13.791 |
| $K_S$ ($\mu g$ PUFA mg POC$^{-1}$) | 0.875 | 4.617 | 0.743 | 1.394 | 0.044 | 0.380 | 0.187 | 0.490 |
| $S_{75\%}$ for $g_{75\%}$ ($\mu g$ PUFA mg POC$^{-1}$) | 2.625 | 13.850 | 2.230 | 4.183 | 0.131 | 1.141 | 0.561 | 1.471 |
| Adjusted $R^2$   | 0.221 | 0.187 | 0.475 | 0.583 | 0.210 | 0.212 | 0.332 | 0.218 |

$g_0$ and eggs$_0$ were calculated prior to curve fitting (mean growth rate and clutch size of $D. pulex$ [n = 3] and $D. magna$ [n = 2]) while $g_\infty$, eggs$_\infty$, $K_S$, $S_{75\%}$ (for both growth and reproduction), and the corresponding adjusted $R^2$ were derived from saturation curves based on modified Monod functions (nonlinear least-square fits, Eq. 2). Total $n$ refers to the total number of data points used to fit the saturation curves (10 different tested concentrations of EPA or ARA in triplicates, i.e., maximum 30 data points). This data correspond to the plots in Fig. 1.
saturation level (Fig. 2b; Table 3). Summary of the estimated parameters for the growth of *D. pulex* and *D. magna*, derived from the bootstrapping procedure, can be found in Table 2(a). Furthermore, we provide the distribution of adjusted $R^2$ values along the estimated asymptotic growth rates $g_\infty$ as a measure of variance explained by the nonlinear least-square fits through bootstrapped growth rate data (Fig. 2a). The asymptotic clutch size and the PUFA threshold concentration were derived from nonlinear least-square fits through bootstrapped reproduction data (Fig. 2c, d). Note that the y-axis in the panels (b, c, d) has a logarithmic scale. Summary of the plots (sample size, median, mean ± standard deviation) and statistical analyses can be found in Tables 2–3, respectively. Different letters indicate significantly different groups (two-way ANOVA).

**C20-PUFA-limited reproduction of *Daphnia* spp.**

Similar to the growth, the reproduction (given as number of eggs per individual) of both *Daphnia* species increased upon EPA and ARA supplement (Fig. 1c, d). However, the proportion of variance explained by the fitted saturation curves along the PUFA concentration gradient was only 21% for EPA- and ARA-limited reproduction of *D. pulex* and 22% and 33% for the reproduction of *D. magna* under EPA and ARA supply, respectively (Table 1). *Daphnia pulex* and *D. magna* reared in C20-PUFA-free treatment produced ~5 and ~9 eggs per individual, respectively (eggs$_0$, calculated as the mean of $n=3$ [D. pulex] and $n=2$ [D. magna] replicates, Table 1). Via the Monod model, we predicted an asymptotic clutch size eggs$_\infty$ of 6.456 and 6.442 (Ind$^{-1}$) for EPA- and ARA-limited reproduction of *D. pulex*, while the predicted asymptotic clutch size was approximately twofold higher for *D. magna* (13.791 and 14.284 eggs Ind$^{-1}$ under EPA and ARA supply, respectively). Hence, we predicted a ~29% increase in the number of eggs produced per individual in *D. pulex* in both experimental lines (EPA vs. ARA supply), while
**Table 2.** (a) Somatic growth rate $g_0$ (d$^{-1}$) for *D. pulex* (egg-bearing, sampled after 7 d) and *D. magna* (egg-bearing, sampled after 8 d) grown on *A. obliquus* without C$_{20}$PUFA supplementation; asymptotic growth rate $g_{\infty}$ (d$^{-1}$), half-saturation constant $K_S$ (in $\mu$g PUFA mg POC$^{-1}$), and PUFA threshold concentration $S_{75\%}$ for growth saturation level $g_{75\%}$ (in $\mu$g PUFA mg POC$^{-1}$) for the EPA- and ARA-limited growth of the two *Daphnia* species. Growth saturation level $g_{75\%}$ corresponds to a reduction of $g_{\infty}$ by 25% relative to the baseline $g_0$. $g_{\infty}$, $K_S$, $S_{75\%}$ for both growth and reproduction, and the corresponding adjusted $R^2$ were derived from nonlinear least-square fits through bootstrapped growth rate and reproduction data (see "Materials and methods" section) and are given both as median and as mean values ± standard deviation. $n$ refers to the total number of data points used to fit the saturation curves (one replicate per each concentration level > 0 $\mu$g PUFA mg POC$^{-1}$ was left out during the bootstrapping procedure), while $N$ refers to the total number of saturation curves fitted after bootstrapping and data cleaning (see "Materials and methods" section). This data corresponds to the plots in Fig. 2.

|       | *D. pulex* |       | *D. magna* |
|-------|------------|-------|------------|
|       | ARA        | EPA   | ARA        | EPA   |
| $n$   | 21         | 21    | 18         | 18    |
| $N$   | 997        | 974   | 1000       | 1000  |
| $g_0$ (d$^{-1}$) | 0.370 | 0.370 | 0.391 | 0.391 |
| $g_{\infty}$ (d$^{-1}$) | 0.041 | 0.412 | 0.049 | 0.449 |
| $K_S$ ($\mu$g PUFA mg POC$^{-1}$) | 0.847 | 5.070 | 0.784 | 1.410 |
| $S_{75\%}$ for $g_{75\%}$ ($\mu$g PUFA mg POC$^{-1}$) | 2.541 | 15.225 | 2.353 | 4.229 |
| Adjusted $R^2$ | Mean ± 1 SD | 0.280 ± 0.077 | 0.221 ± 0.046 | 0.531 ± 0.048 | 0.604 ± 0.074 |
| $n$   | 21         | 21    | 18         | 18    |
| $N$   | 778        | 984   | 779        | 816   |
| eggs$_0$ (Ind$^{-1}$) | 4.556 | 4.556 | 9.250 | 9.250 |
| eggs$_{\infty}$ (Ind$^{-1}$) | 6.498 | 6.461 | 14.399 | 14.073 |
| $K_S$ ($\mu$g PUFA mg POC$^{-1}$) | 0.061 | 0.407 | 0.233 | 0.676 |
| $S_{75\%}$ for eggs$_{75\%}$ ($\mu$g PUFA mg POC$^{-1}$) | 0.182 | 1.220 | 0.698 | 2.028 |
| Adjusted $R^2$ | Mean ± 1 SD | 0.213 ± 0.149 | 1.410 ± 0.952 | 0.821 ± 0.664 | 2.470 ± 2.020 |

In *D. magna* was predicted to increase its reproduction by $\sim$33% and $\sim$35% (from eggs$_0$ to eggs$_{\infty}$) when EPA or ARA is present in infinite amounts, respectively. The estimated threshold concentration $S_{75\%}$ for the 75% reproduction level eggs$_{75\%}$ (i.e., PUFA concentration at which the asymptotic clutch size eggs$_{\infty}$ is reduced by 25% relative to the baseline eggs$_0$) was below 1.5 $\mu$g PUFA mg POC$^{-1}$ for both species and PUFAs (*D. pulex*: $S_{75\%}$ (EPA) = 1.141 $\mu$g mg POC$^{-1}$ and $S_{75\%}$ (ARA) = 0.131 $\mu$g mg POC$^{-1}$; *D. magna*: $S_{75\%}$ (EPA) = 1.471 $\mu$g mg POC$^{-1}$ and $S_{75\%}$ (ARA) = 0.561 $\mu$g mg POC$^{-1}$, Table 1).

Via the bootstrapping procedure, we found a significantly higher asymptotic clutch size eggs$_{\infty}$ produced by *D. magna* compared to *D. pulex* (two-way ANOVA; $p$[Species] < 0.001, Fig. 2c, Tables 2(b), 3), while there were no significant differences between the two PUFA experimental lines (EPA vs. ARA, $p$[PUFA] = 0.754). The interaction PUFA × Species did not have any significant effect on eggs$_{\infty}$ ($p$[PUFA × Species] = 0.766). Finally, the threshold concentration $S_{75\%}$ for the 75% reproduction saturation level was neither significantly affected by the two main factors nor by their interaction (Fig. 2d; Table 3).
The chosen bootstrapped data (were performed for each estimated parameter comparing randomly chosen trials and methods were excluded from the data set due to lack of biological significance).

**Table 3.** Results from two-way ANOVAs: We tested the effect of factors PUFA (EPA vs. ARA), Species (D. pulex vs. D. magna) and their interaction PUFA × Species on the estimated parameters asymptotic growth rate \( g_\infty (d^{-1}) \), \( S_{75\%} \) for growth saturation level \( g_{75\%} \) (in \( \mu \text{g PUFA mg POC}^{-1} \)), asymptotic clutch size \( \text{eggs}_\infty (\text{Ind}^{-1}) \), \( S_{75\%} \) for reproduction saturation level \( \text{eggs}_{75\%} \) (in \( \mu \text{g PUFA mg POC}^{-1} \)), and \( S_{75\%} \) for reproduction saturation level \( \text{eggs}_{75\%} \) derived from nonlinear least-square fits through bootstrapped growth rate and reproduction data (see “Materials and methods” section). In total, 400 trials of two-way ANOVA were performed for each estimated parameter comparing randomly chosen bootstrapped data (n = 3 per group, in total 12 data points). The \( p \) values given in the table correspond to a critical \( p \) value which holds for a statistical power of 0.8 (equivalent to a type II error of 0.2).

| Parameter                      | Factors       | \( p \)   |
|--------------------------------|---------------|-----------|
| Somatic growth \( g_\infty (d^{-1}) \) | PUFA          | 0.298     |
|                               | Species       | <0.001    |
|                               | PUFA × Species| 0.809     |
| \( S_{75\%} \) for \( g_{75\%} \) (\( \mu \text{g PUFA mg POC}^{-1} \)) | PUFA          | 0.096     |
|                               | Species       | 0.137     |
|                               | PUFA × Species| 0.230     |
| Reproduction \( \text{eggs}_\infty (\text{Ind}^{-1}) \) | PUFA          | 0.754     |
|                               | Species       | <0.001    |
|                               | PUFA × Species| 0.766     |
| \( S_{75\%} \) for \( \text{eggs}_{75\%} \) (\( \mu \text{g PUFA mg POC}^{-1} \)) | PUFA          | 0.152     |
|                               | Species       | 0.528     |
|                               | PUFA × Species| 0.805     |

\( p \)-values given in bold represent significance (e.g., significant effect of the factor Species).

Summary of the estimated parameters for the reproduction of D. pulex and D. magna, derived from the bootstrapping procedure, can be found in Table 2(b).

Intersection points of EPA- and ARA-curves for growth and reproduction

Finally, we calculated the intersection points between 1000 randomly chosen fitted saturation curves describing the EPA- and ARA-limited growth (Fig. 3a) and reproduction (Fig. 3b) of D. pulex and D. magna. After data cleaning, 767 for D. pulex and 905 for D. magna intersection points remained for the growth curves, while the final number of estimated intersection points for the reproduction curves was 515 for D. pulex and 603 for D. magna. Although we found large variation in the intersection points within each of the two species, the median values were below 10 \( \mu \text{g PUFA mg POC}^{-1} \). The median intersection point for the growth of D. pulex was at 9.26 \( \mu \text{g PUFA mg POC}^{-1} \), while the median intersection point for the growth of D. magna was at 2.64 \( \mu \text{g PUFA mg POC}^{-1} \). These growth-curves intersection points, however, were not significantly different between the two Daphnia species (Wilcoxon–Mann–Whitney test, \( p = 0.94 \)). Similarly, we did not find a significant difference among the two Daphnia species for the intersection points of the EPA- and ARA-dependent reproduction curves (4.12 and 4.97 \( \mu \text{g PUFA mg POC}^{-1} \) [median values for D. pulex and D. magna, respectively; Wilcoxon–Mann–Whitney test, \( p = 0.82 \)].

**Discussion**

\( C_{20} \)-PUFA-limited fitness of D. magna

In the present study, we show that the dietary availability of both \( \omega \)-3 PUFA (EPA) and \( \omega \)-6 PUFA (ARA) limits the fitness (i.e., both the juvenile somatic growth and reproduction rates) of two different Daphnia species. The results obtained from dose-response growth experiments with the large-bodied D. magna fed on a green alga supplemented with the \( \omega \)-3 PUFA EPA are in line with previous findings. The estimated threshold concentration...
S_{75\%} for the 75\% saturation level (i.e., reduction of asymptotic growth rate by 25\%) of the EPA-limited growth of *D. magna* in our study (4.418 ± 1.576 μg EPA mg POC⁻¹ after bootstrapping procedure; mean ± 1 SD) was in the range of previously published 75\% growth saturation thresholds for this species found at the same temperature (20°C), e.g., 0.7–1.3 μg EPA mg POC⁻¹ (Sperfeld and Wacker 2011) and 5.83–7.33 μg EPA mg POC⁻¹ (Sikora et al. 2016). Furthermore, we observed an increase in the somatic growth rate of *D. magna* when ARA was supplied. This is in line with the results from Schlottz et al. (2014), who observed higher growth rates of *D. magna* fed with an ARA-enriched food mixture of *A. obliquus* and *S. elongatus* compared to a C₂₀₆-PUFA-free diet. Becker and Boersma (2005) also observed an increase in somatic growth of *D. magna* when ARA was supplemented to P-sufficient *A. obliquus* and provided an ARA threshold (lowest ARA concentration at which the growth of *D. magna* was not limited) of only 0.06 mg g⁻¹ dry mass which corresponds to approximately 0.136 μg ARA mg POC⁻¹ when a conversion factor of dry mass to carbon of 0.44 is used (Becker and Boersma 2005, 2010). While Becker and Boersma (2005) found similarly low EPA thresholds (0.02 μg EPA mg⁻¹ dry mass and 0.25 μg EPA mg⁻¹ dry mass, corresponding to 0.05 μg EPA mg POC⁻¹ and 0.57 μg EPA mg POC⁻¹, respectively), their approach and findings were strongly criticized by Brett (2010) and thus may need to be interpreted with caution. We here report ARA saturation thresholds (i.e., S_{75\%} = 2.556 ± 1.195 μg EPA mg POC⁻¹ after bootstrapping procedure; mean ± 1 SD) for the growth of *D. magna* that are considerably higher than previously reported by Becker and Boersma (2005). Furthermore, our data show that equal (not statistically different) amounts of EPA and ARA are required to allow for saturated (i.e., unlimited) growth of *D. magna*. At saturating concentrations, EPA and ARA seem to be equally utilized by *D. magna*, which results in similar asymptotic growth rates.

**C₂₀₆PUFA-limited fitness of *D. pulex***

Our data suggest that also the growth of *D. pulex* is limited by both dietary EPA and ARA availability. The estimated 75\% threshold concentration S_{75\%} for EPA-limited growth of *D. pulex* (2.625 μg EPA mg POC⁻¹ estimated from the raw data and 3.156 ± 2.352 μg EPA mg POC⁻¹ after bootstrapping procedure) was almost 10 times higher than the previously reported EPA-threshold (0.3 ± 0.3 μg EPA mg POC⁻¹) for the 90\% growth saturation (i.e., concentration at which the growth rate is reduced by 10\%) for this species grown on the same food organism (Ravet et al. 2012). The estimated EPA-threshold concentration for the 75\% reproduction saturation level, however, was in the range of EPA-thresholds reported by Ravet et al. (2012) (0.17 ± 0.06 μg EPA mg POC⁻¹ and 1.5 ± 0.6 μg EPA mg POC⁻¹ for the 50\% and 90\% reproduction saturation level, respectively, compared to 1.141 μg EPA mg POC⁻¹ observed in our study). Although the range of ARA supply (0.53–9.2 μg ARA mg POC⁻¹) was similar to the one used in our study, Ravet et al. (2012) did not find any effects of ARA availability on the growth or reproduction of *D. pulex*. In contrast to these earlier findings, we observed similar patterns in the somatic growth rate and clutch size of *D. pulex* when grown on either ARA- or EPA-supplemented *A. obliquus*. To our knowledge, the results of our study have demonstrated for the first time that the growth of *D. pulex* is limited by the availability of a ω₆-PUFA such as ARA. Additionally, we provide evidence for an equal relevance of both tested PUFA for the fitness of *D. pulex*.

**Interspecific variation**

Interspecific variation in growth and reproduction saturation thresholds may affect competition between *Daphnia* species, in particular when essential dietary PUFAs are present in limiting amounts (DeMott 1989). At such conditions, the species with the lowest growth and reproduction saturation threshold for a particular PUFA is suggested to be superior over other species with higher PUFA requirements (von Elert 2004; Brzeziński and von Elert 2007). We expected that EPA and ARA thresholds for saturated growth of the smaller species *D. pulex* would be lower than those for the large-bodied species *D. magna*. However, we only found significant differences in the asymptotic growth rate and reproduction (given as clutch size, i.e., number of eggs per individual) among the two *Daphnia* species, while there were no significant differences between EPA and ARA threshold concentrations for saturated growth and reproduction of the daphnids. This is partly in contrast to the earlier findings of Sikora et al. (2016), who demonstrated that the EPA growth saturation thresholds increase with increasing body size across different *Daphnia* species. However, our findings show a significantly higher asymptotic growth rate of *D. magna* compared to *D. pulex*, in both EPA and ARA experimental lines, which is in accordance with the positively correlated juvenile growth rate and body size of different *Daphnia* species reported by Sikora et al. (2016). Likewise, we also observed a significantly higher clutch size for the larger *D. magna* compared to *D. pulex*, and that the somatic growth rate and reproduction of the two *Daphnia* species, differing in their body size, were limited similarly by both the ω₃-PUFA EPA and the ω₆-PUFA ARA. This indicates that both ω₃- and ω₆-PUFAs are equally relevant for the growth and reproduction of daphnids.

The saturation threshold approach used in our study to access possible interspecific differences between *D. pulex* and *D. magna* did not reveal significant results. We assume that the effect sizes depending on EPA- and ARA-supplementation were too similar to reveal any differences and therefore we suggest the experiments to be repeated at colder temperatures, where possible interspecific differences may be more visible due to a higher requirement for highly unsaturated fatty acids to maintain proper membrane fluidity (Hazel 1995; Valentine and Valentine 2004; Masclaux et al. 2012; Pažík et al. 2012). Nevertheless, we do provide evidence for interspecific differences in the response of the two *Daphnia* species that might be explained exclusively by insufficient amounts of EPA and/or ARA: the growth rate and the clutch size of *D. magna*
along the EPA and ARA concentration clearly followed a saturation curve (between 30% and 50% of the variance was explained by the modified Monod model), while this was not the case for D. pulex. Although the growth rate and clutch size of D. pulex increased when EPA or ARA were present, only a small proportion of the variance of the two response variables was explained by the fitted saturation curve (20–30%). It is important to note, however, that our findings are restricted to only one genotype per species. As shown in previous studies, intraspecific differences in response to PUFA-deficiency and in the body content of single ω3-PUFAs (e.g., EPA) might occur (Brzeziński and von Elert 2007; Sikora et al. 2016; Werner et al. 2018). It will be necessary to test more genotypes per Daphnia species to draw further conclusions on the strength of the effect of insufficient EPA and ARA availability for the fitness of different Daphnia species.

Intersection points of the saturation curves and potential colimitation scenarios

The interpretation of the bootstrapped intersection points of the saturation curves derived from the EPA- and ARA-limited growth and reproduction of D. pulex and D. magna is limited by the lack of information on intraspecific differences. Nevertheless, we report intersection points of EPA- and ARA-saturation curves with median values between 2.64 and 9.26 μg PUFA mg POC$^{-1}$ for both growth and reproduction of the daphnids. As we found intersection points of the EPA- and ARA-reproduction curves to be above the estimated EPA- and ARA-saturation thresholds, we can conclude that they may be of minor importance for competitive interactions. In contrast, the intersection points of EPA- and ARA-growth curves were found to be above the estimated ARA-saturation thresholds for the growth and below the estimated EPA-thresholds. This might indicate that shifts in the relative relevance of ARA and EPA for the growth of Daphnia might occur along the PUFA concentration gradient. Furthermore, the positive fitness response to the addition of both PUFAs provides indication for a potential independent colimitation, i.e., simultaneous limitation of growth or reproduction by both tested PUFAs (sensu Sperfeld et al. 2016). However, an extension of this colimitation scenario necessary to classify EPA and ARA as substitutable or essential resources would require controlled growth experiments with simultaneous supplementation of the diet with both PUFAs along a concentration gradient, i.e., response surface or matrix experiments (Sperfeld et al. 2012, 2016). In nature, this probably plays a minor role, as the ratio of EPA to ARA found in seston is usually higher than 1 (Ahlgren et al. 1997; Müller-Navarra 2006).

As both ARA and EPA serve as precursors for eicosanoids (Heckmann et al. 2008a,b; Schlotz et al. 2016; Garreta-Lara et al. 2018), they play an important role for Daphnia's reproduction and the immune system (Martin-Creuzburg et al. 2010; Schlotz et al. 2014; Fink and Windisch 2018). For example, ARA was shown to improve the survival and reproduction of D. magna exposed to an opportunistic bacterial pathogen (Schlotz et al. 2014). However, in vertebrates, ARA- and EPA-derived eicosanoids have partially opposing effects, where their proinflammatory and anti-inflammatory activity, respectively, serves as the best example (Schmitz and Ecker 2008; Alcock et al. 2012). The possible inhibition of the synthesis of ARA-derived eicosanoids by EPA (Sargent et al. 1999; Schmitz and Ecker 2008) led to the assumption that the actions of eicosanoids in fish physiology depend on the ratio of EPA and ARA in the tissue (Koussoroplis et al. 2011). Nevertheless, both ARA and EPA were shown to be important for the development and physiology of fish (Sargent et al. 1999; Bell and Sargent 2003), which in freshwater systems feed on Daphnia. Thus, the dietary availability of EPA and ARA in nature might not only influence Daphnia's performance, but it could also affect higher trophic levels within lakes.

Conclusion

Overall, our study provides clear evidence that ARA, a ω6-PUFA, limits the fitness of two different Daphnia species to an equal extent as the ω3-PUFA eicosapentaenoic acid. We suggest that together with the ω3-PUFA EPA, ARA availability needs to be considered in further studies on food quality and trophic transfer efficiency within freshwater ecosystems.

Shifts in phytoplankton community composition might result in environmental fluctuations in the dietary availability of ω3- and ω6-PUFAs, as the presence and amount of PUFAs varies among different phytoplankton groups (Lang et al. 2011). Therefore, our findings are of particular importance to better predict and understand the consequences of environmental changes and the ongoing global biodiversity loss for the trophic transfer efficiencies at the phytoplankton-zooplankton interface.

References

Ahlgren, G., L. Lundstedt, M. Brett, and C. Forsberg. 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. J. Plankton Res. 12: 809–818. doi:10.1093/plankt/12.4.809.

Ahlgren, G., W. Goedkoop, H. Markensten, Sonesten, and M. Boberg. 1997. Seasonal variations in food quality for pelagic and benthic invertebrates in Lake Erken - the role of fatty acids. Freshw. Biol. 38: 555–570. doi:10.1046/j.1365-2427.1997.00219.x.

Alcock, J., M. L. Franklin, and C. W. Kuzawa. 2012. Nutrient signaling: Evolutionary origins of the immune-modulating effects of dietary fat. Q. Rev. Biol. 87: 187–223. doi:10.1086/666828.

Becker, C., and M. Boersma. 2003. Resource quality effects on life histories of Daphnia. Limnol. Oceanogr. 48: 700–706. doi:10.4319/lo.2003.48.2.0700.

Becker, C., and M. Boersma. 2005. Differential effects of phosphorus and fatty acids on Daphnia magna growth and
reproduction. Limnol. Oceanogr. 50: 388–397. doi:10.4319/lo.2005.50.1.0388.
Becker, C., and M. Boersma. 2010. Limiting levels of eicosapentaenoic acid: What do we really know? Limnol. Oceanogr. 55: 459–462. doi:10.4319/lo.2010.55.1.0459.
Bell, J. G., and J. R. Sargent. 2003. Arachidonic acid in aquaculture feeds: Current status and future opportunities. Aquaculture 218: 491–499. doi:10.1016/S0044-8486(02)00370-8.
Brett, M. T. 2010. Is a low EPA growth saturation threshold supported by the data presented in Becker and Boersma (2005)? Limnol. Oceanogr. 55: 455–458. doi:10.4319/lo.2010.55.1.0455.
Brett, M. T., D. C. Müller-Navarra, A. P. Ballantyne, J. L. Ravet, and C. R. Goldman. 2006. Daphnia fatty acid composition reflects that of their diet. Limnol. Oceanogr. 51: 2428–2437. doi:10.4319/lo.2006.51.5.2428.
Brzeziński, T., and E. von Elert. 2007. Biochemical food quality effects on a Daphnia hybrid complex. Limnol. Oceanogr. 52: 2350–2357. doi:10.4319/lo.2007.52.6.2350.
Burns, C. W., M. T. Brett, and M. Schallenberg. 2011. A comparison of the trophic transfer of fatty acids in freshwater plankton by cladocerans and calanoid copepods: Fatty acids of herbivorous zooplankton. Freshw. Biol. 56: 889–903. doi:10.1111/j.1365-2427.2010.02534.x.
DeMott, W. R. 1989. The role of competition in zooplankton succession, p. 195–252. In U. Sommer [ed.], Plankton ecology. Springer.
Fink, P., and H. S. Windisch. 2018. The essential omega-3 fatty acid EPA affects expression of genes involved in the metabolism of omega-6-derived eicosanoids in Daphnia magna. Hydrobiologia. doi:10.1007/s10750-018-3675-z. https://link.springer.com/journal/10750/onlineFirst/page/4.
Gaedke, U., and D. Straleva. 1998. Daphnids-Keystone species for the pelagic food web structure and energy flow: A body size-related analysis linking seasonal changes at the population and ecosystem levels. Adv. Limnol. 53: 587–610.
Garreta-Lara, E., A. Checa, D. Fuchs, R. Tauler, S. Lacorte, C. E. Wheelock, and C. Barata. 2018. Effect of psychiatric drugs on Daphnia magna oxylipin profiles. Sci. Total Environ. 644: 1101–1109. doi:10.1016/j.scitotenv.2018.06.333.
Goulden, C. E., and A. R. Place. 1990. Fatty acid synthesis and accumulation rates in daphnids. J. Exp. Zool. 256: 168–178. doi:10.1002/jez.1402560207.
Goulden, C. E., and A. R. Place. 1993. Lipid accumulation and allocation in daphnids cladocera. Bull. Mar. Sci. 53: 9.
Harrison, K. E. 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustacean: A review. J. Shellfish Res. 9: 1–28.
Hezal, J. R. 1995. Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? Annu. Rev. Physiol. 57: 19–42. doi:10.1146/annurev.ph.57.030195.000315.
Heckmann, L.-H., and others. 2008a. Systems biology meets stress ecology: Linking molecular and organismal stress responses in Daphnia magna. Genome Biol. 9: R40. doi:10.1186/gb-2008-9-2-R40.
Kainz, M., M. T. Arts, and A. Mazumder. 2004. Essential fatty acids in the planktonic food web and their ecological role for higher trophic levels. Limnol. Oceanogr. 49: 1784–1793. doi:10.4319/lo.2004.49.5.1784.
Koch, U., E. von Elert, and D. Straleva. 2009. Food quality triggers the reproductive mode in the cyclical parthenogen Daphnia (Cladocera). Oecologia 159: 317–324. doi:10.1007/s00442-008-1216-6.
Koussoroplis, A.-M., A. Bec, M.-E. Perga, E. Koutrakis, G. Bourdier, and C. Desvielles. 2011. Fatty acid transfer in the food web of a coastal Mediterranean lagoon: Evidence for high arachidonic acid retention in fish. Estuar. Coast. Shelf Sci. 91: 450–461. doi:10.1016/j.ecss.2010.11.010.
Lang, I., L. Hodell, T. Friedl, and I. Feussner. 2011. Fatty acid profiles and their distribution patterns in microalgae: A comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biol. 11: 124. doi:10.1186/1471-2229-11-124.
Leonard, A. E., S. L. Pereira, H. Sprecher, and Y.-S. Huang. 2004. Elongation of long-chain fatty acids. Prog. Lipid Res. 43: 36–54. doi:10.1016/S0163-7827(03)00040-7.
Martin-Creuzburg, D., E. von Elert, and K. H. Hoffmann. 2008. Nutritional constraints at the cyanobacteria—Daphnia magna interface: The role of sterols. Limnol. Oceanogr. 53: 456–468. doi:10.4319/lo.2008.53.2.0456.
Martin-Creuzburg, D., A. Wacker, and T. Basena. 2010. Interactions between limiting nutrients: Consequences for somatic and population growth of Daphnia magna. Limnol. Oceanogr. 55: 2597–2607. doi:10.4319/lo.2010.55.6.2597.
Martin-Creuzburg, D., S. Oexle, and A. Wacker. 2014. Thresholds for sterol-limited growth of Daphnia magna: A comparative approach using 10 different sterols. J. Chem. Ecol. 40: 1039–1050. doi:10.1007/s10886-014-0486-1.
Masclaux, H., A. Bec, M. J. Kainz, F. Perriére, C. Desvielles, and G. Bourdier. 2012. Accumulation of polyunsaturated fatty acids by cladocerans: Effects of taxonomy, temperature and food. Freshw. Biol. 57: 696–703. doi:10.1111/j.1365-2427.2012.02735.x.
Monod, J. 1950. La technique de culture continue, théorie et applications. Ann. Inst. Pasteur 99: 390–410.
Müller-Navarra, D. C. 1995a. Biochemical versus mineral limitation in Daphnia. Limnol. Oceanogr. 40: 1209–1214. doi:10.1007/s13131-017-1122-z.
Müller-Navarra, D. C. 1995b. Evidence that a highly unsaturated fatty acid limits Daphnia growth in nature. Arch. Hydrobiol. 132: 297–307.

Müller-Navarra, D. C. 2006. The nutritional importance of polyunsaturated fatty acids and their use as trophic markers for herbivorous zooplankton: Does it contradict? Arch. Hydrobiol. 167: 501–513. doi:10.1127/0003-9136/2006/0167-0501.

Pajk, F., E. von Elert, and P. Fink. 2012. Interaction of changes in food quality and temperature reveals maternal effects on fitness parameters of a keystone aquatic herbivore. Limnol. Oceanogr. 57: 281–292. doi:10.4319/lo.2012.57.1.0281.

Park, S., M. T. Brett, D. C. Müller-Navarra, and C. R. Goldman. 2013. Limitation of polyunsaturated fatty acid concentrations for Daphnia using liposome supplementation. Limnol. Oceanogr. 58: 292–307. doi:10.4319/lo.2013.58.1.0292.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Ravet, J. L., M. T. Brett, and D. C. Müller-Navarra. 2003. A test of the role of polyunsaturated fatty acids in phytoplankton food quality for Daphnia using liposome supplementation. Limnol. Oceanogr. 48: 1938–1947. doi:10.4319/lo.2003.48.5.1938.

Ravet, J. L., and M. T. Brett. 2006. Phytoplankton essential fatty acid and phosphorus content constraints for Daphnia somatic growth and reproduction. Limnol. Oceanogr. 51: 2438–2452. doi:10.4319/lo.2006.51.5.2438.

Ravet, J. L., J. Persson, and M. T. Brett. 2012. Threshold dietary polyunsaturated fatty acid concentrations for Daphnia pulex growth and reproduction. Inland Waters 2: 199–209. doi:10.5268/IW-2.4.546.

Sargent, J., G. Bell, L. McEvoy, D. Tocher, and A. Estevez. 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177: 191–199. doi:10.1016/S0044-8486(99)00083-6.

Schlechtriem, C., M. T. Arts, and I. D. Zellmer. 2006. Effect of temperature on the fatty acid composition and temporal trajectories of fatty acids in fasting Daphnia pulex (Crustacea, Cladocera). Lipids 41: 397–400. doi:10.1007/s11745-006-5111-9.

Schlechtriem, C., M. T. Arts, and I. D. Zellmer. 2006. Effect of temperature on the fatty acid composition and temporal trajectories of fatty acids in fasting Daphnia pulex (Crustacea, Cladocera). Lipids 41: 397–400. doi:10.1007/s11745-006-5111-9.

Schlottz, N., J. G. Sørensen, and D. Martin-Creuzburg. 2012. The potential of dietary polyunsaturated fatty acids to modulate eicosanoid synthesis and reproduction in Daphnia magna: A gene expression approach. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 162: 449–454. doi:10.1016/j.cbpa.2012.05.004.

Schlottz, N., M. Pester, H. M. Freese, and D. Martin-Creuzburg. 2014. A dietary polyunsaturated fatty acid improves consumer performance during challenge with an opportunistic bacterial pathogen. FEMS Microbiol. Ecol. 90: 467–477. doi:10.1111/1574-6941.12407.

Schlottz, N., A. Roulin, D. Ebert, and D. Martin-Creuzburg. 2016. Combined effects of dietary polyunsaturated fatty acids and parasite exposure on eicosanoid-related gene expression in an invertebrate model. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 201: 115–123. doi:10.1016/j.cbpa.2016.07.008.

Schmitz, G., and J. Ecker. 2008. The opposing effects of n–3 and n–6 fatty acids. Prog. Lipid Res. 47: 147–155. doi:10.1016/j.plipres.2007.12.004.

Sikora, A. B., T. Petzoldt, P. Dawidowicz, and E. von Elert. 2016. Demands of eicosapentaenoic acid (EPA) in Daphnia: Are they dependent on body size? Oecologia 182: 405–417. doi:10.1007/s00442-016-3675-5.

Smyntek, P. M., M. A. Teece, K. L. Schulz, and A. J. Storch. 2008. Taxonomic differences in the essential fatty acid composition of groups of freshwater zooplankton relate to reproductive demands and generation time. Freshw. Biol. 53: 1768–1782. doi:10.1111/j.1365-2427.2008.02001.x.

Sperfeld, E., and A. Wacker. 2011. Temperature- and cholesterol-induced changes in eicosapentaenoic acid limitation of Daphnia magna determined by a promising method to estimate growth saturation thresholds. Limnol. Oceanogr. 56: 1273–1284. doi:10.4319/lo.2011.56.4.1273.

Sperfeld, E., D. Martin-Creuzburg, and A. Wacker. 2012. Multiple resource limitation theory applied to herbivorous consumers: Liebig’s minimum rule vs. interactive co-limitation: Co-limitation theory applied to herbivores. Ecol. Lett. 15: 142–150. doi:10.1111/j.1461-0248.2011.01719.x.

Sperfeld, E., and A. Wacker. 2012. Temperature affects the limitation of Daphnia magna by eicosapentaenoic acid, and the fatty acid composition of body tissue and eggs: Temperature and EPA effects on Daphnia. Freshw. Biol. 57: 497–508. doi:10.1111/j.1365-2427.2011.02719.x.

Sperfeld, E., D. Raubenheimer, and A. Wacker. 2016. Bridging factorial and gradient concepts of resource co-limitation: Towards a general framework applied to consumers. Ecol. Lett. 19: 201–215. doi:10.1111/ele.12554.

Stanley, D. W. 2000. Eicosanoids in invertebrate signal transduction systems. Princeton Univ. Press.

Stanley-Samuelson, D. W. 1994. The biological significance of prostaglandins and related eicosanoids in invertebrates. Am. Zool. 34: 589–598. doi:10.1093/icb/34.6.589.

Stanley-Samuelson, D. W., R. A. Jurenka, W. Loher, and G. J. Blomquist. 1987. Metabolism of polyunsaturated fatty acids by larvae of the waxmoth, Galleria mellonella. Arch. Insect Biochem. Physiol. 6: 141–149. doi:10.1002/arch.940060302.

Sterner, R. W., D. D. Hagemeier, W. L. Smith, and R. F. Smith. 1993. Phytoplankton nutrient limitation and food quality for Daphnia. Limnol. Oceanogr. 38: 857–871. doi:10.4319/lo.1993.38.4.0857.

Taipale, S. J., M. J. Kainz, and M. T. Brett. 2011. Diet-switching experiments show rapid accumulation and preferential retention of highly unsaturated fatty acids
in *Daphnia*. Oikos **120**: 1674–1682. doi:10.1111/j.1600-0706.2011.19415.x.

Urabe, J., J. Clasen, and R. W. Sterner. 1997. Phosphorus limitation of *Daphnia* growth: Is it real? Limnol. Oceanogr. **42**: 1436–1443. doi:10.4319/lo.1997.42.6.1436.

Valentine, R. C., and D. L. Valentine. 2004. Omega-3 fatty acids in cellular membranes: A unified concept. Prog. Lipid Res. **43**: 383–402. doi:10.1016/j.plipres.2004.05.004.

von Elert, E. 2002. Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. Limnol. Oceanogr. **47**: 1764–1773. doi:10.4319/lo.2002.47.6.1764.

von Elert, E. 2004. Food quality constraints in *Daphnia*: Inter-specific differences in the response to the absence of a long chain polyunsaturated fatty acid in the food source. Hydrobiologia **526**: 187–196. doi:10.1023/B:HYDR.0000041604.01529.00.

Wacker, A., and E. von Elert. 2001. Polyunsaturated fatty acids: Evidence for non-substitutable biochemical resources in *Daphnia galeata*. Ecology **82**: 2507–2520. doi:10.2307/2679932.

Weers, P., K. Siewertsen, and R. Gulati. 1997. Is the fatty acid composition of *Daphnia galeata* determined by the fatty acid composition of the ingested diet? Freshw. Biol. **38**: 731–738. doi:10.1086/1365-2427.1997.00238.x.

Werner, C., M. Ilic, and E. von Elert. 2018. Differences in heat tolerance within a *Daphnia magna* population: The significance of body PUFA content. Hydrobiologia. doi:10.1007/s10750-018-3769-7. https://link.springer.com/journal/10750/onlineFirst/page/4

Windisch, H. S., and P. Fink. 2018. The molecular basis of essential fatty acid limitation in *Daphnia magna*: A transcriptomic approach. Mol. Ecol. **27**: 871–885. doi:10.1111/mec.14498.

Zehnder, A., and P. R. Gorham. 1960. Factors influencing the growth of *Microcystis aeruginosa* Kütz, emend, Elenkin. Can. J. Microbiol. **6**: 645–660. doi:10.1139/m60-077.

Acknowledgments
The authors would like to thank Michelle Etienne for laboratory assistance, Meike Hahn for statistical advice and Kerri-ann Armstrong (Queen’s University Belfast) for language editing. Thoughtful comments and suggestions from two anonymous reviewers greatly helped to improve this paper. This study was supported by the Deutsche Forschungsgemeinschaft in the project DYNATLOSS (grant DFG FI-1548/6-1) within the DFG Priority Programme 1704 “DynaTrait” to PF.

Conflict of Interest
None declared.

Submitted 25 July 2018
Revised 13 February 2019
Accepted 03 May 2019

Associate editor: Takehito Yoshida