Limnol. Oceanogr., 55(1), 2010, 459–462

Limiting levels of eicosapentaenoic acid: What do we really know?

Claes Becker[a,*] and Maarten Boersma[b]

[a] Sweco Environment, Stockholm, Sweden
[b] Alfred-Wegener-Institut für Polar- und Meeresforschung, Biologische Anstalt Helgoland, Helgoland, Germany

We thank Michael Brett for his thought-provoking comments on our original paper from almost 5 yr ago on the differential effects of phosphorus and fatty acids on *Daphnia magna* growth and reproduction (Becker and Boersma 2005). Brett's comment gave us the opportunity to revisit the relevant literature, investigate the progress made in the meantime, and suggest directions for further research. One of the outcomes of this exercise was that it became very clear to us that progress in this field has been slower than expected given the methodology we have at hand. Moreover, there is a strong need for a comprehensive review on the effects of limiting resources on herbivorous zooplankton, something that we can obviously not deliver here. We do, however, briefly address those points that are in need of careful revision and further research.

In the research field of food quality in aquatic food chains, different schools have propagated various factors determining the flow of energy from primary producers to higher trophic levels, such as cladoceran zooplankton. Some researchers reported that essential fatty acids such as eicosapentaenoic acid (EPA) limit *Daphnia* growth (Müller-Navarra 1995). Other scientists have shown that nutrient stoichiometry (C : N and C : P ratios) is an important food-quality determining factor (Sterner et al. 1998). Other biochemical components of the food such as sterol (von Elert et al. 2003) or amino acid (Guisande et al. 2000) content may also influence its quality. Obviously, there are several factors that may constrain primary consumers and in one part of our original paper we aimed to improve our understanding of the concentration range in which fatty acids (specifically EPA) enhance *Daphnia* growth.

The comment Brett has written on our paper (Becker and Boersma 2005) criticizes the methodology used that is the basis of our figure 1. Although we obviously welcome critique as a part of scientific progress, we were surprised that Brett was the one to deliver it after having repeatedly cited this paper himself without comment (Brett et al. 2006; Ravet and Brett 2006; Persson et al. 2007). Brett's comment (in press) unfortunately missed the opportunity of furthering science as it merely criticizes a small part of a complex paper, without adding any new data or interpretation. Real progress would have been made if Brett had used the opportunity to present the data that were obviously available to him. Müller-Navarra (2008) cites a manuscript of Ravet et al. (unpubl.) of which Brett is a coauthor that contains very specific threshold values for EPA and *Daphnia* growth. We do not understand why Brett did not use the opportunity to compare their new results with the values from our study, and then discuss the observed differences in the light of potential methodological problems.

In our experiments we used the methodology to attach essential fatty acids to algal cells, originally developed by von Elert (2002), which is now well established (Martin-Creuzburg et al. 2006; Wichard et al. 2007). To date, we are the only authors to have studied a concentration gradient of these manipulations. Here we will extend our explanation of our experimental and analytical procedure, but more importantly we will focus on the progress in this interesting research field during the last years to potentially spur new scientific discussions.

In his critique Brett raised several concerns with regard to our figure 1: mystery treatment, limit of detection in fatty acid analysis, repeatability, and statistics.

Mystery treatment—Food suspensions were prepared and replaced daily. The gradient was mixed by diluting (with regard to EPA concentration) enriched algae with control (unenriched algae), by using the diluted suspension for one proportion and further diluting it to achieve the next lower proportion and so on. Food suspensions were kept in 5-liter bottles covered in aluminium foil to prevent algal growth. *Daphnia* growth rates were measured in a flow-through chamber with a flow rate of approximately 1 L d^{-1} over a 3-d period. At day 2 during the 3-d experiment, the remaining food suspensions (after 24 h) were sampled and fatty acids were analyzed; these values were used for figure 1, not the nominal ones. Brett is right in that the measured values of EPA do not correspond well to the expected calculated dilution series. However, even though the expected and measured numbers did not match, the measured concentrations were in the same rank order as our dilution gradient. Thus, we were confident that we had achieved a gradient of fatty acid concentrations. We do not know what made the dilution range larger than expected. One of the reasons could be that we measured the fatty acid concentrations in the remaining medium. Potentially, fatty acids were lost during the 24-h feeding period (e.g., via oxidization or algal metabolism). If so, our measurement represents the lowest available fatty acid concentration that *Daphnia* experienced, which would mean that the threshold values should be adjusted upward.

Limit of detection—With the method we used to identify and quantify fatty acid peaks a blank sample or a fatty acid profile of unenriched *Scenedesmus* algae typically never resulted in an identified peak of EPA. In the dilution range experiment, the gas chromatography software quantified

---

* Corresponding author: claes.becker@sweco.se

peaks at the retention time where EPA peaks occur for all enriched treatments but no peaks were found when analyzing unenriched control algae. However, we concede that we may have overemphasized the importance of the lowest EPA concentration in the first experiment, and maybe we used too strong phrasing when comparing our results to earlier studies. A more conservative interpretation (fitting a saturation curve—the exact shape of which is not important here—through the data) would have been that we had stated that the saturation threshold for EPA on the basis of the first experiment was somewhere between 0.02 and 0.25 μg of EPA (mg dry weight)$^{-1}$, or on the basis of a conversion factor of dry weight to carbon of 0.44, of 0.05, and of 0.57 μg L^{-1} EPA. Using a similar approach for the second experiment would yield a saturation threshold between 0 and 0.61 μg L^{-1} EPA.

Repeatability—Apart from not achieving the same low EPA concentration, the second experiment indicated the same pattern as the first experiment. That is, the lowest EPA concentration supported higher *Daphnia* growth rates than when growing on unenriched control algae. The low growth rates on control algae in the first experiment were disturbing, as was stated in our original paper. Still, the information on the low threshold was intriguing and this information should be of interest to other researchers, stimulating new studies. Unfortunately, our attempt to stimulate new studies was not successful, as we are not aware of any published studies that followed up our study. Brett's comment was not the kind of follow-up we aimed for, as the comment projects the unclear message that the threshold is higher than the 0.05 μg L^{-1} EPA we reported, but does not tell us by how much.

Statistics—The way we analyzed the data is statistically correct. We used an ANOVA that showed significant differences in growth rates between treatments along the concentration gradient. To find out which groups were in fact statistically different from each other we carried out a post hoc comparison. The post hoc test indicated that the unenriched control group differed significantly in growth from the enriched treatments, but that there were no significant differences in growth among the daphniids that received any EPA enrichment. We even used a fairly progressive test to do our post hoc comparisons. Tukey post hoc comparisons would have resulted in even less significant differences. Obviously, using different tests as was done by Brett will lead to different results, but it seems incorrect to adapt the test of choice to the result that is wanted. Of course, the variation in the growth rate in the first experiment was large, leading to the nonsignificant differences in growth for the post hoc test of the enriched treatments.

We will now use the rest of this comment productively, and broadly define where we stand with our knowledge on the relationship of zooplankton growth with fatty acid requirements. In the past, two approaches were used to investigate the relationship between the concentrations of essential fatty acids and growth and reproduction of *Daphnia.* The first is the approach initiated by the original and thought-provoking paper of Müller-Navarra (1995), followed by other papers that took a more or less identical approach (Müller-Navarra et al. 2000; Wacker and von Elert 2001). All these studies collected data on natural seston that was supplied to different *Daphnia* species under standardized conditions, to establish the growth of the animals. Subsequently, measured growth rates were correlated with the seston parameters, and those parameters that yielded the highest correlation coefficients were thought to limit *Daphnia* growth in nature. Although certainly of great interest as a first indication of the processes occurring in the field, there are disadvantages to this approach. The most important one is that the whole argument that EPA is the limiting resource for *Daphnia* growth is based on the comparison of correlation coefficients. Correlating a group of sestonic variables with *Daphnia* growth rates will always yield one highest correlation, but is this meaningful? The fact that Wacker and von Elert (2001) observed the strongest correlation of *Daphnia* growth with the concentration of another fatty acid (α-linolenic acid) illustrates this point. Furthermore, this approach assumes that the factors that limit herbivorous zooplankton production do not change even when seston composition changes completely throughout a growing season, for example. Hence, although very useful to identify potential factors explaining growth of *Daphnia* under natural conditions, there is no mechanistic basis to assume that the substance showing the highest correlation coefficient with the *Daphnia* response is in fact the limiting nutrient. From that point of view, computing threshold saturation values on the basis of such correlations is problematic. However, this is exactly what has been done, and is the basis of the threshold saturation value of 0.8 μg L^{-1} EPA for *Daphnia galeata* as reported by Müller-Navarra (1995).

Another major problem of these studies is the seemingly random switching between absolute amounts of fatty acids per volume and the amounts of fatty acid per unit of carbon (as food). This seems more the result of trying to find the highest correlations of sestonic parameters with *Daphnia* growth rather than being on the basis of a solid mechanistic model. Müller-Navarra et al. (2000) simply state that the correlations of *Daphnia* growth with EPA per milligram of C are higher than those with absolute amounts of EPA, and link this to the feeding conditions (above or below the carbon concentrations that saturate feeding). However, as stated above, there is no mechanistic basis for this; correlations of both quantities with *Daphnia* growth should be similar. Nevertheless, this switch between absolute amounts and concentrations was immediately taken up in the literature (Persson et al. 2007; Gladyshev et al. 2008). We argue that it is essential in future work to address the implications of these different models and clearly base them on a mechanistic understanding of zooplankton feeding.

In addition to our study (Becker and Boersma 2005), other laboratory experiments were carried out, which as a result of the controlled conditions should yield clearer information. Two approaches were chosen: the first one used different algae with different fatty acid contents given alone and in mixtures to obtain food with different fatty acid spectra. In the second approach, specific additions of fatty acids either attached to the algae (von Elert 2002) or given as liposomes (Ravet et al. 2003) were carried out. Unfortunately, the experiments with the additions were mostly of an on–off nature, with very high amounts of EPA and docosahexaenoic acid (von Elert 2002), unclear amounts of extra EPA supplied (von Elert 2004; Ravet and Brett 2006), or mixtures of fatty acids (Weers and Gulati 1997). Interestingly, all of these studies except the one by Ravet and Brett were carried out before we published our paper, but to our knowledge no papers using controlled experiments appeared afterward. Thus, our aim to motivate others to do experimental work on the dependency of *Daphnia* growth on fatty acid concentrations has failed until now.

Using mixtures of different algal species can create gradients of fatty acids (Park et al. 2002). Unfortunately, because of the use of different algae, other differences between the treatments will also be present. Nevertheless, Park et al. (2002) estimated *Daphnia magna* growth to be depressed if ω3-polyunsaturated fatty acid content is below 30 nmol (mg C)$^{-1}$. Combining all their data, this translates to around 1.5 nmol (mg C)$^{-1}$ EPA (0.45 μg [mg C]$^{-1}$). Is this experimental proof for a saturation threshold in this order of magnitude? We think not. By taking different algal species grown under different conditions many other factors in the food will have varied as well, just as was the case in the field studies. Their estimate of the saturation threshold is completely dependent on the relative amounts of fatty acids in the three different algae species, with for example *Synechococcus* sp. supporting the lowest growth rates and the lowest EPA amounts (but detectable in five samples, which had not been reported before). It is well known that the low food quality of *Synechococcus* for *Daphnia* is partly a result of other factors than the fatty acid content of the algae, for example the sterol content (von Elert et al. 2003). In fact, the correlation of the EPA content of the food and the *Daphnia* growth rate when considering *Scenedesmus* only as food is negative, significantly so, when focusing only on the effects of the fatty acids in the food (the subset of algae where P should not be limiting, $C:P < 300$). So, despite all of our joint efforts to find workable saturation thresholds for fatty acids, we have not come very far to date, and apart from our study, nobody as far as we know has kept all other factors constant, manipulating only the fatty acid content of the algae in a gradient approach.

How should we proceed from here? We believe that the time has come to acknowledge that multiple factors limit growth and production of *Daphnia*. We are beyond seeing the world in one dimension, and should acknowledge that elemental limitations as well as biochemical limitations (fatty acids, sterols, and amino acids) can all influence the population dynamics of zooplankton. In fact, the recent studies of Ravet and Brett (2006) and Persson et al. (2007) go in this direction, which is to be applauded. Nevertheless, if we want to understand the exact shape of reaction norms of *Daphnia* species to specific nutritional factors we will have to carry out sophisticated laboratory experiments with graded additions of the substance of interest, which are standard in aquaculture research, but much less so in aquatic ecology. The most difficult aspect of those studies is to find the concentrations of interest, not so high that growth or whatever parameter is measured is saturated already. Recent work of Sperfeld and Wacker (2009), who worked with liposomes and a great number of different concentrations of sterols and fatty acids, should further the field substantially.

We should also start realizing that not all *Daphnia* species behave and react identically to the food and environmental conditions. In all other aspects of *Daphnia* life histories we are very aware that large interspecific as well as intraspecific variation exists (Boersma et al. 1999). Why would this not be the case when it comes to nutritional requirements? Recently, Weider et al. (2004) and Seidendorf et al. (2007) showed this for the effects of nutrient stoichiometry on *Daphnia,* and Brzezinski and von Elert (2007) showed differential responses to the addition of EPA for different *Daphnia* species. There will not be one single value for the requirements for EPA uptake for all species within the genus *Daphnia*. Some species might even be able to synthesize EPA. Both Weers et al. (1997) and von Elert (2002) suggested that for *D. galeata* elongation to EPA from shorter ω-3 fatty acids may indeed be possible. Very recently, Heckmann et al. (2008) identified potential EPA biosynthesis pathways in *Daphnia pulex*. It is unclear whether these pathways will be identified for other species as well. Even so, most authors seem to agree that even though potentially present the process to synthesize EPA is too slow in *Daphnia.* Anderson and Pond (2000) speculated that the necessary enzymes do not need to be present because calanoid copepods (and the same could apply to *Daphnia*) are not normally limited in their EPA uptake, an interesting thought, and certainly in need of more study. So, future progress will lie in trying to understand the variation between and within species, and linking those differences with the biochemistry or life history characteristics of the species.

We would like to thank Michael Brett again for his interest in our work, and his commitment to further the field of nutritional ecology in aquatic systems. It gave us the possibility to identify those areas that are in need of further study, and hopefully this discussion will spawn a renewed interest in this fascinating field of study.

Acknowledgments

We thank Karen Wiltshire, Arne Malzahn, Philipp Fischer, Winfried Lampert, and the anonymous reviewers for their constructive comments on earlier versions of the manuscript.

References

Anderson, T. R., and D. W. Pond. 2000. Stoichiometric theory extended to micronutrients: Comparison of the roles of essential fatty acids, carbon, and nitrogen in the nutrition of marine copepods. Limnol. Oceanogr. **45:** 1162–1167.

Becker, C., and M. Boersma. 2005. Differential effects of phosphorus and fatty acids on *Daphnia magna* growth and reproduction. Limnol. Oceanogr. **50:** 388–397.

Boersma, M., L. De Meester, and P. Spaak. 1999. Environmental stress and local adaptation in *Daphnia magna*. Limnol. Oceanogr. **44:** 393–402.

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.

———, 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.

Brzezinski, T., and E. von Elert. 2007. Biochemical food quality effects on a *Daphnia* hybrid complex. Limnol. Oceanogr. **52:** 2350–2357.

Gladyshev, M. I., N. N. Sushchik, O. P. Dubovskaya, O. N. Makhutova, and G. S. Kalachova. 2008. Growth rate of *Daphnia* feeding on seston in a Siberian reservoir: The role of essential fatty acid. Aquat. Ecol. **42:** 617–627.

Guisande, C., I. Riveiro, and I. Maneiro. 2000. Comparisons among the amino acid composition of females, eggs and food to determine the relative importance of food quantity and food quality to copepod reproduction. Mar. Ecol. Prog. Ser. **202:** 135–142.

Heckmann, L. H., R. M. Sibly, M. Timmermans, and A. Callaghan. 2008. Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. Front. Zool. **5:** 9.

Martin-Creuzburg, D., A. Bec, and E. von Elert. 2006. Supplementation with sterols improves food quality of a ciliate for *Daphnia magna*. Protist **157:** 477–486.

Müller-Navarra, D. C. 1995. Evidence that a highly unsaturated fatty acid limits *Daphnia* growth in nature. Arch. Hydrobiol. **132:** 297–307.

———. 2008. Food web paradigms: The biochemical view on trophic interactions. Int. Rev. Hydrobiol. **93:** 489–505.

———, M. T. Brett, A. M. Liston, and C. R. Goldman. 2000. A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. Nature **403:** 74–77.

Park, S. K., M. T. Brett, D. C. Müller-Navarra, and C. R. Goldman. 2002. Essential fatty acid content and the phosphorus to carbon ratio in cultured algae as indicators of food quality for *Daphnia*. Freshwat. Biol. **47:** 1377–1390.

Persson, J., M. T. Brett, T. Vrede, and J. L. Ravet. 2007. Food quantity and quality regulation of trophic transfer between primary producers and a keystone grazer (*Daphnia*) in pelagic freshwater food webs. Oikos **116:** 1152–1163.

Ravet, J. L., and M. T. Brett. 2006. Phytoplankton essential fatty acid and phosphorus content constraints on *Daphnia* somatic growth and reproduction. Limnol. Oceanogr. **51:** 2438–2452.

———, ———, 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.

Seidendorf, B., M. Boersma, and K. Schwenk. 2007. Evolutionary stoichiometry: The role of food quality for clonal differentiation and hybrid maintenance in a *Daphnia* species complex. Limnol. Oceanogr. **52:** 385–394.

Sperfeld, E., and A. Wacker. 2009. Effects of temperature and dietary sterol availability on growth and cholesterol allocation of the aquatic keystone species *Daphnia*. J. Exp. Biol. **212:** 3051–3059.

Sterner, R. W., J. Clasen, W. Lampert, and T. Weisse. 1998. Carbon : phosphorus stoichiometry and food chain production. Ecol. Lett. **1:** 146–150.

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.

———. 2004. Food quality constraints in *Daphnia*: Interspecific differences in the response to the absence of a long chain polyunsaturated fatty acid in the food source. Hydrobiologia **526:** 187–196.

———, D. Martin-Creuzburg, and J. R. Le Coz. 2003. Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). Proc. R. Soc. London B **270:** 1209–1214.

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

Weers, P. M. M., and R. D. Gulati. 1997. Effect of the addition of polyunsaturated fatty acids to the diet on the growth and fecundity of *Daphnia galeata*. Freshwat. Biol. **38:** 721–729.

———, K. Siewertsen, and R. D. Gulati. 1997. Is the fatty acid composition of *Daphnia galeata* determined by the fatty acid composition of the ingested diet. Freshwat. Biol. **38:** 731–738.

Weider, L. J., K. L. Glenn, M. Kyle, and J. J. Elser. 2004. Associations among ribosomal (r)DNA intergenic spacer length, growth rate, and C : N : P stoichiometry in the genus *Daphnia*. Limnol. Oceanogr. **49:** 1417–1423.

Wichard, T., A. Gerecht, M. Boersma, S. A. Poulet, K. Wiltshire, and G. Pohnert. 2007. Lipid and fatty acid composition of diatoms revisited: Rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success. Chembiochem **8:** 1146–1153.

Associate editor: Everett Fee

Received: 04 March 2009
Accepted: 02 September 2009
Amended: 28 September 2009