COMMENTS

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Is a low EPA growth saturation threshold supported by the data presented in Becker and Boersma (2005)?

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Becker and Boersma (2005) report on the effect of essential fatty acid and phosphorus limitation on *Daphnia* growth and reproduction. Their study reaches several novel and provocative conclusions including in their abstract: "[our] *daphniids have much lower saturation thresholds for growth for* [eicosapentaenoic acid; EPA] *than has been previously described for other* Daphnia *species.*" In their discussion they state: *"the EPA saturation threshold for* Daphnia magna *was very low. If this is a general pattern for daphnids, this suggests that these zooplankters often face an environment above this threshold."* And in their final sentence they conclude: *"it is difficult to understand those studies that found strong correlations between EPA content and* Daphnia *growth above the very low concentrations for saturation that we observed."* Because nearly all lakes sampled so far (e.g., Müller-Navarra et al. 2004) have sestonic EPA concentrations greater than the 0.02 μg EPA (mg dry wt)$^{-1}$ growth saturation threshold reported by Becker and Boersma (2005), their results suggest that EPA limitation of *Daphnia* production may never occur in nature. However, I cannot escape the conclusion that their key findings are not supported by their experimental protocols. There are also inconsistencies in their results and debatable inferences drawn from the data.

Mystery treatments—The authors did not use the EPA treatment levels they claim to have used in their most important experiment. Specifically, in the Methods section they state that their *Scenedesmus obliquus* food cultures were enriched with EPA according to the bovine serum albumin method developed by von Elert (2002). In table 2 they report that this resulted in *Scenedesmus* with an EPA content of 4.5 $\pm$ 1.2 ($\pm$1 SD) μg (mg C)$^{-1}$. In their Methods section they state *"an array of EPA . . . concentrations in the food were obtained through mixing control* [EPA free] *algae with* [EPA] *enriched algae in different proportions: 1.0, 0.5, 0.25, 0.125, and 0 of enriched algae."* Therefore they should have ended up with *Scenedesmus* EPA concentrations of approximately 2.0, 1.0, 0.5, 0.25, and 0.0 μg EPA (mg dry wt)$^{-1}$. (They expressed the EPA concentrations for their algal enrichments as μg EPA [mg C]$^{-1}$ and the concentrations they used in their feeding experiments as μg EPA [mg dry wt]$^{-1}$. Although not explained, I surmise on the basis of the data presented that they used a particulate carbon-to-dry weight conversion factor of $\approx$ 0.44.) However, according the values plotted in their fig. 1, they used a concentration gradient of $\approx$ 2.0, 0.5, 0.25, 0.02, and 0.0 μg EPA (mg dry wt)$^{-1}$. Specifically, this experiment lacked an EPA treatment of $\approx$ 1.0 μg EPA (mg dry wt)$^{-1}$, i.e., the 50% dilution level, and had a lowest level of EPA supplementation that was more than three base 2 dilution levels lower than specified in their methods. No explanation is provided for the missing 1.0 μg EPA (mg dry wt)$^{-1}$ treatment or for the inexplicably present 0.02 μg EPA (mg dry wt)$^{-1}$ treatment. The latter point is critical because the most provocative conclusions of Becker and Boersma (2005) depend entirely on the comparison between the 0.02 μg EPA (mg dry wt)$^{-1}$ and the EPA-free control treatments.

Becker and Boersma have (pers. comm.) stated that the various EPA treatment levels they actually used were based on a series of serial dilutions and that the EPA concentrations reported for their experiments were based on direct determinations via gas chromatography. At the 100% EPA supplementation level they measured 2.0 μg of EPA (mg dry wt)$^{-1}$. This sample was then diluted by a factor of two to obtain an expected EPA concentration 1.0 μg of EPA (mg dry wt)$^{-1}$, but inexplicably they actually obtained a concentration of 0.5 μg of EPA (mg dry wt)$^{-1}$. This sample was then diluted by half to obtain the expected EPA concentration of 0.25 μg (mg dry wt)$^{-1}$. This sample was apparently then diluted in half again to obtain a concentration of 0.02 μg of EPA (mg dry wt)$^{-1}$, which is a factor of six less than expected on the basis of the dilution. These "dilution errors" were not mentioned in the original paper, where they should have been clearly explained. Furthermore, in the original paper they state that the fatty acid samples for their food mixtures were collected *"directly after new food suspensions were prepared,"* but in subsequent communications they indicated that these samples were collected from the remnants of *"food suspension bottles after the 24-h feeding period"* (pers. comm.). They have not explained how often they prepared fresh food suspensions for this 3-d experiment, or how

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often they measured the EPA concentrations for the various dilution levels. It is still unclear whether these dilution errors occured 3 d in succession, or, alternatively, if only one series of EPA food suspensions was prepared and analyzed.

Limit of detection—Becker and Boersma (pers. comm.) indicated that the EPA concentrations reported for their first experiment were based on direct determinations. This also raises the question of what is the pertinent analytical limit of detection (LOD) for their fatty acid analyses in general and their 0.02 μg of EPA (mg dry wt)$^{-1}$ treatment in particular. The most common definition of the LOD is that concentration that produces a signal that is 3 SD greater than an analyte-free blank (Eaton et al. 2005). Given the data presented by Becker and Boersma (2005), it is not possible to determine what an appropriate LOD is for their fatty acid data, which could be calculated from their raw data. However, because they reported on the analytical uncertainty for nine fatty acids that had concentrations $\approx$ 0.02 μg of EPA (mg dry wt)$^{-1}$, i.e., 0.03–0.06 μg of EPA (mg C)$^{-1}$, in their table 2, these data can be used to statistically infer whether a concentration of 0.02 μg of EPA (mg dry wt)$^{-1}$ is close to zero considering the quantification uncertainty for their fatty acid analyses. These nine individual fatty acid concentrations had an average C.V. of 63% $\pm$ 34%. This suggests that an EPA concentration of 0.02 μg (mg dry wt)$^{-1}$ with a sample size of one is analytically indistinguishable from zero, and should have been treated accordingly in their study. This EPA concentration would also represent only 0.03% of the total fatty acids in the food mixture and very few studies even report relative fatty acid values this low. Finally, arguing that EPA must have been present in this treatment on the basis of the growth outcomes is tautological, as the outcome of an experiment cannot be used to validate its design or to prove that a specific EPA concentration was present.

Repeatability—Becker and Boersma (2005) indicated that they repeated the EPA dilution gradient experiment *"because of unexpectedly low* [Daphnia] *growth on the control* S. obliquus*"* treatment during the first experiment. In the methods section they stated that the second EPA dilution experiment *"used a concentration gradient of EPA obtained with the same dilution technique and the gradient as described"* for the first experiment. In the Results section they stated *"the results* [of the first and second experiments] *were the same concerning growth and EPA concentrations; the lowest EPA concentration . . . supported higher growth than the control."* However, it cannot be reasonably argued that the second experiment validated the first (Fig. 1) because that experiment did not support a *"very low"* growth saturation threshold. The follow-up experiment also failed to resolve the unexpectedly low *Daphnia* growth in the control treatment during the first experiment. During the first experiment, *Daphnia* growth in the control treatment (i.e., phosphorus-sufficient *S. obliquus* without EPA supplementation) averaged 0.06 $\pm$ 0.13 d^{-1} ($\pm$1 SD). In the second experiment, *Daphnia* growth in the control

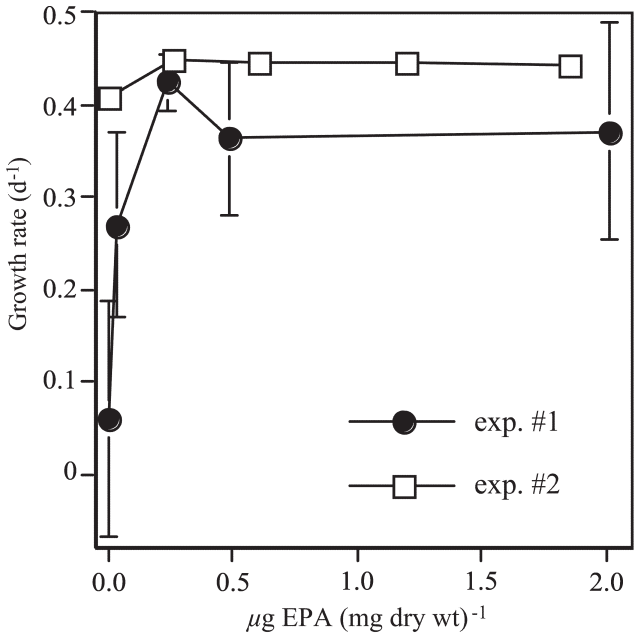

Fig. 1. The results of the first and second EPA supplementation experiments reported in Becker and Boersma (2005). These values were interpolated from figs. 1 and 2 of Becker and Boersma (2005). The error bars represent $\pm$1 SD. These error bars are narrower than the plot symbols for the second experiment. The first experiment utilized five replicates and the second experiment utilized four. The data from the first experiment were manually extracted independently twice and the values obtained varied by $\pm$0.3% and 1.4% ($\pm$1 C.V.) for the mean growth rates and standard errors, respectively.

treatment averaged 0.41 $\pm$ 0.003 d^{-1}. Furthermore, the *Daphnia* grown in P-sufficient *Scenedesmus* in the experiment depicted in the right-hand panel of their fig. 1 also had high growth rates, i.e., 0.46 $\pm$ 0.02 d^{-1}. The fact that *Daphnia* growth in the control treatment for the first EPA supplementation experiment was very low and extremely variable, despite the fact that this clone of *Daphnia magna* has been maintained in the lab on an exclusive diet of *S. obliquus* for over two decades (Lampert 1986), should have caused Becker and Boersma (2005) to reject the results of this experiment. The differences in these two experiments is further emphasized by noting the within-treatment "error" (calculated analogous to a C.V. as the overall average treatment SD : average treatment growth rate) equaled 30% for the first experiment (i.e., 0.09 : 0.30) but was only 1% for the second experiment (i.e., 0.005 : 0.44). By comparison, similar experiments reported in Ravet et al. (2003) and Ravet and Brett (2006) averaged $\approx$ 4% error. Because of the high within-treatment variation and low sample size, the first experiment of Becker and Boersma (2005) also had low statistical power.

Inconsistent statistical reporting—When reporting the results of their first experiment, Becker and Boersma (2005) said *"We found that even the lowest addition of . . . [EPA] increased the growth rates of* D. magna *significantly compared with the controls (analysis of variance [ANOVA]; $p < 0.001$) (fig. 1). Enhancing the . . . EPA contents further-*

Table 1. Treatments means ± 1 SD and the results of key statistical comparisons for the first experiment of Becker and Boersma (2005).

Statistical comparison	First mean	Second mean	Cohen's *d* (effect size)	*t*-value	Probability* one-tailed
Control vs. first EPA	0.06±0.13	0.27±0.10	1.8	2.88	0.0309
First vs. second EPA	0.27±0.10	0.43±0.03	2.1	3.31	0.0159
First vs. second–fourth EPA	0.27±0.10	0.39±0.08	1.3	2.60	0.0270

* Bonferroni corrected.

more had no effect on growth (Newman-Keuls' test).'' I reanalyzed the data extracted from their fig. 1 using ANOVA and post hoc Dunnett t-tests. Initial screening of these data with ANOVA showed that there was at least one significant difference between the various treatments ($p = 0.0001$). Because they stated that the lowest (i.e., first) EPA supplementation level improved growth relative to the control treatment, but further EPA supplementation did not improve growth relative to the first EPA supplementation level, I then compared the various treatments using one-tailed Dunnett *t*-tests. First I compared the growth observed in the control treatment with that in the first EPA supplementation level (the reference group). I then compared the growth observed in the first EPA level to that in the second EPA supplementation level, as well as the first EPA level against the three higher EPA supplementation levels pooled (i.e., second–fourth levels). The Dunnett *t*-tests for all three comparisons were significant (critical $\alpha = 0.05$). Similar results were obtained for successive one-tailed *t*-tests with Bonferroni correction (Table 1).

Since the various post hoc tests weigh the differences between treatment means and within-treatment variability somewhat differently and differ in their conservatism vis-à-vis multiple comparisons, it is possible to use other post hoc tests to obtain somewhat different *p*-values. However, by any measure the differences between the key treatments were of quite similar magnitude. The key point is that when statistical outcome measures are open to multiple interpretations one should be transparent and judiciously cautious when drawing interpretations. Unequivocally stating that the lowest EPA supplementation level was different from the control but no growth improvement was observed for higher supplementation levels (and basing the study's most provocative conclusion on this statement) does not reflect the ambiguous nature of these data.

Conclusions—The results of the first experiment of Becker and Boersma (2005) did not meet several conventional quality-control standards for these types of experiments. For example, *Daphnia* growth in the control treatment for the first experiment was much too low and within-treatment variability was very high. Most importantly, there was a very substantial unexplained deviation between the amount of EPA added to the pivotal treatment in the first experiment and the amount of EPA measured in the sample collected from this food mixture. Furthermore, the EPA concentration reported for this treatment was analytically indistinguishable from zero. These authors also did not present statistical outcome measures that clearly reflected the magnitude of the differences between their most important treatment means. Because of inconsistencies in the experimental methods used, and the results obtained and inferred, the provocative conclusions of Becker and Boersma (2005) regarding extremely low EPA growth saturation thresholds for *Daphnia* growth are not supported by their data.

Future progress—Knowing when particular biochemicals (e.g., fatty acids, amino acids, sterols, elements, aldehydes, protease inhibitors) limit or inhibit zooplankton growth and reproduction is of paramount importance to our understanding of phytoplankton–zooplankton interactions and energy transfer in aquatic systems (Persson et al. 2007). Knowing what these thresholds might be is also essential to obtain a more mechanistic understanding of natural processes, for example, when developing nutrient–phytoplankton–zooplankton models (Arhonditsis and Brett 2004). Studies that attempt to establish specific limiting or inhibiting thresholds should carefully consider how their design influences estimates; for example, sample size, treatment levels, statistical power, and statistical uncertainty are all very important.

The type of functional response (Holling 1959) assumed can also have important consequences when estimating growth-limiting or -inhibiting thresholds. For example, if a type I (linear) Holling's response is assumed, the ''threshold'' has a very intuitive interpretation. In contrast, if type II (asymptotic) or type III (sigmoid) responses are assumed there is no single obvious ''threshold'' value. Type II functional responses in planktonic systems are most commonly mathematically characterized by Michaelis–Menten half-saturation constants. But a half-saturation constant has a very different interpretation from a saturation threshold derived for a type I response. However, any of these responses can be used to infer how likely limitation is to occur in natural systems provided sufficient data are available. It is also important whether a threshold is derived theoretically or empirically (i.e., statistically), as in the latter case threshold estimates will be greatly affected by statistical uncertainty and power. Finally, it is important whether a threshold is defined as that resource quantity or quality level (e.g., the EPA-to-carbon or carbon-to-phosphorus ratios) where food quality declines relative to an optimum or alternatively increases from a minimum.

For example, the carbon-to-phosphorus ratio where phytoplankton food quality is thought to constrain *Daphnia* growth has been derived theoretically assuming a

type I linear response as that phosphorus concentration where growth is first expected to decline relative to optimal phosphorus availability (Urabe and Watanabe 1992). In contrast, Becker and Boersma (2005) determined their EPA-to-carbon (or dry weight) threshold statistically assuming a type II asymptotic response as that EPA concentration where growth first increased relative to an EPA-free control treatment. For obvious reasons, these types of thresholds are not directly comparable. Ultimately, it is more useful to fit food quality and quantity relations to plausible functional responses using appropriate statistical associations (and their uncertainly) as opposed to simply reporting fixed threshold values.

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