1 # Elemental and biochemical nutrient limitation of zooplankton: a meta-

2 analysis

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16 PT, MS, HH, and DvdW conceived and designed the meta-analysis. PT and CK extracted data.

17 PT performed the statistical analyses and wrote the draft manuscript. All authors revised the

18 manuscript and approved its final version for submission.

19 **Data availability statement**

20   The primary data are available from original published studies; the dataset of extracted effect

21   sizes and metadata used for analyses will be uploaded to Dryad upon acceptance; annotated code

22   used for analyses will be uploaded to Zenodo.

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30

31 **Abstract**

32 Primary consumers in aquatic ecosystems are frequently limited by the quality of their food, often

33 expressed as phytoplankton elemental and biochemical composition. Effects of these food quality

34 indicators vary across studies, and the relative importance of elemental (nitrogen and phosphorus)

35 versus biochemical (fatty acid and sterol) limitation in aquatic food webs has been debated. Here

36 we present results of a meta-analysis using >100 experimental studies, which confirms that

37 limitation by N, P, essential fatty acids, and sterols all have significant negative effects on

38 zooplankton performance. However, effects varied by grazer response (growth versus

39 reproduction), specific manipulation, and across taxa. P limitation had greater effects on

40 zooplankton growth than fatty acids, but P and fatty acid limitation had equal effects on

41 reproduction. Furthermore, we show that nutrient co-limitation in zooplankton occurs, that

42 indirect effects induced by P limitation exceed direct effects of mineral P limitation, that effects

43 of nutrient amendments using laboratory phytoplankton isolates exceed those using natural field

44 communities, and that algal physiology mediates zooplankton responses to nutrient limitation.

45 Our meta-analysis reconciles contrasting views about the role of various food quality indicators,

46 and their interactions, for zooplankton performance, and provides a mechanistic understanding of

47 how environmental change affects trophic transfer.

48

49 ## **Introduction**

50 The interface between primary producers and their consumers is marked by high variability in the

51 efficiency of energy transfer, which can constrain the functioning of food webs. This is linked to

52 spatial and temporal variation in both the quantity and quality of food resources available to

53 herbivores across the "phytochemical landscape" (Hunter 2016). While classical models in

54 ecology suggest that density-dependent bottom-up forces (i.e., food quantity) determine energy

55 fluxes to higher trophic levels (Lindeman 1942; Hairston *et al.* 1960), more recent evidence

56 suggests that food quality significantly modifies this relationship and that consumer efficiency

57 depends largely on the quality of producers as well (Sterner & Elser 2002; Arts *et al.* 2009).

58 Specifically, in aquatic systems, trophic transfer has been linked to a small subset of nutrients that

59 explain a large proportion of variation in zooplankton production, including nitrogen (N),

60 phosphorus (P), essential fatty acids (EFAs), and sterols. These are all classified as essential

61 nutrients, meaning that if they are in limiting concentrations in the food, zooplankton often face

62 impaired growth and reproduction, even when edible phytoplankton are high in abundance

63 (Sterner et al. 1993, Brett and Müller-Navarra 1997, Müller-Navarra et al. 2000, Sterner and

64 Elser 2002, Urabe et al. 2002).

65 Despite the known importance of these multiple dimensions of food quality for consumers, there

66 is a long-standing debate regarding whether elemental (i.e., N and P) or biochemical (i.e., EFA

67 and sterol) nutrient limitation is generally more important in shaping zooplankton production,

68 summarized in Gulati and DeMott (1997). Ecological stoichiometry provides a conceptual

69 framework using N and P requirements of producers and consumers to explain trophic

70 interactions, with greater mismatches in algal and consumer C:P ratios in particular causing

71 severe impairment of zooplankton performance (Sterner & Elser 2002). Similarly, variation in the

72   concentration of individual fatty acid molecules in food was shown to explain zooplankton

73   growth and reproduction in eutrophic lakes (Müller-Navarra *et al.* 2000). There are clear

74   physiological explanations for the importance of elemental N, P, individual EFAs, and sterols. In

75   short, N is needed to build proteins, P-rich RNA is required for growth (Elser *et al.* 1996), EFAs

76   regulate membrane fluidity and act as precursors for signaling molecules involved in

77   reproduction (Parrish 2009), and sterols are vital for membrane structure and hormone synthesis

78   (Martin-Creuzburg & von Elert 2009). Despite these clear mechanisms, effects of manipulating

79   the different indicators of nutritional value vary widely across studies and environmental

80   contexts, resulting in a lack of consensus regarding the overall effects of N, P, EFA, and sterol

81   limitation on zooplankton.

82   In addition to the individual effects of elemental and biochemical nutrient limitation, there is

83   evidence that these dimensions of food quality can interact with one another. For example, it has

84   been suggested that EFA limitation is only important under replete food P conditions, and vice

85   versa (Gulati & DeMott 1997). This hypothesis implies a degree of co-limitation in the broad

86   sense, in which the effects of adding one nutrient depend on the availability of another. However,

87   there are many possible forms of co-limitation with diverging implications, including

88   simultaneous co-limitation, independent co-limitation, as well as the closely related concept of

89   serial limitation (see Harpole et al. (2011) and Sperfeld et al. (2016) for a disambiguation of such

90   terms and a guide to the complexity of co-limitation). Moreover, independent co-limitation can

91   be additive, sub-additive, or super-additive, depending upon what effect is induced by co-addition

92   relative to individual addition of nutrients. Whether co-limitation occurs, and in which form it is

93   embodied, however, varies across studies.

94   In addition to co-limitation, elemental and biochemical nutrient levels in the food often directly

95   co-vary with one another, as well as with other aspects of food quality and the environment. For

96   example, P limitation can simultaneously alter the C:P ratio, fatty acid composition and sterol

97   content of phytoplankton (Müller-Navarra 1995; Ahlgren *et al.* 1998; Klein Breteler *et al.* 2005).

98   Simultaneously, it can also affect their cell size, digestibility, colony size, and toxicity (Lürling &

99   Van Donk 1997; Van Donk *et al.* 1997; Brandenburg *et al.* 2020), thus intrinsically linking these

100   distinct dimensions of food quality. This implies a direct dependence between different

101   components of nutritional quality in the case of, e.g., a causative link between P limitation and

102   EFA content or P limitation and digestibility. Therefore, effects of P manipulation may depend

103   on the extent to which indirect effects (i.e., through biochemical compositional changes) are

104   controlled for in the experimental design. This provides one clear example of how the

105   experimental and environmental context may explain variation in nutrient limitation effects

106   across studies. Other influential factors include the taxonomic identity and diversity of study

107   organisms, the traits they express, whether lab isolates or natural phytoplankton communities

108   were used, and the environmental conditions (e.g., temperature, irradiation) used.

109   To reconcile variation in observations to date, and the range of factors that can influence the

110   grazer-phytoplankton interface, we conducted a meta-analysis which quantified the general

111   effects of variation in phytoplankton elemental and biochemical nutrient content on zooplankton

112   grazer performance. We searched the literature for studies that manipulated food quality either

113   directly (e.g., via nutrient supplementation) or indirectly (e.g., across species or natural seston

114   type) while also controlling for confounding effects like food quantity. The included studies were

115   comprised of controlled laboratory experiments using assays where the growth, reproduction, or

116   survival of zooplankton were determined. Field observations or mesocosm studies were included

117   only if they had accompanying assays that disentangled effects of changes in food quantity from

118   food quality. For included studies with direct manipulations and a clear control/treatment

119   structure, we calculated effect sizes as Hedges' *g*, whereas for indirect manipulations and

120   gradient designs we calculated Fisher's *Z*-transformed correlation coefficients as an effect size.

121   As predictors for both approaches, we extracted: molar N:C and P:C ratios, the concentrations of

122   bulk fatty acids and individual EFAs previously reported to be essential (see Table 1 for

123   descriptions), and total sterol concentrations per unit food carbon. Using this database, we tested

124   the following hypotheses:

125   H1: Phytoplankton N:C, P:C, EFA content, and sterol content – as measures for nutritional

126   quality – all individually exert a significant influence on zooplankton performance in terms of

127   growth, reproduction, and survival (H1a), but the effect sizes vary by the zooplankton response

128   type (e.g., growth versus reproduction) and across broad nutrient types (H1b).

129   H2: Zooplankton experience co-limitation by multiple nutrient types, as evidenced by greater

130   effect sizes of co-addition than individual nutrient addition.

131   H3: Other eco-physiological manifestations of food quality (e.g., digestibility) and variation in

132   the environmental and experimental context across studies significantly influence the magnitude

133   of both elemental and biochemical nutrient limitation.

Methods

Data collection and screening

136   Studies were obtained from a Web of Science (www.webofscience.com) search (last accessed

137   February 11, 2021) using the search term: *'((stoichiometry or C:N or C:P or "food quality" or*

138   *"nutritional quality" or "nutritional geometry" or "fatty acid*" or "biochemical compos*" or*

139   *"*sterol*") AND (phytoplankton) AND (zooplankton or herbivor* or consum* or grazer or*

140   *"filter-feed*") AND (growth or product* or defense or grazing or feeding))'.* We also examined

141   the references of previous reviews involving zooplankton food quality (Brett & Müller-Navarra

142   1997; Gulati & DeMott 1997; Sterner & Schulz 1998; Frost *et al.* 2005; Martin-Creuzburg & von

143   Elert 2009; Parrish 2009; Hessen *et al.* 2013; Twining *et al.* 2016; Winder *et al.* 2017) to amend

144   the original search. This yielded a total of 1372 articles which we screened, resulting in a total of

145   122 articles which met all criteria for inclusion. For full documentation of the systematic

146   literature review, see Fig S1 which presents a flow chart of the screening process following

147   PRISMA (preferred reporting items for systematic reviews and meta-analyses) guidelines (O'Dea

148   *et al.* 2021). We included studies that met all the following core criteria: (1) they quantified

149   variation in phytoplankton food quality and/or performed a nutrient supplementation experiment,

150   (2) they quantified zooplankton growth, reproduction, or survival in response to phytoplankton

151   food quality variation, (3) they controlled for confounding effects of food quantity.

152   We also limited the scope of our synthesis to include only published studies with phytoplankton

153   as a food source for zooplankton. In other words, we did not include studies or portions of studies

154   where only heterotrophic bacteria, benthic algae, terrestrial organic matter, etc. were the sole food

155   supply. Similarly, we also did not include studies with benthic grazers and limited the scope to

156   pelagic zooplankton. Lastly, we limited the scope of food quality parameters to include food N:C,

157   P:C, essential fatty acid content, and sterol content (see Table 1 below). Studies measuring only

158   bulk protein, carbohydrates, or lipids were therefore excluded. Here we refer to stoichiometric

159   ratios in the form of molar "nutrient:C" instead of "C:nutrient" as this is more intuitive (higher

160   nutrient:C ratios reflects higher food quality) and thereby comparable to effects of increasing FA

161   and sterol concentrations per unit food carbon. N:C and P:C ratios were log-transformed for all

162   analyses, as non-transformed ratios can lead to biased results (Isles 2020). While definitions of

163   essential fatty acids vary, we included fatty acids defined as essential by Kainz et al. (2004), as

164   well as several closely related ≥18-carbon fatty acids that can be converted to these EFAs, in

165   order to broaden our data beyond only EPA and DHA. Although there is evidence that other

166   nutrients like iron (Lind & Jeyasingh 2018; Jeyasingh & Pulkkinen 2019) and essential amino

167   acids (Fink *et al.* 2011; Koch *et al.* 2013) influence zooplankton production, we did not include

168   these as predictors due to the limited number of experimental case studies.

169   In addition to the suite of food quality and zooplankton performance variables, we also collected

170   information for an array of categorical and numerical variables to act as covariates, provide

171   further context for each study, and explain heterogeneity across studies, to the extent that this

172   information was available. We included the form of food quality manipulation (direct or indirect,

173   see below), the strength of nutrient manipulation, and the nutrient concentration of phytoplankton

174   both pre- and post-manipulation. We considered taxonomy (from broad to species-level) of algae

175   and zooplankton, and noted whether phytoplankton were isolates or natural seston communities.

176   We further retrieved information on the general environment (freshwater versus marine), grazer

177   life stage, food quantity (as mg C L^{-1}), environmental covariates (light, temperature, salinity, pH,

178   pCO$_2$, dissolved inorganic N, P, and Si), and experimental parameters (duration, volume, type of

179   experimental units). Particulate Si:C ratios in diatoms were a covariate of interest, as diatom

180   silification is highly plastic and significantly modifies copepod-diatom interactions (Grønning

181   & Kiørboe 2020; Ryderheim *et al.* 2022); however, this information was notably not reported in

182   studies in our dataset.

183   The manipulation type can be split into three broad categories: direct manipulations, indirect

184   manipulations established via nutrient supply, and indirect manipulations established via other

185   factors including species composition (Table 2). These three levels represent a hierarchy

186   regarding the strength of inferences that can be made to causally link specific nutrients to

187   zooplankton performance. The distinction is particularly important for P manipulation due to

188   known indirect effects of P limitation on phytoplankton food quality. Specifically, P

189   manipulation studies were classified as "direct" if they added a spike in dissolved P supply that

190   was rapidly absorbed by P-limited algae in order to increase algal P:C directly prior to feeding to

191   zooplankton (and change nothing else between treatment and control; see e.g., Plath and Boersma

192   (2001) for a description of this method). Studies were classified as "indirect (via nutrient supply)"

193   if they manipulated nutrient supply over the scale of days to weeks in order to create high and

194   low P:C treatments, as they represent a combination of direct P effects as well as the many

195   indirect physiological effects which can manifest in P-limited algae. Note that fatty acids and

196   sterols only fall within the classifications as "direct" (e.g., direct addition via EFA emulsions or

197   liposomes containing EFAs) or "indirect (other)" (e.g., gradients in EFA due to different species

198   composition or environmental factors).

199   When applicable, we also categorized algae as having important food quality characteristics

200   beyond nutrient content when these were indicated by study authors (e.g., digestion resistance,

201   large size, toxicity). There is a distinction in predicted effects between constitutive (expressed in

202   both high- and low-nutrient conditions) and induced (only expressed with limited nutrients)

203   digestion resistance. Therefore, we differentiated these and classified each case as either

204   constitutive or induced digestion resistance based on descriptions by the authors. All data were

205   extracted from either figures (using WebPlotDigitizer V4.5 (Rohatgi 2021), tables, text, or

206   original raw data provided by authors (when required data was not available in full texts).

207   **Effect size calculations**

208   For the treatment versus control style studies, we calculated Hedges' *g* (a bias-corrected measure

209   of the standardized mean difference) using the function 'escalc' in the R package 'metafor'

210 (Viechtbauer 2010) using package version 3.0-2 and R version 4.0.3 (R Core Team 2020). We

211 calculated Hedges' *g* following Hedges (1983):

212 $$g = \frac{\mu_{replete} - \mu_{limited}}{SD_{pooled}} \times J \qquad \text{where} \qquad J = \frac{\Gamma(m/2)}{\sqrt{m/2} \times \Gamma(\frac{m-1}{2})}$$

213 And $$SD_{pooled} = \sqrt{\frac{(n_{replete}-1) \times SD_{replete}^2 + (n_{limited}-1) \times SD_{limited}^2}{n_{replete} + n_{limited} - 2}}$$

214 Here, $\mu_{replete}$ and $\mu_{limited}$ are the mean zooplankton responses under the most nutrient-replete and

215 most nutrient-limited treatments in each case study, respectively, and SD_{pooled} is the pooled

216 standard deviation (*SD*) with *n* as the number of replicates for each effect size estimate. The term

217 *J* corrects for bias in the estimate of *g* at small sample sizes; Γ denotes the gamma function and *m*

218 denotes the degrees of freedom such that $m = n_{replete} + n_{limited} - 2$. The sampling variance was

219 estimated using the unbiased estimator given in Equation 9 of Hedges (1983), as this is preferable

220 for minimizing bias when small sample sizes are commonly used in studies (i.e., using the setting

221 vtype = "UB" in the metafor `escalc` function (Viechtbauer 2010)). Effect sizes were weighted

222 by the inverse of the sampling variance, such that studies with more precise estimates received

223 greater weight. Hedges' *g* was preferred over log response ratios because negative or zero values

224 of means were common for certain response variables, in which case log response ratios cannot

225 be used.

226 For studies with a correlational/gradient design, we calculated effect sizes as correlation

227 coefficients (Pearson's *r*) for all combinations of nutrient type and zooplankton response

228 category. We then converted these values of *r* into Fisher's *Z*-transformed correlation coefficients

229 (hereafter Z_r), a standard effect size used in meta-analysis to improve distributional properties

230   relative to *r* (Koricheva *et al.* 2013). The *Z*-transformation (Z_r) and its associated sampling

231   variance (v_{Z_r}) are as follows, where *N* is the number data points used per correlation:

232    $$Z_r = \frac{1}{2} ln \left(\frac{1+r}{1-r} \right) \qquad\qquad v_{Z_r} = \frac{1}{N-3}$$

233   Of the 122 studies included in our database, 45 could be placed into both broad design categories;

234   we thus calculated both Hedges' *g* and Z_r for these. The pool of studies therefore contains all

235   categories of direct and indirect manipulations for the correlative (Z_r) approach, while the

236   Hedges' *g* approach includes only direct and nutrient supply-mediated indirect manipulations

237   from which stronger inferences may be made (Table 2), allowing these two datasets to provide

238   complementary information. Agreement between these two distinct effect size methods would

239   add weight to the generality and rigor of the results (shown in Fig. S7).

240   We reversed the sign of effect sizes for a subset of zooplankton responses for which a smaller

241   value indicates greater performance, such that a reduction in e.g., age at maturity or mortality

242   rate, would be coded as a positive effect size. As the estimation of the sampling variance (v_{Z_r})

243   requires 4 data points, only those datasets containing a gradient of ≥ 4 observations were included

244   in the meta-analysis of *Z*-transformed correlation coefficients. For both *g* and Z_r, effect sizes of 0

245   indicate no effect of increased nutrient availability, while positive effect sizes indicate an increase

246   in zooplankton performance with increasing nutrients.

247   **Statistical models**

248   We used both random and mixed effects models using the 'rma.mv' function in the 'metafor' R

249   package in order to calculate weighted effect sizes, to assess broad differences among effects of

250   each nutrient type, and to identify variables that significantly modify the effects of nutrient

251   limitation. To test H1, that limitation by each broad nutrient type is significant, we ran a separate

252 weighted random effects model for each combination of nutrient type (N:C, P:C, overall fatty

253 acids as well as specific EFAs, sterols) and broad zooplankton response type (somatic growth,

254 reproduction, survival, and population growth). For Hedges' g, article ID, zooplankton species,

255 and phytoplankton species were used as random effects to account for non-independence across

256 publications and taxonomy; we specified zooplankton and phytoplankton species as random

257 effects nested within publication. For Z_r, only article ID was used as the random effect, as one

258 effect size could represent gradients across species, and low degrees of freedom precluded the use

259 of zooplankton species as a random effect.

260 For both effect size metrics used, we evaluated H1a based on whether the 95% confidence

261 intervals overlap zero (indicating no overall effect), or do not overlap zero (indicating a

262 significant effect). Similarly, non-overlapping confidence intervals between different nutrient

263 types would indicate differences in the magnitude of nutrient limitation effects (H1b). We tested

264 H2 (that zooplankton experience co-limitation) in two ways: first, by comparing the mean and

265 95% CI of nutrient co-addition to individual nutrient addition (e.g., for studies where both

266 nutrients were factorially manipulated). Second, we used mixed effects models (see below) to test

267 if the concentration of other nutrients significantly influenced the effects of the focal nutrient that

268 was manipulated (e.g., testing if algal P:C ratios influence the magnitude of EFA

269 supplementation effects).

270 To examine how other experimental and environmental covariates may alter effect sizes across

271 different nutrients (H3), we used additive mixed effect models using the following moderators

272 which are commonly reported for all types of nutrient manipulation: experimental duration and

273 volume, food quantity, realm (marine versus freshwater), and morphological dimensions of food

274 quality. Mixed models using factors specific to each nutrient type were used to identify additional

275   influential variables. For instance, with P:C manipulation studies, we also included the

276   manipulation method (direct or indirect P manipulation) as a fixed effect. For all nutrients, a

277   separate model tested whether the initial (pre-supplementation) nutrient levels, as well as the

278   strength of manipulation, influenced effect sizes. Last, we used separate mixed effects models to

279   test for variation among lab versus field phytoplankton communities, phytoplankton taxonomy,

280   and zooplankton taxonomy. In cases with low sample size for each factor, we could not use

281   nested random effects as described above, but instead performed mixed effects models using

282   default settings in the 'rma' function in the 'metafor' package.

283   ## Results

284   **Individual effects of essential nutrient limitation**

285   The results from both the direct and indirect nutrient manipulations in this meta-analysis indicate

286   strong effects of limitation by all the nutrient types we examined. For somatic growth of

287   zooplankton, effect sizes (as Hedges' g) of experimentally increasing N:C ($g = 2.06$, 95% CI:

288   0.94-3.18), P:C ($g = 2.53$, 95% CI: 1.93-3.14), fatty acid content ($g = 1.35$, 95% CI: 0.94-1.77),

289   and sterol content ($g = 7.88$, 95% CI: 1.38-14.4) were all positive and did not overlap 0,

290   indicating significant effects of alleviating their limitation (Fig. 1A, Table S1), and providing

291   clear support for H1a. All broad nutrient types had significant effects on reproduction as well

292   (Fig 1A, Table S1). In addition to the summarized effects of increasing fatty acid content,

293   individual essential fatty acids often had significant positive effects for somatic growth and/or

294   reproduction as well. Overall, similar results were obtained for population growth and survival,

295   yet these responses had relatively limited underlying data compared to somatic growth and

296   reproduction (Fig. S2). Furthermore, these strong effects of nutrient limitation were supported by

297   the correlation results (Fig. 2). Here, effect sizes (as Fisher's Z-transformed correlation) for

298 zooplankton performance in relation to N:C, P:C, fatty acid and sterol content were positive and

299 did not overlap zero for any of the broad nutrient types (shown as squares in Fig. 2), which

300 provides additional support for H1a. The ω3 fatty acids (e.g., ALA, SDA, EPA, total ω3) tended

301 to have strong positive correlations with growth and reproduction, often equal in magnitude to

302 that of N:C and P:C, while correlations for ω6 fatty acids (e.g., LIN, GLA, total ω3) did not differ

303 from zero, indicating no effect. Hedges' *g* and *Zr* were highly congruent with one another for

304 studies where both could be estimated, providing further evidence that support for H1 is robust

305 (Fig. S7).

306 Though all nutrient types had significant effects, the magnitude differed depending on both the

307 nutrient type and the zooplankton response category, supporting H1b (Fig. 1, Fig. 2, Table S1).

308 For somatic growth as response, the grand mean effect size of P:C manipulation was higher than

309 the grand mean for EFA manipulation (Fig 1A). For reproduction as a response, however, effect

310 sizes of P:C and EFA manipulation were equivalent (i.e., overlapping CIs in Fig.1B). In other

311 words, fatty acids appear to be relatively more important for zooplankton reproduction than for

312 somatic growth. Additionally, there is substantial variation for individual fatty acids with

313 different zooplankton responses. Supplementation of ALA, ARA, EPA, and mixed EFAs all had

314 similar effects on somatic growth, but these effects were less than those of P:C manipulations

315 (Fig 1, Table S1). However, with reproduction as the response, increasing the content of ARA

316 and EPA has effect sizes equivalent in magnitude to increasing P:C ratios.

Co-limitation by multiple nutrients

318 Data on co-addition of nutrients from factorially designed experiments was limited to 13 studies,

319 but the results clearly indicate that co-limitation occurs as the mean effect of alleviating

320 limitation of multiple nutrients is substantially greater than for individual nutrients in all cases

321 (Fig. 1). Specifically, a simultaneous increase in P and EFA availability yields a significantly

322 larger effect on zooplankton growth than increasing either nutrient alone. Other co-additions also

323 create larger mean effects than for individual additions, but these are marked by substantial

324 variability and thus have overlapping confidence intervals.

325 Another indicator of co-limitation is the relationship between algal P:C ratio and the strength of

326 other nutrient manipulations like fatty acids. The available data ($n = 9$ studies) show that P:C

327 content significantly modifies the effects of EFA addition, with EFA effect sizes generally being

328 smaller with lower P:C ratios (Fig. 3). EFA addition, however, still had some positive effects

329 even under extreme P-limitation, although effects are much stronger when P is replete.

330 **Effects of other factors on the strength of nutrient limitation**

331 All combinations of added nutrients and response variables were characterized by significant

332 heterogeneity in the responses. Part of this heterogeneity could be explained by moderators

333 reflecting study experimental design and organisms used, providing broad support for H3. For

334 example, the method of P manipulation had a very strong effect on both growth and reproduction

335 responses (Fig. 4). Studies implementing direct limitation of elemental P (i.e., the P-spiking

336 method) had significantly lower effects on zooplankton growth ($g = 1.42$, 95% CI: 0.77-2.08)

337 than those which also allowed for indirect effects, e.g., via physiological changes over the course

338 of algal culturing under different dissolved P supplies ($g = 3.20$, 95% CI: 2.60-3.79).

339 Phytoplankton derived from lab isolates (either mono- or polycultures) had greater nutrient

340 manipulation effect sizes than natural seston (Fig. 5), indicating that there are systematic

341 differences in how complex natural versus simple artificial communities serve as a food base for

342 zooplankton.

343   Results from an additive model reveal further key differences in effects depending upon

344   experimental design and food quality factors beyond nutritional quality (Fig. 6). Variation in the

345   food quantity at which nutrients were manipulated had a significant influence on P:C, EFA, and

346   sterol effect sizes. However, these effects were relatively small in magnitude and varied from

347   negative to positive for different nutrient types. Differences in marine versus freshwater study

348   systems were only detected for sterols. Additionally, the effects of manipulating P:C and EFAs

349   were significantly reduced when algae exhibited morphological traits like constitutive digestion

350   resistance (Fig. 6).

351   Beyond these factors, additional variability in food quality effects can be partially explained by

352   the magnitude of nutrient manipulation, the degree to which nutrients were constrained pre-

353   manipulation, and taxonomy of phytoplankton and grazers. For instance, P:C manipulation is

354   significantly influenced by both the initial (pre-manipulation) P:C ratio, the magnitude of

355   increase, and the interaction between the two (Table S2, Fig S6). For other nutrients, only

356   marginal effects of manipulation strength were detected. Grazer taxonomy also created

357   significant variation in effects. For example, *Bosmina* (a low-P content cladoceran) showed a

358   weaker response to P supplementation compared to *Daphnia*, while rotifers in the genus

359   *Brachionus* had slightly greater responses than *Daphnia* (Fig. S5). Phytoplankton taxonomy

360   provided further variation in effect sizes. For P:C, EFAs, and sterols, adding nutrients to

361   cyanobacteria cultures had greater effects when compared to cryptophytes or green algae. This

362   clearly reaffirms that cyanobacteria are of poor quality while cryptophytes are of superior quality.

Discussion

Individual effects of nutrient limitation

The results of this meta-analysis provide a quantitative summary of the food quality limitations

zooplankton experience in terms of both elemental and biochemical composition of

phytoplankton, two distinct aspects of herbivore nutrition that are typically assessed separately.

Our findings support the hypotheses in recent decades that N, P, fatty acid, and sterol limitation

impede zooplankton growth and reproduction, and may thereby significantly structure

zooplankton communities in nutrient-limited environments. We show that food quantity alone

cannot explain fluxes of energy in aquatic systems, and that food quantity variation was, in fact,

only a marginally influential factor across the controlled experiments in this study (Fig 6).

Though all broad nutrient limitation categories had significant effects (supporting H1a), there

were differences depending on the nutrient type and the response assessed (supporting H1b). In

most cases, P limitation was more evident for somatic growth than other nutrient types. This is in

agreement with the growth rate hypothesis of stoichiometric theory, which postulates that rapid

growth requires greater amounts of P-rich ribosomal RNA (Elser *et al.* 1996; Sterner & Elser

2002). Moreover, fatty acids were shown to be relatively more important for reproduction than

for somatic growth, which also supports previous assertions that fatty acids are essential for

hormone signaling involved in egg production in aquatic invertebrate consumers (Brett and

Müller-Navarra 1997, Parrish 2009, and references therein). In other words, our results suggest

that different nutrients can potentially be relatively more or less limiting throughout the life cycle

of an individual herbivore, with P more likely to limit juvenile growth rates and EFAs more

likely to constrain fecundity of mature adults than to limit juvenile growth. In nature, however,

this will be highly contingent upon the specific nutritional status, and severe limitation by any

individual nutrient can hinder multiple life stages. It is also worth noting that despite a much

387   stronger emphasis in the literature on P limitation effects, we also found N limitation to be

388   significant for zooplankton somatic growth. This suggests that limitation (or co-limitation) by

389   nitrogen, not just phosphorus, is a strong stoichiometric constraint on production of zooplankton

390   that are facing low food N:C ratios, although this may become less frequent with anthropogenic

391   increases of global N:P ratios (Penuelas *et al.* 2020; Peñuelas & Sardans 2022).

Co-limitation and interactive effects of nutrients

393   Our synthesis provides further evidence regarding the extent of co-limitation and interdependence

394   of various dimensions of food quality (H2). Direct tests of co-limitation in this meta-analysis

395   consistently point towards additive effects, whereby adding multiple distinct nutrient types elicits

396   a stronger response than individual manipulations. Combined increases in N:C and P:C, EFAs

397   and P:C, and EFAs and sterols all had larger effects on zooplankton growth than individual

398   nutrient manipulations, which contradicts the idea that any single nutrient tends to be the main

399   limiting factor for zooplankton. This is analogous to widely observed co-limitation of nitrogen

400   and phosphorus for primary producers (Elser *et al.* 2007; Harpole *et al.* 2011). The evidence for

401   co-limitation in zooplankton thus far remains limited, however, and future research on the effects

402   of nutrient co-addition are required to fill this knowledge gap.

403   The interactive effect of both nutrient types is also evident from the observation that responses to

404   EFA manipulations increased with higher P:C ratio, which quantitatively addresses the long-

405   standing hypothesis that fatty acid limitation should be more important when phosphorus is

406   replete (Sundbom & Vrede 1997; Sterner & Schulz 1998; Boersma 2000). These studies, which

407   provide measurements of P:C along with EFA manipulations, illustrate the interdependence of

408   elemental and biochemical nutrients quite well. For example, the threshold for P-limitation in

409   *Daphnia* has been estimated to be a molar C:P ratio of ~150-250 (Anderson & Hessen 2005;

410   Khattak *et al.* 2018); above this ratio, P-limitation should dominate, and below it biochemical

411   nutrients or other limiting factors should become more important. This corresponds to log(P:C)

412   ratios of ca. -5 to -5.5, which is approximately the break point at which predominance of

413   phosphorus limitation versus EFA limitation appear to diverge (see Fig. 3). Specifically, EFA

414   manipulation effects are both higher and more variable than those with P-limited food below this

415   threshold. Also noteworthy is that even under severe P-limitation with C:P ratios near 2,000

416   (logP:C = 7.6), one study still found positive effects of EFA addition (Ferrão-Filho *et al.* 2003).

417   This collectively points to the fact that measurements of multiple aspects of food quality, even

418   when only one nutrient type is the core focus of a particular study, can yield important and novel

419   insights into aquatic food web functioning.

Other factors influencing zooplankton nutritional constraints

421   Several key factors contributed to the variation in the strength of nutrient manipulations

422   (supporting H3), and may reconcile contrasting views on the nature of zooplankton nutrition. The

423   most striking cause of heterogeneity for P-manipulation studies, for example, was the method in

424   which P was manipulated. The direct method using short-term P-spiking immediately before

425   feeding and thus only manipulating the algal P:C ratio had significantly smaller effects compared

426   to the indirect method of manipulating dissolved P in the medium over a longer incubation of

427   days to weeks. The indirect approach includes both direct effects of low P content as well as the

428   complex suite of physiological changes resulting from algal P-stress. Notably, these indirect

429   effects are strong for rotifers (Rothhaupt 1995; Zhou *et al.* 2018) as well as cladocerans,

430   indicating that these effects are not constrained to a single taxon. These indirect effects include

431   induced changes in cell size, cell wall thickness, and digestibility (van Donk & Hessen 1993;

432   Lürling & Van Donk 1997; Lürling *et al.* 1997; Van Donk *et al.* 1997), but also changes in

433   essential fatty acid content (Müller-Navarra 1995; Ahlgren *et al.* 1998; Ravet & Brett 2006;

434   Grzesiuk *et al.* 2018; Bi & Sommer 2020). Moreover, other food quality dimensions that have

435   received less attention so far may be affected. The extent to which fatty acids like EPA are

436   influenced by P limitation varies, however, and can either increase (Bi & Sommer 2020), not

437   change (Park *et al.* 2002), or decline sharply (Müller-Navarra 1995) depending upon

438   phytoplankton taxa. Therefore, we can only speculate as to the most important causes of these

439   indirect effects. In sum, our results confirm that zooplankton can experience direct P limitation,

440   that indirect effects of P-limitation often occur, but that more work is needed to identify the

441   mechanisms by which P-stress induces these indirect effects on zooplankton production.

442   We also found large differences in the effects of nutrient manipulation for natural phytoplankton

443   communities versus laboratory isolates. Here, we can also only make limited inferences as to the

444   exact mechanisms causing these differences. The main hypotheses regarding food quality of

445   phytoplankton involve a) mineral/biochemical content, b) toxicity, c) size/shaped-based grazing

446   resistance, and d) other morphological defenses/digestion resistance (Gulati & DeMott 1997;

447   Sterner & Schulz 1998). As most studies filtered natural seston to include only edible particles (<

448   35-μm fraction), and there were no differences between filtered and unfiltered seston (Fig. 6), we

449   can rule out size in this analysis. However, we cannot rule out any of the other three factors for

450   explaining the large differences between lab and field algae. The relatively low effect sizes of

451   nutrient addition for natural communities suggest three general possibilities: 1) the diverse

452   natural communities were already of high quality and therefore did not increase much in quality

453   with supplementation, 2) zooplankton were limited by algal defenses (e.g. indigestibility,

454   toxicity, morphology) and not nutrients, and/or 3) zooplankton were limited by a mineral or

455   biochemical nutrient not explicitly tested by the authors. Our findings suggest that natural

456   communities may have been of poorer average quality within this dataset, as zooplankton had

457   slightly lower (though still positive) growth rates on non-supplemented natural seston (mean =

458   0.22 d^{-1}, 95% CI: 0.20-0.24) than on non-supplemented isolates (mean = 0.26 d^{-1}, 95% CI: 0.22-

459   0.30). Interestingly, the range in zooplankton somatic growth rates is smaller for natural

460   communities than for isolates (Fig. S8), indicating that these more diverse communities may

461   buffer the most severe effects of nutritional deficiency observed in monocultures. This would

462   support previous findings that phytoplankton biodiversity can decrease the variance in

463   zooplankton production (Striebel *et al.* 2012), possibly increasing the stability of the autotroph-

464   consumer link in food webs. However, such effects clearly depend on the specific array of traits

465   that come with increasing algal diversity (Marzetz *et al.* 2017), and the ability of the grazer to

466   select for high-quality food. We included certain algal traits like digestion resistance as

467   moderators, however these are only included to the extent that authors explicitly acknowledged

468   such factors. The consequences of algal defenses for the observed effects of food nutrition will

469   depend on their response to changes in nutrient availability. For example, if algal defenses

470   increase with nutrient limitation, then the reported effects of nutrient content *per se* will be

471   overestimated, as increased nutrient availability is correlated with reduced defenses. This is in

472   fact seen in the slightly positive influence of induced digestion resistance, compared to the strong

473   negative influence of constitutive digestion resistance, on values of Hedges' *g* (Fig. 6). The full

474   extent to which such traits are linked to nutrient availability, and how they together affect

475   zooplankton performance, remains to be elucidated (Meunier *et al.* 2017).

476   **Nutrient limitation in natural systems**

477   In our analysis, we only included studies which controlled for any confounding effects of the

478   biomass quantity available to zooplankton, which selects for mostly lab experiments, and

479   predominantly those using common study organisms like *Daphnia* and *Scenedesmus* or

480   *Synechococcus* spp. (see sample sizes by taxa in Fig. S4, S5). This largely reflects the biases in

481   this field, where the focus on clear study systems has greatly supported our mechanistic insights

482   into zooplankton nutrient limitation. Nevertheless, that we have "a dearth of knowledge about

483   other anomopods, cyclopoid and calanoid copepods, and rotifers" (Sterner & Schulz 1998)

484   relative to *Daphnia* is as true today as it was in the 1990s.

485   Some of the limitations posed by the controlled laboratory experiments included in this synthesis

486   do not apply for field and mesocosm studies. Therefore, a complementary meta-analysis, using

487   field and mesocosm studies that tested effects of seston food quality (and quantity) on

488   zooplankton production, could provide additional insights based on more field-realistic settings,

489   if (somewhat) controlled for factors like species sorting and food quantity. The relative degree of

490   nutrient limitation in nature is, of course, determined by the distribution of nutrient

491   concentrations in aquatic systems, which varies considerably across sites (Brett *et al.* 2000; Elser

492   *et al.* 2000). At large scales, concentrations of different nutrient types may be highly interactive,

493   especially if P enrichment selects for phytoplankton communities of lower biochemical food

494   quality (Müller-Navarra *et al.* 2004; Taipale *et al.* 2019). The mechanistic results from our meta-

495   analysis will therefore pair well with systematic assessments of elemental and biochemical

496   nutrient concentrations (e.g., field monitoring of nutrients over time and across different lakes).

497   Specifically, our results illustrate the potential magnitude of nutrient limitation effects on

498   zooplankton physiology with phytoplankton species composition ranging from monocultures to

499   natural communities. The extent to which low quality taxa dominate natural systems, combined

500   with the observed nutrient concentrations in a given system, should provide clear predictions

501   regarding the extent of nutrient limitation in nature. However, particularly the combination of

502   field observations across sites with controlled manipulations (e.g., Hartwich et al. (2012)), will

503   allow making strong inferences regarding limitation of zooplankton production in nature.

504   **Knowledge gaps**

505   We identify several recommendations for future research based on our systematic review and

506   meta-analysis. As described above, the need is clear for more information on zooplankton

507   nutrient co-limitation, simultaneous measurements of multiple food quality factors, and how

508   these signals of algal nutritional quality are inherently connected to other functional traits of both

509   producers and consumers. In addition to this, we find our database to be less data-rich for nutrient

510   manipulation effects involving sterols, ω6-EFAs like ARA (Ilić *et al.* 2019), controlled

511   manipulations of nutrients in natural phytoplankton communities, marine systems in general, and

512   interactions between algal defenses (e.g., digestion resistance, silicification) and nutrient content.

513   Therefore, we see clear gaps in our knowledge based on these lines of taxonomy, study system,

514   level of biological complexity, and nutrients examined, which would benefit from additional

515   future research. Moreover, our synthesis emphasizes that integration across disparate nutritional

516   categories provides insights into food web dynamics. Our mechanistic understanding on

517   zooplankton eco-physiology will therefore benefit from focusing on interactive effects of

518   different nutrients on food web structure, as well as the linkages between functional diversity of

519   zooplankton and phytoplankton, and environmental change.

520   **Conclusion**

521   In conclusion, we find that every type of phytoplankton nutritional quality we considered in our

522   meta-analysis significantly influenced growth and reproduction of herbivorous zooplankton. We

523   show that several additional factors beyond low concentrations of nutrients *per se* can explain

524   substantial variation in reported effects of algal food quality on planktonic consumers and

525   therefore variation in the efficiency of aquatic food web functioning.

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696

697   Table 1. Components of food quality investigated in this meta-analysis. Bold denotes essential

698   fatty acids following Kainz et al. (2004). Units used are molar ratios (N:C, P:C) or mass ratios

699   (μg mg C^{-1} for fatty acids and sterols).

Abbreviation	Description
N:C	molar N:C ratio
P:C	molar P:C ratio
LIN	C18:2ω6; linoleic acid
GLA	C18:3ω6; γ-linolenic acid
ALA	C18:3ω3; α-linolenic acid
SDA	C18:4ω3; stearidonic acid
ARA	C20:4ω6; arachidonic acid
EPA	C20:5ω3; eicosapentaenoic acid
DHA	C22:6ω3; docosahexaenoic acid
DHA:EPA	Ratio of DHA:EPA
ω3	total omega-3 fatty acids
ω6	total omega-6 fatty acids
ω3:ω6	ω3:ω6 ratio
PUFA	total polyunsaturated fatty acids
EFA	essential fatty acids (sum of LIN, ALA, ARA, EPA, DHA)
LCEFA	long-chain ($\geq$ 20 C) essential fatty acids (sum of ARA, EPA, DHA)
TFA	total fatty acids
sterol	total sterols

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703 Table 2. Categories of nutrient manipulations used in the meta-analysis.

Nutrient manipulation category	Description	Illustrative studies using each method	Model used in	
			Hedges' g	Zr
Direct	Only the nutrient of interest is manipulated; all else held constant	Plath and Boersma (2001); Zhou et al. (2018) "LP+P" treatment compared to "LP"	✕	✕
Indirect (via nutrient supply)	Nutrient supply is manipulated; however, the manipulation may simultaneously alter other food quality factors	Sterner et al. (1993); Zhou et al. (2018) "HP" treatment compared to "LP"	✕	✕
Indirect (other)	Factor other than nutrient supply is manipulated (i.e., most commonly, species composition)	Müller-Navarra et al. (2000); Mandal et al. (2018)		✕

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Figure captions

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711 Fig. 1. Effect sizes (mean and 95% CI) of nutrient manipulation on zooplankton growth and

712 reproduction from a weighted random effects meta-analysis. Error bars that do not overlap zero

713 indicate significant effects of increasing availability in zooplankton diets. Squares denote overall

714 effects of N:C, P:C, essential fatty acids (EFA), and sterols, as well as their combined addition

715 (below horizontal line); circles denote effects of specific fatty acids. The sample size for each

716 combination of nutrient type and response is shown as the number of unique publications (n) and

717 the number of effect size estimates (k).

718 Fig 2. Effect of nutrient gradients (Z-transformed correlation coefficient, Z_r) for the subset of

719 studies using indirect and/or gradient style manipulations to test effects of nutrient limitation.

720 This dataset includes e.g., studies correlating zooplankton response by algal species, by different

721 environmental conditions, or along nutrient manipulation gradients. Points show mean and 95%

722 CI of effect sizes for each nutrient type, squares denote summary effect sizes, circles denote

723 individual EFA correlations, and the sample size for each combination of nutrient type and

724 response is shown as the number of unique publications (n) and the number of effect size

725 estimates (k).

726 Fig 3. Relationship between variation in log-transformed P:C ratio and the effect size of EFA

727 addition. Effect sizes of EFA manipulation generally increase with greater phosphorus

728 availability, as shown by the `loess` fit curve. The size of each point indicates the reliability of

729 each effect size (i.e., the weighting in the form of inverse variance), and colors indicate different

730 studies. For reference, log(P:C) values of -7, -6, -5, and -4 are equivalent to C:P ratios of ca.

731 1100, 400, 150, and 55, respectively.

732   Fig. 4. The method by which P is manipulated has strong effects on P-limitation effect sizes.

733   "Indirect" indicates that phytoplankton were exposed to differences in P supply during

734   cultivation, which can induce indirect physiological effects of P-limitation (in addition to direct

735   effects), whereas "Direct" indicates a more strictly direct test of elemental P-limitation, as these

736   indirect effects are controlled for by spiking P such that phytoplankton physiology does not

737   change before they are consumed, and only P:C varies between treatment and control. Values of

738   n and k represent number of studies and number of effect size estimates, respectively.

739   Fig. 5. Effect sizes for P:C and essential fatty acid manipulation, separated by zooplankton

740   response category and whether the phytoplankton used were from a natural community or from

741   isolated laboratory strains. Size of points corresponds to the number of studies per effect size

742   estimate.

743   Fig. 6. Effects of additional experimental and morphological factors on nutrient limitation effects.

744   Significant effects are indicated by bolded diamonds. The mean and 95% CI represent results of a

745   mixed effects regression model.

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Figure legend (axis labels and annotations):

Nutrient manipulation (y-axis)

- N:C ratio
- P:C ratio
- Fatty acids
- ALA addition
- SDA addition
- ARA addition
- EPA addition
- DHA addition
- Mixed EFA addition
- Sterols
- N:C and P:C
- P:C and fatty acids
- Fatty acids and sterols

Effect size (Hedges' g) (x-axis)

(A) Somatic growth

- N:C ratio: $n = 11, k = 43$
- P:C ratio: $n = 53, k = 336$
- Fatty acids: $n = 25, k = 216$
- ALA addition: $n = 6, k = 32$
- SDA addition: $n = 1, k = 2$
- ARA addition: $n = 6, k = 26$
- EPA addition: $n = 16, k = 99$
- DHA addition: $n = 3, k = 6$
- Mixed EFA addition: $n = 10, k = 51$
- Sterols: $n = 7, k = 36$
- N:C and P:C: $n = 2, k = 2$
- P:C and fatty acids: $n = 5, k = 9$
- Fatty acids and sterols: $n = 4, k = 5$

(B) Reproduction

- N:C ratio: $n = 15, k = 56$
- P:C ratio: $n = 26, k = 140$
- Fatty acids: $n = 12, k = 56$
- ALA addition: $n = 3, k = 4$
- SDA addition: $n = 1, k = 2$
- ARA addition: $n = 2, k = 5$
- EPA addition: $n = 6, k = 20$
- DHA addition: $n = 2, k = 5$
- Mixed EFA addition: $n = 7, k = 20$
- Sterols: $n = 6, k = 29$
- P:C and fatty acids: $n = 3, k = 7$

Figure showing forest plots of effect sizes (Z-transformed correlation) for nutrient gradients.

(A) Somatic growth

Nutrient gradient	n, k
N:C ratio	n = 22, k=47
P:C ratio	n = 40, k=107
Fatty acids	n = 30, k=290
LIN	n = 13, k=22
GLA	n = 11, k=18
ALA	n = 15, k=27
SDA	n = 15, k=28
ARA	n = 15, k=26
EPA	n = 27, k=55
DHA	n = 16, k=33
n3	n = 10, k=24
n6	n = 9, k=23
PUFA	n = 8, k=15
TFA	n = 10, k=16
sterol	n = 9, k=24

(B) Reproduction

Nutrient gradient	n, k
N:C ratio	n = 14, k=38
P:C ratio	n = 15, k=46
Fatty acids	n = 19, k=303
LIN	n = 10, k=27
GLA	n = 6, k=17
ALA	n = 12, k=31
SDA	n = 11, k=31
ARA	n = 10, k=21
EPA	n = 18, k=44
DHA	n = 14, k=37
n3	n = 9, k=28
n6	n = 8, k=27
PUFA	n = 7, k=23
TFA	n = 7, k=17
sterol	n = 5, k=16

Effect size (Z−transformed correlation)