SYNTHESIS

Elemental and biochemical nutrient limitation of zooplankton: A meta-analysis

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Abstract

Primary consumers in aquatic ecosystems are frequently limited by the quality of their food, often expressed as phytoplankton elemental and biochemical composition. However, the effects of these food quality indicators vary across studies, and we lack an integrated understanding of how elemental (e.g. nitrogen, phosphorus) and biochemical (e.g. fatty acid, sterol) limitations interactively influence aquatic food webs. Here, we present the results of a meta-analysis using >100 experimental studies, confirming that limitation by N, P, fatty acids, and sterols all have significant negative effects on zooplankton performance. However, effects varied by grazer response (growth vs. reproduction), specific manipulation, and across taxa. While P limitation had greater effects on zooplankton growth than fatty acids overall, P and fatty acid limitation had equal effects on reproduction. Furthermore, we show that: nutrient co-limitation in zooplankton is strong; effects of essential fatty acid limitation depend on P availability; indirect effects induced by P limitation exceed direct effects of mineral P limitation; and effects of nutrient amendments using laboratory phytoplankton isolates exceed those using natural field communities. Our meta-analysis reconciles contrasting views about the role of various food quality indicators, and their interactions, for zooplankton performance, and provides a mechanistic understanding of trophic transfer in aquatic environments.

KEYWORDS
cladoceran, copepod, ecological stoichiometry, ecosystem functioning, growth rate, nitrogen limitation, nutrition, phosphorus limitation, traits, trophic interactions

INTRODUCTION

The interface between primary producers and their consumers is marked by high variability in the efficiency of energy and material transfer, which can constrain the functioning of food webs. This is linked to spatial and temporal variation in both the quantity and quality of food resources available to herbivores across the 'phytochemical landscape' (Hunter, 2016). While classical models in ecology suggest that density-dependent bottom-up forces (i.e. food quantity) determine fluxes of matter and energy to higher trophic levels (Hairston

et al., 1960; Lindeman, 1942), more recent evidence suggests that food quality significantly modifies this relationship and that consumer efficiency depends largely on the quality of producers as well (Arts et al., 2009; Sterner & Elser, 2002). Specifically, in aquatic systems, trophic transfer has been linked to a small subset of nutrients that explain a large proportion of variation in zooplankton production, including nitrogen (N), phosphorus (P), essential fatty acids (EFAs) and sterols. These are all classified as essential nutrients, meaning that if they are in limiting concentrations in the food, zooplankton often face impaired growth and reproduction, even when edible phytoplankton are high in abundance (Brett & Müller-Navarra, 1997; Müller-Navarra et al., 2000; Peltomaa et al., 2017; Sterner et al., 1993; Sterner & Elser, 2002; Urabe et al., 2002).

Ecological stoichiometry provides a conceptual framework using N and P requirements of producers and consumers to explain trophic interactions, with greater mismatches in algal and consumer C:P ratios, in particular, causing severe impairment of zooplankton performance (Sterner & Elser, 2002). Similarly, variation in the concentration of individual fatty acid (FA) molecules in food was shown to explain zooplankton growth and reproduction in eutrophic lakes (Müller-Navarra et al., 2000). There are clear physiological explanations for the importance of elemental N, P, individual EFAs and sterols. In short, N is needed to build proteins, P-rich RNA is required for growth (Elser et al., 1996), EFAs regulate membrane fluidity and act as precursors for signalling molecules involved in reproduction (Parrish, 2009), and sterols are vital for membrane structure and hormone synthesis (Martin-Creuzburg & von Elert, 2009). Despite these clear mechanisms, the effects of manipulating the different indicators of nutritional value vary widely across studies and environmental contexts, and we currently lack an integrative overview of the overall effects of N, P, EFA and sterol limitation on zooplankton.

In addition to the individual effects of elemental and biochemical nutrient limitation, there is evidence that these dimensions of food quality can interact with one another. For example, it has been suggested that EFA limitation is only important under replete food P conditions, and vice versa (Gulati & DeMott, 1997). This hypothesis implies a degree of co-limitation in the broad sense, in which the effects of adding one nutrient depend on the availability of another (for a detailed guide to the complexity of co-limitation and serial limitation, see Harpole et al. (2011) and Sperfeld et al. (2016)). Importantly, recent observations of co-limitation provide increasing evidence that simple paradigms of single nutrient limitation (following Liebig's law) do not accurately represent constraints on populations in nature. Whether and how co-limitation occurs, however, varies even across controlled experimental studies. Moreover, the likelihood of co-limitation versus single nutrient limitation in

natural systems depends on whether dominance by certain phytoplankton taxa creates severe constraints for one particular nutrient type, for example during periods of cyanobacteria dominance when levels of fatty acids and sterols can become limiting for zooplankton (Müller-Navarra et al., 2000; Peltomaa et al., 2017).

In addition to co-limitation, elemental and biochemical nutrient levels in the food often directly covary with one another, as well as with other aspects of food quality and the environment. For example, P limitation can simultaneously alter the C:P ratio, fatty acid composition and sterol content of phytoplankton (Ahlgren et al., 1998; Bi & Sommer, 2020; Klein Breteler et al., 2005; Müller-Navarra, 1995). One proposed mechanism for the linkage between P and fatty acids is that severe P limitation reduces the production of membrane phospholipids, which is where the bulk of algal EFAs are located, and instead upregulates non-essential lipid synthesis (Ahlgren et al., 1998). At the same time, P limitation can also affect algal cell size, digestibility, colony size and toxicity (Brandenburg et al., 2020; Lürling & Van Donk, 1997; van Donk et al., 1997), thus intrinsically linking these distinct dimensions of food quality. This implies a direct dependence between different components of nutritional quality in the case of, for example, a causative link between P limitation and EFA content or P limitation and digestibility. Thus far, however, it remains unclear how important strict stoichiometric P limitation is relative to the indirect effects that result from P constraints in natural systems. Moreover, N limitation may also influence the composition of EFAs, though the direction of this effect varies across studies (e.g. Ahlgren & Hyenstrand, 2003; Trommer et al., 2019; Wang et al., 2019). Other factors potentially influencing the effects of limitation by different nutrients include the taxonomic identity and diversity of study organisms, the traits they express (especially those related to digestibility), whether laboratory isolates or natural phytoplankton communities were used, and the environmental conditions (e.g. temperature, irradiation) used.

The overarching goal of our synthesis is to reconcile literature on various food quality indicators, and thereby provide an integrative understanding of the interactive effects of elemental and biochemical nutrient limitation on zooplankton performance. To achieve this, we conducted a meta-analysis which quantified the general effects of variation in phytoplankton elemental and biochemical nutrient content on zooplankton grazer performance. We searched the literature for studies that manipulated food quality either directly (e.g. via nutrient supplementation) or indirectly (e.g. across species or natural seston type) while also controlling for confounding effects like food quantity. Using this database, we tested the following hypotheses:

H1: Phytoplankton N:C, P:C, EFA content and sterol content—as measures for nutritional quality—all

individually exert a significant influence on zooplankton performance in terms of growth and reproduction (H1a), but the effect sizes vary by the zooplankton response type (e.g. growth vs. reproduction) and across broad nutrient types (H1b).

H2: Zooplankton experience co-limitation by multiple nutrient types, as evidenced by greater effect sizes of co-addition than individual nutrient addition.

H3: Other eco-physiological manifestations of food quality (e.g. digestibility) and variation in the environmental and experimental context across studies significantly influence the magnitude of both elemental and biochemical nutrient limitation.

This database of heterogeneous studies allowed us to achieve several goals which can only be done with a meta-analysis, and not through the individual studies previously performed on this topic. For the first time, we (i) directly test the relative effects of N, P, EFA and sterol limitation, and show that their effects are strikingly similar in magnitude, (ii) analyse the causes of heterogeneity across studies and (iii) analyse the uncertainty in our knowledge of each aspect of nutrient limitation or co-limitation.

METHODS

Data collection and screening

Studies were obtained from a Web of Science (www.webofscience.com) search (last accessed February 11, 2021) using the search term: *'((stoichiometry or C:N or C:P or " food quality" or "nutritional quality" or "nutritional geometry" or " fatty acid*" or "biochemical compos*" or "*sterol*") AND (phytoplankton) AND (zooplankton or herbivor* or consum* or grazer or " filterfeed*") AND (growth or product* or defense or grazing or feeding))'.* We also examined the references of previous reviews involving zooplankton food quality (Brett & Müller-Navarra, 1997; Frost et al., 2005; Gulati & DeMott, 1997; Hessen et al., 2013; Martin-Creuzburg & von Elert, 2009; Parrish, 2009; Sterner & Schulz, 1998; Twining et al., 2016; Winder et al., 2017) to amend the original search. This yielded a total of 1372 articles which we screened, resulting in a total of 122 articles which met all general criteria for inclusion, 112 of which could ultimately be included in the meta-analysis. For full documentation of the systematic literature review, see Appendix A/Figure S1 which presents a flow chart of the screening process following PRISMA (preferred reporting items for systematic reviews and meta-analyses)

guidelines (O'Dea et al., 2021) and Appendix D, which briefly describes all included studies. We included studies that met all the following core criteria: (1) they quantified variation in phytoplankton food quality and/or performed a nutrient supplementation experiment, (2) they quantified zooplankton growth, reproduction, or survival in response to phytoplankton food quality variation, (3) they controlled for confounding effects of food quantity by maintaining constant or saturating food concentrations across all food quality treatments within each study.

We also limited the scope of our synthesis to include only published studies with phytoplankton as a component of the food supply for zooplankton. In other words, we did not include studies or portions of studies where only heterotrophic bacteria, benthic algae, terrestrial organic matter, etc. were the sole food supply, but did include studies where the full seston community was used. We focused the scope to phytoplankton because in such experiments the food quality indicator is often well controlled and allows better comparison across treatments, whereas comparing broadly diverging food sources (e.g. detritus) would introduce substantial confounding effects. Similarly, we also did not include studies with benthic grazers and limited the scope to pelagic zooplankton. Lastly, we limited the scope of food quality parameters to include food N:C, P:C, essential fatty acid content and sterol content (see Table 1 below).

Throughout this paper, we report stoichiometric ratios in the form of molar 'nutrient:C' instead of 'C:nutrient' as this is more intuitive (higher nutrient:C ratios reflects higher food quality) and thereby comparable to effects of increasing FA and sterol concentrations per unit food carbon. N:C and P:C ratios were log-transformed for all analyses, as non-transformed ratios can lead to biased results (Isles, 2020); FA and sterol content were not log-transformed as values of zero were common. While definitions of essential fatty acids vary, we included fatty acids defined as essential by Kainz et al. (2004), as well as several ≥18-carbon fatty acids that can be converted to these EFAs (thus making them 'conditionally essential'), in order to broaden our data beyond only EPA and DHA. Although there is evidence that other nutrients like iron (Jeyasingh & Pulkkinen, 2019; Lind & Jeyasingh, 2018) and essential amino acids (Fink et al., 2011; Koch et al., 2013) influence zooplankton production, we did not include these as their effects were analysed in a different set of studies, which would have multiplied the efforts needed. Likewise, studies measuring only bulk protein, carbohydrates, or lipids were excluded as these bulk parameters do not allow to differentiate between the specific essential micronutrients that limit zooplankton.

In addition to the suite of food quality and zooplankton performance variables, we also collected information for an array of categorical and numerical variables to act as covariates, provide further context

for each study and explain heterogeneity across studies, to the extent that this information was available. We included the form of food quality manipulation (direct or indirect, see below), the strength of nutrient manipulation (difference in pre/post nutrient amendment concentrations), and the nutrient concentration of phytoplankton both pre- and post-manipulation. We considered taxonomy (from broad to species-level) of algae and zooplankton, and noted whether phytoplankton were isolates or natural seston communities. We further retrieved information on the general environment (freshwater vs. marine), food quantity (as mg C L^{-1}), environmental covariates (e.g. light, temperature, salinity) and experimental parameters (duration, volume, type of experimental units). However, the extent to which these variables were reported in original studies varied, and our analyses focus on those variables most often clearly described by study authors. Particulate Si:C ratios in diatoms were a covariate of interest, as diatom silicification is highly plastic and significantly modifies copepod-diatom interactions (Grønning & Kiørboe, 2020; Ryderheim et al., 2022); however, this information was notably not reported in studies in our dataset.

The manipulation type can be split into three broad categories: direct manipulations, indirect manipulations established via nutrient supply, and indirect manipulations established via other factors including species composition (Table 2). These three levels represent a hierarchy regarding the strength of inferences that can be made to causally and directly link specific nutrients to zooplankton performance. The distinction is particularly important for P manipulation due to known indirect effects of P limitation on phytoplankton food quality. Specifically, P manipulation studies were classified as 'direct' if they added a spike in dissolved P supply that was rapidly absorbed by P-limited algae in order to increase algal P:C directly prior to feeding to zooplankton (and change nothing else between treatment and control; see, for example, Plath and Boersma (2001) for a description of this method). Studies were classified as 'indirect (via nutrient supply)' if they manipulated nutrient supply over the scale of days to weeks in order to create high and low P:C treatments, as they represent a combination of direct P effects as well as the many indirect physiological effects which can manifest in P-limited algae. Note that fatty acids and sterols only fall within the classifications as 'direct' (e.g. direct addition via FA emulsions or liposomes containing FAs) or 'indirect (other)' (e.g. gradients in FA due to different species composition or environmental factors).

TABLE 1 Components of food quality investigated in this meta-analysis

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
ω3	Total omega-3 fatty acids
ω6	Total omega-6 fatty acids
PUFA	Total polyunsaturated fatty acids
EFA	Essential fatty acids (LIN, ALA, ARA, EPA, DHA)
LCEFA	Long-chain (≥20 C) essential fatty acids (sum of ARA, EPA, DHA)
FA	Fatty acids in general, including essential and non-essential fatty acids
Sterol	Total sterols

Note: Bold denotes essential fatty acids following Kainz et al. (2004), while GLA and SDA are considered important and conditionally essential dietary components. Units used are molar ratios (N:C, P:C) or mass ratios (μg mg C^{-1} for fatty acids and sterols).

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 (most commonly, species composition)	Müller-Navarra et al. (2000); Mandal et al. (2018)		×

When applicable, we also categorised algae as having important food quality characteristics beyond nutrient content when these were indicated by study authors. These morpho-physiological traits include digestion resistance due to gelatinous sheaths and/or thick cell walls, large size of individual cells, or filamentous colony formation, all of which are expected to impede the ability of zooplankton to incorporate algal nutrients. There is a distinction in predicted effects between constitutive (expressed in both high- and low-nutrient conditions) and induced (only expressed with limited nutrients) digestion resistance (DeMott & Van Donk, 2013). Therefore, we differentiated these and classified each case as either constitutive or induced digestion resistance based on descriptions by the authors. All data were extracted from either figures (using WebPlotDigitizer V4.5, Rohatgi, 2021), tables, text or original raw data provided by authors (when required data were not available in full texts).

Effect size calculations

We used two distinct effects sizes, with each allowing us to incorporate data from studies with different experimental designs. For the treatment versus control style studies, where effects of food quality within a study were explicitly separated from other confounding factors, we calculated Hedges' *g* (a bias-corrected measure of the standardised mean difference). For studies in which food quality was manipulated indirectly via changes in species composition or the environment (thus introducing potential confounding factors), we only calculated effect sizes as correlations between each food quality indicator and each metric of zooplankton response.

Specifically, Hedges' *g* was calculated using the function 'escalc' in the R package 'metafor' (Viechtbauer, 2010) using package version 3.0–2 and R version 4.0.3 (R Core Team, 2020). We calculated Hedges' *g* following Hedges (1983):

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

and

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

Here, μ_{replete} and μ_{limited} are the mean zooplankton responses under the most nutrient-replete and most nutrient-limited treatments in each case study, respectively, and SD_{pooled} is the pooled standard deviation (*SD*) with *n* as the number of replicates (i.e. experimental units per treatment) for each effect size

estimate. The term *J* corrects for bias in the estimate of *g* at small sample sizes; Γ denotes the gamma function and *m* denotes the degrees of freedom such that $m = n_{\text{replete}} + n_{\text{limited}} - 2$. The sampling variance v_i was estimated using the unbiased estimator given in Equation 9 of Hedges (1983), as this is preferable for minimising bias when small sample sizes are commonly used in studies (i.e. using the setting vtype = 'UB' in the metafor 'escalc' function (Viechtbauer, 2010)). Effect sizes were weighted by the inverse of the sampling variance (i.e. *weight* = $1/v_i$), such that studies with more precise estimates received greater weight. Hedges' *g* was preferred over log response ratios because negative or zero values of means were common for certain response variables, in which case log response ratios cannot be used.

For studies with a correlational/gradient design, we calculated effect sizes as correlation coefficients (Pearson's *r*) for all combinations of nutrient type and zooplankton response category. We then converted these values of *r* into Fisher's *Z*-transformed correlation coefficients (hereafter Z_r), a standard effect size used in meta-analysis to improve distributional properties relative to *r* (Koricheva et al., 2013), which allows calculation of symmetric confidence intervals for effect sizes. The *Z*-transformation (Z_r) and its associated sampling variance (v_{Z_r}) are as follows, where *N* is the number data points used per correlation:

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

Of the 122 studies included in our database, 45 could be placed into both broad design categories; we thus calculated both Hedges' *g* and Z_r for these. The pool of studies, therefore, contains all categories of direct and indirect manipulations for the correlative (Z_r) approach, while the Hedges' *g* approach includes only direct and nutrient supply-mediated indirect manipulations from which stronger inferences may be made (Table 2), allowing these two datasets to provide complementary information. Agreement between these two distinct effect size methods would add weight to the generality and rigour of the results (shown in Figure S5).

We reversed the sign of effect sizes for a subset of zooplankton responses for which a smaller value indicates greater performance, such that a reduction in, for example, age at maturity or mortality rate, would be coded as a positive effect size. As the estimation of the sampling variance (v_{Z_r}) requires 4 data points, only those datasets containing a gradient of ≥4 observations were included in the meta-analysis of *Z*-transformed correlation coefficients. For both *g* and Z_r, effect sizes of 0 indicate no effect of increased nutrient availability, while positive effect sizes indicate an increase in zooplankton performance with increasing nutrients, and negative effect sizes

indicate reduced performance with increasing nutrients. Since Hedges' g values depend on the mean effect relative to the standard deviation, values can be interpreted in units of standard deviations. For example, Cohen (1988) originally suggested that g values above 0.8 (i.e. an effect size with magnitude of 0.8 standard deviations) indicate strong effects, while lower values indicate weak to moderate effects. As many studies in our analysis are marked by large mean differences between treatments and low variances, values of g in this meta-analysis generally exceed the value of 0.8; thus, any Hedges' g values above 0.8 indicate increasingly strong treatment effects. Interpretation of the magnitude of Z_r, on the other hand, is more similar to interpretation of traditional Pearson correlations. The only difference is that while Pearson's r is bounded from −1 to 1, the Z transformation means that very strong correlations equate nonlinearly to higher absolute values of Z_r. Therefore, a Z_r value over 0.5 can be interpreted as a strong correlation between a food quality indicator and a zooplankton response in our meta-analysis.

Statistical models

We used both random and mixed effects models using the 'rma.mv' function in the 'metafor' R package in order to calculate weighted effect sizes, to assess broad differences among effects of each nutrient type, and to identify variables that significantly modify the effects of nutrient limitation. To test H1, that limitation by each broad nutrient type is significant, we ran a separate weighted random effects model for each combination of nutrient type (N:C, P:C, overall fatty acids as well as specific FAs, sterols) and broad zooplankton response type (somatic growth, reproduction, survival and population growth). For Hedges' g, we used article ID, zooplankton species, and phytoplankton species as random effects to account for non-independence across publications and taxonomy; we specified zooplankton and phytoplankton species as random effects nested within publication. For Z_r, only article ID was used as the random effect, as one effect size could represent gradients across species, and low degrees of freedom precluded the use of zooplankton species as a random effect.

For both effect size metrics used, we evaluated H1a based on whether the 95% confidence intervals overlap zero (indicating no overall effect), or do not overlap zero (indicating a significant effect). Similarly, nonoverlapping confidence intervals between different nutrient types would indicate differences in the magnitude of nutrient limitation effects (H1b). We tested H2 (that zooplankton experience co-limitation) in two ways: first, by comparing the mean and 95% CI of nutrient co-addition to individual nutrient addition (e.g. for studies where both nutrients were factorially manipulated). Second, we used mixed effects models (see below) to test if the concentration of other nutrients significantly influenced the effects of the focal nutrient that was manipulated (e.g. testing if algal P:C ratios influence the magnitude of FA supplementation effects). In this case, we used polynomial regression to account for nonlinearities in the relationship between P:C and FA effects.

To examine how other experimental and environmental covariates may alter effect sizes across different nutrients (H3), we used additive mixed effect models using the following moderators which are commonly reported for all types of nutrient manipulation: experimental duration and volume, food quantity, realm (marine vs. freshwater) and morphological dimensions of food quality. Mixed models using factors specific to each nutrient type were used to identify additional influential variables. For instance, with P:C manipulation studies, we also included the manipulation method (direct or indirect P manipulation) as a fixed effect. For all nutrients, a separate model tested whether the initial (presupplementation) nutrient levels, as well as the strength of manipulation, influenced effect sizes. Last, we used separate mixed effects models to test for variation among laboratory versus field phytoplankton communities, phytoplankton taxonomy and zooplankton taxonomy. In cases with low sample size for each factor, we could not use nested random effects as described above, but instead performed mixed effects models using default settings in the 'rma' function in the 'metafor' package. We assessed publication bias using the functions 'funnel,' 'ranktest' and 'fsn' in the 'metafor' package; we found mixed evidence for publication bias, with funnel plots and fail safe numbers reflecting little to no bias but Kendall tests indicating correlations between effect sizes and precision (Appendix C, Figure S8).

RESULTS

Individual effects of essential nutrient limitation

The results from both the direct and indirect nutrient manipulations in this meta-analysis indicate strong effects of limitation by all the nutrient types we examined. For somatic growth of zooplankton, effect sizes (as Hedges' g) of experimentally increasing N:C (g = 2.06, 95% CI: 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) and sterol content (g = 7.88, 95% CI: 1.38–14.4) were all positive and did not overlap 0, indicating significant effects of alleviating their limitation (Figure 1a, Table S1), and providing clear support for H1a. All broad nutrient types had significant effects on reproduction as well (Figure 1b, Table S1). In addition to the summarised effects of increasing fatty acid content, individual essential fatty acids often had significant positive effects for somatic growth and/or reproduction as
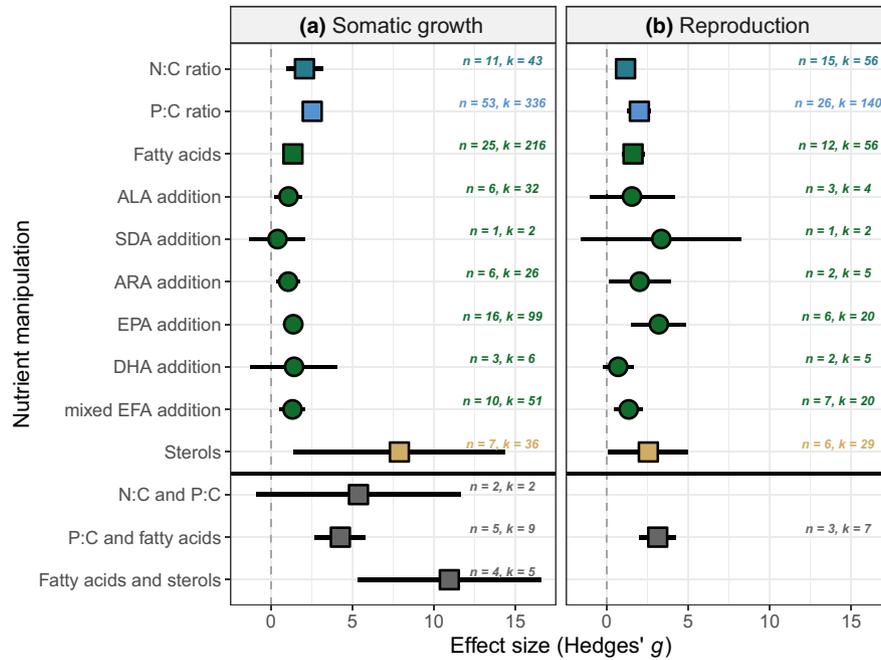
FIGURE 1 Effect sizes (mean and 95% CI) of nutrient manipulation on zooplankton growth and reproduction from a weighted random effects meta-analysis. Error bars that do not overlap zero indicate significant effects of increasing availability in zooplankton diets. Squares denote the overall effects of N:C, P:C, fatty acids and sterols, as well as their combined addition (below horizontal line); circles denote the effects of specific fatty acids. The sample size for each combination of nutrient type and response is shown as the number of unique publications (n) and the number of effect size estimates (k).
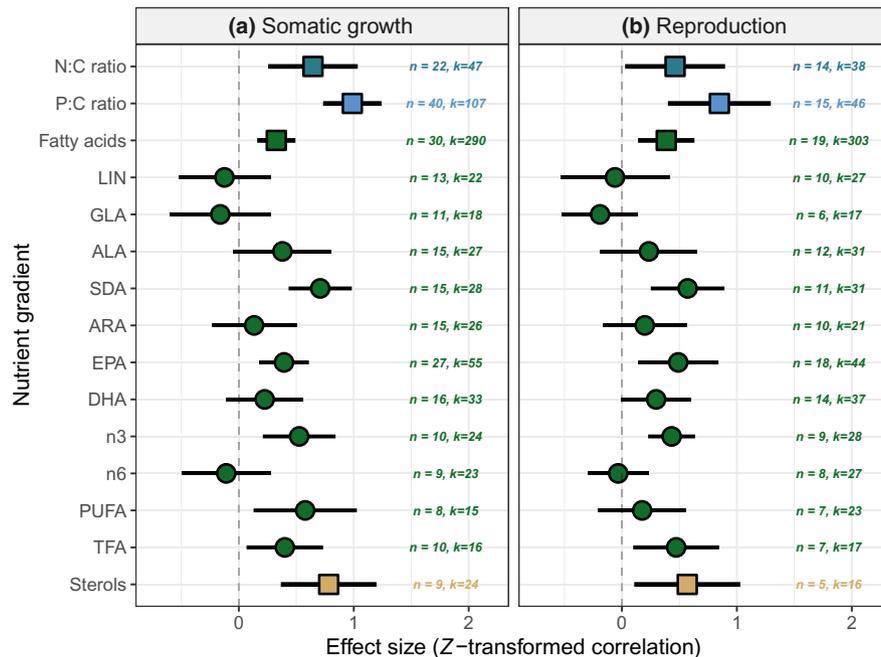
FIGURE 2 Effect of nutrient gradients (Z-transformed correlation coefficient, Z_r) for the subset of studies using indirect and/or gradient style manipulations to test effects of nutrient limitation. This dataset includes, for example, studies correlating zooplankton response by algal species, by different environmental conditions, or along nutrient manipulation gradients. Points show the mean and 95% CI of effect sizes for each nutrient type, squares denote summary effect sizes, circles denote individual FA correlations and the sample size for each combination of nutrient type and response is shown as the number of unique publications (n) and the number of effect size estimates (k).

well. Overall, similar results were obtained for population growth and survival, yet these responses had relatively limited underlying data compared to somatic growth

and reproduction (Figure S2). Furthermore, these strong effects of nutrient limitation were supported by the correlation results (Figure 2). Here, effect sizes (as Fisher's

Z-transformed correlation) for zooplankton performance in relation to N:C, P:C, fatty acid and sterol content were positive and did not overlap zero for any of the broad nutrient types (shown as squares in Figure 2), which provides additional support for H1a. The ω3 fatty acids (e.g. ALA, SDA, EPA, total ω3) tended to have strong positive correlations with growth and reproduction, often equal in magnitude to that of N:C and P:C, while correlations for ω6 fatty acids (e.g. LIN, GLA, total ω6) did not differ from zero, indicating no effect. Hedges' *g* and *Zr* were highly congruent with one another for studies where both could be estimated, providing further evidence that support for H1 is robust (Figure S5).

Though all nutrient types had significant effects, the magnitude differed depending on both the nutrient type and the zooplankton response category, supporting H1b (Figure 1, Figure 2, Table S1). For somatic growth as response, the grand mean effect size of P:C manipulation was higher than the grand mean for FA manipulation (Figure 1a). For reproduction as a response, however, effect sizes of P:C and FA manipulation were equivalent (i.e. overlapping CIs in Figure 1b). In other words, fatty acids appear to be relatively more important for zooplankton reproduction than for somatic growth. Additionally, there is substantial variation for individual fatty acids with different zooplankton responses. Supplementation of ALA, ARA, EPA and mixed EFAs all had similar effects on somatic growth, but these effects were less than those of P:C manipulations (Figure 1, Table S1). However, ARA and EPA supplementation had

effect sizes equivalent in magnitude to increasing P:C ratios for zooplankton reproduction.

Co-limitation by multiple nutrients

Data on co-addition of nutrients from factorially designed experiments were limited to 13 studies, but the results clearly indicate that co-limitation occurs, as the mean effect of alleviating limitation of multiple nutrients is substantially greater than for individual nutrients in all cases (Figure 1). Specifically, a simultaneous increase in P and FA availability yields a significantly larger effect on zooplankton growth than increasing either nutrient alone. Other co-additions also create larger mean effects than for individual additions, but these are marked by substantial variability and thus have overlapping confidence intervals.

Another indicator of co-limitation is the relationship between algal P:C ratio and the strength of other nutrient manipulations like fatty acids. The available data (*n* = 9 studies) show that P:C content significantly modifies the effects of FA addition (Q_M = 25.1, *p*<0.0001), with FA effect sizes generally being smaller with lower P:C ratios (Figure 3). FA addition, however, still had some positive effects even under extreme P-limitation, although effects are much stronger when P is replete. At very high P:C levels where excess P can be inhibitory (i.e. the so-called 'knife edge' effect), there is also evidence for reduced FA addition effects. Additionally, FA addition
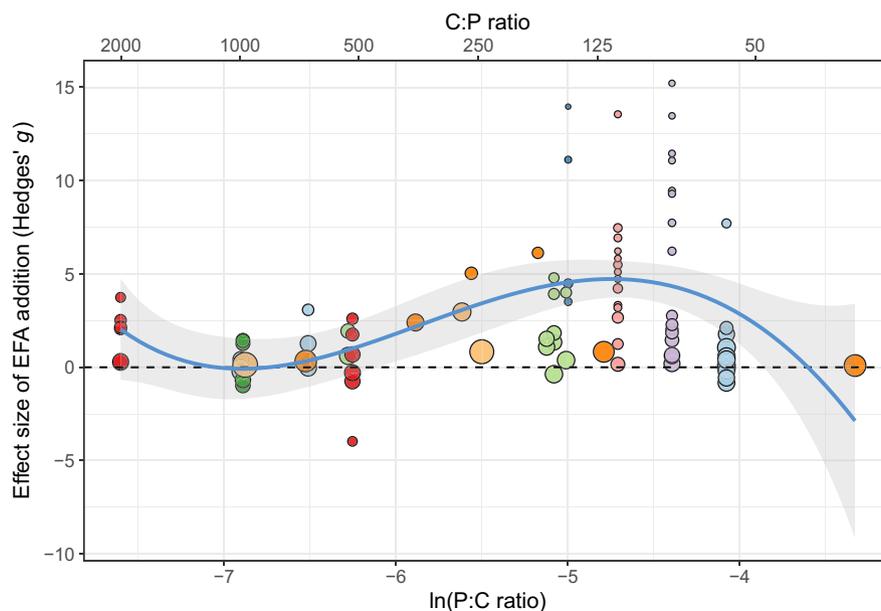

FIGURE 3 Relationship between variation in ln-transformed P:C ratio and the effect size of FA addition (also shown as untransformed C:P ratios for reference). Effect sizes of FA manipulation generally increase with greater phosphorus availability, as shown by the quartic polynomial fit curve, but may also decline with very high P:C. The size of each point indicates the reliability of each effect size (i.e. the weighting in the form of inverse variance), and colours indicate different studies.

effects marginally increased with increasing N:C ratios ($Q_M = 9.2$, $p = 0.03$, see Figure S6).

Effects of other factors on the strength of nutrient limitation

All combinations of added nutrients and response variables were characterised by significant heterogeneity in the responses. Part of this heterogeneity could be explained by moderators reflecting study experimental design and organisms used, providing broad support for H3. For example, the method of P manipulation had a very strong effect on both growth and reproduction responses (Figure 4). Studies implementing direct limitation of elemental P (i.e. the P-spiking method) had significantly lower effects on zooplankton growth ($g = 1.42$, 95% CI: 0.77–2.08) than those which also allowed for indirect effects, for example, via physiological changes over the course of algal culturing under different dissolved P supplies ($g = 3.20$, 95% CI: 2.60–3.79). Phytoplankton derived from laboratory isolates (either mono- or polycultures) had greater nutrient manipulation effect sizes than natural seston (Figure 5), indicating that there are systematic differences in how complex natural versus simple artificial communities serve as a food base for zooplankton.

Results from an additive mixed model reveal further key differences in effects depending upon experimental design and food quality factors beyond nutritional quality (Figure 6). Variation in the food quantity at which nutrients were manipulated had a significant influence on P:C, FA and sterol effect sizes. However, these effects were relatively small in magnitude and varied from negative to positive for different nutrient types. Differences in marine versus freshwater study systems were only detected for sterols. Additionally, the effects of manipulating P:C and FAs were significantly reduced when algae exhibited morphological traits like constitutive digestion resistance (Figure 6).

Beyond these factors, additional variability in food quality effects can be partially explained by the magnitude of nutrient manipulation, the degree to which nutrients were constrained pre-manipulation, and taxonomy of phytoplankton and grazers. For instance, P:C manipulation is significantly influenced by both the initial (pre-manipulation) P:C ratio, the magnitude of increase and the interaction between the two (Table S2, Figure S4). For other nutrients, only marginal effects of manipulation strength were detected.

Phytoplankton and grazer taxonomy also created significant variation in effects. For example, *Bosmina* (a low-P content cladoceran), as well as other cladocerans, showed weaker responses to P supplementation compared to *Daphnia*, while rotifers in the genus *Brachionus* had slightly greater average responses to P limitation than *Daphnia* (Figure 7). There was also evidence that within rotifers, different genera may be more or less prone to P limitation, for example, with *Keratella* showing a weaker response than *Brachionus* to P limitation. We found relatively limited experimental data for copepods, however, which limits the ability to compare the effects of different nutrient limitations.

Phytoplankton taxonomy explained further variation in effect sizes (Figure 8). N limitation had relatively similar effects on zooplankton regardless of the phytoplankton taxon. For P:C, FAs and sterols, however, the strength of nutrient limitation differed strongly depending on which phytoplankton was used. Adding sterols to cyanobacteria cultures in particular had much greater effects
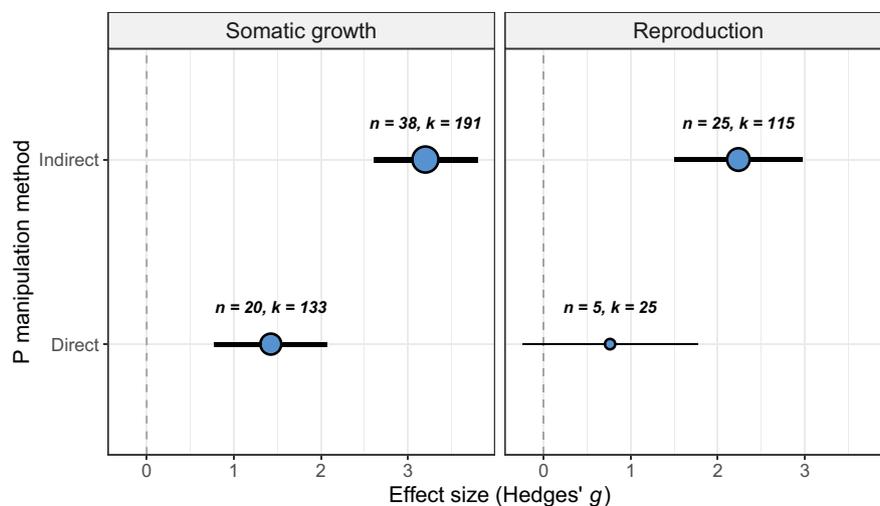

FIGURE 4 The method by which P is manipulated has strong effects on P-limitation effect sizes. 'Indirect' indicates that phytoplankton were exposed to differences in P supply during cultivation, which can induce indirect physiological effects of P-limitation (in addition to direct effects), whereas 'Direct' indicates a more strictly direct test of elemental P-limitation, as these indirect effects are controlled for by spiking P such that phytoplankton physiology does not change before they are consumed, and only P:C varies between treatment and control. Values of *n* and *k* represent the number of studies and the number of effect size estimates respectively.
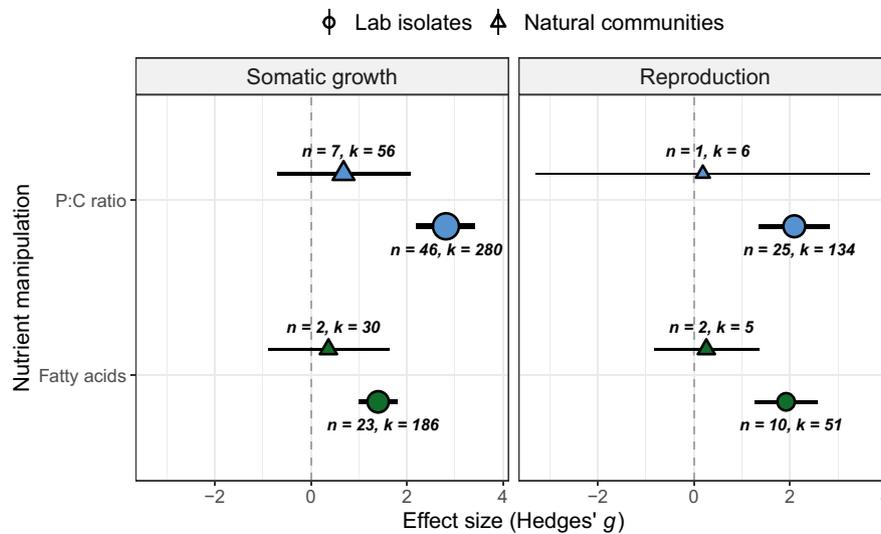
FIGURE 5 Effect sizes for P:C and essential fatty acid manipulation, separated by zooplankton response category and whether the phytoplankton used were from a natural community or from isolated laboratory strains. The size of points corresponds to the number of studies per effect size estimate.
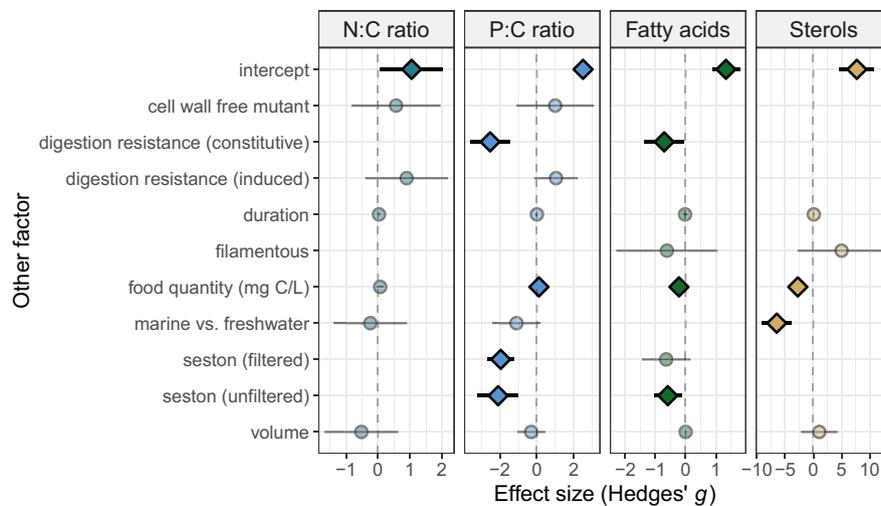

FIGURE 6 Effects of additional experimental and morphological factors on nutrient limitation effects. Significant effects are indicated by bolded diamonds. The mean and 95% CI represent the results of a mixed effects regression model.

when compared to diatoms or green algae. Zooplankton fed on cryptophytes also had reduced P and FA limitation effects compared to other groups. This clearly reaffirms that cyanobacteria are generally of poor quality while cryptophytes are of superior quality.

DISCUSSION

Individual effects of nutrient limitation

The results of this meta-analysis provide a first quantitative integration of food quality limitations experienced by zooplankton in terms of phytoplankton elemental and biochemical composition, as well as their interaction. Our synthesis confirms earlier findings and shows that N, P, fatty acid and sterol limitation generally impede zooplankton growth and reproduction, and may thereby significantly influence the efficiency of zooplankton production in nutrient-limited environments. We describe how these effects vary due to taxonomic differences in zooplankton requirements and phytoplankton nutrient contents. 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, certain fatty acids like EPA were shown to be relatively more important for reproduction than for somatic growth, which also
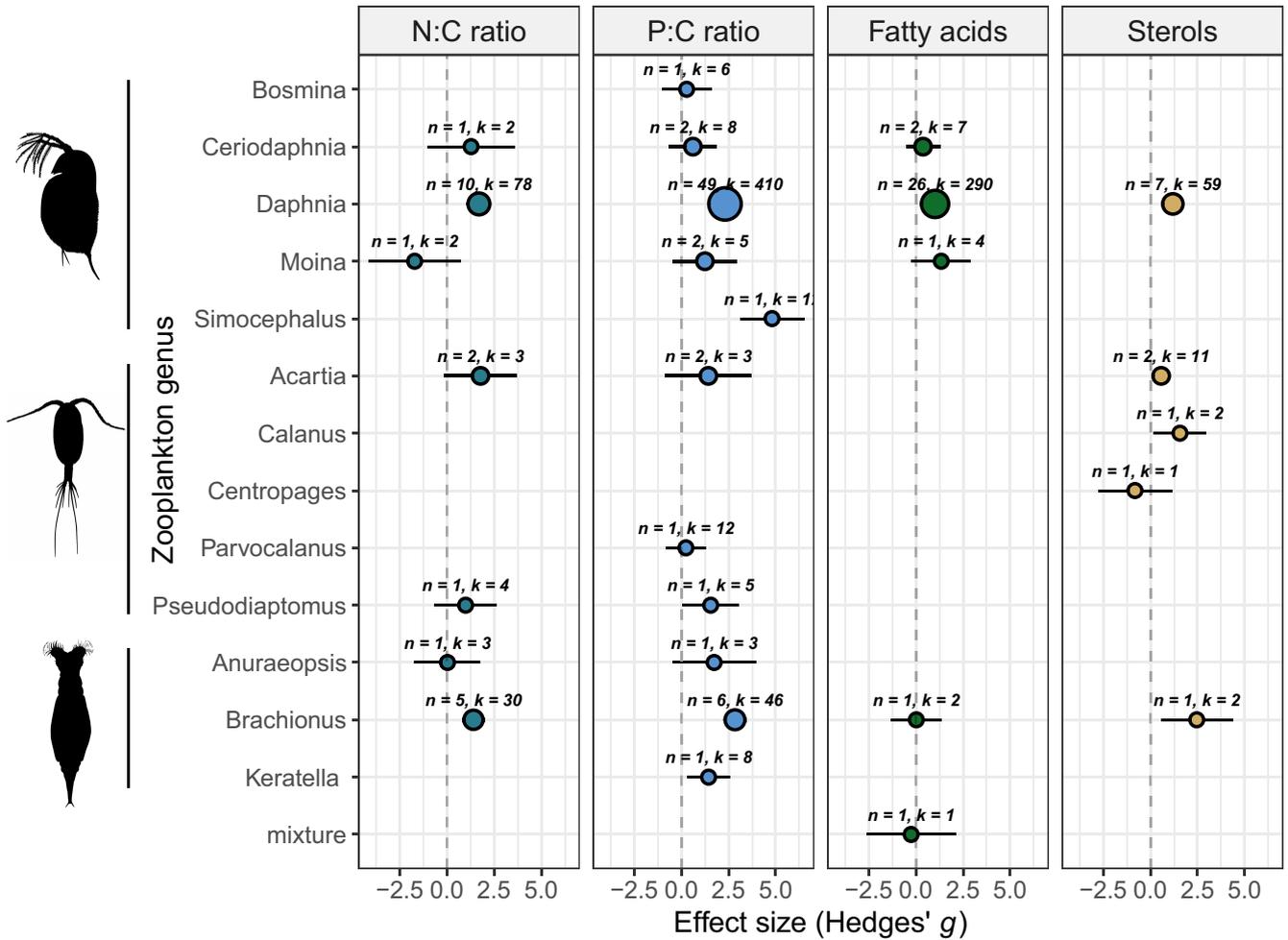
FIGURE 7 Effects of nutrient manipulation differ based on zooplankton genus and broad nutrient type (grouped here by broad zooplankton taxon). Points show the mean and 95% CI of effect sizes for each nutrient type, size of points corresponds to the number of articles (*n*); the number of articles and number of effect size estimates (k) also shown as text.
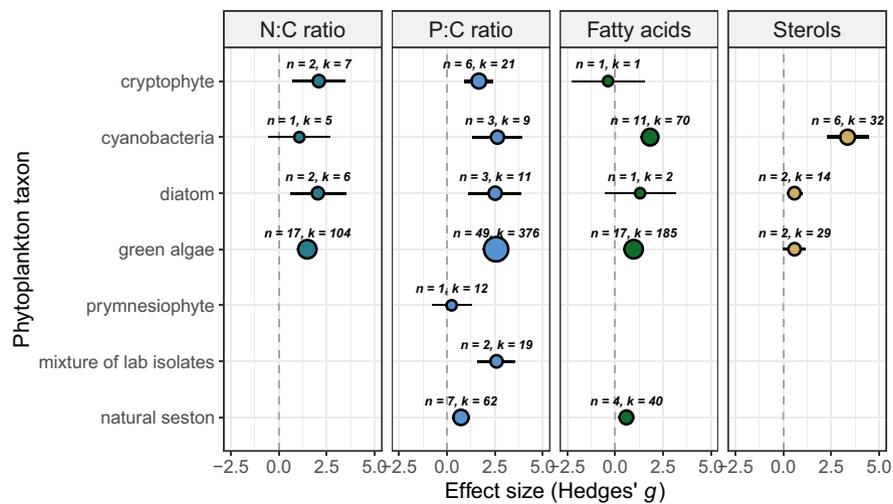

FIGURE 8 Effects of nutrient manipulation differ based on phytoplankton group and broad nutrient type. Points show the mean and 95% CI of effect sizes for each nutrient type, size of points corresponds to number of articles (*n*); number of articles and number of effect size estimates (k) also shown as text.

supports previous assertions that fatty acids are essential for hormone signalling involved in egg production in aquatic invertebrate consumers (Brett & 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 FAs 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 limitations by any individual nutrient can hinder multiple life stages. It is also worth noting that despite a much stronger emphasis in the literature on P limitation effects, we also found N limitation to be significant for zooplankton somatic growth. This suggests that limitation (or co-limitation) by nitrogen, not just phosphorus, is a strong stoichiometric constraint on the production of zooplankton that is facing low food N:C ratios, although this may become less frequent with anthropogenic increases of global N:P ratios (Penuelas et al., 2020; Peñuelas & Sardans, 2022).

Co-limitation and interactive effects of nutrients

Our synthesis provides further evidence regarding the extent of co-limitation and interdependence of various dimensions of food quality (H2). Direct tests of co-limitation in this meta-analysis consistently point towards additive effects, whereby adding multiple distinct nutrient types elicits a stronger response than individual manipulations. Combined increases in N:C and P:C, FAs and P:C, and FAs and sterols all had larger effects on zooplankton growth than individual nutrient manipulations, which contradicts the idea that any single nutrient tends to be the main limiting factor for zooplankton in co-limitation studies to date. This is analogous to widely observed co-limitation of nitrogen and phosphorus for primary producers (Elser et al., 2007; Harpole et al., 2011). The evidence for co-limitation in zooplankton thus far remains limited, however, and future research on the effects of nutrient co-addition are required to fill this knowledge gap. For example, in contrast to the experimental results using laboratory strains that were included in our analysis, eutrophic lakes that are dominated by cyanobacteria are more likely to face single nutrient limitation by sterols (Peltomaa et al., 2017). Only by performing co-limitation experiments using natural communities from lakes and marine systems will we be able to elucidate the existence of co-limitation among elements, FAs and sterols in nature.

The interaction between elements and lipids is also evident from the observation that responses to FA manipulations increased with higher P:C ratio, which quantitatively addresses the long-standing hypothesis that fatty acid limitation should be more important

when phosphorus is replete (Boersma, 2000; Sterner & Schulz, 1998; Sundbom & Vrede, 1997). These studies, which provide measurements of P:C along with FA manipulations, illustrate the interdependence of elemental and biochemical nutrients quite well. For example, the threshold for P-limitation in *Daphnia* has been estimated to be a molar C:P ratio of ~150–250 (Anderson & Hessen, 2005; Khattak et al., 2018); above this ratio, P-limitation should dominate, and below it biochemical nutrients or other limiting factors should become more important. This corresponds to the break point at which predominance of phosphorus limitation versus FA limitation appear to diverge (see Figure 3). Specifically, FA manipulation effects are both higher and more variable than those with P-limited food below this P availability threshold. Also noteworthy is that even under severe P-limitation with C:P ratios near 2000, one study still found positive effects of FA addition (Ferrão-Filho et al., 2003). This collectively points to the fact that measurements of multiple aspects of food quality, even when only one nutrient type is the core focus of a particular study, can yield important and novel insights into aquatic food web functioning.

Other factors influencing zooplankton nutritional constraints

Several key factors contributed to the variation in the strength of nutrient manipulations (supporting H3), and may reconcile contrasting views on the nature of zooplankton nutrition. The most striking cause of heterogeneity for P-manipulation studies, for example, was the method in which P was manipulated. The direct method using short-term P-spiking immediately before feeding and thus only manipulating the algal P:C ratio had significantly smaller effects compared to the indirect method of manipulating dissolved P in the medium over a longer incubation of days to weeks. The indirect approach includes both direct effects of low P content as well as the complex suite of physiological changes resulting from algal P-stress. Notably, these indirect effects are strong for rotifers (Rothhaupt, 1995; Zhou et al., 2018) as well as cladocerans, indicating that these effects are not constrained to a single taxon. These indirect effects include induced changes in cell size, cell wall thickness and digestibility (Lürling et al., 1997; Lürling & Van Donk, 1997; van Donk et al., 1997; van Donk & Hessen, 1993), but also changes in essential fatty acid content (Ahlgren et al., 1998; Bi & Sommer, 2020; Grzesiuk et al., 2018; Müller-Navarra, 1995; Ravet & Brett, 2006). Essential fatty acids are generally expected to decrease in concentrations with P limitation because lower availability of P should decrease the ability of phytoplankton to produce phospholipids that are composed largely of EFAs (Ahlgren et al., 1998). However, this hypothesised effect of P on EFA concentrations is not always observed

empirically. The extent to which fatty acids like EPA are influenced by P limitation varies across studies and can either increase, not change, or decline sharply, possibly depending upon phytoplankton taxa studied (Bi & Sommer, 2020; Müller-Navarra, 1995; Park et al., 2002). Therefore, we can only speculate as to the most important causes of these indirect effects. In sum, our results confirm that zooplankton can experience direct P limitation, and that indirect effects of P-limitation often occur, but that more work is needed to identify the mechanisms by which P-stress in natural systems induces these indirect effects on zooplankton production.

We also found large differences in the effects of nutrient manipulation for natural phytoplankton communities versus laboratory isolates. The main hypotheses regarding food quality of phytoplankton involve (a) mineral/biochemical content, (b) toxicity, (c) size/shaped-based grazing resistance and (d) other morphological defences/digestion resistance (Gulati & DeMott, 1997; Sterner & Schulz, 1998). As most studies filtered natural seston to include only edible particles (<35-μm fraction), and there were no differences between filtered and unfiltered seston (Figure 6), we can rule out size in this analysis. However, we cannot rule out any of the other three factors for explaining the large differences between laboratory and field algae. The relatively low effect sizes of nutrient addition for natural communities suggest three general possibilities: (1) the diverse natural communities were already of high quality and therefore did not increase much in quality with supplementation, (2) zooplankton were limited by algal defences (e.g. indigestibility, toxicity, morphology) and not nutrients and/or (3) zooplankton were limited by a mineral or biochemical nutrient not explicitly tested by the authors. Our findings suggest that natural communities may have been of poorer average quality within this dataset, as zooplankton had slightly lower (though still positive) growth rates on non-supplemented natural seston (mean = $0.22\,\mathrm{d}^{-1}$, 95% CI: 0.20–0.24) than on non-supplemented isolates (mean = $0.26\,\mathrm{d}^{-1}$, 95% CI: 0.22–0.30). Interestingly, the range in zooplankton somatic growth rates is smaller for natural communities than for isolates (Figure S7), indicating that these more diverse communities may buffer the most severe effects of nutritional deficiency observed in monocultures. This would support previous findings that phytoplankton biodiversity can decrease the variance in zooplankton production (Striebel et al., 2012), possibly increasing the stability of the autotroph-consumer link in food webs. However, such effects clearly depend on the extent to which the food quality traits that come with increasing algal diversity guarantee essential nutrient availability (Marzetz et al., 2017), and the ability of the grazer to select for high-quality food, either via particle selection or by selectively foraging on patches of high-quality seston (Schatz & McCauley, 2007). Another possibility is that heterotrophic microbes and terrestrial inputs act to buffer food quality in natural seston (i.e.

more than in laboratory conditions). For example, bacteria contain mineral nutrients and fungi contain EFAs/sterols that could mitigate impacts of low phytoplankton quality (Hessen & Andersen, 1990; Kagami et al., 2007; Taipale et al., 2016).

We included certain algal traits like digestion resistance as moderators, however these are only included to the extent that authors explicitly acknowledged such factors. The consequences of algal defences for the observed effects of food nutrition will depend on their response to changes in nutrient availability. For example, if algal defences increase with nutrient limitation, then the reported effects of nutrient content per se will be overestimated, as increased nutrient availability is correlated with reduced defences. This is in fact seen in the slightly positive influence of induced digestion resistance, compared to the strong negative influence of constitutive digestion resistance, on values of Hedges' *g* (Figure 6). The full extent to which such traits are linked to nutrient availability, and how they together affect zooplankton performance, remains to be elucidated (Meunier et al., 2017).

Nutrient limitation in natural systems

In our analysis, we only included studies which controlled for any confounding effects of the biomass quantity available to zooplankton, which selects for mostly laboratory experiments, and predominantly those using common study organisms like *Daphnia* and *Scenedesmus* or *Synechococcus* spp. (see sample sizes by taxa in Figures 7, 8). This largely reflects the biases in this field, where the focus on clear study systems has greatly supported our mechanistic insights into zooplankton nutrient limitation. Nevertheless, that we have 'a dearth of knowledge about other anomopods, cyclopoid and calanoid copepods, and rotifers' (Sterner & Schulz, 1998) relative to *Daphnia* is as true today as it was in the 1990s.

Some of the limitations posed by the controlled laboratory experiments included in this synthesis do not apply for field and mesocosm studies. Therefore, a complementary meta-analysis, using field and mesocosm studies that tested the effects of seston food quality (and quantity) on zooplankton production, could provide additional insights based on more field-realistic settings, if (somewhat) controlled for factors like species sorting and food quantity. The relative degree of nutrient limitation in nature is, of course, determined by the distribution of nutrient concentrations in aquatic systems, which varies considerably across sites (Brett et al., 2000; Elser et al., 2000). At large scales, concentrations of different nutrient types may be highly interactive, especially if eutrophication selects for phytoplankton communities of lower biochemical food quality (Müller-Navarra et al., 2004; Taipale et al., 2019), an effect which

can be modified by warming (Strandberg et al., 2022). The mechanistic results from our meta-analysis will therefore pair well with systematic assessments of seston elemental and biochemical nutrient concentrations (e.g. field monitoring of nutrients over time and across different lakes and marine systems). Specifically, our results illustrate the potential magnitude of nutrient limitation effects on zooplankton physiology with phytoplankton species composition ranging from monocultures to natural communities. The extent to which low quality phytoplankton taxa dominate natural systems, combined with the observed nutrient concentrations in a given system and the specific nutritional requirements of the prevalent zooplankton taxa, should provide clear predictions regarding the extent of nutrient limitation in nature (Filstrup et al., 2014; Peltomaa et al., 2017). Differences in the ability of cladocerans versus copepods to synthesise fatty acids and sterols, and different body P content among zooplankton, create a landscape of zooplankton nutritional requirement traits; thus dynamic changes in the composition of both phytoplankton and zooplankton may determine which nutrients are limiting at a given space and time. However, particularly the combination of field observations across sites with controlled manipulations using natural phytoplankton communities (e.g. Hartwich et al. (2012)), will allow making strong inferences regarding limitation of zooplankton production in nature given this spatial and temporal heterogeneity.

Our synthesis focuses on zooplankton as a key aquatic consumer group, but the findings have broader implications for consumer–plant interactions. In many ecosystems, herbivores are limited more by the quality than the quantity of their food, and the herbivore-autotroph link has broad implications for the trophic structure of ecosystems (Cebrian, 1999; Shurin et al., 2006). We now know that co-limitation by multiple nutrients tends to be the rule rather than the exception in different ecosystems (Elser et al., 2007; Fay et al., 2015; Harpole et al., 2011); however, a majority of studies do not reflect this and instead only assess individual nutrient limitation. Our synthesis provides yet more evidence that we must move beyond simple paradigms of single nutrient limitation if our knowledge of trophic ecology is to advance.

Knowledge gaps and future directions

We identify several recommendations for future research based on our systematic review and meta-analysis. As described above, the need is clear for more information on zooplankton nutrient co-limitation, simultaneous measurements of multiple food quality factors and how these signals of algal nutritional quality are inherently connected to other functional traits of both producers and consumers. In addition to this, we find our database

to be less data-rich for nutrient manipulation effects involving sterols, ω6-EFAs like ARA (Ilić et al., 2019), controlled manipulations of nutrients in natural phytoplankton communities, marine systems in general and interactions between algal defences (e.g. digestion resistance, silicification) and nutrient content. Therefore, we see clear gaps in our knowledge based on these lines of taxonomy, study system, level of biological complexity and nutrients examined, which would benefit from additional future research. Based on these limitations in the literature to date, we specifically see a critical need for the following types of studies:

1. Manipulative experiments that test the effects of multivariate nutrient limitation in zooplankton. These are clearly under-represented in the literature despite strong evidence that individual nutrient limitation does not adequately describe nature.
2. Manipulative experiments that test the extent of individual and multivariate nutrient limitation using natural phytoplankton and zooplankton communities, in both freshwater and marine habitats. Controlled natural community manipulations can overcome limitations posed by studying laboratory isolates by accounting for a multitude of species and their interactions.
3. Studies investigating the molecular basis of interactions between elemental and biochemical nutrients in phytoplankton and co-limitation in zooplankton. Studies with multivariate designs testing for separate and combined effects of elements and biochemicals, and including the underlying physiology and metabolic processes, will be needed for gaining a mechanistic understanding of planktonic food quality.

Conclusion

In conclusion, we show that phytoplankton N, P, fatty acid, and sterol content all significantly influence growth and reproduction of herbivorous zooplankton, and that evidence for co-limitation of these nutrients is strong. Our meta-analysis further integrates data from the literature thus far to synthesise knowledge of the interactive effects of these distinct food quality indicators. We also show that several additional factors beyond low concentrations of nutrients per se, including taxonomic differences and algal traits like digestion resistance, can explain substantial variation in reported effects of algal food quality on planktonic consumers, and therefore variation in the efficiency of aquatic food web functioning. Our synthesis thus provides an integrative overview of zooplankton nutrition and identifies key research gaps where future work will be particularly impactful for elucidating the drivers of variation in the herbivore-autotroph link in aquatic food webs.

AUTHOR CONTRIBUTIONS

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ele.14125.

OPEN RESEARCH BADGES

This article has earned Open Data and Open Materials badges. Data and materials are available at: https://doi.org/10.5061/dryad.z612jm6fq.

DATA AVAILABILITY STATEMENT

The primary data are available from original published studies; the dataset of extracted effect sizes and meta-data used for analyses is available on Dryad (https://doi.org/10.5061/dryad.z612jm6fq); annotated code used for analyses is available on Zenodo (https://doi.org/10.5281/zenodo.7113003).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.