OIKOS

*Research article*

# Sterol limitation of *Daphnia* on eukaryotic phytoplankton: a combined supplementation and compound-specific stable isotope labeling approach

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**Oikos**
**2024: e10359**
doi: 10.1111/oik.10359
Subject Editor: Steven Declerck
Editor-in-Chief: Dries Bonte
Accepted 11 January 2024

Essential biomolecules can critically influence the performance of consumers. A deficiency in dietary sterols has been shown to constrain the food quality of prokaryotic food sources for aquatic consumers. Here, we assessed the importance of dietary cholesterol for life history traits (survival, growth and egg production) of the freshwater herbivore *Daphnia magna* in supplementation experiments with various sterol-containing eukaryotic phytoplankton diets (dinoflagellates, diatoms, and golden algae). We combined cholesterol supplementation via liposomes with $^{13}$C-labelling of the phytoplankton diets and traced the origin of cholesterol in *Daphnia* using compound-specific stable isotope analysis. All phytoplankton strains used here were rich in polyunsaturated fatty acids (PUFA) but differed in their phytosterol composition. We show that growth and reproduction of *D. magna* can be limited by sterols even when feeding on sterol-containing eukaryotic phytoplankton diets. The impact of cholesterol supplementation on growth and reproduction of *D. magna* differed among phytoplankton diets (strains). The positive effect of cholesterol supplementation was most pronounced on diatom diets. Estimation of source proportions using stable isotopes revealed that *D. magna* preferentially assimilated the supplemented cholesterol rather than synthesizing it from dietary phytosterols. Our experiments suggest that the different responses to cholesterol supplementation on the various phytoplankton diets were unrelated to the suitability of dietary phytosterols to serve as cholesterol precursors but were caused by other biochemical or morphological food quality constraints. The combination of methods applied here could be very useful for uncovering nutritional constraints and thus for assessing the importance of essential biomolecules for the performance of herbivorous consumers.

Keywords: liposomes, nutritional ecology, stable isotopes, sterols, zooplankton

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## Introduction

Food quality crucially influences the performance of herbivorous consumers. Dietary supply of a suit of elemental and biochemical nutrients, such as phosphorus, polyunsaturated fatty acids (PUFA) and sterols affects growth and reproduction of herbivorous zooplankton (Brett 1993, Martin-Creuzburg and von Elert 2004, Ravet and Brett 2006, Martin-Creuzburg et al. 2011, Peltomaa et al. 2017). The amount and the composition of essential nutrients in different phytoplankton species can differ substantially, suggesting that feeding on different phytoplankton groups can pose different nutritional constraints on zooplankton. To explore the significance of dietary sterols and PUFA for the performance of herbivorous zooplankton, previous studies have supplemented cholesterol or single long-chain PUFA to the diet of *Daphnia* (Ravet et al. 2003, Sperfeld and Wacker 2011, Martin-Creuzburg et al. 2014). The poor nutritional quality of prokaryotic food sources (cyanobacteria, bacteria) for *Daphnia* has been attributed to the lack of sterols and long-chain PUFA in prokaryotes (Martin-Creuzburg et al. 2008, 2011, Taipale et al. 2012). There is also evidence that the various phytosterols found in eukaryotic phytoplankton affect the growth of *Daphnia* differently, i.e. they are used with different efficiency (Martin-Creuzburg and von Elert 2004, Martin-Creuzburg et al. 2014). Dietary threshold concentrations for sterol-limited somatic growth of *Daphnia magna* established by supplementing different phytosterols to a sterol-free diet were found to cover a wide range, i.e. from 3.5 to 34.3 μg mg $C^{-1}$ (Martin-Creuzburg et al. 2014). However, the efficiency with which *Daphnia* can utilize phytosterols, occurring naturally in their eukaryotic algal diet, has not been investigated yet.

Cholesterol is the most prominent sterol in animal tissues; it is an indispensable component of cell membranes, serves as a precursor for steroid hormones, and is required as signaling molecule during embryonic development (Harrison 1990, Grieneisen 1994). Since arthropods are incapable of synthesizing sterols de novo, they rely on dietary sterol uptake (Goad 1981). Eukaryotic microalgae contain a wide variety of phytosterols often differing only slightly in their chemical structure (Kritchevsky and Chen 2005, Taipale et al. 2016, Martin-Creuzburg and Merkel 2016). Generally, sterol composition of algae differs among chemotaxonomical groups, but unlike fatty acids and carotenoids, sterol composition also varies within phytoplankton groups (Martin-Creuzburg and Merkel 2016, Peltomaa et al. 2023). Most green algae species contain mainly Δ7-sterols (Taipale et al. 2016, Martin-Creuzburg and Merkel 2016, Peltomaa et al. 2023), which are thought to be used less efficiently by *Daphnia* than the Δ5-sterols commonly found in cryptomonads, diatoms and golden algae (Volkman 1986, Martin-Creuzburg et al. 2014, Peltomaa et al. 2023). Dinoflagellates, which are rich in the physiologically important long-chain PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), contain complex di- and trimethyl sterols (LeBlond and Chapman 2002, Peltomaa et al. 2023). In addition, dinoflagellates often contain cholesterol (Peltomaa et al. 2023), which is also the main body sterol in *Daphnia* and thus well suited to support growth and reproduction (Martin-Creuzburg et al. 2014). Previous studies have shown that diatoms can contain various sterols, of which chalinasterol, cholesterol, campesterol and brassicasterol are the major sterols (Dunstan et al. 2005, Rampen et al. 2010, Martin-Creuzburg and Merkel 2016, Peltomaa et al. 2023). Golden algae (e.g. chrysophytes and synyrophytes) have been reported to contain β-sitosterol, campesterol, stigmasterol, and trace amounts other Δ5-sterols (Taipale et al. 2016, Peltomaa et al. 2023).

Grazing resistance is another important factor influencing the food quality of phytoplankton (Lürling 2021). Phytoplankton have developed a wide variety of different strategies for defending themselves against grazing, which can be divided into three types of defenses: morphological (e.g. cell size and wall structure), physiological (e.g. feeding deterrents), and behavioral defenses (e.g. motility) (Pančić and Kiørboe 2018). These strategies may affect the ability of *Daphnia* to acquire energy or nutrients from defended phytoplankton, which may impair growth or reproduction or result in increased mortality, even though the nutrient content of the phytoplankton is adequate. A previous study using species from seven different phytoplankton groups explored the relative significance of sterols, EPA, and phosphorus in explaining differences in *Daphnia* growth (Peltomaa et al. 2017). Within-taxa differences in food quality have been attributed primarily to differences in edibility and sterol profiles among species since the explored species did not differ much in their fatty acid or carotenoid profiles (Peltomaa et al. 2023). This highlights the importance of considering multiple food quality constraints when exploring phytoplankton–zooplankton interactions.

Supplementation experiments can unequivocally reveal nutrient limitation and provide valuable insight regarding dietary nutrient requirements, but they do not allow researchers to specifically assess the physiological mechanisms underlying the observed limitation. In contrast, $^{13}C$-labeling experiments, in combination with compound-specific stable isotope analyses (CSIA), are an effective way to trace the transfer of individual biomolecules from diet to consumers (Gergs et al. 2015, Taipale et al. 2019, Twining et al. 2020). Tracing $^{13}C$ can also reveal the origin of incorporated carbon and thus can provide information about potential grazing resistance. A combination of nutrient supplementation with $^{13}C$ labeling and CSIA can not only be used to assess potential limitations by single biomolecules, but also to verify the uptake of these biomolecules from various sources. This can provide a deeper understanding of the metabolism of biomolecules and their influence on the growth and reproduction of consumers.

We conducted life table experiments using $^{13}C$-labeled phytoplankton diets from three different algal groups (dinoflagellates, diatoms and golden algae) supplemented with unlabeled cholesterol to explore the role of sterols for growth and reproduction of *D. magna* and to test whether cholesterol addition causes changes to phytosterol and fatty

acid assimilation. The selected phytoplankton strains all contain relatively high amounts of PUFAs, including EPA, but differ in their sterol profiles (Peltomaa et al. 2023), and potentially also in grazing resistance. Differences in sterol composition among and within the studied algal groups were expected to influence growth and reproduction of *Daphnia*.

## Material and methods

A complete description of liposome preparation, sterol and fatty acid analysis can be found in the Supporting information.

### Cultivation of phytoplankton

We performed life table experiments with three different algal strains from three different groups (dinoflagellates, diatoms and golden algae; nine strains in total; Table 1). These specific groups were selected because they differ greatly in phytosterols but included EPA or DHA (Taipale et al. 2013, 2016). However, the selected algal groups differ also in size, shape, and cell wall morphology, which may influence their digestibility.

For the experiments, the algae were grown separately in 2-3-l Erlenmeyer flasks, containing modified woods hole medium (MWC) (Guillard and Lorenzen 1972, Guillard 1975) supplemented with AF6 vitamins (Watanabe et al. 2000). The flasks were all exposed to the same growth conditions, i.e. 16:00/8:00 h light/dark cycle, illumination at 110 ± 2 µE $m^{-2}$ $s^{-1}$, and 18 ± 1°C. However, non-motile diatoms were kept under constant aeration to optimize their growth. Algal cells were harvested during the exponential growth phase of the cultures and used to prepare food suspensions for the experiments and samples for the lipid and stable isotope analyses. Cell sizes were measured from images taken with a Zeiss Axio Vert.A1 microscope (Carl Zeiss) equipped with a Leica MC170 HD digital camera using the Fiji ImageJ tool (Schindelin et al. 2012). Values are presented as the mean of length and width of eight individual cells (Table 1). The purity of the algal strains was verified before and after the experiments under the microscope. Classification is based on the the taxonomy and common names documented in Algaebase (www.algaebase.org) (Guiry and Guiry 2014).

The biochemical food quality of the algal strains was assessed in terms of their phytosterol and PUFA content. Selected algal strains contained different amount of α-linolenic acid (ALA; 18:3ω3), stearidonic acid (SDA; 18:4ω3), eicosapentaenoic acid (EPA; 20:5ω3), and docosahexaenoic acid (DHA; 22:6ω3) from the ω-3 family and linoleic acid (LIN; 18:2ω6) and arachidonic acid (ARA; 20:4ω6) from the ω-6 family. For the analysis of the biochemical composition, 300–600 ml of each algal suspension was centrifuged (300g, 20 min, 15°C), the media was discarded, and the pelleted cells were lyophilized, after which 10 ± 2 mg of each alga was weighed (CP2 P, Sartorius) in a tin capsule for further analysis.

### Liposome preparation

We used a similar preparation method as described in Martin-Creuzburg et al. (2014). In brief, liposomes were prepared from 5 mg $ml^{-1}$ stock suspensions of 1-palmitoy l-2-oleoyl-phosphatidylglycerol (POPG) and 1-palmitoy l-2-oleoyl-phosphatidylcholine (POPC) dissolved in ethanol. Unloaded control liposomes consisted of 3 mg of POPG and 7 mg of POPC, while the loaded liposomes additionally contained 3.33 mg of cholesterol. The cholesterol content of the loaded liposomes was determined via GC-FID (GC-2010 Plus, Shimadzu). A Phenomenex (Torrance, CA, USA)

Table 1. Summary of the selected phytoplankton (group, class, species) and the used abbreviations, strain codes and cell sizes for them (SCCAP = Scandinavian culture collection for Algae and Protozoa; NIVA = Norwegian Institute for Water Research; CCAC = Central Collection of Algal Cultures; CPCC = Canadian Phycological Culture Centre). Sterol index values were calculated using the equations presented by Peltomaa et al. (2017) and an index value below 1 suggests sterol limitation.

| Classification (group, class, species) | Abbreviation | Strain code | Cell size (µm, mean ± SD) | Sterol index |
|---|---|---|---|---|
| Dinoflagellates | | | | |
| Dinophyceae | | | | |
| *Peridinium cinctum* | PCI | SCCAP K-1721 | 35.7 ± 3.4 | 0.45 |
| *Peridinium bipes* | PB | CCAC 1426 B | 55.1 ± 2.9 | 0.35 |
| *Gymnodinium impatiens* | GYM | CCAC 0025 | 26.5 ± 2.8 | 0.57 |
| Diatoms | | | | |
| Mediophyceae | | | | |
| *Cyclotella* sp. | CYC | CCAC 3539 B | 5.8 ± 1.5 | 0.13 |
| Diatomphyceae | | | | |
| *Asterionella formosa* cf. | AF | CCAC 3890 B | 12.8 ± 0.7 | 0.09 |
| Fragilariaphyceae | | | | |
| *Tabellaria* sp. | TAB | CCAC 3717 | 6.9 ± 1.3 | 0.09 |
| Golden algae | | | | |
| Chrysophyceae | | | | |
| *Dinobryon* sp. | DIN | 21320 | 9.4 ± 1.5 | 0.26 |
| *Uroglena* sp. | URO | CPCC 276 | 10.4 ± 2.2 | 0.53 |
| Synurophyceae | | | | |
| *Synura* sp. | SYN | NIVA-5/09 | 9.1 ± 0.8 | 0.38 |

ZB-5HT Inferno (30 m × 0.25 mm × 0.25 µm) was used as a column, and helium was used as the carrier gas.

To ensure that the control group would get an equal number of liposomes as the cholesterol group, the density (liposomes $\mu l^{-1}$) and the size distribution of liposomes in the prepared suspensions was measured using an electronic cell counter (Casy, Omni Life Technologies) equipped with a 45 µm capillary (measurement range of 1.2–30 µm) on the first and the last day of the experiments. The size of the cholesterol and control liposomes (mean ± SD of pre-and post-experiment measurements) were 2.7 ± 0.4 and 2.6 ± 0.5 µm, respectively.

### Life table experiment

The experiments were performed using a clone of *D. magna* (DK-35-9, hereafter referred to as *Daphnia*). The animals were grown individually in 50 ml glass vials containing saturating amounts of *Acutodesmus* sp. at 20 ± 1°C. Both the *Daphnia* and the *Acutodesmus* sp. cultures were originally received from the laboratory of Prof. Dieter Ebert (Institute of Zoology, University of Basel, Switzerland).

To assess the importance of cholesterol for somatic growth and reproduction of *Daphnia*, a set of life table experiments was carried out. The neonates (born within 12 h) were divided individually into 50 ml clear glass vials containing Aachener Daphnien Medium (AdaM) (Kluttgen et al. 1994) to form a total of 18 treatment groups consisting of 24 replicates per group (one control and one test group for all nine different algae treatments). Due to high mortalities obtained in preliminary tests with *G. impatiens* and *P. bipes*, the number of replicates was 48 for *G. impatiens* and 36 (control), and 24 (cholesterol supplement) for *P. bipes*. To minimize maternal effects, juveniles of a specific adult *Daphnia* were equally divided between different diet treatments. Throughout the 18-day experiment, all groups were fed three times a week with a food concentration of 0.1 mg C $day^{-1}$ (2 mg C $l^{-1}$), which is in accordance with the food level suggested by OECD standard (2012) for reproduction tests.

The *Daphnia* of the test groups (denoted as 'Chol') were provided with cholesterol-containing liposomes with an amount equal to 8 µg cholesterol per mg of algal carbon (8 µg mg $C^{-1}$), which roughly equals the threshold for cholesterol-limited growth of *D. magna* (Martin-Creuzburg et al. 2014). The control group (denoted as 'Ctrl') individuals were supplemented with an equal amount of unloaded control liposomes (≈ $6.2 \times 10^8$ liposomes $day^{-1}$). During the experiment, the *Daphnia* were kept in the absence of light at a temperature of 17 ± 1°C.

The carbon content of algae was measured by optical density (IKA Turb 430 IR, Staufen im Breisgau, Germany). This was done by calculating a carbon content factor (CF) for each alga using parallel gravimetric carbon content measurement. CF values were calculated as a mean of two replicates by measuring and recording the optical density of the algae before filtering a volume of 100 ml of it onto a pre-weighed GF/A glass microfiber membrane filter (pore size 1.6 µm, Whatman). Filters were then dried at 105°C for 2 h and weighed again (± 0.001 mg). Dry weights were converted into carbon concentrations (mg C $l^{-1}$) with 50% carbon content estimation based on previous measurements (Taipale et al. 2015), and the final CF value was the carbon content divided by the optical density. The feeding volumes for the algae were calculated using Eq. 1:

$$\text{Feeding volume} = \frac{\text{Feeding amount}}{\text{OD} \times \text{CF}},$$

where the feeding volume is the amount of algae to feed in liters, feeding amount is the amount of algae fed in mg C, OD is the measured optical density of the algae as nephelometric turbidity unit (NTU), and CF is the carbon factor of the algae (mg C $l^{-1}$ $NTU^{-1}$). All the nephelometer measurements were performed in triplicates.

Somatic growth rates (g) were obtained from 6–16 individuals (~2/3 of the surviving population) removed on the 8th day of the experiment and confirmed under the microscope to bear no eggs. These Daphnids were lyophilized, weighed (± 0.001 mg), and stored at −80°C for further analysis. The final somatic growth rate was calculated by using Eq. 2:

$$g = \frac{\left(\ln DW_{t=8} - \ln DW_{t=0}\right)}{t}$$

where DW is the dry weight of the Daphnid biomass at the beginning (t = 0) and the end (t = 8) of the experiment. As the $DW_{t=0}$, we used a calculated mean mass value (n = 30) of 0.00481 mg for a ≈ 6-hour *Daphnia* juvenile, obtained from the same juvenile cohort that was used to start the experiments.

Reproductive capacity was assessed by counting the total number of viable offspring produced by the remaining *Daphnia*, and on the last day of the experiment, clutch sizes were measured under a microscope. Final reproductive outputs are represented as the mean of the sum of total offspring, eggs, and/or embryos produced by the surviving animals during the whole test period. Mortality was recorded as percentages of dead individuals overtime during the 8-day somatic growth experiment. *Daphnia* were considered dead based on the OECD test guideline No. 211 (OECD 2012). Due to high mortality with *G. impatiens*, all the surviving *Daphnia* were collected on the 8th day of the experiment, and consequently, their reproductive capacity was not assessed.

To be able to assess the origin of cholesterol in the body of the animals (i.e. dietary uptake from cholesterol-enriched liposomes versus biosynthesis from dietary phytosterol precursors) in the different diet treatments, all algal strains were enriched with $^{13}C$. The enrichment was achieved by adding 0.5 ml $l^{-1}$ of a $NaHCO_3$ solution (1.26 g $l^{-1}$), consisting of 10% $NaH^{13}CO_3$ (99%, Sigma-Aldrich), to the algal cultures three times a week. The labeling was initiated two weeks before the experiments.

Total fatty acids (TFA) and cholesterol levels were analyzed and quantified using 8-day-old *Daphnia*. To ensure an adequate amount of biomass for the analysis, at least two *Daphnia* individuals were pooled as one replicate.

### Fatty acid analysis

Lipids from the algae and *Daphnia* were extracted using the Folch method (1957) with the ratio of chloroform:methanol:water of 2:1:0.75. Lipids were split into sterols and fatty acids analysis by the ratio of 0.6 and 0.4, respectively. The transmethylation of fatty acids was done using mild acid conditions and using a 50°C water bath for 16 h (Schlechtriem et al. 2008). FAMEs were analyzed with a gas chromatograph (Shimadzu Ultra) equipped with a mass detector (GC-MS) and using a ZB-FAME column (60 m × 0.25 mm × 0.20 µm). FAMEs were identified by using specific ions and their retention times (RT) (Taipale et al. 2016). All the identified FAs from the phytoplankton and *Daphnia* are presented in the Supporting information. Additionally, the full FA profiles for the analyzed phytoplankton cultures are reported as major groups: saturated FAs (SAFA), $C_{16}$ and $C_{18}$ monounsaturated FAs (MUFA), branched-chain FAs (BCFA), and $C_{16}$, $C_{18}$, $C_{20}$ and $C_{22}$ PUFAs in the Supporting information.

### Quantitative and stable isotope analysis of sterols

The formation of trimethylsilyl (TMS) ethers of sterols was made by adding 70 µl of 1% BMSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) reagent (Sigma-Aldrich) to the samples followed by silylation at 70°C overnight. Quantitative and stable isotope analysis of sterols was done using a GC-MSD/IRMS system including Agilent 7890B GC, Agilent 5977B MSD, Elemental GC5 combustion interface, and isoprimePrecision, and using a HP-5MS UI column (30 m × 0.25 × 0.25 µm; Agilent) The $\delta^{13}C$ standard of F8.3 (Indiana University, Bloomington, IN, USA) was used for $\delta^{13}C$ correction of sterols. For the identification of TMS ethers of sterols, we used the prepared standard mixes, RTs, the NIST Mass Spectral Database 11 (http://chemdata.nist.gov), and literature (Rahier and Benveniste 1989, Jones et al. 1994, Goad and Akhisa 1997).

5-α-cholestane was used as the internal standard for all the sterol samples, and the recovery percentages of the phytoplankton samples were 83.8 ± 6.7 (n = 19) and 86 ± 18% (n = 66) for the *Daphnia*.

### Sterol index calculations

We calculated sterol indexes similarly to Peltomaa et al. (2017) based on the threshold values determined by Martin-Creuzburg et al. (2014) for different phytosterols to assess potential sterol limitation of *Daphnia* somatic growth:

$$\text{Sterol index} = \frac{\text{STEa}}{\text{THa}} + \frac{\text{STEb}}{\text{THa}} + \cdots \frac{\text{STEn}}{\text{THn}},$$

where STE is overall sterol content of each sterol (µg STE mg $C^{-1}$) and TH is threshold values. For phytosterols not included in the Martin-Creuzburg et al. (2014) study, we used the available threshold of the structurally most similar phytosterol (based on double bonds and carbon chain length, see supplementary document). A sterol index of < 1 was assumed to indicate a potential sterol limitation.

### Statistical testing and diet mix modeling

Permutational multivariate analysis of variance (PERMANOVA) with a Monte Carlo test was used to examine the significance of the impact of cholesterol on the somatic growth and reproductivity of *Daphnia* (Anderson 2001). PERMANOVA was also used to test the difference in cholesterol levels and PUFA composition between the control and test group Daphnids. All PERMANOVAs were run with an unrestricted permutation of raw data and type III sums of squares and with Euclidean distances of untransformed data using the PRIMER-E software (ver. 7) and PERMANOVA+ add-on. PERMANOVA produces the same test statistic (F-ratio) as standard ANOVA for a single variable and Euclidean distances (Anderson 2001) but has the added benefit of computing p-values via permutation rather than assuming a distribution for the test statistic. All the PERMANOVA results are presented in the Supporting information. The impact of cholesterol on the survival of *Daphnia*, in turn, was tested using R (www.r-project.org) utilizing a parametric survival model *survreg* from the R-package 'survival', similarly as done by Gergs et al. (2014). The confidence level for all the tests was 95%.

We used a mixing model (IsoError ver. 1.04; Phillips and Gregg 2001) to calculate source proportions for the cholesterol concentrations in the *Daphnia* tissues with 95% confidence intervals. IsoError is a two-point source carbon isotope mixing model, where the mean proportion of source A in the mixture is calculated as $(f_A) = \delta_M - \delta_B / \delta_A - \delta_B$, where $\delta_M$, $\delta_A$ and $\delta_B$ denote the mean isotopic signatures ($\delta^{13}C$) from the mixture (M) and sources A and B, respectively (Phillips and Gregg 2001). The error estimates were based on variance obtained in analyzed source and mixture samples, i.e. replicate measurements of control and cholesterol group *Daphnia* (n = 2–5). The $\delta^{13}C$-values of the cholesterol in the *Daphnia* tissues that were used in the calculations are presented in the Supporting information.

## Results

### Biochemical composition of the algal strains

A total of 39 different FAs (Supporting information) and 12 different phytosterols (Supporting information) were detected from the analyzed phytoplankton diets. The total content of PUFAs (Fig. 1a) varied among the selected phytoplankton groups and strains ($p < 0.05$, Supporting information). The total PUFA content among the phytoplankton diets

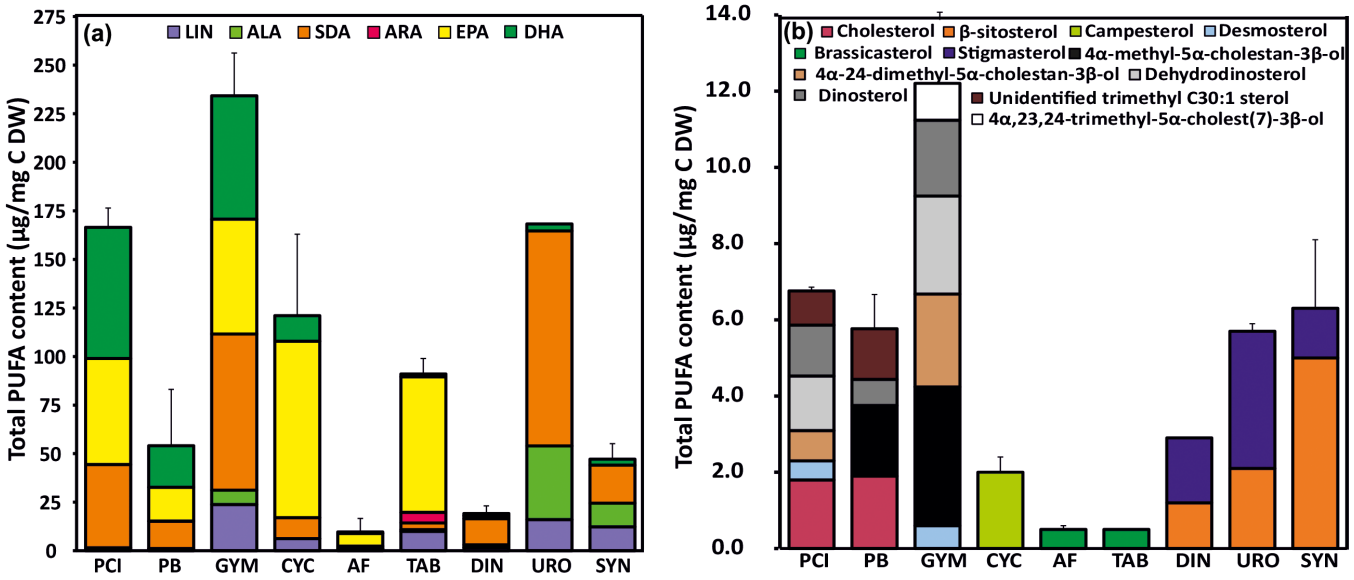


Figure 1. (a) PUFA and (b) phytosterol contents of the used algal diets expressed as µg of PUFA/phytosterol per mg carbon (C) dry weight using a 50% carbon content assumption. Individual biochemical values represent calculated means of duplicate samples, and the error bars represent the obtained standard deviations of total PUFA/phytosterol content. The total contents in phytoplankton strains labeled with same letters are not significantly different (PERMANOVA, $p > 0.05$). Sterol concentrations < 0.5 µg $mg^{-1}$ $C^{-1}$ and PUFA concentrations < 1 µg $mg^{-1}$ $C^{-1}$ are not shown in the charts. Note that the scaling of the x-axis on Fig. a–b are not the same (LIN = linoleic acid; ALA = α-linoleic acid; SDA = stearidonic acid; ARA = arachidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid). The algal strains used here were obtained from different culture collections and full names for abbreviations can be found in Table 1.

ranged from 9.6 to 234 µg $mg^{-1}$ $C^{-1}$, being highest in dinoflagellates and lowest in diatoms and some species of golden algae (Fig. 1a). The lowest amounts of total PUFA were measured from one species of diatom (*A. formosa*) and two species of golden algae (*Dinobryon* sp. and *Synura* sp.). Generally, dinoflagellates were the richest in DHA ($p < 0.05$, Supporting information) but also contained high amounts of SDA and EPA, and moderate amounts of LIN and ALA, but no ARA (Supporting information). Diatoms, in turn, were the richest in EPA and contained moderate to low amounts of LIN, ALA, SDA, ARA and DHA, whereas golden algal diets were the richest in LIN, ALA and SDA but had only trace amounts of EPA and DHA compared to other algal groups and contained no ARA ($p < 0.05$, Supporting information).

Total sterol contents (Fig. 1b) varied across the phytoplankton strains, ranging from 0.5 to 12.2 µg STE mg $C^{-1}$ ($p = 0.001$, Supporting information). Generally, dinoflagellates and golden algal strains had a greater diversity of sterols than the diatom strains ($p < 0.05$, Supporting information), which contained the lowest number of sterols. Among the phytoplankton strains analyzed here, dinoflagellates were characterized by the most diverse sterol profiles. The sterol profiles of dinoflagellates were represented by various methyl-, di- and trimethyl sterols as well as cholesterol and dihydrodino- and dinosterol, which were not found in any of the other phytoplankton strains (Fig. 1a, Supporting information). According to post hoc comparisons, *G. impatiens* has a higher number of sterols compared to other dinoflagellate strains ($p > 0.05$, Supporting information). The total sterol content of diatoms did not differ among species ($p > 0.05$, Supporting information), but they differed in sterol composition. Diatom strains contained mainly either brassicasterol or campesterol, which were not present in any of the other phytoplankton strains, and trace amounts of stigmasterol (Fig. 1b). The primary sterols in all golden algae species were β-sitosterol and stigmasterol, and amongst them, *Uroglena* sp., contained a greater diversity of sterols than *Dinobryon* sp. ($p < 0.05$, Supporting information). Due to a high standard deviation, the total sterol content of *Synura* sp. did not differ significantly from the total sterol content of *Dinobryon*, although the mean total sterol content of *Synura* sp. was even higher than the total sterol content of *Uroglena* sp. (Fig. 1b). The sterol index was < 1 in all cultured strains, suggesting sterol limitation on all strains. The sterol index was lowest for the three diatom strains (~ 0.09, Table 1) and highest for the golden alga *Uroglena* sp. (sterol index = 0.53).

## Mortality

Mortality of *D. magna* ranged from 0 to 83.3% across all diet treatments (Fig. 2, Supporting information), being generally higher on dinoflagellate diets than on diatom or golden algal diets (*survreg* model, $p < 0.05$). Mortality on *G. impatiens* (83.3%) was higher than on any other diet (*survreg* model, $p < 0.001$), and cholesterol supplementation of *G. impatiens* did not reduce the mortality (87.5%). Mortality did not differ among diatom and golden algal diets. Cholesterol supplementation had no impact on the survival of *Daphnia* with any of the other diet treatments (*survreg* model, $p > 0.05$).

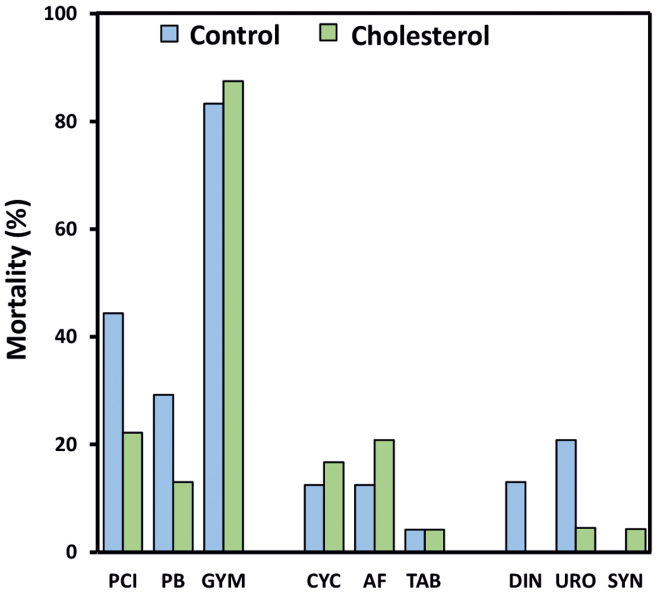


Figure 2. The obtained mortalities of *D. magna* fed with different diet treatments. Mortalities are the percentage of *Daphnia* from the total population that were recorded death during the 8-day somatic growth experiment (n = 24, except for PCI Control n = 36, and for GYM n = 48). Ctrl = non-supplemented control groups and Chol = cholesterol supplemented groups.

### Somatic growth

Somatic growth rates of *Daphnia* differed both among and within algal groups (Fig. 3, Supporting information). In general, somatic growth rates of *Daphnia* were highest with *Synura* sp. and lowest with *G. impatiens* among all unsupplemented diets ($p < 0.05$, Fig. 3, Supporting information). Moreover, *P. bipes* and *Uroglena* sp. supported lower somatic growth than the rest of the algae. Cholesterol supplementation significantly improved somatic growth rates on *P. bipes* (+ 61 ± 43%), *Tabellaria* sp. (+ 54 ± 12%), and *Dinobryon* sp. (+ 22 ± 23%), with respective p-values of 0.004, 0.001 and 0.043 (Fig. 3b, Supporting information). In the other treatments, the responses to cholesterol supplementation ranged from −24 to + 10% compared to the respective non-supplemented control but remained non-significant ($p > 0.05$, Supporting information).

### Reproductive output

The reproductive output of *Daphnia* on unsupplemented diets (Fig. 3c) ranged from 0 to 34 offspring per individual and thus strongly differed among the diets ($p = 0.001$, Supporting information). Generally, diatoms and *Dinobryon* sp. supported higher reproduction than dinoflagellates and the two other golden algal strains ($p < 0.05$, Supporting information). The reproductive output of *Daphnia* was highest on *Dinobryon* sp. and *Cyclotella* sp. and lowest on *Uroglena* sp., *Synura* sp., *P. cinctum* and *P. bipes* (Fig. 3c). Among the diatom diets, the reproductive output of *Daphnia* was similar ($p > 0.05$, Supporting information), but the obtained standard deviation was quite high (Fig. 3c). Cholesterol supplementation (Fig. 3d) increased the reproductive output of *Daphnia* on *A. formosa* (57 ± 40%), *Tabellaria* sp. (71 ± 12%) and *Synura* sp. (98 ± 3%) ($p < 0.05$, Supporting information).

### Impact of cholesterol supplementation on cholesterol metabolism in *Daphnia*

$^{13}$C-labeling of the algal diets resulted in $^{13}$C-labeling of cholesterol in all treatments (Supporting information). Measured cholesterol content in *Daphnia* ranged from 6 to 13.9 μg $mg^{-1}$ $C^{-1}$ among non-supplemented controls (Fig. 4a, Supporting information) and varied between the groups and strains with respective p-values of 0.005 and 0.001 (Supporting information). The cholesterol content of the non-supplemented *Daphnia* was generally highest on dinoflagellate diets (12.9 ± 0.8 μg $mg^{-1}$ $C^{-1}$, Fig. 4a). These individuals contained more cholesterol compared to individuals on diatom (9.3 ± 2.4 μg $mg^{-1}$ $C^{-1}$) or golden algal (10.4 ± 1.3 μg $mg^{-1}$ $C^{-1}$) diets ($p < 0.05$, Supporting information). *Daphnia* feeding on *Tabellaria* sp. contained the lowest amount of cholesterol ($p < 0.05$, Supporting information) followed by *Dinobryon* sp., while the remaining phytoplankton diets resulted in similar higher cholesterol contents in *Daphnia* (excluding *Cyclotella* sp., $p > 0.05$, Supporting information).

The cholesterol content of *Daphnia* ranged from 8.5 to 15.8 μg $mg^{-1}$ $C^{-1}$ among cholesterol-supplemented groups (Fig. 4b) and increased with cholesterol supplementation only on the *Tabellaria* sp. diet (73 ± 15%; $p = 0.001$, Supporting information). In other supplemented treatments, the cholesterol contents in *Daphnia* ranged from being 9% lower to being 23% higher compared to non-supplemented controls. Due to the additional cholesterol from liposome supplementation, the cholesterol content of the *Daphnia* fed *Tabellaria* sp. was similar to that of *Daphnia* feeding on *G. impatiens*, *Dinobryon* sp. and other diatom diets (Fig. 4b). Based on two-source mixing model calculations, the supplemented cholesterol reduced the utilization of algal phytosterols by on average 69 ± 13% among all diets (Fig. 4, Supporting information). The conversion of phytosterols into cholesterol among cholesterol-supplemented treatments was highest on golden algae (39 ± 6%) and lowest on diatom (19 ± 11%) diets (Supporting information). In turn, the relative amount of liposome-derived cholesterol uptake was lowest on *Synura* sp. (54 ± 1%) and highest on *Tabellaria* sp. (95 ± 0%).

### Impact of cholesterol supplementation on PUFA metabolism of *Daphnia*

The total PUFA concentrations of *Daphnia* in non-supplemented controls (Fig. 4c) fed with different phytoplankton diets ranged from 10 to 165 μg $mg^{-1}$ $C^{-1}$ of *Daphnia* dry bodyweight and varied between groups and species ($p = 0.001$, Supporting information). The PUFA contents of the *Daphnia* were highest in golden algal treatments and

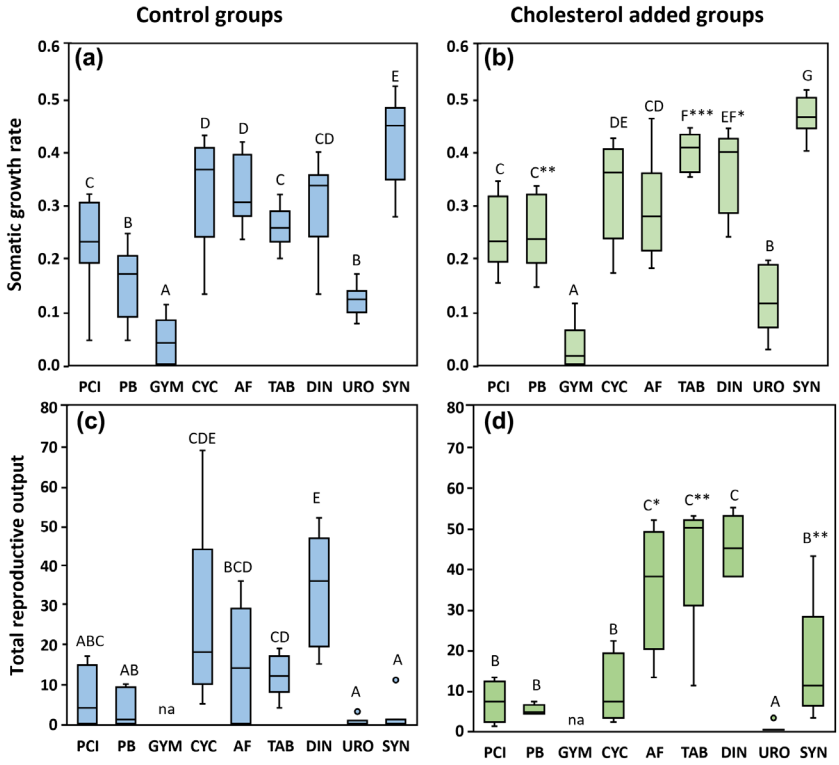


Figure 3. The obtained somatic growth rates ($day^{-1}$) and total reproductive outputs of *D. magna* with all the diet treatments. The center line denotes the 50th percentile, the box contains the 25–75th percentile of the dataset, whiskers mark the 5th and 95th percentiles, and possible outliers are defined as values beyond these outer boundaries and are denoted with dots. The total contents of the data labeled with the same letter are not statistically different (PERMANOVA, $p > 0.05$) and the asterisks indicate the level of significance of cholesterol treatment individuals compared to their respective control treatments. All the statistics are based on PERMANOVAs operated on Euclidean distances and uses the Monte Carlo p-values (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). The n values for different treatments ranged from 6 to 16 in (a)–(b) and from 4 to 8 in (c)–(d) (NA = not assessed). To see a side-to-side comparison of the non-supplemented and supplemented treatments, check the Supporting information.

lowest in dinoflagellate treatments (Fig. 4c). *Daphnia* fed dinoflagellates contained less PUFAs compared to *Daphnia* feeding on diatoms or golden algae, whereas *Daphnia* fed diatoms contained the most EPA, and *Daphnia* fed golden algae contained the most SDA compared to other groups ($p < 0.05$, Supporting information). The *Daphnia* in *P. bipes* and *G. impatiens* treatments contained the lowest amount of PUFAs ($p < 0.05$, Supporting information), whereas *Synura* sp. diet resulted in highest PUFA contents in the *Daphnia* tissues, which was statistically higher compared to all other treatments except *Cyclotella* sp. ($p < 0.05$, Supporting information).

Overall, the impact of cholesterol supplementation on FA metabolization differed among phytoplankton diets (Fig. 4d) causing significant changes to the total PUFA contents in *Daphnia* tissues with four phytoplankton diets: *P. cinctum* (-35 ± 10%), *Cyclotella* sp. (−25 ± 11%), *A. formosa* (−76 ± 22%), and *Tabellaria* sp. (+27 ± 13%) ($p < 0.05$, Supporting information). Most prominently, cholesterol supplementation impacted the PUFA content of *Daphnia* on *Cyclotella* sp. ($p = 0.005$, Supporting information), as the supplementation led to lower amounts of LIN, SDA and EPA ($p < 0.05$, Supporting information). With *P. cinctum*, the differences in total PUFA contents in *Daphnia* tissues between the non-supplemented and supplemented individuals were due to lower amounts of SDA and EPA, while the cholesterol-supplemented *A. formosa* individuals contained less LIN, SDA, ARA and EPA compared to the non-supplemented individuals ($p < 0.05$, Supporting information). The higher PUFA content of cholesterol-supplemented *Daphnia* feeding on *Tabellaria* sp. compared to non-supplemented individuals due to higher amounts of SDA and EPA ($p < 0.05$, Supporting information), which made the *Tabellaria* sp. diet supplemented with cholesterol as good of a source of PUFAs as the cholesterol-supplemented *Synura* sp. diet (Fig. 4d).

## Discussion

For the first time, we were able to track the origin of cholesterol in *Daphnia* using $^{13}C$ labeling of phytosterols and compound-specific stable isotope analyses. Our data show the conversion of various phytosterols to cholesterol, but also that *Daphnia* preferentially takes up supplemented cholesterol rather than converting cholesterol from dietary phytosterols. We also provide evidence that cholesterol

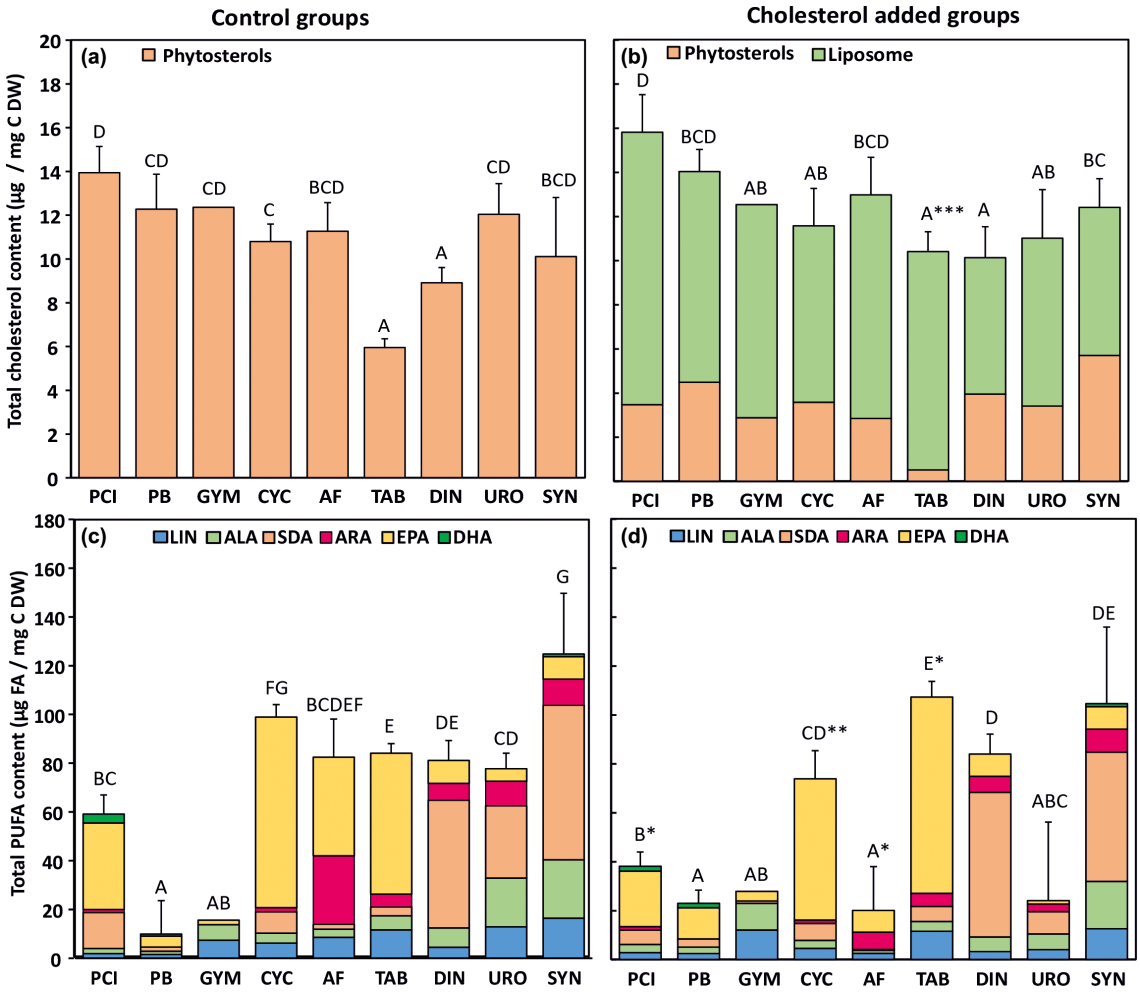


Figure 4. The obtained body cholesterol contents (µg $mg^{-1}$ $C^{-1}$ of *Daphnia* dry bodyweight) of the 8-day *Daphnia* fed with different phytoplankton diets in (a) non-supplemented control treatments and (b) cholesterol supplemented treatments, and the proportions of cholesterol derived from different sources based on diet mix modeling (Phytosterols = fraction of cholesterol synthesized from the assimilated phytosterols; Liposome = fraction of cholesterol assimilated from the liposomes). (c–d) shows the body content (µg $mg^{-1}$ $C^{-1}$) of selected PUFAs (LIN = linoleic acid; ALA = α-linoleic acid; SDA = stearidonic acid; ARA = arachidonic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid) assimilated from the different phytoplankton diets into 8-day *Daphnia* tissues in control and cholesterol supplemented treatments, respectively. Total contents are the mean of replicate samples (n = 3–5; except for GYM groups n = 1) and each replicate contains 2–7 individuals as pooled. The total contents of the data labeled with the same letter are not statistically different (PERMANOVA, $p > 0.05$) and the asterisks indicate the level of significance of cholesterol treatment individuals compared to their respective control treatments. All the statistics are based on PERMANOVAs operated on Euclidean distances and uses the Monte Carlo p-values (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). Error bars represent the obtained standard deviation of total cholesterol/PUFA content of *D. magna*.

supplementation can impact the FA metabolism of *Daphnia*, as evidenced by altered PUFA content. Previous studies with sterol-deficient cyanobacteria have shown the systematic positive impact of cholesterol supplementation on the life history traits of *Daphnia* (Martin-Creuzburg et al. 2008). All phytoplankton strains studied here contained sterols, albeit in relatively small amounts (sterol index < 1), suggesting potential sterol limitation of consumer growth. Our findings revealed that the effect of cholesterol supplementation on the growth and reproduction of *Daphnia* differs among algal strains. Cholesterol supplementation had no effect on the survival of *Daphnia* on any of the algal strains tested here, indicating that the total sterol content of all diets was sufficient for survival. This is in contrast to growth experiments with cyanobacteria, showing that the high mortality of *Daphnia* on cyanobacterial diets can be reduced by cholesterol supplementation (Martin-Creuzburg et al. 2005). Cholesterol supplementation improved the somatic growth of *Daphnia* on one algal strain from each studied algal group (diatom, dinoflagellate, golden algae) and reproduction on two diatom strains and one golden algal strain. Our results suggest that even small changes in sterol or PUFA content in eucaryotic algae may change the limiting biomolecule for growth or reproduction. It should be noted that, based on the

data we have collected, we cannot clearly evaluate all possible factors potentially influencing the growth and reproduction of *Daphnia* on the algal strains tested here. We did not consider the impact of harmful secondary metabolites or other nutritional constraints caused by differences in, for instance, amino acids (Wilson 2003) or elemental nutrients, such as phosphorus or iron (Urabe et al. 1997, Jeyasingh and Pulkkinen 2019), among the phytoplankton diets.

The studied algal strains contained sterols with double bonds at positions $\Delta^5$, $\Delta^{5,22}$ or $\Delta^{5,24(25)}$. The exception was *G. impatiens*, which contained only di- and trimethyl sterols with one or two double bonds. Since $\Delta^5$, $\Delta^{5,22}$ or $\Delta^{5,24(25)}$ sterols have been previously shown to support *Daphnia* growth when supplemented with a sterol-free diet (Martin-Creuzburg et al. 2014), one could assume that all algae, excluding *G. impatiens*, should have been a good source of sterols. $^{13}$C-labeling in combination with CSIA revealed that *Daphnia* was able to synthesize cholesterol from various dietary phytosterols. Generally, the presence of cholesterol in dinoflagellates plays a crucial role in their appropriateness as a sterol source for *Daphnia*. Nevertheless, cholesterol of *Daphnia* became $^{13}$C-enriched when feeding on *G. impatiens* containing exclusively only di- and trimethyl sterols. With our approach, we were able to show that *Daphnia* can convert di- and trimethyl sterols to cholesterol. Two pennate diatoms (*Tabellaria* sp. and *Asterionella* sp.) contained only brassicasterol at detectable concentration, whereas the centric diatom (*Cyclotella* sp.) contained only campesterol. *Daphnia* was able to efficiently use both brassicasterol and campesterol (methyl Δ5-sterol) as cholesterol precursors. Brassicasterol is also common in cryptophytes (Martin-Creuzburg and Merkel 2016, Peltomaa et al. 2023). Two of the three studied golden algae strains belonged to synurophytes (*Synura* sp. and *Uroglena* sp.), and one strain to chrysophytes (*Dinobryon* sp.). All three golden algae contained β-sitosterol and stigmasterol, of which stigmasterol has been reported to have a lower threshold value for growth than β-sitosterol (Martin-Creuzburg et al. 2014). However, *Daphnia* obtained the majority of their body cholesterol from algal phytosterols when feeding on the golden algae, suggesting that *Daphnia* was able to ingest and assimilate phytosterols from any of the golden algal strains used here.

The combination of $^{13}$C-labeling, CSIA, and cholesterol supplementation allowed us to explore the sterol bioconversion capacities of *Daphnia*. In our experiments, *Daphnia* preferentially used the supplemented cholesterol to maintain their body cholesterol content rather than converting cholesterol from phytosterols present in the algal diets. Bioconversion from dietary phytosterols might be energetically more costly than absorbing cholesterol directly from the diet. However, we did not test algae with high sterol content (e.g. cryptophytes with sterol index > 1, Hiltunen et al. 2015, Peltomaa et al. 2017), which could have given a different result. Since the origin of cholesterol between liposome and diet was rather systematical, exceptions from average contributions may suggest differences in the availability or usability of different dietary sterols. For example, when feeding on *Tabellaria* sp., only 5% of the cholesterol found in *Daphnia* originated from the algal diet suggesting low edibility of this diatom algal strains (Fig. 4b). Similarly, the low conversion rates of dietary phytosterols to cholesterol in *Daphnia* feeding on two strains of dinoflagellates (*P. cinctum*, *G. impatiens*) and one strain of diatom *A. formosa* suggest problems in the uptake or bioconversion of dietary phytosterols from these algal strains. Carotenuto and Lampert (2004) did not find any differences in carbon incorporation into *Daphnia* in relation to morphological differences among diatoms, whereas large size was the main limiting factor. However, in contrast to that, our result shows lowered utilization of *Tabellaria* sp., which is most likely related to morphological properties that are unrelated to size since the two pennate diatoms used here did not differ in size. The highest contribution of phytosterol-converted cholesterol was found in *Daphnia* feeding on the golden algae *Synura* sp., which contained a relatively high amount of b-sitosterol and stigmasterol. This suggests that the sterol assimilation strategy was influenced by the total dietary sterol content but also phytosterols of the provided algae.

The total sterol content of all algal strains was relatively low, as compared to previously published data on carbon-related sterol contents of laboratory-cultivated phytoplankton taxa (Martin-Creuzburg and Merkel 2016, Taipale et al. 2016), suggesting that the availability of sterols could have limited *Daphnia* growth on all algal diets. On dinoflagellate diets, somatic growth of *Daphnia* was improved by cholesterol supplementation with only one of the three strains studied *P. bipes*. Even though dinoflagellates contained the highest amount of PUFAs among all tested diets, *Daphnia* feeding on the dinoflagellates contained the lowest amount of PUFAs. This may support a previous study suggesting low digestibility of dinoflagellates (DeMott 1995) and that the high sterol and PUFA content of dinoflagellate was, therefore unavailable to *Daphnia*. Moreover, the substantially higher mortality of *D. magna* feeding on *G. impatiens* implies that this freshwater strain may produce secondary metabolites that are harmful to *Daphnia* and potentially other consumers, as has been shown for a number of marine dinoflagellates, including marine representatives of the genus *Gymnodinium* (Oshima et al. 1993, Giner et al. 2003, Wang 2008). To the best of our knowledge, toxin-producing dinoflagellates have not been documented so far in freshwater, a topic that requires further investigation.

The three diatom strains contained the lowest amount of phytosterols of all algal strains studied here. However, cholesterol supplementation improved the growth of *Daphnia* only while feeding on one of the three diatom strains, i.e. *Tabellaria* sp., suggesting sterol limitation on *Tabellaria* sp. but not on the other two diatom diets. Brassicasterol, the predominant sterol in *Tabellaria* sp. and *A. formosa*, has been shown to be highly efficient in supporting *Daphnia* growth (Martin-Creuzburg et al. 2014). The brassicasterol content of *Tabellaria* sp. and *A. formosa* did not differ. Thus, it remains unclear why the low amounts of brassicasterol were sufficient to cover the sterol requirements of *Daphnia* on *A. formosa* but

not on *Tabellaria* sp., where sterol supplementation revealed sterol limitation. Somatic growth rates of *Daphnia* on the centric diatom *Cyclotella* sp. were high in general and did not further improve upon cholesterol supplementation. The capacity of campesterol, the main sterol found in *Cyclotella* sp., to support *Daphnia* growth has not been studied yet, but the high growth rates, even on unsupplemented *Cyclotella* sp. suggest that this sterol is also efficiently used by *Daphnia*. Since the diatoms studied here were all relatively small, our results do not support the idea of non-edibility of diatoms, even in the case of *Tabellaria* sp. and *A. formosa*.

On the golden alga *Dinobryon* sp., which had the lowest total sterol content of the three golden algae strains tested here, somatic growth rates of *Daphnia* increased upon cholesterol supplementation. Cholesterol supplementation did not improve *Daphnia* growth on *Uroglena* sp. or *Synura* sp. In our experiment, none of the three strains of golden algae formed colonies, so all of them were in a size range suitable for *Daphnia*. The lack of a positive cholesterol supplementation effect combined with the high PUFA content of *Uroglena* sp. suggests that the poor food quality of *Uroglena* sp. observed here is unrelated to the availability of sterols and PUFA. Golden algae is known to contain species that can produce toxins (Kamiya et al. 1979, Boxhorn et al. 1998, Manning and La Claire 2010), which could explain why cholesterol supplementation did not improve growth of *Daphnia* on the *Uroglena* sp. strain used here.

The reproductive capacity of *Daphnia* was low on all dinoflagellate diets, emphasizing the low food quality of dinoflagellates for *Daphnia*. The reproductive capacity of *Daphnia* on golden algae and diatoms differed among strains. Cholesterol supplementation did not improve the reproductive capacity of *Daphnia* on any of the dinoflagellate diets. In contrast, cholesterol supplementation improved the reproductive output of *Daphnia* on one of the diatom diets (*Tabellaria* sp.) and one of the golden algal diets (*Synura* sp.), suggesting that reproduction of *Daphnia* can be limited by sterols. The release from sterol limitation by cholesterol supplementation may have allowed the animals to assimilate substantially more PUFA from the diet in both cases. As a result, it is probable that *Daphnia* reproduction was constrained by sterols and EPA, both of which are known to be important for reproduction (Becker and Boersma 2005, Ravet and Brett 2006, Wacker and Martin-Creuzburg 2007, Martin-Creuzburg et al. 2008). Moreover, it has been reported that the number of eggs per clutch increases linearly with body size in *Daphnia* (Lynch 1980a, b). Therefore, it is interesting that cholesterol supplementation improved the growth of *Daphnia* on *P. bipes* and *Dinobryon* sp. but did not affect reproductive output. Possibly, reproduction was more limited by the lack of PUFAs, e.g. by EPA, than by sterols and vice versa for growth (Martin-Creuzburg et al. 2009). As suggested earlier, with *P. bipes*, the cell size and wall structure could have interfered with ingestion or digestion and thus may have hampered the uptake of PUFAs from the diet. It is worth mentioning, however, that *Daphnia* fed cholesterol-supplemented *P. bipes* contained almost three times more EPA than *Daphnia* fed unsupplemented *P. bipes*, suggesting that the dietary EPA supply on the unsupplemented diet was not sufficient to support higher reproduction. On the other hand, the larger body size may have helped the *Daphnia* to digest the cells more efficiently as the body size can impact the assimilation efficiency of nutrients from the diet (DeMott et al. 2010).

Our experimental approach also allowed us to explore changes in the cholesterol and PUFA content of *Daphnia* in response to cholesterol supplementation. The cholesterol content of *Daphnia* increased upon cholesterol supplementation only on one diet, i.e. *Tabellaria* sp. In this case, cholesterol supplementation also improved somatic growth and reproduction, suggesting that the supplemented cholesterol was directly allocated to somatic growth and egg production. In the same individuals, cholesterol supplementation also increased the body PUFA content of *Daphnia*, suggesting that efficient utilization of dietary PUFA requires adequate cholesterol supply. Interactive effects of co-limiting dietary sterols and PUFA on *Daphnia* somatic growth and reproduction have been described earlier (Martin-Creuzburg et al. 2010). In three of the studied algal strains, however, the body PUFA content decreased upon cholesterol supplementation, highlighting that more studies are required to understand how cholesterol and PUFA content are interconnected in zooplankton.

Previous studies have shown that the growth of *Daphnia* on prokaryotic diets (cyanobacteria, bacteria) is constrained by the lack of sterols (Martin-Creuzburg et al. 2008, 2011). Based on sterol index calculations from 900 boreal lakes, it has been proposed that sterols become limiting when cyanobacteria comprise 40% of the phytoplankton community (Peltomaa et al. 2017). For the first time, we show here experimentally that growth and reproduction of *Daphnia* can be limited by sterols when feeding on eukaryotic algae that otherwise are of high nutritional quality. This suggests that *Daphnia* and potentially other herbivorous cladocerans are more frequently limited by sterols than expected. Previous studies have also emphasized the role of EPA as a potentially limiting nutrient for herbivorous cladocerans in lakes (Müller-Navarra et al. 1995, Ravet et al. 2012). Our study here suggests strong interactive effects between dietary sterols and EPA on the performance of *Daphnia*. Therefore, EPA and cholesterol supplementation studies should be done with natural seston collected from different lakes to understand their interconnection and how each compound limits growth and/or reproduction of *Daphnia* in the field.

Here, for the first time, we tracked the origin of cholesterol in *Daphnia* by combining $^{13}$C-labeling, supplementation and CSIA. Combining these three methods allowed for a better understanding of sterol-mediated metabolic constraints. The transfer of $^{13}$C from dietary phytosterols to the body cholesterol of *Daphnia* revealed that they can biosynthesize cholesterol from the diverse group of phytosterols, including di- and trimethyl phytosterols. Practically, this means that *Daphnia* can use phytosterols from dinoflagellates, golden algae and diatoms as cholesterol precursors. However, the supplemented

cholesterol was preferentially used. Cholesterol supplementation improved *Daphnia* growth and reproduction on at least some of the algal strains used here, showing that sterol limitation is possible even when feeding on eukaryotic algae. Our results show that a combination of $^{13}$C-labeling, compound-specific isotopes, and liposomes efficiently unravel the transfer and fate of dietary sterols and could be used to explore any biomolecules in consumers.

*Acknowledgements* – We thank the laboratory technicians at University of Jyväskylä for their aid in maintaining the phytoplankton and *Daphnia* cultures.
*Funding* – This research was supported by the Academy of Finland grant 333564 awarded to SJT.

### Author contributions

**Miikka Benjami Laine:** Conceptualization (equal); Formal analysis (lead); Methodology (lead); Writing – original draft (equal). **Dominik Martin-Creuzburg:** Conceptualization (supporting); Methodology (supporting); Supervision (supporting); Writing – original draft (supporting). **Jaakko J. Litmanen:** Methodology (supporting); Writing – original draft (supporting). **Sami J. Taipale:** Conceptualization (lead); Data curation (lead); Funding acquisition (lead); Methodology (supporting); Project administration (lead); Supervision (lead); Writing – original draft (equal).

### Data availability statement

All data supporting our results are available in the Supporting information.

### Supporting information

The Supporting information associated with this article is available with the online version.

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