

Supplementation of a diatom diet with cholesterol can enhance copepod egg-production rates

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Abstract

Cholesterol is required for animal growth, yet it cannot be biosynthesized de novo by crustaceans. Dietary sterols are thus necessary for the growth and reproduction of copepods. Sterols vary widely in both composition and concentration in phytoplankton. To explore the potential for sterol limitation of copepod growth, I did experiments with adult copepods feeding on diatoms with and without cholesterol supplementation. Cholesterol was added to the diet by (1) homogenizing the insoluble cholesterol with an ultrasonic disintegrator or (2) preparing gelatin/acacia microcapsules with and without cholesterol using triolein as a carrier. Egg-production rates were measured after 2–4 d on the experimental diets, except in one experiment, in which an 18-d feeding period was used. Egg-production rates increased 1.5–2-fold for *Acartia hudsonica*, *Acartia tonsa*, and *Calanus finmarchicus* when the diatom *Thalassiosira weissflogii* was supplemented with 5–20 $\mu\text{g L}^{-1}$ cholesterol; supplementation at 1 $\mu\text{g L}^{-1}$ had no effect. Nearly three-fold higher rates of egg production were observed with *C. finmarchicus* after feeding for 18 d on the supplemented diet. Cholesterol had a positive effect on egg-production rates when added by either method. Egg hatch rates for *A. hudsonica* were also higher when they were fed *T. weissflogii* supplemented with cholesterol (91% hatched) than when they were fed an unsupplemented diet (40% hatched). Egg production of *Centropages hamatus* feeding on *T. weissflogii* was unaffected by supplementation. Egg-production rates of *A. hudsonica* were unaffected when *Chaetoceros affinis*, *Phaeodactylum tricornutum*, or *Thalassiosira rotula* were supplemented. Higher egg-production rates in supplemented treatments were caused by a decrease in the frequency of small clutches during spawning events rather than an increase in maximum clutch size. These experiments demonstrate the potential for limitation of egg production by dietary sterols when copepods feed exclusively on diatoms.

Determining the factors that control zooplankton production requires a knowledge of limiting conditions such as temperature, predation, and nutrition. Interest in the role of nutrition in zooplankton production has focused at the level of elemental composition of diet (e.g., Sterner 1990; Elser and Hassett 1994), as well as biochemical composition, in particular the role of fatty acids (e.g., Müller-Navarra 1995) and, to a lesser extent, amino acids (Kleppel et al. 1998). Similarly, attention has been directed at factors limiting copepod egg production and hatching success. As with growth, food quality, particularly fatty acid composition, is a key factor in determining egg-production rates on different algal diets (Støttrup and Jensen 1990; Jónasdóttir 1994; Jónasdóttir and Kjørboe 1996). Diatom species have been found to be highly variable in food quality, with some species capable of inhibiting egg productions and/or egg viability (Ban et al. 1997; Paffenhoffer 2002). Allelopathic compounds in some diatom species have been found to be responsible for this inhibition (Miralto et al. 1999, 2003; Pohnert et al. 2002). A range of dietary compounds thus appears to influence copepod reproduction. One biochemical component that has been speculated on (Ederington et al. 1995; Kleppel and Burkart 1995) but never systematically tested as a possible

limiting factor for copepod reproduction is cholesterol, nor have phytosterols that can serve as biosynthetic precursors to cholesterol been studied.

Cholesterol is the most abundant neutral lipid in the plasma membranes of animals and is required for animal growth (Yeagle 1993). Although many invertebrate phyla have some species that can biosynthesize cholesterol de novo and others that cannot, no arthropod species have been found to have the capacity for de novo synthesis (Goat 1981). In the absence of de novo biosynthesis, cholesterol must be acquired through the diet, either directly or by the conversion of certain phytosterols. The sterol composition of phytoplankton is complex, varying considerably among species, with some 200 compounds having been identified from seaweeds, phytoplankton, and animals, mostly of marine origin (Veron et al. 1998). Algal sterols may include cholesterol, a 27-carbon (C27) sterol, as well as longer chain (C28 and C29) sterols, which can be dealkylated to cholesterol by consumers (Teshima 1971). Other sterols, particularly long-chain C30 and higher sterols may not be utilized by animals. Copepods also may be incapable of reducing $\Delta 7$ algal sterols to $\Delta 5$ sterols (Prah et al. 1984). When sterols in formulated diets are inadequate, the growth of decapod crustaceans may be enhanced by the addition of cholesterol to the diet (e.g., Sheen et al. 1994; Teshima et al. 1997). Recently, Von Elert et al. (2003) found that the absence of sterols in cyanobacteria limited the growth rates of the cladoceran *Daphnia galeata*, given that supplementation of the cyanobacteria with cholesterol increased growth rates. To explore the potential for the sterol limitation of zooplankton reproduction, experiments were conducted with adults of four species of copepods feeding on the diatoms *Thalassiosira weissflogii*,

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Thalassiosira rotula, *Phaeodactylum tricoratum*, and *Chaetoceros affinis* with and without cholesterol supplementation.

Materials and Methods

All experiments were conducted at the Mount Desert Island Biological Laboratory (MDIBL) in Salisbury Cove, Maine, except for the experiment with *Acartia tonsa*, which was conducted at Ohio University. Copepods (*Acartia hudsonica*, *Calanus finmarchicus*, and *Centropages hamatus*) were collected from the Damariscotta River estuary, Maine, in 2000 and *A. hudsonica* and *C. finmarchicus* from Frenchman Bay, Maine, in 2001–2002. On returning to the MDIBL laboratory, *A. hudsonica* were sorted after anesthetizing with MS-222 at a concentration of 0.58 g L⁻¹ (Durbin et al. 1990). The MS-222 solution was neutralized to pH 7.0 with NaH₂CO₃ before use. After 24 h, dead or moribund copepods were removed from the containers, and acclimations to the different food types was begun with the remaining animals. *Calanus* and *Centropages* were sorted without anesthetic, and feeding acclimations were begun immediately after sorting. *A. tonsa* were provided by P. Tester from North Carolina and maintained in culture at Ohio University for ~1 month on a diet of *T. weissflogii* and yeast-based Roti-Rich (Florida Aqua Farms) before experiments with the supplemented and unsupplemented *T. weissflogii* were begun. Copepods were maintained in 4-liter polycarbonate containers in a flowing seawater bath (MDIBL, ambient temperature 13–15°C) or a constant-temperature incubator (Ohio University, temperature 18°C). Both females and males were included in the acclimation treatments.

Cultures of *T. weissflogii* (CCMP 1336), *T. rotula* (CCMP 1647), *C. affinis* (CCMP 158), and *P. tricoratum* (CCMP 1327) were obtained from the Center for the Culture of Marine Phytoplankton at the Bigelow Laboratory and maintained in batch cultures in f/2 culture medium. Cultures were maintained in constant-temperature incubators (16°C) at Ohio University and under indirect sunlight at room temperature (~15–20°C) at MDIBL.

Cholesterol, which has very low solubility in water, was added to the diet by (1) homogenization by ultrasonifying the insoluble cholesterol to produce microparticles or (2) preparing gelatin-acacia microcapsules. The prepared cholesterol was added to yield a final concentration of 1–20 µg cholesterol L⁻¹. Microparticles of cholesterol were prepared by homogenizing 50–200 mg of cholesterol in 1 ml deionized water with a 50-W ultrasonic disintegrator for two bursts of 30 s each at maximum intensity. This stock suspension was further diluted to working strength, and both the stock and working-strength suspension were stored frozen at -20°C. The working-strength suspension was thawed and rehomogenized before it was added to experimental containers. To prepare gelatin-acacia microcapsules (adapted from Planas et al. 1990), 2 g acacia was dissolved in 100 ml H₂O and homogenized for 2 min with a Biosonic homogenizer. Lipid (200 µg cholesterol dissolved in 2 g triolein or 2 g triolein alone) was added, the pH was adjusted to 6.5 with NaOH, and the suspension was sonicated for 10 min in an ultrasonic bath. Separately, 2 g of gelatin was added

to 100 ml H₂O (heated to dissolve), and the pH was adjusted to 6.5 with NaOH. The two suspensions were combined, adjusted to pH 5.2 with HCl, and stirred for 5 min at 250 rpm with a magnetic stirrer. The suspension was cooled in an ice bath for 20 min at 100 rpm, adjusted to pH 9.0, and agitated 30 min. After standing for 2 h at 4°C, the suspension was washed three times by centrifuging in 50-ml centrifuge tubes, then decanting and washing with distilled H₂O. A sample of the microcapsules was stained with Nile Red and observed at 400× magnification, to ensure that lipid encapsulation had occurred. The cholesterol and triolein contents of the microcapsules were assayed enzymatically with assay kits (Sigma Chemical). The size ranges of the gelatin/acacia microcapsules and cholesterol microparticles were determined with a Coulter Counter model Z2. Both produced maximum numbers of particles near the detection limit of the aperture tube (equivalent to 2-µm diameter particles). Microcapsules ranged in size up to ~15 µm diameter, with the maximum volume concentration at 8 µm diameter. Cholesterol microparticles were uniformly small, virtually all approximately <5 µm in diameter.

A. hudsonica and *A. tonsa* were fed for 2–3 d on the experimental diets, *C. hamatus* for 4 d, and *C. finmarchicus* for 4 and 18 d in two separate experiments. Algal concentrations were adjusted to ~500 µg C L⁻¹ on the basis of literature values of carbon cell⁻¹ (Støttrup and Jensen 1990 and Ianora and Poulet 1993 for *T. weissflogii* and *T. rotula*, respectively, and carbon determined from cell volume estimates on the basis of an equation from Strathman 1967 for other species). Cholesterol microparticles or cholesterol/triolein microcapsules were added at concentrations of 0–20 µg cholesterol L⁻¹, depending on the experiment. When microcapsules were used, triolein-only microcapsules were added to the control containers. Containers were stirred twice each day, so that the settling of algae during the intervening time would make the concentrations within the containers more variable. After the acclimation period the animals were transferred to 50-mm diameter petri dishes (*Acartia* species) or 250-ml egg separation chambers, which consisted of a 4-cm-diameter tube with 240 µm nitex mesh at the bottom nested inside a 250-ml beaker (*Calanus* and *Centropages*). Petri dish/separation chamber incubations were conducted in a constant temperature incubator at the same temperature as the flowing seawater bath (which varied from 12°C to 15°C over the period of the experiments). After 24 h, eggs were counted; in one experiment, a second set of counts was made after 48 h, in which case eggs from the first 24-h period were removed.

Statistical analysis—Data are given as average ±95% confidence interval. The level of significance for differences among treatments is given above columns, using nonparametric Wilcoxon two-sample or Kruskal-Wallis (for comparisons of ≥3 groups) tests.

Results and discussion

Cholesterol supplementation had a pronounced effect on the egg-production rates of *A. hudsonica* (Figs. 1, 2), *A. tonsa* (Fig. 3A), and *C. finmarchicus* (Fig. 3B,C) fed *T. weiss-*

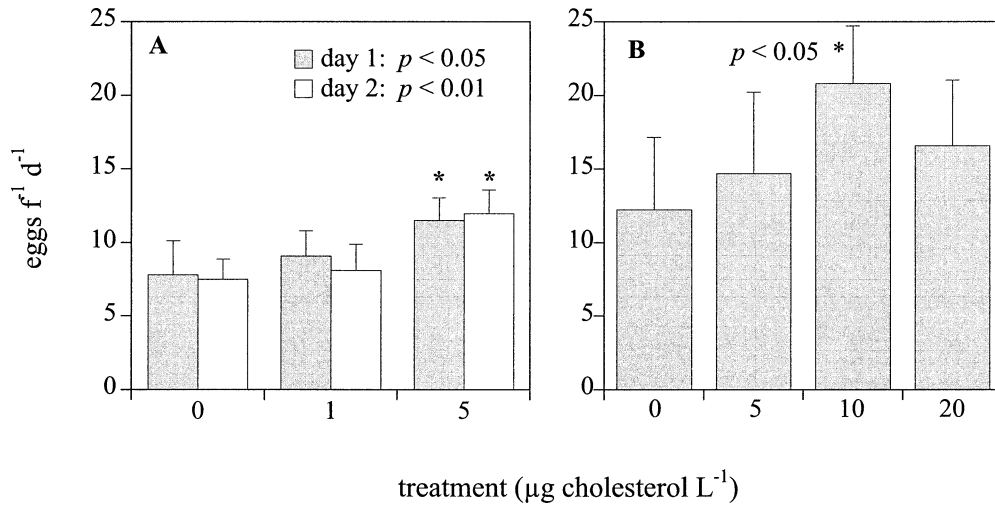


Fig. 1. Egg-production rates of *A. hudsonica* feeding on *T. weissflogii* with and without supplementation with homogenized particulate cholesterol. (A) Supplemented at 0, 1, and 5 μg cholesterol L^{-1} , $n = 9$ per treatment, two copepods per petri dish. Egg-production rates are given for each day of the experiment. (B) Supplemented at 0, 5, 10, and 20 μg cholesterol L^{-1} , $n = 16$ per treatment at 0 and 5 μg L^{-1} and 17 per treatment at 10 and 20 μg L^{-1} , one copepod per petri dish. Egg-production rates are averaged over 2 d. Significant differences were determined with the Kruskal-Wallis nonparametric test, denoted by an asterisk.

flogii. The observed enhancement of egg-production rates in *C. finmarchicus* was maintained over both 4-d (Fig. 3B) and 18-d (Fig. 3C) feeding periods, with a nearly threefold difference in egg-production rates observed after 18 d of exposure to the supplemented diet. A direct comparison between the 4- and 18-d experiments is not possible because of different levels of supplementation (5 and 10 μg chole-

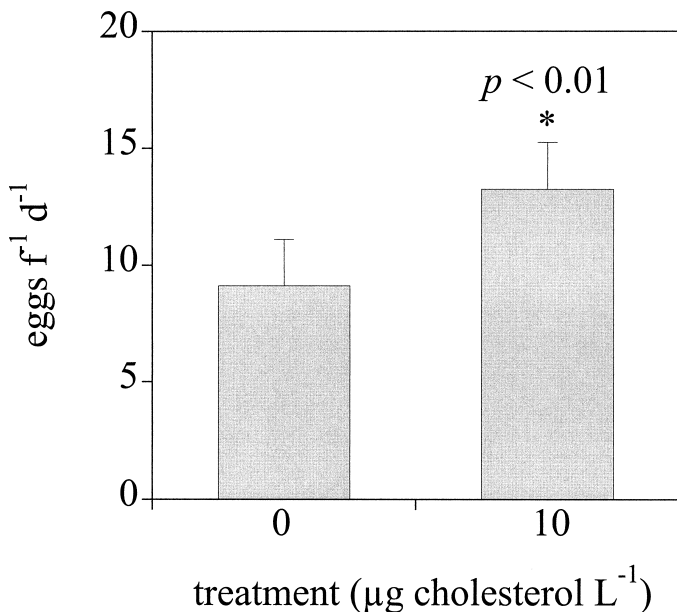


Fig. 2. Egg production rates of *A. hudsonica* feeding on *T. weissflogii* with and without supplementation with cholesterol/triolein microcapsules, $n = 25$ per treatment, one copepod per petri dish. Significant differences were determined with the Wilcoxon two-sample test, denoted by an asterisk.

sterol L^{-1}) and different collection dates of *C. finmarchicus* (2000 and 2002). However, it is clear that the observed enhancement of egg production is sustainable over extended periods when *T. weissflogii* is supplemented with cholesterol and is not caused by a short-term, unsustainable, stimulus of production. Contrary to what was observed with the other three copepod species, cholesterol supplementation of *T. weissflogii* had no significant effect on *C. hamatus* egg production (Fig. 3D).

Egg-production rates of *A. hudsonica* were enhanced by supplementation with either homogenized cholesterol (Fig. 1) or cholesterol microcapsules (Fig. 2). There was no difference in egg-production rates of *A. hudsonica* between the first and second days of the experiment (Fig. 1A). The effect of supplementation was greatest at 10 μg cholesterol L^{-1} (Fig. 1B). Although the difference between 10 and 20 μg L^{-1} was not significant, an eventual decline would be expected, because a high cholesterol content in the diet can inhibit its assimilation (Teshima et al. 1974). Supplementation of the other three diatom species had no effect on *A. hudsonica* egg production (Table 1). Egg production when feeding on *C. affinis* was very low in these experiments, as has been observed previously for *A. tonsa* feeding on *C. affinis* (Jónasdóttir and Kiørboe 1996).

Egg hatch rates were determined for *A. hudsonica* feeding on *T. weissflogii* (see Fig. 1A) and were 40% in the unsupplemented treatment and 91% in the supplemented treatment, as based on eggs pooled from the different treatments. A similar difference (77% \pm 9% supplemented vs. 50% \pm 5% unsupplemented) was observed in another experiment with *A. hudsonica* feeding on *T. weissflogii* (E. L. Crockett and R.P.H. unpubl. data). Both Guisande and Harris (1995) and Jónasdóttir and Kiørboe (1996) found that higher hatch rates were associated with higher egg-produc-

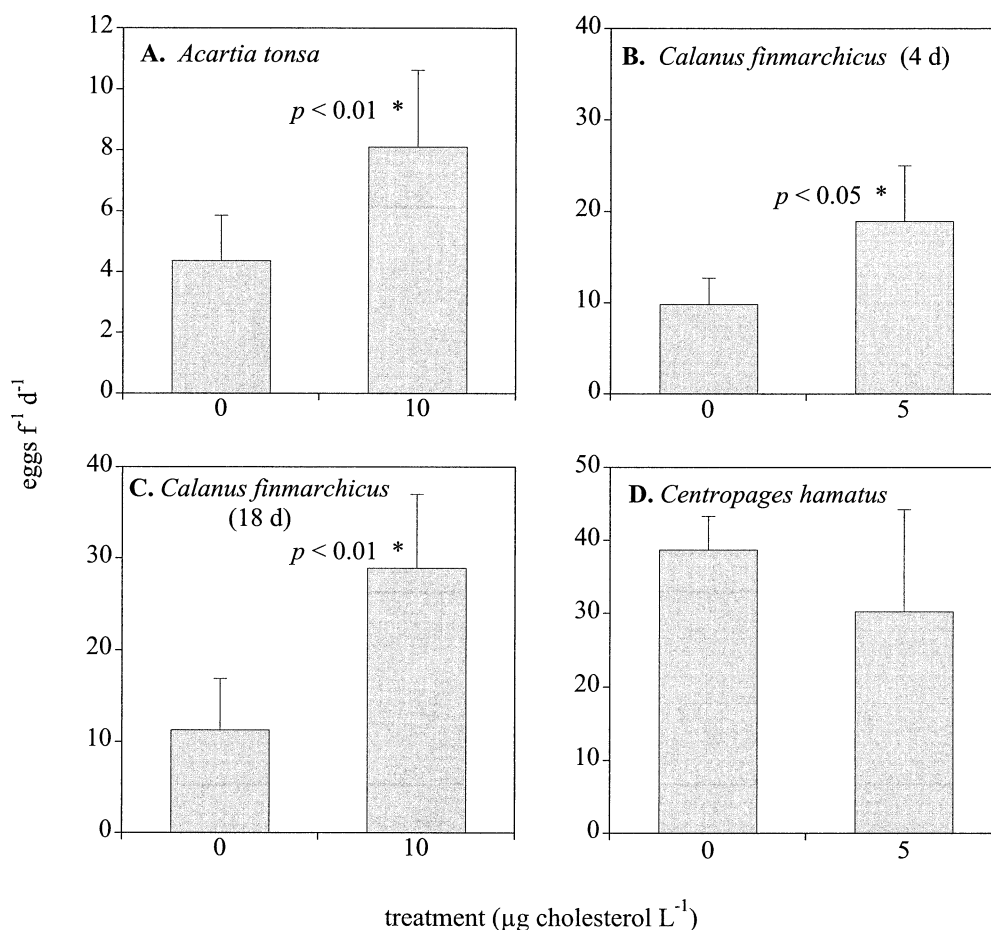


Fig. 3. Egg-production rates of three species of copepods feeding on *T. weissflogii* with and without cholesterol supplementation. (A) *A. tonsa* supplemented with cholesterol/triolein microcapsules, $n = 15$ per treatment, two–three copepods per Petri dish. (B) *C. finmarchicus* after a 4-d feeding trial supplemented with homogenized particulate cholesterol (HPC), $n = 5$ per treatment, two copepods per egg separation chamber. (C) *C. finmarchicus* after an 18-d feeding trial supplemented with HPC, $n = 8$ per treatment, two copepods per egg separation chamber. (D) *C. hamatus* supplemented with HPC, $n = 7$ for unsupplemented treatment, $n = 4$ for supplemented treatment, one–two copepods per egg separation chamber. Significant differences were determined with the Wilcoxon two-sample test, denoted by an asterisk.

tion rates, as was observed in the present studies. Hatch rates have previously been found to vary with diet, apart from the allelopathic effects mentioned earlier. Approximately 20% lower hatch rates were observed by Jónasdóttir and Kjørboe (1996) on slow-growing cultures of *T. weissflogii* than on fast-growing cultures. Hatching success was correlated with fatty acid composition in that study, but sterol compositions were not determined. It should be noted that Ederington et al. (1995) found no effect of egg sterol content on egg hatch rates of *A. tonsa* in an experiment in which two different algal diets produced eggs of differing sterol content. Egg sterol content was not determined in the present supplementation experiments, but it is possible that egg sterol contents on the unsupplemented diet were lower than those observed by Ederington et al. (1995).

A notable feature of the experiments with *A. hudsonica* was the effect of supplementation on the variability in egg

production among individuals. In the three experiments with *A. hudsonica* feeding on *T. weissflogii*, the coefficient of variation was nearly twice as high (1.9 ± 0.3) in the unsupplemented treatments than in the supplemented treatments. Many broadcast spawners, including *Acartia* spp., produce eggs during discrete spawning events or clutches (Runge and Roff 2000). Differences in egg production were not due to differences in maximum clutch sizes among treatments. For instance, in the experiment reported in Fig. 1B, the average egg-production rates for females producing >10 eggs d^{-1} was 20, 21, 24, and 22, respectively, for the 0, 5, 10, and 20 μg cholesterol L^{-1} treatments. However, in this experiment, 8 of 16 individuals in the unsupplemented treatment produced <10 eggs per day (the average of these 8 individuals was 5.8 eggs $f^{-1} d^{-1}$), whereas only 1 of 17 individuals produced <10 eggs (this female produced 8 eggs $f^{-1} d^{-1}$) when supplemented at 10 μg cholesterol L^{-1} . Thus, the dif-

Table 1. Egg production rates of *Acartia hudsonica*. Summary of supplementation experiments with diatoms. Data are eggs female⁻¹ ± 95% confidence interval. Number of replicates is in parentheses; experiments were conducted with 1 copepod per Petri dish. Significant differences determined with the Wilcoxon two-sample test.

Food species	Supplementation level (<i>n</i> eggs f ⁻¹ d ⁻¹)		Significance
	0 µg L ⁻¹	10 µg L ⁻¹	
<i>Chaetceros affinis</i>	4.0±0.8 (34)	2.8±0.8 (30)	NS
<i>Thalassiosira rotula</i>	12.5±2.8 (22)	9.4±1.9 (27)	NS
<i>Phaeodactylum tricornutum</i> (Ex 1)	7.6±2.2 (20)	10.2±2.8 (17)	NS
<i>Phaeodactylum tricornutum</i> (Ex 2)	6.4±1.9 (30)	7.6±1.8 (30)	NS

NS, Not significant.

ference among treatments was due to differences in the occurrence of very small clutches during spawning events and not to differences in maximum clutch sizes.

Consumer sensitivity to sterol limitation is indicated by the lack of response of *C. hamatus* to supplementation of a *T. weissflogii* diet, whereas *A. hudsonica*, *A. tonsa*, and *C. finmarchicus* all exhibited higher egg-production rates when the same diet of *T. weissflogii* was supplemented with cholesterol. It is possible that *C. hamatus* did not ingest the cholesterol particles. However, the high egg-production rates of *C. hamatus* fed *T. weissflogii* without supplementation suggest either that the copepod's demand for sterols are less than that of the other species or that it had body reserves that it could draw on over the short duration of these experiments. In contrast to the present experiments, the related *Centropages typicus* had low egg-production rates with *T. weissflogii* as food, but these rates were increased three- to sevenfold when *T. weissflogii* was supplemented with ciliates (Bonnet and Carlotti 2001). The maximum ciliate-supplemented egg production rates observed by Bonnet and Carlotti (2001) were comparable to the egg-production rates observed in the present study with *C. hamatus* fed *T. weissflogii* alone. Although the enhancement observed by Bonnet and Carlotti was not necessarily caused by sterol composition (e.g., fatty acid composition may play a role), if *C. hamatus* and *C. typicus* have similar sterol requirements, the observed results would be consistent with *C. hamatus* having adequate sterol reserves to allow high egg-production rates on the *T. weissflogii* diet.

These results indicate that, depending on the species involved (both consumer and food), the potential exists for dietary sterol limitation. In three experiments with *Acartia* species feeding on *T. weissflogii*, all demonstrated higher egg-production rates when supplemented with cholesterol. These results are highly repeatable, given that the three experiments shown in Figs. 1A,B and 2 were conducted during consecutive years at MDIBL and thus were not an aberration of the particular stock of copepods or algal culture used (new stock cultures were acquired at the start of each field season). Although *T. weissflogii* has been used in numerous feeding experiments with copepods, it is not an optimal food in terms of growth or egg-production rates. Egg-production rates of *A. tonsa* feeding on *T. weissflogii* were nearly 40% lower than when *Isochrysis galbana* was used as a food source, despite nearly identical rates of carbon ingestion (Støttrup and Jensen 1990). Støttrup and Jensen could not explain the nutritional advantage of *I. galbana* on the basis of fatty acid

content, but, of interest, *I. galbana* has a very high sterol content on a dry-weight basis, with significant amounts of free and esterified cholesterol (Véron et al. 1998). Culture conditions could play a role, given that egg-production rates were nearly twice as high on fast-growing cultures of *T. weissflogii* than on slow-growing cultures (Jónasdóttir and Kiørboe 1996). Egg-production rates followed a similar pattern in the present experiments to rates observed by Jónasdóttir and Kiørboe (1996) with *A. tonsa* after 2–3 d of feeding. Jónasdóttir and Kiørboe found low rates (on a carbon-specific basis) on *C. affinis*, intermediate rates on *P. tricornutum*, *T. rotula*, and slow-growing *T. weissflogii*, and highest rates on *Rhodomonas baltica* and fast-growing *T. weissflogii* (see Table 1 for comparison). Although the growth rate of the algal cultures was not determined in the present experiments, the results of cholesterol supplementation are consistent with the effects of culture growth rates on both the egg-production and hatch rates. Research on the effects of culture conditions on phytoplankton sterols presents a mixed picture. The sterol composition of *P. tricornutum* can be influenced by both temperature and the light spectrum (Veron et al. 1996). However, the sterol composition of other diatom species has been found to be insensitive to differences in culture condition (Ballantine et al. 1979).

If the phytosterols of *T. weissflogii* are inadequate for optimal egg production, either the composition or concentration of the phytosterols could be responsible. The sterols of *T. weissflogii* are dominated by 24-methylene cholesterol (24-methylcholesta-5,24(28)-dien-3β-ol), with significant amounts of 24-methylcholesterol(β) (24-methylcholesta-5-en-3β-ol) and fucosterol (24-ethylcholesta-5,24(28)E-dien-3β-ol) (Gladu et al. 1991), a composition very similar to that of *T. rotula* (Barrett et al. 1995). 24-methylene cholesterol appears to be a characteristic sterol of the genus *Thalassiosira*, generally accounting for ~50% of total sterols (Volkman and Hallegraef 1988). Because *T. rotula* was not improved by supplementation with cholesterol, composition alone is not likely to explain the enhanced egg production of copepods feeding on *T. weissflogii* supplemented with cholesterol.

It is possible, then, that the concentration of phytosterols needs to be considered in assessing the nutritional value of *T. weissflogii* and other phytoplankton. Total sterols as a percentage of dry weight vary considerably among and even within algal species. For instance, eight isolates of the Eustigmatophyte *Nannochloris*, in which cholesterol is the dominant sterol (52–80% of total sterol), have total sterols in the

10–30 fg cell⁻¹ range, whereas one isolate contains 480 fg cell⁻¹ and another contains 1,000 fg cell⁻¹ (Patterson et al. 1994). Similarly, *Chaetoceros* has a 100-fold range in cholesterol content on a dry-weight basis, from 0.3 µg g⁻¹ for *C. calcitrans* to 27.7 µg g⁻¹ for an unidentified *Chaetoceros* species (Tsitsa Tzardis et al. 1993). Given the complexity of algal sterol composition, the potential exists for differences in the assimilation efficiencies of phytosterols in the copepod gut as well as differences in the subsequent conversion efficiencies of phytosterols to cholesterol. Thus, much information is needed to predict when dietary sterol limitation will occur under natural conditions or to incorporate sterols into theoretical approaches to zooplankton nutrition, such as stoichiometric models that incorporate macromolecules that cannot be biosynthesized, such as essential fatty acids, in addition to elemental C, N, and P (Anderson and Pond 2000).

As was noted earlier, cyanobacteria, in which sterols are absent, present a clear case of the dietary sterol limitation of growth in a cladoceran (Von Elert et al. 2003). A heterogeneous diet could overcome the potential for sterol limitation, as could the trophic upgrading described by Klein Breteler et al. (1999), in which the fatty acid and sterol content of *Dunaliella tertiolecta* was “upgraded” in quality by passing through a ciliate. However, diatom blooms could present conditions under which dietary sterol limitation becomes possible. Recent observations of low copepod egg-production rates during diatom blooms (Miralto et al. 2003) raises the possibility that such conditions may be met, and dietary sterols must be considered along with essential fatty acids and allelopathic compounds when considering the deleterious effects of diatoms on copepod production. Although the extent to which dietary sterols are limiting under natural conditions is unknown, it is clear from the results reported here that an understanding of the role of sterols in both consumers and food is needed to fully understand the nutritional limitations of zooplankton.

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