

## Copepod reproduction is unaffected by diatom aldehydes or lipid composition

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### Abstract

We investigated whether reduced reproductive success of copepods fed with diatoms was related to nutritional imbalances with regard to essential lipids or to the production of inhibitory aldehydes. In 10-d laboratory experiments, feeding, egg production, egg hatching success, and fecal pellet production of *Temora longicornis* were measured for six different diatom species as well as for a nondiatom control diet (*Rhodomonas* sp.). The experiments were accompanied by determinations of fatty acids, sterols, and polyunsaturated aldehydes (PUA) in the food. Although diatoms were generally ingested at high rates, they yielded a variable egg production response in copepods, ranging from high egg production in four species (two strains of *Thalassiosira rotula*, *Chaetoceros affinis*, and *Thalassiosira weissflogii*) to low egg production in two species (*Leptocylindricus danicus* and *Skeletonema costatum*). Egg hatching rates decreased after 4 d in all diatom treatments, irrespective of the egg production rate and without any relationship to diatom aldehyde production. Similarly, no evidence was found that diatoms are per se nutritionally inferior to nondiatom food. The lack of a distinct mechanism for the observed inhibitory activity of diatoms suggests that the cause(s) might be more complex. We suggest, as one possible explanation, that hatching-specific nutritional deficiencies might be induced by incomplete digestion following from the low gut passage time of diatoms, as indicated by a strong correlation between egg viability and fecal pellet production.

In the classic paradigm of pelagic productivity, diatom blooms in mid- and high latitudes, and upwelling ecosystems form the base for a short and efficient food chain via zooplankton to large exploitable fish stocks (Cushing 1989; Legendre 1990). This tight coupling of production has been fundamentally questioned by observations of an impaired recruitment success of copepods feeding on high concentrations of diatoms. In several experimental studies, maternal diatom diets reduced the viability of eggs, caused embryonic malformations, and increased larval mortality (Ianora et al. 2003; Paffenhöfer et al. 2005). A number of long-chain (poly)unsaturated aldehydes (PUA) of apoptotic activity have been identified and proposed as blocking agents in copepod embryogenesis (Miralto et al. 1999; Pohnert et al. 2002). They originate from the cleavage of fatty acid precursors by enzymes activated following cell breakage, and they inhibit cell proliferation, cell division,

and phagocytosis in various animal cell types (Romano et al. 2003; Adolph et al. 2004). Diatom toxicity rather than diatom food quality or temperature-limited copepod growth, therefore, may restrain copepod cohort size and cause the frequently observed delay in zooplankton spring development (Miralto et al. 2003; Ianora et al. 2004).

Although laboratory experiments have unequivocally shown that high concentrations of diatoms frequently may cause a reduced fecundity or egg viability in copepods, the ecological significance and underlying mechanism for the diatom effect are still controversial. Some studies suggest an impaired reproduction of copepods associated with various diatom blooms in the field (Miralto et al. 1999; Poulet et al. 2006; Vargas et al. 2006). Despite variable copepod egg production and egg hatching, however, no general negative relationship to diatom biomass has yet been established in various ecosystems (Laabir et al. 1998; Irigoien et al. 2002). Similarly, diatom toxicity as the cause for low egg viability is not without controversy. As an alternative explanation for impaired hatching success, laboratory and field studies have suggested the control of copepod recruitment by essential amino acids, polyunsaturated fatty acids (PUFA), or sterols, leading to the establishment of the “nutritional dietary deficiency hypothesis” (Jónasdóttir et al. 1995; Pond et al. 1996; Hassett 2004). However, the role of lipids or other food constituents in decoupling egg production and egg hatching has still not been clearly demonstrated and also remains controversial (Lacoste et al. 2001; Pohnert et al. 2002; Jónasdóttir et al. 2005).

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Table 1. Cell-specific carbon and nitrogen content, C:N ratio (weight), polyunsaturated aldehyde (PUA), total (tFA) and polyunsaturated fatty acids (16–22 PUFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), and sum of  $\Delta 5$  sterol contents of phytoplankton strains used in grazing experiments with female *Temora longicornis*.

Species/strain	Carbon (pg cell <sup>-1</sup> )	Nitrogen (pg cell <sup>-1</sup> )	C:N	PUA (fmol cell <sup>-1</sup> )	tFA (fg $\mu\text{m}^{-3}$ )	PUFA (fg $\mu\text{m}^{-3}$ )	EPA (fg $\mu\text{m}^{-3}$ )	DHA (fg $\mu\text{m}^{-3}$ )	$\Delta 5$ sterol (fg $\mu\text{m}^{-3}$ )
<i>Rhodomonas</i> sp. (RHO)	98	21	4.6	–	8.9	6.8	0.62	0.53	2.14
<i>Thalassiosira weissflogii</i> (TW)	131	26	5.0	–	3.5	2.0	0.77	0.15	0.03
<i>T. rotula</i> CCMP 1647 (TR 1647)	267	52	5.3	2.27	1.5	0.3	0.09	0.04	0.31
<i>T. rotula</i> CCMP 1018 (TR 1018)	428	97	4.4	–	5.7	1.3	0.33	0.13	0.15
<i>Chaetoceros affinis</i> (CA)	137	15	9.2	–	14.9	2.9	1.19	0.27	0.06
<i>Leptocylindricus danicus</i> (LD)	57	11	5.3	0.04	1.7	0.8	0.24	0.01	0.22
<i>Skeletonema costatum</i> (SC)	18	3	5.7	0.04	1.5	0.5	0.09	0.02	0.15

Recent progress in the characterization of diatom toxins suggests that much of the diatom–copepod controversy results from the lack of proper monitoring of toxicity in experimental studies. Screening of diatom isolates from culture collections has revealed a large variability in PUA concentrations, even among different isolates of the same species (Wichard et al. 2005a). Plasticity in toxin production is, therefore, considered to be a major cause for variable egg hatching success observed in field and laboratory studies (Ianora et al. 2003; Paffenhöfer et al. 2005). Recent field studies that observed a reduction in egg hatching success in conjunction with a strong PUA-producing diatom support this, although the induced effects were very mild (e.g., Pierson et al. 2005).

To test whether low egg hatching success is related to the ingestion of PUA or lipids (PUFA, sterol) with maternal diatom diets, we carried out experiments in which pure cultures of six diatom species or clones were fed to *Temora longicornis*, a typical spring copepod that naturally co-occurs with diatom blooms in the North Sea. In addition to the reproductive success of the copepod, feeding and fecal pellet production were monitored, together with measurements of the elemental and lipid compositions and PUA content of the food species. Although the reproductive success of *T. longicornis* was substantially impaired by all diatom strains tested, no relationship to the PUA or lipid ration of the females could be established. We therefore reject the “diatom toxicity” or the “nutritional deficiency” hypotheses as mechanisms behind the diatom effect.

## Material and methods

**Cultures**—Experiments were conducted using cultured females of the calanoid copepod *Temora longicornis*. Individuals were originally isolated from the central North Sea and kept in the laboratory for more than 10 generations. The species was cultured at 14°C in the dark and fed in excess (>400  $\mu\text{g C L}^{-1}$ ) a mixture of *Rhodomonas* sp., *Thalassiosira weissflogii*, and *Heterocapsa* sp. Recently matured (<5 d) females were collected from the stock culture directly at the start of the experiments.

Algal cultures used as food in experiments were the cryptophyte *Rhodomonas* sp. (RHO, unknown origin, control) and the diatoms *Thalassiosira weissflogii* (TW, unknown origin), *T. rotula* (TR, strains CCMP 1647 and CCMP 1018, Provasoli–Guillard National Centre for Culture of Marine Phytoplankton [CCMP]), *Leptocylindricus danicus* (LD, strain CCMP 469), *Skeletonema costatum* (SC, strain CCMP 1281), and *Chaetoceros affinis* (CA, CCMP 158; Table 1). The strains were grown in 1–2-liter batch cultures at 18°C with a 16 : 8 h light : dark cycle at an irradiance of 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in F/2 (+Si) media. The algae were kept in exponential growth phase and constant density by daily monitoring of cell concentration and dilution of the culture accordingly. The carbon and nitrogen contents of the strains were determined by combustion in a Carlo Erba Analyzer from replicate 5–10-mL samples of each culture. Samples were filtered on combusted Whatman GF/F filters and kept frozen at –80°C until analysis. Samples for the determination of fatty acid, sterol, and polyunsaturated aldehydes (PUA) were taken simultaneously with the setup of the experiments and analyzed as described later. The algae carbon and nitrogen content, C:N ratio, and concentrations of PUA, polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA, 20:5[n-3]), docosahexaenoic acid (DHA, 22:6[n-3]), and  $\Delta 5$  sterols are listed in Table 1.

**Experiments**—At the start of the experiments, 10 females and 2 males of *Temora longicornis* were transferred into 650-mL bottles filled with 0.22- $\mu\text{m}$  filtered seawater and enriched with either *Rhodomonas* sp. or one of the diatom strains. The different food species were fed at a concentration of >500  $\mu\text{g C L}^{-1}$ . Experiments were run in six replicates for a total of 10 d, and daily measurements were taken of egg production and hatching, with three measurements to determine feeding and fecal pellet production rates (days 3, 6, and 8/9). Each day, individuals were transferred to new food solutions. The bottles were placed on a plankton wheel (0.5 rpm) at 17°C and dim light in a walk-in culture chamber.

Eggs were collected daily by concentration on a submerged sieve of 20  $\mu\text{m}$  and counted. Afterward, eggs from each treatment were pooled, carefully rinsed with 0.22  $\mu\text{m}$  filtered seawater into 325 ml bottles and incubated for 3 d until hatching. Eggs for the determination of carbon and nitrogen content were collected from parallel large-scale incubations (30 liters) of females on days 2, 5, and 9, using similar food and temperature conditions as in experiments. A minimum of 300 eggs per sample was analyzed in a Carlo Erba Analyzer, as described already.

For grazing experiments, three additional bottles per food treatment excluding grazers were set up as controls. Replicate samples for cell abundance were analyzed by microscopy (Zeiss Axiovert S100, 100 $\times$  magnification, minimum 300 cells per sample) or using an ELZONE electronic particle counter (Particle Data Inc., minimum 6,000 cells per sample) at the beginning and the end of the 24-h incubations, both from the six replicate bottles containing grazers and from the control bottles. Fecal pellets and eggs in experimental bottles were counted from the remaining volume. At least 30 pellets per treatment were sized using a microscope (Zeiss Axiovert S100, 200 $\times$  magnification) equipped with a digital camera and image-analysis system (Olympus DP12, DP-Soft software) in order to estimate the individual pellet volume and the total volume of feces, assuming a cylindrical shape.

**Calculations**—Feeding rates were calculated according to standard procedures (Frost 1972). Gut passage time was calculated as the inverse of the fecal pellet production rate (Besiktepe and Dam 2002). Due to differences in the volume of fecal pellets produced on diatom diets (resulting from an increase in length by a factor of 1.5–2.2) compared to those produced on a RHO diet, gut passage time may be potentially overestimated when the absorptive area in the gut is passed faster with increasing pellet length. In order to account for this effect of variable pellet size, fecal pellet production rates were also normalized to RHO by multiplying the fecal pellet production by the ratio of pellet volume on diatom diet to pellet volume on RHO diet. The efficiency of egg production was calculated from the ratio of eggs produced to the ingested food in terms of carbon and nitrogen. Since no significant differences in egg size, carbon, and nitrogen content were observed between food species or day of the sample (two-way ANOVA;  $p > 0.05$ ), all measurements were pooled, and common values for carbon and nitrogen were used to determine the efficiencies ( $87 \pm 18 \text{ ng C egg}^{-1}$  and  $19 \pm 4 \text{ ng N egg}^{-1}$ ,  $n = 18$ ).

**Lipid and polyunsaturated aldehyde analysis**—Fatty acid and sterol analysis was conducted with a slight adjustment of standard methods (Klein Breteler et al. 1999). The samples were extracted in KOH : MeOH, including a known amount of an internal standard (23 : 0), and saponified for 35 min at 85°C. After acidification of the sample to pH 3, double distilled water, MeOH, and dichloromethane were added. The sample was treated with ultrasound, and the lipid fraction was drawn off and put through a NaSO<sub>4</sub> column to remove all water traces. Samples were trans-methylated using BF<sub>3</sub> to form fatty acid methyl esters

(FAME), eluted over a silica column with ethyl acetate, and subsequently analyzed on a gas chromatograph-mass spectrometer (GC-MS) (Agilent 6890 with PTV inlet and Agilent 5973 mass selective detector) with an Agilent DB23 (60 m  $\times$  0.25 mm) column using helium as a carrier gas. After analysis of FAMES, the sample was silylated with Bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA) and pyridine and heated at 60°C for 20 min; next, it was analyzed on a GC-MS (as previous) on a Sil-5 (25 m  $\times$  0.32 mm) column using helium as a carrier gas. Retention times were compared to those of known FAME and sterol mixtures. Sterols are presented as the total concentration of three identified  $\Delta^5$  sterols (cholesterol, brassicasterol, and campesterol), while total fatty acid and PUFA (16–22) represent the sum of the concentration of 27 and 14 identified fatty acids, respectively. Extraction of aldehydes was conducted immediately following harvesting by filtration. To quantify PUA release upon cell damage, a protocol based on derivatization of PUA with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA·HCl) and subsequent GC-MS electron impact (EI) analysis was applied (Wichard et al. 2005b).

**Statistics**—Filtration, carbon ingestion, and pellet production rates were tested for differences between the three experiments (days 3, 6, and 9) with a two-way analysis of variance (ANOVA) using day of the experiment and prey type as factors. Since no significant differences were observed ( $p > 0.05$ ), the results were pooled for further analysis. Filtration rate and ingestion of carbon, nitrogen, total fatty acids, PUFAs (total PUFAs, 18–22 PUFAs, EPA, and DHA separately), sterols, and polyunsaturated aldehydes, as well as pellet production rate in number of pellets and in total feces volume, were then tested for differences between the diets using a one-way ANOVA and a Tukey honestly significant difference (HSD) post-hoc test for pairwise comparisons. The dependence of egg production and hatching success on food nutritional components or diatom aldehydes was tested by running a Spearman rank correlation analysis for egg production, hatching success, and ingested carbon, nitrogen, total fatty acids, total and 18–22 PUFAs, DHA, EPA, sterols, and aldehydes, using the pooled data for egg production and ingestion, and the hatching of the last experiment (day 9). All analyses were conducted using the SigmaStat 3.1 statistical package. All data were tested for equal variance and normal distribution and, if necessary, log transformed.

## Results

**Characteristics of algal strains**—Differences in the C : N ratio between algae were small, and values generally varied from 4.4 to 5.7. Only CA was found to be poor in nitrogen and therefore displayed a C : N ratio of 9.2 (Table 1). In comparison to the diatoms, RHO was particularly rich in unsaturated fatty acids and  $\Delta^5$  sterols, and PUFA contributed more than 75% to total fatty acid. The volume-specific fatty acid contents of diatoms ranged from 1.5 to 14.9  $\text{fg } \mu\text{m}^{-3}$  and were with the exception of CA lower than RHO. While PUFA values were rich in TW and

LD (>45%), they contributed only 10–30% to total lipids in other diatoms. Volume-specific sterol concentrations were by a factor of 7–70 lower in diatoms than in RHO. PUA was detected in three diatom strains. The calculated sum of all  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -unsaturated aldehydes (2E, 4E/Z isomeric mixture) in TR CCMP 1647 was 2.27 fmol PUA cell<sup>-1</sup>, while LD and SC contained only 0.04 fmol PUA cell<sup>-1</sup>. The composition of aldehyde was 52% heptadienal, 16% octadienal, 20% octatrienal, and 12% decatrienal in TR CCMP 1647; 41% heptadienal, 43% octadienal, and 16% octatrienal in *S. costatum*; and 21% decadienal and 79% decatrienal in *L. danicus*.

**Egg production and hatching success**—All diatoms tested had a negative but species-specific effect on the reproductive success of *Temora longicornis* (Fig. 1). In four out of six species or clones (TW, TR 1018, TR 1647, CA), egg production initially increased until days 3/4 and fluctuated thereafter around an average egg production of 11–14 eggs female<sup>-1</sup> d<sup>-1</sup> for the rest of the incubation period. In contrast, hatching success decreased from initially high rates (>70%) until day 4 to less than 40% on day 10, with the strongest decrease in TW. In the two other diatom species (LD, SC), both egg production and egg hatching success diminished rapidly after onset of feeding. With these species, egg production never exceeded more than 7 eggs female<sup>-1</sup> d<sup>-1</sup>. Only in control experiments with RHO did egg production remain high (>9 eggs female<sup>-1</sup> d<sup>-1</sup>) throughout the incubation, and egg viability never fell below 80% (Fig. 1B).

**Ingestion and efficiency of egg production**—Filtration rates were largely determined by the size of the algae. They were significantly higher for the two large TR strains but significantly lower for small RHO than the remaining intermediate-sized diatoms (one-way ANOVA,  $F_6 = 49$ ;  $p < 0.001$ ; Tukey HSD,  $p < 0.01$ ; Fig. 2A). Accordingly, daily rations in terms of carbon and nitrogen were also significantly higher in TR1647 and 1018 than in the other algae (ANOVA,  $F_6 = 99$  and 149 for carbon and nitrogen, respectively,  $p < 0.001$ ; Tukey HSD,  $p < 0.001$ ; Fig. 2B,C). Ingestion of lipids was additionally determined by the volume-specific fatty acid and sterol content of the algae (cf. Fig. 2D,E; Table 1). A similar, high daily ration of 18–22 PUFAs of >100 pg d<sup>-1</sup> was ingested by females feeding on a diet of TR1018, CA, and RHO. Significantly lower ingestion rates of <50 pg d<sup>-1</sup> were obtained by females fed the other diets, with particularly small rations for TR 1647, LD, and SC (ANOVA,  $F_6 = 143$ ,  $p < 0.001$ ; Tukey HSD,  $p < 0.01$ ). Ingestion of the three  $\Delta 5$  sterols was high for diets of RHO, TR 1647, TR 1018, and LD (average range 19–46 pg female<sup>-1</sup> d<sup>-1</sup>), but it was significantly lower when females were fed with TW, CA, and SC (ANOVA,  $F_6 = 111$ ,  $p < 0.001$ ; Tukey HSD,  $p < 0.001$ ). Females obtained a high ration of PUA for TR 1647 only (>100 nmol d<sup>-1</sup>; Fig. 2F), whereas PUA ingestion was low in experiments with LD and SC (<15 nmol d<sup>-1</sup>); these three species were the only algae containing detectable amounts of PUA.

Calculations of the efficiency of egg production showed that in RHO and TW, ingested carbon and nitrogen were

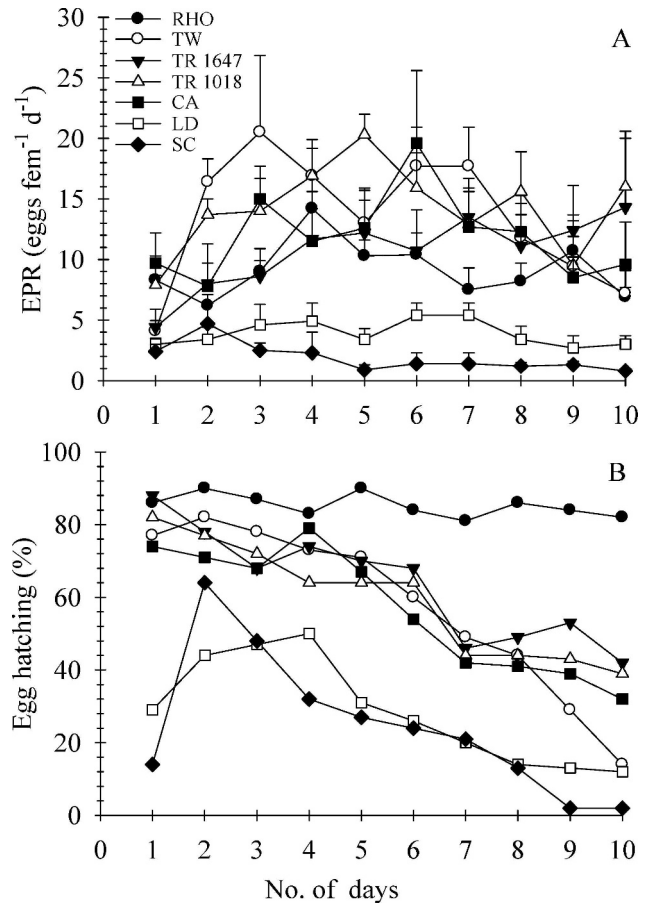


Fig. 1. (A) Egg production rate (eggs female<sup>-1</sup> d<sup>-1</sup> ± SD) and (B) egg hatching success (%) of *Temora longicornis* females feeding a cryptoflagellate (RHO, *Rhodomonas* spp.) or on diatoms (TW, *Thalassiosira weissflogii*; TR, *T. rotula* 1647, 1018; CA, *Chaetoceros affinis*; LD, *Leptocylindricus danicus*; SC, *Skeletonema costatum*) during the 10-d incubation.

utilized with similar efficiency, ranging from 0.19 to 0.23 (Table 2). In CA, a similar high rate was found for nitrogen but not carbon, following from the higher C:N ratio determined for the species. For all other diatoms (TR 1647, 1018, LD, SC), the efficiency for both elements was low and did not exceed 0.08.

**Fecal pellet production and gut passage time**—Fecal pellet production rates ranged from 40 to 80 pellets female<sup>-1</sup> d<sup>-1</sup> (Fig. 3A). Differences in pellet production between diets were significant (ANOVA,  $F_6 = 6.6$ ,  $p < 0.001$ ), and generally higher rates were observed in females feeding on diatoms than on RHO (Tukey HSD,  $p < 0.001$ ). Further, fecal pellets produced on a diet of diatoms were generally much longer and had, by a factor of 1.5 to 2.2, larger volume than those produced on a diet of RHO (Fig. 3B; ANOVA,  $F_6 = 22$ ,  $p < 0.001$ ; Tukey HSD,  $p < 0.001$ ). As a consequence, the total feces production was significantly and about 1.7- to 3.3-fold higher in females feeding on diatoms than in those feeding on RHO (Fig. 3C; ANOVA,  $F_6 = 26$ ,  $p < 0.001$ ; Tukey HSD,  $p < 0.001$ ). There was no direct relationship between the volume excreted and the

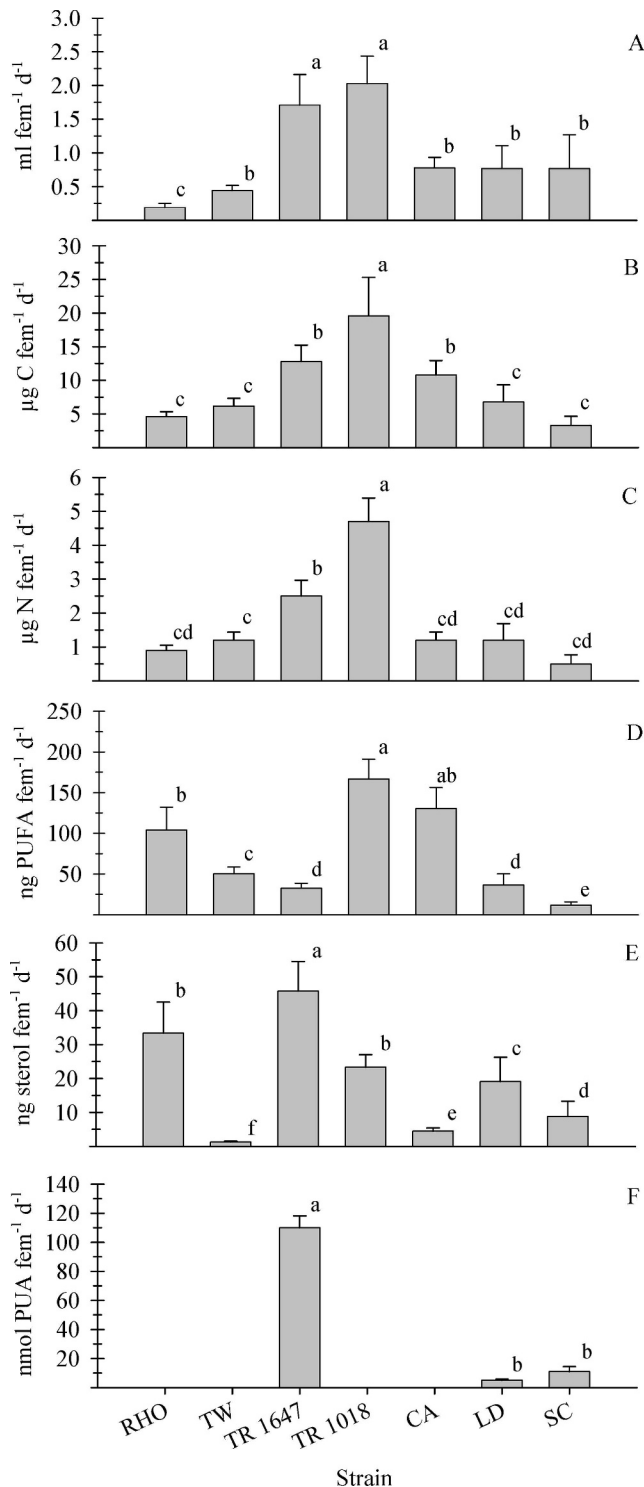


Fig. 2. (A) Filtration rate ( $\text{mL female}^{-1} \text{d}^{-1}$ ) and (B) ingestion of carbon ( $\mu\text{g C female}^{-1} \text{d}^{-1}$ ), (C) nitrogen ( $\mu\text{g N female}^{-1} \text{d}^{-1}$ ), (D) polyunsaturated fatty acids ( $\text{pg PUFA female}^{-1} \text{d}^{-1}$ ), (E) sterol ( $\text{pg sterol female}^{-1} \text{d}^{-1}$ ), or (F) polyunsaturated aldehydes ( $\text{nmol PUA female}^{-1} \text{d}^{-1}$ ) of *Temora longicornis* females feeding on diatoms and RHO (means  $\pm$  SD, abbreviations as in Fig. 1; different letters denote treatments that are significantly different from each other according to Tukey HSD,  $p < 0.05$ ).

volume ingested by females (Table 2). While females defecated roughly more than 45% of the volume ingested with RHO, TW, and CA diets, less than 25% of the ingested volume was egested with other diets. Estimates of the gut passage time, which is directly related to the pellet production rate, showed large differences depending on food type (Table 2). While gut passage time of RHO was on average 32 min, diatoms passed through the gut within significantly shorter periods of 19–24 min (ANOVA,  $F_4 = 20$ ,  $p < 0.001$ ; Tukey HSD,  $p < 0.001$ ). When the larger volume of pellets on the diatom diets is considered in the calculation (see Methods), gut passage time of diatoms decreases to 10 to 17 min (Table 2; ANOVA,  $F_6 = 82$ ,  $p < 0.001$ ; Tukey HSD,  $p < 0.001$ ).

*Relationships among egg production, hatching success, daily rations, and gut passage time*—Egg production of copepods was related to ingestion, as well in terms of carbon, nitrogen, 18–22 PUFAs, EPA, DHA, and total fatty acids (Spearman rank correlation;  $p < 0.05$ ; Fig. 4), although relationships were generally weak. In contrast, hatching success was not related to alga carbon, nitrogen, or to the ingestion of any of the nutritional components or diatom aldehydes (Spearman,  $p > 0.05$ ; Fig. 4A–H), but it was found to correlate significantly with the 22:6(n-3) to 20:5(n-3) fatty acid ratio (22:20 ratio in Fig. 4I; Spearman;  $p < 0.0001$ ). The egg hatching success also showed a significant, time-dependent relationship to the production of feces volume on diatom diets (Fig. 5A;  $r^2 = 0.91$  and 0.88 for days 6 and 9, respectively,  $p < 0.001$ ). The higher the feces production on a particular diatom strain/species, and the longer the females fed on this alga, the stronger was the observed reduction in egg hatching success.

## Discussion

*Impairment of reproduction by diatom aldehydes*—In agreement with previous laboratory investigations (see reviews of Ianora et al. 2003; Paffenhöfer et al. 2005), a species-specific inhibitory effect of diatoms on the reproductive success of *Temora longicornis* was observed. The algae used could be classified into three well-established categories (Ban et al. 1997): strains inducing high egg production but low hatching success (TW, TR 1018, TR 1647, CA), strains inducing both low egg production and hatching (LD, SC), and strains supporting both high egg production and hatching success (control alga RHO). However, similar diatom species have also produced inconsistent effects, with a strong diminution of fecundity or egg viability in one copepod but not another (Ban et al. 1997; Ianora et al. 2003). For instance, egg production and hatching success on a diet of the two strains of TR (1018 and 1647) has already been compared in an earlier investigation using *Calanus helgolandicus* as a test organism, with a different result if compared to the present study (Pohnert et al. 2002). Because only strain TR 1647 was found to produce PUA and to reduce egg viability, the variability in diatom effects has been largely attributed to differences in the PUA content of diatom strains (Ianora et al. 2003; Wichard et al. 2005a).

Table 2. Efficiency of egg production (E-EP) in terms of carbon and nitrogen, ratio of defecated to ingested volume (FP-ING), and gut passage time calculated from pellet production rates (GPT, min) or pellet production rates normalized to a pellet volume of RHO (GPT<sub>RHO</sub>, min; see Methods) (overall averages from experiments on days 3, 6, and 9 ± SD).

Species/strain	E-EP carbon	E-EP nitrogen	Ratio volume FP-ING	GPT (min)	GPT <sub>RHO</sub> (min)
<i>Rhodomonas</i> sp. (RHO)	0.19±0.004	0.21±0.025	0.58±0.041	32±3.4	32±2.2
<i>Thalassiosira weissflogii</i> (TW)	0.22±0.052	0.23±0.051	0.71±0.098	19±1.6	10±0.2
<i>T. rotula</i> CCMP 1647 (TR 1647)	0.07±0.011	0.08±0.012	0.18±0.034	21±2.8	13±1.5
<i>T. rotula</i> CCMP 1018 (TR 1018)	0.05±0.009	0.05±0.009	0.15±0.006	22±2.0	14±0.9
<i>Chaetoceros affinis</i> (CA)	0.11±0.040	0.22±0.075	0.45±0.087	21±1.7	10±0.5
<i>Leptocylindricus danicus</i> (LD)	0.04±0.014	0.06±0.012	0.27±0.058	24±2.8	14±2.4
<i>Skeletonema costatum</i> (SC)	0.06±0.033	0.07±0.047	0.26±0.013	24±3.1	17±2.5

The negative effect of all diatoms on egg viability or of SC and LD on egg production of *Temora longicornis* in our study was clearly not related to PUA production. Three out of six diatom strains tested were toxic in relation to PUA production, but they exerted different inhibitory activity on reproduction (Table 1; Fig. 1). While the mild PUA producers LD and SC reduced egg production, the high fecundity of *T. longicornis* on a diet of the strong PUA-producer TR 1647 contrasts with a potential inhibitory activity of PUA on copepod egg production. This result supports the recent observation that depressed egg production in *Calanus helgolandicus* during diatom blooms in the English Channel was not related to the production of PUA (Poulet et al. 2006). Among the diatoms that allowed a high egg production, the non-PUA producers TW and CA induced the strongest reduction in egg viability, followed by the two TR strains. Previous studies have observed a negative effect of TW (Uye 1996; Ceballos and Ianora 2003), for which the production of any of the various known PUA could not be confirmed yet (Wichard et al. 2005a). Also the earlier results of Pohnert et al. (2002) on the divergent response in the egg viability of *Calanus helgolandicus* to the same TR strains (TR 1647, TR 1018) could not be confirmed. Instead, our results provide a prime example for the inadequacy of aldehyde production in explaining the negative effects on hatching. Despite large differences in the PUA content (Table 1) and in the daily PUA ration of females (Fig. 2F) feeding on the two TR strains, the inhibitory activity of both strains was similar (Fig. 1). Our results, therefore, exclude the production of reactive unsaturated aldehydes as a universal mechanism for the effects of diatoms on both egg production and viability.

**Nutritional inadequacy of diatoms**—The analysis of the elemental and biochemical composition of the food algae also provided no evidence supporting a common dietary deficiency of diatoms. In previous laboratory and field studies, correlations between copepod production or egg hatching and the mineral or lipid composition of bulk seston or phytoplankton diets have been established (Jónasdóttir et al. 1995; Pond et al. 1996). This is supported by some, mostly freshwater, studies, which show an improvement of egg production and growth of zooplankton by the supplementation of diets with PUFA or sterol (Müller-Navarra 1995; von Elert et al. 2003; Hassett 2004), but it remains controversial in marine diatoms, particularly

with regard to their importance in controlling egg hatching (Lacoste et al. 2001; Jónasdóttir et al. 2005; Vargas et al. 2006).

Although the volume-specific PUFA and sterol contents of all diatoms were indeed 2- to 80-fold lower if compared to RHO, they were not generally deficient in bulk lipids, essential sterols, or PUFA, such as cholesterol, EPA, or DHA, which are traditionally known to determine reproductive success in crustaceans (Jónasdóttir and Kiørboe 1996; Klein Breteler et al. 1999; Hassett 2004). Instead, the higher feeding rates observed on diatoms compared to RHO often resulted in similar or even higher daily rations of carbon, nitrogen, sterol, and PUFA. This is clearly reflected in the higher egg production rates on diatom diets (except SC and LD). The significant albeit weak correlation of egg production with the daily ration of minerals and fatty acids suggests that egg production is primarily controlled by the amount of food ingested. However, ingested carbon and nitrogen was not persistently converted into egg production with the same efficiency for females fed diatoms as in females fed a diet of RHO (Table 2). In addition, a quite similar egg production rate in females was realized over a broad range of mineral and lipid rations, varying by a factor of 3 to 4, which suggests that much of the correlation was caused by poor egg production on the diet of SC and LD.

In contrast to egg production, no relation between the daily rations in carbon, nitrogen, or lipid and egg viability was observed. The significant relationship of egg hatching with the 22 : 20 fatty acid ratio, on the other hand, supports studies that indicate that the fatty acid composition of algae rather than the mineral or biochemical ration obtained by females is of some importance in controlling egg viability (Arendt et al. 2005; Jónasdóttir et al. 2005). Ratios larger than 2–2.5 have been reported to promote a high reproductive success in copepods (Arendt et al. 2005; Shin et al. 2003). Other studies, in contrast, could not confirm a general dependence of hatching on the fatty acid composition and reported high hatching rates on diets displaying a 22 : 20 fatty acid ratio smaller than 1, including diatoms (Jónasdóttir and Kiørboe 1996; Broglio et al. 2003). As a consequence, a large variation in egg viability is often observed at low 22 : 20 fatty acid ratios (Jónasdóttir and Kiørboe 1996; Broglio et al. 2003; Jónasdóttir et al. 2005), and this is supported by the large differences in egg viability (20–90%) observed over a narrow range of 22 : 20 fatty acid ratios, ranging from 0.04 to 0.87, in our study.

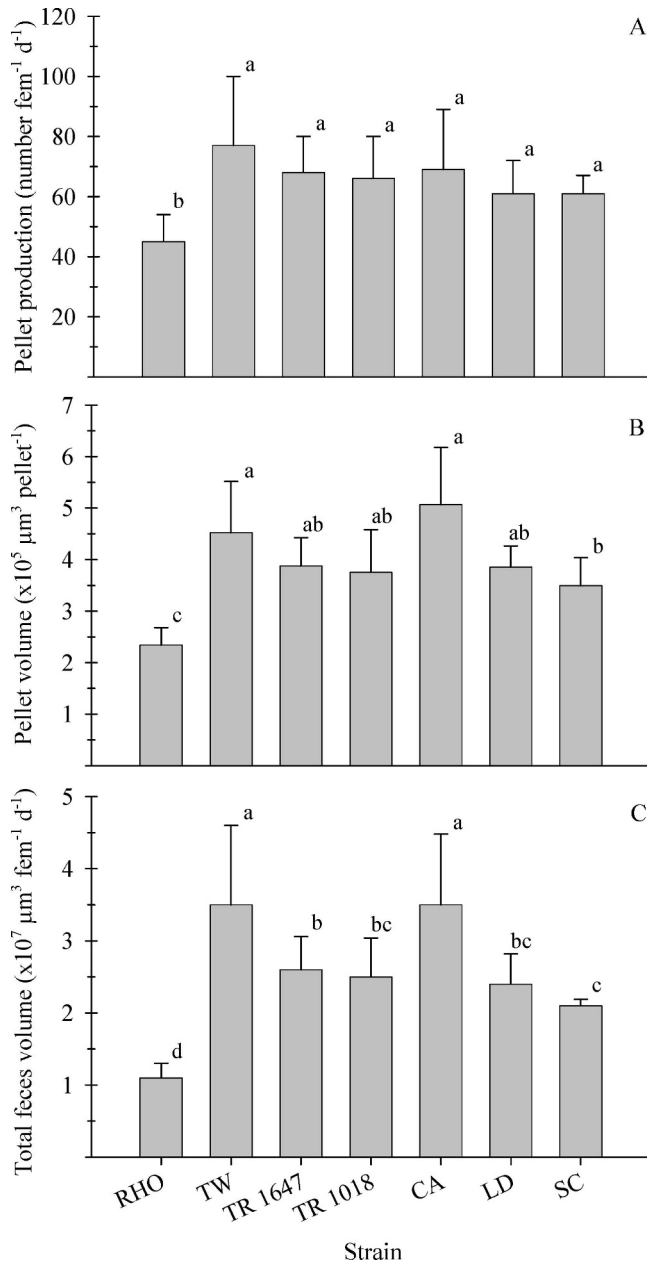


Fig. 3. (A) Number of fecal pellets (pellets female<sup>-1</sup> d<sup>-1</sup>), (B) pellet volume ( $\mu\text{m}^3$  pellet<sup>-1</sup>), and (C) total volume of feces ( $\mu\text{m}^3$  female<sup>-1</sup> d<sup>-1</sup>) produced by *Temora longicornis* females feeding on diatoms or RHO (means  $\pm$  SD, abbreviations as in Fig. 1; different letters denote treatments that are significantly different from each other according to Tukey HSD,  $p < 0.05$ ).

The significance of a correlation between egg viability and the 22 : 20 fatty acid ratio is unknown. The large variability in egg hatching success at low ratios has been interpreted as an indication for a requirement of balanced essential nutrient ratios in the diet (Jónasdóttir and Kiørboe 1996; Broglio et al. 2003). Low hatching, therefore, could indicate a deficiency in an essential food component, although it does not necessarily have to be in a lipid (Jónasdóttir and Kiørboe 1996; Jónasdóttir et al. 2005). In our study, no consistent deficiency in a lipid

compound could be detected, but often the rations were equal to or even higher than those on a diet of RHO. For instance, although females obtained a substantially lower daily ration of sterols with TW and CA than on the other diets, PUFA and sterol rations of females on a diet of TR 1018 were similar to those of females feeding on RHO. Irrespective of this, hatching on diets of all three diatoms was still strongly reduced. This excludes a sterol deficiency of marine diatoms as a mechanism behind the blanket effect of diatoms observed in our study.

*Alternative explanations*—We reject both traditional hypotheses concerning the negative effect of diatoms on egg viability—nutritional deficiency in lipid and aldehyde-induced apoptosis. Nevertheless, egg viability showed a significant, time-dependent decrease on diatom diets. The observed pattern is consistent with other studies that saw no effect of diatoms versus other diets on egg hatching in short-term incubations, whereas longer incubations generally produced a decrease in hatching success (e.g., Ianora et al. 2003). This gradual decrease in hatching could still indicate a depletion or accumulation of other, bioactive or essential compounds controlling egg viability, but it might not necessarily be caused by a single factor. Hitherto unknown toxins might cause the failure of egg production or hatching on diatom diets, as recently suggested by Poulet et al. (2006), but yet lack support from experimental studies and specific chemical analyses.

Apart from lipids and sterols, several other biochemical compounds, which were not included in our measurements, have been suggested as nutritionally essential for copepod reproduction (e.g., Ianora et al. 2003). These compounds include amino acids (AA), proteins, or various vitamins, and their deficiency in the diet may cause a low egg hatching response. Although a lack of essential AA has been shown to affect the reproductive success of copepods (Kleppel and Burkart 1995), support for an AA deficiency of diatoms as the cause for low egg viability is lacking (Laabir et al. 1999). In addition, close similarities in amino acid composition have been found between diatoms and other algae (Brown et al. 1997; Lourenço et al. 1998). Instead, large differences in vitamin content and composition, similar to those in lipids, have been observed among algae, but again diatoms do not appear to be particularly deficient in comparison to other algal classes (De Roeck-Holtzauer et al. 1991; Brown et al. 1997). Therefore, a diatom deficiency in a particular micronutrient does not appear to be plausible for the generic effect of diatoms on egg viability, unless multiple and species-specific deficiencies occur. This requires further investigation due to the limited data that are available.

Deficiencies in the diatom nutritional content, however, might become apparent only during food uptake and digestion. Wichard et al. (2007) recently demonstrated that the enzymatic conversion of PUFA by lipoxygenase and PUA-producing enzymes upon cell disruption caused a rapid depletion of in vitro PUFA levels in the diatom *Thalassiosira rotula* (CCMP 1647) by more than 60%. In contrast, PUFA contents of non-PUA-producing *T. weissflogii* were not affected by cell rupture. Thus, fatty acid

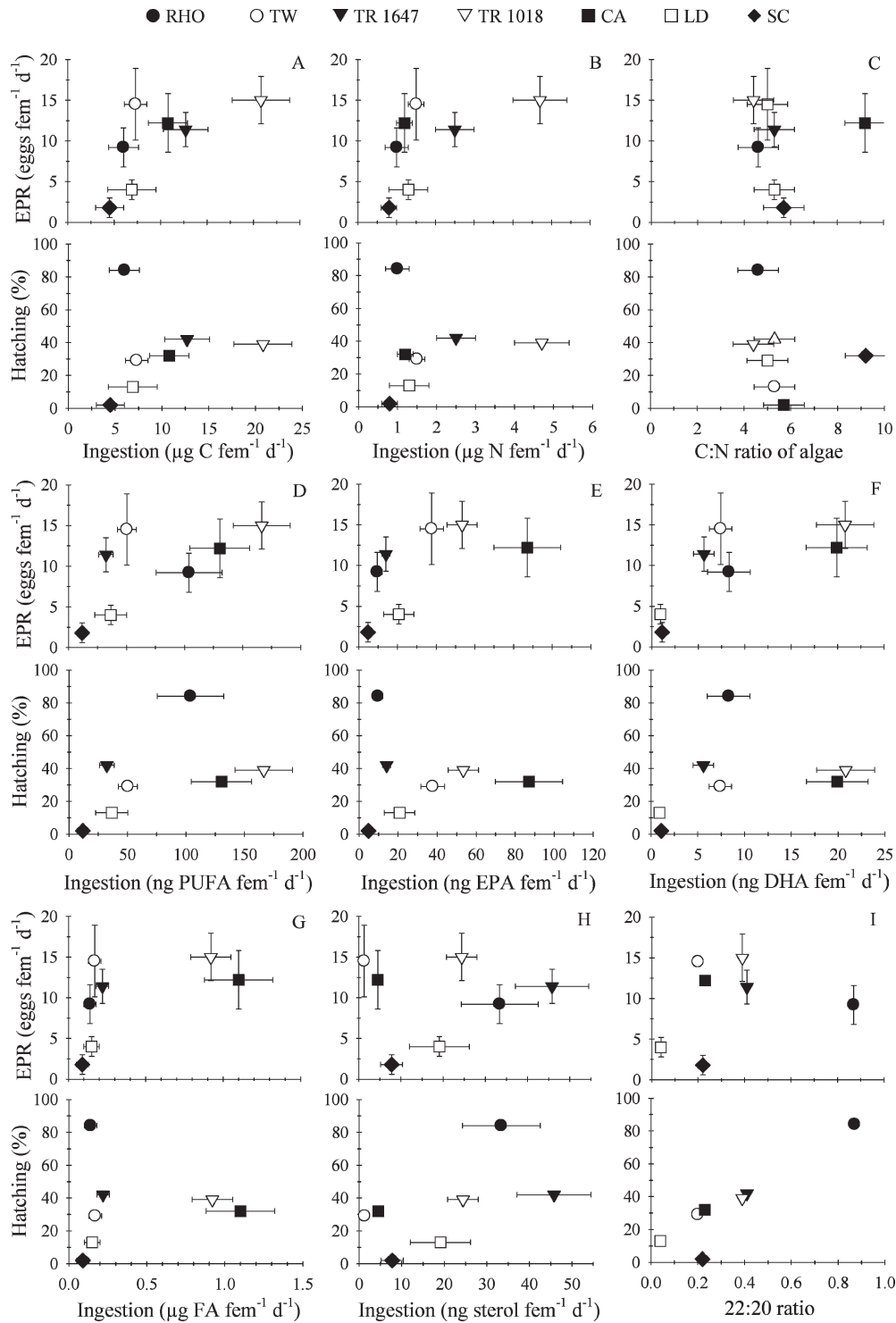


Fig. 4. Relationship between egg production (eggs female<sup>-1</sup> d<sup>-1</sup>, average of days 2–10) or hatching success (%; day 10) and (A) carbon (μg female<sup>-1</sup> d<sup>-1</sup>), (B) nitrogen (μg female<sup>-1</sup> d<sup>-1</sup>), (C) C:N ratio of algae, (D) 18–22 PUFA (ng female<sup>-1</sup> d<sup>-1</sup>), (E) EPA (ng female<sup>-1</sup> d<sup>-1</sup>), (F) DHA (ng female<sup>-1</sup> d<sup>-1</sup>), (G) total fatty acids (FA, μg female<sup>-1</sup> d<sup>-1</sup>), (H) sterol (ng female<sup>-1</sup> d<sup>-1</sup>), and (I) 22:20 (22:6[n-3] vs. 20:5[n-3]) fatty acid ratio of algae ingested by *Temora longicornis* females feeding on diatoms or RHO (means ± SD).

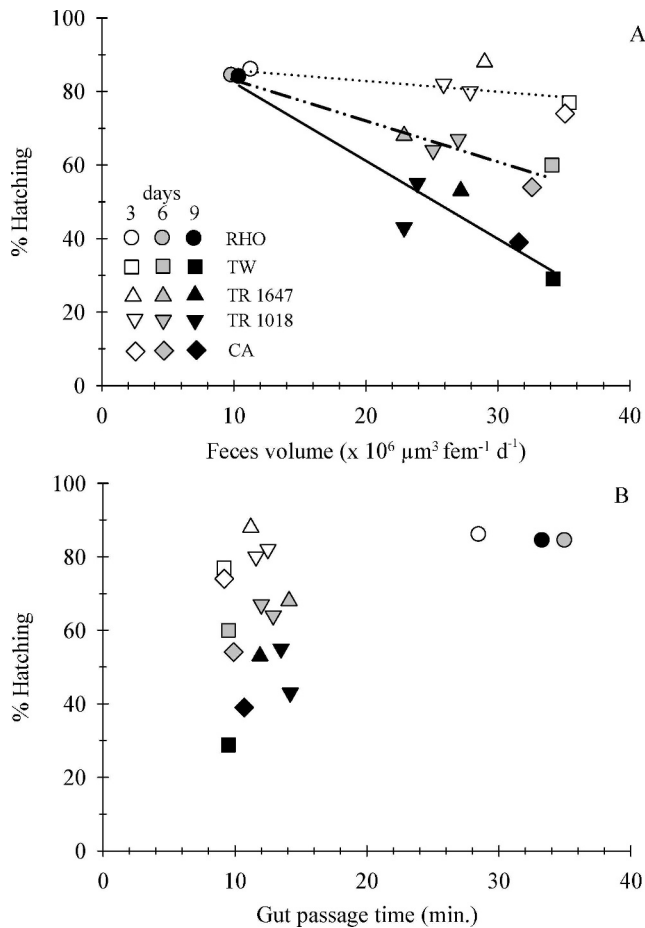


Fig. 5. (A) The relationship of egg hatching and the production of feces (in total volume per day) and (B) gut passage time (corrected for different pellet volume; see Methods) of *Temora longicornis*. Different filling of symbols indicates time span of feeding (open = day 3; dotted line, gray = day 6; dash-dot line; solid = day 9). Regression lines were significant for day 6 ( $r^2 = 0.91$ ,  $F_s = 41.5$ ,  $p < 0.001$ ) and day 9 ( $r^2 = 0.88$ ,  $F_s = 29.4$ ,  $p < 0.001$ ).

levels determined in diatom cultures may not necessarily reflect the effective availability of PUFA for copepods upon ingestion of diatoms producing PUA. Since initial PUFA degradation enzymes were active under physiological conditions in the copepod gut, PUA production could be indirectly responsible for the lack of sufficient PUFA to allow high egg hatching success (Wichard et al. 2007). However, while the enrichment of *T. rotula* by supplementation of EPA increased its concentration in the diet and in the copepod *Temora longicornis* by more than a factor of six and two, respectively, only a small increase in egg hatching was observed. This was interpreted as a stimulation of egg hatching by additional EPA (Wichard et al. 2007). However, these results might also indicate that the enzymatic degradation of PUFA in copepod guts is only minor in comparison to the degradation observed in vitro, or, in accordance with our results, that EPA is probably not controlling and, therefore, also not limiting to egg viability. Furthermore, the mechanism cannot generally account for the low egg viability observed in our study on

diets of non-PUA-producing TR 1018 or CA, which did not appear particularly deficient in PUFA. Finally, egg production rather than egg hatching is expected to depend on the availability of PUFA (Pond et al. 1996; Broglio et al. 2003), but, unfortunately, results on the fecundity of *T. longicornis* were not shown by Wichard et al. (2007).

In our study, the only significant correlation found was between egg hatching and copepod feces production. As a consequence of high feces production, the passage time of diatoms through the gut of *Temora longicornis* was considerably shorter than that of RHO. This is consistent with earlier studies that have demonstrated a higher fecal pellet production of copepods and a lower gut passage time on diatoms than on other food (Besiktepe and Dam 2002; Tirelli and Mayzaud 2005). The shorter residence times of diatoms in the gut of *T. longicornis* might have caused physiological constraints on digestion or assimilation. Recent studies on the fate of phytoplankton minerals, trace metals, or lipids have indicated that the assimilation efficiency of some trace elements and sterols may decrease with a shorter gut residence time, while other compounds, such as PUFAs, are not affected (Harvey et al. 1987; Cowie and Hedges 1996; Xu and Wang 2001). Therefore, it is conceivable that the saturating concentrations at which diatoms are regularly fed to copepods in studies of egg production and egg viability induce high feeding rate and feces production, and short gut-passage time, which thus create imbalances in lipids or unknown compounds important for the reproductive success of copepods. Our results demonstrating a time-dependent effect of feces production on hatching success (Fig. 5) could indicate such imbalances caused by fast gut passage and therefore low assimilation efficiency of the less available (e.g., membrane-bound) elements.

The often observed higher fecal pellet production of copepods fed diatom diets in comparison to flagellates or dinoflagellates intuitively suggests that diatom silica frustules, which are not digested, increase the volume of feces and cause a faster gut evacuation. However, the relation of the egested volume to the ingested volume was higher on the control diet RHO than on the diet of five out of six diatoms (Table 2). Therefore, constraints on digestion or assimilation could result from the denser packing of frustules in pellets on a diatom diet; this requires further investigation.

The suppression of the reproductive success of copepods despite high zooplankton growth rates constitutes a major paradox in copepod–diatom interactions (Ban et al. 1997). Although various studies have been conducted to investigate the negative effect of diatoms on reproduction, the mechanisms, significance, and relevance of the diatom effects on egg viability or the cause for the complete inhibition of copepod reproduction by diatoms such as LD and SC are still unknown. The current lack of a distinct mechanism for the observed inhibitory activity of diatoms suggests that the cause might be more complex, species-specific, and potentially involve multiple factors. We suggest that further studies should focus on the identity of hitherto unknown compounds of antiproliferative activity and on the assimilation of nutritional compounds. Enzymatic degradation or incomplete digestion of essential

nutritional elements could mask the direct relationships between any single nutritional compound and reduction in hatching success.

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