Marine Biology (1995) 121: 533–539

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A comparative study of the inhibitory effect of diatoms on the reproductive biology of the copepod *Temora stylifera*

Received: 19 July 1994 / Accepted: 19 September 1994

Abstract Egg production and viability in the copepod *Temora stylifera* (collected in the Bay of Naples, Italy in 1992) were strongly dependent on food type. A flagellate (*Isochrysis galbana*) diet induced the production of good quality eggs that developed to hatching. By contrast, two diatoms (*Chaetoceros curvisetum, Phaeodactylum tricornutum*) resulted in poor egg quality, with hatching success as low as 20% of total egg production. With the third diatom tested, *Skeletonema costatum*, females produced eggs for only 3 to 4 d, after which time they either became sterile or died. These results are discussed in relation to previous findings regarding the impact of the dinoflagellate *Prorocentrum minimum* and the diatom *Thalassiosira rotula* on the hatching success of *T. stylifera* eggs. Low egg viability was possibly not due to an absence of remating or a deficiency of some specific essential nutrient required for egg development but to the presence of inhibitory compounds blocking cell division during early copepod embryogenesis. This questions the traditional view that diatoms are an important food item regulating copepod secondary production.

Introduction

Food composition plays a major role in the fecundity and hatching success of copepod eggs even though food properties and characteristics responsible for these variations are not well established. Differences in the reproductive responses of copepods have been attributed to subtle differences between diatoms and flagellates such as an absence or presence of cell wall silica, shape and size of cells, palatability or nutrient concentrations per unit cell volume (Hitchcock 1982; Poulet 1983; Huntley et al. 1986; Paffenhöfer 1988). Other studies have stressed the role of environmental factors, such as temperature and nutrient recycling (Kiørboe et al. 1988; Van Rijswijk et al. 1989), or of biological factors such as remating (Wilson and Parrish 1971; Parrish and Wilson 1978; Ianora et al. 1989), in determining fluctuations in reproductive rates. Still other studies have emphasized the importance of essential compounds for growth or reproduction such as fatty acids, proteins or vitamins, to explain the better quality of dinoflagellates or microzooplankton compared to diatoms in inducing higher fecundity (Kleppel et al. 1991; Ianora and Poulet 1993).

Recent studies on *Temora stylifera* and *Calanus helgolandicus* demonstrated that the diatom *Thalassiosira rotula* induced up to 100% egg mortality, due to blockage of egg development, when fed to copepods (Ianora and Poulet 1993) or when eggs were exposed to dense extracts of this diatom (Poulet et al. 1994). On the basis of these results, we proposed an alternative explanation for low hatching success in copepods, instead of food deficiency, namely, the presence of inhibitory substances in diatoms which may block egg embryogenesis. In our paper we present evidence that this inhibition is induced by at least two other species of diatoms but not by a flagellate diet. This is part of a continuing study on the impact of diatoms on the reproductive response of copepods, including viable egg production.

Materials and methods

Temora stylifera specimens were collected in the Bay of Naples, Italy, from July to November 1992, from 0 to 50 m depth, and transported within 1 h from sampling to the laboratory. There, female and male couples were sorted and incubated as individual pairs in crystallizing dishes containing 100 ml ambient bay water for 24 h to determine in situ fecundity and egg viability. After 24 h, couples were transferred to new containers with 100 ml of 0.45-µm filtered seawater enriched with 10 ml of phytoplankton culture. Phytoplankton species tested were the three diatoms, namely, *Chaetoceros curvisetum* (CHA) (length: 22 to 25 µm; width: 6.5 to 7 µm), *Phaeodacty-*

Communicated by M. Sarà, Genova

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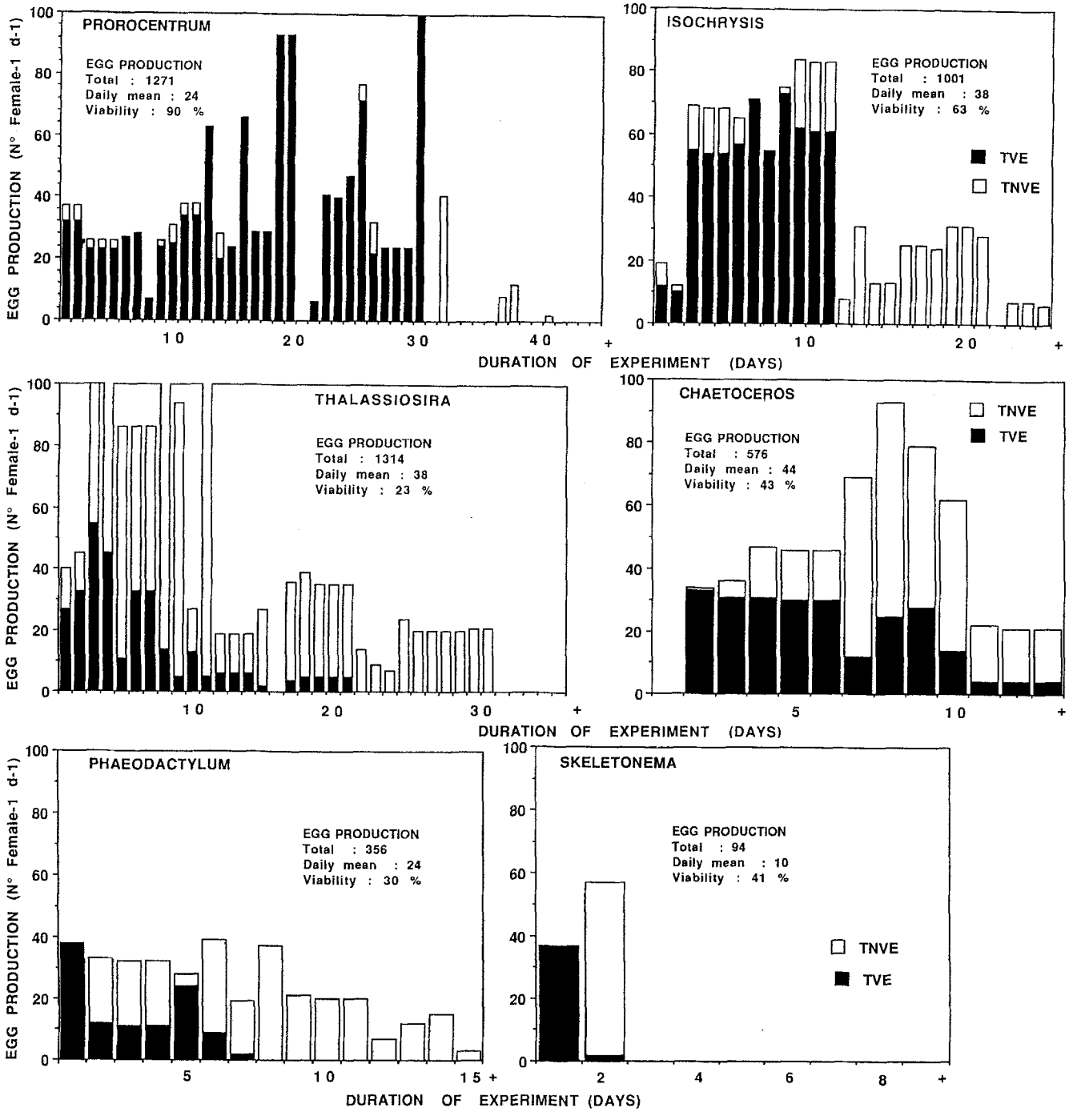

Fig. 1 *Temora stylifera*. Daily egg production and viability for representative couples fed with two non-diatom diets (*Prorocentrum*: *Prorocentrum minimum*; *Isochrysis*: *Isochrysis galbana*) and one of four diatom diets (*Thalassiosira*: *Thalassiosira rotula*; *Chaetoceros*: *Chaetoceros curvisetum*; *Phaeodactylum*: *Phaeodactylum tricornutum*; *Skeletonema*: *Skeletonema costatum*). *Prorocentrum* and *Thalassiosira* are borrowed from Ianora and Pouled 1993. (*TVE* production of viable eggs; *TNVE* production of non-viable eggs; *TVE* + *TNVE* corresponds to total egg production rate; + death of females)

Fig. 2 *Temora stylifera*. Daily egg production and viability for all couples fed with ISO (*Isochrysis galbana*), PRO (*Prorocentrum minimum*) diets and four diatom diets (*SKE*: *Skeletonema costatum*; *THA*: *Thalassiosira rotula*; *PHA*: *Phaeodactylum tricornutum*; *CHA*: *Chaetoceros curvisetum*). PRO and THA data borrowed from Ianora and Poulet 1993. (*TVE* production of viable eggs; *TNVE* production of non-viable eggs; *TVE* + *TNVE* corresponds to total egg production rate)

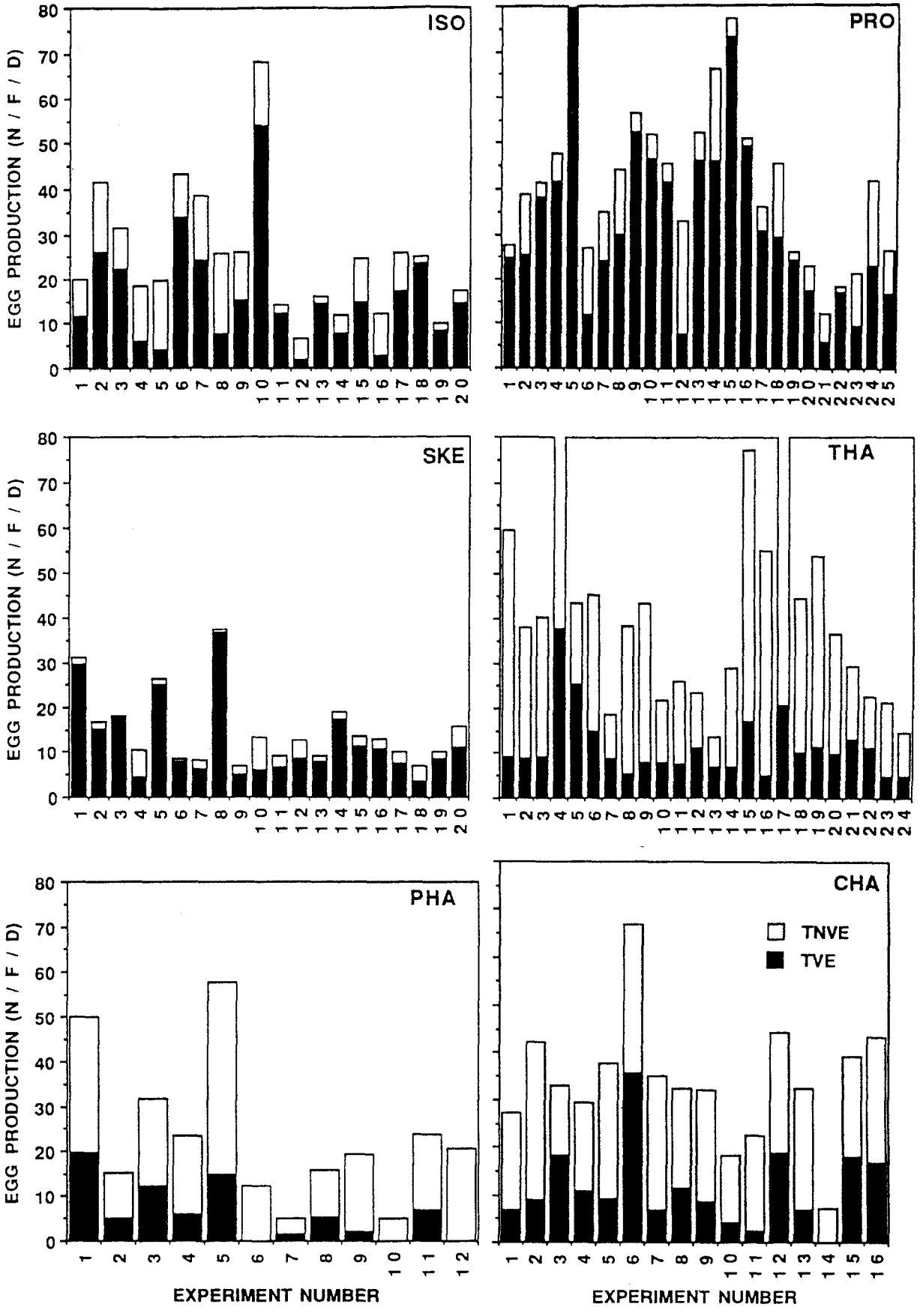
ISO
PRO
SKE
THA
PHA
CHA
TNVE
TVE
EGG PRODUCTION (N / F / D)
EXPERIMENT NUMBER

Table 1 *Temora stylifera*. Summary of results (mean, SD, and statistical tests between grouped values obtained with diatom and non-diatom diets) of feeding/egg production experiments with *T. stylifera* couples fed two non-diatom (*Prorocentrum, Isochrysis*; as in Fig. 1) and four different diatom diets (*Thalassiosira, Skeletonema, Phaeodactylum, Chaetoceros*, same as in Fig. 1) [*N* number of observations; *TE* total eggs female^{-1} d^{-1}; *TVE* total viable eggs female^{-1} d^{-1}; *FP* fecal pellets couple^{-1} d^{-1}; *S* spermatophores male^{-1} d^{-1}; *L* longevity of female (in d); *df* degrees of freedom]

Biological response	Type of food					
	Non-diatom diet		Diatom diet			
	Prorocentrum[a] (*N*=25)	Isochrysis (*N*=20)	Thalassiosira[a] (*N*=24)	Skeletonema (*N*=20)	Phaeodactylum (*N*=12)	Chaetoceros (*N*=16)
TE	40±20	25±14	40±20	15±8	23±16	34±13
TVE	30±20	16±12	10±7	12±9	6±6	11±8
FP	40±20	57±35	100±40	37±17	120±29	103±36
S	0.7±0.6	0.3±0.3	0.4±0.4	0.09±0.15	0.46±0.21	0.43±0.35
L	30±20	24.5±12	20±8	9.8±2.6	17±6.4	13.6±7

Biological response	Type of food		Significance of statistical test	
	Non-diatom diet total (*N*=45)	Diatom diet total (*N*=72)	*F*-test	*t*-test df=115
TE	32.5±17	28±22	*F*=1.67+	*t*=1.17+
TVE	23±16	10±7	*F*=5.22**	*t*=5.15**
FP	48±27	90±30	*F*=1.23+	*t*=7.41**
S	0.5±0.45	0.34±0.28	*F*=2.5**	*t*=2.28*
L	27±16	15.1±6	*F*=1.63+	*t*=3.28**

[a] Borrowed from Ianora and Poulet (1993)
+ Non-significantly different for $P=0.05$ and $F \leq (0.95)$
* Significantly different for $P=0.01$ and $F<(0.95)$
** Highly significantly different for $P \geq 0.001$ and $F<(0.95)$

lum tricornutum (PHA) (length: 21.7 to 28 µm; width: 3.8 to 4 µm), *Skeletonema costatum* (SKE) (length: 14 to 18 µm; width: 3.8 to 4.2 µm) and the prymnophycean *Isochrysis galbana* (ISO) (length: 6.8 to 7.2 µm; width: 3.8 to 4.2 µm). Cell culture concentrations ranged from 10^4 to 10^5 for CHA, PHA, and SKE and from 10^5 to 10^6 cells ml^{-1} for the much smaller flagellate ISO. Final food concentration was, therefore, 10^5 to 10^7 cells crystallizing dish^{-1} copepod^{-1}, depending on the algal cells. Such densities were close to or in excess of daily food requirements reported in the literature. For example, Frost (1985) reports saturation feeding rates for *Calanus pacificus* of 2.88×10^5 cells copepod^{-1} d^{-1} at food concentrations of 3 to 10×10^3 cells ml^{-1}. Phytoplankton were cultured in K-medium for ISO and K+silica for CHA, PHA and SKE (Keller et al. 1987) at 20 °C and on a 12 h light:12 h dark cycle and provided to copepods in exponential or stationary phases of growth.

A total of 20 *Temora stylifera* couples were maintained with ISO, 20 with SKE, 16 with CHA and 12 with PHA. Each day, couples were transferred to new containers with fresh media and a daily record was kept of egg production, egg viability, and number of fecal pellets and spermatophores. Hatching success was determined 48 h after spawning by adding 25 ml of 95% ethyl alcohol and counting nauplii after they had settled on container bottoms. We also distinguished crumpled egg membranes due to cannibalism which were included in the daily tally of egg production but were excluded from the calculation of % viability. All experiments were conducted at 20 °C using a 12 h light:12 h dark cycle and lasted until death of the females. We consider female age as the number of days they were maintained on a given diet, irrespective of their age prior to capture.

In another series of experiments, wild females were sorted in the laboratory in 200-ml crystallizing dishes with ambient by water and eggs were collected as they were spawned. This was done by scanning container bottoms every 10 min with an inverted microscope and transferring newly spawned eggs to 5-ml tissue culture wells. Eggs were immediately exposed to increasing concentrations of extracts of the diatom *Thalassiosira rotula* (THA) (30 to 40 µm in diameter; carbon content: 157.62±15.37 pg cell^{-1}) and the dinoflagellate *Prorocentrum minimum* (PRO) (length: 14 to 22 µm; width: 10 to 15 µm: carbon content: 274.16±2.53 pg cell^{-1}) used to determine egg production and viability in *Temora stylifera* in a previous study (Ianora and Poulet 1993). Increasing concentrations of each phytoplankton extract were diluted in 1 ml of 0.22-µm filtered seawater to give final concentrations in tissue cluster wells corresponding to 10^2 to 10^6 cells ml^{-1}.

Extracts were prepared according to the protocol given by Poulet et al. (1994). Phytoplankton cells in either exponential or stationary growth stages were concentrated by centrifugation at 1000 rpm (Sorvall SS34 centrifuge at 4 °C) for 10 min. The supernatant was removed and phytoplankton pellets were homogenized with a teflon pester and then sonicated three times for 30 s with a Branson sonifier to ensure extraction of cellular contents. Homogenate was recentrifuged at 14000 rpm ($23600 \times g$) for 10 min at 4 °C and used immediately or stored at −20 °C. Prior to use, extracts were filtered through Millipore 0.22-µm disposable filter units to remove debris. Hatching success was determined after 48 h for control eggs and those exposed to phytoplankton extracts.

Results

Daily egg production and hatching success are illustrated in Fig. 1 for a representative *Temora stylifera* female and male couple for each of the food regimes tested (ISO, CHA, PHA and SKE). Fig. 1 also shows the effect of the diatom THA and the dinoflagellate PRO tested on *T. stylifera* couples under similar experimental conditions in a previous

study (Ianora and Poulet 1993). With the non-diatom diets ISO and PRO, spawning was intense and continuous, decreasing until death of the females. Egg viability remained high and stable with time for PRO but decreased after 12 d of incubation with ISO. With the diatoms THA, PHA and CHA, spawning was as intense as with the non-diatom diets, but low egg viability was observed after 3 to 4 d of incubation even though the presence of spermatophores indicated that remating had occurred. With the diatom SKE, egg production was arrested altogether after a few days of incubation, but egg viability was high and mainly reflected previous feeding history of the copepods. With all diets tested, egg production and hatching success were higher at the beginning of the experiments and decreased with increasing female age.

When all data were pooled, THA was the best of the four diatoms tested, inducing the highest production of eggs, whereas the lowest rates were obtained with SKE (Fig. 2). CHA and PHA were also good food items for egg production, inducing as high or higher spawning rates than the flagellate diet ISO. Of the two non-diatoms, PRO was the better diet. In terms of egg viability, non-diatoms were better than diatoms, with the exception of SKE (Fig. 2 and Table 1). Statistical differences among diets indicated no significant differences between non-diatom and diatom diets for total egg production (TE) but significant differences for viable egg production (TVE) with greater hatching success obtained with the non-diatom diets (Table 1).

Also, fecal pellet production differed with diet (Table 1). It was higher with the three diatoms THA, CHA and PHA and lower with the non-diatom diets and with the diatom SKE. Spermatophore production was highest with PRO and lowest with SKE. With regards to longevity, the longest life span was achieved with PRO. The diatom SKE was the worst algae for longevity, paralleling results obtained for egg production.

A summary of the reproductive response of *Temora stylifera* to all diets tested is illustrated in Fig. 3. Highest egg production was obtained with the diatom THA and the dinoflagellate PRO (40 eggs female^{-1} d^{-1}) (Fig. 3 A). Also, egg viability differed between diets. It was always below 50% with the diatoms PHA, THA and CHA compared to the non-diatoms ISO and PRO, where mean hatching success was always greater than 65% (Fig. 3 B). The only exception was SKE, with egg viability as high as with the non-diatom diets. In situ mean egg production (50 eggs female^{-1} d^{-1}) was somewhat higher than with the laboratory diets, but mean egg viability was very similar to the diatom SKE and the non-diatoms PRO and ISO.

We suspected that failure to hatch was due to the presence of inhibitory substances present in diatom but not flagellate diets. To test this assumption, we exposed newly spawned *Temora stylifera* eggs from wild females to increasing concentrations of extracts obtained from the diatom THA and dinoflagellate PRO. Egg development proceeded normally for eggs treated with extracts up to a concentration of 10^4 cells ml^{-1}. Beyond this concentration, development was blocked in eggs treated with THA extracts but proceeded normally in PRO extracts and in control eggs

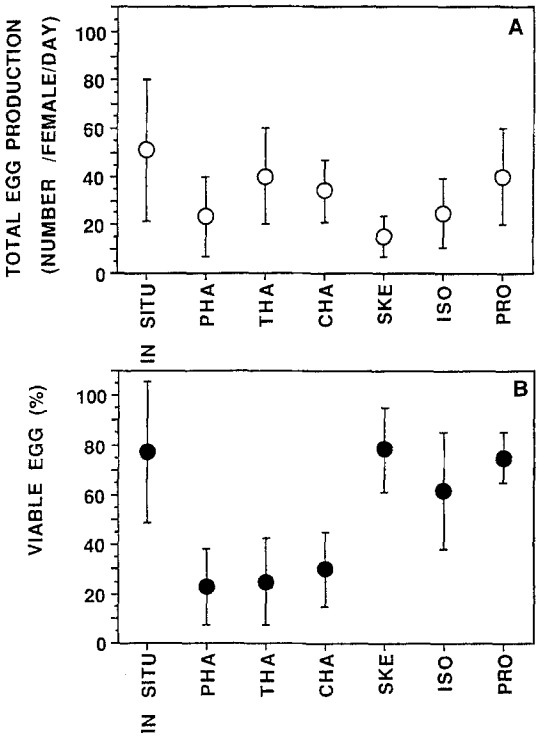

Fig. 3 *Temora stylifera*. **A** Comparison between egg production rates and **B** viability in couples fed diatom (*PHA*: *Phaeodactylum tricornutum*; *THA*: *Thalassiosira rotula*; *CHA*: *Chaetoceros curvisetum*; *SKE*: *Skeletonema costatum*) and non-diatom (*ISO*: *Isochrysis galbana*; *PRO*: *Prorocentrum minimum*) diets. In situ rates refer to initial fertility and egg viability in couples incubated in bay water. Results are mean and SD, as in Table 1. In situ, THA and PRO data borrowed from Ianora and Poulet (1993)

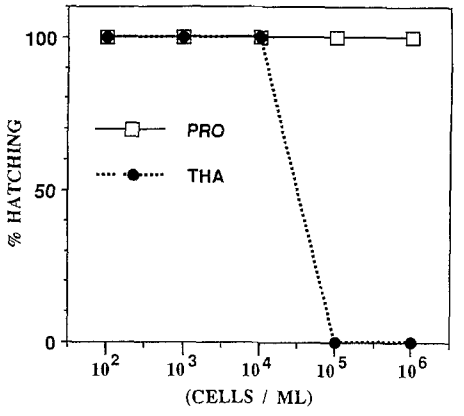

Fig. 4 *Temora stylifera*. Dilution experiments showing % eggs that develop to hatching when exposed to extracts from the dinoflagellate *Prorocentrum minimum* (*PRO*) and the diatom *Thalassiosira rotula* (*THA*). Eggs failed to hatch with THA > 10^4 cells ml^{-1}; development was normal with PRO extracts or for control eggs maintained in filtered seawater

(Fig. 4). In the case of eggs treated with dense THA extracts (≥10^5 cells ml^{-1}), severe cytolysis was observed with degradation of the cytoplasma and surface membrane, and all eggs failed to develop to hatching (Fig. 5 B). With PRO extracts, at all concentrations tested (10^2 to 10^6 cells ml^{-1}),

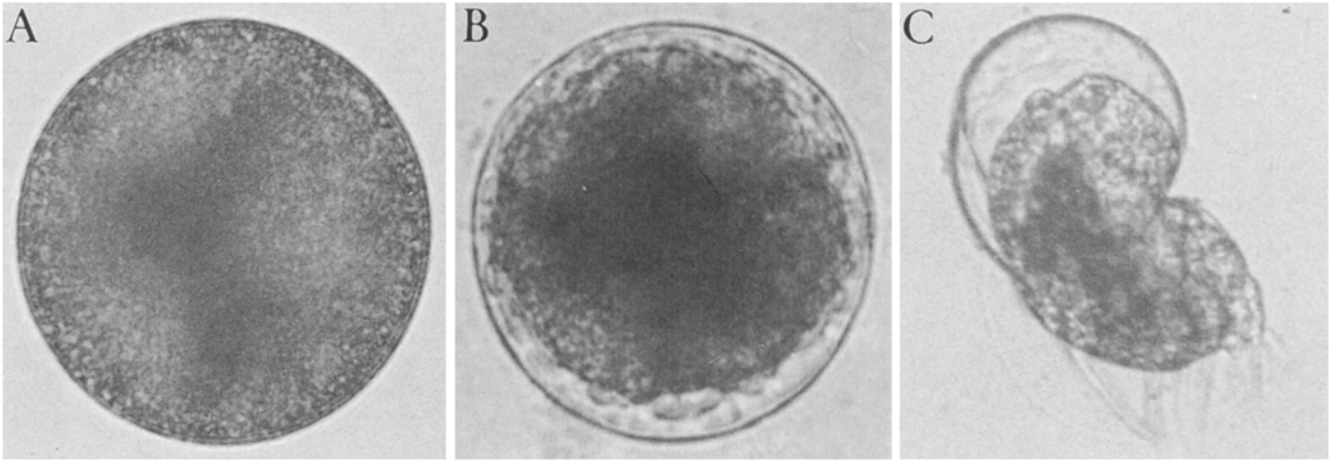

Fig. 5 *Temora stylifera*. Light microscope photograph of a **A** newly spawned viable egg; **B** newly spawned egg exposed to dense extracts (10^6 cells ml^{-1}) of the diatom *Thalassiosira rotula*, which fails to develop to hatching; and **C** egg exposed to dense extracts (10^6 cells ml^{-1}) of the dinoflagellate *Prorocentrum minimum*, which develops normally to hatching

egg development proceeded normally, leading to the hatching of living nauplii (Fig. 5 C). A normal, viable *T. stylifera* egg is shown in Fig. 5 A.

Discussion

The present results indicate that the diatoms offered in our studies are a poor food item for the reproductive biology of copepods. All three diatom species tested induced either lower egg production (SKE) or lower hatching success (CHA, PHA) in *Temora stylifera*, supporting previous results with the diatom THA (Ianora and Poulet 1993). The three diatoms THA, CHA and PHA also induced higher fecal pellet production, implying a poorer efficiency in the assimilation of these diets or the accumulation of more remaining products probably due to an inability to break down diatom silica frustules. With SKE, copepods produced less eggs and fecal pellets because they presumably ate less of this food. The poor quality of SKE was also inferred from the low number of spermatophores produced by males and the shorter life span of females. In all cases, cell concentrations provided to copepod couples were in excess of the range of cells used in saturation feeding experiments (e.g. Frost 1985), indicating that these effects were not due to food limitation.

Poor hatching success with a diatom diet may be due to blockage of egg development caused by unidentified inhibitors likely contained in three diatoms (THA, CHA, PHA) and perhaps also in SKE. The possibility that diatoms contain inhibitory compounds arresting copepod egg development was first described with THA, using both *Calanus helgolandicus* and sea urchin eggs (Poulet et al. 1994). Also in *Temora stylifera*, egg viability was significantly lower with a diatom diet (with the exception of SKE), and blockage of egg development occurred when newly spawned eggs were exposed to dense extracts of the diatom THA but not to the dinoflagellate PRO (Figs. 4, 5).

The mode of action of these inhibitors is still unknown, but we suspect that a possible mechanism of transfer is via feeding, diffusion through the gut epithelium in close contact to the ovary (Blades-Eckelbarger 1986), and accumulation in the oocytes during vitellogenesis. The results obtained with SKE may help to explain this mechanism. SKE was the worst algae tested. The result was sterility or death after only a few days of feeding even though egg viability was high. Assuming that the inhibitors are also present in SKE, the low number of fecal pellets obtained with this diatom may illustrate the link between feeding and egg inhibition.

Another result which lends support to the idea that these inhibitors are accumulated in the gonads is inferred from the high initial viability observed for all diatom diets. This was followed by a progressive diminution in hatching success on successive days, indicating that there may be a "lag phase" of ca. 24 to 72 h between food ingestion and subsequent accumulation in the gonads. The findings from extract experiments also suggest that these compounds are accumulated in the gonads. With diatom extracts, blockade of egg development occurred only at cell concentrations of $\geq 10^5$ cells ml^{-1}, which is much higher than the concentration of cells in naturally occurring diatom blooms or saturation feeding levels reported in the literature (e.g. Frost 1985).

Another possible cause for low hatching rates may be related to the chemistry of the food. Ianora and Poulet (1993) recently explored this possibility by screening 11 biochemical compounds in THA and PRO known to be essential for egg development in other crustaceans. Their results did not indicate substantial differences between these algae. In addition, food was given at libitum levels in our experiments, so that any deficiency of a given essential compound could have been compensated for by feeding, provided that it was not absent from the diet (see Table 2 in Ianora and Poulet 1993). Also, diatoms are known to be a good food for copepod development and growth (Paffenhöfer 1970; Paffenhöfer and Harris 1979) and are widely used in aquaculture. It follows, at this point,

that food characteristics sustaining high egg production and copepod development may differ from those required for viable egg production.

A third possible cause for blockage of egg development may be due to inhibitory compounds originating from virus or bacteria associated with diatoms and not contained in the diatom cells. This is unlikely since Poulet et al. (1994) checked bacterial levels in cultures used for extracts or fed to copepods and found that they were low and the same for both THA and PRO. Also, copepods are not known to feed actively on bacteria (reviewed by Lampert 1987).

Female age also seems to play a fundamental role in the production of fertile eggs. Fig. 1 shows that % viability decreases during the copepods life with all diets tested. However, in the case of diatoms, this reduction seems to occur much earlier, possibly due to unknown substances in the food which affect viable egg production. Female age also affected fecundity, which was much lower towards the end of the female's lifespan, as also observed by other investigators (e.g. Durbin et al. 1992 and references therein).

The copepod literature postulates that food concentration and quality affect fecundity and that non-diatoms are often better food organisms than diatoms for inducing higher egg production (Kleppel et al. 1991; Ianora and Poulet 1993). Fecundity has also been related to phytoplankton characteristics such as accessibility, ornamentation, and shape and size of cells (Gill and Harris 1987) or attributed to copepod feeding ability such as selectivity and digestive enzyme capacity (Mayzaud and Poulet 1978; Mayzaud et al. 1992). These factors are all equally valid to ultimately explain differences in egg production since they induce, in one way or another, food limitation. But they cannot explain low egg viability related to diatoms when fed at libitum to copepods (Figs. 1 to 3, Table 1). On the basis of the present results, and those published elsewhere (Ianora and Poulet 1993; Poulet et al. 1994), the trophic link between primary and secondary production should be re-assessed by distinguishing between the characteristics of food that are good for growth and those which are not favorable to fertility and egg viability.

Acknowledgements This work was supported by a CNRS-CNR grant MDRCI No. 29 and by both Naples and Roscoff Marine Stations. We thank F. Esposito for his technical assistance and for preparing phytoplankton cultures, and G. Gargiulo for the photographs. Special thanks are also due to Dr. M. Montresor for helpful discussions and suggestions.

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