

First evidence of some dinoflagellates reducing male copepod fertilization capacity

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Abstract

Evidence is presented that hatching failure in *Temora stylifera* eggs can depend on poor sperm quality. Three dinoflagellate diets, *Prorocentrum micans*, *Gymnodinium sanguinium*, and *Gonyaulax polyedra*, significantly modified spermatophore production and reduced the fertilization capacity of male sperm after 6–12 d of continuous feeding. Two other diets, the dinoflagellate *P. minimum* and the prymnesiophycean *Isochrysis galbana*, had no effect on hatching success, which remained high (>89%) and stable with time. A reduction in fertilization capacity was neither due to maternal effects nor to male age since hatching success returned to normal upon the introduction of freshly caught wild males or males conditioned with a good diet such as *P. minimum* for the same length of time as couples fed with the poor diets, *P. micans*, *G. sanguinium*, and *G. polyedra*. Confocal microscope images of unhatched eggs colored with a nucleus-specific fluorescent dye confirmed that these eggs had not been fertilized. Experiments with *Calanus helgolandicus* females, which did not require reinsemination and which were fed the same diets that induced hatching failure in *T. stylifera*, showed no change in hatching success with time.

Copepod fecundity has been shown to be strongly related to food type, with some foods that are better than others for promoting higher egg production. High production has been reported with dinoflagellate and ciliate diets (Gill and Harris 1987; Kleppel et al. 1991) since these are estimated to provide more protein, carbohydrates, and lipids than diatoms of equivalent volume (Kleppel 1993). But some dinoflagellates (Huntley et al. 1986) and microzooplankton (Stoecker and Egloff 1987; Sanders and Wickham 1993) appear to be poor food items for copepod production.

Much less is known about how diet affects egg viability. Recent studies have indicated that low hatching success may depend on a nutritionally inadequate diet. Guisande and Harris (1995) demonstrated that hatching success in *C. helgolandicus* increased as egg size and total organic content of the eggs increased. However, Jónasdóttir (1994), Poulet et al. (1995), and Pond et al. (1996) found no significant correlation between egg size and hatching success in *Acartia tonsa* and *C. helgolandicus*. Differences in egg viability were related to the concentration of specific polyunsaturated fatty acids in the diet, in particular to the content of 20:5 ω 3 and 22:6 ω 3 (Kleppel and Burkhardt 1995; Jónasdóttir and Kjørboe 1996), and were negatively correlated with the carbon and nitrogen content of the eggs (Pond et al. 1996).

It has been suggested that low hatching success is related to the presence of antimetabolic compounds in diatoms blocking egg development (Poulet et al. 1994, 1995; Ianora et al. 1995, 1996; Miralto et al. 1995; Uye 1996; Ban et al. 1997). These authors have demonstrated that diatom diets induced the production of abnormal eggs that either failed to develop to hatching or hatched into deformed nauplii. This effect was diatom density dependent (Chaudron et al. 1996) and was

reversible when females were transferred from a diatom to dinoflagellate diet (Laabir et al. 1995; Uye 1996). Inhibition of mitosis has also recently been demonstrated during embryonic development of echinoderms exposed to cell extracts of the diatom *Thalassiosira rotula* (Buttino et al. 1998) and in human bronchopulmonary tumoral cell lines exposed to diatom *Skeletonema costatum* extracts (Bergé et al. 1997).

In most studies of copepod egg viability, emphasis was placed on female egg production and hatching success, with very little information provided on how the potential fertility of males was affected by different algal foods. An absence of remating has been shown to dramatically affect hatching efficiency (Parrish and Wilson 1978), even though not all copepod species require reinsemination to maintain the production of fertile eggs (Marshall and Orr 1952). Here, we report on how some dinoflagellates adversely affected the rate of spermatophore production and sperm fertilization capacity in the copepod *T. stylifera*, a species for which remating has been shown to be necessary to maintain high egg viability (Ianora et al. 1989; Ianora and Poulet 1993).

Materials and methods

T. stylifera specimens were collected in the Bay of Naples on several occasions from June to December 1995–1997 by towing a 250- μ m mesh plankton net obliquely from 0 to 50 m. Samples were transported within 1 h to the laboratory, where males and females were sorted and divided into five groups of 15 couples each. Individual couples were placed in crystallizing dishes containing 100 ml of 0.45- μ m filtered seawater enriched with one of four dinoflagellate diets (*P. micans*: PMIC, *G. sanguinium*: GYM, *G. polyedra*: GON, or *P. minimum*: PMIN) and one flagellate diet (*I. galbana*: ISO) provided individually to couples at final cell concentrations ranging from 10² to 10⁵ cells ml⁻¹, depending on the size and carbon content of the cells (Table 1). Final car-

Acknowledgments

We thank M. Di Pinto and F. Esposito for their technical help. This work was funded by the Stazione Zoologica di Napoli and the CNRS Roscoff Marine Stations.

Table 1. Information on phytoplankton species tested as diets on *T. stylifera* (l, length; w, width; t, thickness).

Phytoplankton	Acronym	Origin	Size (μm)			Volume (μm^3)	Carbon (pg cell $^{-1}$)	Food concentration	
			l	w	t			Cell conc. (ml $^{-1}$)	$\mu\text{g C d}^{-1}$
<i>Prorocentrum micans</i>	PMIC	Mediterranean (Gulf of Naples)	38	30	16	9,549	970	10 ³	9.7
<i>Gymnodinium sanguinium</i>	GYM	N. Atlantic (off Florida)	32	28	13	6,098	658	10 ³	6.5
<i>Gonyaulax polyedra</i>	GON	Mediterranean (Gulf of Naples)	56	40	40	46,910	3,849	2 × 10 ²	7.6
<i>Prorocentrum minimum</i>	PMIN	Mediterranean (Gulf of Naples)	18	12.5	7	825	116	10 ⁴	11.6
<i>Isochrysis galbana</i>	ISO	Mediterranean (Adriatic Sea)	6	4	4	50	10	10 ⁵	10

bon concentrations in containers were therefore more than or equal to food saturation levels given for a similar-sized copepod species, *Centropages typicus* (6.7 $\mu\text{g C d}^{-1}$; Dagg and Grill 1980). Cultures were grown in K medium (Sigma Chemical), at 20°C on a 12:12 light:dark cycle, and given to copepods during exponential growth phases.

Each day, couples from each group were transferred to new containers with fresh dinoflagellate and flagellate media, and a daily tally was kept on the number of eggs, spermatophores, and fecal pellets produced. A daily tally was also kept of crumpled egg membranes due to cannibalism, but these were very few in well-fed animals and rarely comprised >1% of total egg production. Egg viability was determined 48 h after spawning by counting the number of hatched nauplii (see Ianora et al. [1995] methods).

Experiments were also conducted on *C. helgolandicus* females, which are known not to require reinsemination to maintain the production of fertile eggs (Marshall and Orr 1952). Three groups of 15 *C. helgolandicus* females were collected in May 1996 and maintained individually, in the absence of males, with the three diets that induced low hatching success in *T. stylifera* (i.e., GON, GYM, and PMIC); daily tallies were kept of fecundity, egg viability, and fecal pellet production. All experiments were run for 15 d to avoid problems associated with female age and its deleterious effects on egg viability (Ianora et al. 1995, 1996).

To demonstrate that low hatching success of *T. stylifera* eggs was due to a poor efficiency in the fertilization of oocytes caused by reduced sperm quality, three groups of six couples were fed either GON, GYM, or PMIC until $\leq 10\%$ egg viability was reached. Conditioned males were then substituted with freshly caught wild males to determine if this would reinitiate the production of viable eggs by the same females. To test whether reduced fertilizability was due to male age, in a second set of experiments, six couples were fed PMIC and six new males were fed PMIN for the same length of time. When zero hatching success was reached, the preconditioned PMIC males were substituted with males fed the good diet PMIN so that these males were as old as the sterile males.

Finally, to determine whether nonviable eggs had been fertilized, unhatched eggs from *T. stylifera* couples condi-

tioned for several days on each of the three poor diets (PMIC, GYM, and GON) were collected and colored with the vital fluorescent dye specific for cellular nuclei, Hoechst 33342 (Sigma Chemical). Control eggs were obtained from wild females 30 min–2 h after spawning. Stock solutions were prepared by dissolving 5 mg of Hoechst dye in 1 ml of distilled water and diluting this solution to a final concentration of 50 $\mu\text{g ml}^{-1}$ (1:1,000) in 0.45- μm filtered seawater. Two hundred fifty microliters of this solution was added to 750- μl filtered seawater containing batches of 40–50 *T. stylifera* eggs. After 20 min, eggs were washed several times in filtered seawater and then observed at the confocal light microscope (LSM Zeiss 410) equipped with a $\times 40$ water immersion objective and an ultraviolet laser (364 nm λ) used for visualization of the nuclei. Six optical sections were taken at 5- μm intervals, and the digitalized images were processed with a Zeiss software system.

Analyses of variance (Scherrer 1984) were performed using the 15 couples per diet as replicates, and values were averaged over the 15 d of observation to test if diet significantly modified copepod reproductive responses.

Results

The four dinoflagellates PMIC, GYM, GON, and PMIN induced high and variable daily egg production rates in *T. stylifera* females, with mean values ranging from 25.9 to 57.8 eggs $\text{f}^{-1} \text{d}^{-1}$ during the 15-d incubation period (Fig. 1). Of these, PMIN induced egg production rates in *T. stylifera* as high as the other three diets (Fig. 1). Variance analysis indicated no significant difference between means of egg production with PMIN, PMIC, GYM, and GON ($F_c = 1.11 < F_\alpha = 4.19$; $\alpha = 0.01$, $df_1 = 3$, $df_2 = 56$). However, with ISO, mean egg production was almost half (25.9 ± 9 eggs $\text{f}^{-1} \text{d}^{-1}$) that obtained with other diets, even though animals fed well on this food item (55 ± 21.5 fecal pellets d^{-1}); variance analysis showed that mean egg production rates were significantly different between ISO and the four other diets ($F_c = 77 > F_\alpha = 3.6$; $\alpha = 0.01$, $df_1 = 4$, $df_2 = 70$).

Egg viability was initially high with all diets (80–100%), but it diminished rapidly with time, reaching <10% viability

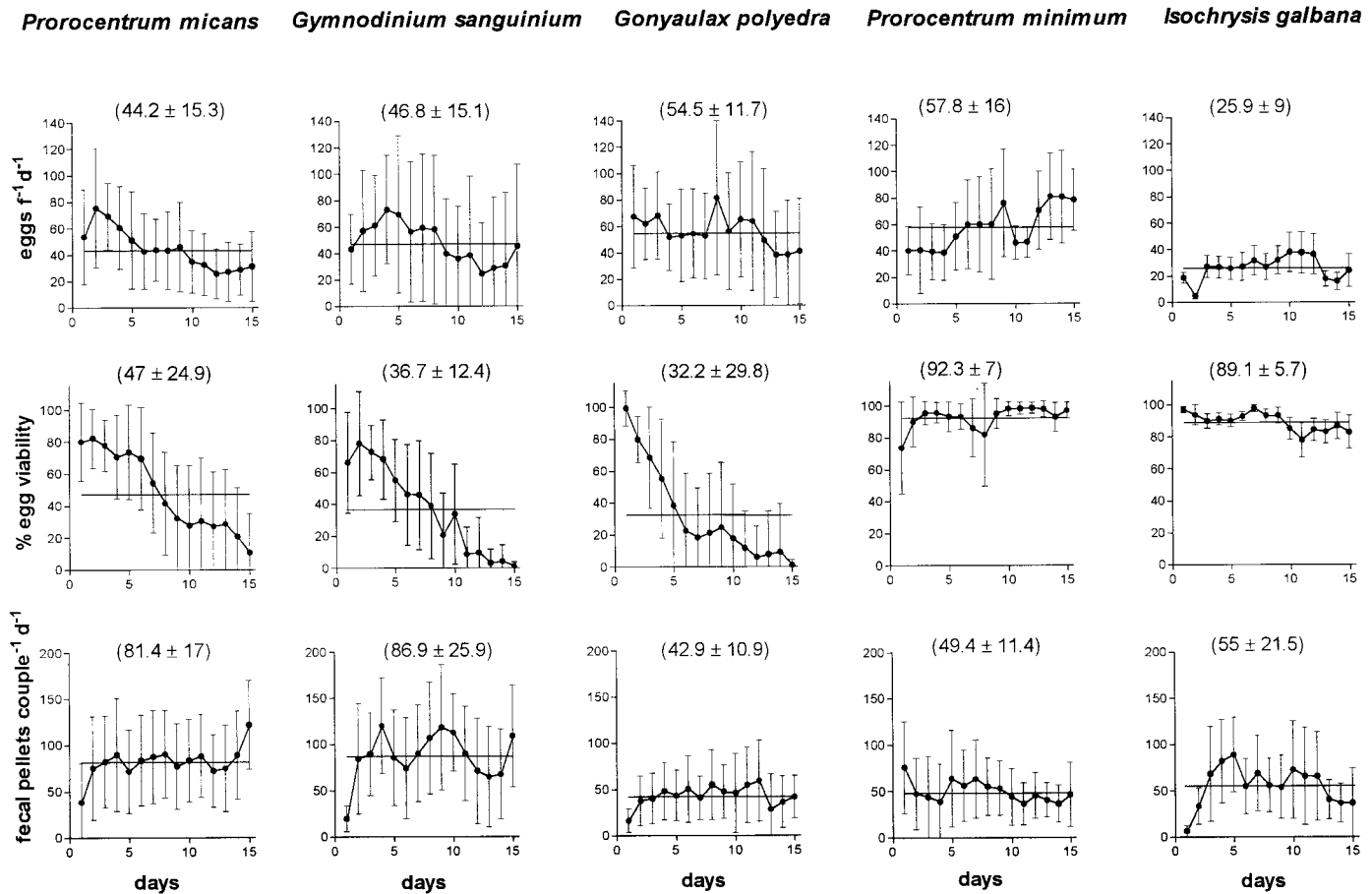


Fig. 1. Effect of four dinoflagellate diets (*P. micans*, *G. sanguinum*, *G. polyedra*, and *P. minimum*) and one flagellate diet (*I. galbana*) on daily egg production, percent egg viability, and fecal pellet production in female and male *T. stylifera* couples. Data are means and SD of $n = 15$ replicate observations. Horizontal bars correspond to total means given in brackets.

within 15 d with PMIC, GYM, and GON. This diminution was much more rapid in the case of GON, for which egg viability decreased to ca. 20% in only 5 d. Egg viability was not significantly different among the PMIC, GYM, and GON diets ($F_c = 0.02 < F_{\alpha} = 5.1$; $\alpha = 0.01$, $df_1 = 2$, $df_2 = 42$). By contrast, egg viability was high and stable over time with both PMIN (92.3%) and ISO (89.1%), but the means observed with these two diets differed significantly from the other three diets tested ($F_c = 69.58 > F_{\alpha} = 3.6$; $\alpha = 0.01$, $df_1 = 4$, $df_2 = 70$). Fecal pellet production also varied with time, depending on the diets (Fig. 1). Variance analysis showed that the means of fecal pellet production were significantly different among the five diets ($F_c = 72.33 < F_{\alpha} = 3.6$; $\alpha = 0.01$, $df_1 = 4$, $df_2 = 70$).

C. helgolandicus responded very differently to PMIC, GYM, and GON (Fig. 2). As opposed to *T. stylifera*, lowest egg production was induced when females were fed PMIC (9.3 ± 5 eggs $f^{-1} d^{-1}$), whereas highest production occurred when females were fed GYM (22.4 ± 6 eggs $f^{-1} d^{-1}$). Variance analysis showed that mean egg production rates ($F_c = 22.08 > F_{\alpha} = 5.1$; $\alpha = 0.01$, $df_1 = 2$, $df_2 = 42$) and fecal pellet production ($F_c = 19.03 > F_{\alpha} = 5.1$; $\alpha = 0.01$, $df_1 = 2$, $df_2 = 42$) were both significantly different among diets. By contrast, egg viability was high and stable with time, with

mean values ranging from 69.6 to 86.2%, showing no significant differences among the three dinoflagellate diets ($F_c = 0.94 < F_{\alpha} = 5.1$; $\alpha = 0.01$, $df_1 = 2$, $df_2 = 42$).

Daily spermatophore production for *T. stylifera* did not diminish over time with any of the five diets tested (Fig. 3). However, means of spermatophore production were significantly different among diets ($F_c = 70.50 > F_{\alpha} = 3.6$; $\alpha = 0.01$, $df_1 = 4$, $df_2 = 70$). Highest production (1.4 spermatophores $m^{-1} d^{-1}$) was observed with PMIN, whereas lowest production occurred when couples were fed GYM (0.6 spermatophores $m^{-1} d^{-1}$), with mean values that were almost half those obtained with the other diets. Interestingly, relatively high spermatophore production with GON (1.2 spermatophores $m^{-1} d^{-1}$) coincided with lowest egg viability, denoting that the number of spermatophores produced was unrelated to the efficacy of hatching success.

Experiments were therefore run to test the fertilizability of male sperm. To do this, couples were maintained on PMIC, GYM, and GON until egg viability had dropped to $< 10\%$, after which males were substituted with freshly caught wild males, and egg viability was closely monitored thereafter. Although there was a great deal of individual variability in the length of time necessary to induce low hatching success (6–12 d), this did not seem to affect the female's

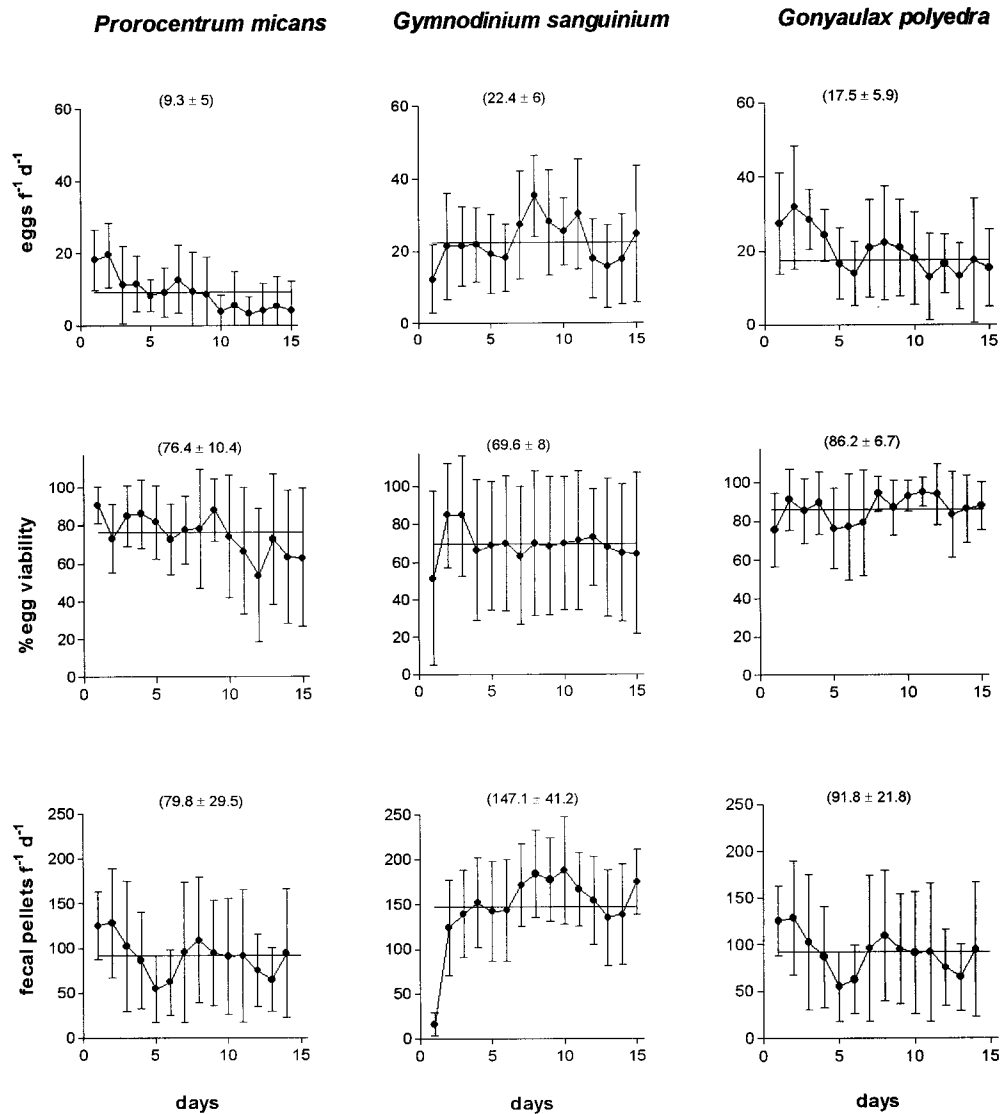


Fig. 2. Effect of three dinoflagellate diets (*P. micans*, *G. sanguinum*, and *G. polyedra*) on daily egg production, percent egg viability, and fecal pellet production in *C. helgolandicus* females. Data are means and SD of $n = 15$ replicate observations. Horizontal bars correspond to total means given in brackets.

response to the presence of new males. In all cases, and with all three diets, the introduction of a new male reinitiated the production of viable eggs from 1 to 2 d later (Fig. 4). Egg viability generally rose back up to $\geq 80\%$ upon the introduction of new males. Control tests were run to make sure that the observed diminution in hatching success was not due to male age. Males conditioned with the good diet PMIN for the same length of time (12 d) as those maintained with females in the poor diet PMIC were switched after hatching success reached $< 10\%$. Again, the introduction of preconditioned PMIN wild males reinitiated viable egg production, which returned to $\geq 70\%$, denoting that reduced viability was not due to male age (Fig. 4).

Unhatched eggs spawned by females maintained with PMIC, GYM, and GON were collected after day 6 and before day 12 (i.e., before the introduction of new males) and

colored with the fluorescent dye Hoechst 33342 specific for cellular nuclei. This was done to test if the eggs spawned by *T. stylifera* couples conditioned on the dinoflagellate diets had been fertilized. Confocal images of 8-h-old eggs show that these were unfertilized, as indicated by the presence of a single pronucleus typical of virgin oocytes (Fig. 5).

Discussion

The results show, for the first time, that hatching success of calanoid copepod eggs can depend on sperm quality. Three dinoflagellate species tested, PMIC, GYM, and GON, reduced the fertilization capacity of *T. stylifera* male sperm within 6–12 d of continuous feeding, depending on the individual males and their response to the diet. Notwithstanding these differences, there was always a rapid reinitiation

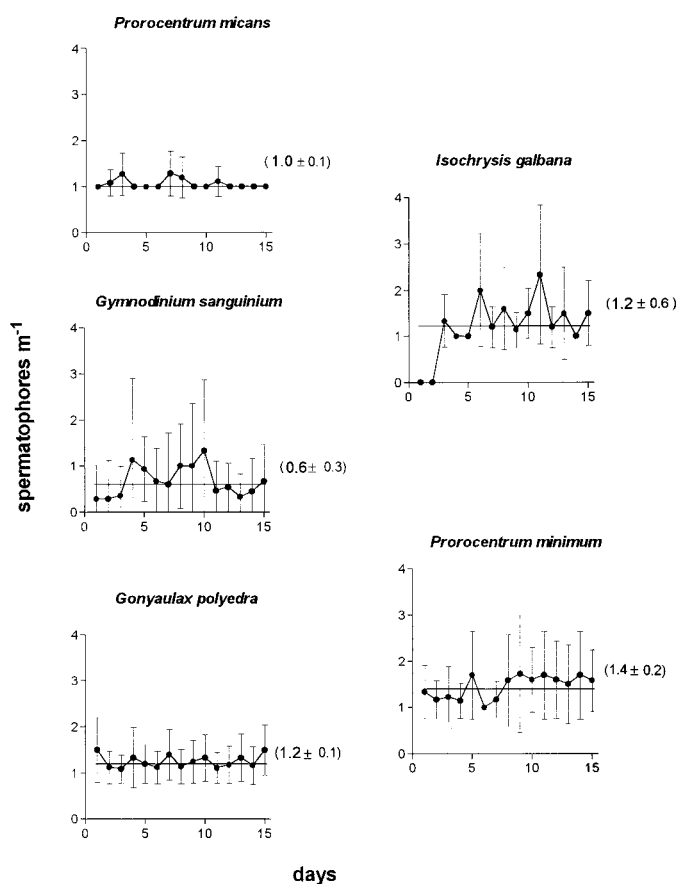


Fig. 3. Effect of four dinoflagellate diets (*P. micans*, *G. sanguinium*, *G. polyedra*, and *P. minimum*) and one flagellate diet (*I. galbana*) on daily spermatophore production in *T. stylifera* males. Data are means and SD of $n = 15$ replicate observations. Horizontal bars correspond to total means given in brackets.

in the production of viable eggs. The observed diminution in hatching success was exclusively due to poor sperm quality and not to maternal effects or male age since hatchability improved when new males were placed in experimental containers.

Very little information is available regarding paternal effects on egg fertilization. In crustaceans, spermatogenesis is known to be under hormonal control regulated by both exogenous factors such as temperature shock, pH, and osmotic pressure (Jayaprakas and Bimal-lal 1996) and endogenous factors such as nutrient reserves associated with food chemical composition. For example, the release of spermatozoa was enhanced when penaeid shrimp were fed with an artificial diet rich in vitamins E or C and containing fish oils (Yano 1995). Jayaprakas et al. (1996) have shown that when cultured male fish were fed diets supplemented with various levels of L-carnitine, growth and reproductive performance improved. Carnitine induced lipid catabolism, leading to a reduction in the lipid content of the fish and to an increase in the percentage viability of the spermatozoa. Bell et al. (1996) found that the phospholipids of fish reared on commercial diets had elevated levels of the fatty acid eicosapentaenoic acid (20:5 ω 3) and that this negatively affected

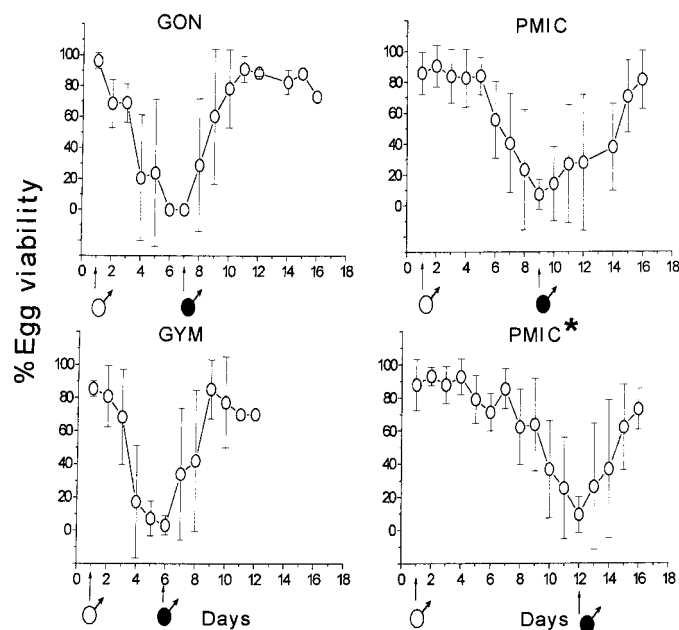


Fig. 4. Daily variations in percent egg viability for *T. stylifera* female and male couples fed either the dinoflagellates *P. micans* (PMIC), *G. polyedra* (GON), or *G. sanguinium* (GYM). When viability was $<10\%$, males (δ) were substituted with freshly caught wild males (\bullet), which reinitiated the production of viable eggs. Daily variations in percent egg viability for *T. stylifera* couples initially fed the dinoflagellate PMIC*; when the viability was $<10\%$, males were substituted with other males conditioned for the same length of time with a good diet, *P. minimum* (PMIN). Data are means and SD of $n = 6$ replicate observations.

sperm viability. Rukmini (1990) found that when laboratory rats were fed diets supplemented with 10% manhua oil rich in the fatty acids palmitic, stearic, oleic, and linoleic acids, all the male rats became sterile in the second generation. On withdrawal of manhua oil from the diet, the males regained their fertility.

All of these studies indicate that diet may affect hormone metabolism and therefore sperm viability, with important consequences on egg survivorship. Changes in fatty acid composition due to an imbalance in the ratio of dietary omega-3 to omega-6 ratio may result in competitive inhibition for available enzymes in the synthesis of arachidonic acid (20:4 ω 6), an important precursor of prostaglandins involved in insect and vertebrate reproduction (Harrison 1990). Whether these compounds are also involved in copepod reproduction is unknown, since studies examining the nutritional requirements of copepods are still in their infancy. Most of the available information of dietary effects on reproduction have focused on females. For example, it has been suggested that maternal nutritional deficiencies may be responsible for reduced egg numbers (Jónasdóttir 1994), smaller egg size (Guisande and Harris 1995), and reduced egg hatchability (Ianora and Poulet 1993). However, information on the nutritional components necessary for male copepod maturation and reproduction is lacking.

Diet is also known to affect copepod fertility. For example, in the present study, there were considerable differences

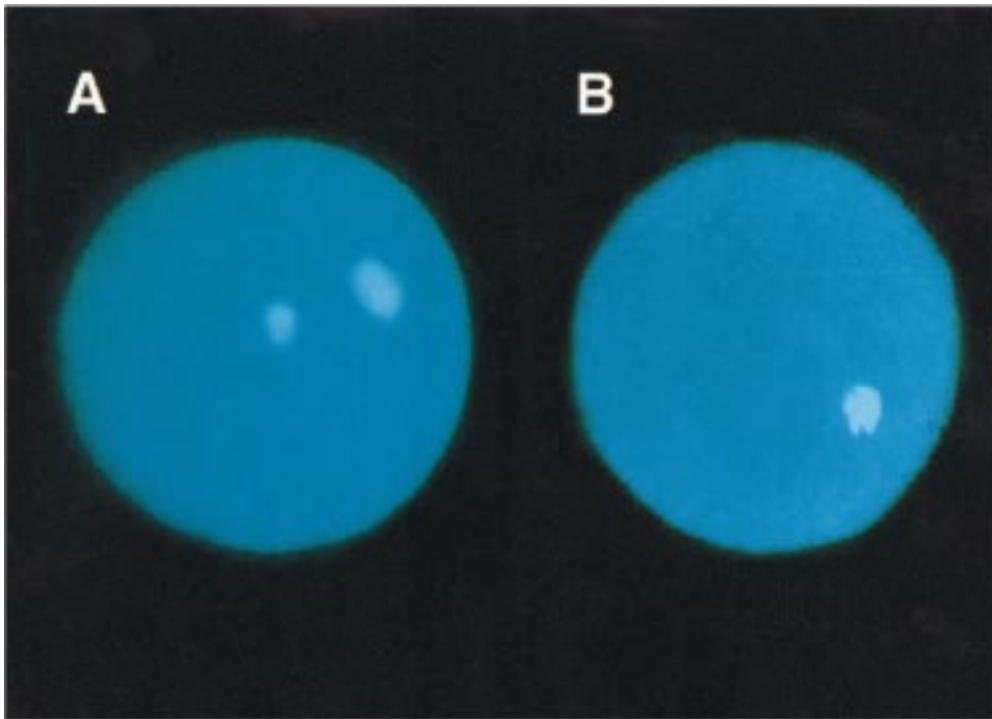


Fig. 5. Confocal microscope images of fluorescent (A) 20-min-old fertilized egg spawned by wild *T. stylifera* females showing female and male pronuclei prior to fusion, and (B) nonviable egg spawned by *T. stylifera* fed one of three dinoflagellate diets (*P. micans*, *G. sanguinum*, or *G. polyedra*) for 2–12 d (see Fig. 4); the egg has not been fertilized, as denoted by the single female pronucleus typical of virgin oocytes.

among diets in their ability to promote higher or lower egg production rates (i.e., in *T. stylifera*, highest production occurred with PMIN and lowest with ISO). There were also considerable interspecific differences in feeding requirements between *T. stylifera* and *C. helgolandicus*. PMIC induced higher egg production in *T. stylifera*, even though both copepod species fed well on this algae, as inferred from fecal pellet counts (Figs. 1, 2). As suggested in a previous study (Miralto et al. 1995), this may reflect differences in assimilation efficiency rather than other food characteristics such as size or taste. That is, *C. helgolandicus* may not assimilate from PMIC the “quota” (sensu Vanni and Lampert 1992) of energy and nutrients needed to equal that obtained from other foods.

In summary, copepod nutrition–reproduction interactions are extremely complex, involving a plethora of compounds, the effects of which have yet to be fully explored. To date, the available evidence indicates that hatching success in copepods may depend on several factors, including remating frequency (Ianora et al. 1989), bioaccumulation of antimetabolic compounds contained in diatoms that block embryonic development (see Ban et al. 1997 and references therein), and maternal (Ianora and Poulet 1993) and paternal (this study) effects related to diet. At this point, it is necessary to distinguish between the compounds responsible for the successful hatching of eggs and those affecting fecundity and postembryonic development. Not only may they differ, but those that may be good for one process may be deleterious for another. In fact, research on diets enriched with lipids

containing polyunsaturated fatty acids indicate that even an overabundance of an essential compound may decrease the beneficial effects of a well-balanced diet (Bell et al. 1996).

Not all dinoflagellates are poor food items for male fertility. Previous experiments with PMIN showed that this was an optimal diet for the hatching success of *T. stylifera* (Ianora and Poulet 1993), *Acartia clausi* (Ianora et al. 1996), and *C. typicus* (Miralto et al. 1995), all of which required reinsemination to maintain the production of viable eggs. ISO has also proved to be a good food item for hatching success in *T. stylifera* (Ianora et al. 1995). In the present study, eggs had not been fertilized because of an inefficiency in the fertilization capacity of male sperm. Spermatophore numbers were sufficiently high with all diets (Fig. 3) to fertilize all of the eggs produced during the 15-d incubation period (Fig. 1), since *T. stylifera* requires reinsemination every 3–4 d to maintain the production of fertile eggs (Ianora et al. 1989). The negative impact on egg viability with the diets PMIC, GYM, and GON therefore differs from the one reported for some diatoms, which contain antimetabolic compounds arresting copepod embryonic development (Poulet et al. 1994, 1995; Uye 1996; Ban et al. 1997; Starr et al. unpubl. data). These studies showed that eggs spawned by females maintained on certain diatom diets were fertilized but underwent abnormal cleavage and failed to develop to hatching. By contrast, in the present study, eggs had not been fertilized due to poor sperm quality. This deterioration seems to occur during the process of sperm maturation (spermatogenesis), since female *C. helgolandicus*,

which stored already mature sperm from wild males, were not affected by diet.

Although the causes of reduced male fertility remain elusive, this study shows that reduced hatching success may also be caused by male fertilization incapacity. This aspect of the biology of copepods, often neglected in reproduction studies, is especially important to consider in those cases in which males are not short-lived and are necessary for the continual reinsemination of females, as is the case in many marine and freshwater copepod genera.

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Received: 24 March 1998

Accepted: 18 September 1998

Amended: 8 October 1998