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Deleterious effect of diatom diets on egg production and hatching success in the marine copepod *Pseudocalanus newmani*

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Abstract: Clutch size and hatching success in the marine calanoid copepod *Pseudocalanus newmani* were examined using two diatom species, *Chaetoceros gracilis* and *Phaeodactylum tricornutum*, and two non-diatom species, *Pavlova* sp. and *Heterocapsa triquetra*, as food. Both clutch size and hatching success varied with food algae after the 3rd clutch. The clutch size was largest with *C. gracilis*, intermediate with *H. triquetra* and *Pavlova* sp., and smallest with *Ph. tricornutum*. Hatching success was significantly lower with the diatom diets than with the non-diatom ones. With a mixed diet of *C. gracilis* and *Pavlova* sp., the clutch size was similar to that with *C. gracilis* or *Pavlova* sp. alone, while the hatching success rate was slightly lower than that with *Pavlova* sp. but higher than that with *C. gracilis*. Eggs exposed to high concentrations of a *C. gracilis* extract at 7×10^7 cells ml^{-1} failed to hatch, but those at the same concentration of *Pavlova* sp. extract successfully hatched at more than 89%. These results suggest the presence of an anti-mitotic substance in these diatoms. The low hatching success on diatom diets might be due to the presence of as yet unidentified embryogenesis-inhibitory-compounds. Furthermore, both the diatom diets included a less amount of 18:3ω3, 18:4ω3 and 22:6ω3 (DHA) fatty acids than the two non-diatom ones. The clutch size was related to neither the inhibitory compounds nor any fatty acids. Probably, nutritional components other than fatty acids in the diets play an important role in determining clutch size. Additionally, feeding the mixed diets of diatom and non-diatom species is considered to reduce the diatom effect on the copepods through lowering the amount of diatoms ingested, as *P. newmani* utilized equally diatom and non-diatom species in its diet.

Key words: diatom effect, copepod, *Pseudocalanus newmani*, clutch size, hatching success

Introduction

Recent laboratory studies using wild-reared populations of copepods under a sufficient food supply have found that either production or hatching success of eggs, or both, decreased when the females were fed on diatoms, but not when fed on non-diatom species (Poulet et al. 1994, 1995; lanora et al. 1995; Miralto et al. 1995; Chaudron et al. 1996; Uye 1996; Ban et al. 1997). Until now, this detrimental effect has been observed in 16 copepod and 17 diatom species from freshwater, estuarine and marine environments in both the Northern and Southern Hemispheres (Ban et al. 1997). It therefore seems that the negative effect of diatom diets on copepod reproduction may be ubiquitous phenomenon, though there are a couple of studies that found no negative effect (Jónasdóttir & Kiørboe 1996; Ban et al. 1997). The importance of diatoms as an abundant and high-quality food source which stimulates copepod reproduction has also been questioned by reexamination of gut contents using pigment analysis (Kleppel 1993; Kleppel et al. 1991). In the traditional marine food chain concept, the spring diatom bloom is considered to support the secondary production of herbivorous zooplankters, such as copepods, and subsequently the growth of fishes and animals at higher trophic positions (e.g. Riley 1947). The new findings described above, however, urge necessity for revision of this 'classic' concept.

There are two explanations as to how diatoms affect copepod reproduction. One is the presence of inhibitory chemical compounds in diatom cells that may block cope-

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pod embryogenesis (Poulet et al. 1994, 1995; Miralto et al. 1995; Ianora et al. 1995, 1996; Uye 1996; Ban et al. 1997). Another one is the lack of nutrients essential for copepod reproduction found in diatom cells. Ambler (1986) found that the egg production rates of *Acartia tonsa* were positively correlated with seston C : N ratios. Jónasdóttir & Kiørboe (1996) showed that hatching of *A. tonsa* eggs was closely correlated with fatty acid composition and contents in the diets. In the field of fish culture, poly-unsaturated fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are important components in the diets for the survival and growth of larval fish (Kanazawa 1993, 1995; Estevez & Kanazawa 1996).

Pseudocalanus newmani is an egg-carrying type of marine calanoid copepod colonizing the coastal regions of boreal waters (Corkett & McLaren 1978; Yamaguchi & Shiga 1997). On the Pacific coast off southwestern Hokkaido, this copepod dominates from early spring to summer, comprising over 80% of the total copepods during the diatom spring bloom in March (Yamaguchi & Shiga 1997; Ban et al. 1998). Therefore, it is important to evaluate the effect of diatoms on the reproductive characteristics of this copepod in order to clarify the role of the diatom bloom in the population dynamics and ecology of this copepod. In this study, we examined clutch size and viability of the eggs of *P. newmani* females, when fed on two diatom and two non-diatom diets, to evaluate the effect of diatoms on fecundity and egg viability. In the previous studies examining the viability of copepod eggs in the laboratory, a single algal diet was usually used. However, copepods can be expected to feed on a wide variety of algae in nature. Therefore, we also evaluated the effect of mixed diets of diatom and non-diatom algae on clutch size and egg viability in *P. newmani*. Furthermore, to clarify what modifies copepod reproduction, we examined the effect of diatom extract on hatching success and analyzed the fatty acid composition of the algae.

Materials and Methods

Collection of experimental animals

Pseudocalanus newmani was collected in a 0–400 m vertical haul using an 80-cm diameter plankton net (mesh size, 0.33 mm) at Stn E-16 (42°09′N, 140°52′E, 500-m bottom depth) off Cape Esan, southwestern Hokkaido in July 1996. The adult males and females isolated from the plankton sample were kept in 1-litter jars containing filtered (Whatman GF/F) seawater (FSW), and fed on *Pavlova* sp. at 2×10^5 cells ml^{-1}, under 10±0.5°C and 12L : 12D conditions for several days before the experiments were carried out.

Effect of algal diets

To examine the effect of different food algae on clutch size and egg viability in *P. newmani*, ten females isolated from the stock culture within a few days of collection were individually placed in 20-ml vials filled with FSW and food algae. Light and temperature conditions were the same as described above. Four different phytoplankton species, *Chaetoceros gracilis* (diameter, 7.3±0.8 μm; height, 4.9±0.3 μm) and *Phaeodactylum tricornutum* (length, 34±10 μm; width, 6.9±2 μm) as the diatom diet, and *Pavlova* sp. (length, 8.6±0.4 μm; width, 4.8±0.9 μm) and *Heterocapsa triquetra* (length, 21±2 μm; width, 13.9±1.2 μm) (Strain No. 7 of National Institute for Environmental Studies) as the non-diatom diet, were used in the experiments. *C. gracilis*, *Ph. tricornutum* and *Pavlova* sp. originated from a culture maintained at the Mori Research Branch of the Hokkaido Fish Hatchery. All the phytoplankton species were grown in f/2 medium at 20±0.5°C, 12L : 12D light cycle, and the late exponential growth phased (ca. 7-d-old) cultures were used for the experiments. The phytoplankton cells from each culture were centrifuged, washed with FSW, their concentrations calculated through hemacytometer counts (Guillard 1978) and used as food. The algal food concentrations used for the experiments were 2×10^5 cells ml^{-1} for *C. gracilis*, 10^5 cells ml^{-1} for *Ph. tricornutum*, 10^3 cells ml^{-1} for *H. triquetra* and 2×10^5 cells ml^{-1} for *Pavlova* sp. in order to be equivalent to 42.1 μg wet-weight ml^{-1}, which is considerably higher than the incipient limiting concentration for the copepod (Corkett & McLaren 1978). An experiment with a mixed diet of *C. gracilis* and *Pavlova* sp. at 4×10^5 cells ml^{-1} (cell/cell, 1 : 1) was also carried out, but the females used were isolated from different stocks, i.e. different date of collection. Each experiment was run for 21 d. The animals were transferred to new vials filled with fresh media every day. Numbers of hatched nauplii and unhatched eggs were counted daily under a binocular microscope at 40× magnification.

Feeding selectivity

To examine feeding selectivity in females of *P. newmani* for non-diatom algae, five adult females were placed in each of five 13-ml screw-capped vials filled with a mixed food suspension including *C. gracilis* and *Pavlova* sp. at 4×10^5 cells ml^{-1} (cell/cell, 1 : 1). Another set of five vials without animals was prepared as a control. The vials were slowly rotated (5 rpm) with a rotator (TAITEC RT-5) in order to suspend the food algae. The experimental period was 12 h in the dark at 10°C, to ensure that the decline in algal concentration due to grazing would not exceed 60% of the initial value. According to Frost (1972), the clearance rate (F, ml female $^{-1}$ h^{-1}) of *P. newmani* for a certain alga was calculated by

$$F = Vg/N,$$

where V is the volume (ml) of the experimental vial and N is the number of *P. newmani* in the vial. The grazing coefficient (g) was calculated from

$$g = [(\ln C_2 - \ln C_1) - (\ln E_2 - \ln E_1)]/(t_2 - t_1),$$

where C_1 and C_2, and E_1 and E_2 are algal cell concentrations in the control vial and the experimental vial, respectively at time t_1 and t_2.

Effect of algal extract

The extracts of *C. gracilis* and *Pavlova* sp. were made with the cells from 8-d-old cultures. The algal cells were centrifuged and resuspended in 20 ml of FSW. After the cell density of the concentrated algal suspension was calculated, the algal cells were sonicated with a sonicator (TOMY SEIKO, UR-20P) for 30 min. The suspension was kept cool during sonication by submerging the beaker containing the sample in an ice bath. The sonicated material was recentrifuged at 20,000 rpm (23,000 g) at 4°C for 10 to 30 min, or until the supernatant was clear. The supernatant was frozen at −20°C until use in the experiments. The extract was thawed, diluted with FSW to the desired concentration, and filtered with a 0.2-μm pore-size Nuclepore filter prior to use. Twenty to thirty adult females of *P. newmani* were placed in 1-liter jars filled with a food suspension of *Pavlova* sp. at 2×10^5 cells ml^{-1}, and incubated at 10±0.5°C and 12L : 12D light cycle for 14 d until the experiments. This was in order to prevent artefacts due to past-feeding history effects in the natural diets. Batches of eggs spawned within 12 h by these females were collected for the experiments. Each batch of eggs was placed in a well (volume, 3 ml) of a 24-well polystyrene tissue culture plate filled with the extract of each algal species at concentrations of equivalent to 10^5 and 7×10^7 cells ml^{-1}, and with FSW as a control. A 1 : 1 (cell : cell) mixture of the extract from *C. gracilis* and *Pavlova* sp. at the equivalent of 14×10^7 cells ml^{-1} was also tested. The plate was incubated at 10±0.5°C, 12L : 12D light cycle. After 4 d, the number of hatched nauplii and unhatched eggs was counted. The experiments for each treatment were done in ten replicates.

Fatty acid composition of the diets

Lipids of the four algae used in the experiments were extracted by the method of Bligh & Dyer (1959) and the total lipid content was determined gravimetrically. Lipid class composition was determined with Iatroscan (Iatron Laboratories, TH-10) spotting chloroform solutions of the extracts on chromarod S-III and using benzene/chloroform/acetic acid (v : v : v, 50 : 20 : 1) as development solvents. Fatty acids were analyzed by gas-liquid chromatography (GLC) after methylation with 7% BF_3-methanol. Fatty acid methyl esters were purified by thin-layer chromatography on Silica gel G plate (Analtech) with n-hexane/diethyl ether (v : v, 85 : 15) for development. GLC analysis of the methyl esters was performed on a gas chromatograph (Shimadzu, GC-14A) equipped with the capillary column Omegawax 320 (Supelco, 30 m×0.32 mm i.d.) and a flame ionization detector. Column temperature was 200°C. Injector and detector temperatures were 250 and 260°C, respectively. The carrier gas was helium. Peak area percentages were measured with an integrator (Shimadzu, C-R6A).

Statistical analysis

Variations among the treatments for four single algal diets between clutch size, interclutch duration, i.e. days from the extrusion of an egg batch to the extrusion of the next, and hatching success were tested with one-way analysis of variance (ANOVA). Multiple comparisons were then conducted using Fisher's protected least significant difference (PLSD) method, when ANOVA indicated significant differences among the treatments. Differences in both clutch size and hatching success between mixed diets of *C. gracilis* and *Pavlova* sp. and the single diet (*C. gracilis* or *Pavlova* sp.) were tested with the *t*-test. Both the data for clutch size and hatching success for the following analyses came from after the 3rd clutch, because no significant differences among the treatments were observed until the 2nd clutch (see Results). Regression analyses was done between some of the fatty acid contents in the diets and clutch size or hatching success in *P. newmani*.

Results

Effect of algal diets

Clutch size of *Pseudocalanus newmani* varied greatly among the treatments (Fig. 1A). When the females were fed with *Chaetoceros gracilis*, clutch size was the largest and

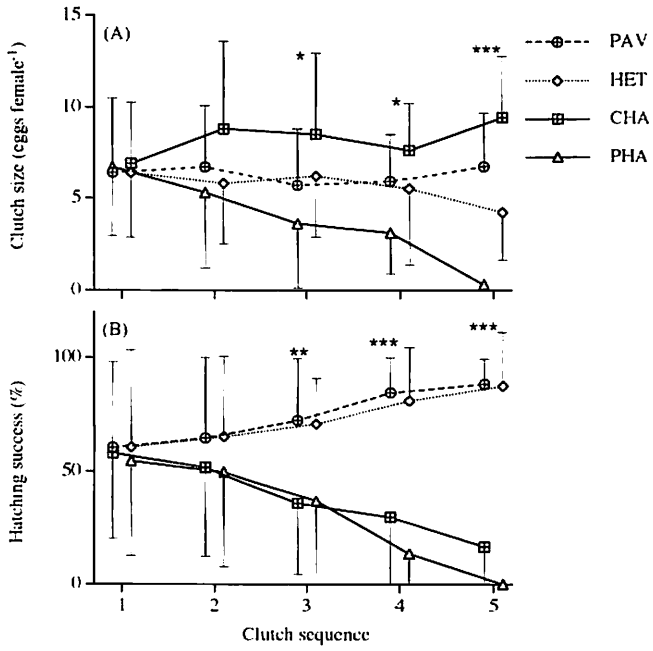

Fig. 1. Effects of four algal diets, the diatoms *Chaetoceros gracilis* (CHA) and *Phaeodactylum tricornutum* (PHA), and non-diatoms *Pavlova* sp. (PAV) and *Heterocapsa triquetra* (HET) on the clutch size (**A**) and hatching success (**B**) in *Pseudocalanus newmani*. Asterisks indicate significant differences at $p<0.05$, *; $p<0.01$, **; $p<0.001$, *** with one-way ANOVA.

Table 1. Results of ANOVA for the effects of algal diets on the number of eggs in each clutch for *Pseudocalanus newmani*. Codes for diets as in Fig. 1.

Clutch sequence	ANOVA			Multiple comparison* by Fisher's PLSD			
	DF	*F*	*p*				
1	3, 36	0.04	>0.05				
2	3, 36	1.55	>0.05				
3	3, 36	3.10	<0.05	CHA	HET	PAV	PHA
4	3, 36	3.89	<0.05	CHA	PAV	HET	PHA
5	3, 36	22.42	<0.0001	CHA	PAV	HET	PHA

* Significant differences are not scored by the same line at significance level, $p<0.05$.

Table 2. Results of ANOVA for the effects of algal diets on the hatching success of each clutch for *Pseudocalanus newmani*. Codes for diets as in Fig. 1.

Clutch sequence	ANOVA			Multiple comparison* by Fisher's PLSD			
	DF	*F*	*p*				
1	3, 36	0.05	>0.05				
2	3, 36	0.45	>0.05				
3	3, 36	3.97	<0.05	PAV	HET	PHA	CHA
4	3, 36	19.41	<0.0001	PAV	HET	CHA	PHA
5	3, 28	27.94	<0.0001	PAV	HET	CHA	PHA

* Significant differences are not scored by the same line at significance level, $p<0.05$.

relatively constant, 7–10 eggs female^{-1}, in all clutches, whereas it gradually decreased with clutch sequence when they were fed with *Phaeodactylum tricornutum*. Clutch sizes with both *Heterocapsa triquetra* and *Pavlova* sp. were intermediate and constant, 4–7 eggs female^{-1}. The differences were statistically significant among the treatments after the 3rd clutch, although the clutch size with *C. gracilis*, *H. triquetra* and *Pavlova* sp. was not significantly different until the 4th clutch (Table 1). For egg-carrying copepods, such as *P. newmani*, egg production rates do not only depend on clutch size but also interclutch duration. Since there were no significant differences in the duration among the treatments for any clutches in this study (ANOVA, DF=3, 36, $F<0.2$, $p>0.3$); mean value is 3.9 d ($n=152$, SD=0.7), the clutch size reflects the egg production rate.

Hatching success of the eggs produced by the females also varied with diet (Fig. 1B). The hatching success percentages for the first clutch were 50–60% for all of the algal diets and no significant differences among the treatments was observed (Table 2). After the 2nd clutch, hatching success with the diatom diets, both *C. gracilis* and *Ph. tricornutum*, gradually declined with clutch sequence, while that with non-diatom diets, both *Pavlova* sp. and *H. triquetra*, increased. The differences were statistically significant among the treatments after the 3rd clutch, although hatching success between *C. gracilis* and *Ph. tricornutum*, and between *Pavlova* sp. and *H. triquetra* were not significantly different for any clutches (Table 2). The food effect was apparent in both clutch size and hatching success after the 3rd clutch, but there was no relationship between the clutch size and the egg viability (Fig. 2).

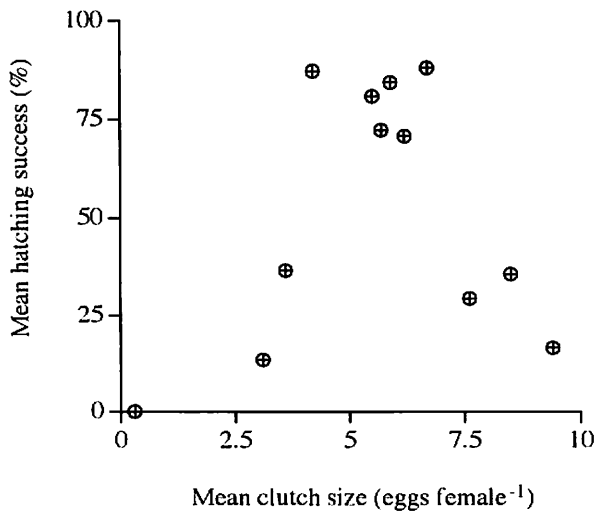

Fig. 2. Relationship between hatching success and clutch size after the 3rd clutch in *Pseudocalanus newmani* females fed on four algal diets.

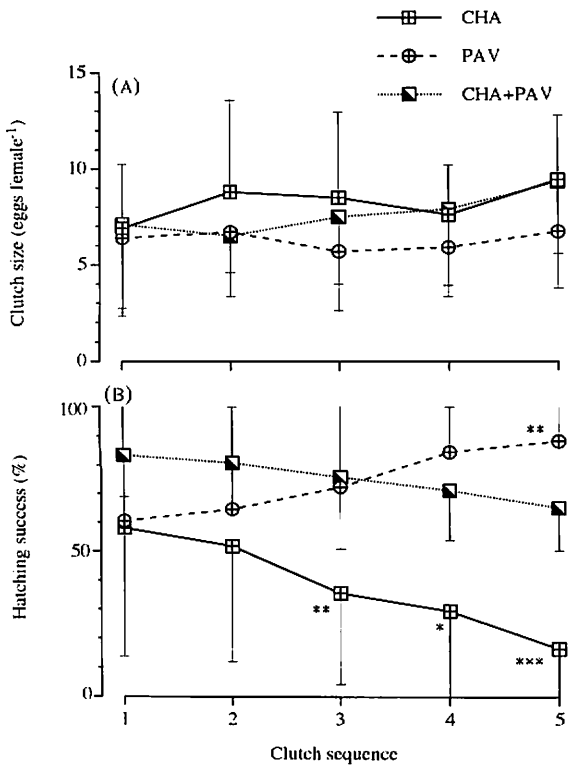

Fig. 3. Clutch size (**A**) and hatching success (**B**) of the eggs of *Pseudocalanus newmani* fed on mixed diets of the diatom *Chaetoceros gracilis* (CHA) and the non-diatom *Pavlova* sp. (PAV). Asterisks indicate significant differences at $p<0.05$, *; $p<0.01$, **; $p<0.001$, *** with *t*-test.

Clutch sizes with a mixed diet of *C. gracilis* and *Pavlova* sp. were 6–10 eggs female^{-1} in all clutches, being not significantly different from *C. gracilis* or *Pavlova* sp. alone (Fig. 3A) (*t*-test, DF=18, $t<1.8$, $p>0.05$). For the first clutch, the hatching success with the mixed diet was higher than that with either *C. gracilis* or *Pavlova* sp. (Fig. 3B). This difference was considered to be due to using females collected on a different date, although the difference was not significant (*t*-test, DF=18, $t<1.3$, $p>0.05$). The hatching success percentages with the mixed algal diet tended to decrease with clutch sequence as with *C. gracilis*, but were always higher than those with *C. gracilis* (Fig. 3B). The differences were statistically significant after the 3rd clutch. The hatching success with the mixed algal diet was lower than that with *Pavlova* sp. after the 4th clutch but the difference was statistically significant at only the 5th clutch.

Feeding selectivity

Adult females of *P. newmani* ingested both algae at similar rates, 0.09 ml indiv.$^{-1}$ h^{-1} for *C. gracilis* and 0.08 ml indiv.$^{-1}$ h^{-1} for *Pavlova* sp., when they were fed on a 1 : 1 (cell : cell) mixture of the algae. This indicates that they did not selectively feed on either of the two algae.

Effect of the algal extracts

No eggs hatched when exposed to either the extract from *C. gracilis* at 7×10^7 cells ml^{-1} or the mixed extract from *C. gracilis* and *Pavlova* sp. at 1.4×10^8 cells ml^{-1}, while more than 80% of the eggs tested hatched in the extract of *C. gracilis* at 10^5 cells ml^{-1} and in *Pavlova* sp. extract at both 10^5 and 7×10^7 cells ml^{-1} (Table 3). The hatching success percentage was significantly lower with *C. gracilis* extract at 10^5 cells ml^{-1} than that in the control (*t*-test, DF=18, $t=2.635$, $p<0.05$).

Relationship between fatty acid composition and clutch size or egg viability

Fatty acid compositions of each of the four algae used in the experiments are shown in Table 4. Percentages of 18 : 3ω3, 18 : 4ω3 and 22 : 6ω3 (DHA) fatty acids were relatively low in both the diatoms as compared to the non-diatom species. There were no such differences between diatom and non-diatom algae for any of the other fatty acids. Proportions of the total fatty acid content to the weight of total lipids were relatively constant among the algae, but the lipid content of algal cells varied greatly among the algae. Therefore, the fatty acid concentration in each food suspension of every algal diet (ng ml^{-1}) was used for the following regression analysis to examine the relationship between the amount of fatty acids and two parameters on copepod reproduction, i.e. clutch size or hatching success. The concentrations of these fatty acids, 18 : 3ω3, 18 : 4ω3 and 22 : 6ω3, in the diets were positively correlated to

Table 3. Effect of the extracts from diatom and non-diatom algae on hatching success in the eggs of *Pseudocalanus newmani*.

Treatment	Equivalent density (cells ml^{-1})	No. of replicates	Hatching success (%) mean±S.D.
Filtered sea water (Control)	0	10	93.4±6.2
Pavlova sp.	10^5	10	91.5±9.8
Pavlova sp.	7×10^7	10	89.7±10.8
Chaetocreos gracilis	10^5	10	82.1±12.2
Chaetoceros gracilis	7×10^7	10	0
C. gracilis+*Pavlova* sp.	14×10^7*	10	0

* 1 : 1 (cell : cell) mixture of the algal extact.

Table 4. Fatty acid composition (% of total fatty acids) of each algal food used in the experiments. Dashes denote undetectable values.

Fatty acids	Food			
	PAV	HET	CHA	PHA
14:0+4,8,12-TMTD*	8.27	4.2	10.51	5
14:1ω5+iso-15:0	0.77	0.42	0.49	0.47
15:0	0.13	—	0.63	0.24
iso-16:0	0.7	0.51	0.53	0.65
16:0	10.35	11.67	13.01	11.99
16:1ω7	12.3	1.64	26.88	22.51
iso-17:0	0.05	0.43	0.93	1.23
anteiso-17:0+16:2ω6	0.37	0.06	3.72	0.55
Phytanic+16:2ω4	0.34	0.03	3.94	4.74
16:3ω4	0.15	0.06	13.74	9.74
16:4ω1	—	—	0.67	0.74
18:0	0.13	1.02	1.26	0.22
18:1ω9	0.49	0.54	2.41	0.64
18:1ω7	0.84	—	0.56	0.25
18:2ω6	1.43	2.46	0.58	1.61
18:3ω6	0.88	0.24	0.24	0.21
18:3ω3	2.42	2.8	0.05	0.61
18:4ω3	12.48	20.54	0.51	1.02
18:5ω3	—	33.02	—	—
20:4ω6	0.43	—	0.92	0.21
20:4ω3	—	—	0.08	0.6
20:5ω3	30.18	0.72	12.96	31.67
24:0	—	—	—	1.34
22:6ω3	12.31	17.16	1.41	1.51
Others	4.98	2.48	3.97	2.25
Total fatty acids (% of total lipids)	42.1	43.4	51	46.2
Total lipids (% of cell weight)	7.73	3.88	0.97	3.68

*4, 8, 12-Trimethyltridecanoic acid.

Table 5. Results of regression analysis, comparing the effect of fatty acids in algal diets on the hatching success and clutch size *Pseudocalanus newmani*.

Dependent variable	Independent variable	Regression analysis			
		N	Slope	r^2	F***
Hatching success (%)	22:6ω3	12	0.401	0.838	51.61**
	18:3ω3	12	2.062	0.75	30.1*
	18:4ω3	12	0.375	0.87	68.25**
	MUFA	12	−0.058	0.02	0.18
	SAFA	12	0.166	0.17	2
	22:20	12	1.63	0.29	4.03
	ω3:ω6	12	3.18	0.21	2.67
Chutch size (eggs female^{-1})	22:6ω3	12	0.002	0.002	0.02
	18:3ω3	12	−0.001	0.001	0.01
	18:4ω3	12	0.001	0.002	0.02
	MUFA	12	−0.013	0.145	1.69
	SAFA	12	−0.012	0.135	1.57
	22:20	12	−0.01	0.01	0.04
	ω3:ω6	12	−0.29	0.3	4.24

*significant difference at $p<0.05$. **significant difference at $p<0.01$. *** to test H_0: slope=0.

hatching success, but not to clutch size (Table 5). More than 75% of the variation in hatching success could be explained by differences in the concentration of each of these three fatty acids in the food suspension. Since hatching success in *Acartia tonsa* was related to the ratio of ω3 to ω6 (ω3:ω6) and 22:6ω3 to 20:5ω3 fatty acids (22:20) and to the concentrations of saturated (SAFA) and mono-unsaturated (MUFA) fatty acids in the diets (Jónasdóttir & Kiørboe 1996), the relationship was also analyzed in this study. However, concentrations of SAFA and MUFA and both the ω3:ω6 and 22:20 ratios in the diets were all unrelated to both clutch size and hatching success in *P. newmani* (Table 5).

Discussion

This study showed that both clutch size and hatching success in *Pseudocalanus newmani* differed greatly with the algal diet and that diatoms led to lower hatching success rates (*Chaetoceros gracilis*) or decreases in both hatching success and clutch size (*Phaeodactylum tricornutum*) as compared with non-diatom diets. During the last 6 years, evidences for the inhibition of copepod reproduction when on diatom diets has accumulated. Ban et al. (1997) examined the diatom effect using 16 species of copepods from freshwater, estuarine and marine environments in the Northern and Southern Hemispheres, and found four categories of responses among the 37 diatom–copepod combinations examined; Category I, the diatom diets reduced both fecundity and hatching success; Category II, reduced hatching success but not fecundity; Category III, reduced fecundity but not hatching success; Category IV, no negative effect on either. Categories I and II were the most frequently observed, i.e. 18 cases in Category I and 11 cases in Category II. In this study, the case for *Ph. tricornutum* corresponds to Category I and that for *C. gracilis* to Category II. Probably, the Category II type response of copepods may delay the discovering the deleterious effect of diatoms on copepod egg viability, because most previous researchers have not measured hatchability of the eggs laid by females reared with diatom diets in the laboratory and collected from the field.

Since suspension-feeding copepods are well known to feed selectively depending on the size, shape and taste of the food particles (e.g. Gliwicz 1980; DeMott 1988a, b), diet unsuitability may lower the ingestion rate of adult copepods and result in a reduction in fecundity. However, our experiments on selective feeding revealed that *P. newmani* females did not necessarily prefer non-diatom algae to diatoms. This means that *P. newmani* females take diatom diets as readily as non-diatom ones, as previously reported

(Ianora & Poulet 1993; Ianora et al. 1996; Miralto et al. 1995; Uye 1996). Thus, the negative effect of diatoms on clutch size and hatching success in *P. newmani* is considered to be caused by either (1) presence of inhibitory chemical compounds that block copepod embryogenesis (Ban et al. 1997), or (2) lack of essential nutrients for copepod reproduction (Jónasdóttir & Kiørboe 1996).

Newly spawned eggs (within 12 h) from the females of *P. newmani* exposed to high concentrations of the diatom extract did not hatch, while eggs exposed to the same concentration of the non-diatom extract hatched successfully in more than 89% of cases. The hatching success percentages at lower concentrations of the diatom extract were also significantly lower than those for the control. Such a dense extract from phytoplankton cells is rich in organic materials and may induce a reduction in dissolved oxygen concentrations in the media during the course of an experiment (e.g. Jónasdóttir & Kiørboe 1996). Although dissolved oxygen in the medium was not measured at the end of each experiment in this study, Miralto et al. (1995) revealed that a concentrated diatom extract blocked copepod embryogenesis both with and without aeration of the media by their procedure that was otherwise the same as in this study. Thus, the present results suggest that the diatom extract tested in this study did in fact inhibit embryogenesis of *P. newmani* eggs. The same results have also been reported for some other copepod–diatom combinations; *Calanus helgolandicus–Thalassiosira rotula* (Poulet et al. 1994), *Calanus helgolandicus–Phaeodacylum tricornutum* (Ban et al. 1997), *Calanus pacificus–Thalassiosira weissflogii*, *Chaetoceros difficilis* and *Ditylum brightwelli* (Uye 1996), *Centropages typicus–T. rotula* (Miralto et al. 1995), *Temora stylifera–T. rotula* (Ianora et al. 1995) and *Acartia clausi–T. rotula* (Ianora et al. 1996). Poulet et al. (1995) revealed that mitotic anomalies occurring during embryogenesis in *C. helgolandicus* that were fed on diatoms were related to erroneous cell division, corresponding to desynchronisation between nuclear division and cytokinesis. Furthermore, extracts of *T. rotula* and *P. tricornutum* also inhibited embryogenesis in sea urchins (Poulet et al. 1994) and cell growth of a human non-small-cell bronchopulmonary carcinoma line (NSCLC-N-6) (Bergé et al. 1997), respectively. The production of such anti-mitotic compounds by diatoms may be ubiquitous (Uye 1996), though the extract compounds remain unidentified.

Another explanation for the reduction in hatching success on diatom diets is the possible poor food quality of diatoms, which may lack nutrients essential for copepod reproduction. The ratio of $\omega 3 : \omega 6$ fatty acids has been suggested as an important indicator of metabolic growth and reproduction processes in some crustaceans (Castell 1982; Harrison 1990; Ahlgren et al. 1990), and in larval growth of bivalves (Webb & Chu 1982; Enright et al. 1986). Ahlgren et al. (1990) obtained the highest growth response for three cladoceran species on a diet of flagellates with a $\omega 3 : \omega 6$ ratio of more than 10. Jónasdóttir & Kiørboe (1996) found that for *Acartia tonsa* the hatch rate of eggs increased with increasing ratios of $\omega 3$ to $\omega 6$ and $22:6\omega 3$ to $20:5\omega 3$ fatty acids, but decreased with increasing concentrations of saturated and mono-unsaturated fatty acids in the diet. In this study, however, such relationships were not readily apparent. The requirement of such nutrients for copepod reproduction is probably species specific.

In this study, more than 75% of the variation in hatching success for *P. newmani* among the treatments can be explained through varying concentrations of each of the three fatty acids, $18:3\omega 3$, $18:4\omega 3$ and $22:6\omega 3$ (i.e. DHA), in the diets. Clutch size, however, was unrelated to the presence or concentrations of any of the fatty acid components. The $18:3\omega 3$ and $18:4\omega 3$ fatty acids have been shown to be related to the egg production rates of *A. tonsa* and *A. hudsonica* (Jónasdóttir 1994), but such relationships have not yet been observed in any other copepods. Although the role of these fatty acids is not well understood, they are thought to be important precursors for prostaglandins in some crustaceans (Castell 1982; Harrison 1990). Docosahexaenoic acid (DHA) is an important component for not only embryogenesis and larval development but also in preventing some diseases in fishes. Kanazawa (1993) has reported that the occurrence of unusual body color among individuals of turbot (Heterosomata), winding vertebra in sea bream *Pagrus major* and sea bass *Lateolabrax japonicus*, and dropsy of sea bream were caused by a shortage of highly unsaturated fatty acids, which also tremendously affected the growth of the larvae. Of these diseases, albinism in Heterosomata is closely associated with retina formation during the larval period, and uptake of DHA has been found to prevent this disease (Kanazawa et al. 1993; Kanazawa 1995; Estevez & Kanazawa 1996). Since the DHA content of *Artemia* fed as the sole diet to cod larvae has a serious impact on the survival and growth of the larvae, an adequate amount of DHA in the diet may be necessary to produce healthy cod larvae (Zheng et al. 1997). In some crustaceans, DHA content has been found to be at 2- to 5-fold higher in eggs and ovaries as compared to the other parts of the female body, suggesting that DHA may play an important role in survival and development during embryogenesis (Hayashi 1976; Sargent & Falk-Petersen 1988). These results suggest that the presence of several poly-unsaturated fatty acids, including DHA, in the diet may be needed for normal copepod reproduction, especially for the embryonic development, but the evidence is still limited. To clarify this issue, further studies are needed.

Some recent studies comparing the chemical composition, other than fatty acids, of marine phytoplankton species suggested that diatoms may be less nutritious for copepods than dinoflagellates. Hitchcock (1982) showed that the lipid, carbohydrate and protein contents in eight diatoms were lower than in eight dinoflagellate species. The nitrogen, amino acid and vitamin C contents in the diatom *Thalassiosira* are less than those of the dinoflagellate *Prorocentrum* (Ianora & Poulet 1993). A lack of these non-lipid nu-

tritional components may be the reason why the *Ph. tricornutum* diet did not support normal clutch sizes in *P. newmani* in this study.

Hatching success with the mixed diet (*Chaetoceros gracilis* and *Pavlova* sp.) was always greater than that with *C. gracilis* alone, but tended to decrease with clutch sequence, being significantly lower than that with *Pavlova* sp. by the 5th clutch (Fig. 3). If any lack of a specific fatty acid, e.g. DHA, in the diatom diet had been sufficiently supplemented by the non-diatom one, i.e. *Pavlova* sp., the hatching success would not have decreased with clutch sequence. This relaxation of the diatom effect, therefore, may not be explained by supplementation of nutrients essential for embryogenesis due to the ingestion of non-diatom alga with diatoms. The results instead lend support to the anti-mitotic compounds in diatom cells, rather than the nutrient limitation hypothesis. Since mixed extracts of *C. gracilis* and *Pavlova* sp. inhibited embryogenesis of *P. newmani* eggs same as that of *C. gracilis*, *Pavlova* sp. may not be an antidote against the anti-mitotic agents in diatoms. Chaudron et al. (1996) found that hatching success in copepod eggs depended on the density of diatoms used as food for the adults; hatching success rates rapidly decreased and hatching was totally inhibited within a few days at high concentrations of diatoms in the diet. However, hatching success rates increased with a decrease in the concentration of diatoms. The amount of diatoms ingested by females of *P. newmani* fed on mixed diets would be half that on the single diatom diet, because the females non-selectively feed on diatom and non-diatom species. Copepods fed on diatoms may accumulate anti-mitotic agents in their oocytes during vitellogenesis (Poulet et al. 1994, 1995). The threshold at which the hypothetical anti-mitotic agents blocked embryonic development was attained after at least 8 d, because the diatom effect occurred after the third clutch and the interclutch duration was mostly 4 d (Fig. 1). Therefore, it can be supposed that it would take at least 16 d for females fed on the mixed algal diet to accumulate the anti-mitotic agents in their oocytes. Thus, relaxation of the diatom effect through ingestion of a mixed diet may be due to a reduction in the amount of diatoms ingested by the females. Likewise, the detrimental effect of diatoms on copepod reproduction may be reduced in the natural environment, because of the many kinds of potential food particles.

In conclusion, this study has revealed the deleterious effect of diatoms on the hatching success of *P. newmani* eggs and it supports the presence of embryogenesis-inhibitory-compounds in the diatoms. Regression analysis between hatching success and fatty acid concentration in the food showed that a lack of some poly-unsaturated fatty acids, including DHA, in the diatoms might also be related to hatching success, though this issue is still an open question because no direct evidence yet exists. Variation in the clutch size on the four algal diets could be explained neither by inhibitory compounds nor any fatty acid components. Probably, other nutritional components in the diets, e.g. nitrogen, proteins, amino acids and vitamins, may play an important role in determining clutch size in *P. newmani*. Results of the mixed algal diet experiment suggested that the diatom effect might be relaxed in nature due to a decrease in the amount of diatoms ingested by females.

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