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Copepod egg production and hatching success is reduced by maternal diets of a non-neurotoxic strain of the dinoflagellate *Alexandrium tamarense*

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ABSTRACT: A unialgal diet of a non-neurotoxic strain of the dinoflagellate *Alexandrium tamarense* strongly modified egg production and hatching success in the copepod *Temora stylifera*, even though grazing rates were relatively constant over time. Both exponential and stationary cultures of *A. tamarense* reduced egg production and hatching success, but the effect of stationary cultures on hatching success was dramatic, with egg viability dropping to 0% after 24 h of feeding. HPLC analyses revealed that the *A. tamarense* clone was non-neurotoxic, with a mean toxin content of about 0.005 fmol per cell, indicating that adverse effects were not due to saxitoxins or neosaxitoxins. ^{1}H-NMR analyses also revealed that diatom-derived PUSCAs (polyunsaturated short-chain aldehydes) were not responsible for reduced hatching rates, since these compounds were absent in *A. tamarense* as compared to the diatom *Skeletonema costatum*. Extracts of *A. tamarense* and the diatom-derived PUSCA 2-trans-4-trans-decadienal were also assessed in terms of biological effects on sea urchin embryo cell divisions. *A. tamarense* did not show anti-mitotic properties, as extracts did not block first-cell cleavage compared to decadienal. However, *A. tamarense* extracts did block fertilization success when sea urchin oocytes were first incubated for 30 min in extracts and then fertilized, as opposed to *S. costatum* extracts, which did not affect normal elevation of the fertilization membrane. This is the first report that dinoflagellates produce antiproliferative compounds, other than PUSCAs, that can potentially reduce copepod egg production and hatching success.

KEY WORDS: Dinoflagellate · Copepods · Egg production · Hatching success · Antiproliferate compounds

INTRODUCTION

Numerous species of diatoms negatively affect copepod reproduction, not only through reduced fecundity, but also and especially through reduced egg hatching success (reviewed by Ianora et al. 2003). Reduced egg viability has recently been linked to the presence of polyunsaturated short-chain aldehydes (PUSCAs) in diatoms that block copepod embryogenesis, thereby acting as anti-herbivory agents (Miralto et al. 1999). These aldehydes are synthesized from polyunsaturated C_{16} and C_{20} fatty acids, which are widespread in this algal class (Pohnert 2000, Pohnert et al. 2002, d'Ippolito et al. 2002). When diatoms become damaged due to feeding, they produce aldehyde within seconds after cell disruption, similar to the wound reaction in higher plants (Rosahl 1996).

In addition to effects on egg hatching, certain diatom diets induce teratogenic effects (i.e. 'birth defects') in newly hatched copepod nauplii, which then have asymmetrical bodies and reduced numbers of feeding appendages (e.g. Poulet et al. 1995). These nauplii die

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within a few hours after hatching. Copepods may ameliorate the negative effects of diatom diets on egg hatching by feeding on alternative food items in a mixed diet (Turner et al. 2001). When this occurs, the effect is not neutralized, but mitigated so that a portion of the eggs develops to hatching.

There is also field evidence that diatom blooms negatively impact copepod egg hatching success. Miralto et al. (2003) showed that during diatom blooms in the northern Adriatic Sea, copepod egg production rates were high, but only 10% of the eggs produced by *Acartia clausi* and *Calanus helgolandicus* were viable. This was compared to post-bloom conditions, when egg production rates were lower, but hatching success was 90%. Mesocosm experiments from the Norwegian coast indicated a drop of >95% in reproductive success of *C. helgolandicus* during a diatom bloom, even though feeding rates were high and alternate prey were available (Nejstgaard et al. 2001). Ban et al. (2000) reported a high incidence (20 to 40%) of abnormal nauplii of *Pseudocalanus newmani* during a diatom bloom in the Sea of Japan, and concluded that the deformities of copepod nauplii were due to maternal feeding on diatoms. In contrast, other studies have not found a negative relationship between hatching viability and diatom concentrations (Ohman & Hirche 2001, Irigoien et al. 2002). This suggests that not all diatom blooms are toxic, toxic effects are lessened by ingestion of co-occurring non-diatom foods, and/or that some copepod species may have evolved mechanisms to detoxify diatom aldehydes in a continuing coevolutionary arms race between plant defenses and animal responses.

Although evidence for diatom chemical defenses against reproductive success of their grazers continues to accumulate (Ianora et al. 2004), it is unclear whether similar mechanisms are found in other phytoplankton groups such as dinoflagellates. Dinoflagellates produce potent neurotoxins that cause shellfish poisoning and adverse effects on grazers such as zooplankton, and these toxins can be transported up the food chain to higher trophic levels (Turner & Tester 1997). However, the often-assumed antipredation role of dinoflagellate neurotoxins is complicated by equivocal observations that these compounds often have no clear adverse effects on zooplankton grazers (Turner et al. 1998a). These toxins may have evolved primarily for other roles that benefit dinoflagellates, such as nitrogen storage, bioluminescence, chromosome structural organization, pheromones for induction of sexuality, or they may simply be vestigial products of archaic pathways for nucleic acid biosynthesis (Cembella 1998). Some dinoflagellate neurotoxins may also be produced by bacterial endosymbionts, rather than by the dinoflagellates themselves (Doucette et al. 1998). Thus, dinoflagellate toxins may be only coincidentally toxic to some phytoplankton grazers.

When dinoflagellate toxins do have adverse effects on consumers, these are mostly neurological effects through sodium channel activation (brevetoxins), sodium channel blockage (saxitoxins) or neuronal depolarisation (domoic acid) (reviews in Baden & Trainer 1993, Turner & Tester 1997, Turner et al. 1998a). Direct adverse neurological effects on grazers such as copepods include reduced ingestion, regurgitation, lethargy or death (Huntley et al. 1986, 1987, Sykes & Huntley 1987, Uye & Takamatsu 1990, Teegarden 1999). There are also reports of adverse effects on reproduction, such as reduced egg production rates (Dutz 1998), or reduced egg hatching success (Turner et al. 1998a,b, Ianora et al. 1999, Frangópulos et al. 2000, Guisande et al. 2002). However, it is still unclear whether these adverse effects on copepod reproduction are due to neurotoxins that also affect mitotic divisions, or whether the adverse effects are caused by other unidentified compounds.

Here we further explore the negative effects of dinoflagellate diets on copepod reproduction. Our working hypothesis was that the dinoflagellates could produce deleterious compounds other than the described neurotoxins. Accordingly, we examined effects of non-neurotoxic clones of the dinoflagellate *Alexandrium tamarense* on filtration and ingestion rates, egg production rates, and egg hatching success in the copepod *Temora stylifera* from the Gulf of Naples, Italy.

MATERIALS AND METHODS

Grazing experiments. Grazing experiments were performed in the summer of 2000 with adult females of *Temora stylifera* collected from the Gulf of Naples and transferred to the laboratory within 1 h of collection. We added 5 females to each of triplicate experimental jars containing 500 ml food suspensions. A control jar with no copepods was treated in the same way as the experimental jars, and an initial sample of each food suspension was preserved with Lugol's solution at the beginning of each experiment. Food suspensions were mixtures of 0.45 µm filtered seawater (FSW) and the non-neurotoxic dinoflagellate *Alexandrium tamarense* in exponential (EXPO) growth at mean (± SD) concentrations of 1.8 (± 0.5) × 10^3 cells ml^{-1}. Equivalent carbon concentrations based on cell size (length: 34.2 µm; width: 32.2 µm) were 1848 pgC $cell^{-1}$, determined using the formulae of Strathman (1967).

Alexandrium tamarense (strain number FE 107; isolated from the Gulf of Naples by F.E. in 1999) was cultured in 2 l glass jars filled with 0.22 µm FSW enriched with *k* medium (Keller et al. 1987) at 20°C and on a

12:12 h dark:light cycle. Food suspensions were prepared daily by performing a cell count in stock cultures and adjusting the concentration by dilution with FSW. Female *Temora stylifera* were transferred daily by gently pouring them into bowls, and then re-pipetting them into freshly prepared food suspensions. After removal of copepods from food suspensions, aliquots of 50 ml of each food suspension were preserved in 2% Lugol's solution. Phytoplankton cell numbers in preserved aliquots were counted in Sedgwick-Rafter cells; at least 400 cells were counted in all cases, ensuring ±10% precision (Guillard 1973). Ingestion and filtration rates were determined from differences in phytoplankton cell concentrations in initial, control and experimental suspensions using the formulae of Frost (1972).

Numerous females were kept in reserve and exposed to the same conditions as experimental ones, including daily switching to fresh food suspensions. Dead experimental females were removed and replaced every day with females from the reserve treatment. Grazing rates for dead copepods were calculated assuming that they had lived for half the experiment. Experimental incubations lasted 22.3 to 24.9 h d^{-1}; incubation bottles were mounted on a rotating wheel (0.5 rpm) in a controlled temperature room at 20°C with a 12:12 h dark:light cycle. Grazing experiments were repeated daily with the same copepods being placed in new food suspensions for 13 consecutive days.

Egg production and hatching experiments. Daily egg production and hatching success in *Temora stylifera* were determined by incubating female and male couples (n = 15) as individual pairs in 100 ml crystallizing dishes containing ambient seawater. Egg production rates, fecal pellet production rates, and hatching success were determined 24 h later. Only couples with high initial egg production rates and hatching success were maintained for successive experiments. These couples were then transferred to new containers with 100 ml of 0.45 µm FSW enriched with *Alexandrium tamarense* in the exponential (EXPO) or stationary (STAT) growth phases. In a first set of experiments, final cell concentrations of EXPO or STAT growth phases targeted 1.6×10^3 cells ml^{-1}, and were therefore similar to cell concentrations used in grazing experiments. In a second and a third set of experiments, final cell concentrations of EXPO or STAT growth phases were 2× and 3× the initial concentrations.

Thereafter, all couples were transferred each day to new containers with fresh cultures provided at EXPO 1×, 2× and 3× or STAT 1×, 2× and 3× initial cell concentrations. 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 95% ethyl alcohol to containers and counting the number of hatched nauplii once they had settled to container bottoms. A daily record was also kept of the number of cannibalised eggs (identified by crumpled egg membranes: see Turner et al. 2001). There were very few cannibalised eggs, since copepods were well fed. All experiments were run for 14 d or until death of the female. If males died before females, they were substituted with freshly caught males. This was done to ensure that non-viable egg production was not due to an absence of remating. Parallel control experiments were run with the non-toxic dinoflagellate *Prorocentrum minimum* in the stationary growth phase with final concentrations (7×10^3 cells ml^{-1}; 177.1 pgC $cell^{-1}$) representing about half the carbon content of *Alexandrium tamarense* (1×); culture media and culture conditions were the same as above. This dinoflagellate species was chosen as control since it promotes high egg production and egg hatching success in *Temora stylifera* (Turner et al. 2001, Ceballos & Ianora 2003); 16 *T. stylifera* couples were incubated as above and a daily record was kept of egg production, hatching success, fecal pellet production and percentage of survival with this diet. All experimental animals were kept in a temperature-controlled chamber at 20°C and on a 12:12 h dark:light cycle.

Toxin analyses. To estimate cell toxin content, *Alexandrium tamarense* cells were cultured in gently aerated 10 l carboys under the conditions described above. Cells in exponential and stationary growth phases were collected on 47 mm diameter Whatman GF/F filters, stored at –20°C in ultracentrifuge plastic tubes and lyophilised. Subsequently, 0.5 ml of 0.05 M acetic acid was added to the lyophilised material and the sample was homogenised using a pipette tip adapted to fit the shape of the vial. The sample was twice shaken followed by freezing. Finally, the extract was centrifuged at 4000 rpm ($400 \times g$) for 10 min twice, after which 200 µl of the supernatant was carefully collected with a Hamilton syringe, and stored at –20°C.

Analysis of paralytic shellfish poisoning (PSP) toxins (C1–4, GTX1–6, neoSTX, STX, dcSTX) by HPLC with fluorescent detection was performed following a modification of the method of Oshima et al. (1989) described by Franco & Fernández (1993). Toxin profiles were determined by injections of an extract of >250 000 cells. Verification of the presence of toxins Cs, GTX 5 and GTX 6 was done by boiling the sample with an equal volume of 0.4 N HCl for 15 min. The eluent of the first isocratic was 95% 0.5 mM sodium octansulfonate in 10 mM ammonium phosphate (pH 7.2) 5% acetonitrile, for the separation of NeoSTX, dcSTX and STX. The eluent for the second isocratic was 1.5 mM sodium octansultonate in 10 mM ammonium phosphate (pH 7) 5% acetonitrile, for the separation of GTXs and dcGTXs. Toxins from the National Research Council of Canada (Halifax) were used as standards.

Toxicity, in saxitoxin equivalents (STXeq), was calculated from HPLC chromatograms, by multiplying toxin concentrations by a toxin-specific conversion factor from Oshima (1995); the conversion factor is based upon empirical mouse bioassay data determined using purified standards, and assuming the conversion factor of 1 mouse unit (MU) = 0.23 µg STXeq for the ddy mouse strain: 567.6 (GTX1), 205.2 (GTX2), 364.3 (GTX3), 414.7 (GTX4), 36.8 (GTX5), 371.9 (GTX6), 3.45 (C1), 54.9 (C2), 7.6 (C3), 32.9 (C4), 571.1 (STX), 527.8 (neoSTX), 293 (dcSTX).

Analysis of amino acids and fatty acids. To estimate their amino acid and fatty acid composition, *Alexandrium tamarense* cells were cultured in 10 l carboys at 17.5°C and on a 12:12 h dark:light cycle. Cells in exponential growth phase were collected on precombusted 13 mm diameter Whatman GF/F filters and stored at –20°C in ultracentrifuge plastic tubes.

Amino acid composition was analysed by HPLC (HPLC) using a Waters Alliance System, a Waters 474 scanning fluorescence detector and a Waters 150 × 3.9 mm Nova-Pack C18 column following the method described by Van Wandelen & Cohen (1997). Amino acid standard H NCI0180 Pierce was used for the identification and quantification of amino acids. We analyzed 15 amino acids: aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, lysine, isoleucine, leucine and phenylalanine.

Total lipids were extracted by the method of Folch et al. (1957). The lipid extract was quantified gravimetrically and kept in a stoppered vessel under nitrogen atmosphere at –30°C until assayed. Analysis of fatty acids was made by gas chromatography (Ruiz-Gutierrez et al. 1992). The samples were saponified by heating for 5 min with 5 ml of 0.2 M sodium methylate and heated again at 80°C for 5 min with 6% (w/v) H_2O in anhydrous methanol. The fatty acid methyl esters thus formed were eluted with n-hexane and analyzed in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and using an Omegawax 320 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 pm film). The initial column temperature was 200°C, held for 10 min, then programmed from 200 to 230°C at 2°C min^{-1}. The injection and detector temperatures were 250 and 260°C, respectively.

The flow rate of helium was 2 ml mm^{-1}, the column head pressure was 250 kPa, and the detector auxiliary flow rate was 25 ml min^{-1}. Peak areas were calculated with a Hewlett-Packard 3390A recording integrator. Individual fatty acid methyl esters were identified on isothermal runs by comparison with standards. Fatty acid methyl esters for which no standard was available were quantified using calibration tables of relative response ratios constructed according to carbon number, using gas chromatography-mass spectrometry (GC-MS). GC-MS was done on a Konik KNK-200 chromatograph interfaced directly to an AEI MS30/70 VG mass spectrometer, using the electron impact mode. The ion source temperature was maintained at 200°C, multiplier voltage was 4.0 kV, emission current was 100 pA and electron energy was 70 eV. The data were processed with a VG 11/250 data system.

Microalgal extraction and NMR analysis. To detect PUSCAs, frozen pellets of *Alexandrium tamarense* (1.3×10^7 cells, 5 g) and *Skeletonema costatum* (5.4×10^9 cells, 4.7 g) were extracted as follows. Microalgae were suspended in 5 ml of distilled water and sonicated for 1 min at 4°C. The suspension was kept at room temperature for 30 min after which 5 ml acetone was added. The sample was sonicated again for 1 min and then centrifuged for 5 min at 1400 × *g*. The supernatant was transferred to a separatory funnel, diluted with distilled water to a final volume of 20 ml, and partitioned 3× against dichloromethane (15 ml). The organic layers were combined, dried over anhydrous Na_2SO_4, filtered on paper and concentrated at reduced pressure (CH_2Cl_2 soluble material). The water phase was added to the sediment obtained from the centrifugation. The resulting mixture was suspended in MeOH and then sonicated for 30 s. The hydro-alcoholic suspension was filtered on paper. The residue was washed with MeOH (10 ml). The combined filtrates were transferred to a conical flask and concentrated at reduced pressure. The viscous residue was diluted with water (9 ml) and partitioned between water, MeOH (12 ml) and $CHCl_3$ (24 ml). The lower layer was recovered and the water phase (upper layer) was processed in the same way twice again. The organic layers were combined and concentrated at reduced pressure ($CHCl_3$-MeOH soluble material). An aliquot of methanolic extract was fractionated in 100% methanol using a Sephadex LH-20 resin. The resulting fractions were tested for biological activity (see next section). The remaining methanolic extract was added to the CH_2Cl_2 soluble material to yield 35.7 mg of extract from *Skeletonema costatum* and 45.6 mg from *Alexandrium tamarense*. *S. costatum* cultures were grown under the same experimental conditions as *A. tamarense* except that the growth medium was *f*/2 (Guillard & Ryther 1962).

The combined extracts were analysed by ^{1}H-NMR carried out on a Bruker Avance DRX at 400 MHz. For the analysis, octadienal and microalgal extracts were dissolved in 0.5 ml $CDCl_3$. Spectra (32 Kbyte) were acquired between 12 and 0 ppm, with an interval delay of 3 s and 512 scans. $CHCl_3$ was used as internal standard at 7.26 ppm.

Sea urchin bioassays. Sea urchins *Sphaerechinus granularis* were collected by SCUBA diving in the Gulf of Naples and transported to the laboratory. The urchins were injected with 0.5 M KCl to induce gamete

ejection; spawned eggs were washed 3× with 0.22 µm FSW and diluted to a final concentration of 3000 eggs ml^{-1}. Concentrated sperm was collected in Eppendorf tubes and 10 µl was diluted in 10 ml of FSW just prior to fertilization. Decadienal solution was prepared by dissolving the commercial aldehyde (SIGMA-Aldrich) in ethanol to a concentration of 0.2 mg ml^{-1}. *Alexandrium tamarense* extract prepared as described above, was initially dissolved in methanol and then transferred to FSW to give a stock solution of 0.2 mg ml^{-1}. Serial dilutions were prepared to give the required experimental concentrations. Approximately 200 oocytes were stocked in 12-well microplates containing 1 ml of medium: FSW alone, solvent blank, and *A. tamarense* extract or decadienal at set concentrations ranging from 0.3 to 5 µg ml^{-1}. After 30 min incubation, oocytes were rinsed 3 times with FSW and fertilized by adding 10 µl of sperm solution. Another group of approximately 200 oocytes were fertilized and transferred soon after in 12-well microplates containing set concentrations of *A. tamarense* and *S. costatum* extracts to test for anti-mitotic activity. Controls and solvent blank were included in all trials. Fertilization success and anti-mitotic activity were determined using an inverted microscope (Axiovert 25, Zeiss) and defined, respectively, as percentage of eggs showing complete elevation of the fertilization membrane, and percentage of first cleavage.

RESULTS

The toxin profile for the *Alexandrium tamarense* strain showed that it was non-neurotoxic with a PSP toxin content of 0.005 ± 0.002 fmol $cell^{-1}$ (mean ± SD; data not shown). The amino acid composition of *A. tamarense* (Table 1) was similar to other non-neurotoxic phytoplankton species (Guisande et al. 1999). The diversity of fatty acids in *A. tamarense* was low compared to other phytoplankton species (Brown et al. 1997), but the content in eicosapentaenoic acid (EPA, 20:5ω3), which is essential for a variety of marine and freshwater zooplankton (DeMott & Müller-Navarra 1997), was high. The fatty acid content of the control diet *Prorocentrum minimum* is very similar (Laabir et al. 2001).

Table 1. *Alexandrium tamarense*. Fatty acid composition (mean weight percentage of total lipids) and amino acid composition (mean weight percentage of total amino acid yield)

Fatty acids	Percent	Amino acids	Percent
14:0	1.1	Aspartic acid	8.1
14:1 ω5	1.1	Serine	6.6
16:0	35.8	Glutamic acid	11.4
16:1 ω9	1.8	Glycine	10.5
18:0	4.3	Histidine	1.2
18:1 ω9	10.3	Arginine	6.2
18:1 ω7	3.4	Threonine	6.1
22:0	10.2	Alanine	12.2
20:5 ω6	2.8	Proline	6.2
20:5 ω3	3.0	Tyrosine	1.3
		Valine	6.1
		Lysine	6.4
		Isoleucine	4.3
		Leucine	9.3
		Phenylalanine	3.8

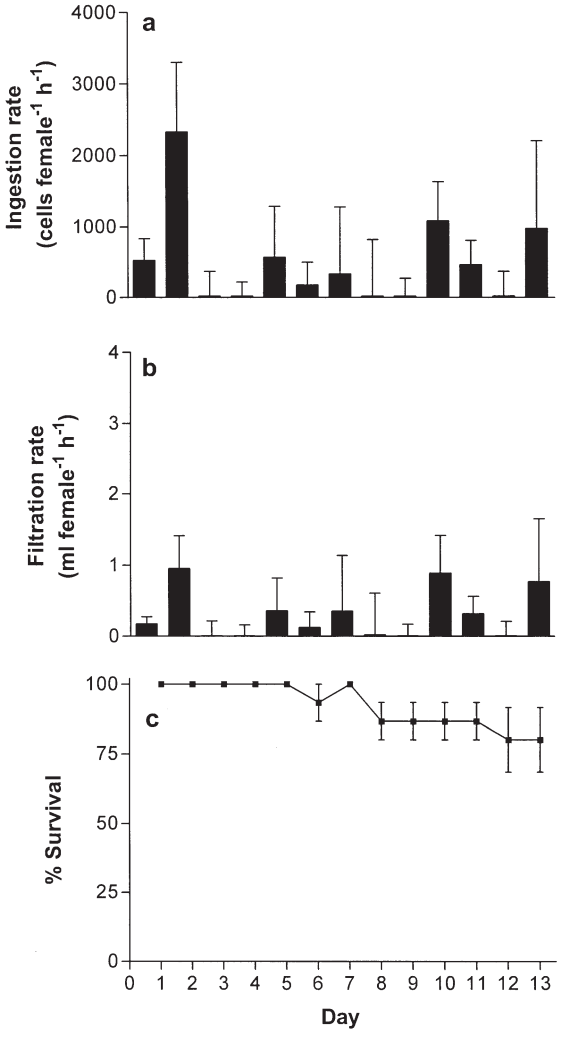

Fig. 1. *Temora stylifera*. (a) Ingestion rates, (b) filtration rates, (c) female survival (%) in copepods offered unialgal cultures of a non-neurotoxic strain of the dinoflagellate *Alexandrium tamarense* for 13 d (mean ± SD of 3 replicates)

Temora stylifera fed willingly on *Alexandrium tamarense*, with ingestion rates comparable to other diets in similar laboratory experiments (Turner et al. 2001). On a day-to-day basis, there was considerable variability in copepod feeding rates over 13 d. Daily mean ingestion rates (Fig. 1a) ranged from 0 to

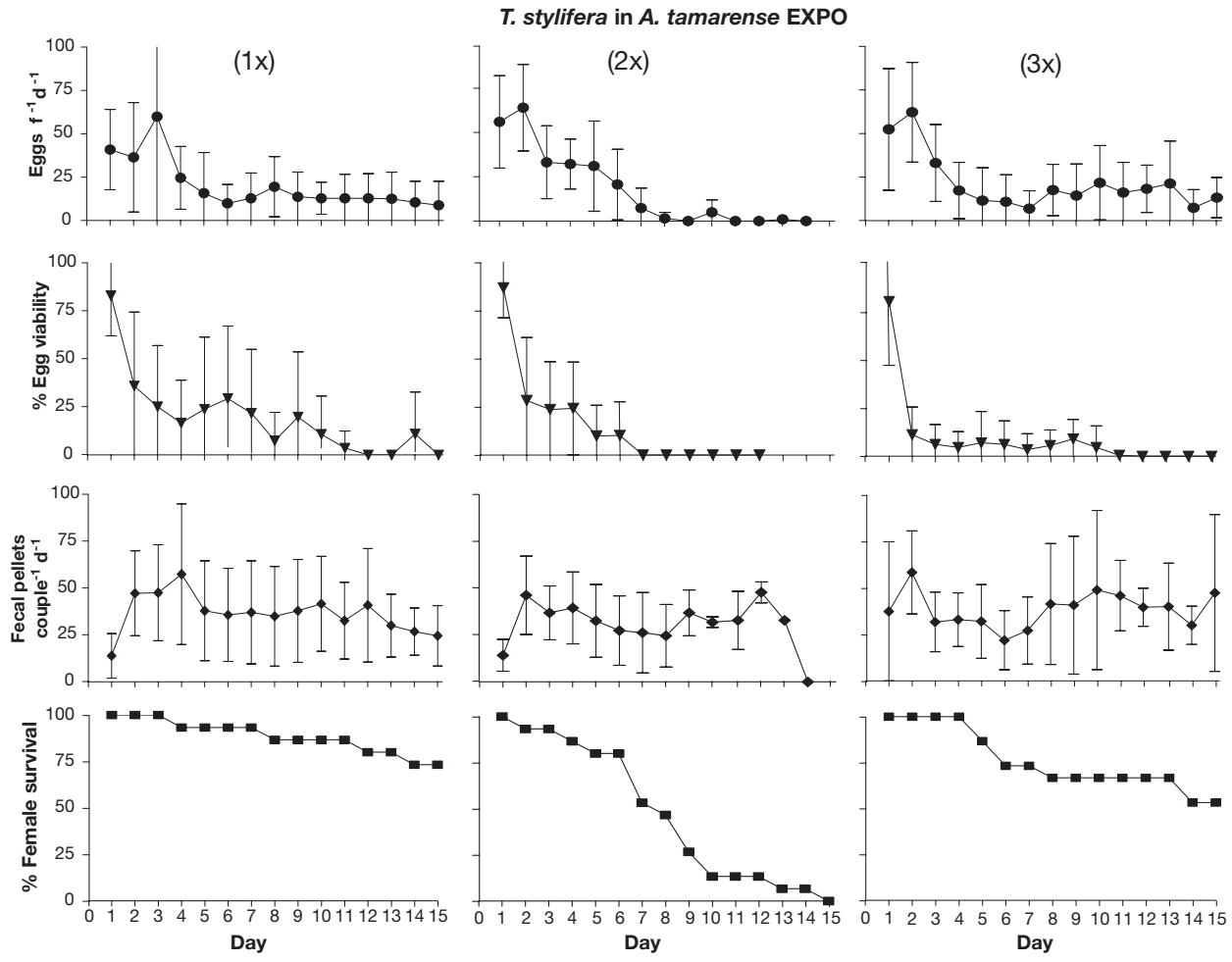

Fig. 2. *Temora stylifera.* Egg production rates per female ($f^{-1}\,d^{-1}$), egg viability (% hatching), fecal pellet production (no. $couple^{-1}\,d^{-1}$) and female survival (%) in copepods offered unialgal exponential phase cultures of a non-neurotoxic strain of the dinoflagellate *Alexandrium tamarense* at cell concentrations of 1×, 2× and 3× initial concentrations

Table 2. *Temora stylifera.* ANOVA repeated measures results on egg production (eggs $female^{-1}\,d^{-1}$), egg viability (%), fecal pellet production (no. $couple^{-1}\,d^{-1}$) and survival (%) during 14 d feeding on exponential and stationary cultures of *Alexandrium tamarense* at 1×, 2× and 3× the initial cell concentration. Treatments with the same letter are not significantly different

	Egg production	Egg viability	Fecal pellet production	Survival
Exponential				
1×	A	A	A,B	A
2×	A	A,B	A,B	C
3×	A	B	A	A,B
Stationary				
1×	A,B	B	A,B	B
2×	B	B	A,B	C
3×	A,B	B	B	C
ANOVA				
F	$F_{5,13} = 5.29$	$F_{5,11} = 8.13$	$F_{5,13} = 3.44$	$F_{5,14} = 28.77$
p	<0.001	<0.001	<0.01	<0.001

2333 cells $female^{-1}\,h^{-1}$ (mean: 498 cells $female^{-1}\,h^{-1}$), and daily filtration rates (Fig. 1b) ranged from 0 to 0.955 ml $female^{-1}\,h^{-1}$ (mean: 0.306 ml $female^{-1}\,h^{-1}$). Daily mean ingestion rates, converted to carbon equivalents ranged from 0 to 4.31 µgC $female^{-1}\,h^{-1}$ (mean = 0.92 µgC $female^{-1}\,h^{-1}$). Copepod survival in grazing experiments was high, with daily means of 80% of the females surviving after 13 d (Fig. 1c).

In all experiments fecundity diminished rapidly with time; by the end of the experiments, egg production rates were <25% of initial values (Figs. 2 & 3). Time-courses of egg production rates were similar in females fed exponential (EXPO) or stationary (STAT) cultures (except for STAT 2×); there were no appreciable differences in egg production rates at increasing food concentrations (Table 2). Egg viability diminished with time in all experiments, and in the EXPO cultures there was a progressive reduction in egg viability from 1× to 2× to 3× initial cell concentrations (Fig. 2, Table 2). Viability in STAT cultures was lower than in

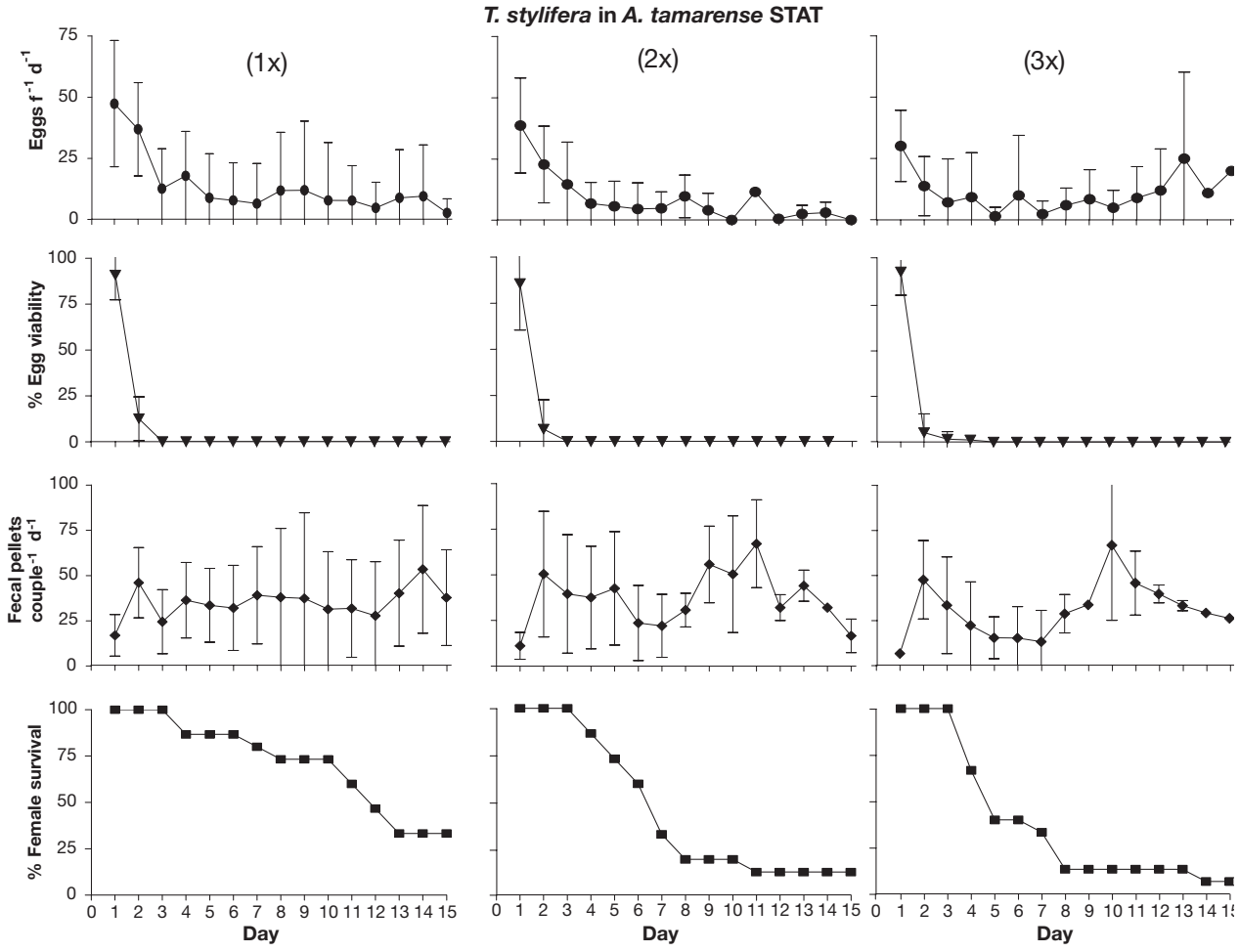

Fig. 3. *Temora stylifera.* Egg production rates per female (f^{-1} d^{-1}), egg viability (% hatching), fecal pellet production (no. $couple^{-1}$ d^{-1}) and female survival (%) in copepods offered unialgal stationary phase cultures of a non-neurotoxic strain of the dinoflagellate *Alexandrium tamarense* at cell concentrations of 1×, 2× and 3× initial concentrations

EXPO 1×, but similar to EXPO 2× and 3× (Table 2), and dropped to 0% after only 24 h of feeding at all 3 algal concentrations (Fig. 3).

Fecal pellet production was generally high and constant with time in both EXPO and STAT cultures, and there were no appreciable differences between low (1×) and high (2× and 3×) cell concentrations, indicating that *A. tamarense* was grazed intensively (Figs. 2 & 3, Table 2). Copepod survival in egg production experiments decreased rapidly with time; <50% of the females survived to Day 15, except at the lowest concentration (1×) EXPO culture. Survival was generally higher in the EXPO than STAT cultures, except for the intermediate concentration (2×) EXPO culture, where all females were dead by Day 15 (Figs. 2 & 3, Table 2). Spermatophore production (not shown) was stable over time and similar to values in other dinoflagellates (Ianora et al. 1999).

When fed with the control food *Prorocentrum minimum*, *Temora stylifera* showed very high egg production rates, which were stable with time, with average values of 61.5 eggs $female^{-1}$ d^{-1} (Fig. 4). Moreover, the time-course fecundity with this control diet was higher than with *Alexandrium tamarense* STAT 1× (paired *t*-test, t_{14} = 11.85, $p < 0.001$). Egg hatching success of females fed *P. minimum* was extremely high (>90%) throughout the 15 d experimental period. Copepods showed very high initial fecal pellet production (>80 pellets $couple^{-1}$ d^{-1}), which decreased after the first day of feeding and remained constant thereafter. This trend was not different from the daily number of fecal pellets produced with a diet of *A. tamarense* (paired *t*-test, t_{14} = 0.46, $p > 0.05$). Copepod survival was always >90% for the entire period (Fig. 4).

To test for the presence of compounds responsible for reduced egg production and hatching success, raw lipophilic extracts of *Alexandrium tamarense* were obtained by successively preparing dichloromethane and chloroform-methanol soluble fractions; these were compared by ^{1}H-NMR analysis to those obtained with

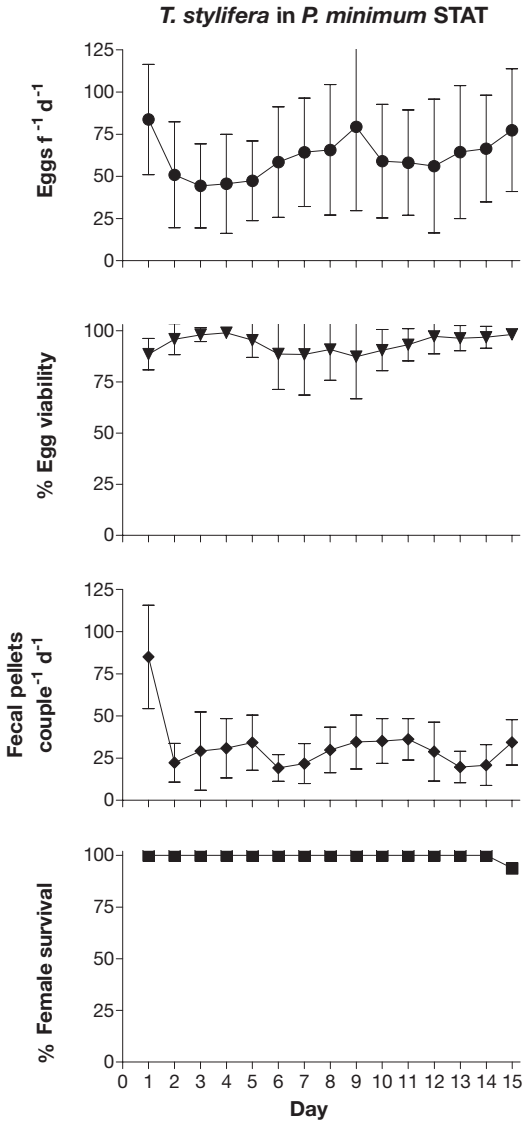

Fig. 4. *Temora stylifera*. Egg production rates per female (f^{-1} d^{-1}), egg viability (%), fecal pellet production (no. $couple^{-1}$ d^{-1}) and female survival (%) in copepods offered unialgal stationary phase cultures of the dinoflagellate *Prorocentrum minimum*

the diatom *Skeletonema costatum*, which has been shown to produce a number of PUSCAs (d'Ippolito et al. 2002, 2003). Commercial octadienal (isomeric mixture of 5% cis and 95% trans) was used as a standard (Fig. 5, top). Examination of ^{1}H-NMR spectra of combined extract of *A. tamarense* (Fig. 5 bottom) unambiguously showed the absence of PUSCA signals at δ 9.62 (d), 9.53 (d), 7.69 (dd), 7.34 (dd), 6.45 (d), 6.43 (d), 6.20 (dd) 6.12 (dd), which are evident in the *S. costatum* combined extract (Fig. 5 center) (where δ = chemical shift, d = doublet, dd = double doublet).

Alexandrium tamarense also differed from PUSCAs in terms of biological activity in the sea urchin bioassay (e.g. Pagano et al. 1986). Of the 2 organic extracts of *A. tamarense*, the one obtained in MeOH was the more active and one of the fractions obtained by its separation on Sephadex LH-20 resin blocked fertilization of sea urchin oocytes at concentrations of 1 µg ml^{-1}; decadienal, a commercially available PUSCA, had little or no effect on fertilization success at any of the concentrations tested (Fig. 6). By contrast, the *A. tamarense* active fraction had little or no effect on sea urchin first-cell cleavage as opposed to decadienal, which blocked cell cleavage at concentrations of 1 µg ml^{-1}, confirming previous studies on the anti-mitotic activity of this aldehyde (d'Ippolito et al. 2002).

DISCUSSION

Even though the dinoflagellate clone used in the present study was 'non-toxic' in that it did not contain saxitoxins or neosaxitoxins, *Alexandrium tamarense* nonetheless produced deleterious effects on reproduction and survival of the copepod *Temora stylifera*. These effects included reduced egg production rates and hatching success. They were not due to reduced grazing, since copepods seemed to feed well upon the dinoflagellate even though there was large variability in daily ingestion rates (possibly due to high algal concentrations). Egg production rates reported here ranged from mean values of 4.3 to 21.7 eggs $female^{-1}$ d^{-1}, which are much lower than those reported for *T. stylifera* when it feeds on other flagellate and diatom species (Ianora et al. 1999, Turner et al. 2001, Ceballos & Ianora 2003). In these previous studies, the highest egg production rates for *T. stylifera* feeding on the dinoflagellates *Gonyaulax polyedra* and *Prorocentrum minimum* for 14 d were 55 and 58 eggs $female^{-1}$ d^{-1}, respectively. Similar values were found in the present study with the control *P. minimum* diet. These results indicate that although *A. tamarense* was 'non-toxic', there were other biochemical characteristics of the food that negatively affected egg production rates in *T. stylifera*.

Similar results have been reported by Dutz (1998), who found a reduction of copepod fecundity in *Acartia clausi* females exposed to the neurotoxic dinoflagellate *Alexandrium lusitanicum*. At concentrations of 1.25 and 1.56 $saxitoxin_{eq}$ $cell^{-1}$, *A. clausi* fed on toxic cells at high rates without effects on copepod survival, but egg production was limited over the range of food concentrations offered. Dutz suggested that the toxins possibly interfered with digestive processes or caused enhanced energy expenditure due to detoxification, resulting in reduced female fecundity.

Reduced egg production rates with *Alexandrium tamarense* occurred even though females fed well on

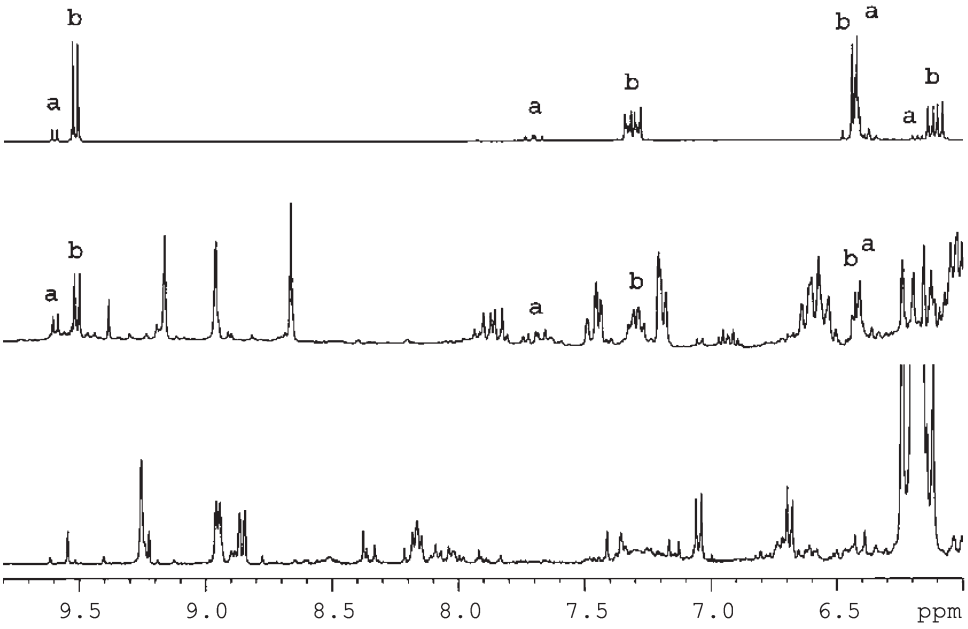

Fig. 5. Downfield region (9.80–6.00 ppm) of the ^{1}H-NMR spectra of lipophilic extracts. Top: commercially available 2,4-octadienal; center: *Skeletonema costatum*; bottom: *Alexandrium tamarense*. Isomers of octadienal: (a) trans-, cis-; (b) trans-, trans-

this dinoflagellate. In fact, ingestion and filtration rates were similar or even higher than those recorded for *Temora stylifera* feeding on diets of the dinoflagellate *Prorocentrum minimum* (Turner et al. 2001). Thus, we found the same reduced fecundity on dinoflagellate diets as reported by Dutz (1998), although he attributed it to saxitoxins, whereas we found that the same effect occurred even when saxitoxins were not present. Hence, such adverse effects on grazers of 'toxic' dinoflagellates may be due to known neurotoxins such as saxitoxins, or to less obvious or unknown compounds.

The reduction of egg hatching success was even more dramatic. In stationary cultures, egg viability was reduced to zero as quickly as 24 h after feeding on *Alexandrium tamarense* at all 3 algal concentrations tested. This was much faster than observed for several species of copepods feeding on various diatoms, where hatching success became reduced after 5 to 10 d of feeding (Poulet et al. 1995, Uye 1996, Turner et al. 2001, Ceballos & Ianora 2003, Ianora et al. 2003). This reduction was not likely due to a nutritional deficiency since *A. tamarense* did not seem to lack any essential amino acids and fatty acids (Table 1). A deficiency in any essential compound in the food will usually produce a negative effect on copepod reproduction after 2 to 3 d (Kleppel et al. 1988, Guisande et al. 1999), and hatching success will decline to 30%, but not to 0%.

If saxitoxins or a nutritional deficiency were not responsible for reduced egg production and hatching success, then what was the cause? Diatom-derived aldehydes were not responsible for adverse effects on hatching, since PUSCAs were absent in *Alexandrium tamarense* as compared to the diatom *Skeletonema costatum*. Diatom PUSCAs include a number of mole-

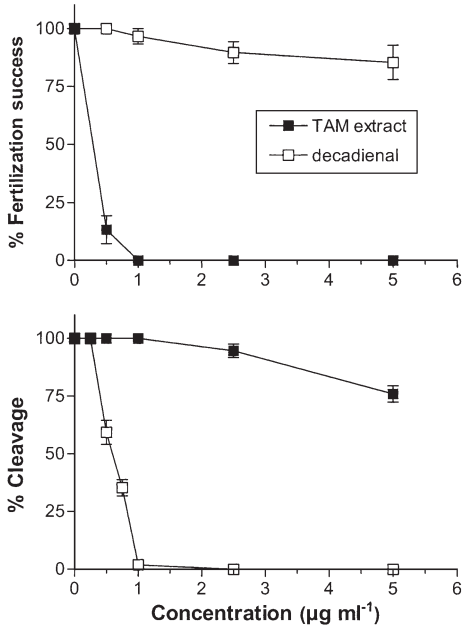

Fig. 6. *Sphaerechinus granularis.* Sea urchin bioassays comparing the effects of lipophilic extracts of the dinoflagellate *Alexandrium tamarense* (TAM) and the diatom-derived aldehyde decadienal

cules sharing the presence of an α,β-unsaturated aldehydic function that is believed to be necessary for the biological activity of these molecules (Adolph et al. 2003). This functional group is easy to detect by proton magnetic resonance spectroscopy (^{1}H-NMR) since it gives rise to a diagnostic signal in the downfield region of the spectrum (Fig. 5). These NMR signals are rather independent of the parts of the molecule that do not affect the chemical shift and multiplicity of the lines. As shown in Fig. 5, the NMR spectra of raw extracts of *A. tamarense* did not reveal any presence of such spectroscopic markers, which on the other hand were clearly discernible in the raw material obtained from the PUSCA-producing diatom *S. costatum* (d'Ippolito et al. 2002). This indicates that toxic aldehydes either do not occur in the dinoflagellate or are present at significantly lower levels than those recorded in diatoms.

Until now, diatom-derived PUSCAs are the only compounds that have been shown to block mitotic divisions in copepod embryos (Miralto et al. 1999, Ianora et al. 2003 and references therein). However, there are several examples of other compounds isolated from marine dinoflagellates that block cell division in the classic sea urchin bioassay. These include goniodomin-A from *Goniodoma pseudogoniaulax* (Murakami et al. 1988), amphidinolide-A from *Amphidinium* sp. (Kobayashi et al. 1986), and okadaic acid and its derivative dinophysistoxin-1, which are common in some species of the genera *Prorocentrum* and *Dinophysis*, respectively (Fujiki et al. 1988). Extracts of *Alexandrium tamarense* may also inhibit egg hatching and larval survival in the scallop *Chlamys farreri* (Yan et al. 2001). In all of these studies, the molecules or extracts tested showed anti-mitotic activity with blockage of cell divisions in developing sea urchin embryos. In the present study, however, there was little or no adverse effect on cell cleavage, but very strong effects on fertilization success when oocytes were incubated for 30 min in *A. tamarense* extracts. A reduction in fertilization capacity has been found for *Temora stylifera* fed with the dinoflagellates *Prorocentrum micans*, *Akashiwo sanguinea* and *Lingulodinium polyedra* (Ianora et al. 1999); however, this effect was due to an inability of sperm to fertilize the oocytes, whereas in the present study an absence of fertilization success was likely due to alterations in the fertilization membrane of female gametes, preventing the sperm from penetrating the oocyte.

Stationary phase cultures generally induced stronger effects on egg production and hatching success than exponential cultures, with hatching success dropping to 0% at all 3 algal concentrations tested. This indicates that the unidentified compounds were being produced in greater quantities in older cultures, as is the case for domoic acid production in *Pseudo-nitzschia multiseries* (Bates 1998). Clearly, these compounds were not feeding deterrents since *Temora stylifera* fed well on this *Alexandrium tamarense* strain, with fecal pellet production rates comparable to other dinoflagellate (*Lingulodinium polyedrum* and *Prorocentrum minimum*) and flagellate (*Isochrysis galbana*) diets (Ianora et al. 1999). Carbon equivalent ingestion rates were also high in comparison to those of our previous studies, although food concentrations were also comparatively high. Nonetheless, there were adverse effects on other life history parameters such as fecundity, egg viability and adult female survivorship, even though such effects are probably attenuated during natural *A. tamarense* blooms since the algal concentrations tested here are 5× greater than *in situ*.

Why do microalgae produce such compounds if they do not serve to deter feeding in their predators? According to chemical defense theory, there should be a selective survival advantage for species that can 'protect' themselves from grazers, allowing such species to grow explosively, because the growth of the populations of their predators has been suppressed. This could occur through the production of chemicals that not only deter feeding activity, but also reduce population growth. For example, reactive aldehydes of diatoms reduce egg production and/or hatching success, thereby sabotaging copepod population growth and, consequently, predator grazing impact. In the case of *Alexandrium tamarense*, the defense machinery is potentially even more effective, because female longevity is reduced as well.

A paradox of dinoflagellate toxins such as the saxitoxins is that they primarily poison organisms that are not the direct grazers of the dinoflagellates, such as fish, marine mammals and birds, and humans (reviewed by Turner & Tester 1997, Cembella 2003). These toxins often have little or no adverse effects on direct dinoflagellate grazers such as copepods and other zooplankton organisms. Thus, an antipredation role of dinoflagellate toxins might serendipitously favor grazers rather than dinoflagellates, since copepods may sequester these compounds from dinoflagellates (Turner et al. 2000, Tester et al. 2001), and ingested toxins possibly act as defenses to deter predation on copepods by fish and other zooplanktivorous consumers. Conversely, dinoflagellates may produce metabolites, other than reactive aldehydes, that directly impact predator fitness by reducing fertilization capacity. Although the chemical nature of these molecules remains elusive, our findings indicate that as in the case of diatoms, dinoflagellates may produce antiproliferative compounds which can suppress copepod population growth.

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