PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2006) **273**, 1673–1680
doi:10.1098/rspb.2006.3502
Published online 10 April 2006

Copepods induce paralytic shellfish toxin production in marine dinoflagellates

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Among the thousands of unicellular phytoplankton species described in the sea, some frequently occurring and bloom-forming marine dinoflagellates are known to produce the potent neurotoxins causing paralytic shellfish poisoning. The natural function of these toxins is not clear, although they have been hypothesized to act as a chemical defence towards grazers. Here, we show that waterborne cues from the copepod *Acartia tonsa* induce paralytic shellfish toxin (PST) production in the harmful algal bloom-forming dinoflagellate *Alexandrium minutum*. Induced *A. minutum* contained up to 2.5 times more toxins than controls and was more resistant to further copepod grazing. Ingestion of non-toxic alternative prey was not affected by the presence of induced *A. minutum*. The ability of *A. minutum* to sense and respond to the presence of grazers by increased PST production and increased resistance to grazing may facilitate the formation of harmful algal blooms in the sea.

Keywords: *Alexandrium minutum*; *Acartia tonsa*; induced resistance; paralytic shellfish poisoning

1. INTRODUCTION

Paralytic shellfish poisoning (PSP) is a threat to marine aquaculture and shellfish consumers around the world. PSP is caused by planktonic dinoflagellates of the genera *Alexandrium*, *Gymnodinium* and *Peridinium*. These microscopic phytoplankton produce paralytic shellfish toxins (PST), a group of highly potent neurotoxic alkaloids among which saxitoxin was the first to be discovered (Schantz *et al.* 1975). PST may cascade through the food web and intoxicate higher trophic levels and occasional mass mortalities of marine mammals and seabirds have been attributed to PST (Landsberg 2002). Humans are mainly intoxicated through consumption of contaminated filter feeders, e.g. mussels. If the dose is high enough, PST is lethal.

The ultimate cause for PST production is not clear (Cembella 2003), but based on the inherent toxicity of the molecules and the negative effects that PST containing phytoplankton have on some grazers, PST has been suggested to act as a protection against grazers (Cembella 2003). Studies on the effects of PST-containing prey on consumers have shown variable results (Turner & Tester 1997) ranging from non-detectable in some copepods (Colin & Dam 2003) to decreased feeding (Teegarden 1999), decreased fecundity (Guisande *et al.* 2002*a*), delayed development (Frangopulos *et al.* 2000) and direct mortality in others (Colin & Dam 2003). Furthermore, the copepods *Calanus finmarchicus* (Turriff *et al.* 1995), *Eurytemora herdmanii* and *Acartia tonsa*, as well as the barnacle *Semibalanus balanoides* (Teegarden 1999; Teegarden *et al.* 2001) have been shown to avoid feeding on PST-containing prey when offered together with a non-toxic food option. Even though feeding deterrence is the best supported role for PST production, most previous studies on the factors determining the level of PST production have focused on the physical surroundings of the dinoflagellates. The most apparent pattern from these studies is that nitrogen depletion results in low cell specific toxicity whereas nitrogen repletion results in high cell specific toxicity (John & Flynn 2002). If growth is impaired by factors other than nitrogen, cell specific toxin content often increases. This relationship is best established for phosphate limitation (John & Flynn 2002) but has also been described for turbulence (Juhl *et al.* 2001) and salinity stress (Grzebyk *et al.* 2003).

The effects of biological interactions on PST production are less well known. If the primary function of PSTs is to protect the producers against grazers, the optimal defence theory predicts that cell specific toxin content should correlate to the probability of attack (Rhoades 1979). The chemical defences of many vascular plants are induced by the presence of grazers or airborne chemical cues related to grazing (Karban & Baldwin 1997; Tollrian & Harvell 1999). Induced chemical defences have also been shown to occur in seaweeds in response to direct grazing (Pavia & Toth 2000) as well as to waterborne chemical cues (Toth & Pavia 2000). Morphological and life-history changes induced by biological threats, such as spine formation (Lurling & Beekman 1999), colony formation (Hessen & Van Donk 1993) and induction of temporary cysts (Toth *et al.* 2004), have been described in diverse groups of planktonic organisms (Kats & Dill 1998). Furthermore, injury induced cleavage of dimethylsulphonopropionate into dimethylsulphide (DMS) and acrylate that deters protozoan consumers has been suggested to occur in the bloom-forming coccolithophorid *Emiliana huxleyi* (Wolfe *et al.* 1997), and the formation of terratogenic aldehydes from fatty acid precursors is

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Received 12 December 2005
Accepted 29 January 2006

induced by injury in several diatoms (Miralto *et al.* 1999). However, the natural function of acrylate and DMS, as well as terratogenic aldehydes, has been questioned (Irigoien *et al.* 2002; Strom *et al.* 2003). Signal induced toxin production has previously been reported for the limnic cyanobacteria *Microcystis aeruginosa*, which was shown to produce more microcystins upon encounter with grazers or waterborne cues from grazers (Jang *et al.* 2003). However, the inducing grazers used in the direct grazing experiment died during the course of the experiment, regardless of the microcystin content of *M. aeruginosa*. Moreover, the effects of the increased microcystin content were not tested on any grazer species, making it impossible to conclude whether or not the induction was adaptive. A recent investigation has shown that the presence of the copepod grazer *A. tonsa* in *Alexandrium minutum* cultures results in a higher cell specific toxin content compared to copepod-free control cultures (Guisande *et al.* 2002*b*). One possible explanation for this observation is that the toxin production is induced by the presence of grazing copepods. However, some marine copepods, including *A. tonsa*, are known to discriminate against prey with higher PSP content (Teegarden 1999). Selective grazing on the least toxic cells in a population of *A. minutum* would also result in higher average cell specific toxicity, and the result may be indistinguishable from induced toxin production. In order to separate these two mechanisms, the toxin producing cells need to be exposed to potential inducing signals without being subjected to direct grazing. Alternatively, if cells are exposed to direct grazing, the possible effect of selective grazing on the overall cell specific toxicity needs to be controlled for.

In this study, we tested the hypothesis that copepod grazing can induce increased PST production by exposing dinoflagellates of the species *A. minutum* to waterborne cues from the copepod grazer *A. tonsa*. Furthermore, in order to examine if any induced changes in PST content would be correlated with an increased resistance to grazing, we performed a subsequent feeding preference experiment, where we specifically tested the hypothesis that induced *A. minutum* cells, with a higher PST content, would be relatively less preferred food compared to non-induced *A. minutum* cells.

2. MATERIAL AND METHODS

(a) *Organisms and culture conditions*

The marine dinoflagellates *A. minutum* (strain # 83, strain synonyms: CCMP 113 Al and Al 1V) and *Prorocentrum micans* (strain # K35, strain synonym: CCMP 1589) were obtained from the Göteborg University Marine Algal Culture Collection. Cultures were grown in sterile cotton-plugged Erlenmeyer flasks (250 ml) by inoculating a few millilitres of the cultures into fresh K medium (Leftley *et al.* 1987) every second week. The copepod grazer, *A. tonsa*, was obtained from a culture held at Kristineberg Marine Research Station (Sweden) and was reared for several generations on a phytoplankton diet consisting of the diatoms *Thallasiosira pseudonana*, *Skeletonema costatum* and the prymnesiophyte *Isochrysis galbana* before the experiments were started. All experiments were conducted in K medium diluted 10 times to avoid artefacts due to unnaturally high nutrient concentrations (John & Flynn 2002). Phosphate was reduced to 25% of the initial formula to avoid nitrogen-limited conditions, which are known to inhibit PST production (John & Flynn 2002). Experimental containers and dinoflagellate cultures were incubated in a temperature-controlled incubator at 18 °C with 150 μmol m^{-2} s^{-1}, 14 : 10 h light : dark cycles, provided by fluorescent tubes.

(b) *Induction experiment*

The aim of the induction experiment was to investigate whether *A. minutum* cells can detect and respond to copepod-related waterborne chemical cues by inducing an increased production of PST. The experiment was carried out in glass bottles (120 ml) containing cages made from polyethylene centrifuge tubes (50 ml) with a heat-sealed nylon-mesh bottom (mesh size 10 μm). The cell concentration in the *A. minutum* culture was determined using a particle counter (Elzone 180 XY) before addition to the experimental containers. At the start of the experiment, the *A. minutum* culture (70 ml, 1750 cells ml^{-1}) was added to each glass bottle and the cages were immersed in the cultures. The cages contained either pure *A. minutum* culture (30 ml, 1750 cells ml^{-1}, controls), freshly prepared diluted K medium (30 ml) with three adult female *A. tonsa* (starving copepods) or *A. minutum* culture (30 ml, 1750 cells ml^{-1}) with three adult female *A. tonsa* (grazing copepods). During the experiment, cages were gently lifted two-thirds of the immersed length five times each day to assure water exchange between the *A. minutum* cultures outside the cages and the different treatments inside the cages. Each treatment (control, starving copepods and grazing copepods) was replicated four times, i.e. a total of 12 glass bottles were used in the induction experiment. With the experimental set-up used in the induction experiment, *A. minutum* cells kept outside the cages received waterborne cues from the different treatments inside the cages. Fluids ran freely between the compartments, but the organisms were restricted to their original compartment by the mesh in the bottom of the cages.

After 3 days when the experiment was terminated, the cages were removed from the glass bottles and the content of each compartment (outside and inside the cages) was transferred individually to clean beakers. Each compartment was rinsed three times with filtered seawater and the rinses were also included in the sample to avoid loss of cells in the transfer. The weight of the fluid samples in the beakers was determined and the exact volume of the samples was calculated using the mass density of the medium. Mean diameter of cells in the control and grazing copepod treatments was measured under a light microscope (200× magnification) to control for possible grazer induced changes in cell volume. The algal cell concentration in each compartment was determined with the particle counter and the number of cells (n) in each compartment was calculated according to the formula $n = V \times C$, where V is the volume of the sample and C is the concentration of cells obtained by the particle counter. Five millilitres of each sample from outside the cages (i.e. the cultures receiving waterborne cues) was removed, filtered (0.45 μm) and frozen for analysis of dissolved nutrients (see §2*e*). The weight of the samples from outside the cages was redetermined after removal of fluid for cell counts and nutrient analysis and the total number of *A. minutum* was calculated as described above. After calculation of total cell number in each sample, the cells were filtered onto 25 mm GF/A filters for further toxin analysis. Filters were lyophilized and stored at −80 °C until toxin extraction was performed (see §2*e*). The net growth rate

(μ) of the *A. minutum* cultures both outside and inside the cages was calculated using the formula

$$\mu = \frac{\ln C_t - \ln C_0}{\Delta t}, \tag{2.1}$$

where C_t is the cell concentration at the end of the experiment, C_0 is the cell concentration at the start of the experiment and Δt is the elapsed time in days.

(c) *Density dependence experiment*

An additional induction experiment was carried out to evaluate the density-dependent effects of copepod grazing on the cell specific PST content of *A. minutum*. The copepod densities in this experiment were chosen to reflect more commonly occurring densities of *A. tonsa* in natural populations and ranged from 4 to 16 individuals l^{-1}. The *A. minutum* culture (480 cells ml^{-1}) was divided between 12 Ehrlenmeyer flasks (500 ml in each flask). The flasks received 0, 2, 4 or 8 ($n=3$) *A. tonsa* females and were incubated in the temperature and light controlled incubator. To ensure an effective spreading of the inducing cues in the larger flasks (compared to the first induction experiment), we excluded the cages, i.e. the *A. minutum* cells were exposed to direct grazing. Given the magnitude of the previously observed toxin induction and the presumption that only small fraction of the *A. minutum* cells would be consumed during the course of this experiment, we assumed that selective feeding could most likely be excluded as a significant explanatory factor for the predicted increase in PST concentration. A 50 ml sample was extracted after 5 days and cell specific PST content was determined as described in §2*b*.

(d) *Feeding preference experiment*

Induced resistance to copepod grazing was investigated in a feeding preference experiment, where *A. tonsa* was allowed to choose between different food items. Since it is not possible to visually discriminate between induced and control *A. minutum* cells, the dinoflagellate *P. micans* was used as a non-toxic food alternative (cf. Guisande *et al.* 2002*b*). *P. micans* is similar to *A. minutum* in size and motility, suggesting that *A. tonsa* presented with a mixture of equal numbers of *A. minutum* and *P. micans* would experience similar encounter rates with both prey types. The copepods in the feeding preference experiment were provided with either a combination of induced *A. minutum* and *P. micans* or a combination of control *A. minutum* and *P. micans*.

Induced and control *A. minutum* cells were prepared by culturing *A. minutum* with or without the presence of *A. tonsa* for 3 days prior to the experiment. *A. minutum* cultures in diluted K medium (2000 cells ml^{-1}) were placed in two large (500 ml) glass bottles and 15 adult female *A. tonsa* were added to one of the bottles. The *A. minutum* cultures were exposed to the same environmental conditions as the cultures used in the induction experiment. At the end of the pretreatment period, a sample (100 ml) was collected from each bottle for toxin analysis (see §2*b*). Each of the two *A. minutum* cultures was mixed with a *P. micans* culture to a final concentration of 600 *A. minutum* cells ml^{-1} and 600 *P. micans* cells ml^{-1} (i.e. a total of 1200 cells ml^{-1}). Each of the algal mixtures was divided into 24 glass bottles (60 ml, i.e. a total of 48 bottles) and five adult female *A. tonsa* individuals were added to half of the bottles. All bottles were incubated on a plankton wheel (1 r.p.m.) for 3 days to cover both fast (e.g. behavioural rejection and physical incapacitation; Colin & Dam 2003) and slow (e.g. associative learning) mechanisms that may result in discriminate feeding of the copepods. At the termination of the experiment, a sample of 300 µl from each replicate bottle was preserved in Lugol's solution and the number of *A. minutum* and *P. micans* was counted under an inverted microscope. The ingestion rate of each algal species was calculated according to the formula described in (Frost 1972)

$$I = \bar{C}\frac{Vg}{n}, \tag{2.2}$$

where $\bar{C}$ is the mean cell concentration during the experiment calculated as

$$\bar{C} = \frac{C_0(e^{(\mu-g)\Delta t}-1)}{\Delta t(\mu-g)}, \tag{2.3}$$

where C_0 is the initial concentration of *A. minutum* or *P. micans*, μ is net growth rate in control bottles calculated as in equation (2.1) but with Δt expressed in hours, V is the volume of the bottle, Δt is the elapsed time in hours, n is the number of copepods and g is the grazing coefficient calculated from each flask with grazers as

$$g = \mu - \frac{(\ln C_t - \ln C_0)}{\Delta t}, \tag{2.4}$$

where C_t is the final concentration of *A. minutum* or *P. micans*, respectively, in grazed replicates. The feeding preference was analysed by comparing the proportion of ingested *A. minutum* cells in the diet of the copepods.

(e) *Chemical analyses*

The nitrate and phosphate concentration in the medium at the end of the induction experiment were quantified with a continuous-flow automated system (TRAACS 800, Bran-Luebbe, Germany) according to standard colorimetric procedures (Strickland & Parsons 1972). PST samples were extracted with 1.5 ml 0.05 M acetic acid (aq) through four consecutive freeze–thaw cycles. The extract was filtered (GF/A) and frozen in HPLC glass vials until analysis with high performance liquid chromatography with fluorescence detection (HPLC-FD; Asp *et al.* 2004). HPLC analyses were carried out on a Hitachi-7000 system equipped with a RP8 Column (Supelco Discovery C8, 5 µm, 150 mm, ID = 4.6 mm). PST-standards (saxitoxin, decarbamoylsaxitoxin, neosaxitoxin and gonyautoxin (GTX) 1–4) were purchased from NRC-CNRC, Halifax, Canada. The *A. minutum* strain used here is only known to produce GTX 1–4 (Franco *et al.* 1994). However, to exclude the possible presence of *N*-sulfocarbamoyl toxins (C-toxins), a subset of the toxin samples was hydrolysed and analysed for changes in corresponding carbamate toxin concentrations. Since only GTX 1–4 were detected in our samples, an isocratic elution with 50 mM ammonium-phosphate buffer (pH 7.1) and 2 mM sodiumheptanesulfonate at 0.8 ml min^{-1} was used to separate the toxins. After the separation, toxins were oxidized with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9.0, 0.4 ml min^{-1}) in a PEEK capillary (10 m, 80 °C). The oxidation was terminated with 0.5 M acetic acid (0.4 ml min^{-1}) before fluorescent detection at $\lambda_{ex}=330$ nm, $\lambda_{em}=390$ nm.

(f) *Statistical analysis*

Data on the total cell specific toxin content and the net growth rate of *A. minutum* outside cages (i.e. the cells that

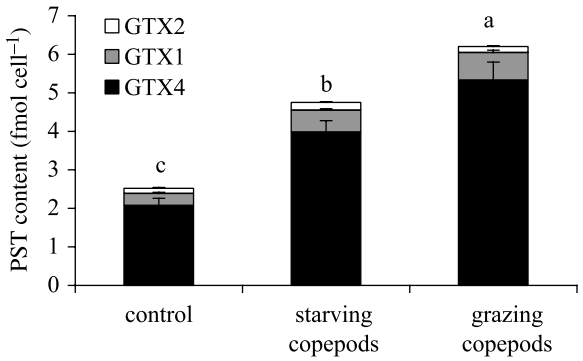

Figure 1. Paralytic shellfish toxin (GTX 1, 2 and 4) content (fmol cell^{-1}) in *A. minutum* that received waterborne cues from different treatments (control, starving copepods, grazing copepods) outside cages in the induction experiment. The GTX 3 content was close to the detection limit in all treatments and is not visible in the figure. Letters above bars indicate significant differences in total PST content among treatments based on the SNK multiple comparisons test ($p<0.05$). Error bars show +s.e.m. ($n=4$).

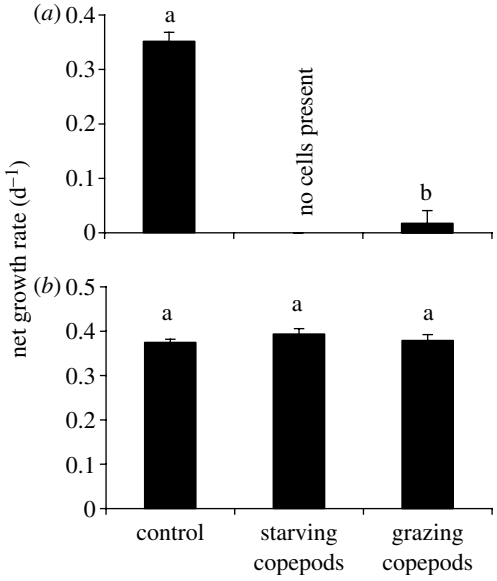

Figure 2. Net growth rate, μ, (d^{-1}) of *A. minutum* cells (*a*) exposed to the different treatments (control, starving copepods, grazing copepods) inside cages and (*b*) that received waterborne cues from the different treatments outside cages. Letters above bars indicate significant differences among treatments based on the SNK multiple comparisons test ($p<0.05$). Error bars show +s.e.m. ($n=4$).

received waterborne cues) in the induction experiment were statistically analysed using a one-way analysis of variance (ANOVA) with treatment as a fixed three-level factor. Cell specific toxin content data from the density dependence experiment was analysed with a one-way ANOVA with treatment as a fixed four-level factor. Statistically significant differences between mean-values were compared using the Student–Newman–Keul's (SNK) multiple comparison procedure (Underwood 1997). Data on the mean diameter of cells, net growth rate of the *A. minutum* cultures inside cages in the induction experiment and on the proportion of ingested *A. minutum* in the feeding preference experiment were statistically analysed with Student's *t*-test. A one-tailed test was used in the feeding preference experiment because the hypothesis was that the copepods would feed less on induced *A. minutum* cells compared to control cells. Prior to all statistical analyses the data was tested for homogeneity of variances with Cochran's test. No heterogenous variances were detected and therefore, data were not transformed.

3. RESULTS

(a) *Induction experiment*

The nutrient analysis at the end of the induction experiment showed that the medium in all the experimental treatments contained more than 40 μM nitrate and 7 μM phosphate indicating that these nutrients did not limit the growth of the *A. minutum* cells. Four different PSTs, GTX 1–4, were detected in the *A. minutum* cells (figure 1). No derivates of C-toxins were detected after hydrolysis of a subset of the toxin samples. Data on the total (GTX 1–4) cell specific toxin content showed that there was a statistically significant difference between *A. minutum* cells exposed to waterborne cues originating from the different treatments (ANOVA, $F_{2,9}=22.8$, $p<0.001$). When mean values were compared with the SNK procedure, the total toxin content in *A. minutum* cells exposed to cues from cages containing copepods (starving and grazing copepods) was found to be significantly ($p<0.05$) higher compared to controls (figure 1). Furthermore, the cells exposed to cues from cages with grazing copepods had a significantly ($p<0.05$) higher total toxin content compared to cells exposed to cues from starving copepods (figure 1). There were only minor differences in the toxin composition of *A. minutum* cells exposed to the different treatments. GTX 4 was the dominating toxin in all samples, followed by GTX 1. Together, GTX 4 and GTX 1 constituted more than 90% of the total toxin content. GTX 2 was the third most common toxin and GTX 3 was present in trace amounts (less than 0.5% of total toxin content) in all samples (figure 1).

There was a clear and statistically significant decrease in the net growth rate of *A. minutum* cells grazed by copepods compared to the control cells inside the cages (*t*-test, $t_{\mathrm{d.f.}=6}=11.8$, $p<0.001$), reflecting that the copepods were actively feeding during the induction experiment (figure 2*a*). However, there was no statistically significant difference in the net growth rate between *A. minutum* cultures exposed to waterborne cues from the different treatments in the induction experiment (ANOVA, $F_{2,9}=0.79$, $p=0.48$, figure 2*b*). Furthermore, there was no statistically significant difference (*t*-test, $t_{\mathrm{d.f.}=6}=1.53$, $p=0.18$) in the mean spherical diameter of the *A. minutum* cells exposed to the different treatments (controls versus grazing copepods, 22.2 ± 0.312 and 21.8 ± 0.296 μm, mean±s.e.m., respectively). Consequently, the statistically significant difference in total toxin content between *A. minutum* cultures exposed to different waterborne cues from control and grazing copepods (figure 1) was not due to changes in *A. minutum* cell volume or net growth rate.

(b) *Density dependence experiment*

The presence of *A. tonsa* resulted in increased cell specific PST in all treatments compared to controls (ANOVA, $F_{3,8}=24.5$, $p<0.05$, figure 3). The increase was significantly ($p<0.05$) stronger in the highest copepod density (16 copepods l^{-1}) compared to the intermediate concentrations (4 and 8 copepods l^{-1}). The growth rate averaged

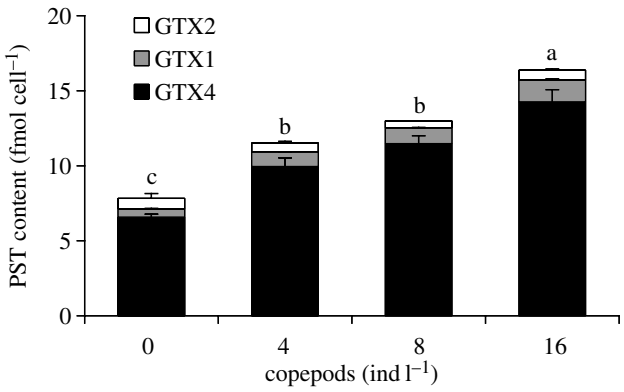

Figure 3. Cell specific content of GTX 1, 2 and 4 (fmol cell^{-1}) in *A. minutum* exposed to direct grazing from the different densities of *A. tonsa* indicated by the category axis. Letters above bars indicate significant differences among treatments based on the SNK multiple comparisons test ($p<0.05$). Error bars show +s.e.m. ($n=3$).

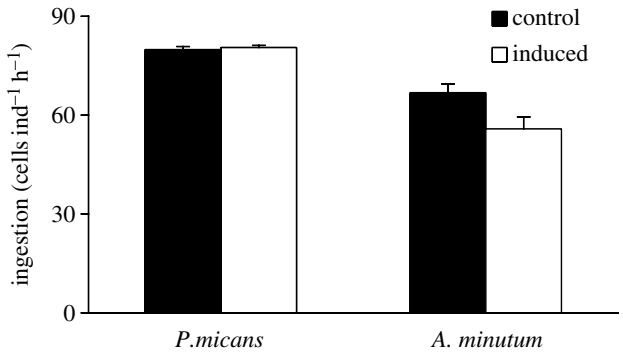

Figure 4. Ingestion of algae (cells copepod^{-1} h^{-1}) by the copepod *A. tonsa* offered a simultaneous choice between either control *A. minutum* and *P. micans* (black bars) or induced *A. minutum* and *P. micans* (white bars) in the feeding preference experiment. Error bars show +s.e.m. ($n=12$). (NB, the statistical analysis was performed on the proportion of ingested control and induced *A. minutum* in relation to the ingested *P. micans* and not on the absolute ingestion of different prey item.)

0.40 ± 0.008 (mean$\pm$s.e.m.) and did not differ between treatments (ANOVA, $F_{3,8}=1.06$, $p=0.42$). Thus, the observed 47–109% increase in cell specific PST content cannot be attributed to copepods feeding selectively on *A. minutum* with low cell specific PST content.

(c) *Feeding preference experiment*

Toxin samples taken from the preconditioned *A. minutum* cells at the start of the feeding preference experiment showed that the total cell specific PST content (GTX 1–4) was higher in induced *A. minutum* cells (9.7 ± 0.2 fmol cell^{-1}, mean$\pm$s.e.m.) than in control *A. minutum* cells (1.5 ± 0.05 fmol cell^{-1}, mean$\pm$s.e.m.). *A. tonsa* ingested a significantly lower proportion of *A. minutum* cells when offered a choice between induced *A. minutum* and the non-toxic alternative prey *P. micans*, compared to when the copepods were offered a choice between control *A. minutum* and *P. micans* (*t*-test, $t_{\mathrm{d.f.}=22}=2.31$, $p=0.015$, figure 4). The significant difference in the proportion of ingested *A. minutum* was not due to a difference in the ingestion of *P. micans* cells (figure 4). Therefore, we conclude that *A. tonsa* ingested fewer induced compared to control *A. minutum* and that the ingestion of *P. micans* was not affected by the presence of induced *A. minutum* cells (figure 4).

Copepod survival was 96% in the induction experiment, 95% in the density dependence experiment and 98% in the feeding preference experiment.

4. DISCUSSION

The results of the present study show that *A. minutum* cells are able to detect and respond to waterborne signals from a natural enemy by inducing an increased production of PSTs. Furthermore, the increased PST content of the algal cells was correlated to an increased resistance of *A. minutum* to grazing by the copepod *A. tonsa*. Inducible algal PST production that is correlated with an increased resistance to grazing has, to our knowledge, not been demonstrated in previous studies on grazer–phytoplankton interactions, and these findings may have important implications for our understanding of the formation of harmful algal blooms. Increased resistance to grazers is not only associated with the direct benefit of reduced biomass losses, but it may also result in a competitive advantage. Resistant phytoplankton species or clones will have higher net growth rates compared to less resistant species or clones when grazing pressure is intense, resulting in a greater ability to exploit available resources. In unicellular organisms with the potential of rapid exponential growth, even small changes in resistance to important grazers may rapidly propagate to large differences in net growth rate and competitive abilities among different clones and species of prey. The magnitude of such an increased competitive advantage will depend on the mechanism causing the induced resistance against grazers. If grazers are able to detect and behaviourally reject induced cells prior to ingestion, biomass losses are reduced, and at the same time grazing pressure is likely to shift from the toxic species to less- or non-toxic competitors (Guisande *et al.* 2002*b*). In contrast, if grazers are unable to distinguish between toxic and less/non-toxic cells prior to ingestion, consumption of the toxic species would be required to physically incapacitate the grazer and hence reduce grazing. This might seem to contradict the principles of natural selection, since the apparent selective unit, the cell, becomes extinct when it is ingested. However, phytoplankton species, including *Alexandrium* species, commonly reproduce primarily through asexual division, and an entire bloom may theoretically consist of only one or a few clones. Increased toxin production could therefore provide a benefit by reducing the grazing pressure on nearby genetically identical cells (Wolfe 2000). However, physical incapacitation of grazers through ingestion of toxic cells would also benefit less toxic or non-toxic prey species in terms of a reduced overall grazing pressure.

The results from the feeding preference experiment indicate that behavioural rejection, rather than physical incapacitation, was the main mechanism behind the increased resistance against grazers in induced *A. minutum* cells. Physical incapacitation would have resulted in decreased ingestion rates of the non-toxic alternative prey (*P. micans*), but this was not supported by the results from the feeding preference experiment (figure 4). The outcome of the feeding preference experiment is in agreement with the findings from a previous study where

A. tonsa was shown to be able to discriminate between different species of *Alexandrium* with different toxin content, with a significant preference for the least toxic species (Teegarden 1999; but see also Colin & Dam 2003). As far as we know, the present study is the first investigation using a single phytoplankton clone with variable PST content in order to investigate the feeding preference of grazing copepods. However, the relationship between the PST content of induced cells and resistance against grazers is correlative and algal traits other than cell specific PST content may still confound the results. Nevertheless, the problem with confounding factors is highly reduced compared to previous experiments, in which different phytoplankton clones or species have been used to compare the effects of different cell specific toxin content on biological interactions (Teegarden 1999). In order to further investigate the possible role of PSTs as chemical defences against grazers, more direct tests are needed, e.g. by specifically manipulating the PST concentrations in artificial food particles. PSTs have previously been shown to reduce the feeding activity of the harpacticoid copepod *Tigriopus brevicornis* when mixed with diatom diets (Shaw *et al.* 1997). However, the feeding deterrent effect found by Shaw *et al.* (1997) was due to addition of dissolved PSTs to the diatom cultures, and not to experimental manipulation of the PST content within the diatom cells. As pointed out in a review by Hay (1996), assessing effects of metabolites on consumers by dissolving them into water have little ecological relevance and should be avoided. To increase the ecological relevance of feeding experiments with toxic phytoplankton and inducible defences, it is also important that alternative foods, besides the toxic organisms, are presented simultaneously to the grazer. In this study, the copepods were offered a natural alternative food choice (*P. micans*) and it was found that induced *A. minutum* cells were ingested at a significantly lower rate than non-induced cells in the presence of the alternative food. The increased resistance of induced cells might not have been detectable if no alternative food would have been presented. Obviously, the outcome could also have been different if more food sources had been offered simultaneously, in order to mimic more natural conditions. Under such conditions, the induced resistance effects may be reduced due to dilution of toxicity by ingestion of alternative food sources, but it may also be reinforced by an increased availability of alternative choices to the dinoflagellate cells with increased toxicity.

Acartia spp. and other copepods of similar size are often dominating in the pelagic, marine metazoan fauna (Turner 2004) and dinoflagellates (including *Alexandrium* spp.) constitute a major component of the copepod diet (e.g. Calbet *et al.* 2003). Copepod abundances may vary by orders of magnitude on both temporal and spatial scales. For example, the density of copepods in temperate regions is often two orders of magnitude higher in summer than in winter (Turner 2004). Furthermore, zooplankton commonly show diurnal migration patterns resulting in dramatic daily changes in copepod densities in the photic zone. Copepods may also aggregate around food patches (Tiselius 1992) or form swarms (Byron *et al.* 1983). It is thus most likely that copepods exert a strong and highly variable selection pressure on dinoflagellates, including *A. minutum*, and that the basic prerequisites for the evolution of inducible defences are fulfilled in the interaction between copepods and dinoflagellates (Karban & Baldwin 1997). Accordingly, we could detect a strong inducible chemical response, as well as a significantly increased grazing resistance, in *A. minutum* when it was exposed to intense grazing by copepods. The density of *A. tonsa* females used in the induction and feeding preference experiments was in the upper end of what is normally occurring, even if similar and even higher densities of copepods have been observed in the field (Durbin & Durbin 1981; Cervetto *et al.* 1993). However, the results from the density dependence experiment show that increased cell specific PST content was induced also at lower, more commonly encountered, densities of copepod grazers and that the response was positively correlated to the density of grazing copepods.

The waterborne chemical cue(s) responsible for the induced production of PST in *A. minutum* was present in both copepod treatments (starving and grazing copepods), but the PST induction was more pronounced in *A. minutum* cells exposed to cue(s) from actively feeding copepods. The difference in response between algae exposed to cue(s) from the starving and feeding copepods may have resulted from lower release rate of the cue(s) caused by lower metabolic rate in starving copepods compared to grazing copepods. It could, however, also have been caused by additional cues being released from algal cells fractured by grazing. Molecules that may function as chemical cues in aquatic environments are either general compounds from primary metabolism, e.g. excretory compounds in urine and faeces or body fluids from damaged tissues (Atema 1995) or metabolites that are specifically produced to function in information conveyance, e.g. small peptides (Zimmer & Butman 2000). A number of these compounds (especially excretory products) may change the nutrient availability and physiological conditions for primary producers, without having cue-specific interactions with receptors. A change in nutrient availability (especially the availability of nitrogen) is known to affect the production of PST in *Alexandrium* sp. cultures (John & Flynn 2002). For instance, it has been shown that *A. tamarense* produce significantly more PST when cultured using ammonium rather than nitrate as nitrogen source (Leong *et al.* 2004). *A. tonsa* partly excretes nitrogen in the form of ammonium, but given the background level of nutrients in the experimental medium, the excretion rate from the copepods used in the induction experiment was most likely orders of magnitude too low to significantly contribute to the nitrogen metabolism of *A. minutum* (cf. Miller & Glibert 1998). The results found here parallel previous findings on induced colony formation in the unicellular freshwater green algae *Scenedesmus* spp., where the inducing chemical cue(s) are specifically associated with the grazing activity of herbivorous zooplankton (*Daphnia* spp.), and not to general excretory products (e.g. urea) or nutrients (reviewed in Lurling 2001). However, in contrast to our findings for PST production in *A. minutum*, colony formation in *Scenedesmus* spp. is only induced by signals from actively feeding, and not by starving, grazers (van Holthoon *et al.* 2003).

The present study is, to our knowledge, the first report of an infochemical-mediated induction of toxin production coupled to an increased resistance to grazers

among marine phytoplankton. Behavioural rejection of induced cells constitutes a direct increase in the fitness of more toxic cells, providing a potentially important mechanism behind the formation of harmful algal blooms. Given the importance of grazing as a selective force in the pelagic ecosystem (Smetacek 2001), the growing body of evidence of the widespread distribution of induced responses (Tollrian & Harvell 1999) and the amplitude of the effects these responses may have (Hay & Kubanek 2002), induced responses may well be an important factor in determining the structure and composition of pelagic ecosystems, including the formation of algal blooms.

We are grateful to L. Nguyen and W. Stolte for information on toxin analysis and P. Jonsson, J. Havenhand and three anonymous reviewers for comments on earlier drafts of the manuscript and Karen Wilson for linguistic advice. Financial support was provided by EU through the European Regional Development Fund, Object 2 West Sweden, the foundation of Hasselblad, Carl Trygger and Wåhlström and the Swedish research Council for Environment, Agriculture Sciences and Spatial Planning (contract 21.0/2003-1122).

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