Marine dinoflagellates show induced life-history shifts to escape parasite infection in response to water-borne signals

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Many dinoflagellate species form dormant resting cysts as a part of their life cycle, and in some freshwater species, hatching of these cysts can be delayed by the presence of water-borne signals from grazing zooplankton. Some marine dinoflagellates can form temporary cysts, which may function to resist unfavourable short-term environmental conditions. We investigated whether the marine dinoflagellate *Alexandrium ostenfeldii* is able to induce an increased resistance to the parasitic flagellate *Parvilucifera infectans* by forming temporary cysts. We performed several laboratory experiments where dinoflagellates were exposed either to direct contact with parasites or to filtered water from cultures of parasite-infected conspecifics (parasite-derived signals). Infection by *P. infectans* is lethal to motile *A. ostenfeldii* cells, but temporary cysts were more resistant to parasite infection. Furthermore, *A. ostenfeldii* induced a shift in life-history stage (from motile cells to temporary cysts) when exposed to parasite-derived water-borne signals. The response was relaxed within a couple of hours, indicating that *A. ostenfeldii* may use this behaviour as a short-term escape mechanism to avoid parasite infection. The results suggest that intraspecific chemical communication evoked by biotic interactions can be an important mechanism controlling life-history shifts in marine dinoflagellates, which may have implications for the development of toxic algal blooms.

Keywords: dinoflagellate; induced resistance; parasite; water-borne signal

1. INTRODUCTION

Plants and animals have evolved a range of behavioural, morphological and chemical traits that render them more resistant to predator and parasite attacks. Theory predicts that the evolution of inducible defences is favoured when the attack risk is unpredictable, there is a fitness cost involved in the production of the defence and reliable environmental signals are available (Karban & Baldwin 1997; Tollrian & Harvell 1999). If the consequence of an attack is lethal to an organism, selection should strongly favour the evolution of an ability to sense and respond to signals received before a direct encounter with the attacking organism. There are numerous examples of the induction of defences in aquatic animals in response to water-borne signals emitted either from predators or from wounded conspecifics (reviewed in Chivers & Smith 1998; Kats & Dill 1998). Increasing evidence also shows that chemical signals induce defences in multicellular marine algae (Toth & Pavia 2000; Arnold *et al.* 2001; Potin *et al.* 2002; G. B. Toth and H. Pavia, unpublished data). Furthermore, colony formation (Lüring 2001) and excystment from resting cysts (Hansson 1996; Rengefors *et al.* 1998) in freshwater phytoplankton are affected by signals emitted from herbivorous planktonic organisms. In contrast to the accumulating evidence that a wide range of aquatic organisms are capable of both sensing and responding to herbivore- or predator-related water-borne signals, studies on parasite-induced defence reactions in

algae are still scarce (but see Potin *et al.* 1999). To our knowledge, no previous studies have investigated the role of water-borne signals in the interaction between parasites and marine unicellular algae (but see Tillmann & John 2002).

Alexandrium ostenfeldii is a paralytic shellfish toxin (PST)-producing marine dinoflagellate that has a widespread geographical distribution in Arctic and temperate waters (Østergaard Jensen & Moestrup 1997). The life cycle of *A. ostenfeldii* includes motile vegetative cells, haploid gametes, diploid zygotes, resting cysts and temporary cysts (figure 1a–c). In contrast to the dormant resting cysts, the temporary cysts are not products of sexual reproduction and can hatch within a few hours (Anderson & Wall 1978; Doucette *et al.* 1989). The formation of temporary cysts can be induced by changes in environmental variables, such as light, temperature, salinity, and trace-metal and nutrient concentrations (Anderson & Wall 1978), and has been suggested as a short-term strategy to resist temporary adverse environmental conditions (Østergaard Jensen & Moestrup 1997; Anderson & Wall 1978; Doucette *et al.* 1989). When temporary cysts are formed, the motile cells lose the three outermost layers of the amphiesma, while the pellicular layer becomes thicker and underlain by granular material (Doucette *et al.* 1989). The pellicular layer in *A. ostenfeldii* temporary cysts probably contains cellulose or a cellulose-like substance, since it stains with calcofluor (Østergaard Jensen & Moestrup 1997). *Parvilucifera infectans* is a parasitic protist belonging to the phylum Perkinsozoa. It can infect a number of different dinoflagellate species, including *A. ostenfeldii*, *A. fundyense*, *A. tamarense*, *Prorocentrum micans* and *Heterocapsa triquetra* (Norén *et al.* 1999). The

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Figure 1. The life cycles of *Alexandrium ostenfeldii* and *Parvilicifera infectans*. (a) During sexual reproduction, the motile *A. ostenfeldii* cells undergo meiosis and form haploid gametes. When the gametes fuse, diploid swimming zygotes are formed. The zygotes are transformed into resting cysts, which may sink to the bottom sediment and be dormant for long periods. When the environmental conditions are favourable, the resting cysts hatch and give rise to new motile vegetative cells. (b) The motile cells can divide clonally, and give rise to genetically identical motile cells. (c) The motile cells rapidly form temporary cysts if the environmental conditions are unfavourable. The temporary cysts can hatch within a few hours. (d) The *P. infectans* zooids are the infectious stage and penetrate the motile dinoflagellate cells in the girdle between the amphiesmal plates. The infected cells rapidly lose the flagella and, after *ca.* 1 day, developing parasite sporangia are visible in the cells as spherical bodies. As the sporangia mature, the content of the dinoflagellate cell is consumed, and eventually new parasite zooids are released, leaving an empty sporangium behind.

infection process, which has been described in detail by Norén *et al.* (1999), is summarized in figure 1*d*. Infection results in the death of the dinoflagellate and probably occurs when the *P. infectans* zooids penetrate the motile cells through the girdle between the amphiesmal plates (G. B. Toth and F. Norén, personal observations).

We tested several hypotheses to investigate the potential function of temporary cysts as an inducible defence in *A. ostenfeldii*. Since the temporary cysts of *A. ostenfeldii* lack the girdle structure, and since the pellicular layer of the amphiesma becomes thicker during temporary-cyst formation, we suggest that dinoflagellate temporary cysts are more resistant to parasite infection than are the motile cells. More specifically, we propose that *P. infectans* should be able to infect and kill motile *A. ostenfeldii* cells in culture, but that the temporary cysts should be more resistant to direct parasite infection. This experiment included exposing *A. ostenfeldii* cultures directly to active *P. infectans* sporangia. Furthermore, since infection is lethal to *A. ostenfeldii*, we suggest that chemical signals from dinoflagellate cultures infected with *P. infectans* should induce an increase in temporary-cyst formation in uninfected algal cultures. This hypothesis was tested either by keeping infected and uninfected *A. ostenfeldii* cultures in the same container, separated by a filter through which molecules could pass, or by adding filtrates from infected *A. ostenfeldii* cultures to uninfected cultures.

2. MATERIAL AND METHODS

(a) *Culturing procedures*

Non-axenic stock cultures of *A. ostenfeldii* (strain K287; Scandinavian Culture Collection of Algae and Protozoa, Copenhagen) were maintained in sterile cotton-plugged 500 ml Erlenmeyer flasks containing autoclaved natural seawater-based f/2-Si medium with a salinity of 26 ‰. Each flask contained *ca.* 250 ml of stock culture and was placed in a climate-controlled room at 18 °C, with a light regime of 14 L : 10 D at a photon irradiance rate of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$. By transferring small volumes of the *A. ostenfeldii* stock cultures to new flasks with fresh medium at different times, we had continuous access to cultures in different growth phases, which were used in the experiments (see §§ 2*b–d*).

Parvilicifera infectans sporangia were isolated from mixed plankton samples concentrated from surface water (0–5 m depth) outside Kristineberg Marine Research Station on the Swedish west coast. Parasite stock cultures were initiated by transferring mature *P. infectans* sporangia from the mixed plankton samples to cultures of *A. ostenfeldii* in the exponential growth phase. After initiation, the parasites were allowed to infect the dinoflagellates for 2 days, and an inoculum of parasite culture was transferred to new *A. ostenfeldii* cultures. This procedure was repeated several times before parasite cultures were used in the experiments (see Norén *et al.* (1999) for further details of the infection process of *P. infectans*). Owing to the large dilution of the initial mixed plankton samples using this procedure, contamination by substances from natural seawater in the experiments was highly unlikely. The *P. infectans* stock cultures were kept in a refrigerator (+8 °C and dark) to keep the parasite sporangia inactive before use in the experiments. Several hours before the experiments started, the parasite cultures were removed from the refrigerator and placed on a laboratory bench at room temperature (between 18 and 20 °C). This procedure stimulates the parasite sporangia to release active zooids.

The experiments in the present study were all carried out using Nunc multidishes with a pit volume of 2 ml. The Nunc dishes were kept under the same conditions as the *A. ostenfeldii* stock cultures (18 °C, with a light regime of 14 L : 10 D at a photon irradiance rate of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$). At the start of the experiments, 1 ml of *A. ostenfeldii* stock culture for each replicate was transferred into a separate Nunc well, which probably caused some of the motile cells to form temporary cysts. However, the transfer procedure from stock culture to experimental container was the same for controls and for experimental treatments and should not affect the outcome of the experiments. Furthermore, the cells in the *A. ostenfeldii* stock cultures were not homogeneously distributed in the Erlenmeyer flasks, and this may have contributed to the slight differences in cell numbers at the start of the first experiment with direct parasite infection (figure 2*b*). However, this difference will result in a more conservative test of the hypothesis stated in the first experiment. In the following experiments, we gently swirled the cultures before transfer to the Nunc wells, to make the distribution of the *A. ostenfeldii* cells in the medium more homogeneous. After transfer of *A. ostenfeldii* stock culture to the Nunc wells, the treatments were added and the cells and cysts were counted at various times (see descriptions of the separate experiments in §§ 2*b–d*).

(b) *Direct parasite infection*

To investigate whether temporary cysts of *A. ostenfeldii* are more resistant to *P. infectans* infection than are motile cells, an

Figure 2. Direct parasite infection. (a) *Alexandrium ostenfeldii* temporary cysts (%), and (b) total number of uninfected *A. ostenfeldii* cells ml⁻¹ in control (open bars) algal cultures and in cultures exposed to direct contact (shaded bars) with parasites (*Parvilucifera infectans*) at different sampling times. Different letters above bars indicate significant differences between means based on the Student Newman-Keuls multiple comparisons test ($p < 0.05$). Error bars show + s.e.m. ($n = 6$).

infection experiment where the dinoflagellate cells were exposed to direct contact with parasite zooids was conducted. We used *A. ostenfeldii* stock cultures in the stationary growth phase, since they had a high initial percentage of temporary cysts (44% of total cell number). The stock culture had an initial density of, on average, 2500 uninfected cells ml⁻¹ (see figure 2b), and was transferred to the Nunc wells as described in § 2a. Parasites were added to half of the wells at a ratio of approximately one parasite sporangium per 75 *A. ostenfeldii* cells; the remaining wells were kept as controls. Both the *A. ostenfeldii* temporary cysts and the parasite sporangia sank to the bottom of the wells, and could be monitored directly under an inverted light microscope. However, parasite sporangia disintegrate after zooids are released, and therefore only the numbers of temporary cysts and motile cells were monitored at three times during the experiment (0, 36 and 60 h; $n = 6$; total sample size of 36 wells). Uninfected temporary cysts were separated from infected *A. ostenfeldii* cells and developing parasite sporangia by the absence of the round body described by Norén *et al.* (1999). Data for the proportion of uninfected temporary cysts and the total number of uninfected cells in the wells were statistically analysed in a two-way analysis of variance (SuperANOVA, Abacus Concepts, Inc.)

with treatment (two levels) and time (three levels) as fixed orthogonal factors.

(c) Continuous exposure to parasite-derived chemical signals

For the induction of temporary-cyst formation to function as an effective escape strategy, there should be reliable water-borne signals that the dinoflagellates can sense and respond to before they come into direct contact with parasite zooids. To test this, we kept uninfected and parasite-infected *A. ostenfeldii* stock cultures in the same container, separated by a filter through which molecules, but not parasite zooids, could pass. We used an *A. ostenfeldii* stock culture in the exponential growth phase with an initial density of, on average, 1500 uninfected cells ml⁻¹. The *A. ostenfeldii* stock culture was transferred to Nunc wells as described in § 2a, and polycarbonate tissue-culture inserts (pore size of 0.4 µm, pit volume of 0.5 ml) containing 0.4 ml of either the same *A. ostenfeldii* stock culture as outside the inserts (control), or *P. infectans* stock culture (treatment), were placed inside the wells. The density of parasite sporangia in the *P. infectans* stock culture was not quantified, but parasite sporangia at various stages of maturity (including some that were releasing active zooids) were observed before the experiment started. After 50 h, the inserts were removed and *A. ostenfeldii* temporary cysts were counted directly under an inverted light microscope in the Nunc wells. The total cell number in the cultures was determined after fixation in Lugol's solution ($n = 2$; total sample size of four wells). Data were statistically analysed with *t*-tests.

(d) Momentary exposure to parasite-derived signals

To study further the timing of the induction and a possible relaxation of temporary-cyst formation in *A. ostenfeldii*, filtrates from uninfected and parasite-infected stock cultures were added to *A. ostenfeldii* cultures at the start of the experiment. *Alexandrium ostenfeldii* stock culture in the exponential growth phase was transferred to Nunc wells as described in § 2a, and filtrate (200 µl, filter pore size of 0.2 µm) from either the *A. ostenfeldii* stock culture (control) or the *P. infectans* stock culture (treatment) was added. We used an *A. ostenfeldii* stock culture in the exponential growth phase with an initial density of, on average, 3500 cells ml⁻¹. As described in § 2c, the density of parasite sporangia in the *P. infectans* stock culture was not quantified by counting, but cultures were observed to contain active parasite sporangia. The numbers of temporary cysts and motile cells were monitored 2 and 8 h after addition of filtrates as described in § 2c ($n = 4$; total sample size of 24 wells). Data were statistically analysed using a two-way analysis of variance with treatment (two levels) and time (three levels) as fixed orthogonal factors.

Prior to all statistical analyses, data were tested for homogeneity of variances with Cochran's test and transformed when required (Underwood 1997). Significant differences between mean values were analysed using the Student Newman-Keuls (SNK) multiple-comparisons test (Underwood 1997).

3. RESULTS

(a) Direct parasite infection

Data on the percentages of temporary cysts and the total numbers of uninfected cells in *A. ostenfeldii* cultures exposed to direct contact with *P. infectans* were log transformed before statistical analysis to fulfil the assumption

Figure 3. Continuous exposure to water-borne signals. (a) *Alexandrium ostenfeldii* temporary cysts (%), and (b) total number of *A. ostenfeldii* cells ml⁻¹ in control algal cultures and in cultures continuously exposed to water-borne signals from *A. ostenfeldii* infected with *Parvilucifera infectans* after 50 h. Error bars show + s.e.m. ($n = 2$).

of homogeneity of variances. There was a statistically significant interaction between different treatments and sampling times for both the percentage of temporary cysts (ANOVA: $F_{2,30} = 53.23$, $p < 0.0001$) and the total number of uninfected cells (ANOVA: $F_{2,30} = 275.2$, $p < 0.0001$), when *A. ostenfeldii* cells were exposed to direct contact with *P. infectans*. Infected *A. ostenfeldii* cells and parasite sporangia in different stages of development were observed only in the wells where parasites were added and not in the control wells, which contained only temporary cysts and motile cells throughout the experiment. All uninfected cells present in the parasite-exposed cultures 36 and 60 h after inoculation with active parasite sporangia were temporary cysts (figure 2a), showing that all motile *A. ostenfeldii* cells had either been infected or transformed into temporary cysts. Furthermore, the total number of uninfected cells in parasite-exposed cultures decreased significantly during the course of the experiment (figure 2b). The percentage of temporary cysts in the control cultures had decreased after 36 h (figure 2a), while the total number of uninfected cells had increased (figure 2b), showing both that some temporary cysts hatched and that the cells divided. However, after 60 h the percentage of temporary cysts in the control cultures had increased (figure 2a), and the total cell number had decreased (figure 2b).

(b) Continuous exposure to parasite-derived chemical signals

The *A. ostenfeldii* cultures that continuously received water-borne signals from parasite-infested conspecifics formed twice as many temporary cysts as did algal cultures exposed to water-borne signals from uninfected conspecifics (t -test: $t_{1,2} = 23.5$, $p = 0.040$; figure 3a). The results clearly show that motile *A. ostenfeldii* cells are able to sense and respond to parasite-derived water-borne signals by forming temporary cysts. No statistically significant difference in the total number of cells was detected between control cultures and cultures receiving parasite-derived signals (t -test: $t_{1,2} = 0.191$, $p = 0.705$; figure 3b).

(c) Momentary exposure to parasite-derived chemical signals

Data for percentages of temporary cysts in *A. ostenfeldii* cultures exposed to one momentary addition of parasite-derived chemical signals were log-transformed before

Figure 4. Momentary exposure to water-borne signals. (a) *Alexandrium ostenfeldii* temporary cysts (%), and (b) total number of *A. ostenfeldii* cells ml⁻¹ in cultures exposed to a momentary addition of filtrate from cultures with either uninfected conspecifics (control: open bars) or conspecifics infected with *Parvilucifera infectans* (momentary cues: shaded bars) at different sampling times. The dilution resulting from the addition of the filtrate has been taken into account. Different letters above bars indicate significant differences between means based on the Student Newman-Keuls multiple comparisons test ($p < 0.05$). Error bars show + s.e.m. ($n = 4$).

statistical analysis to fulfil the assumption of homogeneity of variances. There was a statistically significant interaction between different treatments and sampling times (ANOVA: $F_{2,18} = 4.62$, $p = 0.024$). The percentage of *A. ostenfeldii* temporary cysts in wells exposed to filtrate from parasite-infested conspecifics increased significantly after 2 h (figure 4a). However, 8 h after the addition of filtrate, this effect had disappeared and the percentage of temporary cysts in the *A. ostenfeldii* cultures exposed to parasite-derived signals was not significantly different from that of the control cultures, where it remained low throughout the experiment (figure 4a). The total numbers of *A. ostenfeldii* cells differed significantly between sampling times (ANOVA: $F_{2,18} = 7.10$, $p = 0.005$). The total number of cells had decreased by an average of 18% in both control cultures and cultures receiving parasite-derived signals when the experiment was terminated (figure 4b).

4. DISCUSSION

The parasitic flagellate *P. infectans* was recently described (Norén *et al.* 1999), and presently we know of

only one other study concerning the ecology of either *P. infectans* or a closely related species (Erard-Le Denn *et al.* 2000). Parasite infection was observed in a range of dinoflagellate species, but the authors did not differentiate between different life-history stages in the dinoflagellate cultures and therefore no conclusions can be drawn about differences in resistance between motile cells and temporary cysts from these previous studies (Norén *et al.* 1999; Erard-Le Denn *et al.* 2000). By contrast, the results of the present study show that the motile-cell stage of *A. ostenfeldii* is very sensitive to *P. infectans* infection, while temporary cysts are more resistant. When active *P. infectans* sporangia were added to *A. ostenfeldii* cultures that contained a mixture of motile cells and temporary cysts, all motile cells were infected within 36 h, and the only uninfected *A. ostenfeldii* cells left in the cultures were temporary cysts. However, the decrease in the total number of uninfected cells (i.e. temporary cysts) in parasite-exposed cultures during the experiment indicates that temporary cysts may also be infected by *P. infectans*. It should be noted that the environment in the experimental wells was highly artificial: the temporary cysts were confined within a small container and continuously exposed to parasite zooids. If the *A. ostenfeldii* cells can stay as temporary cysts for only a limited period of time, an alternative explanation for the disappearance of temporary cysts from the parasite-exposed cultures may be that some temporary cysts hatched during the experiment and became infected by *P. infectans* as motile cells. Regardless of the mechanism behind the disappearance of temporary cysts from parasite-exposed cultures, the results show that the temporary cysts are more resistant to parasite infection than are the motile cells. Therefore, if *A. ostenfeldii* cells are able to perceive the presence of parasites before a direct encounter, they may use temporary-cyst formation as a morphological escape to avoid infection when exposed to parasite attacks.

The results from the experiment where *A. ostenfeldii* were exposed continuously to water-borne substances from parasite-exposed conspecifics show that the motile cells have the ability to sense and respond to the presence of *P. infectans* before a direct encounter. To our knowledge, the results presented here are the first to demonstrate that a marine dinoflagellate species can sense water-borne chemical signals and respond to them by inducing a shift in life-history stage. Previous studies have found that shifts in the life-history stages of freshwater dinoflagellates can be affected by water-borne chemical signals from herbivorous zooplankton (Hansson 1996; Rengefors *et al.* 1998). However, the response of these dinoflagellate species was delayed rather than induced by the zooplankton signals, since the resting cysts hatched with a lower frequency when filtrates from zooplankton cultures were added to the water (Hansson 1996; Rengefors *et al.* 1998). There was also a higher hatching frequency of cysts in natural populations in lakes that contained few zooplankton than in lakes where zooplankton were abundant (Hansson 1996). Furthermore, induced morphological defences in response to herbivore-associated water-borne signals have been found in species of the freshwater green algal genus *Scenedesmus* (reviewed by Lüring 2001). In these studies, *Scenedesmus* formed colonies (coenobia) when exposed to signals from actively feeding herbivorous

zooplankton, which rendered the algae more resistant to grazing by small zooplankton.

Defences that are expressed early in ontogeny can result in high costs resulting from lost opportunities for resource acquisition throughout the lifetime of the organism (Herms & Mattson 1992; Karban & Baldwin 1997). Such opportunity costs can be severe, especially for exponentially growing organisms and if there is competition for resources with other species. In a natural situation, with intense competition for light and nutrients among different species and clones of phytoplankton, it is likely that temporary cysts are costly for *A. ostenfeldii* to produce, as they proliferate at a much slower rate than the motile cells (Garces *et al.* 1998). Therefore, if temporary-cyst formation is costly, there should be a high selection pressure on *A. ostenfeldii* to evolve mechanisms for a rapid relaxation of the defence. The results from the experiment where motile *A. ostenfeldii* cells were exposed to one momentary addition of filtrate from parasite-exposed cultures show that temporary-cyst formation is a short-term response, since temporary cysts hatched within a few hours. This could either reflect a cost associated with temporary-cyst formation, or be caused by a breakdown of the active signal. However, since we do not currently know either the mechanism behind the induction of temporary-cyst formation in *A. ostenfeldii*, or the chemical nature or origin (dinoflagellate or parasite) of the signal, we cannot differentiate between these two alternative explanations.

The induced resistance to *P. infectans* infection through temporary-cyst formation found in the present study may have evolved as a response to a signal that is leaking from infected neighbouring cells, or as a response to a dinoflagellate signal released with the specific intention of warning surrounding cells. The notion of 'talking trees' that send information to neighbours through airborne signals has been heavily criticized, since there can be no selective advantage to individual plants of dispensing information to conspecifics (Karbon & Baldwin 1997; but see Arimura *et al.* 2000; Dolch & Tschardt 2000; Karban *et al.* 2000). However, phytoplankton grow through mitotic division of individual vegetative cells, and group selection does not need to be invoked when discussing an active release of signals to warn other dinoflagellate cells. If a large proportion of the surrounding cells share the same genome, signal conveyance in a dinoflagellate bloom can, from an evolutionary viewpoint, be compared with systemic signalling in a multicellular plant (Karbon & Baldwin 1997; Tollrian & Harvell 1999) or with quorum sensing in bacterial biofilms (e.g. Shapiro 1998; Crespi 2001; Miller & Bassler 2001). Research on the appearance and development of toxic algal blooms in marine systems has previously been heavily focused on the importance of abiotic factors, mainly levels of nutrients such as nitrogen and phosphorous. The results presented here show that dinoflagellates are clearly capable of sensing and responding to biotic environmental signals, which implies that intercellular communication evoked by biotic interactions may also be an important factor influencing the development of algal blooms.

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