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Transcriptomic profiling of *Alexandrium fundyense* during physical interaction with or exposure to chemical signals from the parasite *Amoebophrya*

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Abstract

Toxic microalgae have their own pathogens, and understanding the way in which these microalgae respond to antagonistic attacks may provide information about their capacity to persist during harmful algal bloom events. Here, we compared the effects of the physical presence of the parasite *Amoebophrya* sp. and exposure to waterborne cues from cultures infected with this parasite, on gene expression by the toxic dinoflagellates, *Alexandrium fundyense*. Compared with control samples, a total of 14 882 *Alexandrium* genes were differentially expressed over the whole-parasite infection cycle at three different time points (0, 6 and 96 h). RNA sequencing analyses indicated that exposure to the parasite and parasitic waterborne cues produced significant changes in the expression levels of *Alexandrium* genes associated with specific metabolic pathways. The observed upregulation of genes associated with glycolysis, the tricarboxylic acid cycle, fatty acid β-oxidation, oxidative phosphorylation and photosynthesis suggests that parasite infection increases the energy demand of the host. The observed upregulation of genes correlated with signal transduction indicates that *Alexandrium* could be sensitized by parasite attacks. This response might prime the defence of the host, as indicated by the increased expression of several genes associated with defence and stress. Our findings provide a molecular overview of the response of a dinoflagellate to parasite infection.

Keywords: dinoflagellate, ecological genomics, harmful algae, host–parasite interaction, RNA-seq

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Introduction

Phytoplanktonic organisms are important primary producers that play an essential role in food webs and energy fluxes in marine ecosystems; however, certain toxic phytoplankton species can form harmful algal blooms. The dinoflagellate *Alexandrium* is one of the best known bloom-forming and toxin-producing species responsible for paralytic shellfish poisoning (PSP) outbreaks (Hallegraeff 1993; Cembella 2003; Anderson *et al.* 2012), and some studies show that the associated toxins act as defence compounds against copepod grazers (Selander *et al.* 2006; Wohlrab *et al.* 2010; Yang *et al.* 2010). In addition to toxins, the dinoflagellates, *Alexandrium*, can also produce allelochemicals that affect species interactions due to cell lysis of potential grazers

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and/or competitors (Tillmann & John 2002; Tillmann *et al.* 2008; John *et al.* 2014). However, very little is known about the roles of the toxic and allelochemical compounds produced by *Alexandrium* as defence against pathogens (Anderson *et al.* 2012).

Microalgae can be infected by a broad variety of organisms, such as viruses, bacteria and parasites (Kim 2006; Chambouvet *et al.* 2008; Velo-Suárez *et al.* 2013). The parasites *Amoebophrya ceratii* (Syndiniales) and the host *Alexandrium fundyense* (Gonyaulacales) are both Dinophyceae (Alveolata) (Guillou *et al.* 2008). Field studies show that a large variety of host species are infected by the parasite *Amoebophrya*, including a wide taxonomic range of harmful dinoflagellates (Siano *et al.* 2010; Park *et al.* 2013; Li *et al.* 2014). There is also evidence that *Amoebophrya* infections play a pivotal role in controlling host mortality and can regulate the dynamics of dinoflagellate blooms at high infection rates (Coats *et al.* 1996; Chambouvet *et al.* 2008); these effects coincide with the life cycle transition of the host *Alexandrium* from vegetative division to sexual fusion (Velo-Suárez *et al.* 2013).

Together with grazing by microzooplankton, parasite infection is an important top-down control mechanism for bloom-forming dinoflagellates (Montagnes *et al.* 2008). *Amoebophrya* is a model parasitic organism that can be cocultured with its host, *Alexandrium,* in the laboratory with an infective cycle of approximately 4 days (Lu *et al.* 2014). Infection by *Amoebophrya* is initiated by penetration of the parasitic dinospores into the host cells (Cachon 1964; Miller *et al.* 2012). Once inside the cytoplasm or nucleus (depending on the specific host and parasitic strains), the parasite starts to feed (the trophont stage). The trophont increases in size until sequential nuclear divisions and flagellar replications ultimately form an intracellular and multicellular 'beehive' stage inside the cytoplasm or nucleus of the host cell (the sporocyte) (Cachon 1964). The mature sporocyte ruptures the cell wall of the host, and most develop into a short-lived vermiform stage that soon divides into numerous free-living infectious dinospores (Coats & Bockstahler 1994; Coats & Park 2002).

Chemical signals can determine feeding behaviour, habitat selection and induced defences in a wide range of aquatic organisms (Chivers & Smith 1998; Hay 2009). In marine species, the induction of defence can also be triggered in response to waterborne cues emitted by predators (Chivers & Smith 1998; Toth & Pavia 2000; Roberts *et al.* 2011). A recent study investigated the potential mechanisms involved in chemically mediated predator–prey interactions, and accumulating evidence suggests that the marine dinoflagellate *Alexandrium* can recognize and distinguish predators and respond when exposed to waterborne cues from conspecific, threatening copepod grazers (Selander *et al.* 2006; Wohlrab *et al.* 2010; Roberts *et al.* 2011). A single study has described the ability of the dinoflagellate *Alexandrium ostenfeldii* to form temporary cysts as a response to waterborne cues from the parasitic Perkinsozoa *Parvilucifera infectans* (Toth *et al.* 2004).

Relatively, few studies have examined the transcriptomic responses of dinoflagellates infected with parasites (Bachvaroff *et al.* 2009). Consequently, little is currently known about the importance of chemical cues that may prime host responses towards parasites. The objective of this study was to investigate the molecular mechanisms that underlie the responses of *Alexandrium* to parasite infection and to discriminate these responses from those elicited by parasitic waterborne cues. To this end, we used RNA sequencing (RNA-seq) to compare the transcriptional responses of the host *Alexandrium* to the presence of the parasite *Amoebophrya* and waterborne chemical cues from this parasite. Annotation of the final data revealed that a large number of genes associated with this host–parasite interaction are involved in energy conversion metabolic pathways, signal transduction and defence mechanisms.

Materials and methods

Cultures

The *Amoebophrya* (AT5.2) parasite strain [the term parasitoid is also used (Lu *et al.* 2014)] was isolated from *Alexandrium* cells sampled from the Gulf of Maine, USA (Chambouvet *et al.* 2011), and was used to infect the *Alexandrium fundyense* strain [formerly described as *A. tamarense* (John *et al.* 2014)] isolated from the North Sea coast of Scotland (Alex5; RCC3037) (Tillmann *et al.* 2009). To understand the host–parasite mechanisms, we needed a strain, which can be reliably infected with high rates. We tested the prevalence of the *Amoebophrya* strain to different population and strains of *Alexandrium* in our laboratory and identified no difference in the infection percentage of each population. Alex5; RCC3037 was chosen in this study, because its infection percentage by the parasite *Amoebophrya* strain was among the highest ones. This strain of *Alexandrium* has also the advantage that its genetic/genomic background is well studied (Alpermann *et al.* 2009; Wohlrab 2013) and it does not produce allelochemicals which may affect the host–parasite infection (Tillmann *et al.* 2009). The life cycle of the parasite was 4 days. All cultures were grown at 15 °C in K-medium (Keller *et al.* 1987), with cool-white fluorescent lamps providing photon irradiation of 150 μmol/m^2/s on a light: dark cycle of 14 h:10 h.

Influence of parasitism on the host

The parasite infection experiment covered one complete parasite life cycle (4 days) and included three harvesting time points (0, 6 and 96 h). Triplicate exponential phase cultures of *Alexandrium* (400 mL) at a concentration of approximately 1000 cells/mL were prepared in 500 mL Erlenmeyer flasks. Triplicate cultures containing the host only (400 mL *Alexandrium* at a concentration of 1000 cells/mL) served as no-parasite controls (−P). Portions (100 mL) of the *Alexandrium* cultures were used for RNA extraction at the 0-h time point. The remaining cultures were used for the parasite infection experiment. Infection of the host culture was performed following the procedures of Coats and Park (Coats & Park 2002). Infective parasite dinospores were harvested from infected host cultures on Day 4 by gravity filtration through a 10-μm pore size mesh. The harvested dinospores were examined under a microscope to ensure the absence of host cell contamination and were then inoculated immediately into the triplicate *Alexandrium* cultures at a parasite: host ratio of 10:1 (Lu *et al.* 2014). The cultures were incubated for 6 or 96 h after adding the dinospores. At each time point, samples collected from the triplicate cultures of *Alexandrium* parasite-infected treatment (+P) and no-parasite control (−P) were used for fixation and cell counting, parasite prevalence assessment, RNA extraction and sequencing, and PSP toxin analysis.

Influence of waterborne cues on the host

To examine the response of *Alexandrium* to waterborne cues from the parasites, and to discriminate the potential wounding impact from the response to lysed *Alexandrium* cells, three different incubations were performed: (i) parasite-infected waterborne medium (+WP): the host was treated with medium by gravity filtration through a 0.2-μm pore size mesh from *Alexandrium* cells that had been infected by parasites; (ii) lysed host cells (+A): the host was treated with medium from host cells that had been lysed using ultrasound for 2 min; and (iii) No-parasite *Alexandrium* waterborne medium; used as control (−WP): the host was treated with filtered medium from an exponentially growing control *Alexandrium* culture. *Alexandrium* cultures (400 mL) at a concentration of approximately 1000 cells/mL were incubated in nine 500-mL Erlenmeyer flasks (three per incubation type). In each experiment, the medium with waterborne cues was replenished at the 24-, 48- and 96-h time points. The experiment examining the effect of waterborne cues on the host *Alexandrium* also covered one complete parasite life cycle (4 days) and included the same three harvesting time points (0, 6 and 96 h) as the previous parasite infection experiment. At each time point, samples were collected from each culture and used for fixation and cell counting, RNA extraction and sequencing, and PSP toxin analysis.

Fixation and growth rate calculation

Samples (10 mL) from each experiment were fixed with Lugol's solution (10 g of potassium iodide and 5 g of iodine in 100 mL of distilled water) at a final concentration of 2% (Tillmann *et al.* 2009), and three 1 mL aliquots were counted under an inverted microscope (Axiovert 200M; Zeiss, Göttingen, Germany) after sedimentation in chambers. A minimum of 400 cells per sample were counted. Parasite infection and the release and survival of dinospores was followed by examining the persistence of the natural autofluorescence of the parasite under a microscope (Axiovert 200M) (Coats & Bockstahler 1994). The growth rate of *Alexandrium* was calculated using the following formula (Guillard 1973; Tillmann *et al.* 2009), where μ is the growth rate (d^{-1}), t is the sampling day, and N_1 and N_2 are the abundances of *Alexandrium* at t_1 and t_2, respectively:

$$\mu = \frac{1}{t_2 - t_1} \ln \frac{N_2}{N_1}$$

RNA preparation, library construction and sequencing

Total RNA was extracted from parasite-infected (+P), parasite control (−P), waterborne cue-treated (+WP), lysed host cell-treated (+A) and waterborne cue control cultures (−WP) at three time points (0, 6 and 96 h). The 100 mL samples was centrifuged at 4 °C for 10 min. The supernatants were decanted, and the resulting cell pellets were resuspended immediately in 1 mL of hot (60 °C) TriReagent (Sigma-Aldrich, Steinheim, Germany), according to the manufacturer's protocol. RNA purification, including on-column DNA digestion, was performed using the RNeasy Kit (Qiagen, Hilden, Germany). The quality and quantity of the RNA were determined using a NanoDrop ND-100 spectrometer (PeqLab, Erlangen, Germany) and a RNA Nano Chip assay on a 2100 Bioanalyzer device (Agilent Technologies, Böblingen, Germany). For the construction of the reference transcriptome, aliquots of the triplicate RNA samples from each experiment were pooled and sequenced as a 100-bp paired-end Illumina library. The raw reads were assembled to yield the *Alexandrium* transcript reference sequences. Each RNA sample was then sequenced independently as a 50-bp single-end Illumina library for the expression analyses (Table S1, Supporting information).

Libraries were prepared using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. To obtain longer molecules for paired-end sequencing, the fragmentation time was reduced to 4 min. The libraries were quality checked and quantified using a Bioanalyzer 2100 device and a DNA Chip assay and then sequenced using a HiSeq2000/2500 instrument (high-output mode) in either single-read/50 cycle or paired-end/2 × 100 cycle mode (Bentley *et al.* 2008). Multiplexing was performed using three, four or five libraries per lane. Sequence information was extracted using the CASAVA v1.8.2 software (Illumina) in FASTQ format. The analysis produced 33 data sets for single-end sequencing and six data sets for paired-end sequencing (Table S1, Supporting information).

Analysis of RNA-seq data

No complete genomes of *Alexandrium* species are currently available; therefore, an *Alexandrium* reference transcriptome was constructed by merging sequence information from three sources: (i) a de novo transcriptome of *Alexandrium* (122 219 contigs; Table S2, Supporting information), which was assembled using the CLC Genomics Workbench (CLC Bio, Aarhus, Denmark) with default settings from paired-end Illumina RNA-seq reads of six *Alexandrium* samples at the 6- and 96-h time points and single-end reads at the 0-h time point (Table S1, Supporting information); (ii) 44 024 expressed sequence tags of *Alexandrium* from our internal database (Wohlrab 2013); and (iii) an expressed sequence tag data set containing 29 995 *Alexandrium* contigs from a previous host–parasite infection study (Lu *et al.* 2014). The overlapping and identical contigs from these data sources were merged to generate a total of 147 835 unique transcripts in the *Alexandrium* reference transcriptome (Fig. S1, Supporting information). All contigs were mapped to the parasite *Amoebophrya* genome sequence data (SRP067624) of the same strain. Matching sequences were excluded from the data set.

Table S1 (Supporting information) provides an overview of the number of RNA-seq reads per *Alexandrium* sample examined. After quality control, a total of 1.3 billion reads were aligned to the *Alexandrium* reference transcriptome using the CLC Genomics Workbench to obtain the read counts using default settings. To determine differential gene expression, the read counts were analysed using the DESeq package in R (Anders & Huber 2010). Size factor estimation and normalization were performed using the 'estimateSizeFactors' and 'estimateDispersions' functions, respectively. Differentially expressed contigs were detected by a negative binomial test using the 'nbinomTest' function. Transcripts with a false discovery rate-adjusted *P*-value < 0.05 were considered statistically significant and used for annotation.

Genes that were significantly differentially expressed between treated and untreated *Alexandrium* samples were annotated by homology searches following the Trinotate annotation suite guidelines (Grabherr *et al.* 2011; Haas *et al.* 2013). Putative orthologs were predicted from reciprocal best BLAST hits; peptide sequences were predicted using the Trinity TransDecoder package; and protein families, signal peptides and transmembrane domains were identified using Pfam (Punta *et al.* 2012), SignalP (Petersen *et al.* 2011) and TMHMM (Krogh *et al.* 2001), respectively. The data were compared to the eggNOG/GO (Powell *et al.* 2012), Gene Ontology (Ashburner *et al.* 2000) and KEGG (Kanehisa *et al.* 2012) databases. The differentially expressed *Alexandrium* genes were translated into amino acid sequences using the Virtual Ribosome package (http://www.cbs.dtu.dk/services/VirtualRibosome), and the Batch Web CD search tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was used to assign eukaryotic orthologous groups (KOGs). Significant enrichments of the transcripts were tested by calculating the *P*-value from a hypergeometric distribution at the background level of all KOGs (Subramanian *et al.* 2005). KOGs were considered significantly enriched for a given experimental time point when the test statistics gave a *P*-value < 0.05.

PSP toxin analysis

Samples (50 mL) of treated and untreated cultures were used for PSP toxin analyses, which were performed as described previously (Krock *et al.* 2007). Briefly, the 50 mL cell culture was centrifuged for 15 min at 3220 ***g***. The supernatant was discarded, and the cell pellet was added to 1 mL of sterile seawater, transferred to a 2 mL tube and centrifuged for 10 min at maximum speed. After removing the seawater, the pellet was transferred to a tube containing 0.9 g of Lysing Matrix D (Thermo Savant, Illkirch, France). The cells were homogenized by reciprocal shaking in a Bio101 FastPrep instrument (Thermo Savant) at speed 6.5 for 45 s. The samples were then centrifuged for 15 min at 4 °C and 13 000 ***g***. The supernatant was passed through a spin filter (pore size 0.45 mm) by centrifugation for 30 s at 3000 ***g***. The filtrate was analysed by high-performance liquid chromatography with fluorescence detection (Krock *et al.* 2007).

Results

Growth of Alexandrium

At late growth stages, the growth rates of *Alexandrium* treated with the *Amoebophrya* parasite (+P) or parasitic waterborne cues (+WP) were lower than those of their corresponding controls (parasite control;-P and waterborne cue control;-WP, respectively) (Fig. 1 and Table S3, Supporting information). The differences between the growth rates of *Alexandrium* in the three treatment groups (+P, +WP and +A) were significant (ANOVA: $f = 10.85$, $P < 0.05$). Compared with that of the parasite control culture (−P), the growth rate of the parasite-infected culture (+P) was reduced significantly at the 24-h (ANOVA: $f = 16.55$, $P < 0.05$), 72 h (ANOVA: $f = 43.41$, $P < 0.01$) and 96-h (ANOVA: $f = 109.5$, $P < 0.01$) time points. Similarly, compared with that of the waterborne cue control (−WP), the growth rate of the parasitic waterborne cue-treated culture (+WP) was reduced significantly at the 48-h (ANOVA: $f = 17.85$, $P < 0.05$), 72-h (ANOVA: $f = 16.25$, $P < 0.05$) and 96-h (ANOVA: $f = 12.68$, $P < 0.05$) time points. By contrast, the growth rate of the culture exposed to the lysed *Alexandrium* cells (+A) (adding more potential organic food supply) was significantly higher than that of the control at the early infection stages (6 h, ANOVA: $f = 21.21$, $P < 0.01$; 24 h, ANOVA: $f = 54.42$, $P < 0.01$) (Table S3, Supporting information).

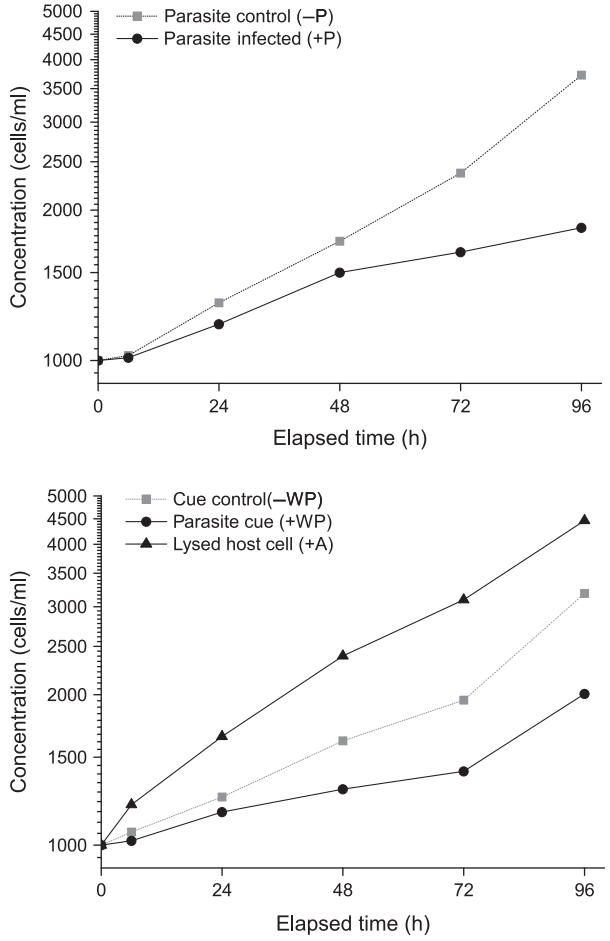


Fig. 1 Growth of *Alexandrium* in the treated (parasite-infected, +P; waterborne parasite cue-treated, +WP; and lysed host cell-treated, +A) and control (parasite control, −P; and cues control, −WP) groups at the indicated time points.

Differentially expressed genes

A total of 14 882 genes were significantly differentially expressed (adjusted P-value < 0.05) between the treated and corresponding control samples at the 6- and 96-h time points (Fig. 2). At the early infection stage (6 h), the parasite (+P) induced lower differentially expressed genes compared to other conditions. Interestingly, parasite cue (+WP) and cue control (−WP) induced a relatively important number of upregulated genes (123) compared to the host-lysed cell (+A) (40 upregulated and 83 downregulated).

At the late infection stage (96 h), a large number of genes were differentially expressed in *Alexandrium* treated with the parasite (+P) or parasitic waterborne cues (+WP), whereas treatment with lysed host cells (+A) produced few changes in gene expression (two genes only). Hosts infected by parasites (+P) responded differently compared to hosts induced by parasite cues (+WP), by having much more downregulated genes (5701 compared to 215, respectively), but similar numbers of upregulated genes (with similar transcripts for about half of them between the two conditions).

Functional categorization of Alexandrium genes at the 6-h and 96-h time points

Of the 14 882 contigs corresponding to differentially expressed genes, a putative function was identified for 9680 of them, among which 7121 were classified into functional categories according to KOGs (Fig. 3). At the early infection stage (6 h), only a few or no up or downregulated genes were detected in the parasite-infected samples. Only 28 of the 123 upregulated genes could be classified into KOG categories. Of these most were found in the categories 'translation, ribosomal structure and biogenesis (32%)', 'inorganic ion transport and metabolism (11%)' and 'general function prediction (14%)' categories.

At the late infection stage (96 h), treatment of *Alexandrium* with parasites or parasitic waterborne cues resulted in the upregulation of a large number of genes

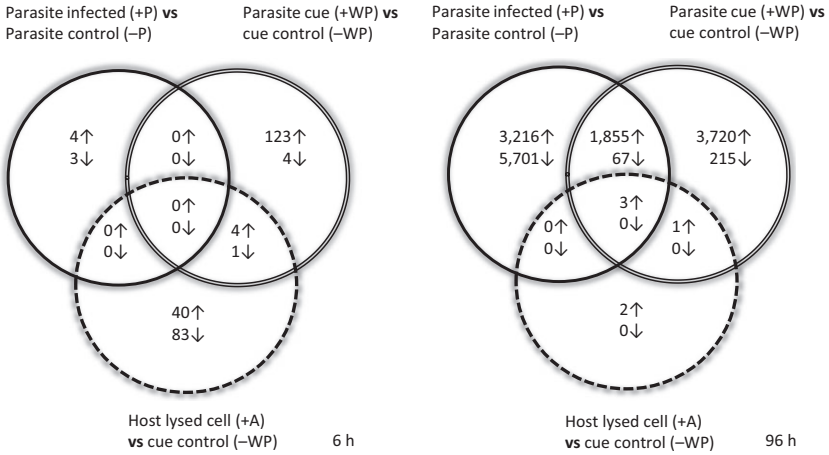
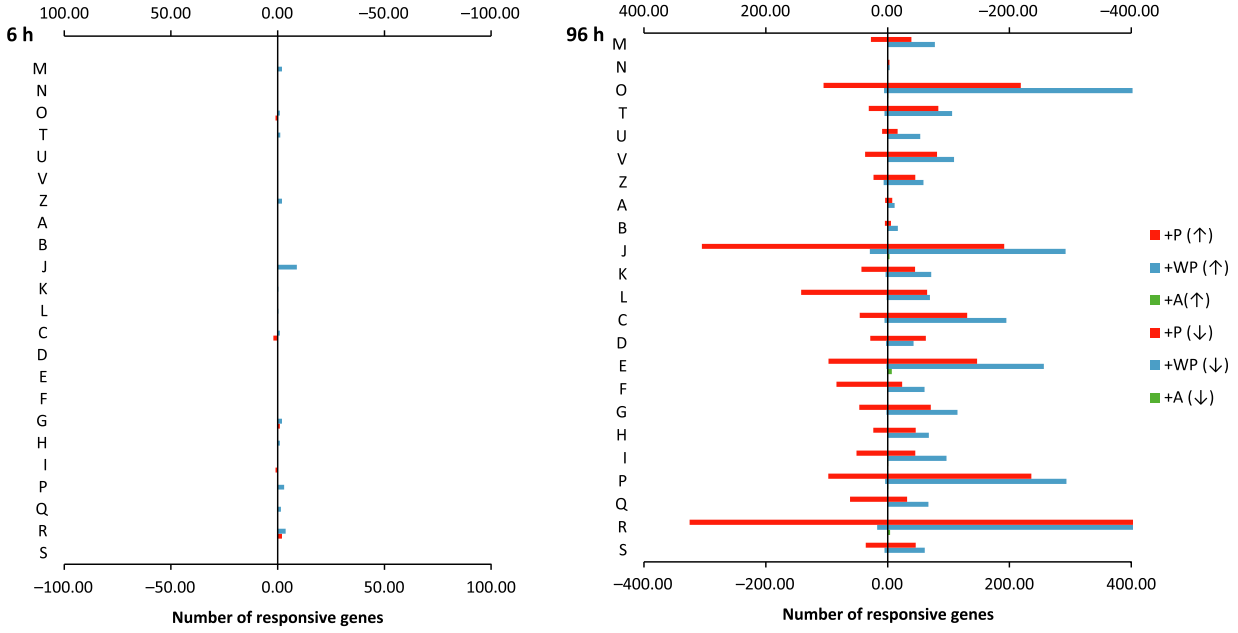


Fig. 2 Differential gene expression in *Alexandrium* treated with parasites (+P), waterborne cues from the parasites (+WP) or lysed host cells (+A).



Cellular processes and signalling

[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[O] Post-translational modification, protein turnover, chaperones
[T] Signal transduction mechanisms
[U] Intracellular trafficking, secretion and vesicular transport
[V] Defence mechanisms
[W] Extracellular structures
[Y] Nuclear structure
[Z] Cytoskeleton

Metabolism

[C] Energy production and conversion
[D] Cell cycle control, cell division, chromosome partitioning
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport and catabolism

Information storage and processing

[A] RNA processing and modification
[B] Chromatin structure and dynamics
[J] Translation, ribosomal structure and biogenesis
[K] Transcription
[L] Replication, recombination and repair
[R] General function prediction
[S] Function unknown

Fig. 3 KOG category distributions of *Alexandrium* gene sequences that were uniquely upregulated (↑) or downregulated (↓) in the parasite-infected (+P), or waterborne cue-treated (+WP) or host-lysed samples (+A) compared with the corresponding control samples at the 6-h and 96-h time points.

(Fig. 3). The significantly enriched transcripts (those with *P*-values < 0.05) were sorted from the hypergeometric distribution (the complete list is provided in Table S4, Supporting information). The majority of the enriched and upregulated genes were assigned to the following KOG categories: 'post-translational modification, protein turnover, chaperones' [parasite-infected only: 10%, $P < 0.05$; parasite-infected and parasite

waterborne cue-treated (common genes): 17%, $P < 0.01$; parasite waterborne cue-treated only: 14%, $P < 0.05$]; 'translation, ribosomal structure and biogenesis' [parasite-infected only: 7%, $P < 0.01$; parasite-infected and parasite waterborne cue-treated (common genes): 23%, $P < 0.01$; parasite waterborne cue-treated only: 11%, $P < 0.01$]; and 'general function prediction [parasite-infected only: 20%, $P < 0.01$; parasite-infected and parasite waterborne cue-treated (common genes): 14%, $P < 0.05$; parasite waterborne cue-treated only: 18%, $P < 0.01$] (Table S4, Supporting information).

Transcripts that were upregulated in response to parasite infection only were enriched in the 'signal transduction mechanisms' (8%, $P < 0.01$), 'cell cycle control, cell division, chromosome partitioning cell' (6%, $P < 0.01$) and 'transcription' (6%, $P < 0.05$) categories. The genes that were upregulated in response to treatment with threatening parasitic waterborne cues only were mainly included in the 'amino acid transport and metabolism' (5%, $P < 0.01$) and 'lipid transport and metabolism' (5%, $P < 0.01$) categories. Genes that were commonly upregulated by parasite infection and waterborne cues at the 96-h time point were enriched in the 'energy production and conversion' (9%, $P < 0.01$) and 'replication, recombination and repair' (3%, $P < 0.01$) categories.

We observed a marked downregulation of transcription in response to parasite infection alone at the 96-h time point (5701 transcripts). Most of these genes were included in the 'translation, ribosomal structure and biogenesis' (29%, $P < 0.01$); 'replication, recombination and repair' (11%, $P < 0.01$); 'nucleotide transport and metabolism' (9%, $P < 0.01$); 'post-translational modification, protein turnover, chaperones' (6%, $P < 0.01$); and 'general function prediction' (12%, $P < 0.01$) categories (the full list is provided in Table S4, Supporting information).

Genes of particular interest

The numbers and functional annotations of the genes that were differentially expressed at the 6- and 96-h time points are compared in Table 1. Among the functional categories identified, we examined transcriptional changes in genes associated with metabolic pathways for energy production, photosynthesis, signal transduction, reactive oxygen species (ROS) and defence mechanisms. A full list of the regulated genes, with annotations, is provided in Appendix S1 (Supporting information).

Among the 1855 genes that were commonly upregulated at the 96-h time point in the +P and +WP treatments, we observed a significant enrichment ($P < 0.01$) of those involved in energy supply. More specifically, these genes were involved in glycolysis, fatty acid β-oxidation, the tricarboxylic acid cycle, oxidative phosphorylation and the glyoxylate cycle (Table 1). Consistent with the observation that the breakdown of fatty acids (e.g. their β-oxidation) is enhanced following parasitic infection, the genes involved in fatty acid biosynthesis (such as those encoding the malonyl-acyl carrier protein and S-malonyltransferase) were downregulated in *Alexandrium*. Notably, core components of the photosystems, including six subunits of the photosystem II complex and photosystem I P700 chlorophyll *a* apoprotein A, were induced in response to parasite infection alone (Table 1).

In *Alexandrium* infected with the parasite or exposed to the waterborne cues, we observed differential regulation of signal transduction-related genes ('signal transduction mechanisms' category; $P < 0.01$), such as serine/threonine kinases and genes involved in calcium, mitogen-activated protein kinase and Ras signalling, as well as secondary metabolism-related genes. The differentially expressed serine/threonine kinases are highlighted in Table 1 (the full list is provided in Table S5, Supporting information), along with further potential defence-related ROS scavenging enzymes (the full list is provided in Table S6, Supporting information).

PSP toxin content of Alexandrium

At both the 6- and 96-h time points, the PSP toxin content (fmol/cell) of *Alexandrium* cells in the +P and +WP treatments was slightly lower than that of the corresponding control cultures (Fig. 4); however, the differences were not statistically significant (Students *t*-test, $P > 0.05$). The control cultures did not show a significant change in PSP toxin content over time.

Discussion

The results presented herein demonstrate that the dinoflagellates, *Alexandrium,* can mount a strong response to the parasite, *Amoebophrya*, as revealed by changes in gene expression. At the early stage (6-h time point), neither parasite infection nor exposure to parasitic waterborne cues caused a marked regulation of genes (Fig. 2). It is likely that the level of threat from the parasite, in terms of time or intensity, was too low to trigger the main response of the host at the 6-h time point. Infection with parasites resulted in a massive downregulation of *Alexandrium* genes (5701) after 96 h, whereas exposure to waterborne cues did not trigger a similar pattern of downregulation, suggesting that a direct interaction between the parasite and its host is necessary for this phenomenon. The

Table 1 Gene content and annotation summaries. The numbers of differentially expressed genes with predicted functions in *Alexandrium* at 6 h and 96 h after infection with *Amoebophrya* or treatment with parasitic waterborne cues or lysed host cells.

		6 h						96 h							
		+P		+WP		+A		+P		Overlap of +P and +WP		+WP		+A	
		Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Significantly differentially expressed transcripts (adj $p < 0.05$)		4	3	123	4	40	83	3216	5701	1855	67	3720	215	2	0
Annotated transcripts		0	1	73	2	27	26	1796	3267	1566	56	2935	123	2	0
Energy production	ko00010 Glycolysis/Gluconeogenesis	0	0	0	0	0	0	5	10	13	0	18	2	0	0
	ko00620 Pyruvate metabolism	0	0	0	0	0	0	5	8	7	0	14	0	0	0
	ko01200 Carbon metabolism	0	0	0	0	0	0	12	24	28	0	42	2	0	0
	ko01230 Biosynthesis of amino acids	0	0	0	0	0	0	11	23	17	0	28	4	1	0
	ko01212 Fatty acid metabolism	0	0	0	0	0	0	4	11	9	0	18	0	0	0
	ko00020 Citrate cycle (TCA cycle)	0	0	0	0	0	0	3	7	13	0	15	0	0	0
	ko00190 Oxidative phosphorylation	0	0	2	1	0	0	10	13	29	0	23	0	0	0
	ko00630 Glyoxylate and dicarboxylate metabolism	0	0	0	0	0	0	4	5	7	0	9	0	0	0
Photosynthesis	ko00195 Photosynthesis	0	0	0	0	0	0	9	3	0	0	0	1	0	0
	ko00710 Carbon fixation in photosynthetic organisms	0	0	0	1	0	0	3	5	9	0	10	2	0	0
	ko00860 Porphyrin and chlorophyll metabolism	0	0	1	0	0	0	3	5	0	0	2	0	0	0
	ko00030 Pentose phosphate pathway	0	0	0	0	0	0	2	5	6	0	8	1	0	0
Signal transduction	ko00260 Glycine, serine and threonine metabolism	0	0	0	0	0	0	5	6	7	0	13	1	0	0
	ko04020 Calcium signalling pathway	0	0	1	0	0	0	10	6	8	0	11	1	0	0
	ko04010 MAPK signalling pathway	0	0	1	0	0	0	13	14	9	0	13	0	0	0
	ko04014 Ras signalling pathway	0	0	0	0	0	0	6	6	9	0	7	1	0	0
	ko01110 Biosynthesis of secondary metabolites	0	0	2	0	0	0	28	52	52	0	96	3	1	0
	PF00069 Pkinases domain (Serine/threonine protein kinase)	0	0	1	0	0	0	51	46	24	0	39	2	0	0
	ROS scavenging enzymes	0	0	0	0	0	0	7	17	17	0	37	1	0	0
Cell cycle	ko04110 Cell cycle	0	0	0	0	0	0	9	23	10	1	18	2	0	0

MAPK, mitogen-activated protein kinase; TCA, tricarboxylic acid.

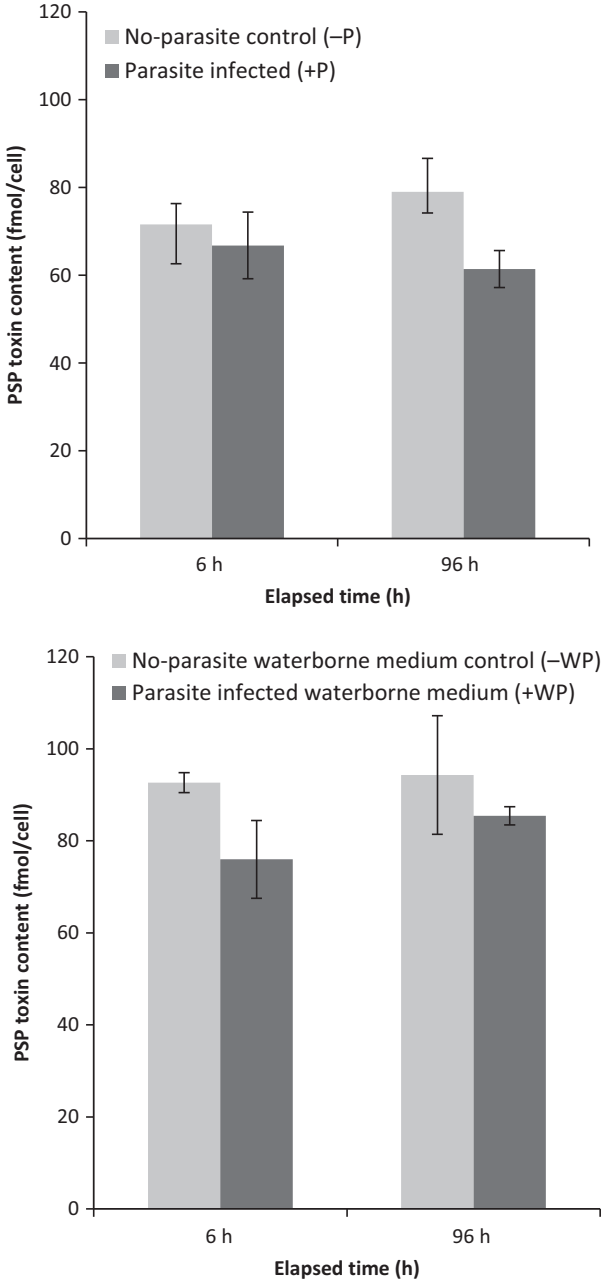


Fig. 4 PSP toxin content of *Alexandrium* in the parasite-infected (+P), parasite waterborne medium-treated (+WP) and corresponding control groups (−P and −WP) at the 6-h and 96-h time points. No significant differences were detected (ANOVA).

observed downregulation of genes could be due to an ongoing degradation of the host cell structures; however, the simultaneous upregulation of more than 3000 genes in the parasite-infected samples argues against this scenario. On the other hand, exposure to parasitic waterborne cues also elicited the upregulation of an approximately equal number of transcripts as direct parasite infection, indicating that the host is capable of sensing its parasite. The remarkable overlap of upregulated genes between these two conditions indicates that the stimulation of signal transduction chains by waterborne cues alone could prime the host's defences or induce host's adaptive responses to the parasite activity. While the observed downregulation of genes could be the consequence of host decay, the induction of genes is not likely a side effect of such and thus represents an active response to the parasites attack. The apicomplexan parasites, *Toxoplasma* and *Plasmodium* included, provide efficient infection strategies to subvert host cell processes, avoid clearance by the defence mechanisms and modulate the metabolic pathways of the host (Plattner & Soldati-Favre 2008). Therefore, this parasite-driven activity could result in expression changes related to host adaptive responses to parasite-initiated effects. We summarized our major findings in Fig. 5 and describe the differentially expressed genes involved in energy production, signal transduction and defence mechanisms in the following sections.

Functional categorization of processes in Alexandrium

Energy production. The increased expression of pathway components associated with energy production at the 96-h time point after infection of *Alexandrium* with the parasite or exposure to waterborne cues indicates an increased energy demand for defence in the host. A KEGG pathway analysis depicted a complete oxidation of carbohydrates and fatty acids by the tricarboxylic acid cycle in the mitochondria of *Alexandrium*, as well as ATP generation, and may reflect the costs necessary for defence, as observed in land plants (Livaja *et al.* 2008). Alternatively, the high demand of energy might also be induced by the parasite to get resources for its intracellular development and reproduction, in agreement with studies in *Toxoplasma gondii* (Tenter *et al.* 2000; Danne *et al.* 2013). And one could speculate that the ATP generation observed here might be induced by a rapid ATP depletion in host cells after the parasite infection. However, photosynthetic activity, which is increased after parasite infection but is not inducible by waterborne cues, might hint at the requirement for enhanced energy needs and oxygen production from light in defending the host cells.

Genes related to fatty acid biosynthesis, such as the malonyl-acyl carrier protein, were downregulated in parasite-infected cells at the 96-h time point. Acyl carrier protein is the core protein involved in fatty acid synthesis (Mazumdar & Striepen 2007). Our results indicate that fatty acid synthesis in the plastid was inhibited, whereas β-oxidation of fatty acids was

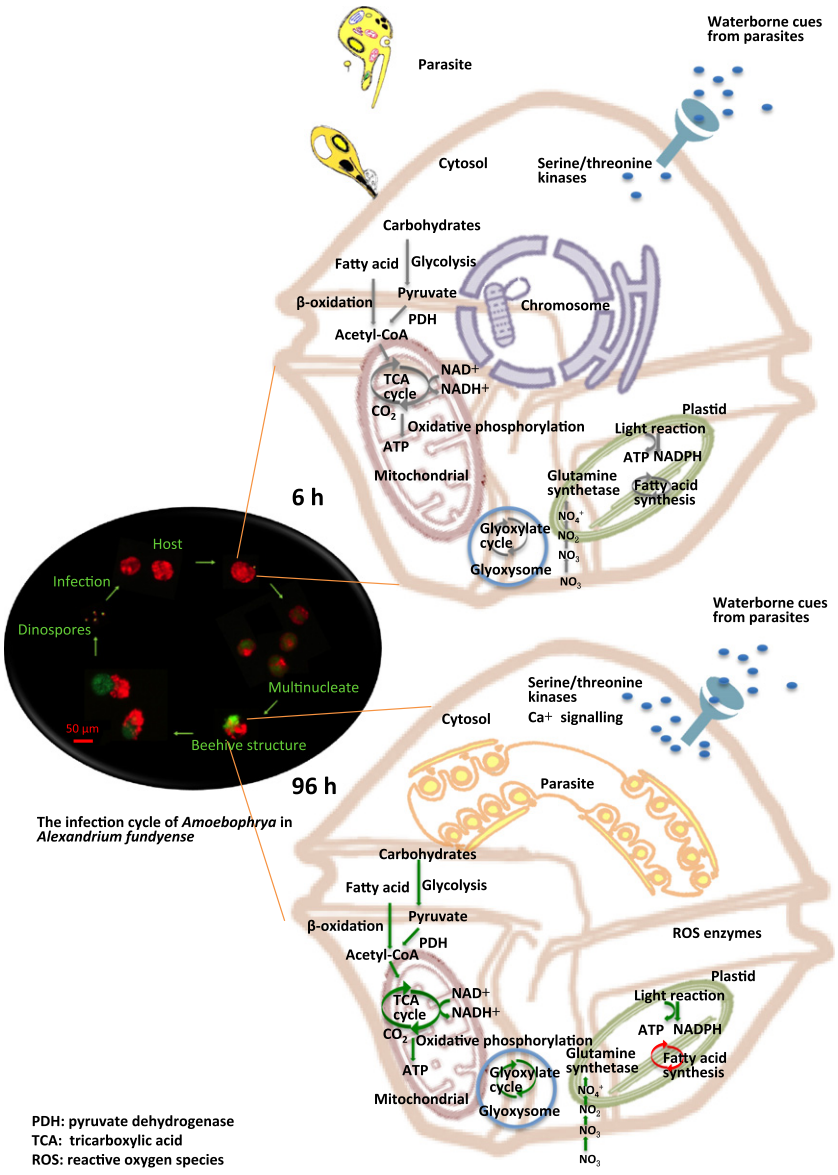


Fig. 5 Altered cellular activity in *Alexandrium fundyense* infected by the parasite *Amoebophrya*. The presence of the parasite and waterborne cues from the parasite results in upregulation (green arrows) of energy production processes involved in photosynthesis, ATP synthesis through glycolysis and fatty acid production, and downregulation (red arrows) of fatty acid synthesis. At the same time, the activation of calcium-mediated signal transduction and enzymes correlated with ROS production indicates that *Alexandrium* could perceive chemical cues from the parasite and induce defence mechanisms.

induced, either reflecting the increased energy requirement of the infected host or the response induced by the parasite to fulfil itself. This finding is consistent with that reported for the response of the coral *Acropora cervicornis* to pathogen infection (Libro *et al.* 2013), as well as those of other organisms affected by grazing (Wohlrab *et al.* 2010; Flöthe *et al.* 2014). In addition, the regulation of fatty acid coding genes, as major components of cell membranes, may be an indicator of cell growth machinery. We identified the downregulation of genes involved in cell cycle in response to parasite infection at 96 h (Table 1), which implies that parasite infection could inhibit host cell division process. By contrast, cell growth-related genes were observed to be upregulated at 96 h when infected by parasite or exposed to waterborne cues. This phenomenon is likely to be induced by the parasite in order to make use of this resource for its own reproduction and growth, because simultaneously cell division- and proliferation-related genes were highly expressed in the parasite *Ameobohrya* at the time of fast cell growth and nuclear division (Lu *et al.* 2014).

Signal transduction and defence mechanisms. Calcium signalling and the activities of several protein kinases seem to be important for dinoflagellates as direct responses to the parasite and may also act to prime the cell towards a parasite attack. Calcium and calmodulin, which were differentially regulated in parasite-infected *Alexandrium*, are key components of signal transduction pathways and are involved in stress responses of various environmental stress conditions (Scandalios 2005)

but also in cell cycle control in plants and marine phytoplankton (Jingwen *et al.* 2006). The second messenger cAMP regulates cell cycle progression in the dinoflagellates, *Crypthecodinium cohnii* (Lam *et al.* 2001; Wurzinger *et al.* 2011); although it may be important for the dinoflagellate host–parasite interaction, little is currently known about the significance of calcium signalling in photosynthetic dinoflagellates compared with heterotrophic dinoflagellates or parasitic apicomplexan (Verret *et al.* 2010; Plattner *et al.* 2012). A recent study showed that the expression levels of calcium-dependent protein kinases and serine/threonine kinases are altered as a defensive response to copepod grazers (Wohlrab *et al.* 2010).

Exposure of *Alexandrium* to parasites or parasitic waterborne cues induced the upregulation of genes involved in the production of ROS (Tables 1 and S6, Supporting information), pointing towards ROS production as a key defence mechanism or a response to ROS spreading in the cytosol due to internal membrane damage. ROS, such as oxygen ions and peroxides (H_2O_2), are a by-product of cell metabolism; however, their overproduction in marine organisms can cause oxidative damage and irreversibly alter DNA, proteins and lipids (Halliwell & Gutteridge 1985; Lesser 2006). A recent study showed that a high production of ROS by *Alexandrium catenella* under stress conditions may play the key role of fish gill damage in Chilean fjords (Mardones *et al.* 2015). As such, ROS can have direct negative effects on intracellular parasites. ROS production occurs in *Alexandrium* during temperature increases (Jauzein & Erdner 2013) and has been observed in diverse marine organisms exposed to environmental abiotic stressors such as UV or heat shock (Lesser 2012); hence, it is considered an indicator of stress-related pathways. Flores *et al.* (Flores *et al.* 2012) found that the addition of ROS enzymes increased the survival of both the ciliate *Tiarina fusus* and the heterotrophic dinoflagellate *Polykrikos kofoidii* after exposure to *Alexandrium*, suggesting that ROS may be indirectly correlated to the toxicity of *Alexandrium* to protists. Here, several ROS enzymes, including superoxide dismutase, peroxidase and catalase, were upregulated in response to parasite infection or exposure to parasitic waterborne cues. Superoxide dismutase catalyses the conversion of the superoxide radical (O_2^-) to peroxides (H_2O_2), whereas peroxidase and catalase convert H_2O_2 to water (Apel & Hirt 2004). Photosynthesis is an additional source of ROS in plants (Foyer & Shigeoka 2011). Our finding that photosynthesis-related genes were upregulated after direct contact with the parasite, but were not induced by exposure to parasitic waterborne cues, suggests that ROS play an important role in the defence mechanism.

PSP toxin distribution

The absence of a significant change in the PSP toxin content of *Alexandrium* after parasite infection or exposure to parasitic waterborne cues (Fig. 4) indicates that there is no effect of the parasite on PSP toxin production in this species. To date, PSP toxins have been shown to act as potential defence compounds against metazoan (copepod) grazers (Selander *et al.* 2006; Wohlrab *et al.* 2010; Yang *et al.* 2010), but do not act as defence compounds against unicellular heterotrophs (Tillmann & John 2002; Tillmann & Hansen 2009). The phylogeny and mode of action ('intracellular grazer') of *Amoebophrya* more closely resemble those of unicellular protistan grazers than metazoan copepods; therefore, it is likely that PSP toxins do not serve as defence compounds towards protistan parasites in *Alexandrium*. Bai *et al.* (2007) performed similar experiments using *Amoebophrya* sp. and a toxic strain of *Karlodinium veneficum* and found that the parasite did not actively catabolize the host's toxins. However, little is currently known about the impact of parasitism on *Alexandrium* toxin production, and our study investigated the effect on only one strain of *Alexandrium.* Repeated exposure of different *Alexandrium* strains and analysis of the effects of PSP toxins during parasite infection remain to be explored. In addition, we observed the upregulation of genes involved in secondary metabolism in response to parasite infection or parasitic waterborne cues (Table 1). Aside from the known PSP toxins and the unknown allelochemicals (Tillmann & John 2002; Ma *et al.* 2009), these secondary metabolites may also be involved in the defence against parasites during the infection cycle.

Conclusions

The results presented herein reveal that the dinoflagellate *Alexandrium fundyense* undergoes specific alterations in gene expression in response to infection or exposure to waterborne cues from the parasite *Amoebophrya.* A large number of genes were downregulated, mainly due to parasite infection and damage to host cell structures. By contrast, the upregulation of genes in cells treated with parasitic waterborne cues affected the host's defence mechanisms, in particular energy production involved in photosynthesis, ATP synthesis through glycolysis and fatty acid production, calcium-mediated signal transduction and ROS production. Taken together, these data suggest that dinoflagellate parasite infection and chemical cues from the parasites can trigger a powerful defence response in dinoflagellate hosts. It would be beneficial to have further protein biochemistry or proteomics analysis in order to

investigate the changes on the transcriptome level measured in this study reflected in protein mechanisms.

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Y.L. and U.J. designed the research; Y.L. performed experiments; Y.L., S.W. and M.G. analysed the data; and Y.L., S.W., M.G., G.G, L.G. and U.J. wrote the manuscript.

Data accessibility

RNASeq raw sequences generated using Illumina have been submitted to SRA at NCBI under the Accession no. SRP067590. *Amoebophrya* genome sequence data: NCBI SRA: SRP067624.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Full annotated list of *Alexandrium* genes that were up- or down-regulated at the 6 h and 96 h time points (.xlsx).

Table S1 Summary of the 1.3 billion Illumina RNAseq reads (single-end and paired-end approaches) from *Alexandrium* samples in the treated (parasite-infected, parasitic waterborne cue-treated, and lysed host cell-treated) and control (parasite and cue controls) groups.

Table S2 The de novo *Alexandrium* transcriptome, which was assembled (using the CLC Genomics Workbench) from six samples at the 6 h and 96 h time points and single-end reads at the 0 h time point (nos. 1 to 3 in Table S1).

Table S3 Growth rates of infected and healthy *Alexandrium* cultures.

Table S4 KOG enrichment of up- and down-regulated genes at the 96 h time point.

Table S5 Up- and down-regulated genes coding for proteins with serine/threonine kinase activity or calcium-binding domains.

Table S6 Up- and down-regulated genes encoding ROS enzymes.

Fig. S1 The *Alexandrium* master reference (147 835 transcripts), which was constructed from (i) a de novo transcriptome of *Alexandrium* (122 219 contigs), (ii) 14 801 transcripts from 44 024 expressed sequence tags of *Alexandrium* from NCBI, and (iii) 10 815 transcripts from 29 995 expressed sequence tags from an *Alexandrium* vs. *Amoebophrya* infection study.