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Trait changes induced by species interactions in two phenotypically distinct strains of a marine dinoflagellate

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Populations of the toxigenic marine dinoflagellate *Alexandrium* are composed of multiple genotypes that display phenotypic variation for traits known to influence top-down processes, such as the ability to lyse co-occurring competitors and prospective grazers. We performed a detailed molecular analysis of species interactions to determine how different genotypes perceive and respond to other species. In a controlled laboratory culture study, we exposed two *A. fundyense* strains that differ in their capacity to produce lytic compounds to the dinoflagellate grazer *Polykrikos kofoidii*, and analyzed transcriptomic changes during this interaction. Approximately 5% of all analyzed genes were differentially expressed between the two *Alexandrium* strains under control conditions (without grazer presence) with fold-change differences that were proportionally higher than those observed in grazer treatments. Species interactions led to the genotype-specific expression of genes involved in endocytotic processes, cell cycle control and outer membrane properties, and signal transduction and gene expression regulatory processes followed similar patterns for both genotypes. The genotype-specific trait changes observed in this study exemplify the complex responses to chemically mediated species interactions within the plankton and their regulation at the gene level. The ISME Journal (2016) 10, 2658–2668; doi:10.1038/ismej.2016.57; published online 19 April 2016

Introduction

In the classic article entitled ‘The paradox of the plankton,’ Hutchinson (1961) addresses the question of how species can coexist in a relatively isotropic or unstructured environment, competing for the same resources. This apparent paradox derives from the traditional view that ‘bottom-up’ processes lead to competition for abiotic resources (for example, light and nutrients) and are the main driving force in shaping the structure of plankton communities. The failure of resource competition alone to account for the observed species heterogeneity and niche differentiation in plankton has led to an increased appreciation of species interactions, considered ‘top-down’ processes, as a potential driving force for annual species succession (Margalef, 1978; Smetacek *et al.*, 2004; Murray *et al.*, 2011). The recognition of the role of species interactions in paradigms of successional processes on seasonal and annual time scales has underscored the fundamental importance of such interactions in co-evolutionary processes determining species diversity (Smetacek, 2001; Hamm *et al.*, 2003; Smetacek *et al.*, 2004).

The co-evolutionary mechanisms involved in the maintenance of species diversity are even more

complex when considering the coexistence of multiple genotypes that exhibit fitness differences in the laboratory under controlled environmental conditions. This phenomenon has been named ‘the second paradox of the plankton’ (Hebert and Crease, 1980; Fox *et al.*, 2010). Different genotypes within one population can differentially express traits, such as cell size, cell shape and the production of secondary metabolites. These differentially expressed traits may also include integrative biochemical mechanisms, leading to variation in intrinsic growth rates. Differences in these parameters that result from genotypic variation influence both intra- and inter-specific interactions (Van Donk *et al.*, 1999).

There are few well-defined model marine phytoplankton species for which phenotypic and genotypic diversity have been explored at the population level. In all cases to date, in-depth analyses using molecular markers have revealed a high degree of intra-population diversity within dominant phytoplankton groups, including dinoflagellates (Alpermann *et al.*, 2010), coccolithophorids (Iglesias-Rodríguez *et al.*, 2006) and diatoms (Rynearson and Armbrust, 2000). Among 77 clonal strains of a population of the toxigenic marine red-tide dinoflagellate *Alexandrium*, no repeated sampling of any genotype was observed using microsatellite and amplified fragment length polymorphism markers (Alpermann *et al.*, 2010).

These *Alexandrium* genotypes also exhibit wide intra-population phenotypic variation (Alpermann *et al.*, 2010) in the production of secondary metabolites,

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including paralytic shellfish toxins, a group of potent neurotoxic alkaloids responsible for toxic effects on marine organisms and human consumers of seafood (Anderson *et al.*, 2012), and allelochemical compounds of uncertain structural affinity with lytic effects (Tillmann and John, 2002; Fistarol *et al.*, 2004; Hattenrath-Lehmann and Gobler, 2011).

On a per cell basis, populations exhibit wide variation in lytic potency (Alpermann *et al.*, 2010; Hattenrath-Lehmann and Gobler, 2011). Lytic allelochemicals affect both putative competitors and predators, and such genotype-specific species interactions are expected to affect community structure for both competitive and bi-trophic systems. Furthermore, mutual facilitation, whereby interspecific interactions occur between individuals with different genotypes that vary in lytic capacity, provides a benefit to one species, without negatively affecting other genotypes (John *et al.*, 2015).

In our study, we resolved species interactions among *A. fundyense* clones at the genotypic level. We utilized a comparative approach whereby a lytic (*Alex2*) and non-lytic (*Alex5*) strain of *A. fundyense* were exposed to waterborne cues from a protistan grazer. The two strains were selected based on previously established differences in their lytic capacity, as quantified by a whole-cell bioassay using the cryptomonad *Rhodomonas* (Tillmann *et al.*, 2009). For all tested cell concentrations, *Alex2* showed a high lytic capacity in the *Rhodomonas* bioassay, whereas *Alex5* showed no lytic effect (Tillmann *et al.*, 2009). We used a functional genomics approach to (1) assess the differences between the two *A. fundyense* strains and (2) screen for traits that differ among genotypes during exposure to a heterotrophic grazer. Our results provide insight into traits that are selectively induced and may hence be beneficial with respect to fitness under pressure owing to biotic interactions.

Materials and methods

Alexandrium fundyense and *Polykrikos kofoidii* cultures
Two strains of *A. fundyense*, *Alex2* (lytic) and *Alex5* (non-lytic), previously classified as *A. tamarense* Group 1 (John *et al.*, 2014), were isolated in May 2004 from the North Sea coast of Scotland (Alpermann *et al.*, 2010). Clonal isolates were grown in natural seawater-based (salinity ~33) K-medium (Keller *et al.*, 1987) in a temperature- and light-controlled culture chamber (18 °C, 14-h:10-h light–dark cycle, ~150 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

The culture of the heterotrophic dinoflagellate *Polykrikos kofoidii* was established from a plankton net tow (20 μm) in 2009 in coastal waters of Scotland. The stock culture was maintained in K-medium (Keller *et al.*, 1987) in culture flasks on a plankton wheel (1 r.p.m.) at 15 °C under low light (10–20 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The *Polykrikos* cultures were fed the thecate dinoflagellate *Lingulodinium polyedrum* (CCMP 1738). A subculture used for experimental inoculation was starved for ~1 day so that no dinoflagellate food algae were present.

Experimental design

Axenic, mid-exponential phase cultures of *A. fundyense* were washed three times with sterile-filtered seawater and diluted to a concentration of ~1000 cells ml^{-1} . The washing steps ensured the exchange of culture media containing lytic substances before the experiments. *P. kofoidii* cultures were diluted to a concentration of ~40 cells ml^{-1} .

A cage culture technique was used to separate *Alexandrium* strains from mixed *Polykrikos/Alexandrium* cultures to ensure RNA isolation from only the target *Alexandrium* and to avoid cross-hybridization on the microarray. The cages (50 ml polypropylene tubes containing a 10 μm plankton mesh at the bottom) were placed in 250 ml glass flasks. For both *Alexandrium* strains, 24 flasks were set-up, including three replicates of controls and three replicates of species interaction treatments for each harvesting time point (24, 48, 72 and 96 h). Each flask received 125 ml of the *Alex2* or *Alex5* culture diluted with 125 ml of K-medium to a concentration of ~500 cells ml^{-1} . Cages were submerged in the flasks, and each treatment cage was inoculated with 1 ml of *Polykrikos* culture (~40 cells) and 1 ml of *Alex5* culture (~1000 cells). Control flasks received 1 ml of *Alex5* cultures within the cage. Cages were slowly moved up and down three times a day to promote the exchange of medium and dissolved compounds (including allelochemicals) between the two compartments. Experiments were terminated after 24, 48, 72 and 96 h; the first 24 h was considered to be an acclimation phase because dinoflagellates are sensitive to mixing (Berdalet *et al.*, 2007) and often show a prolonged lag-phase after cell culture transfer.

Cell harvesting

Cages were removed from the experimental flasks and submerged in 50 ml glass flasks filled with sterile-filtered seawater to 30 ml. The contents of the cages were mixed, and 10 ml of culture suspension was preserved in acid Lugol's iodine solution for cell counts.

The flasks containing the surrounding *Alexandrium* cultures were also mixed, and 10 ml of the culture was preserved in acid Lugol's iodine solution for cell counts. Afterward, the culture was poured over a 10 μm nylon mesh sieve to collect the *Alexandrium* cells. The 10 μm nylon mesh was back-washed with sterile seawater into a 50 ml collection tube and immediately centrifuged at 4 °C for 5 min to obtain cell pellets for RNA isolation.

Cell pellets for RNA samples were mixed with 1 ml of hot (60 °C) TriReagent (Sigma-Aldrich, Steinheim, Germany), transferred to a 2 ml cryovial containing acid-washed glass beads, vortex-mixed for 1 min to shear the cells and subsequently frozen in liquid nitrogen for storage at –80 °C until RNA extraction.

Total RNA isolation

Frozen cell pellets fixed with TriReagent were lysed using a Bio101 FastPrep instrument (Thermo Savant,

Illkirch, France) at maximum speed (6.5 m s^{-1}) for $2 \times 45 \text{ s}$. Total RNA was isolated following the Sigma-Aldrich TriReagent protocol recommendations, with linear polyacrylamide (Ambion, Life Technologies, Carlsbad, CA, USA) as a co-precipitant. Protocol details are described by Lu *et al.* (2014). RNA quality and integrity were verified with a RNA Nano-Chip Assay on a 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, CA, USA). The RNA yield and concentration were checked with a NanoDrop ND-100 spectrometer (PeqLab, Erlangen, Germany). Only high-quality RNAs with minimum absorption ratios at $260/280 \text{ nm} \geq 2$ and $260/230 \text{ nm} \geq 1.8$ with corresponding intact ribosomal peaks were used for subsequent analyses.

454 Sequencing and complementary DNA library construction

Alex2 and *Alex5* RNA samples for each treatment were pooled, and $10 \mu\text{g}$ of total RNA was used to prepare a normalized complementary DNA library for GS FLX Titanium sequencing. Complementary DNA library construction was carried out by Vertis Biotechnology AG (Freising-Weihenstephan, Germany). In total, 521 578 reads were assembled into 19 107 contigs longer than 500 bp, with an average coverage of ~ 27 reads per contig. The contigs were compared with an existing gene library from *A. fundyense* by a reciprocal best-BLAST hit search. This resulted in a final selection of 29 224 unique contigs for the microarray design.

Microarray design and hybridizations

A standardized in-house working pipeline for microarrays was used to assure reproducible sample processing and robust data analysis. Microarray probes (60-mers) were designed with the Agilent eArray online platform (<https://earray.chem.agilent.com/earray/>). Microarray hybridizations were designed to compare RNA from treatments and controls with respect to a reference pool of RNAs. The RNA reference pool consisted of equal volumes of subsamples of all RNAs obtained from the control treatments, and was used as a common control baseline to minimize hybridization biases (Novoradovskaya *et al.*, 2004).

Labeling reactions were prepared from 200 ng of total RNA with the Agilent two-color Low Input Quick Amp Labeling Kit and the RNA Spike-In Mix (Agilent Technologies). In brief, RNA was reverse transcribed into complementary DNA with T(7) promoter primers and labeled fluorescently by linear amplification into complementary RNA with cyanine-3-CTPs or cyanine-5-CTPs according to the manufacturer's protocol.

Dye incorporation rates and complementary RNA concentrations were determined with a NanoDrop ND-100 spectrometer (PeqLab). Hybridizations were carried out on $8 \times 60 \text{ K}$ microarray slides with 300 ng of cyanine-3- and cyanine-5-labeled complementary

RNA at 65°C for 17 h and were washed according to the manufacturer's protocol. Microarrays were scanned with an Agilent G2565AA Scanner (Agilent Technologies).

Raw data processing was performed using Agilent Feature Extraction Software version 9.1.3.1 (FE); quality monitoring was performed using the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07. Differentially expressed genes were detected using the Agilent GeneSpring GX software platform version 12. Hybridization results for biological triplicates were subjected to multiple comparison tests with a two-way analysis of variance (ANOVA) to determine the effects of treatment and time. Screening for differentially expressed genes for both genotypes under control conditions was performed in the same way. Genes were considered differentially expressed when P -values were < 0.05 . The data set was then reduced to include only genes for which expression changes exceeded 1.5-fold. The microarray design and hybridizations were MIAME compliant, and the experimental raw data were deposited in a MIAME-compliant database (ArrayExpress accession: E-MTAB-4060).

Gene set enrichment analysis

All sequences represented on the microarray were subjected to a hidden Markov model-based search for Pfam protein families (Finn *et al.*, 2008). Sequences were first translated *in silico* (Wernersson, 2006) and subsequently online batch-processed to search for Pfam matches (<http://pfam.sanger.ac.uk/>). Upregulated Pfam families were tested for significant enrichment by calculating P -values from a hypergeometric distribution (Subramanian *et al.*, 2005). Pfam families were considered significantly enriched at a given experimental time point when the P -value was < 0.05 .

Results

Differentially expressed genes between genotypes

In an analysis of the variability in gene expression patterns between the two *Alexandrium* genotypes under control conditions, we found that $\sim 5\%$ of all genes represented on the microarray were differentially expressed at all time points. Among these, 923 genes were always more highly expressed in *Alex2* cultures, and 584 were more highly expressed in *Alex5* cultures. For these differentially expressed genes, we observed maximum fold-change differences between control cultures of 385 for *Alex2* and 977 for *Alex5* (Figure 1). The top 10 differentially expressed genes for each strain and their assigned Pfam protein families are listed in Table 1 (*Alex2*) and Table 2 (*Alex5*).

We analyzed differentially expressed genes between representative lytic (*Alex2*) and non-lytic (*Alex5*) genotypes, specifically for the occurrence of Pfam protein family annotations with known involvement in secondary metabolite production (Medema *et al.*, 2011), and found that 24 genes in these protein families were more highly expressed in the lytic *Alex2* than in

Figure 1 Differentially expressed genes between control cultures of the two *Alexandrium fundyense* genotypes. Fold-changes and total number of differentially expressed genes for *Alex2* (left) and *Alex5* (right).

Table 1 Ten most highly overexpressed genes in *Alex2* control cultures (in comparison with *Alex5*) and overexpressed genes coding for proteins potentially involved in secondary metabolism

Contig	Mean FC value	Pfam protein family name	Attributed function
<i>Top 10 overexpressed genes in Alex2</i>			
contig11508	385.70	—	—
contig47146	232.12	—	—
Atam01000	199.42	—	—
contig01736	188.40	Ion channel	Ion flux control
Atam27888	172.62	—	—
Atam09090	164.73	—	—
contig48538	144.33	—	—
contig14000	141.05	—	—
contig54109	140.65	—	—
Atam19896	133.97	—	—
<i>Prominent secondary metabolite protein coding genes overexpressed in Alex2</i>			
contig46061	28.56	Glycosyl transferase family 8	Transfer of glycosyl groups
contig27559	9.43	ABC transporter transmembrane region	Transport
Atam22087	5.87	FAD binding domain	Redox reactions
contig50076	5.44	ABC-2 type transporter	Transport
contig53666	5.41	Glycosyl transferase family 8	Transfer of glycosyl groups
contig03340	4.17	Glycosyl transferase family 8	Transfer of glycosyl groups
contig30821	3.81	Major facilitator superfamily	Transport
contig33504	3.79	Major facilitator superfamily	Transport
Atam04504	2.93	Aldo/keto reductase family	Redox reactions
contig29413	2.90	Aldo/keto reductase family	Redox reactions
contig47324	2.82	Taurine catabolism dioxygenase TauD, TfdA family	Redox reactions
contig51994	2.69	Aminotransferase class-III	Special, transaminase activity
contig05529	2.68	Zinc-binding dehydrogenase	Redox reactions
contig06697	2.66	Acyl-CoA dehydrogenase, middle domain	Redox reactions
Atam18919	2.55	Glycosyl transferase family 8	Transfer of glycosyl groups
contig08880	2.52	ABC-2 type transporter	Transport
Atam25893	2.49	ABC transporter	Transport
contig02047	2.47	SnoaL-like polyketide cyclase	Polyketide synthase
contig18051	2.22	ABC transporter transmembrane region	Transport
Atam05470	2.19	Cytochrome P450	Redox reactions
contig21528	2.17	Methyltransferase domain (2)	Methyl group transfer
contig14651	2.09	Beta-ketoacyl synthase, C- and N-terminal domain	Polyketide synthase
contig40268	2.09	Oxidoreductase family, C-terminal alpha/beta domain	Redox reactions
contig41426	1.82	Glycosyl transferase family 8	Transfer of glycosyl groups

The table includes fold-change (FC) values and the assigned Pfam families for each gene, as well as the attributed function of each annotatable gene.

the non-lytic *Alex5* (Table 1). By comparison, 13 genes coding for proteins involved in secondary metabolism were more highly expressed in *Alex5* than in *Alex2* (Table 2). However, none of these genes were differentially expressed in response to grazers (Supplementary File S1).

Cage experiment results

Exposure of both *Alexandrium* genotypes to *Polykrikos* grazing had different effects on conspecifics (Figures 2 and 3). When the mixture of *Polykrikos* and non-lytic *Alex5* cultures was surrounded by lytic *Alex2* cultures, the number of *Alexandrium* cells inside the cage

Table 2 Ten most highly overexpressed genes in *Alex5* control cultures (compared with *Alex2*) and overexpressed genes coding for proteins potentially involved in secondary metabolism

Contig	Mean FC value	Pfam family name	Assessed function
<i>Top 10 overexpressed genes in Alex5</i>			
contig52451	977.12	Fatty acid desaturase	Unsaturated fatty acid synthesis
contig10253	318.80	—	—
contig22596	205.62	—	—
contig22292	156.37	—	—
contig03283	143.74	—	—
contig14894	78.45	—	—
58180097	59.28	—	—
contig05597	51.09	EF-hand domain pair (2)	Calcium-ion binding
contig22152	45.17	—	—
Atam02515	42.39	—	—
<i>Prominent secondary metabolite protein coding genes overexpressed in Alex5</i>			
contig48765	10.39	Methyltransferase domain	Methyl group transfer
Atam13914	5.12	Methyltransferase domain	Methyl group transfer
Atam35211	4.65	EamA-like transporter family	Transport
Atam25791	3.31	Acetyltransferase (GNAT) family	Acetyl group transfer
Atam23762	2.94	Cytochrome P450	Redox reactions
contig33556	2.86	Aldo/keto reductase family	Redox reactions
contig34741	2.04	ABC-2 type transporter	Transport
contig01330	1.99	Taurine catabolism dioxygenase TauD, TfdA family	Redox reactions
Atam01694	1.88	NAD-dependent epimerase/dehydratase family	Redox reactions
contig06638	1.84	EamA-like transporter family	Transport
contig16321	1.80	ABC-2 type transporter	Transport
contig37655	1.79	Periplasmic binding protein	Transport
Atam12516	1.76	Acyltransferase family	Acyl group transfer

The table includes fold-change (FC) values and the assigned Pfam families for each gene, as well as the attributed function of each annotatable gene.

Population growth rates (% increase after 96 h)	<i>Alex2</i>	<i>Alex5</i>
Outside cage	156 ± 5	141 ± 26
Control	165 ± 10	144 ± 13
Inside cage	139 ± 38	14 ± 65
<i>P. kofoidii</i>	98 ± 111	268 ± 19

Figure 2 Growth rates for *Alexandrium fundyense* and *Polykrikos kofoidii* and schematic representation of the experimental set-up. The relative growth rates of *Alex2* and *Alex5* treatment versus control cultures, as well as for *P. kofoidii* cultures, are given as percentage cell population increases over 4 days. The experimental set-up shows the partition of *A. fundyense* and *P. kofoidii* cells in the cage and a surrounding compartment.

Figure 3 Cell concentration of *Alex5* and *Polykrikos kofoidii* inside the cage. Panel (a) shows the cell concentrations and standard deviations ($n = 3$) for *Alex5* and *P. kofoidii* when surrounded by *Alex2* cultures; panel (b) shows the cell numbers and s.d. ($n = 3$) for *Alex5* and *P. kofoidii* when surrounded by *Alex5* cultures.

increased significantly over time (Figures 2 and 3a; ANOVA, $P=0.015$). The *Polykrikos* cell concentration inside the cage in this treatment did not increase over time (ANOVA, $P=0.196$).

By contrast, exposure of the *Polykrikos/Alex5* mixture to non-lytic *Alexandrium* cultures resulted in a significant increase in *Polykrikos* cells (Figures 2 and 3b; ANOVA, $P<0.001$). The abundance of *Alexandrium* cells inside the cage did not change significantly over time (ANOVA, $P=0.793$).

Differentially expressed genes with respect to grazer presence

The number of upregulated genes increased by >9-fold for the lytic *Alex2* during the exposure period (Table 3). In the non-lytic *Alex5* cultures, the number of upregulated genes doubled over time (Table 3).

The genes that code for enriched Pfam family proteins were assigned to categories (A: secondary metabolic processes; B: cell cycle control and genetic information storage and processing; C: intracellular vesicle-associated processes; and D: signal transduction processes) and are summarized in Figure 4. Genes assigned to category B showed a similar increase over time in transcript abundance for both strains, but differed with respect to their Pfam family annotations (Figures 4 and 5). Categories A, C and D showed differences in frequency over time in both strains, for transcript numbers and assigned gene products (Figures 4 and 5).

Discussion

We observed that species interactions induce gene expression changes in a genotype-specific manner. These genotype-specific responses may be influenced by the differences in the allelochemical properties of both strains as only one is lytic. At least under the experimental conditions, this suggests that the lytic strain *Alex2* constructs an ecological niche that is distinct from that of the non-lytic strain *Alex5*. This suggests that genotype-specific interactions and associated trait variation within a population maintain disequilibrium among genotypes and can, therefore, among other mechanisms, explain the 'paradox of the plankton'. The presence of paralytic shellfish

poisoning toxins appears to have a negligible or neutral effect with respect to the species interactions investigated here (Senft-Batoh *et al.*, 2015).

Inherent genotype-specific transcription patterns

In our transcriptome analysis, ~5% of all tested genes differed between the two *Alexandrium* strains under control conditions and we detected unusually high fold-change values for dinoflagellates (Figure 1). Among treatments, we detected maximal gene expression differences of ~15-fold (*Alex2*) and ~77-fold (*Alex5*), and in the strain comparison between controls, we observed maximal differences of ~385-fold (*Alex2*) and ~977-fold (*Alex5*). Dinoflagellates have a tendency to expand particular genes via tandem DNA repeats, and high transcript abundances are correlated with the genomic abundance of such genes (Bachvaroff and Place, 2008). Further studies are needed to determine whether the observed differences in transcriptional activity are caused by genome expansions. If indeed this is the case, different traits could arise by the selective duplication of particular genes in dinoflagellates. mRNA recycling mechanisms proposed for dinoflagellates (Slamovits and Keeling, 2008) could contribute to the manifestation and diversification of traits via gene duplication.

The distinct genetic programs that are apparently active in the two strains may also be related to differences in cues originating from conspecific strains in the cage, that is, *Alex2* is surrounded by the conspecific *Alex5* and in the experimental set-up with *Alex5*, no other strain is present. Indeed, complex strain-specific interactions between co-cultured genotypes have been observed (Van Gremberghe *et al.*, 2009), and these interactions may be facilitated and/or initiated by differential gene expression. Both mechanisms (cues from conspecifics and potential strain-specific gene expansions in the genome) could have caused the observed differences in gene expression

Table 3 Gene expression analysis for the combined treatment with both *Alexandrium fundyense* strains

Genotype	24 vs 48 h	24 vs 72 h	24 vs 96 h	Common upregulated at all time points
Alex2	604	959	5724	244
Alex5	496	1177	909	61

The table displays the numbers of upregulated genes for comparisons at various time points after exposure for each *A. fundyense* strain to the cage containing interacting *P. kofoidii* cells and conspecific *A. fundyense*. The last column shows the number of genes that were upregulated at all points.

Figure 4 Differentially expressed genes and their time-dependent abundances for *Alex2* and *Alex5* in response to grazer presence. Genes presented here only exhibited changes in expression in the grazer treatment and not in the respective control. Selected differentially expressed genes are grouped into categories (a–d). The area of a circle represents the number of genes per category and per time point. (a) Secondary metabolic processes; (b) cell cycle control and genetic information storage and processing; (c) intracellular vesicle-associated processes; (d) signal processing and modification.

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exhibit changes in expression over time (Figure 4, category C). Changes in dissolved and particulate organic matter may have triggered the expression of these genes. The lytic effects of *Alex2* increase the levels of dissolved organic matter and particulate organic matter in the growth medium, which may be used as an additional nutritional source. The molecular genetic basis of dissolved organic matter/particulate organic matter uptake in marine protists has only been described in a metatranscriptomic study (Lin *et al.*, 2010; Lin, 2011). In our data set, we found several genes associated with vesicle transport processes that were significantly enriched in treatments where cells were lysed. Genes necessary for the formation of clathrin-coated vesicles were significantly enriched at every experimental time point for the lytic *Alex2* strain (Figure 5). Clathrin-coated vesicles sequester solutes or receptor-bound molecules from the surrounding medium and subsequently develop into lysosomes, which are digestive compartments with an acidic pH (Alberts *et al.*, 2008). Proton-translocating inorganic pyrophosphatases may have an important role in acidifying these digestive compartments. We found a significant enrichment and an increase over time in genes encoding such inorganic pyrophosphatases for the lytic *Alex2* (Figure 5). Inorganic pyrophosphatases are proton pumps distinct from F-, P- and V-ATPases that utilize inorganic pyrophosphate hydrolysis as a driving force for H⁺-ion movement across the membrane (Rea and Poole, 1993). In general, inorganic pyrophosphate is considered a waste product of anabolism (Pérez-Castiñeira *et al.*, 2001), and the use of inorganic pyrophosphate, rather than ATP, to acidify lysosomes would therefore be an efficient and energetically favorable recycling process in *Alexandrium* cells to sequester nutrients derived from endocytosis.

Prior investigations of endocytosis in marine heterotrophic protists revealed that protein kinases (Hartz *et al.*, 2008) and lectins (Wootton *et al.*, 2007) can be directly linked to phagocytosis. Genes that clustered into category D in *Alex2* included those for lectins (C-type and Ricin-B-type), which were significantly enriched in the expression analysis after 48 and 96 h (Figure 5). In addition to phagocytosis, lectins have an important role in cell–cell recognition as well as receptor-mediated and clathrin-dependent endocytosis (Roberts *et al.*, 2006; Varki *et al.*, 2009). In addition, *Alex2* exhibited a significant enrichment for genes associated with other adhesive (MAM) receptors after 48 and 96 h (Figure 5), and these loci may contribute to endocytotic processes. Genes for ion channels and protein kinases accounted for the majority of genes assigned to category D at 96 h in the lytic *Alex2*. Such an increase in the expression of ion channels can be directly connected to endocytosis, as a necessary mechanism to maintain intracellular ion homeostasis. Owing to the increased expression of genes for ion channels and protein kinases, we observed an

increased abundance of genes assigned to category D (72 h) for the non-lytic *Alex5* strain (Figure 5), although the enrichment of protein families involved in endocytotic processes was not as strong as it was for the lytic *Alex2* strain (Figure 4). *Alex5* cultures may, however, still utilize organic compounds excreted from the heterotrophic grazer.

In *Alex2*, we detected increased expression of motor proteins, kinesins, and, predominately, dyneins, including associated motor units (Figure 5). This pattern strongly indicates the establishment of intracellular trafficking routes and could be directly associated with vesicular transport and endosome and lysosome organization and maintenance. Furthermore, after 96 h, we observed increased expression of acidifying V-ATPases and inorganic pyrophosphatases. These observations provide evidence that endocytotic processes were strongly induced and maintained over time in the lytic *Alex2* strain (Figures 4 and 5).

Protein families that clustered into category D for *Alex2* and, to a lesser extent, for *Alex5* included several types of ligand-binding receptors (Figure 5). These proteins are associated with receptor-mediated endocytosis and glycoprotein and glycolipid modifications, which can alter the properties of outer glycosylated membrane surfaces. The biochemical compositional change of the glycosylated membrane surface determines important traits, such as self-recognition, non-self discrimination and ‘cell taste’ (Gahmberg, 1981; Wolfe, 2000), and thus could have a strong cascading effect on further interactions with conspecifics, competitors and/or predators.

Signal transduction processes

Significantly enriched protein families involved in signaling processes provide molecular genetic evidence for signal transduction via G-protein-coupled receptors, as proposed by Hartz *et al.* (2008). Cascades induced by ligand binding to G-protein-coupled receptors lead to increased levels of diacylglycerol (DAG), an important intracellular secondary messenger (Alberts *et al.*, 2008). Our data show that DAG kinase accessory domains are significantly enriched in both *Alexandrium* strains after 48 h and additionally after 72 h in the lytic *Alex2* strain (Figure 5). DAG kinase drives the conversion of DAG to phosphatidic acid (PA), which is involved in a wide range of biotic (for example, pathogen related) and abiotic stress responses in plants (Arisz *et al.*, 2009). In higher plants, PA has therefore emerged as an important lipid secondary messenger with direct effects on vesicular trafficking and membrane surface charge. Our transcriptome data suggest that similar mechanisms may operate in less well-studied marine protists. The involvement of PA signaling in biotic interactions between marine protists may therefore be an important link between the activation of G-protein-coupled receptors and subsequent

responses. Our expression data indicate that the conversion of DAG into PA takes place in both *Alexandrium* strains, underpinning the importance of G-protein-coupled receptors and DAG/PA signaling as an intracellular response inducer in biotic interactions.

Transcriptional regulation

The number of significantly enriched genes associated with protein families involved in information storage and processing and/or cell cycle control increased over time for both *Alexandrium* strains (Figure 4, category B). For *Alex2*, this category primarily comprised protein families that act on several levels of transcriptional and translational control. The increased expression after 96 h was predominantly explained by an increase in RNA-binding proteins, which act on post-transcriptional regulation of gene expression. In addition, several genes linked to protein families with transcription factor properties were enriched. We observed a co-occurring expression pattern at all time points in *Alex2*, and at 96 h in the *Alex5* treatment in which there was an upregulation of genes associated with protein families involved in DNA m⁶adenine methylation and the primary translation initiation factor If4e, which binds the cap structure of mRNAs (Figure 5). M⁶adenine methylation is an epigenetic regulation process in prokaryotes and lower eukaryotes that differs from cytosine methylation because it activates transcription, whereas cytosine methylation suppresses transcription (Hattman, 2005). Given the low abundance of histones in the dinoflagellate genome (Lin, 2011) and their importance in transcriptional control by opening the chromatin structure (Zhang and Reinberg, 2001), epigenetic processes like m⁶adenine methylation may be a convergent mechanism (with respect to histone acetylation) in dinoflagellates and could potentially have profound effects on the observed changes in transcriptional regulation in response to species interactions.

Cell division and growth-related processes

Protein families clustered in category B for the non-lytic *Alex5* indicated an influence on cell cycle progression (Figures 4 and 5). Protein families involved in cell cycle control were significantly enriched, followed by an upregulation of ribosomal protein (*RP*) genes after 96 h (Figure 5). The synthesis of ribosomal proteins in lower eukaryotes, such as yeasts, is often linked to nutrient availability and stress-related signals (Powers *et al.*, 2004). Studies in yeast reveal that the *RP*-gene regulon is activated via the protein kinase A pathway (Jorgensen *et al.*, 2004), which regulates a range of growth-related processes. Our transcriptomic enrichment data provide evidence that a protein kinase A-activated transcription regulator (AKAP7_NLS) is expressed after 48 h, concurrent with the expression of proliferation cell nuclear antigen (*PCNA*) and a cyclin domain-containing gene (Figure 5). This pattern is

followed by the expression of additional genes encoding proliferation cell nuclear antigens after 72 h, along with several genes that encode DNA replication, modification and repair proteins. Finally, after 96 h, the transcriptional accumulation of *RP*-genes was apparent (Figure 5). *RP*-gene expression also increased in the first 48 h in the lytic strain *Alex2*, followed by an enhanced expression of genes coding for proteins involved in transcriptional and translational control (Figure 5). Investment in new ribosomes provides a platform for faster growth, if all other essential components are available (Warner, 1999). Protein kinase A-mediated transcriptional regulation along with *RP*-gene transcription and cell cycle gene expression exemplifies how cellular growth may be internally regulated on a molecular basis in *Alexandrium*. However, external cues derived from biotic interactions also have the potential to restructure the internal growth rhythm and cellular processes. This influence of chemical cues derived from species interactions on phytoplankton growth was recently shown for a diatom by Amin *et al.* (2015).

Conclusions

Our comparative transcriptional analysis indicates that a heterotrophic protistan grazer induced changes in several traits in the here investigated *Alexandrium* strains that differ in their capacity to facilitate the lysis of potential grazers or competitors. This study thus provides new insight into chemically mediated processes involved in plankton species interactions and their regulation. The increased expression of genes involved in endocytotic processes, particularly in the lytic *Alex2*, suggests that this strain preferably switches to organic matter as an alternative food source, even under favorable laboratory conditions where ample inorganic nutrients and light are available. The production of lytic compounds thus not only provides a competitive advantage via grazing protection, but it may also have a positive feedback effect by providing a selective advantage to out-compete other prey organisms under grazing pressure via increased uptake of organic matter.

Based on the gene expression data, additional traits that changed in a strain-specific manner in response to species interactions included properties of the outer glycosylated membrane, which could influence further interactions. Such strain-specific trait alterations indicate a complex interplay within community ecological processes. Thus, depending upon 'who interacts with whom and when,' there may be short-term differences in the outcome of species interactions, which set the stage for the survival of dominant genotypes in seasonal succession processes. Future investigations should therefore account for how specific genotypes alter the community structure and also determine the impact of phenotypic diversity of multiple genotypes on a given community composition.

Conflict of Interest

The authors declare no conflict of interest.

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