



Transcriptomic Response to Feeding and Starvation in a Herbivorous Dinoflagellate

Ewelina T. Rubin*, Shu Cheng†, Amanda L. Montalbano, Susanne Menden-Deuer and Tatiana A. Rynearson

Graduate School of Oceanography, The University of Rhode Island, Kingston, RI, United States

OPEN ACCESS

Edited by:

Alison Buchan,
The University of Tennessee,
Knoxville, United States

Reviewed by:

Javier del Campo,
University of Miami, United States
Yong Jiang,
Ocean University of China, China

*Correspondence:

Ewelina T. Rubin
ewelina_rubin@uri.edu

† Present address:

Shu Cheng,
University of Arizona Cancer Center,
University of Arizona, Tucson, AZ,
United States

Specialty section:

This article was submitted to
Aquatic Microbiology,
a section of the journal
Frontiers in Marine Science

Received: 29 January 2019

Accepted: 24 April 2019

Published: 14 May 2019

Citation:

Rubin ET, Cheng S,
Montalbano AL, Menden-Deuer S
and Rynearson TA (2019)
Transcriptomic Response to Feeding
and Starvation in a Herbivorous
Dinoflagellate. *Front. Mar. Sci.* 6:246.
doi: 10.3389/fmars.2019.00246

Grazing by heterotrophic protists influences plankton population dynamics, community composition, and the flux of carbon through marine planktonic food webs. To gain insight into the molecular underpinnings of grazing in dinoflagellates, a group of important heterotrophic protists, we used a RNA-Seq approach to investigate the transcriptomic response of *Oxyrrhis marina* under fed and starved conditions with three different phytoplankton prey (*Isochrysis galbana* and two strains of *Heterosigma akashiwo*). In response to fed and starved conditions, 1,576 transcripts were significantly differentially expressed in *O. marina*. Fed *O. marina* cells upregulated transcripts involved in the synthesis of essential fatty acids and storage carbohydrates suggesting that the predator was food satiated and excess glucose was being stored as an energy reserve. Transcripts encoding voltage-gated ion channels were also upregulated during grazing, and they are known to be involved in the detection of mechanical stimuli and the regulation of swimming behavior in several eukaryotic protists. Fed *O. marina* cells upregulated kinases, which can dictate cell shape changes and may be associated with phagocytosis. During starvation, upregulated *O. marina* transcripts included those involved in the degradation of energy-storage molecules like glucan 1,4- α -glycosidase and those involved in antioxidant activities and autophagy, like acid ceramidase that are associated with the digestion of polar lipids present in cell membranes. Starved *O. marina* also upregulated transcripts with high similarity to proton pumping proteorhodopsins suggesting that this heterotrophic protist may supplement its energy requirement during starvation with a light harvesting mechanism. Although herbivorous grazing is a pivotal transformation in the C cycle, logistical constraints limit our investigations of environmental and biological drivers. The molecular signals identified here provide new insights into the metabolic regulation of feeding and starvation in marine heterotrophic protists and can fuel hypothesis-driven research into predators' metabolic response to prey availability.

Keywords: microzooplankton, grazing, RNA-Seq, differential expression, starvation, transcriptome, NASA EXPOTS

INTRODUCTION

Grazing by unicellular eukaryotic herbivorous marine plankton (i.e., microzooplankton, heterotrophic protists) on phytoplankton constitutes the single largest loss process of primary production in the marine environment and is important in mediating plankton population dynamics and community composition, and ultimately the flow of energy and material throughout marine planktonic food webs (Tillmann, 2004; Schmoker et al., 2013; Weisse et al., 2016). Heterotrophic protists are the main consumers of marine phytoplankton biomass, consuming on average over 60% of daily phytoplankton production (Calbet and Landry, 2004). Moreover, these herbivores are important prey for larger zooplankton and thus serve as important trophic links in marine food webs (Calbet and Saiz, 2005; Campbell et al., 2009; Löder et al., 2011). The population dynamics of marine plankton are disproportionately affected by heterotrophic protists, because these predators have growth rates that can equal or exceed the growth rates of their phytoplankton prey (Hansen et al., 1997). As a result, heterotrophic protists can influence biogeochemically and ecologically significant events, such as slowing or preventing the formation of phytoplankton blooms (Sherr and Sherr, 2009) including blooms formed by harmful and toxic phytoplankton (Jeong et al., 2003; Harvey and Menden-Deuer, 2012). Despite the key importance of heterotrophic protists in marine food webs and global biogeochemical processes, considerable knowledge gaps persist in understanding the metabolic processes involved in protistan herbivory.

The feeding process of heterotrophic protists involves physiologically and metabolically complex mechanisms, including chemical and mechanical perception involved in prey selection and capture, as well as ingestion and digestion (Montagnes et al., 2008). Chemical recognition and prey selectivity are important steps in the feeding process (Roberts et al., 2011) and some of the key receptors involved in that process have been identified (Roberts et al., 2006; Hartz et al., 2008). In addition, light is thought to be involved in maintaining circadian cycles of the feeding process (Jakobsen and Strom, 2004) and enhance digestion rates (Strom, 2002). Some heterotrophic species have also been shown to have unique starvation capacities, with dinoflagellates able to survive starvation for weeks at a time (Strom, 2002; Menden-Deuer et al., 2005; Calbet et al., 2013; Anderson and Menden-Deuer, 2017).

The metabolic responses of heterotrophic protists to food availability are relatively unknown and most molecular studies have focused on examining gene content. For example, transcriptomes of the heterotrophic dinoflagellate *Oxyrrhis marina* revealed extensive gene redundancy, evidence of lateral gene transfer, and functional gene annotations similar to eukaryotic protists, including other dinoflagellates (Lowe et al., 2011; Lee et al., 2014). Genes potentially involved in predation, such as phagotrophy, were not identified likely because the predators were maintained on dissolved organic matter instead of prey cells. Interestingly, a comparison of gene content in a mixotrophic alga and a heterotroph (both ciliates) did not reveal significant differences in gene content in relation to trophic mode

(Santoferrara et al., 2014). One study that examined differentially expressed genes and thus potential metabolic differences in feeding status focused on a mixotrophic alga either feeding on prey or photosynthesizing (Liu et al., 2015). Differentially expressed genes in that study were associated primarily with prey-derived nutrient uptake, such as iron from bacterial prey and organic nitrogen from ciliate prey.

Here we used a metatranscriptome approach (simultaneous sequencing of predator and prey transcriptomes) to examine the metabolic responses of a heterotrophic protist to feeding and starvation treatments. We conducted a set of feeding and starvation experiments followed by transcriptome analyses of metabolic changes in a heterotrophic protist fed different phytoplankton species and strains followed by starvation. We selected the marine heterotrophic dinoflagellate *O. marina* because it has a wide geographic distribution, consumes a broad range of prey taxa, and has been previously used as a model organism for studying feeding activities in heterotrophic protists (Montagnes et al., 2011; Roberts et al., 2011; Yang et al., 2011; Guo et al., 2013). Two prey species were selected in this study: the raphidophyte *Heterosigma akashiwo* and the prymnesiophyte *Isochrysis galbana*, to find common metabolic responses to grazing regardless of prey type. The metabolic response of the predator to different phytoplankton species, strains, and concentrations was evaluated using transcriptome-wide profiles for fed and starved *O. marina* cells. This approach revealed several metabolic pathways and cellular processes that were affected by prey availability, including signal transduction, biosynthesis of energy reserves, and self-digestion or autophagy during food scarcity. To our knowledge, the present study is a first to investigate the transcriptomic response of a herbivorous predator during phagotrophy on phytoplankton prey.

MATERIALS AND METHODS

Plankton Cultures and Feeding Experiments

The heterotrophic dinoflagellate *O. marina* was grown in 0.2 μm sterile-filtered autoclaved seawater (FSW), and three phytoplankton prey types were grown in FSW enriched with F/2 nutrients without silicate (Guillard and Ryther, 1962). All cultures were kept at 15°C on a 12:12 h light:dark cycle. All plankton strains were deposited to the National Center for Marine Algae, formerly the Culture Collection of Marine Phytoplankton (CCMP) and include two strains of the raphidophyte: *H. akashiwo* 1 (CCMP3107) and *H. akashiwo* 2 (CCMP3374), and a prymnesiophyte *I. galbana* (CCMP1323). All cultures were xenic and grown exponentially before the start of the experiments.

Four experiments of *O. marina* cultures were established in triplicate 1 L polycarbonate bottles. There were three feeding treatments, one per phytoplankton prey type, and a fourth treatment representing a starved condition. The feeding treatments were established by adding between 500 and 600 ml of the predator culture so that the starting concentration of

the predator was: 300, 800, and 1,000 cells ml⁻¹ in the feeding treatments containing *H. akashiwo* 1, *H. akashiwo* 2, and *I. galbana*, respectively. Between 400 and 500 ml of prey was added to the same culture bottles. Aiming for saturating prey concentrations, prey types were added at different concentrations because of their cell size. The starting concentration of the larger prey species was 13,000 cells ml⁻¹ (both strains of *H. akashiwo*) and 200,000 cells ml⁻¹ for the smaller *I. galbana*. The starved treatment of *O. marina* was achieved by keeping the predator without prey for 4 days after an initial 3 day feeding period with the *I. galbana* prey. Similar to the *I. galbana* feeding treatment, the starved treatment was initiated with the addition of *O. marina* at 1,000 cells ml⁻¹ and *I. galbana* at 200,000 cells ml⁻¹ and incubated for 7 days total. Cell concentrations in all experimental bottles (for both the predator and prey) were determined at the beginning and end of the experiment by fixing 1 ml aliquots of each treatment bottle with 2% acid Lugol's solution (Menden-Deuer et al., 2001). Cell concentrations were determined using a Sedgewick-Rafter counting cell and an Eclipse E800 microscope (Nikon).

After 3 days (for the feeding treatments) and 7 days (for the starved treatment), all cells (predator and prey) were harvested by vacuum filtration on 1-μm pore size filters (GE Water and Process Technologies). The same total number of cells (different volumes depending on the concentration) was harvested from each replicate, ensuring that each replicate was equally represented in the sequencing sample. There were between 3.9 and 6.6 million *O. marina* cells per filter depending on the treatment (Table 1). Biomass on filters was flash-frozen in liquid nitrogen and stored at -80°C until further processing.

In addition to the feeding and starved treatments, all prey types were cultivated individually without the predator. This was done so that the individual prey transcriptomes could be subtracted from the mixed predator-prey transcriptomes generated from the feeding and starved treatments. To generate robust assemblies of prey transcriptomes, all prey items were grown individually, in triplicate, at different light and salinity conditions. Cells were harvested at the mid-point of exponential growth and stored as described above (Tables 1, 2), but the actual transcriptomic response of the prey to these treatments was not a subject of this study and was not used for differential expression analysis.

RNA Extraction and Transcriptome Sequencing

Triplicates of each experimental treatment were pooled into one sample at the time of cell harvest. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, United States) according to the manufacturer's protocol, with the following exceptions: cells were lysed using a bead-beater homogenizer in a solution of lysis buffer and 0.5 mm zirconia/silica beads (BioSpec, United States) until the solution looked homogenous (~1 min). Next, QIAshredder columns (Qiagen, United States) were used to remove cell debris. To remove DNA, the RNase-free DNase set (Qiagen, United States) was followed by an addition DNA-digestion using the Turbo DNA-free kit (Ambion, United States),

both according to the manufacturer's instructions. The RNA was quantified in duplicate using the Qubit Fluorometer (Invitrogen, United States). All transcriptomes (Table 1) were sequenced as part of the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) using methods described in Keeling et al. (2014).

Bioinformatics Analyses

The transcriptome assemblies for the three species and strains used in the experiments were obtained from <https://imicrobe.us/> (Keeling et al., 2014; Table 2). Since *O. marina* treatments also contained prey when harvested, the joined assembly of all *O. marina* treatments (hereinafter referred to as the metatranscriptome) contained transcripts from both predator and prey.

To find and remove prey transcripts, 53,041 contigs from the metatranscriptome were subjected to blastn homology searches (cutoff of the expected value or the *E*-value = 10⁻⁵, Altschul et al., 1990) against the contigs from the prey-only assemblies (*H. akashiwo* 1 assembly, *H. akashiwo* 2 assembly, *I. galbana* assembly, Table 2). Metatranscriptome contigs matching any of the prey contigs at the percent nucleotide identity ≥79.5% were considered to represent prey contigs and removed from the metatranscriptome, thus leaving behind only *O. marina* contigs. To determine the 79.5% nucleotide identity threshold for prey removal, highly conserved contigs from the metatranscriptome annotated as actins (23 contigs), alpha and beta-tubulins (17 contigs), elongation factors (20 contigs), and 40S ribosomal proteins (84 contigs) were subjected to a blastn search against our prey-only assemblies and the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database. We found that a subset of the selected, highly conserved contigs returned blast hits with nucleotide identities between 79.5 and 100% to our prey species or to species closely related to our prey (e.g., other haptophytes *Emiliania huxleyi* and *Prymnesium parvum*). A second subset matched the contigs in our prey-only assemblies with nucleotide identities below 79.5% and at the same time returned NCBI blast hits to *O. marina* or species closely related to *O. marina* (e.g., *Perkinsus marinus*, *Symbiodinium microadriaticum*, and other dinoflagellates). Because the selected metatranscriptome contigs represented well-known protein coding sequences (such actin and tubulin genes) that are conserved across phyla, the threshold of 79.5% was assumed to be applicable to the remainder of the metatranscriptome contigs with less conserved nucleotide identity. After the removal of the prey associated contigs, the assembled, prey-free *O. marina* transcriptome was used in further analyse including mapping and gene expression estimates.

Raw reads for the experimental samples (*O. marina* – three feeding treatments and one starvation treatment) were downloaded from the NCBI's Short Read Archive (accession number listed in Table 1) and quality trimming and filtration was accomplished using Trimmomatic using a sliding window threshold of quality score equal to 20 for every three nucleotides (Bolger et al., 2014). The high-quality reads were mapped to the assembled, prey-free *O. marina* transcriptome using Bowtie (Langmead et al., 2009) and transcript quantification

TABLE 1 | Experimental summary including strain identification, experimental conditions, number of cells filtered, transcriptome identification numbers for both the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) and the National Center for Biotechnology Information (NCBI) sequence read archive and the number of paired reads in each transcriptome.

Species/strain	Experimental conditions (salinity in PSU)	Number of cells filtered (predator; prey)	MMETSP number	NCBI sample-run accession number	Number of paired reads (2 * 50 bp)
<i>Oxyrrhis marina</i> CCMP3375	Fed on <i>H. akashiwo</i> 1	4.1×10^6 ; 3.0×10^5	468	SRR1296900	25,389,999
	Fed on <i>H. akashiwo</i> 2	3.9×10^6 ; 9.1×10^5	469	SRR1296901	31,049,832
	Fed on <i>I. galbana</i>	6.6×10^6 ; ND	470	SRR1296903	28,638,609
	Starved after being fed on <i>I. galbana</i>	3.6×10^6 ; ND	471	SRR1296907	37,949,715
<i>Heterosigma akashiwo</i> 1 CCMP3107	Salinity 29, sampled in light	none : 2.9×10^6	409	SRR1296775	13,864,482
	Salinity 15, sampled in light	none : 2.9×10^6	410	SRR1296776	19,140,554
	Salinity 29, sampled in dark	none : 2.9×10^6	411	SRR1296777	16,176,764
<i>Heterosigma akashiwo</i> 2 CCMP3374	Salinity 29, sampled in light	none : 2.9×10^6	414	SRR1296797	17,370,776
	Salinity 15, sampled in light	none : 2.9×10^6	415	SRR1296798	17,703,099
	Salinity 29, sampled in dark	none : 2.9×10^6	416	SRR1296799	40,620,574
<i>Isochrysis galbana</i> CCMP1323	Salinity 29	none : 1.6×10^7	595	SRR1296904	20,748,923
	Salinity 33	NA	943	SRR1300331	25,600,062
	Salinity 33	NA	944	SRR1300332	22,767,092

ND, not detectable; NA, not available.

TABLE 2 | Transcriptome assembly information.

Assembly name	Assembled MMETSP sample IDs	Number of contigs	N50*	B1000** (%)	B2000** (%)
Metatranscriptome	468,469,470,471	53,041	1,500	69.6	33.0
<i>H. akashiwo</i> 1	409,410,411	27,342	1,112	55.3	20.4
<i>H. akashiwo</i> 2	414,415,416	50,467	1,204	58.1	25.0
<i>I. galbana</i>	595,943,944	45,931	1,847	80.1	45.3
<i>O. marina</i> fed on <i>H. akashiwo</i> 1	468	31,235	1,353	65.9	24.9
<i>O. marina</i> fed on <i>H. akashiwo</i> 2	469	51,092	1,430	67.6	30.2
<i>O. marina</i> fed on <i>I. galbana</i>	470	32,594	1,360	66.1	25.9
<i>O. marina</i> starved	471	30,682	1,395	68.0	27.2

Assemblies were generated by the National Center for Genome Research as a part of the MMETSP (Keeling et al., 2014) and were downloaded from <https://imicrobe.us/>.

*N50 – the contig length above which 50% of bases are part of the assembly. **B1000 and B2000 indicate the percentage of bases involved in contigs with at least 1000 and 2000 bp in length, respectively.

was determined using RSEM (Li and Dewey, 2011). Differential expression (DE) was determined using the Analysis of Sequence Counts tool (ASC, Wu et al., 2010) using transcripts per million (TPM) counts which were generated by the RSEM tool. The ASC tool is a Bayesian statistical approach that was specifically developed for pooled biological replicates and RNA-Seq statistical analysis without replication (Wu et al., 2010). Functional annotation of *O. marina* differentially expressed transcripts was accomplished by blastx searches (Altschul et al., 1990) against NCBI's non-redundant protein database (*E*-value cut off 10^{-5}), followed by the import of blastx results into the Blast2GO software (Conesa et al., 2005) to obtain Gene Ontology (GO) (Ashburner et al., 2000) annotations, InterPro conserved protein domains (Zdobnov and Apweiler, 2001), and KEGG (Kyoto Encyclopedia of Genes and Genomes) enzyme searches (Kanehisa et al., 2016). GO enrichment analysis was carried out using the agriGO web-based tool (Du et al., 2010; Tian et al., 2017).

RESULTS

Growth Response of *O. marina* to Food Availability

Oxyrrhis marina grazed all three phytoplankton prey types and exhibited positive growth, reaching between $4.0\text{--}4.7 \times 10^3$ cells ml^{-1} at the time of harvest on day 3 (Figure 1A). Specific growth rates for *O. marina* feeding on *I. galbana* were $0.69 \pm 0.05 \text{ d}^{-1}$ and feeding on *H. akashiwo* 1 and *H. akashiwo* 2, $1.27 \pm 0.02 \text{ d}^{-1}$ and $0.74 \pm 0.01 \text{ d}^{-1}$, respectively. *O. marina* in the starved treatment had an average specific growth rate over the 7-day incubation period of $0.26 \pm 0.02 \text{ d}^{-1}$, due to an initial rapid increase in cell numbers over the first 3 days followed by 4 days of little to no growth due to starvation. In the grazing treatments, evidence of active grazing was observed in the rapid decrease of prey from between 1.28×10^4 and 2×10^5 cells ml^{-1} at the start of the experiment, to below 10^3 cells ml^{-1} after 3 days

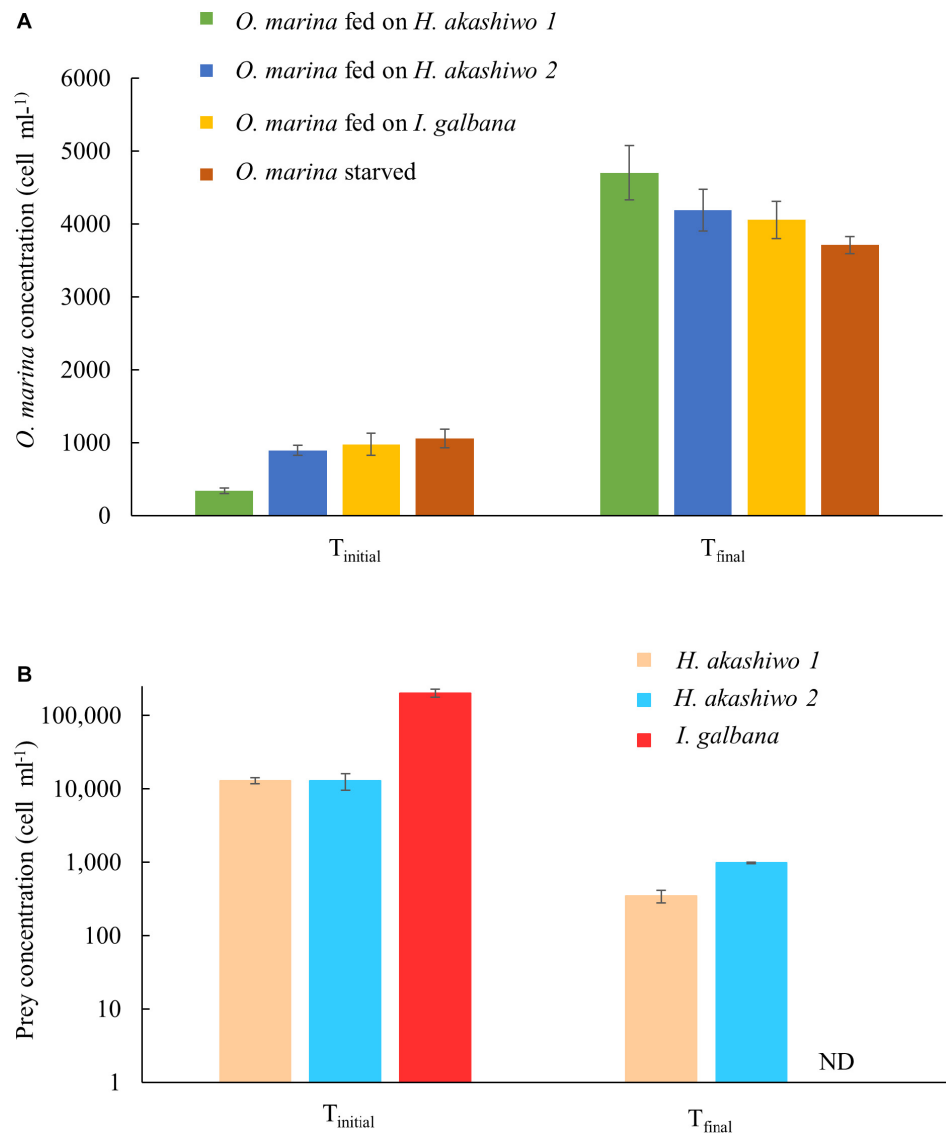


FIGURE 1 | Predator and prey cell concentrations at the start (T_{initial}) and end (T_{final}) of the experiment for the predator species *O. marina* (A) and the phytoplankton prey species: *H. akashiwo* strain 1, *H. akashiwo* strain 2, and *I. galbana* (B). Cell harvests at T_{final} occurred on day 3 for the fed treatments and day 7 for the starved treatment. ND indicates cell concentration below detection for *I. galbana* ($<1 \text{ cell ml}^{-1}$).

of grazing (Figure 1B). The abundance of *I. galbana* in the fed treatment was very low on day 3 and was not detectable using our counting methods; the concentration was conservatively estimated at $<1 \text{ cell ml}^{-1}$.

Removal of Prey Transcripts From the Metatranscriptome

A combined assembly was generated using all *O. marina* treatments (metatranscriptome, Table 2). This combined assembly was compared with the prey-only transcriptome assemblies (Table 2) using BLAST, percent identity, and hit coverage, yielding 7,488 transcripts (14% of the assembly) which were identified as homologous to sequences in the

prey transcripts (Figure 2). The numbers of transcripts in the combined assembly matching prey transcripts were 5,260, 5,398, and 2,503 for *H. akashiwo* 1, *H. akashiwo* 2, and *I. galbana*, respectively. It should be noted that $>80\%$ of those prey-transcript matches showed nucleotide sequence identity $>95\%$ thus almost certainly belonging to the prey. More transcripts of *H. akashiwo* than *I. galbana* were found in the combined assembly (Figure 2), likely because *O. marina* grazed *I. galbana* more effectively and very few *I. galbana* cells remained in the harvested biomass used for the sequencing of those samples (Figure 1 and Table 1). The remaining 86% of transcripts in the combined assembly (metatranscriptome) were identified as belonging to *O. marina* (Figure 2). The prey-free transcripts were used for mapping and gene expression analyses.

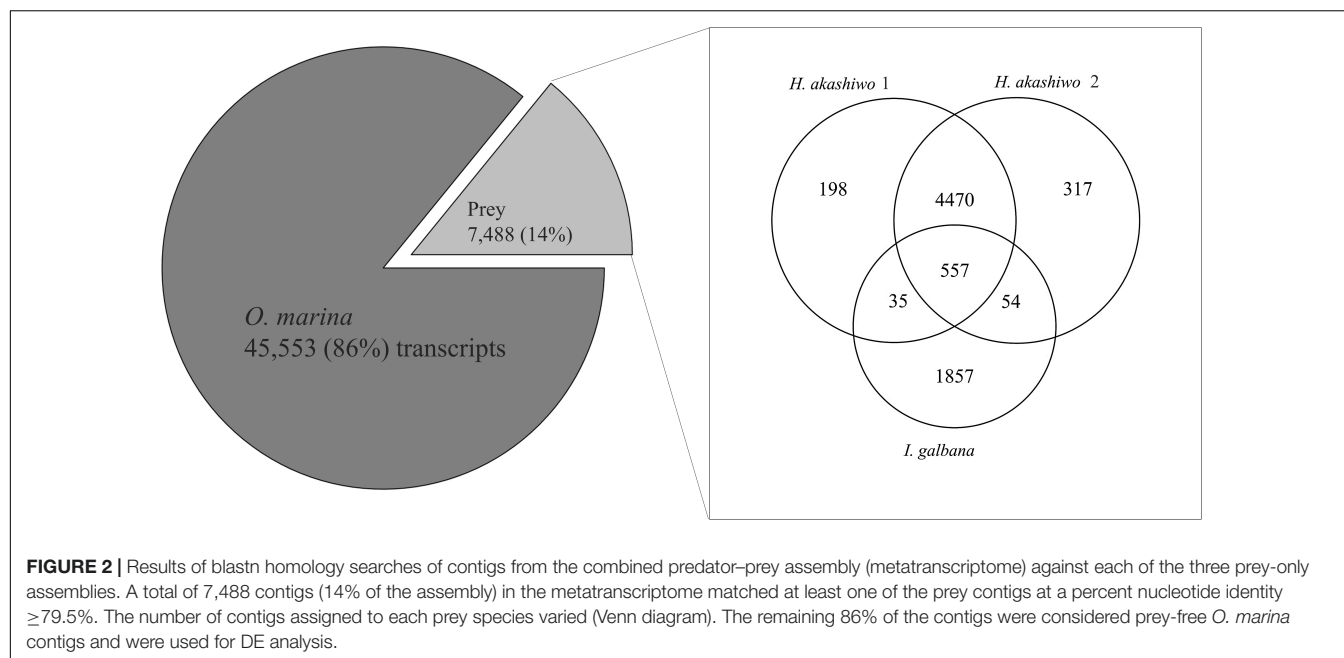


TABLE 3 | The results of quality filtration and mapping of sequence reads to *O. marina* transcripts.

Sample	MMETSP number	Number of paired reads (2 * 50 bp)	Number of paired reads after quality filtration	Number of paired reads mapped to the <i>O. marina</i> transcriptome			
				Mapped		Unmapped	
<i>O. marina</i> fed on <i>H. akashiwo</i> 1	468	25,389,999	24,299,926	20,934,193	86%	3,365,733	14%
<i>O. marina</i> fed on <i>H. akashiwo</i> 2	469	31,049,832	29,842,133	23,901,292	80%	5,940,841	20%
<i>O. marina</i> fed on <i>I. galbana</i>	470	28,638,609	27,603,463	23,420,022	85%	4,183,441	15%
<i>O. marina</i> starved	471	37,949,715	36,430,302	31,831,996	87%	4,598,306	13%

Differentially Expressed (DE) Transcripts in Response to Feeding and Starvation Conditions

Between 80 and 87% of quality-filtered reads in the *O. marina* single prey assemblies mapped to *O. marina* contigs identified in the combined assembly (Table 3). The abundance of *O. marina* transcripts under the starved treatment was compared to each of the three fed treatments representing the three phytoplankton prey types. Overall, 1,576 differentially expressed (DE) transcripts were identified, including 972 with higher expression in the fed *O. marina* cells and 604 with higher expression in the starved cells (Table 4). The 972 transcripts that were upregulated in fed *O. marina* cells included 39 transcripts that were upregulated in all fed treatments, regardless of prey type (Figure 3A and Supplementary File S1 – Grazing genes). Of those, 20 could be functionally annotated and included genes putatively identified as glycogen synthase, sugar epimerase, phosphoglucosyltransferase, actin, three kinases, acyl-CoA dehydrogenase, kinesin, and two transcription factors (for a complete list see Supplementary File S1 – Grazing genes).

An additional 203 transcripts were upregulated when *O. marina* was fed *H. akashiwo* regardless of the strain

(Figure 3A). When *O. marina* was feeding on *H. akashiwo* 1 and 2, 499 and 153 transcripts, respectively, were uniquely upregulated. In addition, there were 42 uniquely upregulated transcripts in *O. marina* cells when it was feeding on *I. galbana*.

When *O. marina* was starved, 604 transcripts were upregulated, including 64 transcripts that were upregulated regardless of the prey treatment selected as the control comparison (Figure 3B, complete list in Supplementary File S2). Additional starvation-induced transcripts were identified when comparing starved *O. marina* cells to those fed on *H. akashiwo* 1 (271 transcripts), *H. akashiwo* 2 (325 transcripts), and *I. galbana* (62 transcripts) (Figure 3B). A GO term, Interpro protein domain, or KEGG enzyme could be assigned to 311 transcripts in the fed treatments (32%, Supplementary File S1) and 286 transcripts in the starved treatments (52%, Supplementary File S2).

Transcripts in *O. marina* cells with a significantly higher expression when the predator was feeding included those encoding enzymes involved in carbohydrate and fatty acid synthesis, metabolism, and transport (Figure 4). In addition, fed *O. marina* cells had higher expression of transcripts encoding several ion channels responsible for the detection of mechanical stimuli, and multiple proteins involved in motility, cytoskeletal

TABLE 4 | Number of significantly differentially expressed transcripts (posterior probability ≥ 0.950 and fold change ≥ 2) in *O. marina* cells fed with three different prey types (two strains of *H. akashiwo*: *H. akashiwo* 1, *H. akashiwo* 2, and *I. galbana*) in comparison to *O. marina* cells kept in starvation for 4 days after prey depletion.

	Number of transcripts with significantly higher abundance during	
	Feeding	Starvation
<i>O. marina</i> fed <i>H. akashiwo</i> 1 vs. <i>O. marina</i> starved	775	335
<i>O. marina</i> fed on <i>H. akashiwo</i> 2 vs. <i>O. marina</i> starved	397	389
<i>O. marina</i> fed on <i>I. galbana</i> vs. <i>O. marina</i> starved	117	126
Total unique	972	604

organization, cell shape, and cell division (Figure 4). Transcripts in *O. marina* cells with a significantly higher expression when the predator was starving included upregulation of light harvesting proteins, enzymes involved in degradation of storage molecules, and enzymes involved in autophagy and cell redox homeostasis and the stress response (Figure 5).

Examination of the most frequent protein domains (InterPro domains, Table 5) identified among DE transcripts highlighted the importance of certain enzymes and transporters in the feeding and starvation processes. Many more kinase protein domains (IPR011009, IPR000719, IPR008271) were identified among DE transcripts from the fed cells than among DE transcripts from the starved cells (Table 5). On the other hand, the rhodopsin protein domains (IPR001425), pepsinogens also known as aspartyl peptidase domains (IPR001461), and cathepsins (IPR000668) were identified among DE transcripts from the starved cells but not among DE transcripts from the fed cells (Table 5). Similarly, a

majority of the ion transporter domains (IPR005821, IPR013122, IPR004481, IPR004837) were identified among DE transcripts from the fed cells but not among DE transcripts from the starved cells (Table 5).

The results of GO enrichment analysis revealed that 31 GO terms related to carbohydrate metabolism were overrepresented in the fed *O. marina* cells. These included GO terms such as glycolysis, gluconeogenesis, monosaccharide catabolic process, hexose catabolic processes, and several other functionally related terms as well as terms describing voltage-ion channels including “divalent metal ion transport” (complete list in Table 6). The GO terms that were enriched in the starved *O. marina* cells included “peptidase activity” and “oxidoreductase activity” which are terms related to enzymes involved in autophagy and oxidative stress (GO:0008233, GO:0016491 in Table 6).

The examination of predicted KEGG enzymes among the DE transcripts revealed specific enzymes involved in the metabolic processes mentioned above (Figure 6 and Supplementary Files S1 and S2). DE enzymes involved in lipid, carbohydrate, amino acid, and energy metabolism, and other enzymes were identified. Overall, a higher number of DE transcripts involved in lipid and energy metabolism (such as carbon fixation) were identified under starvation, whereas during feeding, a higher number of DE transcripts involved in carbohydrate metabolism were identified (Figure 6). In addition, a higher number of kinases were differentially expressed during feeding, but a higher number of peptidases were differentially expressed during starvation (Figure 6).

Metabolic pathways for carbohydrate and lipid synthesis were examined in detail. KEGG enzymes involved in carbohydrate biosynthesis were mostly upregulated in feeding *O. marina* cells (Figure 7). Several enzymes of the gluconeogenesis pathway including three different fructose-bisphosphate aldolases (EC:4.1.2.13), 6-phosphofructokinase (EC:2.7.1.11), phosphohexose isomerase (EC:5.3.1.9), and

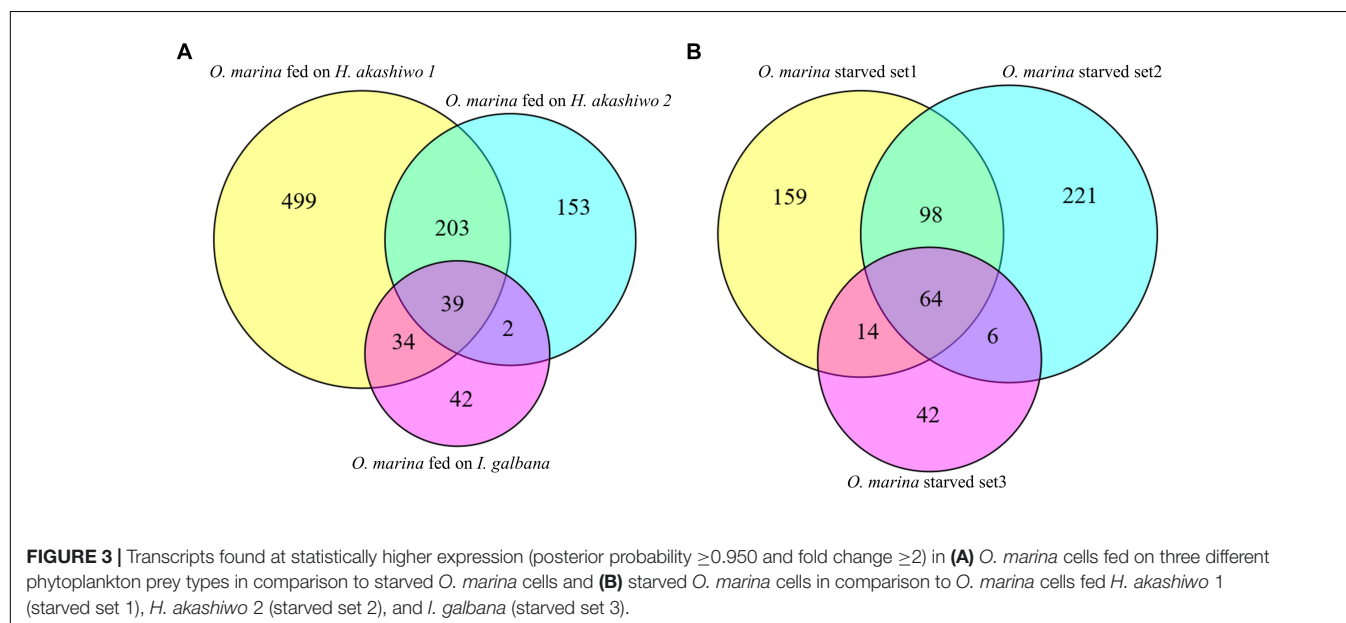




FIGURE 4 | A heat map representing 73 transcripts that were up-regulated in *O. marina* cells fed on *H. akashiwo* 1 (Ha1), *H. akashiwo* 2 (Ha2), and *I. galbana* (Ig) compared to starved *O. marina* cells. Numbers inside boxes indicate fold change with each prey treatment and the number of asterisks next to each transcript indicates the number of prey that were significantly differentially expressed. Names, contig IDs, and annotations of the transcripts can be found in **Supplementary Files S1, S2**.

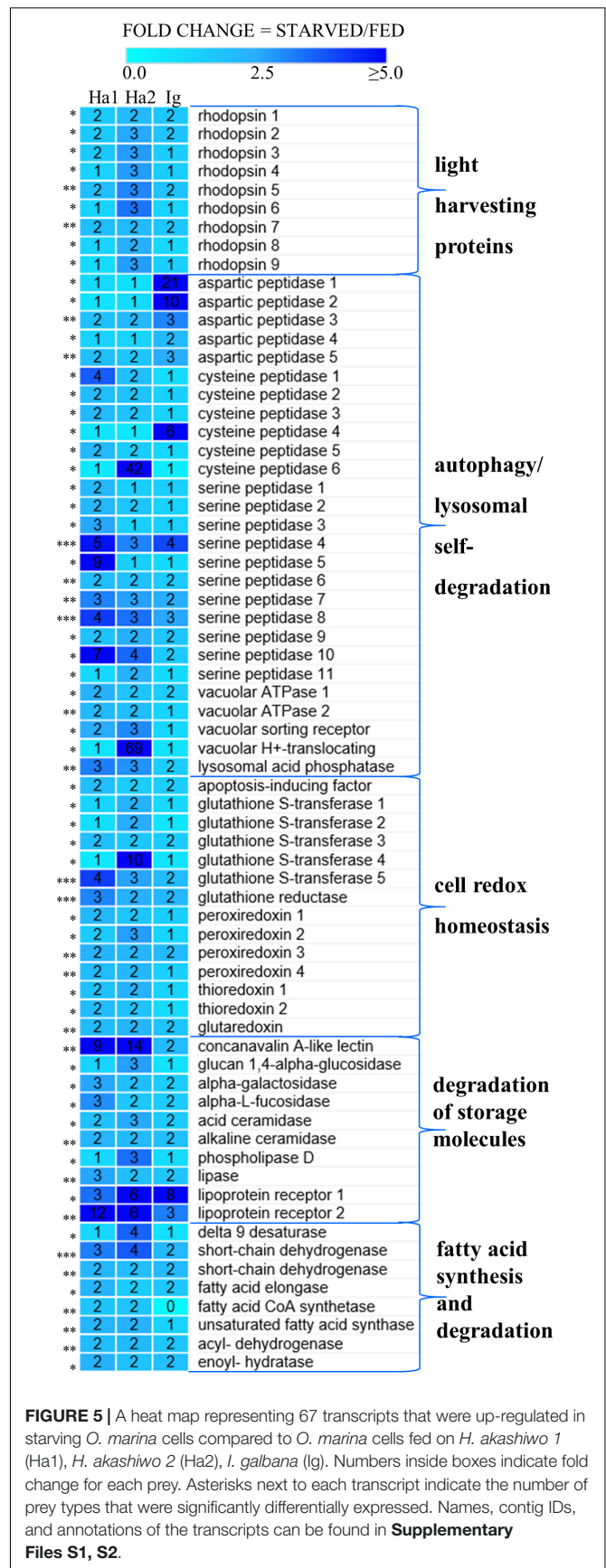


FIGURE 5 | A heat map representing 67 transcripts that were up-regulated in starving *O. marina* cells compared to *O. marina* cells fed on *H. akashiwo* 1 (Ha1), *H. akashiwo* 2 (Ha2), *I. galbana* (Ig). Numbers inside boxes indicate fold change for each prey. Asterisks next to each transcript indicate the number of prey types that were significantly differentially expressed. Names, contig IDs, and annotations of the transcripts can be found in **Supplementary Files S1, S2**.

phosphoglucomutase (EC:5.4.2.2) were upregulated in the fed cells. In addition, the enzyme responsible for the synthesis of glucose polymers (either starch or glycogen), the glycogen branching enzyme (EC:2.4.1.18) was upregulated in fed *O. marina* cells. Further, enzymes involved in the production of trehalose (EC:3.1.3.12), D-xylose (EC:3.2.1.37), and the nucleotide sugar, GDP-mannose (EC:4.2.1.47) were also upregulated during feeding. Only one enzyme in the carbohydrate metabolism pathway was found to be upregulated in the starved cells and it was a glycogen degrading enzyme, the glucan 1,4- α -glucosidase (gamma amylase, EC:3.2.1.3).

Kyoto Encyclopedia of Genes and Genomes (KEGG) enzymes involved in lipid metabolism were significantly DE during starvation (Figure 8). Specifically, two enzymes involved in the conversion of fatty acids to alcohols, the aldehyde dehydrogenase (EC:1.2.1.3) and the alcohol dehydrogenase (EC:1.1.1.1), were upregulated during starvation. The upregulation of ceramidase (EC:3.5.1.23) and alpha-galactosidase (EC:3.2.1.22) during starvation suggests degradation of sphingolipids or lipids inside the cell membranes. Further, the upregulation of the beta-ketoacyl acyl carrier protein (ACP) reductase (EC:1.1.1.100) and stearoyl-CoA 9-desaturase (EC:1.14.19.1) in starved cells suggests accumulation of oleic acid. In contrast, changes in lipid metabolism during grazing included the upregulation of the transcript encoding the monoacylglycerol lipase (EC:3.1.1.23) and two enzymes involved in fatty acid synthesis (fatty acid synthase, EC:2.3.1.85 and enoyl-[ACP] reductase, EC:1.3.1.9).

DISCUSSION

Significant shifts in gene expression were observed between *O. marina* cells that were fed and starved. These data provide insights into the molecular underpinnings of the feeding and starvation processes. We found 3.4% of the transcriptome (1576 *O. marina* contigs) to be regulated on the transcriptional level which is in general agreement with other dinoflagellates (reviewed by Roy et al., 2018) and other eukaryotic organisms (e.g., Dyhrman et al., 2012; Bochenek et al., 2013; Liu et al., 2015). Here, the transcriptomic response of the predator feeding on high prey concentrations included 972 upregulated transcripts revealing cellular and metabolic processes indicative of active grazing. These active grazing processes included genes associated with (1) detection of mechanical stimuli and regulation of swimming patterns, (2) adjustment of cell shape and cytoskeletal re-arrangement, (3) regulation of cell division, and (4) starch and fatty acid synthesis. In response to the starvation treatment, 604 transcripts were found to have higher expression, revealing physiological changes and metabolic adjustments taking place in starving cells. The predator's molecular response to starvation conditions included higher expression of genes associated with (1) light harvesting proteins called rhodopsins, (2) degradation of storage molecules, including fatty acids, lipids, and polysaccharides (most likely starch), (3) lysosomal self-degradation of organelles, and (4) signs of oxidative stress.

The two feeding stages contrasted here highlighted differences in growth between active feeding and starvation. Under high

prey concentrations, *O. marina* grew at rates similar to those previously reported in other studies using the same prey species (Jeong et al., 2003). Starving cells were alive and actively swimming at the time of harvest. This behavior, coupled with previous observations of extended starvation capacity in *O. marina* (Anderson and Menden-Deuer, 2017), suggests that the cells were starving but not undergoing senescence.

At the end of the experiment, the active feeding treatments contained more *H. akashiwo* cells than *I. galbana*, which was below detection ($<1 \text{ cell ml}^{-1}$). The difference in prey abundance was reflected in the transcriptome. When the predator was feeding on *H. akashiwo*, there was a sevenfold higher number of significantly differentially expressed transcripts compared to when it was feeding on *I. galbana*. The cell concentration of the different prey types in fed treatments during harvest provides a possible explanation for the difference in upregulated transcripts between species. During the 3-day experiment, *O. marina* consumed *I. galbana* more rapidly than either *H. akashiwo* strain. As a result, *O. marina* may have adjusted its metabolism to a reduced level of prey at the time of cell harvest. In contrast, *H. akashiwo* was still present at the time of harvest, suggesting active feeding and growth. Given the differences observed in the number of upregulated transcripts, we have not focused on the dependence of gene regulation on prey type but instead focused on an overall description of metabolic processes related to feeding and starvation in *O. marina*.

Energy Acquisition and Storage During Grazing

Oxyrrhis marina has an intricate set of swimming behaviors, allowing for the search, detection, and capture of prey items (Roberts et al., 2011). We identified a set of genes that may be involved in prey detection and phagocytosis. When feeding on *H. akashiwo*, *O. marina* cells exhibited several upregulated transcripts encoding voltage-gated potassium and calcium transmembrane channels. Voltage-gated ion channels are involved in detection of mechanical stimuli and in the regulation of swimming behavior in eukaryotic protists including *Paramecium* (a ciliate), *Chlamydomonas* (a green alga), *Stylonychia* (a ciliate), and others (Naitoh and Eckert, 1969; Catterall, 1995; Martinac et al., 2008). It is possible that similar voltage-gated ion channels might allow *O. marina* to detect prey movement and regulate its swimming patterns during prey chase and capture. In contrast to high food availability treatments, during starvation none of the voltage-gated channels were upregulated possibly because there is no need for the intricate regulation of swimming, maneuvering, and prey detection during prey capture.

After prey detection and capture, *O. marina* uses phagocytosis to ingest its prey. It has been hypothesized that *O. marina* phagocytosis might coincide with an increased activity of kinases (Roberts et al., 2011), which are commonly responsible for signal transduction processes and activation of enzymes via phosphorylation. We found that a large set of kinases are in fact significantly upregulated during feeding suggesting their involvement in the regulation of cellular processes related to

TABLE 5 | Most frequent protein domains found among significantly differentially expressed transcripts from RNA-Seq analysis of *O. marina* cells in culture conditions with (fed) and without (starved) different phytoplankton prey types.

Treat.	InterPro ID	Number of transcripts	Name	Function
Fed	IPR011009	26	Protein kinase-like domain	Signal transduction, activation of enzymes by phosphorylation
Fed	IPR000719	24	Protein kinase domain	Signal transduction, activation of enzymes by phosphorylation
Fed	IPR008271	17	Serine/threonine-protein kinase	Signal transduction, activation of enzymes by phosphorylation
Fed	IPR000595	8	Cyclic nucleotide-binding domain	Nucleotide binding
Fed	IPR029058	8	Alpha/beta-hydrolase fold	Hydrolytic enzyme
Fed	IPR005821	7	Ion transporter domain	Transmembrane ion transport
Fed	IPR001623	6	dnaJ domain	Molecular chaperon/protein refolding
Fed	IPR000504	6	RNA recognition motif domain	RNA-binding/alternative splicing
Fed	IPR023214	5	Haloacid dehydrogenase	Protein superfamily/variety of functions
Fed	IPR023366	5	ATP synthase subunit alpha-like domain	ATP synthesis
Fed	IPR001404	4	Heat shock protein Hsp90 family	Molecular chaperon/protein refolding
Fed	IPR000771	4	Fructose-bisphosphate aldolase	Glycolysis/glyconeogenesis
Fed	IPR013122	4	Polycystin cation channel, PKD1/PKD2	Cation transmembrane transfer
Fed	IPR009091	4	Regulator of chromosome condensation	Regulation of chromosome condensation
Fed	IPR000791	3	Acetate transporter GPR1/FUN34/SatP family	Acetate transmembrane transport
Fed	IPR003593	3	AAA+ ATPase domain	Diverse cellular processes
Fed	IPR004000	3	Actin family	Formation of cytoskeletal filaments
Fed	IPR004481	3	Sodium/potassium/calcium exchanger	Transmembrane ion transport
Fed	IPR006694	3	Fatty acid hydroxylase	Fatty acid metabolism
Fed	IPR013217	3	Methyltransferase type 12	Diverse cellular processes
Fed	IPR027038	3	Ran GTPase-activating protein	Transport between the nucleus and cytoplasm
Fed	IPR004837	3	Sodium/calcium exchanger membrane region	Transmembrane ion transport
Fed	IPR000408	3	Regulator of chromosome condensation, RCC1	Regulation of chromosome condensation
Starved	IPR001425	7	Rhodopsin	Retinal-binding proteins that provide light-dependent ion transport
Starved	IPR001461	7	Pepsinogen	Lysosomal proteolysis
Starved	IPR020471	7	Aldo/keto-reductase	NADPH-dependent oxidoreductases: aldehyde reductase, aldose reductase
Starved	IPR000884	5	Thrombospondin type-1 (TSP1) repeat	Multimeric multidomain cell surfaces glycoproteins
Starved	IPR007201	5	RNA-recognition motif	C-terminal RNA recognition motif
Starved	IPR029058	4	Alpha/beta-hydrolase fold	Domain present in a number of hydrolytic enzymes
Starved	IPR000668	4	Cathepsin L1-like	Lysosomal proteolysis
Starved	IPR002110	3	Ankyrin repeat domain	Protein-protein interaction motifs
Starved	IPR013126	3	Heat shock protein 70	Molecular chaperon/protein refolding

feeding. We also detected several highly expressed transcripts involved in the regulation of cytoskeleton organization, cell shape, and polarity during feeding. This agrees with the previous direct observation of reorganization of microtubular bands in *O. marina* cells during phagocytosis. The involvement of cytoskeletal changes (e.g., actin remodeling) during phagocytosis is a fundamental process of eukaryotic cells (May and Machesky, 2001; Fletcher and Mullins, 2010). The transcriptomic response revealed here allowed us to link phagocytosis to specific transcripts encoding protein components of the cytoskeleton in dinoflagellates. These phagocytosis-related transcripts could be used in future studies to examine the molecular regulation of food engulfment in dinoflagellates.

Following prey ingestion by phagocytosis, the prey is digested inside food vacuoles that become infused with digestive enzymes (Mast, 1947; Öpik and Flynn, 1989; Hausmann, 2002). Interestingly, no upregulated digestive enzymes in response to grazing were identified in this study. These enzymes, however,

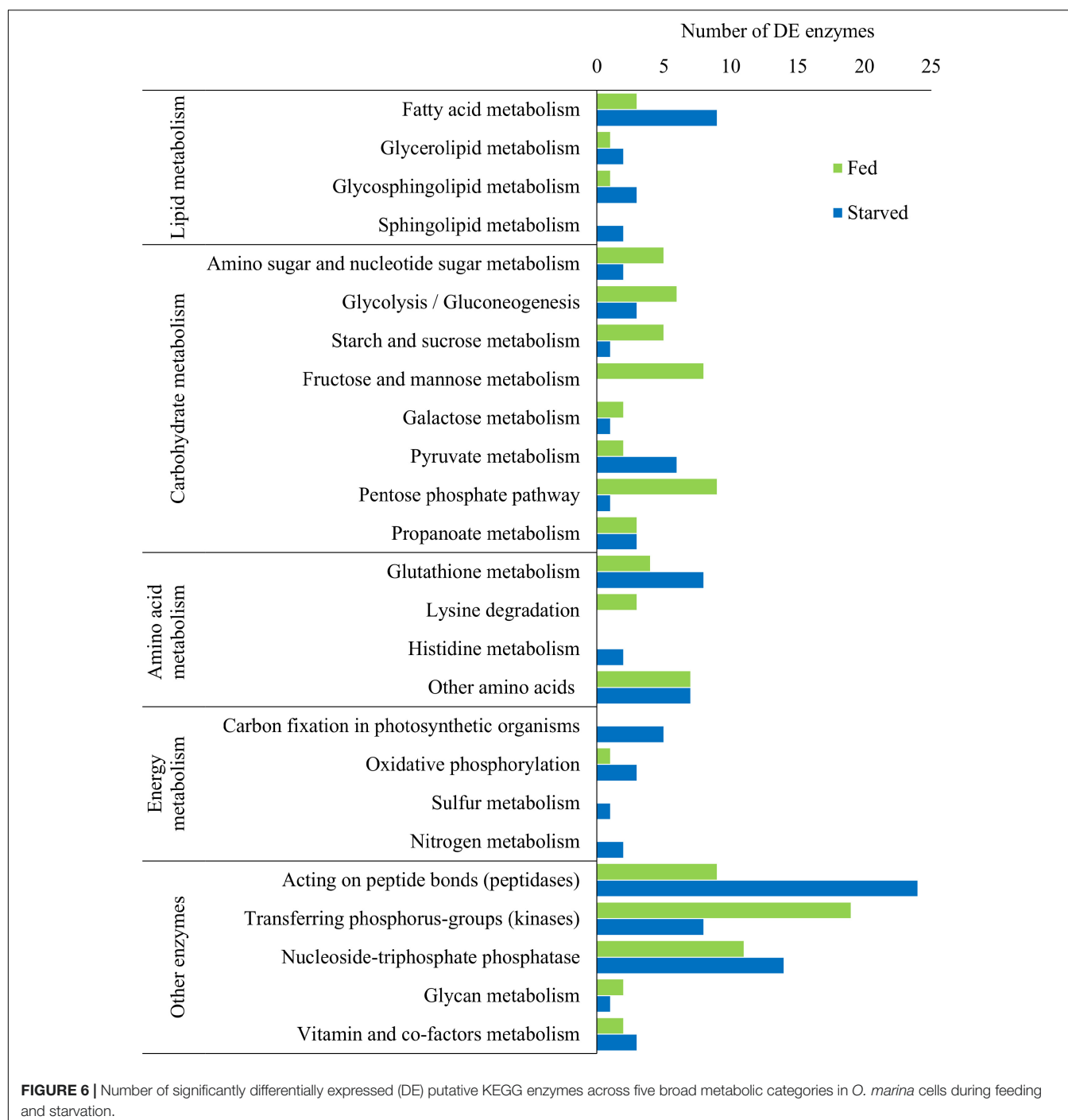
might be regulated on the post-transcriptional level (Roy et al., 2018) or they are in the group of transcripts that were differentially expressed but not functionally annotated. Further studies are required to address the expression of digestive enzymes.

Actively feeding *O. marina* cells increased the expression of genes associated with the synthesis of polysaccharides including starch. The biosynthesis of starch is well documented, and primary (short-term) energy storage of excess glucose is found in nearly all organisms across all kingdoms (Zmasek and Godzik, 2014). Storage polysaccharides are synthesized by all organisms from bacteria to animals (Ball and Morell, 2003; Zmasek and Godzik, 2014). Glycogen is found in most animals, fungi, and bacteria (including cyanobacteria and archaeobacterial species), while starch is only found in photosynthetic eukaryotes including non-photosynthetic dinoflagellates (Ball and Morell, 2003). The increased expression of genes associated with polysaccharide synthesis suggests that the predator was food satiated and excess

TABLE 6 | Result of the GO enrichment analysis based on the RNA-Seq derived 1576 significantly differentially expressed transcripts found in *O. marina* cells subjected to two culture treatments: feeding and starvation.

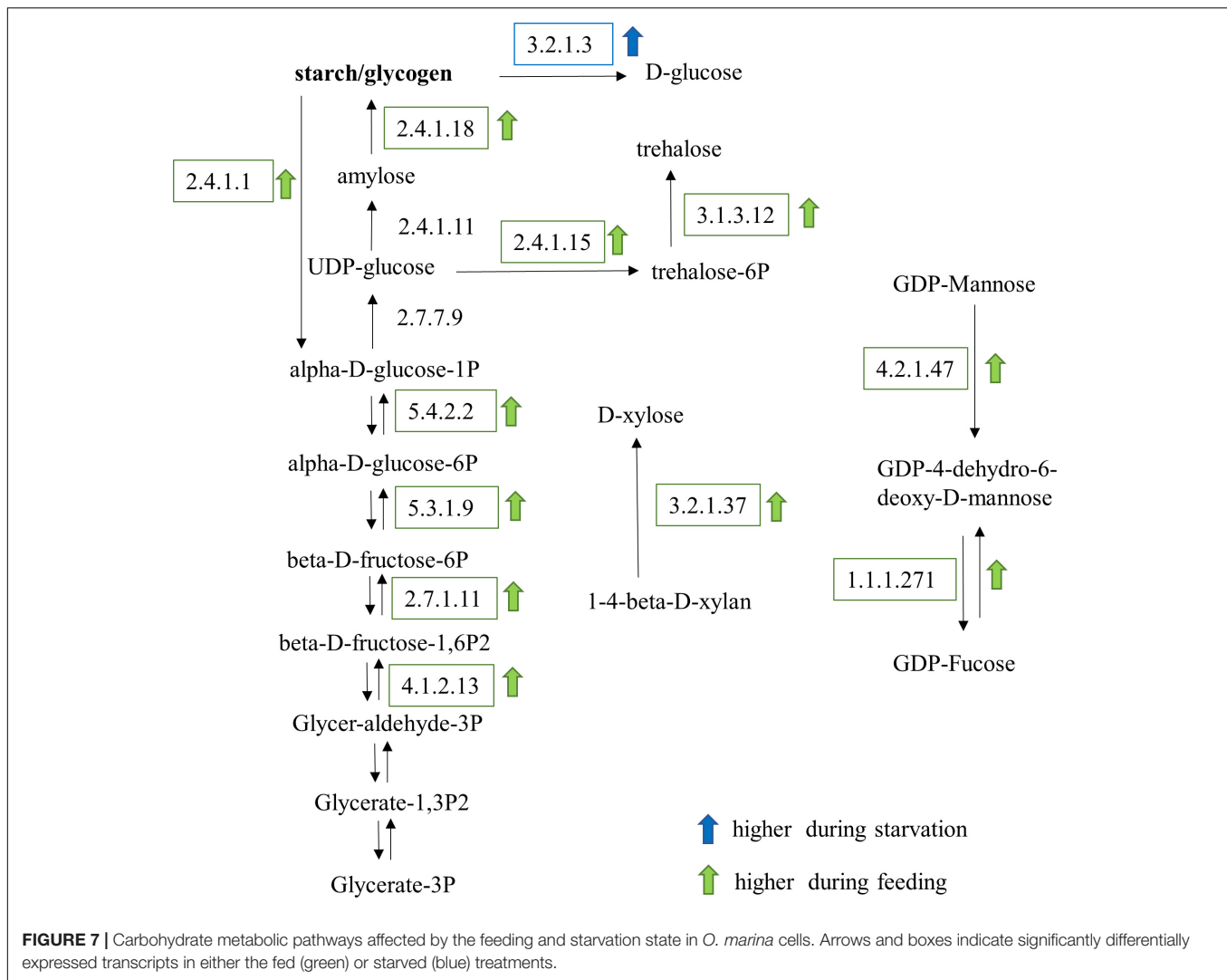
Treat.	GO number	GO type	GO terminology	P-value	FDR
Fed	GO:0004332	F	Fructose-bisphosphate aldolase activity	0.0000	0.0002
Fed	GO:0008773	F	[Protein-PilI] uridylyltransferase activity	0.0000	0.0016
Fed	GO:0019319	P	Hexose biosynthetic process	0.0000	0.0085
Fed	GO:0016051	P	Carbohydrate biosynthetic process	0.0000	0.0085
Fed	GO:0016832	F	Aldehyde-lyase activity	0.0000	0.0028
Fed	GO:0006094	P	Gluconeogenesis	0.0000	0.0140
Fed	GO:0006096	P	Glycolysis	0.0001	0.0230
Fed	GO:0034637	P	Cellular carbohydrate biosynthetic process	0.0002	0.0400
Fed	GO:0046364	P	Monosaccharide biosynthetic process	0.0003	0.0410
Fed	GO:0006090	P	Pyruvate metabolic process	0.0003	0.0450
Fed	GO:0046165	P	Alcohol biosynthetic process	0.0005	0.0600
Fed	GO:0016830	F	Carbon-carbon lyase activity	0.0005	0.0540
Fed	GO:0006007	P	Glucose catabolic process	0.0007	0.0700
Fed	GO:0019318	P	Hexose metabolic process	0.0007	0.0700
Fed	GO:0019320	P	Hexose catabolic process	0.0009	0.0780
Fed	GO:0070588	P	Calcium ion transmembrane transport	0.0010	0.0830
Fed	GO:0006006	P	Glucose metabolic process	0.0015	0.1100
Fed	GO:0046365	P	Monosaccharide catabolic process	0.0016	0.1100
Fed	GO:0070569	F	Uridylyltransferase activity	0.0019	0.1700
Fed	GO:0009144	P	Purine nucleoside triphosphate metabolic process	0.0022	0.1500
Fed	GO:0046164	P	Alcohol catabolic process	0.0031	0.1900
Fed	GO:0006091	P	Generation of precursor metabolites and energy	0.0033	0.1900
Fed	GO:0005996	P	Monosaccharide metabolic process	0.0057	0.3000
Fed	GO:0009141	P	Nucleoside triphosphate metabolic process	0.0058	0.3000
Fed	GO:0006816	P	Calcium ion transport	0.0067	0.3300
Fed	GO:0032787	P	Monocarboxylic acid metabolic process	0.0076	0.3500
Fed	GO:0007059	P	Chromosome segregation	0.0079	0.3500
Fed	GO:0044275	P	Cellular carbohydrate catabolic process	0.0082	0.3500
Fed	GO:0044282	P	Small molecule catabolic process	0.0087	0.3500
Fed	GO:0008168	F	Methyltransferase activity	0.0090	0.5700
Fed	GO:0070838	P	Divalent metal ion transport	0.0094	0.3700
Starved	GO:0006352	P	Transcription initiation	0.0000	0.0089
Starved	GO:0016491	F	Oxidoreductase activity	0.0002	0.0530
Starved	GO:0055114	P	Oxidation reduction	0.0002	0.0560
Starved	GO:0055086	P	Nucleobase, nucleoside, and nucleotide metabolic process	0.0009	0.1900
Starved	GO:0008483	F	Transaminase activity	0.0011	0.1300
Starved	GO:0016769	F	Transferase activity, transferring nitrogenous groups	0.0012	0.1300
Starved	GO:0034404	P	Nucleobase, nucleoside, and nucleotide biosynthetic process	0.0017	0.2500
Starved	GO:0034654	P	Nucleobase, nucleoside, nucleotide, and nucleic acid biosynthetic process	0.0019	0.2500
Starved	GO:0034641	P	Cellular nitrogen compound metabolic process	0.0023	0.2500
Starved	GO:0070011	F	Peptidase activity, acting on L-amino acid peptides	0.0030	0.2400
Starved	GO:0044248	P	Cellular catabolic process	0.0036	0.3400
Starved	GO:0006091	P	Generation of precursor metabolites and energy	0.0049	0.3700
Starved	GO:0006414	P	Translational elongation	0.0051	0.3700
Starved	GO:0008233	F	Peptidase activity	0.0062	0.3400
Starved	GO:0016765	F	Transferase activity, transferring alkyl or aryl (other than methyl) groups	0.0064	0.3400
Starved	GO:0044281	P	Small molecule metabolic process	0.0078	0.4300
Starved	GO:0009117	P	Nucleotide metabolic process	0.0078	0.4300
Starved	GO:0006753	P	Nucleoside phosphate metabolic process	0.0078	0.4300

GO types: F, molecular function; P, biological process. FDR, false discovery rate.



glucose was being stored as energy reserve. In particular, GO-enrichment analysis showed that out of 31 GO terms that were overrepresented in the transcripts during feeding, 14 described carbohydrate metabolic processes including glycolysis and gluconeogenesis. In addition, the key enzyme involved in the synthesis of long glucose polymerase, the starch/glycogen synthase (EC:2.4.1.18), was found to be upregulated in fed *O. marina* cells. This enzyme was upregulated in *O. marina* cells feeding on all three prey types suggesting that *O. marina* cells

use the starch as an energy storage molecule regardless of the type of phytoplankton it feeds on. Interestingly, the glucan 1,4-alpha glycosidase (EC:3.2.1.3) that is directly involved in the degradation of starch/glycogen was upregulated in the starved *O. marina* cells revealing the potentially important role that the starch/glycogen molecule is playing in the alternating feeding and starvation metabolic states. Starch/glycogen are primary (short-term) energy storage molecules as their conversion to glucose requires short and simple reactions (Scialdone and Howard,



2015). Enzymes for the production of starch have been found in other dinoflagellates (Butterfield et al., 2013) indicating a common pathway for this group of protists and their common ability to store energy in carbohydrates.

Survival During Starvation: Utilization of Stored Energy Reserves and Autophagy

During starvation conditions, organisms generally utilize energy reserves such as those stored in glycogen and lipids, breaking them down using the process of autophagy (Kaur and Debnath, 2015). Here, starving *O. marina* upregulated enzymes putatively involved in the degradation of energy-storage molecules (polysaccharides and lipids), presumably to cope with the lack of food availability. For example, in starved *O. marina*, there was increased expression of a transcript encoding glucan 1,4-alpha-glycosidase (EC:3.2.1.3, aka amylase), used for the digestion of stored starch/glycogen molecules (Ball and Morell, 2003; Zmasek and Godzik, 2014). Additional enzymes, presumably responsible for the degradation of various molecules, such as an alpha-galactosidase (Weignerová et al., 2009; Katrolia et al., 2014), an

acid ceramidase (Canals et al., 2011), and a lipase (Svendsen, 2000; Thakur, 2012) were also upregulated in starved cells. The upregulation of acid ceramidase (EC:3.5.1.23) suggests that the starving cells might be digesting polar lipids (sphingolipids) present in cell membranes (Futerman and Hannun, 2004). Thus, this enzyme could also be involved in autophagy or self-digestion, specifically in the digestion of organellar membranes (Kaur and Debnath, 2015).

Autophagy is a well-documented and common response to starvation among many eukaryotic organisms. It is defined as a controlled and selective self-degradation of cytoplasmic and organellar components in order to preserve minimal cellular functions (Sinai and Roepe, 2012; Kaur and Debnath, 2015). Autophagy is a type of programmed cell death that is different than apoptosis (Schwartz et al., 1993), by which nutrient limited cells can recycle cellular compounds to sustain limited cell functions (Sinai and Roepe, 2012). In the current study, the process of autophagy was indicated by upregulation of lysosomal peptidases. In addition, two vacuolar ATPases and a vacuolar hydrogen ion pyrophosphate, which ensure activation of

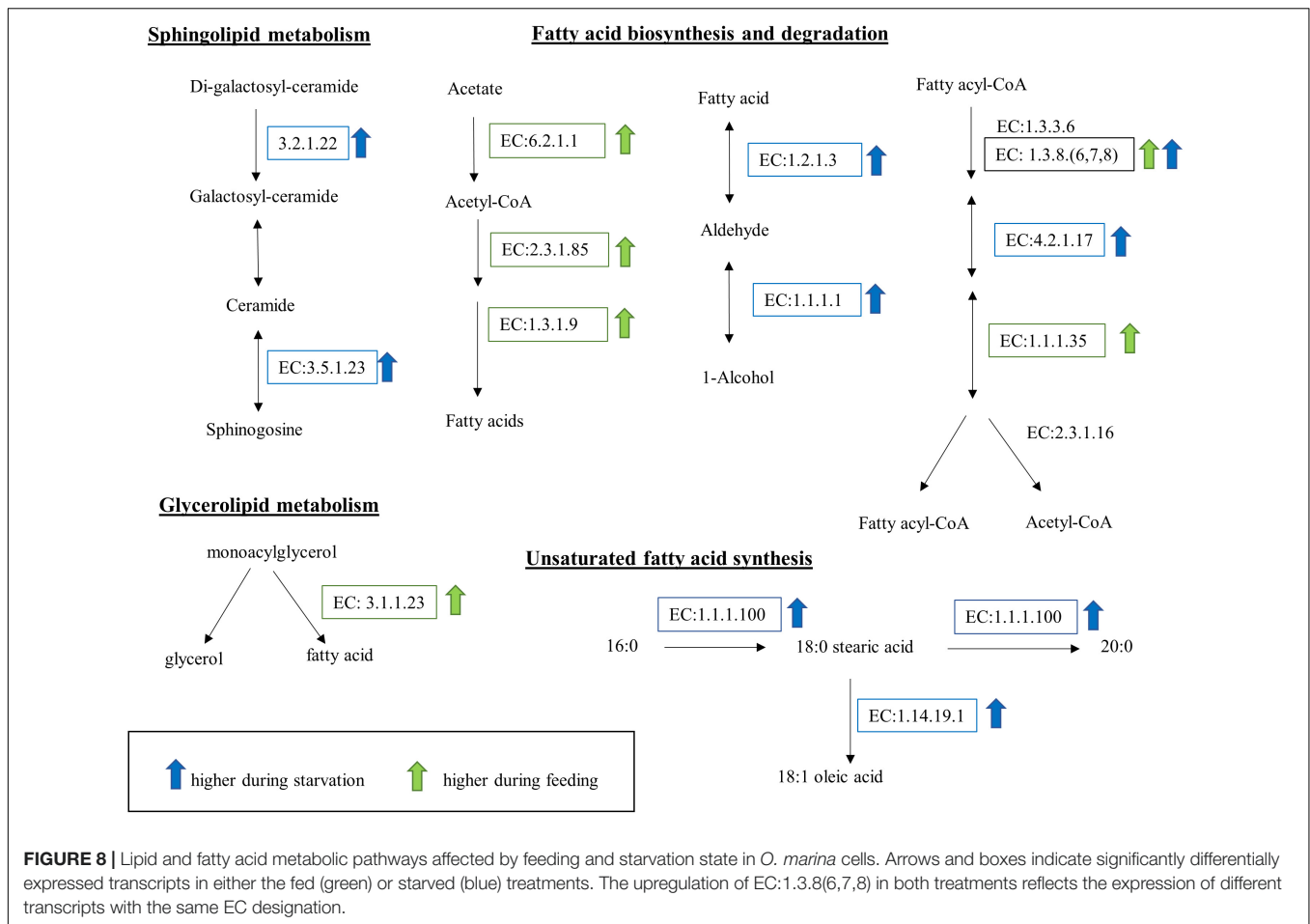


FIGURE 8 | Lipid and fatty acid metabolic pathways affected by feeding and starvation state in *O. marina* cells. Arrows and boxes indicate significantly differentially expressed transcripts in either the fed (green) or starved (blue) treatments. The upregulation of EC:1.3.8(6,7,8) in both treatments reflects the expression of different transcripts with the same EC designation.

peptidases by lysosomal acidification (Holliday, 2014), were also found at higher abundance during starvation.

The molecular signs of autophagy reported in this study agreed with the direct observations of starved *O. marina* cells, which become smaller in size, transparent, and sometimes deformed (Anderson and Menden-Deuer, 2017). The process of autophagy as well as the signaling pathways that control it are well-described in mammalian and yeast cells, where it co-occurs with oxidative stress and cell-redox mediation (Reggiori and Klionsky, 2002; Lee et al., 2012; Navarro-Yepes et al., 2014; Filomeni et al., 2015; Kaur and Debnath, 2015). Thus, it is possible that a higher abundance of transcripts encoding for several enzymes involved in an antioxidant activity and cell redox homeostasis, including three peroxiredoxins, two thioredoxins, and five components of the glutathione redox system, in the starved *O. marina* cells are also related to autophagy. Further, transcripts coding for aldo-keto reductases were up-regulated during starvation and these enzymes work in collaboration with other oxido-reducing proteins to ensure redox transformation of common metabolites (Chang et al., 2007; Barski et al., 2008; Chang and Petrash, 2008; Penning, 2015).

Starved *O. marina* cells also upregulated enzymes involved in unsaturated fatty acid synthesis (EC:1.1.1.100, EC:1.14.19.1). These enzymes are specifically involved in the conversion of saturated fatty acid to unsaturated fatty acids, specifically the

production of oleic acid, a monounsaturated fatty acid (C18:1). Although it is counterintuitive for a starving cell to expend energy synthesizing molecules, it is possible that oleic acid is an essential fatty acid for *O. marina* survival. Although this hypothesis requires further testing, oleic acid concentrations in *O. marina* cells have been shown to increase after several days of starvation (Yoon et al., 2017), supporting our speculation.

Starving *O. marina* cells also showed significant increases in the expression of rhodopsins. ATP-generating light-activated rhodopsins (aka proteorhodopsins) have been documented to support metabolic activities in various archaea and bacteria (Fuhrman et al., 2008). In most eukaryotic microorganisms, rhodopsins (a chromophore-retinal binding proteins) function as a photoreceptor (Kikukawa et al., 2012). However, evidence has emerged for proteorhodopsin-mediated phototrophy in some eukaryotes. For example, Slamovits et al. (2011) showed that *O. marina* possesses proteorhodopsins which are located in cytoplasmic structures resembling an endomembrane system. *O. marina* proteorhodopsins are phylogenetically similar to both sensory and proton-pumping proteorhodopsins (Slamovits et al., 2011) and show increased expression over time when cells were incubated without prey (Guo et al., 2014). In addition, the authors found 40 distinct rhodopsin genes in the *O. marina* transcriptome including some that showed phylogenetic grouping to sensory rhodopsins although the majority

of them belonged to the proton-pumping proteorhodopsins (Slamovits et al., 2011). Here, nine different transcripts showing the highest similarity to the proton pumping proteorhodopsins were upregulated in *O. marina* cell during starvation suggesting that the *O. marina* may be supplementing its energy requirement using this light harvesting mechanism.

CONCLUSION

Heterotrophic dinoflagellates must identify, capture, and digest prey in a generally dilute ocean. The data from this study provide insight into the molecular basis of feeding and response to starvation states in heterotrophic dinoflagellates. For example, voltage-gated transmembrane channels appear to be involved in the complex swimming behavior of dinoflagellates which allows them to detect, pursue, and capture prey. The active grazing process also requires a large set of enzymes involved in the signal transduction process as well as the regulation of cytoskeletal organization and cell polarity. This study also suggests that rhodopsins allow some dinoflagellates to utilize light energy during times of food scarcity. In addition, the regulated and organized process of self-digestion (autophagy) allows dinoflagellates to sustain minimum cellular functions by recycling cellular organic matter, helping to explain their ability to survive long periods of starvation (Menden-Deuer et al., 2005). Furthermore, the feeding and starvation experiments revealed that the primary short-term storage of extra glucose produced during periods of high food availability is a long polymer of glucose, mostly likely starch (Ball and Morell, 2003), which might be used during the first few days of food shortage. The results from this study are fundamental building blocks for further studies that will develop new tools for measuring protist grazing *in situ*, a notorious challenge. For example, the formation and digestion of starch/glycogen could be used as a tool to measure dinoflagellate response to pulses of prey in the water column. It is our hope that ultimately the molecular signals measured here can be used to provide broad-scale, high resolution measurements

of grazing in marine microbial food webs and identify the environmental and biological drivers of grazing, the major loss factor of marine primary production.

AUTHOR CONTRIBUTIONS

SM-D and TR designed the study. AM conducted the experiments. ER and SC conducted the bioinformatics analysis. ER, TR, and SM-D wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was supported by the Rhode Island Science and Technology Advisory Council (to SM-D and TR) and NASA grant 80NSSC17K0716 (to SM-D and TR) as part of the Export Processes in the Global Ocean from Remote Sensing (EXPORTS) field campaign. This research was funded in part by the Gordon and Betty Moore Foundation through Grant 2637 to the National Center for Genome Resources, where samples were sequenced and assembled. This study was conducted using the Center for Computation and Visualization, Brown University, supported in part by NSF EPSCoR research infrastructure improvement awards EPS-1004057 and OIA-1655221.

ACKNOWLEDGMENTS

We thank B. Hurwitz for developing and maintaining the iMicrobe database.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2019.00246/full#supplementary-material>

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2
- Anderson, S. R., and Menden-Deuer, S. (2017). Growth, grazing, and starvation survival in three heterotrophic dinoflagellate species. *J. Eukaryot. Microbiol.* 64, 213–225. doi: 10.1111/jeu.12353
- Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556
- Ball, S. G., and Morell, M. K. (2003). From bacterial glycogen to starch: understanding the biogenesis of plant starch granule. *Annu. Rev. Plant Biol.* 54, 207–233. doi: 10.1146/annurev.arplant.54.031902.134927
- Barski, O. A., Tipparaju, S. M., and Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab. Rev.* 40, 553–624. doi: 10.1080/03602530802431439
- Bochenek, M., Etherington, G. J., Koprivova, A., Mugford, S. T., Bell, T. G., Malin, G., et al. (2013). Transcriptome analysis of the sulfate deficiency response in the marine microalga *Emiliania huxleyi*. *New Phytol.* 199, 650–662. doi: 10.1111/nph.12303
- Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170
- Butterfield, E. R., Howe, C. J., and Nisbet, R. E. R. (2013). An analysis of dinoflagellate metabolism using EST data. *Protist* 164, 218–236. doi: 10.1016/j.protis.2012.09.001
- Calbet, A., Isari, S., Martínez, R. A., Saiz, E., Garrido, S., Peters, J., et al. (2013). Adaptations to feast and famine in different strains of the marine heterotrophic dinoflagellates *Gyrodinium dominans* and *Oxyrrhis marina*. *Mar. Ecol. Prog. Ser.* 483, 67–84. doi: 10.3354/meps10291
- Calbet, A., and Landry, M. R. (2004). Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. *Limnol. Oceanogr.* 49, 51–57. doi: 10.4319/lo.2004.49.1.0051
- Calbet, A., and Saiz, E. (2005). The ciliate-copepod link in marine ecosystems. *Aquat. Microb. Ecol.* 38, 157–167. doi: 10.3354/ame038157
- Campbell, R. G., Sherr, E. B., Ashjian, C. J., Plourde, S., Sherr, B. F., Hill, V., et al. (2009). Mesozooplankton prey preference and grazing impact in the western

- Arctic Ocean. *Deep Sea Res. Part II* 56, 1274–1289. doi: 10.1016/j.dsr2.2008.10.027
- Canals, D., Perry, D. M., Jenkins, R. W., and Hannun, Y. A. (2011). Drug targeting of sphingolipid metabolism: sphingomyelinases and ceramidases: drug targeting, sphingomyelinases and ceramidases. *Br. J. Pharm. Chemoth.* 163, 694–712. doi: 10.1111/j.1476-5381.2011.01279.x
- Catterall, W. A. (1995). Structure and function of voltage-gated ion channels. *Annu. Rev. Biochem.* 64, 493–531. doi: 10.1146/annurev.bi.64.070195.002425
- Chang, Q., Griest, T. A., Harter, T. M., and Mark Petrash, J. (2007). Functional studies of aldo-keto reductases in *Saccharomyces cerevisiae*. *BBA Mol. Cell Res.* 1773, 321–329. doi: 10.1016/j.bbamcr.2006.10.009
- Chang, Q., and Petrash, J. M. (2008). Disruption of aldo-keto reductase genes leads to elevated markers of oxidative stress and inositol auxotrophy in *Saccharomyces cerevisiae*. *BBA Mol. Cell Res.* 1783, 237–245. doi: 10.1016/j.bbamcr.2007.08.008
- Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674–3676. doi: 10.1093/bioinformatics/bti610
- Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res.* 38, W64–W70. doi: 10.1093/nar/gkq310
- Dyhrman, S. T., Jenkins, B. D., Rynearson, T. A., Saito, M. A., Mercier, M. L., Alexander, H., et al. (2012). The transcriptome and proteome of the diatom *Thalassiosira pseudonana* reveal a diverse phosphorus stress response. *PLoS One* 7:e33768. doi: 10.1371/journal.pone.0033768
- Filomeni, G., De Zio, D., and Cecconi, F. (2015). Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ.* 22, 377–388. doi: 10.1038/cdd.2014.150
- Fletcher, D. A., and Mullins, R. D. (2010). Cell mechanics and the cytoskeleton. *Nature* 463, 485–492. doi: 10.1038/nature08908
- Fuhrman, J. A., Schwalbach, M. S., and Stingl, U. (2008). Proteorhodopsins: an array of physiological roles? *Nat. Rev. Microbiol.* 6, 488–494. doi: 10.1038/nrmicro1893
- Futerman, A. H., and Hannun, Y. A. (2004). The complex life of simple sphingolipids. *EMBO Rep.* 5, 777–782. doi: 10.1038/sj.embor.7400208
- Guillard, R. R. L., and Ryther, J. H. (1962). Studies of marine planktonic diatoms: I. *cyclotella nana* (Hustedt) and *detonula confervacea* (Cleve) gran. *Can. J. Microbiol.* 8, 229–239. doi: 10.1139/m62-029
- Guo, Z., Zhang, H., and Lin, S. (2014). Light-promoted rhodopsin expression and starvation survival in the marine *Dinoflagellate Oxyrrhis marina*. *PLoS One* 9:e114941. doi: 10.1371/journal.pone.0114941
- Guo, Z., Zhang, H., Liu, S., and Lin, S. (2013). Biology of the marine heterotrophic *Dinoflagellate Oxyrrhis marina*: current status and future directions. *Microorganisms* 1, 33–57. doi: 10.3390/microorganisms1010033
- Hansen, P. J., Bjørnsen, P. K., and Hansen, B. W. (1997). Zooplankton grazing and growth: scaling within the 2–2,000- μ m body size range. *Limnol. Oceanogr.* 42, 687–704. doi: 10.4319/lo.1997.42.4.0687
- Hartz, A. J., Sherr, B. F., and Sherr, E. B. (2008). Using inhibitors to investigate the involvement of cell signaling in predation by marine phagotrophic protists. *J. Eukaryot. Microbiol.* 55, 18–21. doi: 10.1111/j.1550-7408.2007.00297.x
- Harvey, E. L., and Menden-Deuer, S. (2012). Predator-induced fleeing behaviors in phytoplankton: a new mechanism for harmful algal bloom formation? *PLoS One* 7:e46438. doi: 10.1371/journal.pone.0046438
- Hausmann, K. (2002). Food acquisition, food ingestion and food digestion by protists. *Jap. J. Protist.* 35:11.
- Holliday, L. S. (2014). Vacuolar H⁺-ATPase: an essential multitasking enzyme in physiology and pathophysiology. *New J. Sci.* 2014, 1–21. doi: 10.1155/2014/675430
- Jakobsen, H. H., and Strom, S. L. (2004). Circadian cycles in growth and feeding rates of heterotrophic protist plankton. *Limnol. Oceanogr.* 49, 1915–1922. doi: 10.4319/lo.2004.49.6.1915
- Jeong, H. J., Kim, J. S., Yoo, Y. D., Kim, S. T., Kim, T. H., Park, M. G., et al. (2003). Feeding by the heterotrophic *Dinoflagellate Oxyrrhis marina* on the red-tide raphidophyte *Heterosigma akashiwo*: a potential biological method to control red tides using mass-cultured grazers. *J. Eukaryot. Microbiol.* 50, 274–282. doi: 10.1111/j.1550-7408.2003.tb00134.x
- Kanehisa, M., Sato, Y., and Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* 428, 726–731. doi: 10.1016/j.jmb.2015.11.006
- Katrolia, P., Rajashekhar, E., Yan, Q., and Jiang, Z. (2014). Biotechnological potential of microbial α -galactosidases. *Crit. Rev. Biotechnol.* 34, 307–317. doi: 10.13109/07388551.2013.794124
- Kaur, J., and Debnath, J. (2015). Autophagy at the crossroads of catabolism and anabolism. *Nat. Rev. Mol. Cell. Biol.* 16, 461–472. doi: 10.1038/nrm4024
- Keeling, P. J., Burki, F., Wilcox, H. M., Allam, B., Allen, E. E., Amaral-Zettler, L. A., et al. (2014). The marine microbial eukaryote transcriptome sequencing project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. *PLoS Biol.* 12:e1001889. doi: 10.1371/journal.pbio.1001889
- Kikukawa, T., Tamogami, J., Shimono, K., Demura, M., Nara, T., and Kamo, N. (2012). “Photo-induced proton transfers of microbial rhodopsins,” in *Molecular Photochemistry – Various Aspects*, ed. S. Saha (InTech). doi: 10.5772/34105
- Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25. doi: 10.1186/gb-2009-10-3-r25
- Lee, J., Giordano, S., and Zhang, J. (2012). Autophagy, mitochondria and oxidative stress: cross-talk and redox signaling. *Biochem. J.* 441, 523–540. doi: 10.1042/BJ20111451
- Lee, R., Lai, H., Malik, S., Saldarriaga, J. F., Keeling, P. J., and Slamovits, C. H. (2014). Analysis of EST data of the marine protist *Oxyrrhis marina*, an emerging model for alveolate biology and evolution. *BMC Genomics* 15:122. doi: 10.1186/1471-2164-15-122
- Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323
- Liu, Z., Jones, A. C., Campbell, V., Hambricht, K. D., Heidelberg, K. B., and Caron, D. A. (2015). Gene expression in the mixotrophic prymnesiophyte, *Prymnesium parvum*, responds to prey availability. *Front. Microbiol.* 6:319. doi: 10.3389/fmicb.2015.00319
- Löder, M. G. J., Meunier, C., Wiltshire, K. H., Boersma, M., and Aberle, N. (2011). The role of ciliates, heterotrophic dinoflagellates and copepods in structuring spring plankton communities at Helgoland Roads, North Sea. *Mar. Biol.* 158, 1551–1580. doi: 10.1007/s00227-011-1670-2
- Lowe, C. D., Mello, L. V., Samatar, N., Martin, L. E., Montagnes, D. J., and Watts, P. C. (2011). The transcriptome of the novel dinoflagellate *Oxyrrhis marina* (Alveolata: Dinophyceae): response to salinity examined by 454 sequencing. *BMC Genomics* 12:519. doi: 10.1186/1471-2164-12-519
- Martinac, B., Saimi, Y., and Kung, C. (2008). Ion channels in microbes. *Physiol. Rev.* 88, 1449–1490. doi: 10.1152/physrev.00005.2008
- Mast, S. O. (1947). The food-vacuole in *Paramecium*. *Biol. Bull.* 92, 31–72. doi: 10.2307/1537967
- May, R. C., and Machesky, L. M. (2001). Phagocytosis and the actin cytoskeleton. *J. Cell Sci.* 114, 1061–1071.
- Menden-Deuer, S., Lessard, E., and Satterberg, J. (2001). Effect of preservation on dinoflagellate and diatom cell volume, and consequences for carbon biomass predictions. *Mar. Ecol. Prog. Ser.* 222, 41–50. doi: 10.3354/meps222041
- Menden-Deuer, S., Lessard, E., Satterberg, J., and Grünbaum, D. (2005). Growth rates and starvation survival of three species of the pallium-feeding, thecate dinoflagellate genus *Protoperdinium*. *Aquat. Microb. Ecol.* 41, 145–152. doi: 10.3354/ame041145
- Montagnes, D., Barbosa, A., Boenigk, J., Davidson, K., Jürgens, K., Macek, M., et al. (2008). Selective feeding behaviour of key free-living protists: avenues for continued study. *Aquat. Microb. Ecol.* 3, 83–98. doi: 10.3354/ame01229
- Montagnes, D. J. S., Lowe, C. D., Roberts, E. C., Breckels, M. N., Boakes, D. E., Davidson, K., et al. (2011). An introduction to the special issue: *Oxyrrhis marina*, a model organism? *J. Plankton Res.* 33, 549–554. doi: 10.1093/plankt/fbq121
- Naitoh, Y., and Eckert, R. (1969). Ionic mechanisms controlling behavioral responses of *paramecium* to mechanical stimulation. *Science* 164, 963–965. doi: 10.1126/science.164.3882.963
- Navarro-Yepes, J., Burns, M., Anandhan, A., Khalimonchuk, O., del Razo, L. M., Quintanilla-Vega, B., et al. (2014). Oxidative stress, redox signaling, and

- autophagy: cell death versus survival. *Antioxid. Redox. Sign.* 21, 66–85. doi: 10.1089/ars.2014.5837
- Öpik, H., and Flynn, K. J. (1989). The digestive process of the dinoflagellate, *Oxyrrhis marina* Dujardin, feeding on the chlorophyte, *Dunaliella primolecta* butcher: a combined study of ultrastructure and free amino acids. *New Phytol.* 113, 143–151. doi: 10.1111/j.1469-8137.1989.tb04700.x
- Penning, T. M. (2015). The aldo-keto reductases (AKRs): overview. *Chem. Biol. Interact.* 234, 236–246. doi: 10.1016/j.cbi.2014.09.024
- Reggiori, F., and Klionsky, D. J. (2002). Autophagy in the eukaryotic cell. *Eukaryot. Cell* 1, 11–21. doi: 10.1128/EC.01.1.11-21.2002
- Roberts, E. C., Wootton, E. C., Davidson, K., Jeong, H. J., Lowe, C. D., and Montagnes, D. J. S. (2011). Feeding in the dinoflagellate *Oxyrrhis marina*: linking behaviour with mechanisms. *J. Plankt. Res.* 33, 603–614. doi: 10.1093/plankt/fbq118
- Roberts, E. C., Zubkov, M. V., Martin-Cereceda, M., Novarino, G., and Wootton, E. C. (2006). Cell surface lectin-binding glycoconjugates on marine planktonic protists. *FEMS Microbiol. Lett.* 265, 202–207. doi: 10.1111/j.1574-6968.2006.00484.x
- Roy, S., Jagus, R., and Morse, D. (2018). Translation and translational control in *Dinoflagellates*. *Microorganisms* 6:30. doi: 10.3390/microorganisms6020030
- Santoferrara, L. F., Guida, S., Zhang, H., and McManus, G. B. (2014). De novo transcriptomes of a mixotrophic and a heterotrophic ciliate from marine plankton. *PLoS One* 9:e101418. doi: 10.1371/journal.pone.0101418
- Schmoker, C., Hernández-León, S., and Calbet, A. (2013). Microzooplankton grazing in the oceans: impacts, data variability, knowledge gaps and future directions. *J. Plankt. Res.* 35, 691–706. doi: 10.1093/plankt/fbt023
- Schwartz, L. M., Smith, S. W., Jones, M., and Osborne, B. A. (1993). Do all programmed cell deaths occur via apoptosis? *Proc. Natl. Acad. Sci. U.S.A.* 90, 980–984. doi: 10.1073/pnas.90.3.980
- Scialdone, A., and Howard, M. (2015). How plants manage food reserves at night: quantitative models and open questions. *Front. Plant Sci.* 6:204. doi: 10.3389/fpls.2015.00204
- Sherr, E., and Sherr, B. (2009). Capacity of herbivorous protists to control initiation and development of mass phytoplankton blooms. *Aquat. Microb. Ecol.* 57, 253–262. doi: 10.3354/ame01358
- Sinai, A. P., and Roepe, P. D. (2012). Autophagy in apicomplexa: a life sustaining death mechanism? *Trends Parasitol.* 28, 358–364. doi: 10.1016/j.pt.2012.06.006
- Slamovits, C. H., Okamoto, N., Burri, L., James, E. R., and Keeling, P. J. (2011). A bacterial proteorhodopsin proton pump in marine eukaryotes. *Nat. Commun.* 2:183. doi: 10.1038/ncomms1188
- Strom, S. (2002). Novel interactions between phytoplankton and microzooplankton: their influence on the coupling between growth and grazing rates in the sea. *Hydrobiologia* 480, 41–54.
- Svensen, A. (2000). Lipase protein engineering. *BBA Protein Struct. M* 1543, 223–238. doi: 10.1016/S0167-4838(00)00239-9
- Thakur, S. (2012). Lipases, its sources, properties and applications: a review. *Int. J. Sci. Eng. Res.* 3:29.
- Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., et al. (2017). agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Res.* 45, W122–W129. doi: 10.1093/nar/gkx382
- Tillmann, U. (2004). Interactions between planktonic microalgae and protozoan grazers1. *J. Eukaryot. Microbiol.* 51, 156–168. doi: 10.1111/j.1550-7408.2004.tb00540.x
- Weignerová, L., Simerská, P., and Křen, V. (2009). α -Galactosidases and their applications in biotransformations. *Biocatal. Biotransfor.* 27, 79–89. doi: 10.1080/10242420802583416
- Weisse, T., Anderson, R., Arndt, H., Calbet, A., Hansen, P. J., and Montagnes, D. J. S. (2016). Functional ecology of aquatic phagotrophic protists – concepts, limitations, and perspectives. *Eur. J. Protisto.* 55, 50–74. doi: 10.1016/j.ejop.2016.03.003
- Wu, Z., Jenkins, B. D., Rynearson, T. A., Dyhrman, S. T., Saito, M. A., Mercier, M., et al. (2010). Empirical bayes analysis of sequencing-based transcriptional profiling without replicates. *BMC Bioinformatics* 11:564. doi: 10.1186/1471-2105-11-564
- Yang, Z., Jeong, H. J., and Montagnes, D. J. S. (2011). The role of *Oxyrrhis marina* as a model prey: current work and future directions. *J. Plankt. Res.* 33, 665–675. doi: 10.1093/plankt/fbq112
- Yoon, E. Y., Park, J., Jeong, H. J., and Rho, J.-R. (2017). Fatty acid composition and docosahexaenoic acid (DHA) content of the heterotrophic dinoflagellate *Oxyrrhis marina* fed on dried yeast: compared with algal prey. *ALGAE* 32, 67–74. doi: 10.4490/algae.2017.32.3.5
- Zdobnov, E. M., and Apweiler, R. (2001). InterProScan - an integration platform for the signature-recognition methods in interPro. *Bioinformatics* 17, 847–848. doi: 10.1093/bioinformatics/17.9.847
- Zmasek, C. M., and Godzik, A. (2014). Phylogenomic analysis of glycogen branching and debranching enzymatic duo. *BMC Evol. Biol.* 14:183. doi: 10.1186/s12862-014-0183-2

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Rubin, Cheng, Montalbano, Menden-Deuer and Rynearson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.