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RESEARCH ARTICLE

Gene expression characterizes different nutritional strategies among three mixotrophic protists

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One sentence summary: Transcriptomes of three mixotrophic protists under light and dark conditions reveal differences among protists with various nutritional strategies.
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ABSTRACT

Mixotrophic protists, i.e. protists that can carry out both phototrophy and heterotrophy, are a group of organisms with a wide range of nutritional strategies. The ecological and biogeochemical importance of these species has recently been recognized. In this study, we investigated and compared the gene expression of three mixotrophic protists, *Prymnesium parvum*, *Dinobyron* sp. and *Ochromonas* sp. under light and dark conditions in the presence of prey using RNA-Seq. Gene expression of the obligately phototrophic *P. parvum* and *Dinobryon* sp. changed significantly between light and dark treatments, while that of primarily heterotrophic *Ochromonas* sp. was largely unchanged. Gene expression of *P. parvum* and *Dinobryon* sp. shared many similarities, especially in the expression patterns of genes related to reproduction. However, key genes involved in central carbon metabolism and phagotrophy had different expression patterns between these two species, suggesting differences in prey consumption and heterotrophic nutrition in the dark. Transcriptomic data also offered clues to other physiological traits of these organisms such as preference of nitrogen sources and photo-oxidative stress. These results provide potential target genes for further exploration of the mechanisms of mixotrophic physiology and demonstrate the potential usefulness of molecular approaches in characterizing the nutritional modes of mixotrophic protists.

Keywords: mixotrophic protist; gene expression; *Prymnesium*, *Dinobryon*, *Ochromonas*

INTRODUCTION

Planktonic protists, along with other microorganisms, play important roles in aquatic ecosystems globally. Traditionally, these species have been divided into two categories based on their nutrition modes and ecological roles: phototrophic forms that serve as primary producers, and heterotrophic forms that feed on bacteria and protists. However, mixotrophic protists that can carry out both phototrophy and heterotrophy are also found in many taxonomic groups including chrysophytes (Holden and Boraas 1995), haptophytes (Hansen and Hjorth 2002), cryptophytes (Laybourn-Parry, Marshall and Marchant 2005), dinoflagellates (Stoecker 1999) and ciliates (Esteban, Fenchel and Finlay 2010) among others (Stoecker 1998; Caron 2000; Burkholder, Gilbert and Skelton 2008; Raven *et al*. 2009; Stoecker *et al*. 2009). Mixotrophic protists have been reported as abundant and sometimes dominant components of many different marine and freshwater plankton communities (Bird and Kalff 1986; Sanders 1991, 2011). Research in recent decades has shown that the prevalence and ecological importance of mixotrophic protists has been significantly under-appreciated, and has raised questions regarding the utility of classic food web models based on

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the ‘plant/animal’ dichotomy to adequately capture energy utilization and elemental flow (Flynn *et al.* 2013). Thus, recognition of the significant role of mixotrophic protists in the global carbon cycle is improving (Mitra *et al.* 2014; Cropp and Norbury 2015; Worden *et al.* 2015).

Among mixotrophic protists, physiological dependence on phototrophy or heterotrophy can vary greatly. Some taxa rely primarily on phototrophy and use heterotrophy to supplement phototrophic ability, while others are predominantly heterotrophic and resort to phototrophy only when prey become scarce. Many others exhibit nutritional strategies between these two extremes (Jones 1994). As a consequence, mixotrophic protists are often divided into several categories based on their specific nutritional strategies. Jones (1997, 2000), Stoecker (1998) and Mitra *et al.* (2016) created conceptual models that categorized mixotrophic protists based on their primary mode of nutrition, and the role(s) that their supplemental nutritional mode might play, for example, providing essential nutrients such as nitrogen, phosphorus and trace elements, or serving as an alternative source of energy.

Past research on mixotrophic protists has largely involved experimental investigations of their changes in growth, prey ingestion and photosynthetic activity in relation to the availability of various nutrients, prey type or light (for example, Caron *et al.* 1993; Sanders *et al.* 2001; Carvalho and Granéli 2010). Little is known at the molecular level regarding the metabolic responses of mixotrophic protists to changing nutrient or light conditions. Recently, the application of next generation sequencing technology has been used to study gene expression in mixotrophic protists (Santoferrara *et al.* 2014; Liu *et al.* 2015a,b). In this study, we investigated how gene expression of three mixotrophic protists with different nutritional strategies responded when grown in the light and dark. Our main goal was to identify potential similarities and distinctions in gene expression across three taxonomically and physiologically different mixotrophic protists.

Prymnesium parvum is a prymnesiophyte (haptophyte) alga that is obligately phototrophic. *Prymnesium parvum* is capable of axenic, phototrophic growth in inorganic media with growth rates of up to 0.8 d^{-1} (Granéli *et al.* 2012; Hambright *et al.* 2014). In addition to robust phototrophic growth, however, *P. parvum* also displays ferocious predatory behavior (Tillmann 2003). It has been reported that nitrogen and phosphorus contributed by heterotrophy can account for 43%–78% of the cellular N and P acquisition in *P. parvum* (Carvalho and Granéli 2010). Yet, heterotrophy has little effect on the growth rate of *P. parvum* (Carvalho and Granéli 2010; Liu *et al.* 2015a), and *P. parvum* cannot grow or survive in the dark, even when prey are present at high abundance (Brutemark and Granéli 2011), and only when supplemented with extremely high concentrations of a few specific organic compounds such as glycerol (Rahat and Jahn 1965).

Dinobryon sp. is a chrysophyte alga. Past research on *Dinobryon* spp. indicates that most species are obligately phototrophic, yet with well-developed ability to consume bacteria. *Dinobryon cylindricum* is an obligate phototroph that ingests and requires bacterial prey for growth (Caron *et al.* 1993). Studies on *D. sertularia* (Jones and Rees 1994b) and *D. divergens* (Jones and Rees 1994a) also found that they are obligate phototrophs with heterotrophic behaviors demonstrated by particle ingestion. The relative contribution of phototrophy and heterotrophy appears to vary with species. It has been estimated that one natural *Dinobryon* population obtained more than 50% of its carbon from bacterivory (Bird and Kalff 1986), while phototrophy contributed 75% of organic carbon during the growth of *D. cylindricum* in culture (Caron *et al.* 1993). *Dinobryon* spp. are often grouped together with *P. parvum* as primarily phototrophic mixotrophs (Stoecker 1998; Fig. S1, Supporting Information).

Ochromonas sp. is also a chrysophyte alga. Some *Ochromonas* species such as *O. minima* (Nygaard and Tobiesen 1993) and *Ochromonas* sp. CCMP 583 (Keller *et al.* 1994) have been found to be obligately phototrophic (they do not grow in the dark), while studies on other species or strains indicate that they are primarily heterotrophic (Fig. S1, Supporting Information). Most reports on *Ochromonas* species indicated that they require either dissolved organic carbon or bacterial prey for their growth (Andersson *et al.* 1989; Rothhaupt 1996; Sanders *et al.* 2001; Foster and Chrzanowski 2012). Autotrophic growth of *O. danica* on inorganic medium was recently reported, although it grew much more rapidly when bacteria were available as prey (Wilken, Schuurmans and Matthijs 2014). Analysis of the photosynthetic machinery of *O. danica* showed reduced pigment content and increased PSI:PSII ratio when prey are available, suggesting that it favors heterotrophy over phototrophy (Wilken, Schuurmans and Matthijs 2014). Additionally, growth and survival of some *Ochromonas* species in continuous darkness has been documented (Gibbs, Cheng and Slankis 1974; Fenchel 1982).

An experimental study was conducted with strains of these three mixotrophic (phagotrophic) phytoflagellates to investigate changes in gene expression as a consequence of the light regime. Cultures of each were initially grown in a 12:12 h light:dark cycle, and sampled during exponential phase in the middle of the light cycle, and after incubation in an extended dark period (24 h). Comparisons among the transcriptomes under light/dark conditions showed significant distinctions between the obligately phototrophic (*Prymnesium, Dinobryon*) and primarily heterotrophic (*Ochromonas*) species. Transcriptome data also indicated differences in nutritional strategies between the obligately phototrophic species. Expression patterns of genes in specific pathways are described and the physiological implications of these observations are discussed.

METHODS AND MATERIALS

Organisms and cultures

Prymnesium parvum strain UOBS-LP0109 (Texoma1) was isolated from Lake Texoma, OK, USA. *Dinobryon* sp. strain UTEX-LB2267 was obtained from the UTEX Culture Collection (http://www.utex.org) and *Ochromonas* sp. strain CCMP 1393 was obtained from the NCMA Culture Collection (https://ncma.bigelow.org). All cultures were grown with their attendant bacterial flora. *Prymnesium parvum* was grown in L1 medium minus silica at a salinity of 18 ppt, *Dinobryon* sp. in DYV medium (both media recipes found at https://ncma.bigelow.org) and *Ochromonas* sp. in filtered sterile seawater with 0.01% yeast extract.

Three cultures of ~2 L each were grown at 18°C in a 12h:12h light/dark regime. *Prymnesium parvum* and *Dinobryon* sp. were grown under a light intensity of ~300 $\mu E\ m^{-2}\ s^{-1}$, while *Ochromonas* sp. was grown under a light intensity of ~70 $\mu E\ m^{-2}\ s^{-1}$. Cultures were sampled once every few days to monitor growth by counting cells using a Palmer-Maloney chamber after fixing 1 mL of culture with 1% formalin. For each culture, when growth reached late exponential phase (determined by cell density in the culture), about half of the culture was harvested at approximately the sixth hour of the light period as the ‘light treatment’ sample (Fig. S2, Supporting Information). The remaining culture was immediately placed in the dark at the same temperature for 24 h, after which, cells were harvested

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as the 'dark treatment' sample. Approximately 4×10^9, 6×10^6 and 1×10^9 cells were collected for each sample for *P. parvum*, *Dinobryon* sp. and *Ochromonas* sp., respectively. The length of the dark incubation (24 h instead of 12 h) was chosen in order to distinguish changes in gene expression in response to light regime change and internal diel gene expression patterns that could exist in these three organisms.

RNA extraction, cDNA production and sequencing

Cell collection, cDNA production and sequencing procedures were as previously described (Liu *et al.* 2015a). Briefly, cells were spun down at 3200 rcf for 15 min at 15°C, and the pellets were collected. RNA was extracted from the pellet using a Ribopure kit (Ambion, Foster city, CA), treated with DNase (Sigma, St. Louis, MO), cleaned and concentrated using RNA Clean and Concentrator-25 (Zymo Research, Irvine, CA). RNA was quantified and quality-controlled and sent to the National Center for Genome Resources. cDNA libraries were constructed using TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) and sequenced on Illumina HiSeq 2000 which generated 2×50 bp (paired-end) reads as part of the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling *et al.* 2014). The original sequences are publicly available from NCBI Sequence Read Archive under accession number SRA166613 and sample IDs MMETSP0815 and MMETSP1083, for light and dark treatment of *P. parvum*; MMETSP0019_2 and MMETSP0020_2, for light and dark treatment of *Dinobryon* sp.; MMETSP0004_2 and MMETSP0005, for light and dark treatment of *Ochromonas* sp.

Bioinformatic analyses

For each species, sequences from both treatments were combined and *de novo* assembled. Assembly procedures were as previously described (Liu *et al.* 2015a). Briefly, all sequences were quality-checked using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Short reads were first assembled using ABySS (Simpson *et al.* 2009) at four different k-mer settings of 19, 25, 31 and 37. The resulting four assemblies were merged using Trans-ABySS (Robertson *et al.* 2010). Redundant contigs were removed using CD-Hit-EST (Li and Godzik 2006). Contigs were further assembled using CAP3 (Huang and Madan 1999), and assembled contigs were scaffolded using ABySS. Gap closing was attempted with GapCloser (Luo *et al.* 2012). Scaffolds with unfilled gaps were broken into contigs. CD-Hit-EST was used again to remove redundant contigs.

Annotation procedures of the assembled transcriptomes were also similar as previously described (Liu *et al.* 2015a). All contigs were searched against SILVA database (Quast *et al.* 2013) using BLAST to identify rRNA contigs. Protein-coding genes were predicted from non-rRNA contigs using ESTscan (Iseli, Jongeneel and Bucher 1999). Genes were annotated using an e-value cutoff of 1e−5 based on a variety of database searches including HMMER searches against pfam and Tigrfam databases, and BLAST searches against NCBI nr database. KEGG terms for genes were obtained using KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas/). Annotations obtained from pfam and Tigrfam searches, followed by KEGG terms were given priority when assigning annotations to genes over hits in the NCBI nr database. Annotations of some genes were manually inspected and curated.

For each species, reads were mapped back to the assembled transcriptome using BWA (Li and Durbin 2009). Correctly aligned read pairs were counted using a custom PERL script. Statistical comparison of the light and dark treatments for each species was carried out using the 'exact test' function of edgeR (Robinson, McCarthy and Smyth 2010) with common dispersion set at 0.1. *P*-values were adjusted to false discovery rate using p.adjust in R software v. 3.1.0 (Benjamini and Hochberg 1995). Only genes with adjusted *P*-values smaller than 0.05 were deemed as having significantly different expression levels between treatments. Three different culture media were chosen for the three organisms to achieve optimal growth. Because of this, comparisons of gene expression levels were conducted separately for each organism between light and dark conditions, not between any two organisms.

Predicted protein sequences of all three species were compared using OrthoMCL (Li, Stoeckert and Roos 2003) with percent match cutoff at 30% and inflation setting at 1.5 to generate homologous protein clusters. Other cutoffs such as 40% and 50% were also tested. They produced slightly less homologous protein clusters but the pattern of similarity among the three organisms was very similar regardless of the cutoff.

RESULTS

Overview of the transcriptomes

The assembled transcriptome of the three species ranged from 40 to 48 Mbp and contained between 24 000 and 41 000 predicted genes. Among them, *P. parvum* had the largest transcriptome and the most genes, while *Dinobryon* sp. and *Ochromonas* sp. had transcriptomes of similar sizes (Table 1). Homologous protein cluster analyses showed that the two chrysophytes had

Table 1. Summary of the transcriptomes of *P. parvum*, *Dinobryon* sp. and *Ochromonas* sp.

	P. parvum	*Dinobryon sp.*	*Ochromonas sp.*
No. of read pairs (light)[a]	33 615 796	32 734 851	23 409 391
No. of read pairs (dark)[a]	24 261 976	20 489 824	26 646 967
Transcriptome assembly	53 985 contigs 48.70 Mbp N50 = 1378 bp	43 853 contigs 43.47 Mbp N50 = 1634 bp	39 552 contigs 40.33 Mbp N50 = 1663 bp
Predicted genes	41 719 genes 35.84 Mbp	24 423 genes 26.20 Mbp	26 689 genes 26.68 Mbp
Percent reads mapped back to assembly (light)	74.8%	72.0%	79.3%
Percent reads mapped back to assembly (dark)	74.5%	75.8%	79.9%

[a]After quality filtering.

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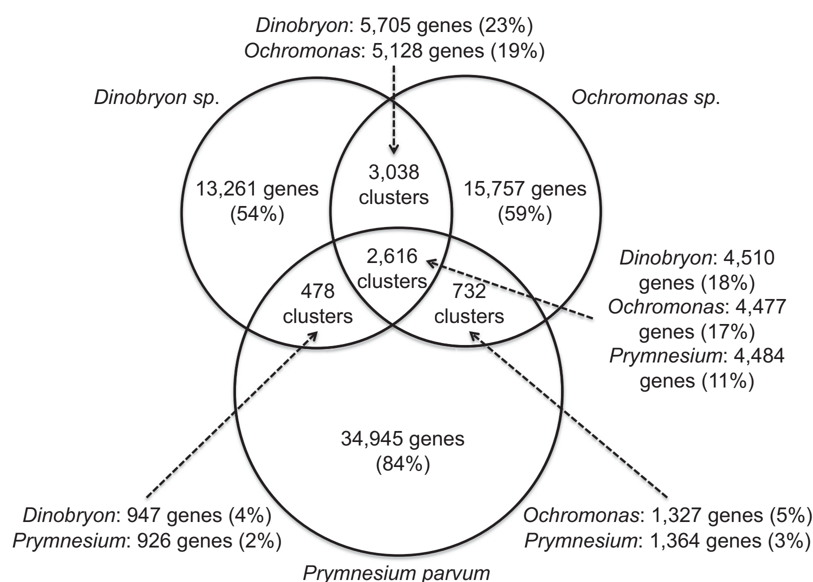

Figure 1. Summary of homologous gene clusters among *P. parvum*, *Dinobryon* sp. and *Ochromonas* sp. Numbers in parentheses indicate the percentage of all genes in that organism.

Table 2. Summary of gene contents of *P. parvum*, *Dinobryon sp.* and *Ochromonas sp.* in key metabolic pathways derived from transcriptome assemblies. Gene presence/absence was based on KEGG annotations of predicted genes from the transcriptomes. 'Complete' indicates that all genes necessary for pathways to function were detected, but alternative genes might be missing.

	P. parvum	*Dinobryon sp.*	*Ochromonas sp.*
Glycolysis/gluconeogenesis	*Complete*	*Complete*	*Complete*
TCA cycle	*Complete*	*Complete*	*Complete*
Fatty acid biosynthesis/metabolism	*Complete*	*Complete*	*Complete*
Nucleotide biosynthesis	*Complete*	*Complete*	*Complete*
Ala/Asp/Glu metabolism	*Complete*	*Complete*	*Complete*
Gly/Ser/Thr metabolism	*Complete*	*Complete*	*Complete*
Val/Leu/Ile biosynthesis	*ilvC missing*	*acetolactate synthase missing*	*acetolactate synthase missing*
Lys biosynthesis	*Complete*	*Complete*	*Complete*
Arg/Pro metabolism	*Complete*	*Complete*	*argG missing*
His metabolism	*hisB missing*	*hisBCG missing*	*hisAB missing*
Tyr/Phe/Trp biosynthesis	*trpEFG, pheA/C, tyrA missing*	*trpF, pheA/C, tyrA missing*	*trpF, pheA/C, tyrA missing*
Nitrate/nitrite reduction	*Complete*	*Complete*	*Not found*
Thiamine biosynthesis	*Not found*	*Not found*	*Not found*
Biotin biosynthesis	*bioABF found*	*only bioA was found*	*only bioA was found*
Cobalamin biosynthesis	*Not found*	*Not found*	*Not found*
Riboflavin biosynthesis	*Complete*	*Complete*	*Complete*
Vitamin B6 biosynthesis	*Complete*	*Complete*	*Complete*
NAD/NADP biosynthesis	*Complete*	*Complete*	*Complete*
CoA biosynthesis	*Complete*	*Complete*	*Complete*

much more similar gene contents to each other than to *P. parvum* (Fig. 1). Between 11% and 18% of the genes were shared by all three transcriptomes. *Dinobryon* sp. and *Ochromonas* sp. shared additional 19%–23% more genes with each other. In comparison, the genes shared by *P. parvum* with either of the chrysophytes were $\leq$5%. *Dinobryon* sp. and *Ochromonas* sp. each had 54% and 59% unique genes, respectively, while 84% of *P. parvum* genes had no homologs in the other two species.

The minor percentage of the genes shared by all three species were genes necessary for known essential metabolic pathways such as glycolysis, TCA cycle and nucleotide biosynthesis. Most of the genes necessary for the biosynthesis of all amino acids were found in all three transcriptomes with only a few missing genes (Table 2). The genes necessary for synthesizing thiamine and cobalamin were missing from all three taxa. All three transcriptomes contained large numbers of unique genes. More than 60% of those genes had no similarity to any database used in this study, and therefore had no functional annotation. The most notable general difference in the gene contents of these three algae was that *Ochromonas* sp. did not have the genes responsible for nitrate/nitrite reduction that were found in the other two algal species (Table 2).

Overview of differentially expressed genes

The transcription levels of *Ochromonas* sp. genes were very similar between the light and dark treatments (Fig. 2). Only 77 genes had significantly higher expression levels in the light treatment

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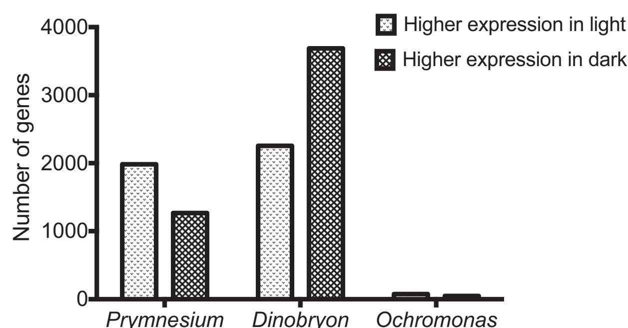

Figure 2. Numbers of *P. parvum*, *Dinobryon* sp. and *Ochromonas* sp. genes that were differentially expressed between light and dark treatments.

while 48 genes had higher levels in the dark treatment. In comparison, *P. parvum* and *Dinobryon* sp. had between 1267 and 3688 genes that were differentially expressed between the treatments (Fig. 2). The list of differentially expressed genes and the homologous gene clusters were examined together in search for homologous genes of different species that exhibited similar transcriptional behaviors. Very few genes were similarly expressed between *Ochromonas* sp. and the other two species because of the small number of differentially expressed genes in the latter species. Significant similarities were found between *P. parvum* and *Dinobryon* sp. genes that were more highly expressed in the light treatment. A total of 145 out of 1984 *P. parvum* genes and 149 out of 2256 *Dinobryon* sp. genes belonged to homologous gene clusters. On the other hand, the numbers of homologous genes that were more highly expressed in the dark treatment were much smaller (29 out of 1267 for *P. parvum* and 46 out of 3688 for *Dinobryon* sp.).

Differentially expressed *Ochromonas* sp. genes

Five of the 77 *Ochromonas* sp. genes that were more highly expressed in the light are related to photosynthesis. Three encode chlorophyll A-B binding proteins, and two others encode protoporphyrin IX magnesium-chelatase, an enzyme involved in the synthesis of chlorophylls. There were three other genes that are related to iron–sulfur cluster chemistry. One of them encodes a periplasmic protein inducible by low iron. The other two encode hydrogenase-like iron–sulfur assembly proteins. On the other hand, among 48 genes that were more highly expressed in the dark, six were involved in fatty acid biosynthesis. A gene encoding aureochrome-like protein was also more highly expressed in the dark (Table 3).

Central carbon, nitrogen and phosphorus metabolism of *P. parvum* and *Dinobryon* sp.

Chlorophyll A-B binding proteins of both *P. parvum* and *Dinobryon* sp. were more highly expressed in the light treatment, similar to the observation in *Ochromonas* sp. (Fig. 3; Tables S1 and S2, Supporting Information). Most of glycolysis/gluconeogenesis genes of both species were not differentially expressed between light and dark treatments (Tables S1 and S2, Supporting Information). Glucose kinase, which only participates in glycolysis, was more highly expressed in the light treatment in both species. Most of the TCA cycle genes were not differentially expressed (Tables S1 and S2, Supporting Information). Genes that interconnect glycolysis/gluconeogenesis and the TCA cycle had different expression patterns between the two species. Phosphoenolpyruvate (PEP) carboxykinase of *P. parvum* had higher expression level in the dark treatment, while PEP carboxylase of *P. parvum* was more highly expressed in the light treatment. In comparison, these two genes were not differentially expressed in *Dinobryon* sp. (Fig. 3; Tables S1 and S2, Supporting Information).

Three key genes in the nitrogen metabolism of *P. parvum* and *Dinobryon* sp. exhibited similar expression patterns. Glutamate dehydrogenase of both species, which catalyzes release of nitrogen from amino acids, had higher expression levels in the dark treatment. Glutamate synthase of both species had higher expression levels in the light treatment. Glutamine synthetase of *P. parvum* was more highly expressed in the light treatment, but that of *Dinobryon* sp. was not differentially expressed

Table 3. Notable *Ochromonas sp.* genes that were differentially expressed between the light and dark treatments.

ID	Gene annotation	FPKM (read pairs)[a]		Log2 light/dark
		Light	Dark	
	Genes more highly expressed in light treatment			
22928	Chlorophyll A-B binding protein	19.8 (308)	0.84 (15)	4.6
31218	Chlorophyll A-B binding protein	4.6 (52)	0.08 (0)	5.9
2419	Chlorophyll A-B binding protein	75.3 (1119)	11.5 (196)	2.7
9485	Protoporphyrin IX magnesium-chelatase	27.6 (609)	2.8 (70)	3.3
69141	Protoporphyrin IX magnesium-chelatase	2.2 (44)	0.13 (3)	4.1
11784	Low iron-inducible periplasmic protein	2315 (17700)	69.9 (613)	5.0
8359	Hydrogenase-like FeS assembly protein Nar1	30.2 (1317)	2.5 (127)	3.6
27506	Hydrogenase-like FeS assembly protein Nar1	26.2 (334)	2.0 (29)	3.7
	Genes more highly expressed in dark treatment			
2004	Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase	0.36 (12)	6.7 (259)	– 4.2
300	Malonyl CoA-(acyl-carrier-protein) transacylase	0.84 (19)	8.6 (221)	– 3.3
70609_1	Beta-ketoacyl-(acyl-carrier-protein) synthase II	7.8 (227)	65.0 (2160)	– 3.1
71230_1	Enoyl-(acyl-carrier-protein) reductase I	2.6 (76)	21.5 (716)	– 3.1
69052_1	Fatty acid desaturase	3.3 (85)	26.0 (777)	– 2.9
23159_1	3-oxoacyl-(acyl-carrier-protein) reductase	16.7 (334)	108.6 (2486)	– 2.7
4099	Aureochrome2-like protein	1.3 (56)	11.2 (534)	– 3.1

[a]FPKM refers to fragments (read pairs) per kb gene per million mapped fragment. Numbers in parentheses indicate numbers of read pairs aligned to the gene. If that number was zero, it was changed to 1 when calculating FPKM to avoid division by zero when comparing FPKM between light and dark treatments.

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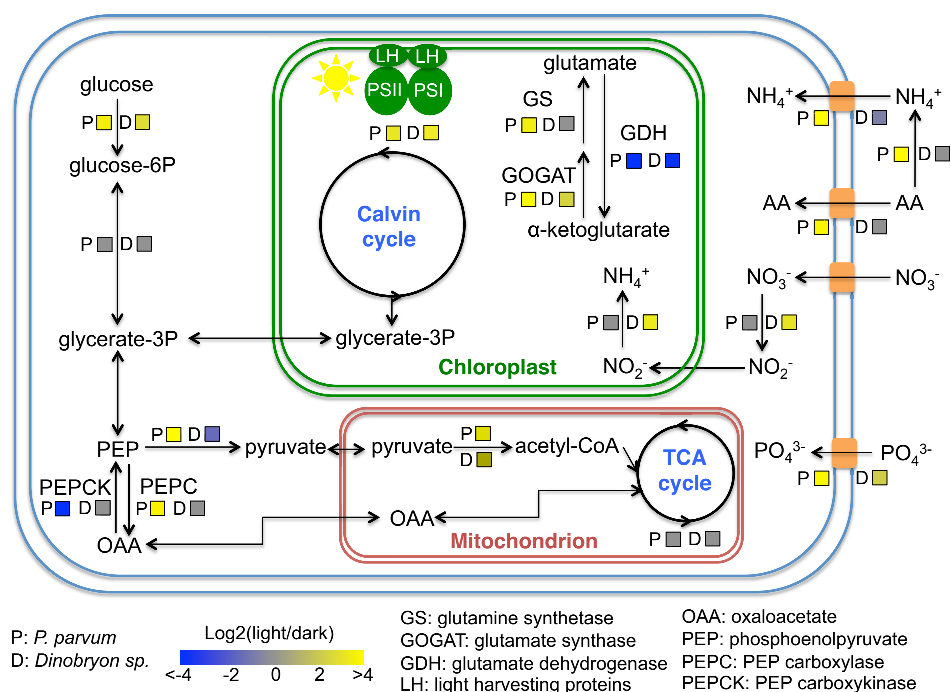

Figure 3. Expression patterns of *P. parvum* and *Dinobryon* sp. genes involved in central carbon metabolism and inorganic nitrogen and phosphorus uptake. If more than one gene involved in the same reaction was differentially expressed, the average of their expression pattern is shown. A list of the genes plotted and their read counts and FPKM values can be found in Tables S1 and S2, Supporting Information.

(Fig. 2; Tables S1 and S2, Supporting Information). These two latter enzymes together catalyze the incorporation of nitrogen into amino acids. Genes involved in inorganic nitrogen uptake of both species were in general more highly expressed in the light treatment. However, the specific genes that were differentially expressed were different between the two. In *P. parvum*, ammonium transporters had expression levels hundreds of times greater in the light treatment than in the dark, while its genes for nitrate/nitrite reduction were not differentially expressed. In *Dinobryon* sp., three ammonium transporters were differentially expressed, one higher in the light treatment, the other two higher in the dark treatment. Its nitrate reductase, nitrite transporter and nitrite reductase genes all had higher expression levels in the light treatment. In addition, a periplasmic amino acid oxidase and an amino acid transporter were more highly expressed in *P. parvum*. No such observation was made in *Dinobryon* sp. (Fig. 3; Tables S1 and S2, Supporting Information).

Two genes encoding proteins involved in inorganic phosphate transport were expressed at higher levels in the light treatment in both species (Fig. 3; Tables S1 and S2, Supporting Information). They were a sodium-dependent inorganic phosphate transporter and a protein belonging to the Pho88 protein family, which is involved in inorganic phosphate transport in yeast (Yompakdee *et al.* 1996).

Other gene expressions in *P. parvum* and *Dinobryon* sp.

Prymnesium parvum and *Dinobryon* sp. exhibited similar transcriptional responses in the dark relating to several functions and pathways that are involved in two fundamental aspects of growth. First, similar expression patterns were observed for genes involved in protein biosynthesis in both species. These genes include those involved in amino acid biosynthesis (Fig. 4A), those responsible for delivery of amino acids to the ribosome, which are aminoacyl-tRNA synthetases (Fig. 4B), and ribosomal proteins (Fig. 4C). In addition, expression patterns of genes involved in the synthesis of nucleic acids of both species were similar to those observed for protein biosynthesis genes. Those genes included genes involved in nucleotide biosynthesis (Fig. 4D), those involved in DNA replication, which were DNA primases, polymerases and replication factors (Fig. 4E), and those involved in transcription, which were RNA polymerases (Fig. 4F).

Dinobryon sp. had several pathways that also had higher expression levels in the light treatment, but their *P. parvum* counterparts were not differentially expressed between the two treatments. They included genes involved in quinone biosynthesis and a gene encoding alternative oxidase (Fig. 4G), genes involved in folate biosynthesis and one carbon metabolism (Fig. 4H), and genes encoding different subunits of vacuolar type proton ATPase (Fig. 4I).

DISCUSSION

The gene expression of three mixotrophic protists, *P. parvum*, *Dinobryon* sp. and *Ochromonas* sp. sampled during the light period and after incubation in the dark for 24 h was compared in this study. The primary nutritional modes of the three organisms, as indicated by the transcriptome data obtained, are consistent with previously established understandings based on culture experiments of the same or related species and strains (Fig. S1, Supporting Information).

Little change was observed in the *Ochromonas* sp. transcriptome after dark incubation, suggesting that its metabolism barely changed and may have continued its growth during the 24-h dark period. Photosynthesis seemed to have a limited impact on its metabolism when prey were available. On the other hand, dark incubation led to differential expression of

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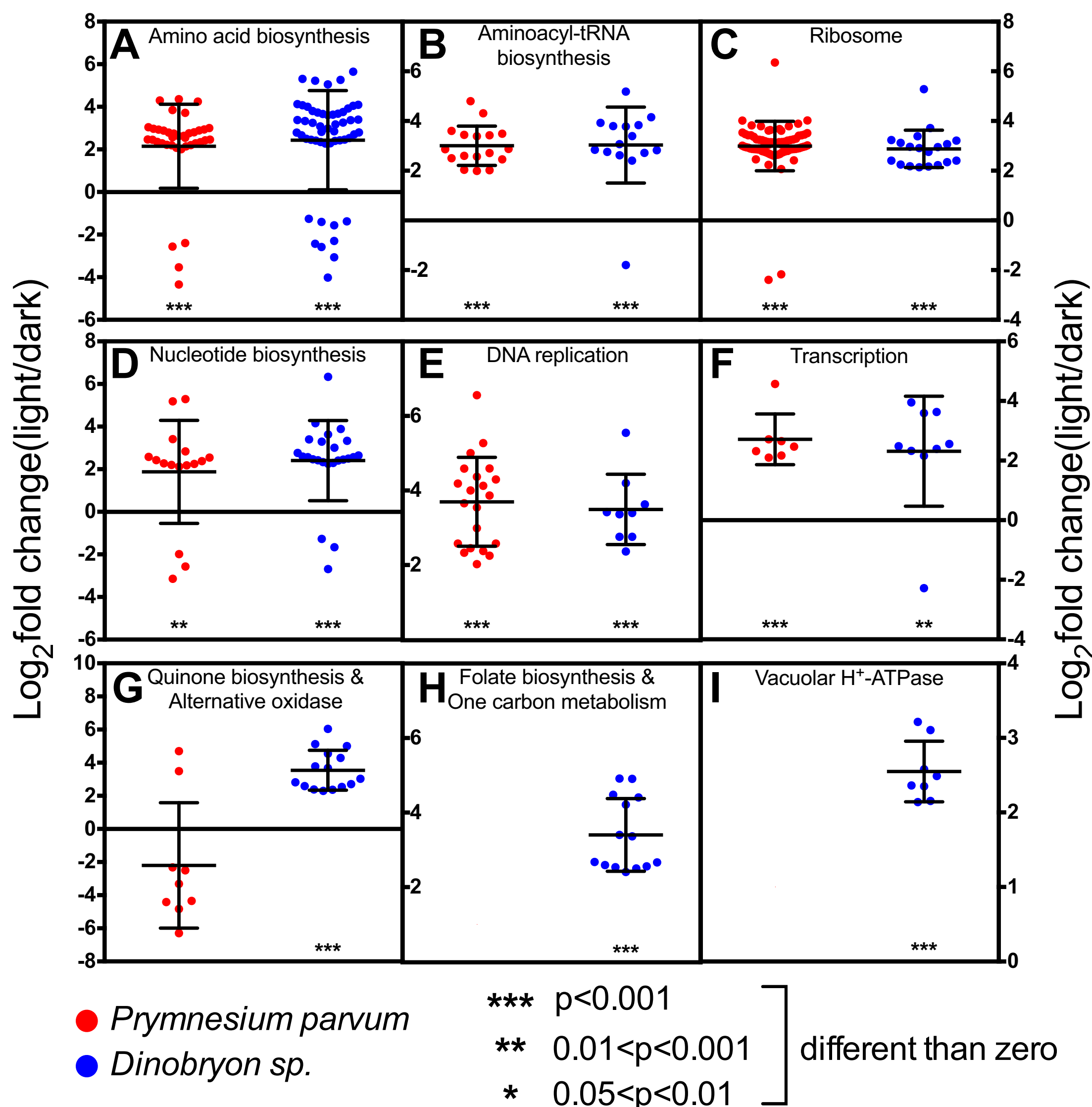

Figure 4. Expression patterns of *P. parvum* and *Dinobryon* sp. genes involved in select pathways. Log2 of fold changes (light/dark) was plotted. Each dot represents a single gene. Only differentially expressed genes are plotted. Error bars represent means ± standard deviation. For each pathway of each organism, a t-test was carried out to check whether the subset of data was significantly different from zero, and P-values are indicated by asterisks at the bottom of each panel. A list of the genes plotted and their read counts and FPKM values can be found in Tables S1 and S2, Supporting Information.

thousands of genes in both *P. parvum* and *Dinobryon* sp. (Fig. 2). Genes involved in several aspects of the biosynthesis of nucleic acids and proteins were more highly expressed in the light treatment in both *P. parvum* and *Dinobryon* sp. (Fig. 4A–F). This indicates that removal of light for 24 h altered their growth significantly. There were also changes in inorganic nitrogen and inorganic phosphorus uptake genes in both species between light and dark treatments (Fig. 3), suggesting much less need for those nutrients in the dark, presumably because of curtailed growth. These observations support the perception that *P. parvum* is primarily phototrophic (Brutemark and Granéli 2011), and that the *Dinobryon* sp. strain used in this study, like many other *Dinobryon* species, is also primarily phototrophic. Similar large-scale differential gene expression between light and dark periods of diel cycles has been previously reported in exclusively phototrophic protists (Ashworth *et al.* 2013; Zones *et al.* 2015). In contrast, the *Ochromonas* sp. strain is primarily heterotrophic (Sanders *et al.* 2001).

Different nutritional strategies of *P. parvum* and *Dinobryon* sp.

Transcriptome data showed many similarities between the two primarily phototrophic mixotrophs, *P. parvum* and *Dinobryon* sp., but they also shed light on the subtle differences in their nutritional strategies. PEP carboxykinase, which catalyzes an anaeplerotic reaction to the TCA cycle, and PEP carboxylase, which catalyzes a cataplerotic reaction, connect the glycolysis pathway and the TCA cycle. Their expression patterns have important implications on the carbon source for a species. The direct product of photosynthesis is glycerate-3-phosphate, an intermediate in the glycolysis pathway. If photosynthesis is the main carbon source, glycolysis intermediates will be distributed to the TCA cycle whose intermediates lead to many other metabolites such as amino acids. Conversely, if a species mostly depends on prey as its carbon source, the flow between glycolysis and the TCA cycle would change dramatically. Catabolism of amino

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acids, which account for the majority of the dry weight of cells, generates largely intermediates in the TCA cycle, which will be distributed to other pathways to make other molecules. In *P. parvum*, PEP carboxykinase had higher expression in the dark treatment while PEP carboxylase had higher expression in the light treatment (Fig. 3). This suggests that the carbon source for *P. parvum* changed significantly between light and dark treatments. In other words, photosynthesis was the main carbon source for *P. parvum* in the light, and when photosynthesis was disabled in the dark, organic carbon from prey, which previously plays a minor role, became the only carbon source. This is consistent with a previous study that showed *P. parvum* used CO_2 rather than carbon from prey in the light (Brutemark and Granéli 2011). However, significant decrease in expression levels of genes related to growth (Fig. 4A–F) indicated that *P. parvum* did not use organic carbon as a replacement of photosynthesis but rather for generating energy for cell maintenance in the dark. This is also consistent with previous results that *P. parvum* incorporated nitrogen and phosphorus in the dark, but not carbon (Brutemark and Granéli 2011).

On the contrary, PEP carboxykinase and PEP carboxylase were not differentially expressed in *Dinobryon* sp. (Fig. 3). This observation implies that the carbon source for *Dinobryon* sp. did not change significantly as a consequence of the change in light regime. There are two logical explanations to this observation. One is that photosynthesis was not a major carbon source even in the light. This speculation is contradicted by multiple studies that have shown that 50%–75% *Dinobryon* spp. cellular carbon originates from photosynthesis (Bird and Kalff 1986; Caron *et al.* 1993). The other and more probable explanation is that *Dinobryon* sp. used a combination of both organic and inorganic carbon in the light, and consumption of organic carbon from prey dramatically decreased along with photosynthesis in the dark. Other data in this study supported this explanation. Vacuolar type proton ATPase genes of *Dinobryon* sp. had much lower expression in the dark treatment (Fig. 4I). Vacuolar type proton ATPase pumps protons into phagosomes to lower their pH for the digestion of food. The expression patterns of these suggest that the digestion of prey in *Dinobryon* sp. phagosomes decreased in the dark. This explanation is consistent with previous results that show the ingestion rate of *D. cylindricum* sharply decreased to near zero in the dark (Caron *et al.* 1993).

Based on data from this study, heterotrophy appears to serve different roles in *P. parvum* and *Dinobryon* sp. For *P. parvum*, prey are a source of supplementary nutrition and energy. Organic carbon is not a major carbon source; prey does not help *P. parvum* grow faster (Liu *et al.* 2015a); organic carbon serves as an alternative energy source for *P. parvum* in the dark. This suggests that limitation imposed by photosynthetic output, i.e. the need for organic carbon, is not the reason for the predatory behavior of *P. parvum*. It seems more likely that *P. parvum* consumes prey for the nitrogen and phosphorus contained in them. *Prymnesium parvum* has been shown to be more toxic when nitrogen or phosphorus limit population growth (Granéli and Johansson 2003; Hambright *et al.* 2014) and it incorporates significant amounts of nitrogen and phosphorus both in the light (Carvalho and Granéli 2010) and the dark (Brutemark and Granéli 2011). *Dinobryon* sp., on the other hand, seemed to treat prey as an essential nutrient. Organic carbon from prey contributed to the growth of *Dinobryon* sp., but when photosynthesis was disabled, prey consumption dramatically decreased along with the uptake of inorganic nitrogen and phosphorus. It is possible that *Dinobryon sp.* requires both inorganic and organic carbon to grow, or that prey are a source of essential micronutrient(s) for *Dinobryon* sp.

Other notable gene expression patterns

Expression patterns of nitrogen uptake genes also indicated a possible difference in preferred nitrogen source between *P. parvum* and *Dinobryon* sp. In *P. parvum*, ammonium transporter genes and genes encoding a periplasmic amino acid oxidase and an amino acid transporter had much higher expression in the light, while the nitrate/nitrite reduction genes were not differentially expressed (Fig. 3). The periplasmic amino acid oxidase gene is homologous to a gene in *Chlamydomonas reinhardtii*, which has been shown to oxidize extracellular amino acids to release ammonium (Vallon *et al.* 1993). The tighter control on the uptake genes for ammonium and amino acid suggests that they were the main source of nitrogen for *P. parvum*. This is consistent with previous results that showed the preference of amino acids over urea and nitrate and extracellular use of amino acids (Lindehoff, Granéli and Glibert 2011).

Dinobryon sp. showed greater regulation between light and dark treatments for nitrate/nitrite reduction genes (Fig. 3). The meaning of this observation is less clear. It could imply that nitrate/nitrite reduction was the more prominent nitrogen source for *Dinobryon* sp. However, this disagrees with previous studies that reported preference for ammonium over nitrate in several *Dinobryon* species (Lehman 1976). The expression patterns of nitrate/nitrite reduction genes might simply be explained by the change in light because nitrate/nitrite reduction depends on the reductants produced by photosystem I. Regardless, the differences between nitrogen uptake gene expression between *P. parvum* and *Dinobryon* sp. imply that preferences for ammonium and amino acids are much stronger in *P. parvum* than in *Dinobryon* sp.

Quinone biosynthesis genes and a gene encoding alternative oxidase were more highly expressed in the light by *Dinobryon* sp. (Fig. 4G). Alternative oxidase catalyzes an alternative respiratory pathway that bypasses complexes III and IV in the mitochondrion, and is an important mechanism for protecting respiratory and photosynthetic electron transport chains from over-reduction (Vanlerberghe and McIntosh 1997). Alternative oxidase plays a crucial role in chloroplast protection under stress such as high light (Xu, Yuan and Lin 2011), and its gene expression could be stimulated by reactive oxygen species (Wagner 1995). Increased biosynthesis of quinone/quinol effectively increases the capacity of the electron transport chain, thereby reducing the risk of its over-reduction. Genes involved in folate biosynthesis and one carbon metabolism, which uses tetrahydrofolate as its cofactor, had similar expression patterns in *Dinobryon* sp. (Fig. 4H). One carbon metabolism is a part of many different pathways and functions, and one of them is photorespiration (Wingler *et al.* 2000; Jabrin *et al.* 2003). Photorespiration, acting as an energy sink, is another mechanism to prevent the over-reduction of the photosynthetic electron transport chain (Wingler *et al.* 2000). These observations together imply that *Dinobryon* sp. may have experienced photo-oxidative stress under the current experimental conditions.

An *Ochromonas* sp. gene encoding low iron inducible periplasmic protein was more highly expressed in the light treatment (Table 3). This gene is homologous to FEA1 of *C. reinhardtii*, which has been shown to facilitate iron uptake (Narayanan *et al.* 2011). The result implies *Ochromonas* sp. takes up more iron in the light. Iron is incorporated into iron–sulfur proteins that are involved in various functions such as photosynthesis and redox control. Two iron–sulfur assembly proteins had similar expression patterns in this study (Table 3). These two genes are homologous to the yeast Nar1 gene, which is essential for assembly of

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iron–sulfur clusters in the cytosol (Balk *et al.* 2004). Recently, it has been shown that Nar1 may play a role in the regulation of sensitivity to oxygen (Fujii *et al.* 2009). This implies that *Ochromonas* sp. also may have experienced photo-oxidative stress under the current experimental condition, yet exhibiting this effect through different gene expression than observed in *Dinobryon* sp. A group of fatty acid biosynthesis genes were more highly expressed in the dark treatment. A gene encoding aureochrome, a blue light receptor and transcription regulator found in photosynthetic stramenopiles (Takahashi *et al.* 2007), also had higher expression in the dark treatment (Table 3). The exact function of this regulator is still unclear. However, it has been shown recently that overexpressed aureochrome stimulates lipid accumulation in yeast (Huang *et al.* 2014). This information is consistent with the expression patterns of the fatty acid biosynthesis genes observed in the present study, and implies that *Ochromonas* sp. might shift its metabolism in the dark toward making more lipids, possibly as storage product.

In this study, we were able to confirm the primary nutritional modes of three mixotrophic protists through light/dark comparisons of their transcriptomes. Our data demonstrate the effectiveness of transcriptomics approaches in indicating primary nutrition modes of mixotrophic protists. We are hopeful that similar molecular approaches would reveal nutritional strategies of other mixotrophic protists that are poorly understood. These data also offered more detailed implications on the physiology of these organisms, especially the difference between the predominantly phototrophic species *P. parvum* and *Dinobryon* sp. in terms of their utilization of prey in light and dark. Specific genes identified in our study could potentially be used as target genes in field studies of mixotrophic protists to document their specific nutritional activities in nature. Our study offered a first glimpse into the transcriptional responses of different mixotrophic protists to light and darkness. Further studies with more species and more time points throughout the light regime could add much more to our understanding of the ecophysiology of mixotrophic protists. For example, it would be interesting see if and when the changes in gene expression observed in this study are manifested over a typical diel light cycle in *P. parvum* or *Dinobryon* sp., and whether those changes are observed *in situ*. This study illustrates the potential to use molecular approaches to investigate mixotrophic protists, especially those difficult to culture, as RNA-Seq technologies including metatranscriptomics mature and become more accessible while cultivation remains difficult and unpredictable. Mixotrophic protists with different nutritional strategies obviously play different ecological roles. Understanding those nutritional strategies is essential for accurately modeling their behaviors and biogeochemical roles.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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