Nitrogen-Deprivation Elevates Lipid Levels in *Symbiodinium* spp. by Lipid Droplet Accumulation: Morphological and Compositional Analyses

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Abstract

Stable cnidarian-dinoflagellate (genus *Symbiodinium*) endosymbioses depend on the regulation of nutrient transport between *Symbiodinium* populations and their hosts. It has been previously shown that the host cytosol is a nitrogen-deficient environment for the intracellular *Symbiodinium* and may act to limit growth rates of symbionts during the symbiotic association. This study aimed to investigate the cell proliferation, as well as ultrastructural and lipid compositional changes, in free-living *Symbiodinium* spp. (clade B) upon nitrogen (N)-deprivation. The cell proliferation of the N-deprived cells decreased significantly. Furthermore, staining with a fluorescent probe, boron dipyrromethene 493/503 (BODIPY 493/503), indicated that lipid contents progressively accumulated in the N-deprived cells. Lipid analyses further showed that both triacylglycerol (TAG) and cholesterol ester (CE) were drastically enriched, with polyunsaturated fatty acids (PUFA; i.e., docosahexaenoic acid, heneicosapentaenoic acid, and oleic acid) became more abundant. Ultrastructural examinations showed that the increase in concentration of these lipid species was due to the accumulation of lipid droplets (LDs), a cellular feature that have previously shown to be pivotal in the maintenance of intact endosymbioses. Integrity of these stable LDs was maintained via electronegative repulsion and steric hindrance possibly provided by their surface proteins. Proteomic analyses of these LDs identified proteins putatively involved in lipid metabolism, signaling, stress response and energy metabolism. These results suggest that LDs production may be an adaptive response that enables *Symbiodinium* to maintain sufficient cellular energy stores for survival under the N-deprived conditions in the host cytoplasm.

Introduction

*Symbiodinium* spp., a unicellular dinoflagellate, is commonly found in mutualistic associations with invertebrates such as corals and anemones, and able to transfer more than 90% of its photosynthetically fixed carbon to the host cytoplasm in which it resides [1]. This intracellular symbiosis (i.e. the endosymbiosis) has been the focus of intensive research due to its pivotal role in maintaining the health of corals and homeostasis of the marine ecosystem.

Numerous studies have highlighted the crucial role of nutritional status and nutrient transfer in these endosymbioses [2]. The supply of organic carbon by *Symbiodinium* to the hosts and the recycling of essential nutrients during such associations have contributed to the success of coral reefs in nutrient-limited tropical seas [3]. On the other hand, the growth of *Symbiodinium* is dependent on nutrients from various sources including exogenous seawater, host catabolism, and host heterotrophy [4,5]. Nitrogen, which is one of the most important essential nutrients, can be excreted as ammonium by the host [6].

The host metabolism plays a significant role in regulating the nutritional status of its endosymbionts (i.e. the symbiotic *Symbiodinium*) [7]. For instance, it has been shown that the endosymbionts were present in nutrient-limited environments [8,9], particularly with respect to nitrogen sources. Using the infrared microspectroscopy, it has been shown that the concentration of nitrogenous compounds was significantly lower in endosymbionts, compared to that of free-living *Symbiodinium* [9]. It suggests that the cytoplasm of the host cell may be nitrogen-deficient and alter various physiological of the endosymbiont. In examining the effect of nutrient supplement of host anemones on their endosymbionts, Zhu and colleagues have observed that there was an increase formation of lipid droplets (LDs) in endosymbionts after 45 days of nutrient starvation on host sea anemones [10]. However, the cellular and molecular mechanisms of this regulation remain to be elucidated.

Nitrogen deprivation represents an important source of stress for microalgae, which causes various changes in cellular metabolism and development. For example, treatments with nitrogen deprivation tend to elevate lipid production in algae [11,12], such as increases of cellular triacylglycerols (TAGs) stored in the cytoplasmic lipid droplets (LDs) [13]. TAGs, which are composed primarily of saturated and monounsaturated fatty acids, can be
efficiently packed into the cell and generate more energy than carbohydrates upon oxidation, thus constituting the best reserve for rebuilding the cell upon returning to homeostatic conditions [14]. The proteins associated with these LDs have been the focus of investigations in order to elucidate the mechanism of LD formation [15]. Recent studies have shown that the LD-associated protein in Chlorella cells belong to the caeleosin family [16,17]. Another lipid droplet protein, the major lipid droplet protein (MLDP), was also revealed in the LDs of various green microalgae including Chlamydomonas reinhardtii, Haematococcus pluvialis [18,19] and Dunaliella [20].

In order to elucidate the mechanism that allows the endosymbionts to adapt to the nitrogen-limited environment of cnidarian hosts, the present study aimed to first examine the cellular response of free-living Symbiodinium (clade B) to nitrogen deprivation treatment. To the best of our knowledge, the effect of nitrogen deprivation in free-living Symbiodinium spp. has never been described, mainly due to the relative difficulty of cultivating Symbiodinium spp. with synthetic culture media [21]. This is an unfortunate knowledge dearth, as nitrogen is an important nutrient required for the metabolism of Symbiodinium [22]. Under nitrogen deprivation, the specific goal here was to examine the changes in cellular biology, including the proliferation, lipid contents and ultrastructure in free-living Symbiodinium spp. Results show that there is increased formation of LDs, a phenomenon similar to that occur in symbiotic Symbiodinium. Subsequently, LDs of these N-deprived cells were purified for biochemical analyses for proteins and lipids. Results of the present study should provide important information in elucidating possible regulatory mechanisms underlying LDs formation in nitrogen-deficient environments such as those in the symbiotic association with Cnidaria.

Materials and Methods

Symbiodinium culture and the nitrogen-deprivation treatment

The free-living Symbiodinium spp. (clade B) used in this study were originally isolated from the sea anemone Aiptasia pulchella [9]. They were cultured in the f/2 medium [23] in filtered seawater (FSW) at room temperature under a photosynthetically active radiation (PAR) of 40 μmol m⁻² s⁻¹ in a 12-h light/12-h dark (12L/12D) cycle. Media were prepared with two different concentrations of nitrogen (as NaNO₃). Two new batch cultures were grown, one in nitrogen-deficient f/2 medium (no NaNO₃ was added to the medium) and the other in nitrogen-sufficient f/2 medium (0.882 mM NaNO₃).

Symbiodinium clade identification

The genetic identity (18S rDNA) of the cultured Symbiodinium was examined by PCR-RFLP [Polymerase chin reaction-Restriction fragment length polymorphism] analysis [24], and shown to be from clade B. Symbiodinium DNA was extracted using a plant genomic DNA extraction miniprep system (VIOGENE, Taipei). Basically, Symbiodinium nuclear small subunit (n18S-rDNA) was amplified by PCR) from 3 replicate extracts of each of the two cultures using the primers, ssz5 (an equimolar mixture of the oligonucleotides 5'-GGAGTTATATTAGTATGGCTAC-TGC-TACC-3' and 5'-GGAGTTATATTAGTATGGCTAGGT-GCTGCTAC-3') and ssz3 (5'-AGCACTGCGCTAGTCCGA-TATTAACCAGG-3') and digested with the restriction enzyme, Tag 1 and Sau3A I (Promega, USA); Digestion products were separated by electrophoresis on 1.5% 0.3x TAE (Amresco, USA) agarose gels, to generate the RFLP pattern. RFLP pattern analysis was compared to the literature [24] to assign each culture to one of the established Symbiodinium n18S-rDNA RFLP clades.

Cell density analysis

Symbiodinium proliferation was examined with hemocytometer-based cell counting. Cell densities were determined daily by placing an aliquot of well-mixed culture suspension on a Neubauer hemocytometer (Marienfeld, Germany) under a Axioskop 2 Plus microscope (Zeiss, Germany) connected to a CCD (charge-coupled device) camera (Photometrics, USA).

Chlorophyll a and protein determinations

Symbiodinium were harvested by centrifugation at 12000 g for 10 min from the control and nitrogen-deprived culture. For each replicate during the analysis, a volume of culture containing 5×10⁹ cells was used. Chlorophyll a was extracted in 90% acetone (v/v), and their amount was estimated spectrophotometrically as previously described [23]. The protein content was determined using the BCA protein assay kit (Invitrogen, USA) on the same samples used for chlorophyll quantification.

The isolation of lipid droplets (LDs) from nitrogen-deprived Symbiodinium

LDs were isolated from the nitrogen-deprived culture (1L) on days 5 and 7 by first homogenizing at 4°C in a “grinding buffer” (0.6 M sucrose in 10 mM sodium phosphate buffer, pH 7.5) according to a published procedure [26]. After filtration, each 8-ml portion of the homogenate was placed at the bottom of a 12-ml centrifuge tube, and 2-ml of “floation buffer” (0.4 M sucrose in 10 mM sodium phosphate buffer, pH 7.5) was layered on top. The tube was centrifuged at 35,000×g for 60 min in a swinging-bucket rotor centrifuge (Beckman Coulter, USA). LDs on the top layer were collected and re-suspended in the “detergent washing solution” (0.4 M sucrose, 0.1% Triton X-100 in 10 mM sodium phosphate buffer, pH 7.5) to remove non-specifically associated proteins. After a further centrifugation, LDs on the top were collected and then re-suspended in the floating buffer to remove excess detergent. The washing step was repeated two more times. Finally, the purified LDs were re-suspended in the grinding buffer and stored at −20°C until further analysis.

Structural integrity of Symbiodinium LDs

The structural integrity of LDs isolated from Symbiodinium was assessed by examining the surface properties (steric hindrance and electrostatic repulsion) that accounted for the aggregation of LDs without fusion at pH 6.5 [26]. Symbiodinium LDs suspended in 5 mM sodium phosphate buffer, pH 7.5 or 6.5, were kept at 23°C for 6 hrs. To confirm that the steric hindrance was provided by surface proteins, a 2-ml preparation of Symbiodinium LDs was subjected to trypsin (2.5 μg; bovine pancreas type III, Sigma, USA) digestion at 37°C for 30 min.

Lipid analyses

All solvents for lipid analyses were analytic grade. Lipid contents of 3 replicates from the nitrogen-sufficient f/2 medium and nitrogen-deprived culture Symbiodinium cells were extracted by the Bligh and Dyer procedure [27]. Neutral lipids in the LDs isolated from Symbiodinium cells were extracted with 150 μl of chloroform/methanol (2:1, v/v). After centrifugation, the lower chloroform fraction was collected for the analysis by thin layer chromatography (TLC) [Anatech, USA] with the solvent system modified from previous reports [28,29]. Briefly, TLC was first developed to the Rf=1 position in hexane. The plate was air-dried and then
developed to the top (Rf = 1) in benzene. The plate was air-dried and then developed to the top (Rf = 0.5) in hexane: diethyl ether: acetic acid (70:30:1 v/v/v/v). The lipid visualization on TLC plates was performed by staining with 0.03% Coomassie blue R 250 (Sigma, USA) dissolved in 20% methanol containing 0.5% acetic acid [30]. Concentrations of individual lipid species were then quantified using the Metamorph Image Processing system (Molecular Devices Inc., Toronto, Canada) based on calibration curves of individual lipid standards (Wax ester: Sigma-Aldrich, USA; TAGs [mixed triacylglycerides: tricaprin, tricaprylin, trilaurin, tristearin, tripalmitin]; Sigma-Aldrich, USA; Choles-
terol: Avanti Polar Lipids, USA; CE: Sigma-Aldrich, USA) co-run on the same TLC plate. To analyze the phospholipids in extracted lipids, chloroform: acetic acid: methanol: water (70:25:5:2, v/v/v/v) was used as the TLC solvent system.

Analyses of fatty acid composition in cellular TAGs by gas chromatography (GC)-mass spectrometry (MS)

*Symbiodinium* cells collected after five days of culture in either nitrogen-enriched (control) or nitrogen-deprivation f/2 medium were dried by lyophilization. Total lipids in dried cells were extracted and separated by TLC as previously described. TAGs on TLC plates was first identified by 0.01% primuline spraying (in 80% acetone), and then extracted by the Bligh and Dyer procedure [27]. Isolated TAGs were first saponified in 1N sodium hydroxide-methanol solution for 15 min at 100 °C. After hexane extraction, the fatty acid methyl esters were analyzed on a gas chromatograph (GC, Varian CP-3800) and a mass spectrometer (Varian 320 MS) operated in full scan mode; scan range from 100 to 450 m/z. The column was a Saturn GC/MS (Varian, USA) to identify fatty acids. For Western blotting, proteins were transferred from SDS-PAGE onto a nitrocellulose membrane in a Trans-Blot system (Bio-Rad, USA) according to the manufacturer’s instructions and visualized on a ChemiDoc XRS+ (Bio-Rad, USA). In-gel digestion of the lipid droplet proteins in *Symbiodinium* spp.

Five protein bands of *Symbiodinium* lipid droplet resolved by SDS-PAGE were manually excised from the gel and ground into pieces. After washing with 50% acetonitrile and 50% acetonitrile/25 mM ammonium bicarbonate, the protein was reduced and alkylated at 56°C for 45 min in 10 mM dithiothreitol and 55 mM iodoacetamide in 25 mM ammonium bicarbonate, followed by overnight in-gel digestion with 0.1 µg in 15 µl of TPCK-treated modified porcine trypsin (Promega, USA) in the same buffer at 37°C. The supernatant containing tryptic peptides was combined with two more extracts of the gel by 50% acetonitrile/5% formic acid. The sample was analyzed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) and MALDI-MS/MS. All data were acquired by quadrupole-time-of-flight (Q-TOF) hybrid mass spectrometers (Micromass Q-ToF Ultima, Manchester, UK, and Applied Biosystems QSTAR, USA), in which 2-cyano-4-hydroxycinnamic acid was used as the matrix. The low-energy collision-induced dissociation MS/MS product ion spectra acquired from Q-TOF Ultima and QSTAR were analyzed by Micromass ProteinLynx™ Global Server 2.0 and Applied Biosystems BioAnalyst™ data processing software, respectively. For protein identification, the acquired MS/MS spectra were automatically searched against the NCBI nr database using the Mascot search program (www.matrixscience.com) restricted to all entries taxonomy. The mass tolerance parameter...
was 20 ppm, the MS/MS ion mass tolerance was 1 Da, and up to one missed cleavage was allowed. Variable modifications considered were methionine oxidation and cysteine carboxamidomethylation. Positive identification of proteins was confirmed by observation of at least one of the following criteria: (i) the total number of matched peptides (mps) is more than 2, or (ii) the mps equals 2 with two different matched peptides, or (iii) the MOWSE score has to be higher than 70 which indicates identity or extensive homology (p<0.05).

Statistical analysis
All statistical analyses were performed using SigmaStat 3.5 (Systat software, Chicago, IL, USA). The results were expressed as mean±SD (standard deviation of the mean).

Results
Nitrogen-deprivation induces lipid content increase in Symbiodinium
To examine the effects of nitrogen deprivation on Symbiodinium growth and lipid accumulation, free-living Symbiodinium in f/2 medium at the early stationary phase were transferred to nitrogen-free f/2 medium. As shown, cells cultivated in the nitrogen-deprived medium proliferated more slowly than those cultivated in the normal medium, particularly after three days of experimentation (Fig. 1A).

The lipid accumulation in nitrogen-deprived Symbiodinium could be visualized by staining the cells with a neutral lipid specific dye BODIPY 493/503. In Symbiodinium grown in control medium, little lipid accumulation was observed as shown by very dim BODIPY staining (Fig. 1B). On the contrary, BODIPY fluorescence gradually increased in cells with the nitrogen-deprivation treatment (Fig. 1B; day 1 to day 9), indicating an increase of lipid accumulation.

The change in lipid content during nitrogen-deprivation treatment was then analyzed by TLC (Fig. 2). In the nitrogen-deprivation culture, Symbiodinium began to accumulate neutral lipids including TAGs and CEs (cholesterol esters); both of which were very low (<1 pg per cell) in control culture (Fig. 2A). Concentration of both TAGs and CEs increased to reach maximal levels (168.57±4.93 and 13.5±0.52 pg cell⁻¹, respectively) after seven days of nitrogen-deprivation treatment (Table 1).

As the amount of TAG was significantly increased by nitrogen-deprivation treatment, their fatty acid compositions in control and nitrogen-deprivation treatment for five days were further analyzed by GC-MS (Fig. 3). In the control group of Symbiodinium, four major fatty acids were identified, such as C14:0, C16:0, C18:0 and C22:1. Among them, C16:0 and C18:0 were the most abundant, occupying approximately 38% and 32% of total fatty acids, respectively. After cultivation in nitrogen-deprived medium for 5 days, the percentages of C14:0 and C16:0 remained unchanged. Nevertheless, C18:0 drastically decreased from about 32% to 6.4%. On the other hand, apart from the C12:0 and C22:0, the

Figure 1. Effect of nitrogen-deprivation on the cell proliferation and lipid accumulation in Symbiodinium. (A) Growth of Symbiodinium cells cultivated in control versus nitrogen-deprivation media. The data represents mean ± SD (n=3). (B) The visualization of neutral lipid accumulation using BODIPY 493/503 in control vs. nitrogen-deprived cultures. Scale bar, 10 μm.
amount of numerous polyunsaturated fatty acids (PUFAs) such as C16:1, C18:1, C18:2, C20:5, C21:5 and C22:6, increased significantly. In other words, the proportion of PUFAs over saturated fatty acids (SFA) in TAGs increased in Symbiodinium cells under the nitrogen-deprived condition.

Ultrastructural changes in Symbiodinium by nitrogen-deprivation

Morphological changes of Symbiodinium after nitrogen-deprivation treatment were investigated by TEM (transmission electron microscopy). First, the average cell size (diameter) of Symbiodinium cultivated in nitrogen-deprivation medium at day 5 and 7 increased significantly to 7.35±0.86 μm and 6.96±0.96 μm, respectively (versus 6.54±1.02 μm in control) (see Table 2). Secondly, the change in cell size was concurrent with cell wall thickness changes (Table 2 and Fig. 4). Specifically, the thickness of the cell wall increased three fold over the controls, from 0.08±0.04 μm to 0.24±0.06 μm at day 5, and then decreased to 0.17±0.05 μm at the 7th day (see also the arrows in Figs. 4A, 4C, and 4E). Thirdly, there were 83.24% and 86.1% reductions in chlorophyll a concentration relative to the controls in nitrogen-deprivation cells at day 5 and 7, respectively (Table 2). Finally, there were significant changes in the size and number of LDs after nitrogen-deprivation treatment (Table 2 and Fig. 4). A number of large LDs accumulated in the cytosol of Symbiodinium (Figs. 4C and 4E) in nitrogen-deprivation samples. The LDs formation was greatly induced by nitrogen-deprived at day 5, as shown in Figs. 4C-D. Furthermore, the sizes of these LDs significantly increased from 0.72±1.02 μm² to 2.90±1.88 μm² after 7 days of the nitrogen-deprivation treatment (Table 2). Moreover, numerous inclusion bodies, which are OsO4 staining-negative, appeared inside the LDs at the 7th day of nitrogen-deprivation treatment (see the arrowhead in Fig. 4F).

Analysis of LD-associated proteins and lipids during the nitrogen-deprivation

LDs were isolated from the cultured Symbiodinium incubated in nitrogen-deprivation medium for 5 days. It showed that the LDs purified from the Symbiodinium cells maintained as individual particles in a medium of pH 7.5 at 23°C (Fig. 5A). An aggregation of these LDs was induced by lowering the pH of the medium to 6.5; most of these aggregates did not coalesce when left overnight.
at 23°C (Fig. 5B). After the trypsin treatment, LDs coalesced, floating rapidly and forming a transparent layer on the top of the reaction solution (Fig. 5C). Therefore, the structural integrity of the purified LDs was presumably maintained via the electrostatic repulsion and steric hindrance, in a manner similar to the stable LDs isolated from the *Chlorella* cells [16]. Furthermore, there was significant amount of membrane phospholipids (PLs) in purified LDs but not the lower layer of fractions after the detergent washing (see the “Materials and methods” section) (Fig. 5D). This further indicates that the purified LDs have maintained their membrane integrity during the purification process.

The purity of the purified LDs was confirmed based on their absence of a chloroplast-specific protein, RuBisCO, by western blotting according to a published procedure [33] (Fig. 6A). The high lipid contents of the purified LDs were confirmed by strong BODIPY 493/503 staining (Fig. 6B). The TLC analysis showed...
that the purified LDs contained mainly neutral lipids such as TAGs and CEs (see Fig. 2B), which are both the major lipid component in whole cells as shown in Fig. 2A.

Five major LD-associated protein bands with an estimated molecular weight ranging from 17 to 84 kDa (Fig. 6A) were excised and analyzed by mass spectrometry. Seven proteins were identified, with a variety of cellular functions including lipid metabolism (“Sterol transfer protein” from the protein band 5, MW~17 kDa), signaling (“14-3-3 protein” from the protein band 4, MW~19 kDa), stress responses (“Heat shock protein HSP90” from the protein band 1, MW~84 kDa and “Osmotically inducible protein Y” from the protein band 3, MW~28 kDa and “ADP-ribosylation factor (ARF)” from the protein band 4, MW~19 kDa), energy metabolism (“Mitochondria ATP synthase F1 alpha subunit-like protein 1” from the protein band 2, MW~59 kDa and “GTP-binding protein” from the protein band 4, MW~19 kDa) (Table 3).

**Discussion**

**Effects of nitrogen-deprivation on *Symbiodinium* proliferation and morphology**

The deprivation of nitrogen, one of the nutrient limitations that critically affect the cellular metabolism, has been shown to induce lipid accumulations in numerous strains of microalgae [13,34]. *Symbiodinium* spp. is an unique marine microalgae with two living status, either freely living in open ocean or symbiotically residing inside the gastrodermal cells of most marine eukaryotic such as sea anemones and corals [1]. Here, we have shown for the first time that free-living *Symbiodinium* accumulate lipids in the form of LDs when the nitrogen source of the culture medium was deprived. Positive growth of *Symbiodinium* spp. was observed after 3 days of the cultivation either under the normal condition or nitrogen starvation. However, it was observed that the slow growth pattern of *Symbiodinium* and accumulation of the LDs occurred under the nitrogen-deprivation (Fig. 1A). This has been reported in several algae that accumulated carbon metabolites under nitrogen-deficient condition, resulting in slow growth and lipid accumulation [35,36]. However, the nitrogen starvation may also induce an abrupt loss of electron transport capacity in the photosystem II, resulting in the change of the photochemical efficiency (Fv/Fm) and growth of *Symbiodinium* [37].

The ultrastructural observation indicated that, after the 7th day of nitrogen deprivation, numerous inclusion bodies started to appear in the LDs (Figs. 4E and 4F). The present study also demonstrated that there was a decreased lipid accumulation in cells on day 9 (Fig. 1B). It was presumed that during day 7 to day 9, *Symbiodinium* used the lipid in order to survive since the reserved nitrogen source had been used up. After 9 days of nitrogen starvation, the *Symbiodinium* cells entered the death phase (Fig. 1A).

Neutral lipids including TAG and CE, are the major lipid species in these LDs. Moreover, concurrently with drastic increases in size and number of LDs, detailed ultrastructural examinations indicated that the increases in cell size and cell wall thickness are typical features induced by the nitrogen deprivation (see Fig. 4 and Table 2). These morphological observations are similar to those reported on the morphological changes in green alga *Scenedesmus obtusiusculus* by nutrient starvation [38,39]. The increases in cell size and cell wall thickness were probably due to the nutrient deficiency, which led to a delay in cell cytokinesis and metabolism.

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**Table 2. Influence of duration after nitrogen depletion on *Symbiodinium* cells.**

|                         | Control               | Starvation (Day 5) | Starvation (Day 7) |
|-------------------------|-----------------------|--------------------|--------------------|
| Cell size (diameter, µm) | 6.54±1.02* (n=86)     | 7.35±0.86b (n=123) | 6.96±0.96b (n=110) |
| Cell wall (thickness, µm) | 0.08±0.04* (n=63)     | 0.24±0.06a (n=115) | 0.17±0.05* (n=109) |
| Chl a/protein (µg 100cell^-1) Reduction of Chl a (%) | 83.27±0.398 0% | 3.98±0.011 95.22% | 3.30±0.011 96.03% |
| LDs size (area, µm^2) | 0.72±1.02* (n=117)     | 2.66±1.61b (n=436) | 2.90±1.88c (n=497) |

Impact of the nutrient regime in cell pattern between the normal growth and nitrogen starvation (i.e Cell size, LD size, Chl a, Cell wall) as analyzed by ANOVA. Upper level a-c denote statistical significance different between control growth, day 5(starvation) and day 7(starvation), respectively (P<0.001). Cell wall and cell size: n = number of cell analyzed, LD size: n = number of LDs analyzed.

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Under severe nutrient limitation, cell division-arrested phytoplanktons were found to accumulate glycoproteins and other carbon contents in starch granules. As also shown in the present study, the proliferation of N-deprived *Symbiodinium* decreased significantly in comparison to the cells grown in normal medium (Fig. 1A). Similar inhibitory effects of nitrogen limitation on the proliferation and morphology of *Symbiodinium* in hospite have also been reported previously in two hermatypic corals (*Porites porites* and *Montastrea annularis*) and a sea anemone (*Aiptasia pallida*) [21,42].

The Carbon/Nitrogen (C/N) relocation during the nitrogen-deprivation and its implication for the endosymbiotic regulation

The decreased proliferation rates in most microalgae due to nitrogen deprivation have been reported to be concurrent with increases of total lipid levels [43,44]. This was also confirmed by...
the present study, indicating that there is a reduced synthesis of new membrane constituents, in the nitrogen-deprived Symbiodi-
nium. Here, the cell machinery shifted its metabolism to synthesize higher levels of TAGs and CE fats for storage in LDs (Figs. 2–4).

The depletion of nitrogen could break the balance of carbon (C) and nitrogen (N) availability, switching the cellular metabolism toward the synthesis of carbon-containing compounds (i.e. lipids and/or carbohydrates); which then eventually leads to a higher cellular C:N ratio [45,46]. For example, the increase of lipid in microalgae upon nitrogen-deficiency could result from the partition of the excess carbon to intracellular lipid storage [47]. As shown in the present study, under nitrogen-deprived environment of free-living Symbiodinium, excess carbon allocates to the lipid pool and results in lipid accumulation as a form of LDs. Other environmental stresses, such as irradiance and various ecological changes, could also impact the C:N allocation in marine diatoms [48,49].

Besides amino acids, ammonium and nitrate have been shown to be sources of nitrogen in cultured and symbiotic Symbiodinium [6,50]. The investigation using the synchrotron radiation spectroscopy has further confirmed that symbiotic Symbiodinium (i.e. in hostite) contained less nitrogen compounds and more lipids than free-living Symbiodinium [9]. It has indicated the cytosol of the gastrodermal cell could be a nitrogen-limited environment, by which the symbiotic association with endosymbionts is regulated [51]. It is feasible that, by a mechanism of nitrogen limitation, cnidian hosts regulate their symbiotic Symbiodinium to elevate lipid contents and store in a form of LDs [5,8,9].

The nitrogen-deprivation induced quantitative and qualitative changes of lipids in Symbiodinium

The most abundant fatty acid components in TAGs of Symbiodinium cultivated in the standard I/2 media (Fig. 3) were found to be myristic acid (C14), palmitic acid (C16), stearic acid (C18) and erucic (22:1), representing a fatty acid composition similar to that of higher plants [52]. These fatty acids are also common in other algae [19]. Palmitic acid (C16) and docosahexaenoic acid (C22:6) were the most abundant fatty acids in TAGs of nitrogen-deprived Symbiodinium cells. It has been proposed that TAG biosynthesis in microalgae may consist of three steps: (1) formation of acetyl coenzyme A in the cytoplasm, (2) elongation and desaturation of the carbon chain of fatty acids in the chloroplast, and (3) synthesis of TAG in the endoplasmic reticulum [53]. In the second step, C16 and C18 fatty acids are formed prior to the production of other long-chain fatty acids or PUFAs, which requires further elongation and desaturation. Therefore, the change of C16:0 and C18:0 levels in nitrogen-deprived Symbiode-
nium documented herein may imply the accelerated synthesis and accumulation of PUFAs in the LDs, such as C16:1, C18:1, C18:2, C20:3, C20:5, C21:3, C22:1, and C22:6. PUFAs are of the utmost importance for human metabolism [54]. For example, the long-chain docosahexaenoic acid (DHA) provides significant health benefits to the human population, particularly in preventing cardiac diseases such as arrhythmia, stroke and high blood pressure [55,56]. Accordingly, an increase in the PFA production by microalgae has been shown to be important for many nutritional and pharmaceutical purposes [57].

The proteomics of LDs

Using the neutral lipid probe BODIPY 493/503, we were able to visualize the LDs isolated from cultured Symbiodinium, with 0.5–
1 μm in diameter (Fig. 6B). Oil bodies of similar sizes (0.5–2 μm diameter) have previously been reported in plant seeds [15]. However, the protein compositions of LDs between the Symbio-
dinium and other plant seeds are obviously different, indicating distinct regulatory mechanisms [58]. For example, other LD-related proteins, such as oleosin, have been discovered to be abundant in microalgae and plant seeds [16,19,59]. Both N- and C-terminal domains of oleosins have been proposed to reside on the LD (or so-called “Oil Bodies”) surface to stabilize this organelle via steric hindrance and electronegative repulsion [60]. Neverthe-
less, oleosins were not identified in the LDs of Symbiodinium spp, as shown in this study, demonstrating a different formation mechanism during the endosymbiosis. As a consequence, the examination of protein compositions of LDs in nitrogen-deprived free-living Symbiodinium should provide new insights to elucidate the LD formation mechanism. Besides proteins responsible for lipid and energy metabolisms, other LD-associated proteins involving in signaling (14-3-3 protein and ARF) and stress response (HSP90 and the osmotically inducible protein Y) were also identified (see Table 3). The 14-3-3 proteins are a ubiquitous group of signaling proteins that involve in many regulatory functions [61] such as carbohydrate and lipid metabolisms in plants [62]. Furthermore, both ARF and GTP-binding proteins are key regulators in membrane trafficking, and thus play pivotal role in LDs biogenesis [63].

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Author Contributions

Conceived and designed the experiments: PLJ CSC. Performed the experiments: PLJ BP. Analyzed the data: PLJ CSC. Contributed reagents/ materials/analysis tools: PLJ CSC. Wrote the paper: CSC PLJ.

References

1. Mascartine L (1980) Uptake, retention, and release of dissolved inorganic nutrients by marine alga-invertebrate associations. In: Cook CB, editor. In: Cellular interactions in symbiosis and parasitism. Ohio state, pp. 229–244.
2. Berda CA, Lucas JS, Yellowlyes D (1993) Nutrient limitation in the giant clam: zooxanthellae symbiosis: effect of nutrient supplements on growth of the symbiotic partners. Mar Biol 117:655–664.
3. Wang JT, Douglas AE (1998) Nitrogen recycling or nitrogen conservation in an alga-invertebrate symbiosis? J Exp Biol 211:2453–2453.
4. Sumanu-Froelich A, Floun MBQ (1984) Effects of feeding frequency and symbiosis with zooxanthellae on nitrogen metabolism and respiration of the coral Acanthus danae. Mar Biol 81:153–162.
5. Steen RG (1986) Evidence of heterotrophy by zooxanthellae in symbiosis with Aiptasia pulchella. Biol Bull 170:267–278.
6. Kahaw V, Dobinkor Z, Achint Y, Falwoski PG (1989) Ammonium metabolism in the zooxanthellae coral Sphylora pistillata. Proc R Soc Lond B Biol Sci 236:325–337.
7. Gordon BR, Leggat W (2010) Symbiodinium-invertebrate symbioses and the role of metabolomics. Mar Drugs 8: 2546–2568.
8. Cook CB, D'Silva CF (1987) Are natural populations of zooxanthellae ever nutrition-limited? Symbiosis 4:199–211.
9. Peng SE, Chen CS, Song YP, Huang HT, Jiang PL, et al. (2012) Assessment of metabolic modulation in free-living versus endosymbiotic Symbiodinium using synchrotron radiation-based infrared microspectroscopy. Bio Let. 23:434–437.
10. Zhu B, Pan K, Wang G (2010) Effects of host starvation on the symbiotic dinoflagellates from sea anemone Stichodactyla mertensii. Mar Ecol 31:15–23.
11. Piorecek M, Baxach KH, Pohl P (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochem 23:207–216.
12. Li X, Hu HY, Yang J (2010) Lipid accumulation and nutrient removal properties of a newly isolated freshwater microalgae, Scedesmus sp. LXY, growing in secondary effluent. New Biotech 27:59–63.
32. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
33. Fuchs B, Schiller J, Sub R, Schurenberg M (2007) A direct and simple method of sphingolipid synthesis inhibitors on silica gel thin-layer plate. Anal Biochem 36:851–861.
34. Rowan R, Powers DA (1991) Molecular genetic identification of symbiotic algae. In: Birkeland C, editor. In Life and Death of Coral Reefs. New York: Plenum Press, pp.26–60.
35. Tsen JTC, Feng CC, Cheng IH, Chen ECF, Chin JMH (1997) A new method for seed oil body purification and examination of oil body integrity following germination. J Biochem 121:762–768.
36. Zhang H, Hou Y, Miranda L, Campbell AD, Nancy R, et al. (2007) Spliced leader RNA trans-splicing in dinoflagellates. Proc Natl Acad Sci 11:4618–4623.
37. Bligh EG, Dyer WJ (1959) A rapid method for total lipid extraction and determination. Can J Biochem. Physiol. 37:911–917.
38. Buchs B, Schiller J, Stahl R, Schurenberg M (2007) A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) to thin layer chromatography (TLC) for the analysis of phospholipids from egg yolk. Anal Biochem 369:827–834.
39. Ahe A (1998) Modification of the Cosmasi brilliant blue staining method for sphingolipid synthesis inhibitors on silica gel thin-layer plate. Anal Biochem 269:149–150.
40. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
41. Bigogno C, Khozin-Goldberg I, Adlerstein D, Cohen Z (2002) Biosynthesis of arachidonic acid in the oleaginous microalga Haematococcus pluvialis (Chlorophyceae). Lipids 36:851–861.
42. Marubini F, Davies PS (1996) Nitrate increase zoanthellae population density and reduces skeletogenesis in corals. Mar Bio 127:319–328.
43. Takagi M, Watanabe K, Yamaberi K, Yoshida T (2000) Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of Nannochloris sp. UTEX LB 1999. Appl Microbiol Biotechnol. 54:112–117.
44. Griffiths MJ, Harrison STL (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J Appl Physiol 21:493–507.
45. Reitan KI, Rainuzzo JR, Olsen Y (1994) Effect of nutrient limitation on fatty acid composition and lipid content of marine microalgae. J Phycol 30:972–9.
46. Lynn SG, Kilham SS, Kreger DA, Interlandi SJ (2000) Effect of nutrient availability on the biochemical and elemental stoichiometry in the freshwater diatom Stephanocococcus minutulus (Bacillariophyceae). J Phycol 36:310–22.
47. Martin T, Oswald O, Graham IA (2002) Arabidopsis seedling growth, storage lipid mobilization and gene expression in response to carbon:nitrogen availability. Plant Physiol 128:472–81.
48. Palucci M, Ratti S, Giordano M (2011) Ecological and evolutionary implications of carbon allocation in marine phytoplankton as a function of nitrogen availability; a Fourier transform infrared spectroscopy approach. J Phycol 47:313–323.
49. Norici A, Bazzoni AM, Paggetti A, Raven JA, Giordano M (2011) Impact of irradiance on the C allocation in the coastal marine diatom Skeletonema marinoi Sarno and Zinzongo. Plant Cell Environ 34:1666–1677.
50. Carroll S, Blanquet RS (1984) Alanine uptake by isolated zoanthellae of the mangekyojellyfish, Cassiopea xamachana. I. Transport mechanisms and utilization. Biol Bull 166:409–418.
51. Cocozza A, Nielt XF (1991) C/N ratio in response to nitrogen supply and light quality in Uva rigida C. Agath. (Chlorophyta:Ulvophyceae). Sci Mar 55:405–411.
52. Minzangi K, Kaaya AN, Kansiime F, Tabuti JRS, Samvura B, et al. (2011) Fatty acid composition of seed oil from selected wild plants of kahuzi-biega national park and surroundings, democratic republic of congo. African Journal of Food Science 5:219–226.
53. Deng X, Fei X, Li Y (2011) The effects of nutritional restriction on neutral lipid accumulation in Chlamydomonas and Chlorella. Afr J Microb Res 5:260–269.
54. Simopoulos AP (2002) Importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother 56:365–379.
55. Romieu I, Telliez-JoMMI, Lazo M, Manzano-Patino A, Cortez-Lugo M, et al. (2005) Omega-3 fatty acid prevents heart rate variability reductions associated with moderate cigarette smoke. Am J Hypertens 18:134–140.
56. Van Schacky C (2008) Omega-3 fatty acid: antiarrhythmic, proarrhythmic or both? Curr Opin Clin Nutr Metab Care 11:94–99.
57. Perea H, Barreira L, Figueredo F, Custódio L, Vezzoto-Duarte C, et al. (2012) Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. Mar Drugs 10:1920–1935.
58. Tsen JTC, Wang MMC, Chen JCF, Lin LJ, Chen MCM (2003) Seed oil body proteins: oleosin, oleosin and, cereolosin. Curr Topics Biochem Res 5:133–139.
59. Huang NL, Huang MD, Chen TL, Huang AH (2013) Oleosin of subcellular lipid droplets evolved in green algae. Plant Physiol 161:1862–1874.
60. Chen DH, Chyan CL, Jiang PL, Chen CS, Tsen JTC (2012) The same olein isoforms are present in oil bodies of rice embryo and aleurone layer while cereolosin exists only in those of the embryos. Plant Physiol Biochem 60:1082–1083.
61. Ferl RJ, Manak MS, Reyes MF (2002) The 14-3-3s. Genome Biol 3:1–7.
62. Prescha A, Swiedrych A, Biernat J, Szopa J (2001) Increase in lipid content in potato tubers modified by 14-3-3 gene overexpression. J Agric Food Chem 49:3638–3643.
63. Tan R, Wang W, Wang S, Zun L, et al. (2013) Small GTPase Rab40l associates with lipid droplets and modulates the biogenesis of lipid droplets. PLoS One 8:e63213.
64. Pratx WA (2007) Non-vesicular sterol transport in cells. Prog Lipid Res 46:297–314.
65. Yang L, Ding Y, Chen Y, Zhang S, Hao C, et al. (2012) The proteomics of lipid droplets: structure, dynamics, and functions of the organelle conserved from bacteria to humans. J Lipid Res 53:1245–1253.
66. Bartz R, Neemann J, Zehner K, Serrero G, Chapman DK, et al. (2007) Evidence that mono-ADP-ribosylation of CBP1/BARS regulates lipid storage. Mol Biol Cell 18:3015–3025.
67. Zhang H, Hou Y, Miranda L, Campbell AD, Nancy R, et al. (2007) Sphered leader RNA trans-splicing of CA12. Proc Natl Acad Sci 104:4620–4623.
68. Silby MW, Cerdote-Tarraga AVM, Vernikos GS, Giddens SR, Jackson RW, et al. (2009) Genomic and genetic analyses of diversity and plant interactions of Pavlova fusca. Genome Biol 10:R31.
69. Danse JC, Waller RF (2011) Analysis of dinoflagellate mitochondrial protein sorting signals indicates a highly stable protein targeting system across eukaryotic diversity. J Mol Biol 408:643–653.
70. Fischer W, Windhager L, Rohrer S, Zeiller M, Kahlbrandt A, et al. (2010) Strain-dependent IV secretion system and genomic island transfer. Nucleic Acids Res 38:6089–6101.