Resting cell formation in the marine diatom *Thalassiosira pseudonana*

Guangning Wang[1#], Lu Huang[1#], Shanshan zhuang[1#], Fang Han[1#], Qianqian Huang[1], Mengyuan Hao[1], Guifang Lin[1], Longnan Chen[1], Biying Shen[1], Feng Li[1], Xuesong Li[1], Changping Chen[1], Yahui Gao[1, 2], Thomas Mock[3*], Junrong Liang[1*]

[1]School of Life Sciences, Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, Xiamen Key Laboratory of Plant Genetics, Xiamen University, Xiamen 361102, China. [2]State Key Laboratory of Marine Environment Science, Xiamen University, Xiamen 361102, Fujian, China. [3]School of Environmental Sciences, University of East Anglia (UEA), Norwich Research Park, Norwich, United Kingdom

*Corresponding authors: T.Mock@uea.ac.uk; sunljr@xmu.edu.cn

#These authors contributed equally to this work.

Junrong Liang: ORCID: 0000-0001-7130-4253

Thomas Mock: ORCID: 0000-0001-9604-0362

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Summary

- Resting cells represent a survival strategy employed by diatoms to endure prolonged periods of unfavourable conditions. In the oceans,-many diatoms sink at the end of their blooming season and therefore need to endure cold and dark conditions in the deeper layers of the water column. How they survive these conditions is largely unknown.
- We conducted an integrative analysis encompassing methods from histology, physiology, biochemistry, and genetics to reveal the biological mechanism of resting-cell formation in the model diatom *Thalassiosira pseudonana*. Resting-cell formation was triggered by a decrease in light and temperature with subsequent catabolism of storage compounds. Resting cells were characterised by an acidic and viscose cytoplasm and altered morphology of the chloroplast ultrastructure.
- The formation of resting cells in *T. pseudonana* is an energy demanding process required for a biophysical alteration of the cytosol and chloroplasts to endure the unfavourable conditions of the deeper ocean as photosynthetic organisms. However, most resting cells (> 90%) germinate upon return to favorable growth conditions.

Keywords: cytoplasmic acidification, diatom, metabolic reprogramming, resting cell formation, stress resistance

Introduction

As most photosynthetic unicellular organisms, diatoms require light for growth, so they typically thrive and frequently form blooms in the euphotic layers of marine and freshwater ecosystems. Hence, their productivity contributes significantly to the global carbon cycle and therefore the structure of food web and global climate. However, when diatom blooms demise or when the environment becomes unfavourable, the cells tend to sink into deeper water or the sea floor, where they must endure longer periods of darkness at lower temperatures. Thus, biological mechanisms exist such as catabolism of stored carbon (e.g., lipids) (Schaub *et al.*, 2017; Kennedy *et al.*, 2019), mixotrophy (Rivkin & Putt, 1987; McMinn & Martin, 2013), and the formation of resting stages (Sicko-Goad *et al.*, 1989; Taylor & McMinn, 2002; McMinn & Martin, 2013). which enable diatoms to survive these conditions Resting stages generally consists of two fundamental types, namely resting cells and spores, which have been frequently observed in diatom species inhabiting temperate and polar oceans. Examples include resting cells of *Thalassiosira* (e.g. *T. rotula*, *T. nordenskzoddii,* and *T. scotia*) and *Sheletonema*, as well as resting spores of *Chaetoceros*, *Bacteriastrum*, *Leptocylindrus* and *Thalassiosira* (e.g. *T. australis*, *T constricta*, and *T gramda*) (Lund, 1971; Du Preez & Bate, 1992; Cameron, 1995; McQuoid *et al.*, 2002; Sugie & Kuma, 2008). Diatom resting stages not only contribute significantly to carbon export from surface oceans, but they also play critical roles in the global ocean ecosystem by serving as "seed banks", which can rejuvenate and initiate new seasonal blooms upon returning to the euphotic zones of the ocean under more favourable growth conditions (Sicko-Goad *et al.*, 1986; Itakura *et al.*, 1997; Ishikawa & Furuya, 2004; Ellegaard & Ribeiro, 2018).

Resting stages are common for numerous microbes because they enable them to endure unfavourable growth conditions in a dormant life-cycle stage characterised by a significant reduction of metabolic activity (McQuoid & Hobson, 1996; Lennon & Jones, 2011; Ellegaard & Ribeiro, 2018; Pelusi *et al.*, 2020). Diatom resting stages compared to vegetative cells are generally characterised by a reduction in respiration rates (≥ 20% lower) and photosynthesis (≥ 4% lower) (Anderson, 1975; Kuwata *et al.*, 1993). These data are comparable to other microorganisms which show metabolic rates reduced by at least ~30% under conditions of dormancy (Storey & Storey, 1990; Withers & Cooper, 2010).

This metabolic suppression facilitates the reduction of energy consumption necessary for long-term survival under adverse conditions.

Metabolic suppression is typically accompanied by structural modifications in appearance, organelles, and sometimes the cytoplasm (Bradshaw *et al.*, 1998; Boon *et al.*, 2001; Van Bodegom, 2007). Different organisms exhibit distinct resting stages with various species-specific structural modifications. Examples include dinoflagellate cysts, cyanobacteria akinetes, chlorophyte akinetes, bacterial spores, and plant seeds. Diatom spores display unique morphological features. They are characterized by a thicker species-specific external modification of the silica cell wall, such as observed in spores of *Chaetoceros* species (Ishii *et al.*, 2011). On the other hand, diatom resting cells share a phenotypic appearance similar to their vegetative counterparts and are often overlooked due to their similarity (Ishii *et al.*, 2012; Matsubara *et al.*, 2022), resulting in limited knowledge about them. However, several diatom resting cells exhibit morphological alterations in chloroplasts that appear more condensed and rounded, such as observed in *Cymbellonitzschia diluviana*, *Asteroplanus karianus*, and *Biddulphia alternans* (Sicko-Goad *et al.*, 1989; McQuoid & Hobson, 1996; Jewson *et al.*, 2006; Ishii *et al.*, 2012; Matsubara *et al.*, 2022). Condensed cytoplasm has also been reported in resting cells of *Skeletonema costatum* (McQuoid, 2005). Despite providing initial insights into the biology of diatom resting cells through histological data analysis, our understanding of their physiological and cytoplasmic properties remains incomplete.

The genera *Thalassiosira* comprises numerous species capable of forming resting cells and/or spores (McQuoid & Hobson, 1996; McQuoid, 2005). The potential ability of the model diatom *Thalassiosira pseudonana* to form resting cells is supported by their capability to resume growth after being buried for over two years in surface sediments (McQuoid, 2005). However, there is a general lack of information regarding the mechanism underpinning resting cell formation and subsequent germination in diatoms despite their important role especially in coastal ecosystems. To address this knowledge gap, resting cells of *T. pseudonana* were induced by exposure of early stationary phase cultures to total darkness at 4°C for ≥ 84 days (Fig. **1**). We conducted an investigation into the morphological, physiological, cytoplasmic, and biochemical characteristics of resting cells during their formation. This study was accompanied by transcriptome profiling

(RNAseq) to gain first insights into the underlying molecular mechanisms involved in diatom resting cell formation (Fig. **1**). Our results revealed that the process of *T. pseudonana* resting cell formation is characterized by histological alterations in organelles such as plastids, significant acidification within the cytoplasm, increased viscosity of the cytoplasm and chloroplasts, as well as catabolism of stored compounds. Furthermore, our results demonstrate that these changes are crucial for the development of resting cells and their ability to withstand harsh environmental conditions in the long term. A germination experiment showed that the process of forming resting cells is reversible.

Materials and Methods

Culturing and the induction of resting cells

The diatom *T. pseudonana* (Hustedt) Hasle et Heimdal, CCMP1335, was maintained in f/2 medium at 20 ± 1 °C under a 12:12-h light:dark cycle at 50 μmol photons m^{-2} s^{-1}. To induce resting cells, vegetative cells were cultured in 5L glass flasks containing 4 L of f/2 medium. The preliminary study demonstrated successful induction of vegetative cells into resting cells at various growth stages, including the exponential and stationary phases. Here, the cells from the early stationary growth phase, when the cell count is at its highest, were harvested and concentrated in fresh f/2 medium (to replicate a nutrient-rich environment similar to that found in deep sea conditions) to achieve a concentration of 3×10^6 cells mL^{-1}. Subsequently, they were maintained in complete darkness at 4 °C for a minimum duration 84 days. All experiments described herein were conducted in triplicate unless otherwise specified. The resting phase induced in different culture stocks were utilized for different analyses. The detailed information is presented in Table S1.

The germination assessment of resting cells

The resting cells, which were maintained in cold and darkness for 90 and 180 days respectively, were utilized for germination assessment. These cells were collected and re-suspended in fresh f/2 medium. The culture conditions remained consistent with those employed for vegetative cells. Cell density and photosynthetic efficiency (Fv/Fm) were measured at intervals of either 12 or 24 hours.

Cell physiology

The cell density was monitored using a hemocytometer under light microscopy (LM). Cell-cycle analysis was performed as previously described (Hildebrand *et al.*, 2007). Flow cytometry (BD Fortessa, U.S.) at the PE channel was used to monitor the fluorescence of cells labeled with propidium iodide (PI). ModfitLT software was utilized to calculate the percentage of cells in different phases of the cell cycle. Cell viability was estimated through a double-staining method (Thamatrakoln *et al.*, 2012), where PI and annexin-V (Vazyme) were employed to detect dead cells and cells undergoing apoptosis, respectively. A flow cytometer (BD Fortessa, USA) at PE and FITC channels was used for determining cell activity. Gating and data analysis were conducted using FlowJo analytical software. The respiration rate measurement followed the protocol by Li et al. (Li *et al.*, 2017). Photosynthetic efficiency (Fv/Fm) of cells was assessed using a Walz Phyto-PAM after dark incubation of cultures for approximately 15 minutes.

Cell morphology and histology

LM images of cells were acquired using a Leica DM4B microscope (Leica, Germany). Transmission electron microscopy (TEM) was performed according to Luo et al. (Luo *et al.*, 2014). Visualization of cellular and subcellular structures was achieved utilizing an H-7800 electron microscope (HITACHI, Japan).

Intracellular pH (pHi)

The membrane-permeable dye BCECF-AM (Beyotime Institute of Biotechnology) was utilized for measuring pHi, as described in previous studies (Dixon *et al.*, 1989; Guo *et al.*, 2022; Schnipper *et al.*, 2022). This dye exhibits a stable intensity upon excitation from 400- 440 nm, enabling the use of this wavelength for radiometric normalization (Loiselle & Casey, 2010). Additionally, the dye demonstrates a pH-sensitive change after excitation at 488 nm, which is distinct from the pH-insensitive excitation wavelength at the range of 400-440 nm. To obtain a radiometric measurement of pHi, emission was recorded at 535 nm using both a Zeiss LSM780 laser confocal microscope system (Zeiss, Germany) and a flow cytometer (Quanteon, USA), following excitation at wavelengths of 405 nm and 488 nm respectively. Subsequently, pHi values were determined by calculating the

ratio between emissions at pH-sensitive (488 nm) and pH-insensitive (405nm) wavelengths. Nigericin (Shanghai Yuanye Bio-Technology Co., Ltd., P. R. China), capable of balancing extracellular and intracellular pH levels, was employed to establish a standard curve with BCECF-AM-loaded dye across five different pH values: namely 6, 6.5, 7, 7.5, and 8. Notably, a final concentration of nigericin at 10 μM was added to samples five minutes prior to measurement.

To assess the impact of cytoplasmic acidification on respiration rate and photosynthesis, 10 mL aliquots containing approximately 2 $\times 10^6$ cells mL^{-1} of vegetative cells were harvested by centrifugation at 3000 g for 3 min, followed by re-suspension in 2 mL f/2 medium. The medium's pH was adjusted to 8.1 and 6 .0 using HCl. Subsequently, respiration rate and photosynthetic rate were measured within 1 .5 h to evaluate the effects of extracellular acidification on these processes. Nigericin (final concentration 50 nmol L^{-1}) was added to f/2 medium containing cells with a pH value of 7.0 and 6.0 to achieve corresponding pHi values. Respiration rate and photosynthetic rate measurements were conducted 10 min later to evaluate the effects of cytoplasmic acidification (pHi, 6). The respiration rate was determined using a Clark-type oxygen electrode (Hansatech) by measuring oxygen consumption rates in the dark (△R) at 4 ℃. Oxygen evolution rates were estimated (△L) based on changes in the oxygen content under light conditions at 50 μmol photons m^{-2} s^{-1} and 4 ℃. Additionally, the photosynthetic rate was calculated as △L minus △R, along with Fv/Fm measurements (Li *et al.*, 2017).

Cell biochemistry

To quantify ATP and the ratio of AMP to ATP, cell samples (40 mL) were harvested by centrifugation (3000 g, 4℃ for 5 min). The supernatant was removed and the pelleted cells were stored at -80℃ for ATP measurements. The ATP content was measured using an ATP kit (Solarbio, China) according to the manufacturer's instructions. The AMP/ATP ratio was determined in quintuplicate cell samples according to Bennette (Bennette *et al.*, 2011). The relative values of AMP and ATP were measured by LC-MS/MS analysis, which was performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) and UPLC BEH Amide column coupled with Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). The AMP/ATP ratio was calculated using the relative values of AMP and ATP.

To estimate the total amount of cellular carbohydrates and proteins, cell samples (50 mL for one) were harvested for total carbohydrate and protein concentrations using a "Total carbohydrate assay kit" (Sigma, NO. MAK104) and the Bradford protein kit (Solarbio), respectively. The carbohydrate and protein concentrations were estimated using a microplate reader (Tecan spark, Switzerland) at 490 nm and 595nm, respectively. Amino acids (AAs) were extracted and analysed as described in Chen et al. (Chen *et al.*, 2014). HPLC (Agilent 1200, USA) was used for amino acid analysis. AA standards (sigma) were used for peak identification and subsequent quantification. Fatty acids (FAs) were extracted and analysed using a modified method of Tang et al. (Tang *et al.*, 2011). AutoSystem XL GC/TurboMass MS (Perkin Elmer, Germany) with CP-Wax52CB column (30m×0.25mm×0.25 μm) was used for FA analysis by GC-MS. For triglycerides (TAG), cells were stained with boron-dipyrromethene (Beyotime Institute of Biotechnology, China) as previously described (Lin *et al.*, 2017). Stained lipid bodies were visualised under a fluorescence microscope (Leica DM4B, Germany) and TAG content was determined using a flow cytometer at the FITC channel (BD Fortessa, USA).

Stable VCs-eGFP (vegetative cells expressing eGFP) and RCs-eGFP (resting cells expressing eGFP) cell lines

The pTpNR/GFP and pTpfcp/nat plasmids were fused to generate the pTpNR-GFP/fcpNat (-KpnI) vector, as described by Scheffel et al. (Scheffel *et al.*, 2011). Subsequently, the pTpNR-GFP/fcpNat (-KpnI) plasmid was introduced into *T. pseudonana cells* (cultured in f/2 medium) using the biolistic PDS-1000/He particle delivery system (Bio-Rad), following the protocol outlined by Poulsen et al. (Poulsen *et al.*, 2007). The transformed strains were screened using ClonNAT antibiotics to establish a stable line of VCs-eGFP cells. RCs-eGFP refers to resting cells derived from VCs-eGFP that have been incubated under cold and dark conditions for 90 days.

Viscosity of cytoplasm and chloroplast

To evaluate the changes in viscosity of cytoplasm and chloroplast, fluorescence recovery after photo-bleaching (FRAP) experiments were conducted on *T. pseudonana* VCs-eGFP and RCs-eGFP using a water lens on a Celldiscoverer 7 (Zeiss) system. The

cells were placed in confocal dishes and covered with a 1% agarose gel. Imaging of the cells was performed for either 290s or 120s at a maximum rate of one frame per every 10s or 4s respectively. Following two pre-bleach frames, the fluorescence signal from a specific region of interest was bleached using lasers emitting at wavelengths of 488 nm (for cytoplasm) and 561nm (for chloroplast), both set at full power. The recovery of the bleached region was measured over a period of either 270s or 108s. ZEN 2.3 software along with EasyFRAP software (https://easyfrap.vmnet.upatras.gr/) were utilized to obtain raw fluorescence values and normalize the data respectively.

RNA sequencing, data analysis, and quantitative qRT-PCR

The RNA extraction, RNAseq, and data analysis procedures were performed following the protocol described by Yuan et al. (Yuan *et al.*, 2021). Pairwise comparisons were utilized to identify differentially expressed genes (DEGs) on day 84 compared to the control group (vegetative cells, day 0), using a threshold value of log2-converted fold change ≥ 2 or ≤ -2 with a false discovery rate ≤ 0.05. The KEGG enrichment analysis was conducted according to a previously published study (Luo *et al.*, 2014). Q-PCR primers (Table S2) were designed based on mRNA sequences obtained from NCBI using oligo7 software. Q-PCR experiments were conducted using the GoTaq® RT-qPCR System (Promega), following the manufacturer's instructions. The amplification efficiency of primers was determined through the standard curve method and LinRegPCR as instructed. For calculation of differential expression, triplicates were used for each gene along with two internal reference genes (gene IDs 7445148 & 7445884), according to Bromke et al.'s study (Bromke *et al.*, 2013).

Results

Formation of resting cells

During the time course of resting cells induction (days 1-84), the cell cycle was predominantly arrested in the G1 phase, at least starting from day 14 (Fig. S1). The cell density exhibited a reduction of approximately 60% (Fig. **2a**), indicating that not all vegetative cells possess the ability to transition into resting cells and consequently experienced mortality. However, following the demise of some cells, the remaining

population demonstrated a high level of viability as evidenced by PI and annexin V staining (Fig. **2b**, Fig. S2). An increasing number of cells displayed morphological modifications in their chloroplasts, which became more condensed and rounded while still maintaining the morphological features observed in vegetative cells (Fig. **2c**). Considering that this characteristic has been describe previously (Sicko-Goad *et al.*, 1989; McQuoid & Hobson, 1996; Jewson *et al.*, 2006; Ishii *et al.*, 2012; Matsubara *et al.*, 2022), we postulate that this cell type represents a resting cell. The proportion of these resting cells progressively increased over time, peaking at approximately 94% on day 84 post induction (Fig. **2d**). TEM images revealed significant alterations in thylakoid membrane structure within resting cells characterized by an absence of stacking, contrasting with the highly regular patterns observed in vegetative cells (Fig. S3).

The PSII photochemical quantum yield (Fv/Fm) exhibited a continuous decline until day 112 (Fig. **2e**). Concurrently, the respiration rate experienced a significant decrease starting from day 42 (Fig. **2f**). By day 84, when approximately 94% of cells exhibited condensed and round chloroplasts, the level of metabolic suppression aligns with the widely accepted definition of resting cells observed across various phytoplankton species (Kuwata *et al.*, 1993; Deng *et al.*, 2017), with only 21% of the initial respiration rate and less than 10% of the initial Fv/Fm. This finding substantiates our hypothesis that the presence of condensed and round chloroplast serves as an indicator for resting cells in *T. pseudonana*. Furthermore, we noted variations in the extent of metabolic suppression between populations of resting cells on day 70 and day 84, both consisting of over 90% resting cells (Fig. **2d,f**). This observation suggests that morphological alterations in chloroplasts serve as an early indication for physiological dormancy. Therefore, we classify the resting cells on day 70 and earlier as young resting cells, whereas those on day 84 and later are considered more mature dormant cells (mature resting cells). Additionally, an extended period of dormancy is likely to result in a gradual intensification of dormancy within these resting cells, as evidenced by strongly reduced Fv/Fm on day 112 compared to day 84 (Fig. **2e**).

The mature resting cells, which were exposed to cold and dark conditions for 90 and 180 days respectively, were selected to evaluate their germination capability. After subsequently cultivation under optimal growth conditions (e.g., nutrient repletion, light),

the cell density increased over time (Fig. **3a,d**). The proportion of vegetative cells gradually increased (Fig. **3b,e**) and Fv/Fm gradually returned to its optimum level (Fig. **3c,f**). Thus, the germination experiments revealed that the majority of the resting cells were able to completely recover. However, cells exposed to 90 days of cold and darkness recovered faster compared to those exposed to a longer period (180 days). This suggests that the time in dormancy appears to be equivalent to the time required to completely recover from it.

The physicochemical characteristics of resting cells

The intracellular pH (pHi) of resting and vegetative cells was measured ratiometrically using a pH-sensitive dye called BCECF-AM. A laser confocal microscope, capable of distinguishing between vegetative cells and resting cells based on the morphology and fluorescence characteristics exhibited by their chloroplasts, revealed a significant decrease in pHi for both young and mature resting cells compared to vegetative cells (Fig. **4a,b**; Fig. S4). Regardless of the day on which they were formed, the pHi of resting cells remained at ca. pH 6. Extracellular pH levels remained within the range of 7.5-7.8 (Fig. S4) throughout this period. This suggests that *T. pseudonana*'s resting cells are characterized by an acidic cytoplasm, which appears to preceding physiological dormancy.

Cytoplasmatic acidification was accompanied by a higher level of viscosity as evidenced through slower kinetics of GFP diffusion in the cytoplasm of resting cells (Fig. **4c,d**). Furthermore, even after exposure to cold and darkness for 25 days, the cytoplasmic viscosity of vegetative cells remained lower than that of young resting cells, suggesting that the reduction in cytoplasmic viscosity is characteristic to young and mature resting cells rather than solely induced by cold and darkness alone (Fig. S5). Additionally, the resting cells exhibited a noticeable increase in chloroplast viscosity (Fig. **4e,f**), which possibly can be attributed to morphological alterations and therefore might contribute to the observed decline in Fv/Fm.

The cells that germinated from a resting state exhibited elevated intracellular pH levels (Fig. **4g**), as well as reduced cytoplasmic and chloroplast viscosity compared to their initial resting state (Fig. **4h,i**). This finding further confirms that alterations in cytoplasmic pH and viscosity are specific characteristics of resting cells.

The relationship between intracellular acidification, viscosity and metabolic activity

Employing nigericin that artificially lowered the cytoplasmic pH in vegetative cells, revealed that an acidic cytoplasm (pH 6) contributes to the suppression of respiration and photosynthesis (Fig. **5a**). This implies that intracellular acidification plays a role in reducing metabolic activity as observed in resting cells.

The photo-bleaching assays showed a significant increase in both cytoplasmic and chloroplastic viscosity within vegetative cells upon artificial reduction cytoplasmic pH (Fig. **5b,c**). This suggests that intracellular acidification also leads to an elevation in overall cellular viscosity. After removing nigericin and replacing it with regular f/2 medium at pH 8.0, cytoplasmic viscosity returned to normal levels (Fig. **5d**). These findings confirm a correlation between intracellular acidification and changes in cellular viscosity, while cell viability remained unaffected by nigericin treatment (Fig. S6).

The biochemical characteristics of resting cells

Cellular ATP concentrations dropped on day 56 and subsequently remained low, ultimately reaching approximately 22% of initial levels by day 84 (Fig. **6a**). Concurrently, the AMP to ATP ratios increased over this period, peaking on day 84 with an approximate twenty-fold increase (Fig. **6b**). Thus, resting cells possess a reduced ATP content but elevated levels of AMP causing the AMP/ATP ratio to increase, which is indicative of energy deficiency (González *et al.*, 2020).

In terms of metabolic alterations during the formation of resting cells, the total cellular carbohydrate levels decreased to approximately 50% of their initial levels within the first 14 days (Fig. **6c**). This decline was accompanied by a gradual decrease in free amino acids, which reached approximately 50% of their initial concentrations on day 45 (Fig. **6d**). Therefore, it can be inferred that both carbohydrates and amino acids were likely catabolized during resting cell formation. However, the concentration of neutral lipids (triacylglycerols) exhibited an upward trend until day 70 (Fig. **2d**), followed by a subsequent decline until day 84 (Fig. **6e**). This transition was accompanied by a reduction in total fatty acids (both saturated and unsaturated) and proteins (Fig. **6f,g**). The observed differences between young and mature resting cells may indicate that the catabolism of

neutral lipids, fatty acids, and proteins plays a crucial role in the maturation of resting cells. Consequently, mature resting cells are characterized by lower concentrations of carbohydrates, free amino acids, triacylglycerols, fatty acids, and proteins. These findings suggest that *T. pseudonana* metabolizes various compounds as sources of energy during the process of forming resting cells.

Differential gene expression during resting cell formation

To gain insights into the regulation of metabolic pathways underpinning the physiology of resting cells, we performed transcriptome sequencing of mature resting cells (day 84) in comparison to vegetative cells (day 0). We identified 4,142 differentially expressed genes (DEGs) in resting cells compared to their vegetative counterparts, which means that a significant proportion of genes in *T. pseudonana* (> 30%) changed their expression as a consequence of resting-cell formation. Quantitative RT-PCR (qPCR) of 11 randomly selected genes confirmed the transcriptome data (Fig. S7). KEGG enrichment analysis revealed that the majority of up-regulated DEGs were significantly associated with processes such as oxidative phosphorylation, fatty acid degradation, carbon metabolism, secondary metabolite biosynthesis, valine, leucine and isoleucine (BCAA) degradation, metabolic pathways, and photosynthesis (Fig. **7**). Conversely, a significant proportion of down-regulated DEGs were found to be involved in amino sugar/nucleotide sugar metabolism and aminoacyl-tRNA biosynthesis. This transcriptome profiling aligns with our biochemical measurements indicating that resting cells exhibit low levels of free amino acids, fatty acids, proteins and total carbohydrates; thus suggesting a consistent correlation.

Specifically, there was a significant increase in the expression levels of 38 genes associated with amino acid degradation pathways including tryptophan, lysine, tyrosine proline and BCAA degradation (Fig. S8-11; Table S2). Hence, it is not surprising to observe rapid decreases in amino acids since free amino acids may undergo degradation into acetyl-CoA α-ketoglutarate succinyl-CoA fumarate or oxaloacetate for utilization in the TCA cycle for energy production.

Genes involved in the degradation of fatty acids were activated. Four genes (gene ID 7442404, 7447201, 7451792, 7449925) that participate in all steps of the fatty acids β-oxidation degradation pathway exhibited significant upregulation ranging from

approximately 4 to 39-fold (Fig. S12a). This transcript profile was consistent with the observed reduction in fatty acid contents. However, before degradation can occur through the β-oxidation process in peroxisomes or mitochondria (Xu *et al.*, 2013), fine-tuning is required to regulate their entry into this pathway for degradation. The repression observed in carnitine acyltransferase expression (7447893 & 7442239), a key enzyme involved in fatty acid shuttling (Fig. S12a), was consistent with partial depletion rather than complete exhaustion of total fatty acids in resting cells (Fig. **4a**). In contrast, the downregulation of β-ketoacyl-ACP synthase (gene ID 7442001, 7447264, 7445965) and enoyl-ACP reductase (gene ID7444050), key enzymes involved in fatty acids biosynthesis, was consistent with the absence of further accumulation of fatty acids during resting cell formation (Fig. S12b).

Repressed expression of 14 genes encoding aminoacyl-tRNA synthetase, essential enzymes for translation (Fig. S13), was consistent with the observation that there was a decrease in protein content in resting cells. Additionally, the expression of genes involved in chrysolaminarin biosynthesis, such as chrysolaminarin synthase and β-1,6-transglycosylase, showed significant downregulation. Furthermore, the endo-glucanase responsible for catalyzing chrysolaminarin degradation to glucose also exhibited decreased expression levels. These findings were in line with the observed reduction in total carbohydrate levels. Thus, the transcriptional data demonstrated that resting cells of *T. pseudonana* repressed anabolic but activating catabolic pathways.

Discussion

Diatoms possess a multitude of unique physiological and metabolic characteristics that enable them to withstand adverse conditions (Jewson *et al.*, 2006; Ellegaard & Ribeiro, 2018; Ishii *et al.*, 2022) . Our study has demonstrated that *T. pseudonana*, a representative diatom, is capable of generating resting cells as a mechanism for enduring prolonged periods of low temperatures and darkness. To the best of our knowledge, this is the first experimental evidence providing insights into the underpinning biological mechanisms of resting cell formation in diatoms. We have employed histological, cytoplasmic, biochemical, and transcriptomic analyses in conjunction with the photo-bleaching assay to gain insights into the intrinsic characteristics of resting cells and propose a model elucidating the underlying mechanisms through which diatom cells enter a resting state

(Fig. **8**). These findings therefore represent a foundation for advancing this field and they build bridges to ecological and physiological research on the role of diatom resting stages in an ecosystem context.

Resting cells of *T. pseudonana* display conserved characteristics

The resting cells of *T. pseudonana* demonstrated significant metabolic suppression, halted growth, and morphologically altered plastids characterized by condensed spherical chloroplasts - common features among other diatom resting cells (Sicko-Goad et al., 1989; McQuoid & Hobson, 1996; Jewson et al., 2006; Ishii et al., 2012; Matsubara et al., 2022). Moreover, they exhibited distinct attributes not previously reported in any diatom resting cells but observed in other organisms: prominent intracellular acidification (with a pHi value of ca. 6), elevated viscosity levels in the cytoplasm and chloroplasts, as well as elevated AMP/ATP ratios. Intracellular acidification has been observed during *Bacillus subtilis* dormancy (with a pHi range of 6.0±0.3) and in yeast spores (pHi 5.8) and diapause stages of marine brine shrimp (pHi 6.3) (Busa & Crowe, 1983; van Beilen & Brul, 2013; Munder et al., 2016). Reduced intracellular pH in yeast spores contributes to the preservation of the native structure of proteins and their oligomeric states (Petrovska *et al.*, 2014). This is because the pH-dependent macromolecular assembly of proteins does not result in protein denaturation and therefore facilitates rapid recovery from pH-induced changes within the cytoplasm (Munder et al. 2016). Moreover, *Dictyostelium* cells utilize significant cytosolic acidification as a signaling mechanism to inhibit fluid-phase endocytosis under hyperosmolarity, contributing to their survival (Pintsch *et al.*, 2001). This work in distantly related microbes resembles what we have seen in *T. pseudonana* resting cells and therefore might represent conserved mechanisms to ensure the survival of resting cells in microbial eukaryotes regardless of their main metabolic mode. However, the mechanism underlying sustained metabolic activity, albeit at a reduced level, in the presence of a low pHi remains elusive (Lennon & Jones, 2011; Speers-Roesch *et al.*, 2018; Persson *et al.*, 2020; Lebenzon *et al.*, 2022; Reeve *et al.*, 2022). Nevertheless, acidification has been observed to cause widespread macromolecular assembly of proteins and trigger a transition of the cytoplasm to a solid-like state with increased mechanical stability and therefore viscosity (e.g. (Munder et al., 2016; Plante et al., 2023). Addtionally, elevated

AMP/ATP ratios as observed in *T. pseudonana* resting cells have been observed to be characteristic for reduced metabolism and embryonic development within *Austrofundulus limnaeus* and *Artemia franciscana* (Hand et al., 2011; Patil et al., 2013). These results suggest that dormancy traits have evolved independently and therefore might be conserved in species across the tree of life including diatoms.

Underlying mechanisms of resting-cell formation

Revealing the underlying mechanisms responsible for inducing a resting state in *T. pseudonana* cells is crucial for understanding their ability to adapt to fluctuating environments. Our study has demonstrated that intracellular acidification can contribute to suppressed metabolism. Similar observations have been reported for various organisms, including *Chlamydomonas*, zooplankton embryos, yeast spore, and cancer cells (Hand, 1998; Munder *et al.*, 2016; Boedtkjer & Pedersen, 2020; Pang *et al.*, 2023). Previous studies in yeast, human, and plant cells have demonstrated that inadequate levels of intracellular energy levels can result in intracellular acidification (Cassel *et al.*, 1986; Yoshida *et al.*, 1989; Munder *et al.*, 2016; Wang *et al.*, 2022). For example, a decrease in ATP levels has the potential to impede the activity of tonoplast H^+-ATPase, a crucial player in proton efflux from the cytoplasm, ultimately leading to intracellular acidification (Yoshida *et al.*, 1989). Furthermore, it has been observed that a decline in ATP content triggers an increase in intracellular viscosity within yeast cells (Persson *et al.*, 2020). Henceforth, cellular energy deficiency may be considered one of the primary factors inducing dormancy. Resting cells of *T. pseudonana* exhibit indications of energy deficiency during their formation process as evidenced by an elevated AMP/ATP ratio and decreased ATP concentration. Consequently, energy deficiency potentially contributes to the intracellular acidification of *T. pseudonana* and triggers the formation of resting cells.

Compared to vegetative cells, mature resting cells of *T. pseudonana* exhibit significantly reduced levels of carbohydrates and free amino acids, as well as relatively lower levels of triacylglycerols, fatty acids, and proteins. Transcriptional data further confirms that *T. pseudonana* resting cells undergo modified metabolic pathways involving the inhibition of anabolic pathways and activation of catabolic pathways for energy production. Similar energy-related metabolic modifications have also been observed in the

formation of diatom spore and dinoflagellate cysts (Guo *et al.*, 2021; Pelusi *et al.*, 2023). For instance, the diatom *Chaetoceros socialis* upregulates catabolic pathways, such as the tricarboxylic acid cycle, glyoxylate cycle, and fatty acid beta oxidation to use lipids as an energy source (Pelusi *et al.*, 2023). These findings collectively suggest that a substantial amount of energy derived from cellular stored compounds is required for the formation of a state of dormancy. The dynamic changes in stored compounds during *T. pseudonana* resting cell development also indicate that carbohydrates and amino acids may be essential for the transition from active to resting states, while fatty acids and triacylglycerols serve as primary energy sources for mature resting cells; both stages necessitate an adequate energy supply. This catabolism of stored compounds in a certain order may facilitate metabolic shifts necessary for coping with unfavorable conditions and ensuring survival during resting cell formation.

Finally, our results have led us to propose a hypothesis regarding the formation of resting cells in *T. pseudonana* and possibly other diatom species because this process appears to be universal (Fig. **8**). According to our hypothesis, the combined stresses of cold and darkness result in significant energy expenditure and reorganization of energy-related metabolism, ultimately leading to an energy-deficient state of cells. Consequently, this energy deficit state induces intracellular acidification which is associated with an increase in cytosolic viscosity and metabolic suppression, ultimately culminating in the formation of mature resting cells. Therefore, both storage compounds and protons play crucial roles in the development of resting cells. Thus, our study provides insights into the characteristics of *T. pseudonana* resting cells and advances our understanding of the molecular mechanisms involved in resting cell formation in diatoms.

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Competing interests

The authors declare no competing interests

Author contributions

J.R.L designed the project. G.N.W, L.H, S.S.Z, F.H, Q.Q.H, M.Y.H, G.F.L, L.N.C, B.Y.S, and F.L performed the experiments. G.N.W, L.H, S.S.Z, F.H, X.S.L, C.P.C, Y.H.G, T.M, and J.R.L contributed to data analysis. J.R.L, T.M., G.N.W, L.H, M.Y.H and S.S.Z wrote the manuscript. All authors reviewed and approved the manuscript.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper, the Supplementary Materials, and/or NCBI repository, bioproject PRJNA1043413 https://www.ncbi.nlm.nih.gov/sra/PRJNA1043413.

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Figures legends

Fig. 1 Experimental design for studying resting cell formation in *T. pseudonana*.

Fig. 2 Physiological and morphological characteristics of *T. pseudonana* during resting cell formation. **(a)** Temporal changes in cell density. **(b)** Cell viability assessment. **(c)** Morphology analysis of chloroplasts in vegetative cells (VC, left) and resting cells (RC, right), based on fluorescence microscope images. The upper and lower panels display light images and chlorophyll fluorescence for VC and RC, respectively. **(d)** Percentage of cells exhibiting chloroplast alterations. **(e)** Quantum yield (Fv/Fm) **(f)** Measurement of respiratory rate. Note: An asterisk denotes values that were found to be significantly different ($P < 0.05$), as determined by t-test comparison with vegetative cells.

Fig. 3 Germination of resting cells subjected to cold and dark conditions for 90 and 180 days, respectively. **(a, d)** Cell density during the process of germination. **(b, e)** Cell density of vegetative and resting cells during germination. **(c, f)** Quantum yield (Fv/Fm) during germination.

Fig. 4 Intracellular pH and viscosity of resting (RCs) and vegetative cells (VCs). **(a)** A comparative analysis of intracellular pH levels is conducted between RCs on days 90 and 160, as well as VCs on day 0. **(b)** Intracellular pH of RCs on days 30, 50, 72, and 102 in comparsion to VCs on days 30 and 50. **(c)** Fluorescent images show GFP-labeled cells prior to photobleaching, t=0 s immediately following photobleaching, and after a duration of 270s in the cytoplasmic region. **(d)** The fluorescence recovery curves after photobleaching (mean ± standard error mean [s.e.m]) are shown for VCs-eGFP and RCs-eGFP, n=5. **(e)** Fluorescent images display cells pre-photobleaching, t=0 s immediately post-photobleaching, and following a duration of 270 s in the chloroplast region. **(f)** The fluorescence recovery curves after photobleaching (mean ± standard error) of VCs, RCs, and RCs-eGFP in the chloroplast region are illustrated using n =5 replicates. The yellow rectangle box represents the designated region of Interest (ROI), used for bleaching analysis. Scale bar :5μm. **(g)** An examination is conducted to compare the intracellular pH

levels between RCs at 0, 12, 60, and 72 hours after germination and VCs at 12, 60, and 50 hours after germination. **(h)** After germination, the fluorescence recovery curves after photobleaching (mean ± standard error) are shown for VCs-eGFP and RCs-eGFP with n=5 replicates in the cytoplasmic region. **(i)** After germination, the fluorescence recovery curves after photobleaching (mean ± standard error) are shown for VCs and RCs with n=5 replicates in the chloroplast region.

Fig. 5 Metabolic and photo-bleaching assay (FRAP) following artificial adjustment of intracellular pH. **(a)** The effects of artificial adjustment of intracellular pH on respiration and photosynthesis in *T. pseudonana* vegetative cells. **(b)** Fluorescence recovery curves after photobleaching (mean ± standard error) of VCs-eGFP, RCs-eGFP, and VCs-pH 6.0+N (VCs-eGFP cells under nigericin + pH 6 treatment) in the cytoplasmic region (n=5). **(c)** Fluorescence recovery curves after photobleaching (mean ± standard error) of VCs-eGFP, RCs-eGFP, and VCs-pH 6.0+N in the chloroplast region (n=5). **(d)** Fluorescence recovery curves after photobleaching (mean ± standard error) of the cells under different treatments (n=5). VCs-eGFP served as the control group; VCs-pH 6.0+N represented VCs-eGFP cells treated with nigericin + pH 6 for 5 minutes; VCs-pH 8.0 indicated replacement of extracellular solution with f/2 medium (pH ≈ 8.0) after treating the cells with nigericin + pH 6 for approximately 50 minutes followed by culturing under light for 20 minutes.

Fig. 6 Energy generation and storage compounds during the formation of resting cells. **(a)** ATP content, **(b)** AMP to ATP ratio, **(c)** total carbohydrate content, **(d)** total free amino acid content, **(e)** TAG content, **(f)** contents of total fatty acids including saturated and unsaturated fatty acids, and **(g)** total protein content. An asterisk denotes values that were significantly different from vegetative cells as determined by t-test analysis with a significance level of $P < 0.05$.

Fig. 7 The KEGG term analysis of up-regulated DEGs **(a)** and down-regulated DEGs **(b)** in resting cells.

Fig. 8 A conceptual model depicting the process of resting cell formation in *T. pseudonana*.

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