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# Population and molecular responses to warming in *Netzelia tuberspinifera* – An endemic and sensitive protist from East Asia

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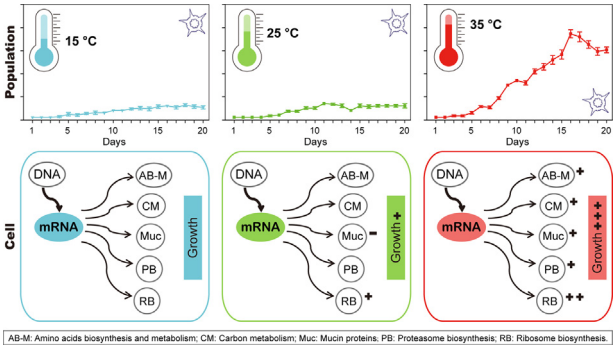
## HIGHLIGHTS

- An East Asian endemic protist species was successfully cultured in lab.
- Temperature drives the geographical and seasonal patterns of *N. tuberspinifera*.
- Biosynthesis of ribosomes was triggered in *N. tuberspinifera* from 15 to 25 and 35 °C.
- Molecular processes of growth and reproduction were triggered from 25 to 35 °C.

## GRAPHICAL ABSTRACT

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## ABSTRACT

With the effects of global warming becoming ever more obvious, biodiversity conservation is facing severe challenges. Currently, a deeper understanding the mechanisms of the effects of warming on sensitive species has become an important topic in aquatic biodiversity and ecological management. Our study first overcame the "challenge" for a sensitive indicator species (*Netzelia tuberspinifera*, an endemic testate amoeba species in East Asia) of culturing under laboratory conditions, and then explored its molecular response mechanisms to warming using transcriptomic analysis. Our data indicate that temperature mainly drove the geographical and seasonal variation of *N. tuberspinifera* populations. Transcriptomic results indicate that when the temperature is <25 °C, rising temperature triggers the biosynthesis of ribosomes; while the temperature is >25 °C, it triggers molecular processes related with cell division, test formation and general biomass increase. However, once the temperature exceeds 40 °C, *N. tuberspinifera* is unable to survive. Following from these results, the distribution of *N. tuberspinifera* might expand towards higher altitude or latitude regions under global warming. For the first time, our study showed direct evidence for sensitive protozoa species that presents a very narrow adaptation mechanism to local climate. Our work provides fundamental data for regional biodiversity conservation and scientific reference in subtropical and tropical waterbodies.



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## 1. Introduction

Global warming is expected to affect organism distribution and habitat associations, potentially leading to a significant decline of sensitive species (Pounds et al., 2006; AghaKouchak et al., 2014; Trisos et al., 2020). The surface temperature is predicted to increase by 2 °C until the mid-21st century, in which biodiversity will be facing severe challenges (Sheridan and Bickford, 2011; García-Robledo et al., 2016; Gao et al., 2019; Barbarossa et al., 2021). The decrease or loss of biodiversity threatens ecosystem functions, such as stability, invasion resistance, nutrient cycling, carbon retention and so on (Chapin et al., 2000; Hooper et al., 2012; Pennekamp et al., 2018). Therefore, deeper understanding the response of aquatic organisms (especially sensitive species) to warming has become a core topic in biodiversity and ecology. However, much work in this area has concentrated on larger multicellular organisms; and unfortunately, such works are lacking in single-celled protozoa, especially for freshwater ecosystems.

Arcellinida testate amoebae are a monophyletic group of amoeboid protozoa widely distributed in lakes and reservoirs (Patterson and Kumar, 2000; Wang et al., 2020). They play an important role in the biogeochemical cycles and energy flow of aquatic ecosystems and have extremely important ecological and evolutionary status among protists (Yang et al., 2006; Mitchell et al., 2008; Wilkinson, 2008; Lahr et al., 2019). In previous limnological studies, Arcellinida testate amoebae have been used as indicators to reveal changes in different environmental factors such as temperature, pH, and salinity (Patterson and Kumar, 2000; Mattheeussen et al., 2005; Roe and Patterson, 2014; Ndayishimiye et al., 2019). Recently, Ndayishimiye et al. (2019) developed temperature transfer functions of testate amoebae on freshwater ecosystems across China to gain insight into the ecological integrity and vulnerability to global warming. *Netzelia tuberspinifera* (basionym *Difflugia tuberspinifera*) is an endemic sensitive species of East Asia (Yang et al., 2004; Gomaa et al., 2017) and played an important role in the temperature transfer functions (Ndayishimiye et al., 2019). From datasets of 88 lakes and reservoirs in China, we found that the occurrence and distribution of *N. tuberspinifera* were significantly related to latitude, elevation and temperature (Yang et al., 2006; Liu et al., 2010; Ju et al., 2014; Ndayishimiye et al., 2019; Wang et al., 2021), indicating it is mainly distributed in subtropical and tropical waterbodies with relatively low latitude, low elevation and high temperature (Fig. S1). Previous studies suggested that changes of temperature can significantly shape the seasonal patterns of *N. tuberspinifera* population dynamics, while population density in surface water peaked in summer and autumn, and decreased to very low densities in winter and spring (Han et al., 2008; Wang and Han, 2008; Liu et al., 2010; Han et al., 2011).

In recent years, gene expression studies based on transcriptomic data have been applied mostly to monitor metabolic responses to perturbations in both prokaryotic and eukaryotic microorganisms (Caron et al., 2017; Schaum et al., 2018; Lu et al., 2019; Zhu et al., 2020). Fortunately, the advent of transcriptomic can provide an accurate and effective technique to explore the molecular mechanism of response to warming in *N. tuberspinifera*. The experiment, however, require the establishment of pure cultures to test different conditions, which render the work particularly challenging with *N. tuberspinifera*, which had not previously been successfully cultured in the laboratory.

In this study, we established a successful protocol for culturing *N. tuberspinifera* in laboratory and explored the effect of temperature on its growth along a temperature gradient from 4 to 40 °C which temperature setting based on the successive samples of *N. tuberspinifera* population in a subtropical reservoir (Tingxi Reservoir, Xiamen, southeast China). Then, we compared the transcriptomes of cells grown under three temperature levels (15, 25 and 35 °C). Our study aimed at answering the following questions: 1) How does warming affect growth and propagation of *N. tuberspinifera* through the setting of different temperature gradients; 2) Which metabolic pathways are triggered in *N. tuberspinifera* by warming.

## 2. Materials and methods

### 2.1. Sample collection and environmental data

Field sampling was conducted in the lacustrine zone close to the dam of Tingxi Reservoir (24°48′ N, 118°08′ E) in Xiamen, southeast China. Tingxi Reservoir provides an important drinking-water supply for Xiamen City, ensures flood control and irrigation for the surrounding cultures. It was built on a tributary of Dongxi River, the largest river of Xiamen City (Gao et al., 2019). Xiamen has a subtropical monsoon climate with annual mean temperature 20.7 °C and annual mean precipitation 1335.8 mm (Yang et al., 2017). A total of 60 L surface water samples were collected every 10 days from January 2016 to December 2018. Sediment samples were also collected using a sediment-trap (Fig. S2). Water temperature was measured *in situ* using a multiparameter water quality analyzer (Hydrolab DS5, Hach Company, Loveland, CO, USA) at 0.5 m interval from surface to bottom waters (Fig. S2). The temperature ranged from 14.3 to 32.1 °C for surface water and varied from 14.3 to 21.7 °C for bottom water in Tingxi Reservoir in this study (Fig. 1b, Fig. S2). The population of *N. tuberspinifera* was counted in an Utermöhl chamber using an inverted microscope (Motic AE31, Xiamen, China); densities were expressed in individuals per L (Fig. 1c). We calculated pairwise Spearman's rank correlation between the density of *N. tuberspinifera* and water temperature. We also applied generalized additive model (GAM) to evaluate the effect of water temperature on the density of *N. tuberspinifera* population. The GAM was fitted using the "mgcv" R package (Wood, 2016).

### 2.2. Culture of N. tuberspinifera

We isolated living individuals of *N. tuberspinifera* from fresh samples (plankton from surface water) under the inverted microscope using a narrow pipette. Single individuals of *N. tuberspinifera* were cleaned with sterile water, and then inoculated into 24-well cell culture trays containing culture medium. The medium includes *Scenedesmus obliquus* (Chlorophyta) as preys (obtained from Institute of Hydrobiology, Chinese Academy of Sciences), sterile quartz sand (diameter < 64 μm, collected from sediment of the reservoir) and *in situ* water. The *in situ* water was pre-filtered through 0.22 μm pore-size polycarbonate membrane (47 mm diameter; Millipore, Bedford, MA, USA) to remove unwanted particles and microorganisms. *S. obliquus* has been selected as the food source after many trials. Sand grain size was also optimized for test building. When in culture medium without sand grain or the sand grain size over 64 μm, *N. tuberspinifera* cannot maintain a normal growth.

Cultures of *N. tuberspinifera* were observed under light microscope every day. In order to investigate the response of *N. tuberspinifera* to a range of temperatures, we cultured *N. tuberspinifera* separately under seven temperature levels (from 4 to 40 °C) (Fig. 1e). Cultures were left to grow for 20 days. The temperature setting was based on the research of *N. tuberspinifera* population in a subtropical reservoir (*i.e.* Tingxi Reservoir) over three years.

### 2.3. Determination of key temperatures for transcriptomics

Our observations on *in vitro* growth of *N. tuberspinifera*, combined with our field observations from Tingxi Reservoir determined our choices on the experimental temperatures for building transcriptomes. Accordingly, we selected three temperature levels (Fig. 1): 15, 25 and 35 °C, respectively. Here, we considered 15 °C as approximately the lowest temperature water usually reaches in Tingxi Reservoir, 25 °C as the lowest temperature at which *N. tuberspinifera* is firstly observed in surface water in the field each year, and the 35 °C as the optimal temperature for growth in the laboratory (Fig. 1). First, we conducted simultaneous expansion cultivation at the optimal temperature (35 °C) to obtain enough individuals on which experiments could be

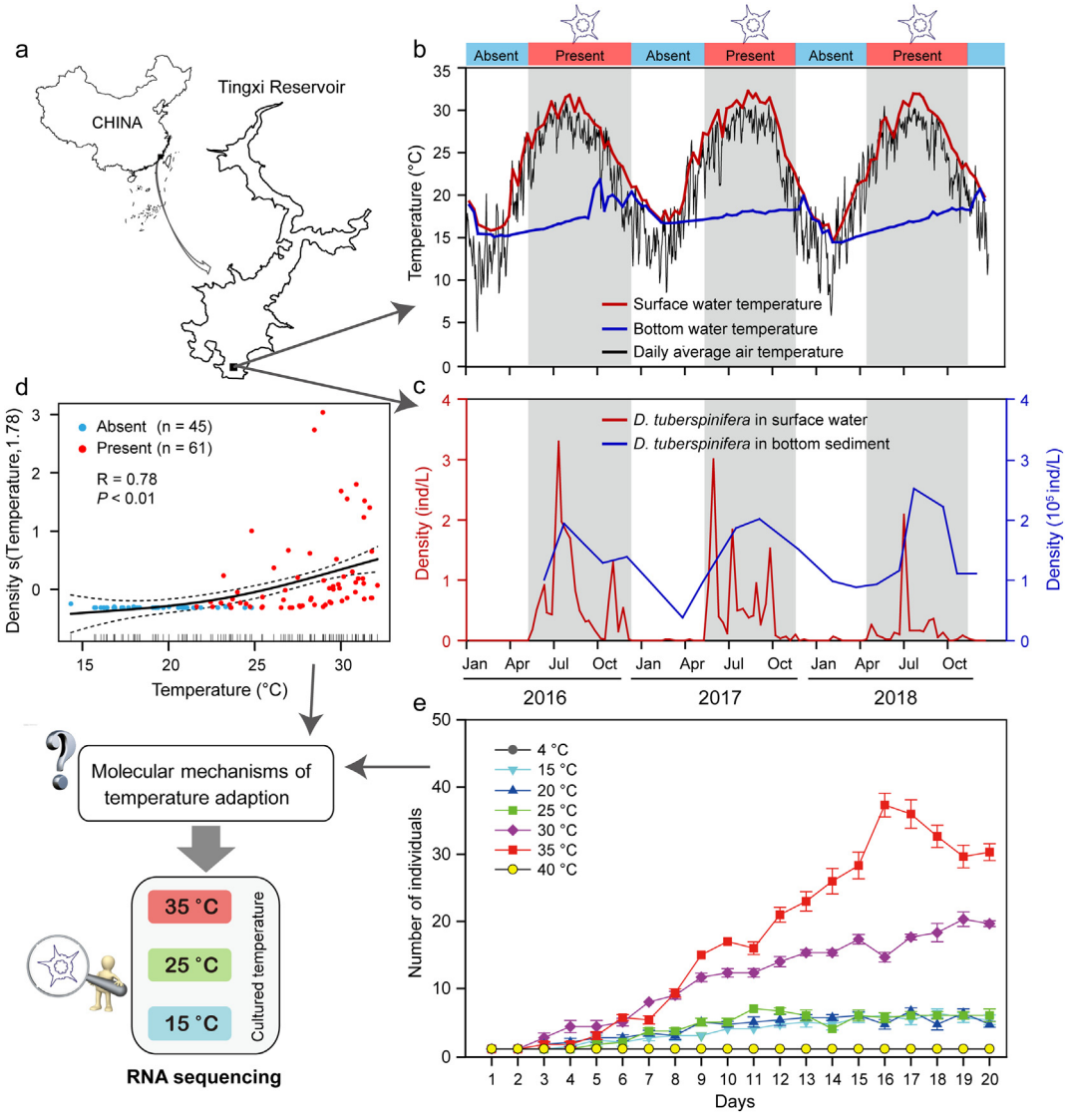


**Fig. 1.** Relationship between *Netzelia tuberspinifera* and temperature. a) Location of Tingxi Reservoir in southeast China. b) Temporal variation of air temperature and water temperature (surface and bottom) in Tingxi Reservoir. c) Density of *N. tuberspinifera* in surface water and bottom sediment over three years. d) Relationship between the density of *N. tuberspinifera* and surface water temperature. The black dashed lines indicate the 95% confidence intervals. e) The growth curve of *N. tuberspinifera* along temperature gradient including seven different levels. Data are showed as mean with standard error.

performed. Second, three biological replicates were used in each temperature treatment, and each sample included about 2000 cells. The culture experiments were conducted in culture plates (diameter: 5 cm) for 24 h at 15, 25 and 35 °C, respectively. To minimize contamination of transcriptomes with RNA from preyed organisms, *N. tuberspinifera* was not fed throughout the experimental period under three-temperature treatment. Each of cultures was filtered within 1 min through 64 μm pore-size membrane, then concentrated prior to total RNA extraction.

### 2.4. RNA isolation and sequencing

After harvesting the cells (each sample with 2000 cells), samples were immediately collected in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and frozen at -80 °C. RNA was extracted according to the manufacturer's protocol. In this isolation, ethanol precipitation instead of an RNA binding column was used (Mraz et al., 2009; Pan et al., 2018). RNA degradation and contamination were monitored on 1% agarose gel electrophoresis. The concentration, integrity and quality of RNA were checked by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Due to difficulties encountered in extracting large amounts of RNA, we used a micro-transcriptome sequencing approach in this study. The NEBNext® Ultra™ RNA Library Prep Kit (New England Biolabs, Boston, MA, USA) and the total RNA of per sample were used to generate a paired-end RNA-sequencing library following the manufacturer's instructions. The libraries were purified (AMPure XP system) and quantified using the Agilent Bioanalyzer 2100 system and then sequenced on an Illumina HiSeq platform (Illumina Inc., San Diego, CA, USA).

### 2.5. Bioinformatics

Raw reads for RNA sequencing were cleaned up by trimming adapter sequences, and then filtered the low quality reads (Q-score < 30 for ≥50% of nucleotides in each read). We removed the poly-N containing reads (≥ 5% of nucleotides in each read) using a proprietary program (Pan et al., 2018; Liang et al., 2019). The Q20, Q30, GC content and sequence duplication level of the clean data were calculated. The high-quality clean data were used in the downstream analyses (Table S1).

Transcriptome assembly was performed using Trinity (min_kmer_cov = 2, and all other parameters set as default) (Grabherr et al., 2011). After all the filtrations, we analyzed the completeness of the transcriptome by BUSCO analysis using an eukaryotic database (Ribeiro et al., 2020). The assemblies were annotated by the NCBI non-redundant nucleotide sequence database (NT), Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COG), Swiss-Prot database (Swiss-Prot), Kyoto Encyclopedia of Genes and Genomes (KEGG), Protein families database (Pfam) and NCBI non-redundant protein sequence database (NR), respectively, to predict the function of unigenes (Table S2) with a cutoff e-value of $1 \times 10^{-5}$ (Pan et al., 2018). Raw sequence data of mRNA have been deposited into the GEO databank of NCBI under the accession number GSE165834.

### 2.6. Differentially expressed genes

Differential gene expression levels were compared among 15 *vs.* 25 °C, 25 *vs.* 35 °C and 15 *vs.* 35 °C, respectively, using the R packages of "DESeq" (Anders and Huber, 2010; Love et al., 2014). Genes showing |log2 (fold change)| > 1 and FDR < 0.01 (adjusted *P*-value) at any of the three comparison groups were defined as differentially expressed genes (DEGs). Adjusted *P*-value was determined based on the Benjamini and Hochberg multiple-testing correction used the "p.adjust" method in R (Benjamini and Hochberg, 1995). We used integrated smoothness estimation for each explanatory variable and sample as a random factor. We performed a principal component analysis (PCA) of the covariance matrix to visualize the genes differentially expressed with varying temperature using the "ggrepel" R package. The results can reveal the difference of *N. tuberspinifera* in three temperature treatments based on DEGs (Pan et al., 2018).

### 2.7. KEGG enrichment analysis of differentially expressed genes

KEGG identification of each annotated gene was obtained from both the UniProt database and the GhostKOALA service (Kanehisa and Goto, 2000; Kanehisa et al., 2016). KEGG is a database resource intended to facilitate the assignation of high-level gene functions and utilities in a variety of biological systems, such as the cells, organisms, and ecosystems (meta-transcriptomes), using molecular-level information, especially large-scale molecular datasets generated by genome sequencing technologies (https://www.genome.jp/kegg/). The statistical enrichment of DEGs in KEGG pathways was assessed using the "phyper" function in R software (3.6.3). In addition, KEGG pathway categories were selected for KEGG enrichment analyses from the following categories: organismal systems, metabolism, genetic information processing, environmental information processing and cellular processes. Adjusted *P*-value <0.05 as cutoff was used to yield statistically significant gene enrichment for important pathways (Strimmer, 2008; Liang et al., 2019).

### 2.8. Protein-protein interaction networks

Protein-protein interactions were inferred using the software STRING (version 9.1) based on the DEGs (Franceschini et al., 2013). Here, we used the functional homology of interactional score over 300 as a threshold to define the significant protein-protein interactions in the networks.

### 2.9. Gene expression validation

Total RNA was extracted as described for DEGs library preparation and sequencing. We selected nine key DEGs identified by transcriptomic approach for real-time quantitative reverse transcription PCR (RT-qPCR) analysis following the previous procedure (Pan et al., 2018). The 500 ng of each RNA sample was reversely transcribed to cDNA using a Primescript™ RT reagent Kit (TaKaRa Biotech, Dalian, China). The qPCR reaction mixture consisted of 1 μL cDNA (~10 ng, each sample) with 0.6 mM each primer, 10 μL 2 X SYBR premix Ex Taq II and 7.8 μL dd$H_2O$. The amplification was carried out in triplicate with a thermal profile of 5 min at 95 °C, followed by 40 cycles of 20 s at 94 °C, 20 s at 51 °C, and 10 s at 72 °C. Thermal cycling was used for the reaction mixture on LightCycler 96 (LightCycle® 480, Roche, Basel, Switzerland). The amplification and the data analysis were carried out with a RT-qPCR detection system on the Roche LightCycler 480 software. We quantified the expression of nine selected genes using the 18S rRNA gene as a reference for normalization, these genes were selected based on their higher differential expression level (Table S3). Calculations of the average crossing point (Cp) values, standard deviations, and resulting expression ratios for each target gene were determined. The primer sets are listed in Table S3.

## 3. Results

### 3.1. Dynamics of N. tuberspinifera population in subtropical reservoir and in vitro

In the plankton, *N. tuberspinifera* population showed a significant seasonal cycle with highest densities in summer and autumn from January 2016 to December 2018 (Fig. 1c, Fig. S2). In sediment, *N. tuberspinifera* population exhibited a very similar seasonal pattern with highest densities also in summer and autumn (Fig. 1c). Surface water temperatures ranged throughout seasonal cycles from 14.3 to 32.1 °C in Tingxi Reservoir. Individuals of *N. tuberspinifera* could be observed in plankton in the summer when temperature reached 25.5 ± 0.7 °C, and remained below detection level in the winter below 21.2 ± 0.3 °C over three years (Fig. 1b, Fig. S2). In the surface waters of Tingxi Reservoir, *N. tuberspinifera* population density was strongly correlated with water temperature as evidenced by GAM analysis; the density of the *N. tuberspinifera* population was significantly and positively related to water temperature (Spearman's correlation, $n = 106$, $R = 0.78$, $P < 0.01$) (Fig. 1d). Over three years, the density of *N. tuberspinifera* in water was highest between 28.5 and 30.9 °C in Tingxi Reservoir.

*In vitro*, we found the *N. tuberspinifera* was usually in a cyst or dormant state and did not grow normally at 4 °C. Growth rates were very low at 15–25 °C (corresponding to field observations) and peaked at 35 °C. However, 40 °C was lethal to *N. tuberspinifera* (Fig. 1e).

### 3.2. Transcriptomic response to temperature

We generated approximately 47.27–54.16 millions of 150 bp pair-end reads through RNA sequencing in each sample for the three temperature treatments (15, 25 and 35 °C) (Table S1). The *de-novo* assembly yielded 53,743 total unigenes with an average length of 765 bp and 37,581 coding sequences (Fig. S3), as well as 89% of transcriptome completeness by BUSCO analysis. About 74.38% of all unigenes could be matched to genes documented in public databases (Fig. S4, Table S2).

A total of 2805 DEGs were detected, among which 361, 1876 and 1944 DEGs were detected for 15 *vs.* 25 °C, 25 *vs.* 35 °C and 15 *vs.* 35 °C comparisons, respectively (Fig. 2). Principal component analysis (PCA) showed that the biological replicates of each temperature exhibited the highest similarity between themselves, thus corroborating the reproducibility and reliability of the mRNA sequence data (Fig. 2b). We observed a higher number of down-regulated genes (254) than up-regulated genes (107) of DEGs when temperature increased from 15 to 25 °C. The number of up-regulated genes increased drastically from 25 to 35 °C (up-regulated: 1795; down-regulated: 81) and between 15 and 35 °C (up-regulated: 1904; down-regulated: 40) comparisons (Fig. 2d, Table 1). The genes encountered corresponded to five KEGG gene categories which are: organismal systems, metabolism, genetic information processing, environmental information processes and cellular processes (Fig. S5). The organismal systems- and metabolism-

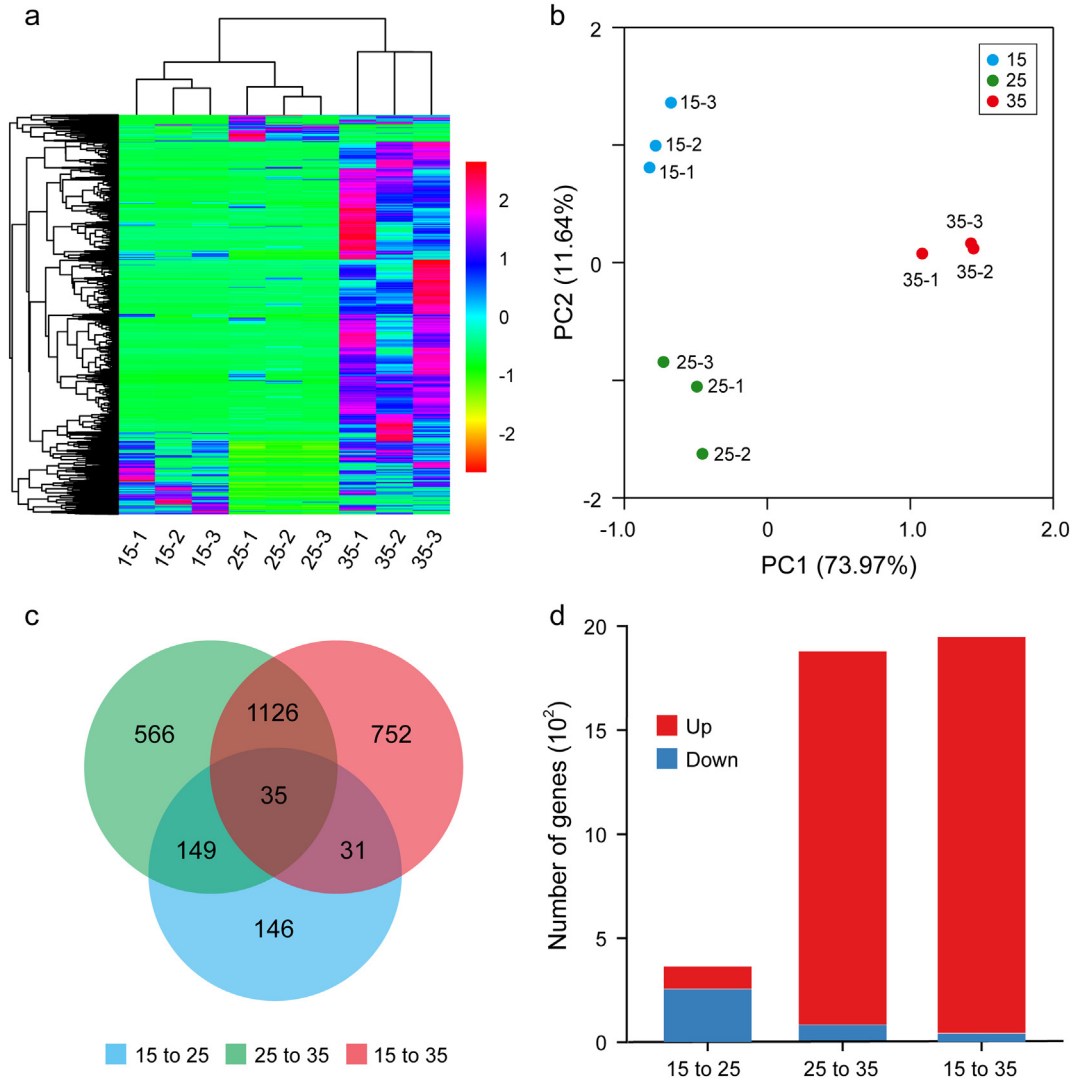


**Fig. 2.** The differentially expressed genes of *Netzelia tuberspinifera* based on the transcriptomic dataset. a) Heatmap showing the differentially expressed genes among three-temperature treatments. b) Principal component analysis of the differentially expressed genes counts for each treatment. Each circle represents one sample, each treatment has three replicates. Three biological replicates in each temperature: 15-1, 15-2, 15-3 for *N. tuberspinifera* cultured at 15 °C; 25-1, 25-2, 25-3 for *N. tuberspinifera* cultured at 25 °C; 35-1, 35-2, 35-3 for *N. tuberspinifera* cultured at 35 °C. c) Venn diagram showing the number of differentially expressed genes that are unique and shared among the three temperature treatments. d) The number of differentially expressed genes in each comparison group. The up and down indicate up-regulated and down-regulated genes, respectively.

associated genes were the most commonly up-regulated genes especially in the comparisons of 25 to 35 °C and 15 to 35 °C.

### 3.3. Identification of metabolic pathways associated with temperature rising

The KEGG enrichment of DEGs revealed the metabolic pathways signifcantly associated with temperature increase (Table 2). The genes encountered corresponded to five KEGG gene categories which are: organismal systems, metabolism, genetic information processing, environmental information processes and cellular processes based on KEGG enrichment (Fig. S6). In the 15 to 25 °C comparison, proteins associated with ribosome assembly and functioning were significantly up-regulated, particularly the genes of the large subunit ribosomal protein L32e (*RP-L32e*) and the small subunit ribosomal protein S12e (*RP-S12e*) (Fig. 3, Table 2). On the contrary, the synthesis and secretion of mucin proteins were significantly down-regulated from 15 to 25 °C, including the genes for mucin-2 (*MUC2*) and mucin-5 AC (*MUC5AC*) (Fig. 3b). However, these genes were up-regulated again in the 25 to 35 °C comparison.

Only ribosome synthesis pathway was significantly up-regulated in all comparisons. Most pathways were up-regulated in the comparisons 25 to

**Table 1**
Description and interpretation of the cells and differentially expressed genes in *Netzelia tuberspinifera* with three-temperature comparisons.

| General description | 15 to 25 °C | 25 to 35 °C | 15 to 35 °C |
|---|---|---|---|
| Temperature | up | up | up |
| Growth rate | 0 | up | up |
| Up-regulated expressed genes | 107 | 1795 | 1904 |
| Down-regulated expressed genes | 254 | 81 | 40 |

Up: up-regulated.

**Table 2**
The KEGG enrichment analysis for the up- and down-regulated differentially expressed genes in three temperature comparisons, respectively.

| Group | | Number of pathways | Gene counts | Q-value |
|---|---|---|---|---|
| From 15 to 25 °C | up | 1 | 7 | <0.05 |
| | down | 1 | 35 | <0.05 |
| From 25 to 35 °C | up | 17 | 441 | <0.05 |
| | down | 9 | 74 | <0.05 |
| From 15 to 35 °C | up | 12 | 395 | <0.05 |
| | down | 0 | 0 | <0.05 |

Up: up-regulated, down: down-regulated.

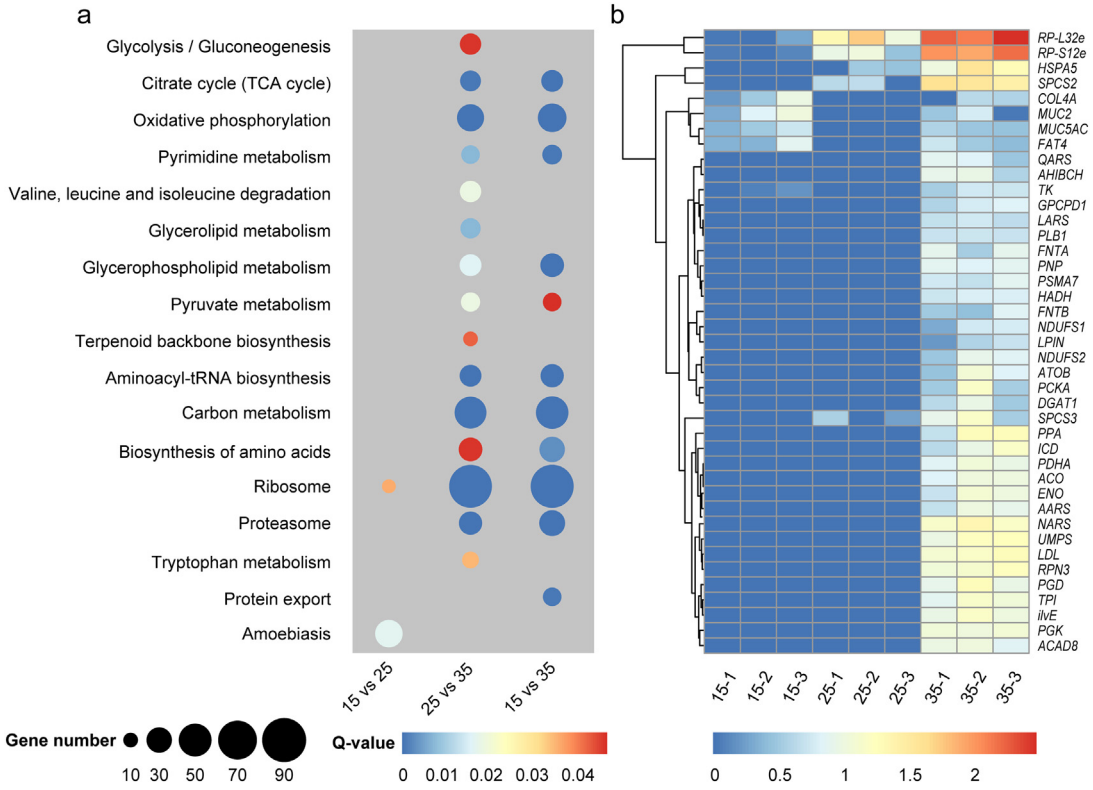


**Fig. 3.** Important KEGG pathways and key genes in *Netzelia tuberspinifera* that response to increasing temperature. a) Significant enrichment pathways based on KEGG enrichment analysis for each pair comparison group (Q-value <0.05). b) Heatmap illustrating the expression level of differentially expressed genes in all samples exposed to different temperatures. 15: cultured at 15 °C; 25: cultured at 25 °C; 35: cultured at 35 °C. For abbreviation of genes see Table S4.

35 °C and 15 to 35 °C; we did not observe any down-regulated pathway in the 15 to 35 °C comparison (Table 2). A majority of significantly up-regulated genes were related to general cell metabolism at higher temperatures (Fig. 3a, Fig. 4). Analogously, the number of genes for carbon metabolism and ribosome pathways was higher than other pathways both in 25 to 35 °C and 15 to 35 °C comparisons. However, glycolysis/gluconeogenesis, valine, leucine and isoleucine degradation, glycerolipid metabolism, terpenoid backbone biosynthesis and tryptophan metabolism were significantly up-regulated only in the 25 to 35 °C comparison, and protein export genes only in the 15 to 35 °C comparison (Fig. 3a).

We further selected those highly represented genes whose expression was most specifically correlated to temperature increase to be illustrated in a heat map (Fig. 3b). Particularly strongly up-regulated genes with increasing temperature were: pyruvate dehydrogenase E1 component alpha subunit (*PDHA*) in glycolysis/gluconeogenesis pathway, inorganic pyrophosphatase (*PPA*) in oxidative phosphorylation pathway, isocitrate dehydrogenase (*ICD*) and phosphoenolpyruvate carboxykinase-ATP (*PCKA*) in citrate cycle, and aconitate hydratase (*ACO*) in carbon metabolism. In glycerolipid and glycerophospholipid metabolisms pathways, the diacylglycerol o-acyltransferase 1 (*DGAT1*) and phospholipase B1, membrane-associated (*PLB1*) were particularly up-regulated. In addition, we found some genes related to mitosis in the DEGs although the increase in their upregulation was only marginally significant. Those genes exhibited a higher expression level at 35 °C than at 25 and 15 °C (Fig. S7), an expected result since culture growth reaches a peak at 35 °C.

### 3.4. Network of protein-protein interactions for differentially expressed genes

We did not detect any network module of protein-protein interactions in the 15 *vs.* 25 °C comparison, perhaps due to a smaller number of DEGs enriched by KEGG database (Fig. S8). For 25 *vs.* 35 °C and 15 *vs.* 35 °C comparisons, however, both networks of protein-protein interactions exhibited a strongly modularized structure. These modularized structures suggested the three co-expression patterns for differentially expressed functions that were (1) the genes for ribosome synthesis, (2) the genes for energy metabolism and biomass cycles (*e.g.*, glucose and lipid metabolism), and (3) the genes implicated in interactions between these last two categories (Fig. S8).

### 3.5. Transcriptomic data validation by qPCR

To validate the gene expression data obtained from RNA sequencing, nine genes were selected for RT-qPCR (for detail see Table S3). The RT-qPCR results showed a strong correlation with the data of RNA sequencing data ($R = 0.945$, $P < 0.001$, Fig. 5).

## 4. Discussion

Based on datasets of 88 waterbodies in China and one subtropical reservoir over three years, we found that the occurrence and distribution of *N. tuberspinifera* across space and time were significantly related to temperature. However, demographic fluctuations in *N. tuberspinifera*, as an example of temperature-sensitive species, were only inferred by observation without solid causal relationship with metabolic processes under different temperatures (Wang et al., 2021). Here, we first established clonal cultures of *N. tuberspinifera* and further used a comparative transcriptomic analysis with three temperature treatments to reveal the molecular mechanisms associated with different warming treatments, as well as the molecular basis for the increasing growth rates with elevated temperature in the field.

### 4.1. Temperature drives spatial and temporal distribution variations of *N. tuberspinifera* population

*N. tuberspinifera* was mainly distributed in subtropical and tropical waterbodies, which are characterized by relatively low latitude, low

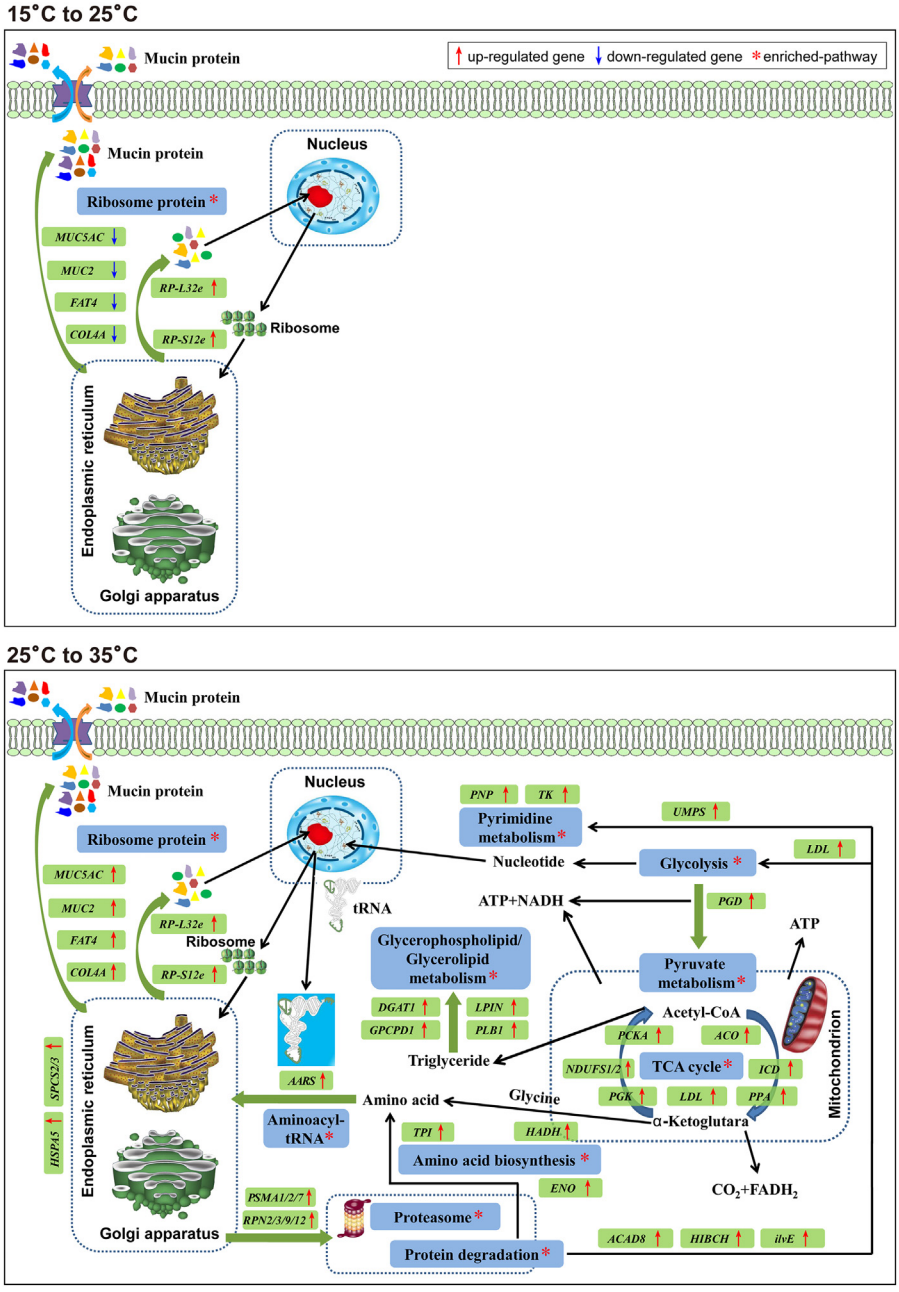


**Fig. 4.** Schematic representation of biological pathways in *Netzelia tuberspinifera* affected by increasing temperature. Green box represents the gene; blue box represents the KEGG pathway. The arrows or asterisks in the box represent the regulation of genes or significantly enriched pathways compared by 15 to 25 °C and 25 to 35 °C, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

elevation and high temperature (Yang et al., 2006; Liu et al., 2010; Ndayishimiye et al., 2019; Wang et al., 2021). Further, marked seasonal cycle patterns were already known in *N. tuberspinifera* populations in subtropical reservoirs (Han et al., 2008; Wang and Han, 2008; Liu et al., 2010; Han et al., 2011). However, the exact reasons for these demographic fluctuations remained unclear. Liu et al. (2010) speculated that the seasonal cycle pattern was perhaps driven by temperature. However, Han et al. (2008) suggested that food abundance was the strongest driver for the seasonal patterns observed in the water column, rather than temperature (Han et al., 2008). In our field survey, we observed a significant correlation between the abundance of *N. tuberspinifera* population and water temperature ($n = 106$, $R = 0.78$, $P < 0.01$). In fact, *N. tuberspinifera* is a generalist predator that can prey on algae, rotifers and cladocerans (Han et al., 2008; Han et al., 2011; Wang et al., 2015). In Tingxi Reservoir, we found that the density of *N. tuberspinifera* varied along the full seasonal cycle between 0 and 3.4 ind/L in the plankton, and disappeared in winter despite the abundant presence of preys (Gao et al., 2019). This indicates that prey abundance is probably not the key factor that shapes the seasonal occurrence and abundance cycles of *N. tuberspinifera* population, but suggests that temperature may play that role.

Our cultivation experiment along seven temperature levels further corroborated this idea, as food (*S. obliquus*) was provided and it availability did not vary in all replicates at the beginning of culture-experiments. Life has adapted to thrive under much of the range of ambient temperatures found on the Earth, but most species have specialized to live at a narrow subset of Earth's full temperature range (Pörtner et al., 2014). Species generally have higher intrinsic growth rates under optimal conditions including temperature (Liang et al., 2019). In our study, substantial population growth was observed only

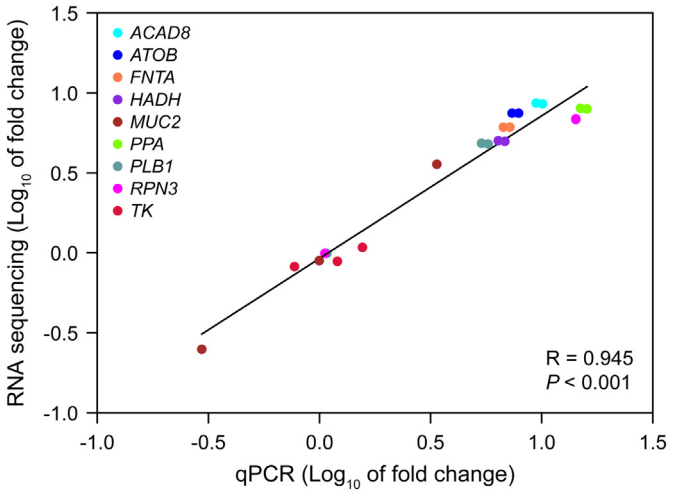


**Fig. 5.** Spearman's correlation between RNA sequencing and qPCR results for the nine selected genes. Each point represents a value of fold change in a gene. Fold change values were $\log_{10}$ transformed. For abbreviation of genes in Table S3.

between 25 °C and slightly below 40 °C, the latter being lethal; optimal growth was observed at 35 °C. Surface water temperatures varied from 14.3 to 32.1 °C in Tingxi Reservoir during the period 2016 to 2018, which means that optimum temperatures had not been reached during this period. *N. tuberspinifera* can be thus considered as a stenothermophilic protist (Boag et al., 2018), *i.e.* which grows best within a narrow temperature range.

### 4.2. Gene expression triggered by temperature in N. tuberspinifera from 15 to 25 °C

At 15 °C, both field and *in vitro* observations showed no growth, which corresponds well to the low gene expression levels observed. From 15 to 25 °C, while the growth curves still exhibited non-significant difference, the transcriptome provided evidence for metabolic activity. The biosynthesis of ribosomes was significantly up-regulated including seven related genes which mainly regulated the biosynthesis of large and small subunit ribosomal proteins. Ribosome synthesis prepares the cell for the synthesis of further proteins (Brimacombe, 1992) that would be needed for increased activity later in the season when water temperature further increases. This suggests also that a temporal drop in the temperatures is probably needed for the triggering the next steps in *N. tuberspinifera* life cycle.

Interestingly, the synthesis and secretion of mucin proteins showed a significant down-regulation from 15 to 25 °C. Mucin proteins have been found to be related to a wide range of functions, including cyst formation in protists (Tomita et al., 2018), as well as the regulation of biomineralization processes in molluscs, echinoderms and brachiopods, for example *Lingula* (Luo et al., 2015). *N. tuberspinifera* might keep a dormant state at <15 °C, which is consistent with the increased production of proteins present in cysts during an inactive part of the life cycle. At 25 °C, cells metabolism starts to activate and mucins are down-regulated to prepare cells for division. However, mucins are up-regulated again from 25 to 35 °C, as biomineralization is again required to build new shells for daughter-cells. Like other members of genus *Netzelia*, *N. tuberspinifera* builds its test at least in part with self-secreted mineral elements, unlike the genus *Difflugia* (Netzel, 1972; Gomaa et al., 2017). Here, our results may contain only part of mucins involved in life-related activities of *N. tuberspinifera*. Further research including immunocytochemical observations will be required to confirm this hypothesis.

### 4.3. Response mechanism in N. tuberspinifera from 25 to 35 °C

We observed an increased expression level for genes related to cell metabolism and division from 25 to 35 °C, thus corroborating both field and *in vitro* observations that showed increased division rates and emphasizing the thermophilic metabolism of *N. tuberspinifera*. The number of DEGs in the 25 *vs.* 35 °C comparison (1876) was far higher than in the 15 *vs.* 25 °C comparison (361); about 96% of all DEGs found in this study exhibited an up-regulation from 25 to 35 °C. Here, we found that genes corresponding to main cellular metabolic pathways were differentially expressed under these conditions, like ribosome biosynthesis, amino acids metabolism (tryptophan metabolism and valine, leucine, and isoleucine metabolism), synthesis of proteasome and carbon metabolism (glucose and lipid metabolisms).

These genes, among others, are required for biomass building, which corresponds well with the higher cell-division rates observed. Amino-acid metabolism related genes like tryptophan, valine, leucine, and isoleucine metabolism were significantly up-regulated in *N. tuberspinifera* from 25 to 35 °C. In line, the accelerated biomass building requiring more protein synthesis, the large subunit ribosomal protein and the small subunit ribosomal proteins were massively up-regulated. Moreover, the synthesis of proteasomes also showed a significant up-regulation in *N. tuberspinifera* from 25 to 35 °C, probably having an effect on the clearance of misfolded proteins, breakdown of regulatory proteins, or any other functions (Gille et al., 2003).

Cell metabolism-related genes were also up-regulated under higher temperatures. Carbon metabolism (*e.g.* glucose and lipid metabolisms) was significantly up-regulated from 25 to 35 °C. Similarly, glycolysis, oxidative phosphorylation and citrate cycle were significantly up-regulated in the glucose metabolism, from 25 °C to 35 °C, leading to more ATP production. Lipid metabolism is another important part of the carbon metabolism, which can provide original or intermediate materials for glucose and other metabolic processes (Walther and Farese, 2012). Here also, both glycerolipid and glycerophospholipid metabolisms were significantly up-regulated from 25 to 35 °C, and used in cell membrane production before cell division. Overall, temperature increase might trigger an increase in both biomass production through cell division and an increase in the metabolism of *N. tuberspinifera* from 25 to 35 °C.

Moreover, we found that proteasome-related genes were more active in gene co-expression modules, especially the 26S proteasome regulatory subunit (*RPN*) and 20S proteasome subunit (*PSMA*) gene clusters. A general gene co-expression pattern emerged correlated with rising temperature, *i.e.* carbon metabolism, amino acid metabolism and proteasome synthesis. These results indicate these genes may have a synergistic effect on gene functions that are triggered simultaneously when *N. tuberspinifera* increases metabolic rate and divides rapidly. The increase in metabolism and population size observed under warming can now be further confirmed by observations on mitosis-associated genes. Indeed, higher expression levels of these genes in *N. tuberspinifera* occurred at 35 °C than at 25 and 15 °C. This result further confirms the strong relationship between life cycle and temperature increase in *N. tuberspinifera*.

Intriguingly, the genes related to mitosis were found in *N. tuberspinifera*, but the typical and unequivocal meiosis genes of testate amoebae weren't found. Sexual phenomena in amoebae or protozoa have been reported, although they were earlier considered asexual. Recently, Hofstatter et al. (2018) found that most amoebozoans performed mixis, recombination, and ploidy reduction through canonical meiotic processes, among which some testate amoebae were confirmed such as *Arcella gibbosa* and *Difflugia bryophila* (Hofstatter et al., 2018). Therefore, the meiosis may also be present in *N. tuberspinifera*, however, more evidence is needed.

## 5. Conclusions

Our study demonstrated the preponderant role of temperature in the geographical occurrence and seasonal variation of *N. tuberspinifera*

populations in China. The occurrence and distribution of *N. tuberspinifera* were mainly in subtropical and tropical waterbodies with high temperature. The narrow dependence on marked seasonal patterns certainly plays an important role in the dispersal capacity of this conspicuous East Asian endemic species. Indeed, while high temperatures (> 25 °C) are needed to trigger molecular processes related with cell division, test formation and general biomass increase; ribosome production might require colder temperatures. Therefore, *N. tuberspinifera* requires a subtropical monsoon influenced climate to thrive; its distribution southwards is probably limited by the lack of colder season. On the other hand, the need for a long warm season prevents the species from expanding northwards. Environmental filters depending on climate are known to efficiently prevent global distribution of testate amoebae (Smith and Wilkinson, 1986), as suggested for some lineages of the soil species complex *Hyalosphenia papilio* (Singer et al., 2019). In our case, we show for the first time direct evidence for a testate amoeba that presents very narrow adaptations to a local climate.

In addition, as a biological indicator species, *N. tuberspinifera* exhibited the highest growth rate at 35 °C, but cannot survive at 40 °C. Such a sensitivity to natural temperature cycles suggests that the occurrence and distribution of *N. tuberspinifera* might be shifted towards higher altitudes/latitudes in a global warming environment. Given the position of *N. tuberspinifera* in the lacustrine food web as an abundant microbial top predator (Han et al., 2008; Wang et al., 2015), its removal from the community would probably disturb the local biotic interactions network which may then lead to a loss of ecosystem functions and services.

## Data availability

The transcriptomic data are available at the GEO database of NCBI (the accession number GSE165834).

## CRediT authorship contribution statement

J.Y. conceived the idea and designed the experiments. W.W., J.N. and H.C. collected the samples and determined the environmental parameters. W.W. isolated and cultured the *N. tuberspinifera*. W.W. and K.R. performed the RNA sequencing and bioinformatics. W.W. and J.N. measured the population density. W.W., J.Y. and X.G. analyzed data. W.W. and J.Y. wrote the first draft of the manuscript. E.L., D.L. and H.Q. reviewed and revised the manuscript. All authors contributed to and have approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150897.

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