

# A constitutive stress response is a result of low temperature growth in the Antarctic green alga *Chlamydomonas* sp. UWO241

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## Abstract

The Antarctic green alga *Chlamydomonas* sp. UWO241 is an obligate psychrophile that thrives in the cold (4–6°C) but is unable to survive at temperatures  $\geq 18^\circ\text{C}$ . Little is known how exposure to heat affects its physiology or whether it mounts a heat stress response in a manner comparable to mesophiles. Here, we dissect the responses of UWO241 to temperature stress by examining its growth, primary metabolome and transcriptome under steady-state low temperature and heat stress conditions. In comparison with *Chlamydomonas reinhardtii*, UWO241 constitutively accumulates metabolites and proteins commonly considered as stress markers, including soluble sugars, antioxidants, polyamines, and heat shock proteins to ensure efficient protein folding at low temperatures. We propose that this results from life at extreme conditions. A shift from 4°C to a non-permissive temperature of 24°C alters the UWO241 primary metabolome and transcriptome, but growth of UWO241 at higher permissive temperatures (10 and 15°C) does not provide enhanced heat protection. UWO241 also fails to induce the accumulation of HSPs when exposed to heat, suggesting that it has lost the ability to fine-tune its heat stress response. Our work adds to the growing body of research on temperature stress in psychrophiles, many of which are threatened by climate change.

## KEYWORDS

cold adaptation, heat stress, metabolism, psychrophile, transcriptome

## 1 | INTRODUCTION

Perennially cold environments, such as polar and alpine regions, are one of the world's largest ecosystems. Phototrophic microbes, many of which are obligate cold extremophiles (psychrophiles), are the dominant primary producers in these habitats and the base of virtually all low temperature food webs (Christmas, Anesio, & Sánchez-Baracaldo, 2015; Lyon & Mock, 2014; Margesin, 2008; Morgan-Kiss, Priscu, Pocock, Gudynaite-Savitch, & Huner, 2006). Most studies on psychrophily focus on the biochemical characteristics that enable these organisms to thrive at permanently low temperatures, including

cold-adapted proteins and increases in membrane fluidity, as well as the presence of ice-binding proteins, antifreeze proteins and cryoprotectants (reviewed in Siddiqui et al., 2013; De Maayer, Anderson, Cary, & Cowan, 2014). But a distinguishing feature of psychrophiles is not an exceptional ability to grow at low temperatures. Indeed, many land plants, green algae and cyanobacteria survive and grow at both cold and warm temperatures and are therefore not psychrophilic (Chang, Bräutigam, Hüner, & Ensminger, 2021; Hüner et al., 2012; Huner, Öquist, & Sarhan, 1998; Öquist & Huner, 2003; Tang & Vincent, 1999; Yamori, Hikosaka, & Way, 2014). Rather, it is the inability of psychrophiles to survive at moderate (mesophilic)

temperatures ( $\geq 20^{\circ}\text{C}$ ) that distinguishes them from cold-tolerant species (Morita, 1975).

*Chlamydomonas* sp. UWO241 is one of the most comprehensively studied algal psychrophiles and an up-and-coming model for photosynthetic adaptation to extreme environments (Cvetkovska, Hüner, & Smith, 2017; Dolhi, Maxwell, & Morgan-Kiss, 2013; Morgan-Kiss et al., 2006). This alga resides at a depth of 17 m within the water column of the perennially ice-covered Antarctic Lake Bonney (Neale & Priscu, 1995), where it faces several environmental challenges including constantly low temperatures ( $\sim 5^{\circ}\text{C}$ ), high salinity (0.7 M), extreme shading ( $5\text{--}15\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ), high oxygen concentrations (200% air saturation levels), low phosphorus levels (N:P  $\sim 1,000$ ), and seasonal extremes in photoperiod. While this environment is extreme in many aspects, it is also very stable. The perennial ice-cover prevents wind-driven water mixing and promotes a vertically stratified environment where the salinity, nutrient levels and temperature profiles remain extraordinarily constant year-round (Obryk, Doran, Hicks, McKAY, & Priscu, 2016; Spigel, Priscu, Obryk, Stone, & Doran, 2018).

In green algae, heat stress affects most cellular processes, including the fluidity of biological membranes, metabolism, enzyme activities, and protein homeostasis. To maintain cellular function and prevent irreversible damage, green algae induce a heat stress response (HSR) that involves arrest of active cell growth, a switch from regular to stress metabolism, compositional remodelling of membrane lipids, and maintenance of protein homeostasis (Schroda, Hemme, & Mühlhaus, 2015). One of the first responses to heat stress is the increased transcription of genes encoding heat shock proteins (HSPs), highly conserved molecular chaperones that engage in nascent protein synthesis as well as folding and transport (Gupta, Sharma, Mishra, Mishra, & Chowdhuri, 2010; Lindquist & Craig, 1988; Vierling, 2003; Wang, Vinocur, Shoseyov, & Altman, 2004). HSPs were first described in relation to heat shock in *Drosophila* (Ritossa, 1962), but are now known to be important in both normal homeostatic growth (Lindquist & Craig, 1988) as well as during several abiotic and biotic stresses (Park & Seo, 2015; Vierling, 2003; Wang et al., 2004). Their function has been extensively studied in the model green alga *Chlamydomonas reinhardtii* (Nordhues, Miller, Mühlhaus, & Schroda, 2010; Schroda et al., 2015; Schroda & Vallon, 2009), where their expression is regulated by a single heat shock transcription factor (HSF1) (Schmollinger et al., 2013). The HSR in *C. reinhardtii* is rapidly initiated, with increased HSP expression seen within the first 25 min of exposure to  $42^{\circ}\text{C}$  and robustly maintained for at least 24 hr (Hemme et al., 2014; Légeret et al., 2016; Mühlhaus, Weiss, Hemme, Sommer, & Schroda, 2011).

Life in the perpetual cold has shaped the physiological make-up of UWO241, which has been mostly studied at the level of its photosynthetic machinery, as reviewed in (Cvetkovska et al., 2017) and subsequently discussed in (Cook et al., 2019; Kalra et al., 2020; Szyszka-Mroz et al., 2019). This alga is unable to grow above  $18^{\circ}\text{C}$ , but little is known how exposure to temperatures above this upper growth limit affects its physiology. It been shown that exposure to  $24^{\circ}\text{C}$  is lethal but cell death occurs slowly, and the effects are reversible in the first 12 hr (Possmayer et al., 2011). Short-term exposure to  $24^{\circ}\text{C}$  resulted

in the cessation of cell growth, inhibition of PSII photochemical efficiency and expression of the molecular chaperone HSP22A, and longer exposures led to cell death (Possmayer et al., 2011). In addition, two key photosynthetic proteins in UWO241, ferredoxin (Cvetkovska et al., 2018) and the chloroplast kinase STT7 (Szyszka-Mroz et al., 2019) were shown to be specifically adapted to low temperatures, with higher activities in the cold but at the expense of increased sensitivity and loss of activity at more moderate temperatures when compared to their mesophilic homologs from *C. reinhardtii*. While it is clear that UWO241 experiences stress at moderate temperatures, it is currently unknown whether this psychrophile mounts an HSR in a manner comparable to its mesophilic relatives. In this work, we compare UWO241 to the classical model and reference organism *C. reinhardtii* (Salomé & Merchant, 2019), whose responses to both cold and heat stress have been characterized in detail at the level of the genome, transcriptome and metabolome (reviewed in (Ermilova, 2020; Schroda et al., 2015)). To gain insight into the systemic response of UWO241 to temperature stress, we examined the growth, primary metabolome and transcriptome of UWO241 under steady-state low temperature and heat stress conditions. The recent sequencing of the UWO241 genome (Zhang, Cvetkovska, Morgan-Kiss, Hüner, & Smith, 2021) has placed UWO241 in an excellent position for comparative sequence analysis with other green algae models, including *C. reinhardtii* (Cvetkovska et al., 2017; Possmayer et al., 2016). Using this new resource, we investigated the presence of HSP genes in the UWO241 genome and their responsiveness to heat stress using RNA-Seq and protein immunoblotting. Our work contributes to a better understanding of psychrophilic stress biology, a question that is gaining in importance since polar environments are particularly threatened by current patterns of global climate change (Kennicutt et al., 2019; Xavier et al., 2016).

## 2 | MATERIALS AND METHODS

### 2.1 | Strains and growth conditions

*Chlamydomonas* sp. UWO241 and *Chlamydomonas reinhardtii* (cc-1,690) were grown axenically in Bold's Basal Medium (BBM) supplemented with 10 mM NaCl. All cultures were aerated continuously with ambient air filtered by a  $0.2\ \mu\text{m}$  filter in 250 mL glass growth tubes suspended in thermo-regulated aquaria. A continuous growth irradiance of  $130\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$  was generated by fluorescent tubes (Sylvania CW-40) and measured with a quantum sensor attached to a radiometer (Model LI-189; Li-Cor). Algal cultures were exposed to heat stress by transfer to aquaria maintained at  $24^{\circ}\text{C}$  (UWO241) and  $42^{\circ}\text{C}$  (*C. reinhardtii*). Mid-log cultures were used in all experiments.

### 2.2 | Growth and cell death kinetics

Cell growth was estimated by measuring optical density at 750 nm ( $\text{OD}_{750}$ ) and chlorophyll concentration in algal cultures over time

(Possmayer et al., 2011). The concentrations of chlorophyll *a* and *b* were measured spectrophotometrically at 647 and 664 nm (Cary 50 Bio; Varian, USA) and calculated as described (Jeffrey & Humphrey, 1975). Maximum growth rates were calculated using natural log transformation of the optical density values during the exponential phase. Culture viability was assayed by resuspending pelleted algal cells in 0.5% (w/v) Evans Blue solution, incubating for 30 min and removing the unbound dye by washing with BBM medium. Dye bound to dead cells was solubilized in 50% (v/v) methanol and 1% (w/v) SDS, and extracted by incubation at 50°C for 30 min. The suspension was centrifuged (16,000 g, 3 min), and absorbance was measured spectrophotometrically at 600 nm. The absorbance of cells treated with 1% (v/v) chloroform (100% death) was equivalent to values obtained after prolonged exposure to heat. Light microscopy was carried out using a Zeiss Axioimager Z1 Microscope (Carl Zeiss AG, Germany) at the Integrated Microscopy Facility, The Biotron, Western University.

### 2.3 | Gas chromatography-mass spectrometry and metabolomic analysis

Algae were grown in three biological replicates at 4, 10 and 15°C and sampled at a steady-state temperature or after 6-hr exposure to 24°C. Cells were harvested by centrifugation (6,000 g, 5 min), washed once with fresh medium, flash-frozen in liquid N<sub>2</sub>, and stored at -80°C. Metabolite extractions, chromatography and quality processing were performed at the West Coast Metabolomics Center (UC Davis, CA, USA), following a previously established protocol optimized for retention and separation of primary metabolite classes (amino acids, carbohydrates, sugar acids, sterols, aromatics, nucleosides, amines and miscellaneous compounds (Fiehn et al., 2008)). Mass spectra were processed using BinBase and analysed as described in (Fiehn, Wohlgemuth, & Scholz, 2005). Metabolites were identified based on their mass spectral characteristics and GC retention times by comparison with compounds in a plant and algae reference library (West Coast Metabolomics Center). Peak heights for the quantification ion at the specific retention index corresponding to each metabolite were normalized by the sum of peak heights in the sample. Normalized data were processed by cube root transformation followed by range scaling. All statistical analyses were performed by the Metaboanalyst 4.0 software suite (Chong et al., 2018) and included principal component analysis (PCA), analysis of variance (ANOVA), heatmap and clustering analysis using Ward's linkage for clustering and Pearson's correlation as a measure of dissimilarity.

### 2.4 | RNA sequencing and transcriptomic analysis

Nucleic acids were isolated from frozen cell pellets using a modified CTAB protocol (Possmayer et al., 2011). RNA concentration was determined using a Nanodrop2000 (Thermo Fisher Scientific), and integrity was assessed with a 2,100 Bioanalyzer (Agilent Technologies,

USA). RNA library preparation and sequencing were performed by Genome Quebec (Montreal, QC, Canada). Libraries were generated from 250 ng of total RNA. Poly-A mRNA was isolated with the NEB-Next Poly(A) mRNA Magnetic Isolation kit (NEB, USA). Reverse transcription was performed with the NEBNext RNA First Strand Synthesis kit (NEB) and second strand synthesis with the NEBNext Ultra Directional RNA Second Strand Synthesis kit (NEB). Libraries were prepared using the NEBNext Ultra II Library Prep Kit for Illumina (NEB) and were sequenced with 100 base paired-end reads on an Illumina HiSeq4000 platform (Illumina, San Diego, USA).

For gene expression analysis, the RNA-Seq reads were mapped to the UWO241 assembled genome (Zhang, Cvetkovska, et al., 2021) (Accession number GCA\_016618255.1) using HISAT2 (Kim, Langmead, & Salzberg, 2015) and counted against the predicted gene models using HTSeq-count v0.11.3 (Anders, Pyl, & Huber, 2015). Stringtie v2.1.5 was used to generate expression estimates from the SAM/BAM files created by HISAT2 (Pertea, Kim, Pertea, Leek, & Salzberg, 2016). Samtools v1.11 was used to read and write Illumina RNA-Seq alignments in the SAM and BAM files. The total number of aligned reads was normalized by gene length and sequencing depth and expressed as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) as a measure of the expression level for each gene. Differentially expressed genes (DEGs) were determined by Ballgown v2.22.0 (Pertea et al., 2016) and edgeR v2.22.0 (Robinson, McCarthy, & Smyth, 2010). Genes were sorted according to their log<sub>2</sub> (read counts)-transformed values. The Generally Applicable Gene-set Enrichment (GAGE v2.40.1) package in R (Luo, Friedman, Shedden, Hankenson, & Woolf, 2009) was used to perform pathway analysis based on genes that were assigned Chlamydomonas Entrez IDs. The parameter 'same.dir' in GAGE was set in True and significantly regulated pathways were defined as those enriched sets of genes with a *p*-value < 0.05. To generate the heatmap expression profiles of HSPs, hierarchical clustering using the Euclidean distance method was performed within each subfamily using the ComplexHeatmap R package. Venn diagrams were constructed using an online tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

### 2.5 | Identification of HSP genes in green algal genomes

The UWO241 genome (Zhang, Cvetkovska, et al., 2021) was screened for the presence of HSP genes using *C. reinhardtii* HSP protein sequences (Phytozome v12.1) and conserved domains typical for HSPs from the Pfam database (Mistry et al., 2021) as queries. Putative HSP genes in UWO241 were identified through a tBLASTn search ( $e$ -value <  $e^{-10}$ , bit-score > 100). The results were manually inspected for redundant sequences and to ensure correct gene structure annotation. The presence of conserved HSP domains was confirmed using Pfam and NCBI Conserved Domain Database (Lu et al., 2020). The gene names are based on the closest *C. reinhardtii* homologs, and multiple homologous sequences were numbered in order of discovery (e.g., HSP70A-1). Only genes supported by transcriptomic data are

reported. The genomes of other green algae were obtained from GenBank (*Chlamydomonas* sp. ICE-L (Zhang et al., 2020); *Chlamydomonas eustigma* (Hirooka et al., 2017); *Gonium pectorale* (Hansch et al., 2016); *Chlorella sorokiniana* (Arriola et al., 2018)) or Phytozome V12.0 (*Dunaliella salina* (Polle et al., 2017); *Volvox carteri* (Prochnik et al., 2010); *Coccomyxa subellipsoidea* (Blanc et al., 2012)) and similarly screened. Only full-length genes were used in downstream analyses. Multiple sequence alignments were performed using ClustalW (Sievers et al., 2011) implemented through Geneious Prime (Biomatters Ltd, Auckland, New Zealand). Cladograms and phylogenetic trees were inferred based on protein alignments using FastTree v.2.1 with the Neighbor-Joining method and the Jukes-Cantor genetics distance model (Price, Dehal, & Arkin, 2010), and annotated in iTOL v6 (<https://itol.embl.de/>). The bootstrap values for each branch reflect the percentage on 1,000 replicate trees. Subcellular localization was predicted by four independent software: TargetP2.0 (Armenteros et al., 2019), Predotar (Small, Peeters, Legeai, & Lurin, 2004), WoLF PSORT (Horton et al., 2007) and LOCALIZER (Sperschneider et al., 2017).

## 2.6 | SDS-PAGE and immunoblotting

Algal cells were harvested by centrifugation (5,000 g, 5 min), resuspended in 0.1 M Na<sub>2</sub>CO<sub>3</sub> containing 0.1 M DTT, and frozen at -20°C for 1 hr. Protein samples were solubilized with 5% (w/v) SDS and 30% (w/v) sucrose, and heated to 85°C for 5 min. The protein content was quantified using a protein quantification kit (Pierce BCA Protein Assay Kit, ThermoFisher Scientific) and loaded on equal protein basis. Electrophoresis and protein transfer was performed as described before (Szyzka-Mroz, Pittcock, Ivanov, Lajoie, & Hüner, 2015). Membranes were probed with primary antibodies raised against *C. reinhardtii* and specific for HSP70A (AS08371; 1:3,000), HSP70B (AS06175; 1:10,000), HSP90A (AS08346; 1:1,500), HSP90C (AS06174; 1:5,000) (Agrisera, Vännäs, Sweden), and CPN60A (1:5,000) (a kind gift from M. Schroda) predicted to bind specifically to their closest homologs in UWO241 ( $\geq 90\%$  epitope homology, personal communication). All proteins were confirmed to be the expected size (data not shown). HRP-conjugated secondary antibody was used (1:10,000; Sigma-Aldrich), and antibody-protein complexes were visualized using enhanced chemiluminescence detection reagents (GE Healthcare). Protein amounts were quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MA, USA). Protein alignments between UWO241 and *C. reinhardtii* major HSPs used in the immunoblotting analyses are shown in Figure S7.

## 3 | RESULTS

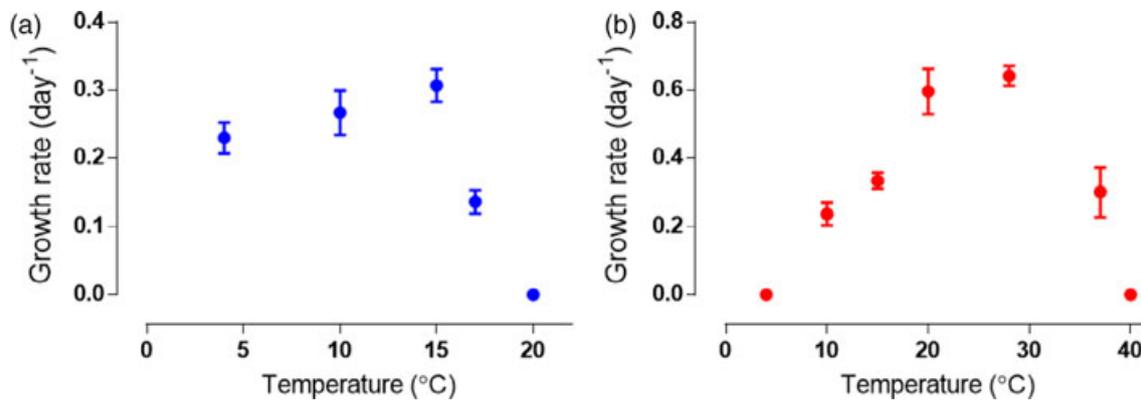
### 3.1 | The growth and metabolic make-up of UWO241 and *C. reinhardtii* at low temperature

In its native environment, UWO241 is exposed to year-round low temperatures (4–6°C). A culturing temperature of 4°C supported a

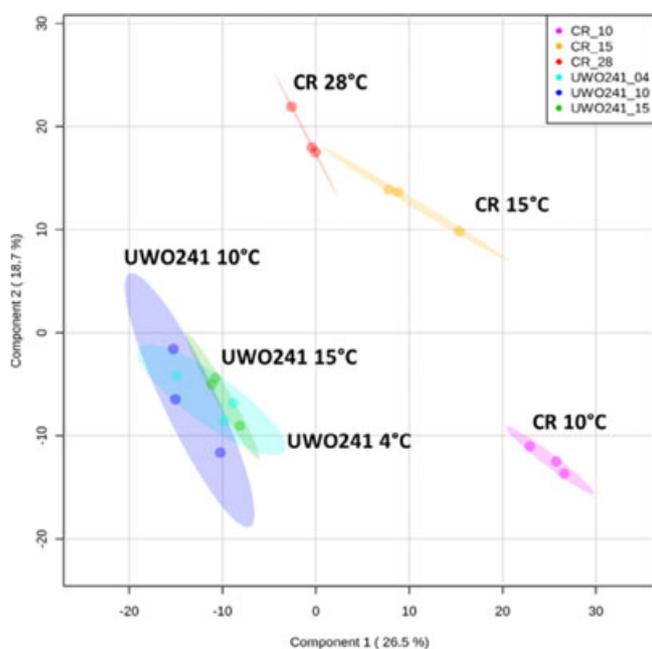
robust growth ( $\sim 0.22$  day<sup>-1</sup>), but maximal growth rates were achieved at 10–15°C (0.25–0.30 day<sup>-1</sup>). As an obligate psychrophile, UWO241 did not grow at 20°C (Figure 1a). In accordance with previous reports (Schroda et al., 2015), *C. reinhardtii* exhibited maximal growth rates at 20–28°C ( $\sim 0.6$  day<sup>-1</sup>) and lower rates at temperatures both higher and lower than this range (Figure 1b) and was unable to grow <10°C. *C. reinhardtii* cultures were able to grow at 10 and 15°C, albeit at a decreased rate (0.25–0.32 day<sup>-1</sup>) compared to growth at 28°C (Figure 1b), but notably the rates were similar to those of UWO241 between 10 and 15°C (Figure 1a).

Untargeted analysis of the primary metabolome of both species acclimated to different temperatures (4, 10, 15°C – UWO241; 10, 15, 28°C – *C. reinhardtii*) detected 771 unique metabolites, 163 of which were positively identified based on their mass spectra and retention times (Kind et al., 2009). PCA analysis revealed that the metabolic status of *C. reinhardtii* was dependent on the culturing temperature and differed between both components. This contrasted with UWO241 where temperature had minimal effects on the overall metabolic status, and we observed only nominal separation between the cultures grown at different temperatures (Figure 2). Differentially accumulated metabolites (DAMs) were defined as those exhibiting a twofold change (FC) in accumulation between treatments ( $p < 0.01$ , ANOVA, Tukey's post hoc). The metabolome of *C. reinhardtii* responded strongly to growth temperature with 273 (35%) DAMs at 10°C compared to the optimal temperature of 28°C. This response was temperature-dependent, with cultures at 15°C exhibiting a similar metabolite profile but with a decreased magnitude of the response (72 DAMs, 9%) (Figure 3a). The metabolite profile of UWO241 was similar regardless of the culturing temperature. Cultures at 10 and 15°C had only 78 (10%) and 48 (6%) DAMs when compared to 4°C (Figure 3a). These findings indicate that the mesophile grown at low temperatures adjusts its metabolome as an acclimation to cold, while the primary metabolome of the psychrophile is less affected by culturing temperature.

Next, we analysed the 163 primary metabolites with positively identified chemical signatures by comparing all samples to the metabolome of *C. reinhardtii* grown at 28°C (Figure 3b; Data S1). We present the 20 metabolites that have the largest differences in abundance between the treatments (Table 1). Carbohydrates and glycerol are well-known cryoprotectants in cold-adapted plants and algae (Leya, 2013; Roser, Melick, Ling, & Seppelt, 1992; Su et al., 2016; Tulha, Lima, Lucas, & Ferreira, 2010). We observed high accumulation of several carbohydrates (trehalose, maltose, and fructose) and glycerol metabolism intermediates in *C. reinhardtii* grown at 10°C. UWO241 accumulated these compounds constitutively, regardless of culturing temperature (Figure 3b; Data S1). Carboxylic acids and TCA cycle intermediate accumulation showed a strong dependence on growth temperature and was increased at the lowest temperature for both algae. Notably,  $\alpha$ -ketoglutarate ( $\alpha$ -KG), 3-phosphoglycerate (3-PGA), and phosphoenolpyruvate (PEP) showed the highest increases in abundance in UWO241 grown at 4°C (FC 48.1, 39.8, and 18.0, respectively). Lactic acid is the exception: Its abundance is significantly increased in the mesophile (FC 70.2 at 10°C) but decreased in



**FIGURE 1** The maximal growth rate of exponentially growing algal cultures at various temperatures. (a) *Chlamydomonas* sp. UWO241 (b) *Chlamydomonas reinhardtii*. Data are the means  $\pm$  SD of at least six biological replicates [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 2** Principal component analysis (PCA) of the primary metabolome of the two *Chlamydomonas* species acclimated to different steady-state temperatures. *C. reinhardtii* was grown at 10°C (magenta; CR\_10), 15°C (orange; CR\_15) and 28°C (red; CR\_28). *UWO241* was grown at 4°C (cyan; UWO241\_04), 10°C (blue; UWO241\_10) and 15°C (green; UWO241\_15). The analysis includes all 771 quantified metabolites separated along the first two principal components that explained the largest degree of variation in the datasets, and the 95% confidence interval for each treatment [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

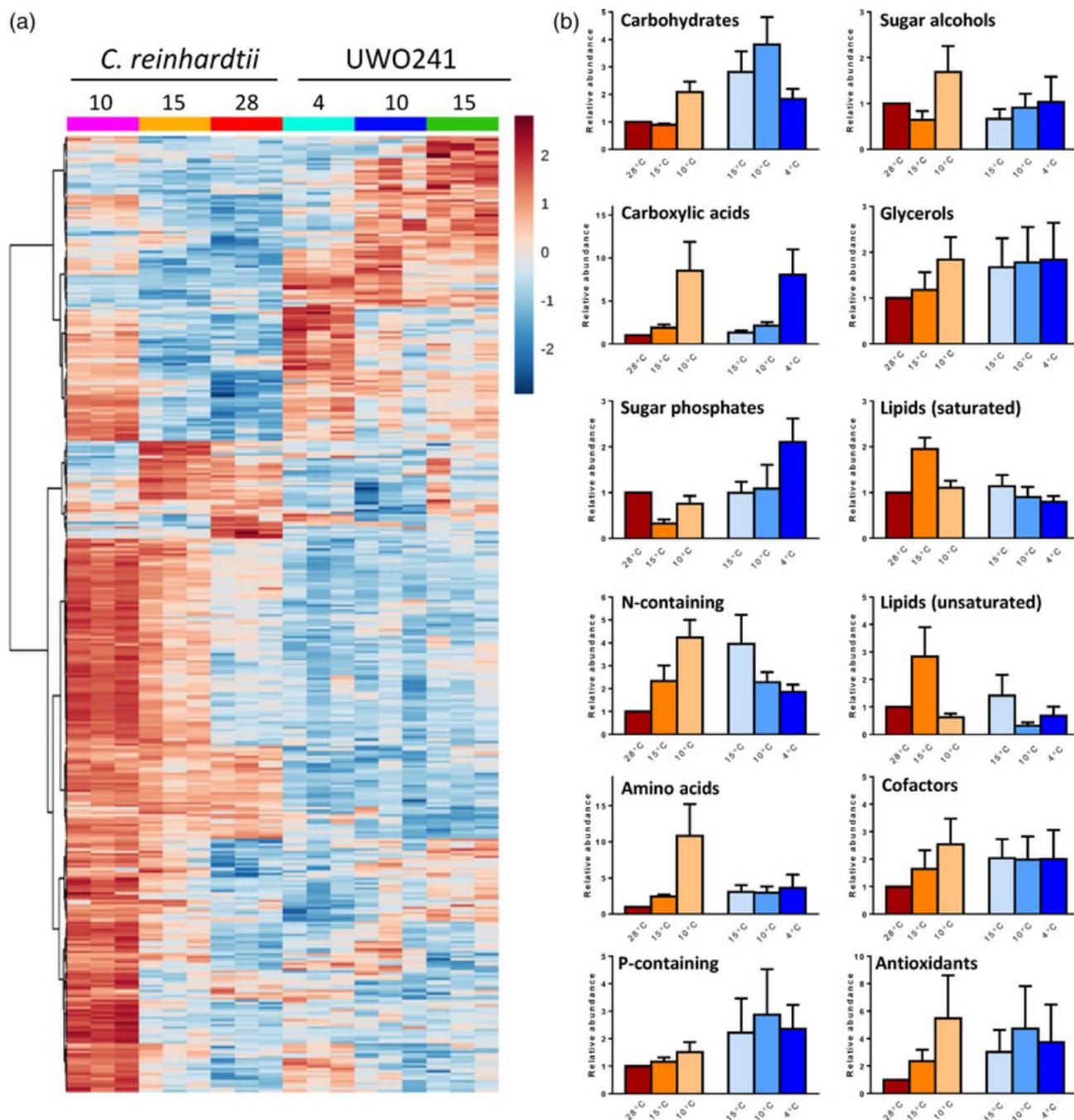
the psychrophile (FC 6.7 at 4°C). We also detected high accumulation of dehydroascorbic acid in both *C. reinhardtii* at 10°C (FC 19.7) and *UWO241* at all temperatures (FC 12.4–22.5) (Table 1). Threonic acid, a product of ascorbate catabolism (Debolt, Melino, & Ford, 2007), showed a similar pattern, suggesting an important role for the ascorbate pathway during low temperature growth.

We observed species-specific differences in the primary metabolomes. Glucose, which has known roles in osmotolerance and cold

stress (Demmig-Adams, Garab, Adams III, & Govindjee, 2014; Taïbi et al., 2018), was present at lower levels in *UWO241* compared to *C. reinhardtii*, regardless of the temperature (FC 24–81). Sugar alcohols are important molecules in cold-stress tolerance in plants and algae (Leya, 2013; Roser et al., 1992); their accumulation was increased in *C. reinhardtii* but not in *UWO241* (Figure 3b), although both species showed a significant decrease in several sugar alcohols (mannitol and galactinol; Table 1) at low temperatures. Amino acid metabolism was significantly affected by low temperature in *C. reinhardtii* and nearly all detected amino acids increased in abundance. Again, we did not observe this in *UWO241*, and amino acid abundance was largely unchanged or decreased (Figure 3b; Table 1; Data S1). An exception is the non-proteinogenic amino acid ornithine, which accumulated at low temperatures in both species. In *C. reinhardtii*, this accumulation was temperature-dependent (higher at 10°C than at 28°C, FC 9.7), whereas in *UWO241*, it is constitutively high at all temperatures (FC 33.5–34.7; Table 1). N-containing compounds, including those involved in purine and pyrimidine metabolism, exhibited cold-dependent accumulation in *C. reinhardtii*; however, we observed the opposite trend in *UWO241* where N-compounds accumulated at higher levels at 15°C when compared to 4°C (thymidine, Figure 3b, Table 1; Data S1). Altogether, we suggest that these data reflect a metabolic switch in the primary metabolism of *UWO241* due to life in a perennially cold environment. *UWO241* appears to retain an active energy metabolism at low temperatures, which fuels the constitutive accumulation of stress-related compounds across a range of growth temperatures.

### 3.2 | Highest growth rates do not correlate with resistance to heat stress in *UWO241*

To test whether growth temperature affects heat stress sensitivity, *UWO241* and *C. reinhardtii* were exposed to non-permissive temperatures (24 and 42°C, respectively). To ensure sufficient but non-lethal stress, we based these treatments on previous heat stress studies in *UWO241* (Possmayer et al., 2011) and *C. reinhardtii* (Hemme



**FIGURE 3** Differences in the primary metabolome of *C. reinhardtii* and UWO241, acclimated at different steady-state temperatures. (a) Heat map showing the relative changes in metabolite abundances between growth temperatures in the two algal species. Only metabolites, which are significantly different are shown (392 metabolites, ANOVA,  $p < 0.01$ ). In each treatment, three biological replicates are represented using a colour-based metabolite profile as indicated (red – increase in abundance; blue – decrease in abundance). Hierarchical clustering is based on Euclidean distances and Ward's linkage. (b) Relative abundance of metabolites classified based on their chemical nature. Only metabolites, which were positively identified based on their GC-MS spectra and retention times, were taken into consideration. In this analysis, the metabolite abundance corresponding to *C. reinhardtii* grown at 28°C was arbitrarily set to 1 and all other treatments were compared to this sample [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

et al., 2014; Légeret et al., 2016), which showed that the response of *C. reinhardtii* to 42°C was broadly comparable to the that of UWO241 to 24°C. To quantify the effects of heat stress, we measured cell death using Evans Blue dye that accumulates in cells with damaged membranes (Figure S1). UWO241 cultured at 10 and 15°C

(temperatures that lead to the fastest growth), completely lost viability after 72 hr exposure. In contrast, UWO241 cultures grown at 4°C (with slower growth) were more resistant to 24°C exposure and suffered only 57% cell death at 72 hr (Figure 4a). Cell structures and chlorophyll were detected under the light microscope only in the 4°C-

**TABLE 1** The 20 metabolites that show the largest differences in abundance between the *C. reinhardtii* and UWO241 acclimated to different steady-state temperatures. The metabolite abundance corresponding to *C. reinhardtii* grown at 28°C was arbitrarily set to 1, and all other treatments were compared. The numbers represent FC as an average between three biological repeats, with (–) representing a decrease and a (+) representing an increase in abundance. The values that are significantly different in comparison to the control sample are marked with \* ( $p < 0.01$ , ANOVA, Tukey's post hoc test). The main pathways that each metabolite is involved in were designated using the KEGG database (not an exhaustive list)<sup>a</sup>

Metabolite	Class	KEGG pathway	<i>C. reinhardtii</i>		UWO241		
			15°C	10°C	15°C	10°C	4°C
Glucose	Carbohydrate	cre00010; cre00030	+1.06	+1.69*	–32.16*	–24.07*	–81.47*
α-Ketoglutarate	Carboxylic acid	cre00020; cre00053; cre01230	–1.02	+2.25	+1.73	+7.97*	+48.15*
3-phosphoglycerate	Carboxylic acid	cre00010; cre00030; cre01230; cre00523	–1.18	+7.34*	+4.16*	+3.16*	+39.83*
Phosphoenolpyruvate	Carboxylic acid	cre00020; cre00010; cre00710; cre01230	+1.57	+4.46*	+1.20	+2.38*	+18.02*
Lactic acid	Carboxylic acid	cre00620; cre00010; cre00051	+6.94*	+70.16*	–13.21*	–10.80*	–6.68*
Galactinol	Sugar alcohol	cre00052	–4.69*	–2.11*	–56.68*	–54.75*	–15.57*
Mannitol	Sugar alcohol	cre00051	–2.94	+1.09	–9.28*	–16.40*	–57.30*
Ornithine	Amino acid	cre01230	+2.85	+9.68*	+13.47*	+34.71*	+18.06*
Histidine	Amino acid	cre01230	+6.76*	+22.94*	+3.67	+8.87	+2.32
Glutamine	Amino acid	cre01230; cre00230, cre00240; cre00910	+4.61	+35.20*	+2.88	+3.39	+1.46
Lysine	Amino acid	cre01230	+7.25*	+27.98*	+5.18*	+3.31	–1.18
Cysteine	Amino acid	cre01230; cre00920	–2.04	+1.66*	–34.05*	–27.90*	–11.42*
Aspartic acid	Amino acid	cre01230; cre00710; cre00760	+1.71	+2.29*	–2.37*	–17.92*	–22.44*
N-acetylglutamate	Amino acid (derivative)	cre00220	+4.13*	+20.62*	+1.69	+1.34	+4.93*
Thymidine	N-containing	cre00240	+1.21	+2.03	+25.98*	+4.61*	+4.68*
Xanthine	N-containing	cre00230	+1.84*	+13.59*	–59.69*	–25.96*	–27.19*
Ergosterol	Lipid	cre00100	+6.25	–18.11*	+3.53	–11.00*	–15.94*
Linoleic acid	Fatty acid	cre01040	–1.89*	–8.60*	–24.23*	–17.48*	–42.83*
Dehydroascorbic acid	Antioxidant	cre00053; cre00480	+3.25	+19.68*	+12.41*	+22.70*	+20.12*
Threonic acid	Sugar acid	cre00053	+7.34*	+201.97*	+39.90*	+21.88*	+84.65*

<sup>a</sup>KEGG Partways: cre00010 – Glycolysis/Glyconeogenesis; cre00020 – TCA cycle; cre00030 – Pentose phosphate pathway; cre00051 – Fructose and mannose metabolism; cre00052 – Galactose metabolism; cre00053 – Ascorbate and alderate metabolism; cre00100 – Steroid biosynthesis; cre01040 – Biosynthesis of unsaturated fatty acids; cre01230 – Biosynthesis of amino acids; cre00220 – Arginine biosynthesis; cre00230 – Purine metabolism; cre00240 – Pyrimidine metabolism; cre00480 – Glutathione metabolism; cre00523 – Glycerolipid metabolism; cre00710 – Carbon fixation in photosynthetic organisms; cre00760 – Nicotinate and nicotinamide metabolism; cre00620 – Pyruvate metabolism; cre00910 – Nitrogen metabolism; cre00920 – Sulphur metabolism.

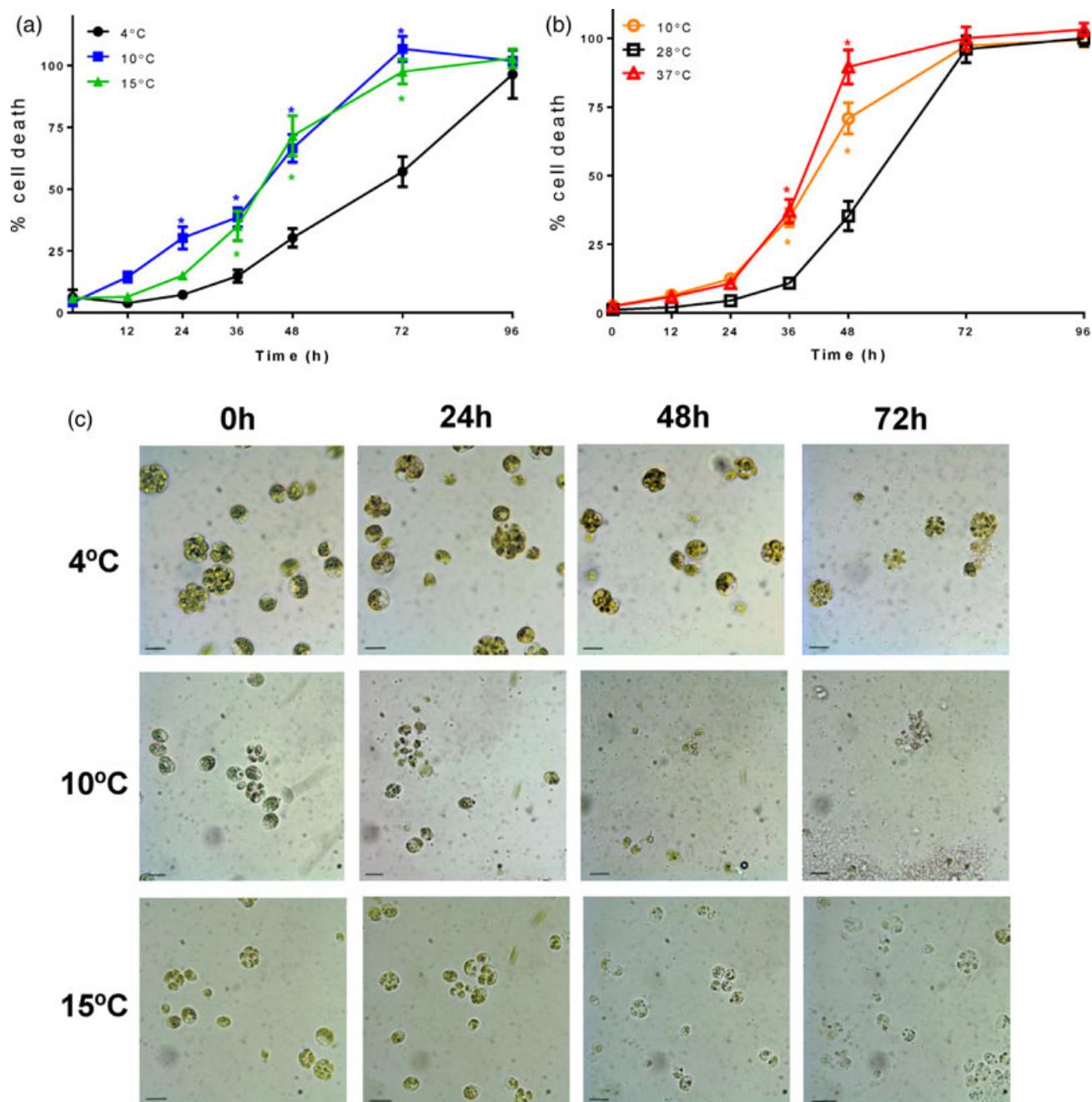
grown UWO241 after 72-hr exposure to 24°C, but not in the 10 and 15°C ones (Figure 4c). *C. reinhardtii* was most resistant to 42°C when initially grown at of 28°C with fastest growth rates, showing 30% cell death at 48 hr. Cultures acclimated to lower (10°C) and higher (37°C) temperature were more sensitive to heat exposure and rapidly lost viability (70 and 90%, respectively) by 48 hr. All cultures appeared to be completely dead after 72-hr exposure (Figure 4b). Similar patterns of chlorophyll loss (Figure S2) and cell structure were confirmed by light microscopy (Figure S3).

### 3.3 | Heat shock-induced global metabolomic and transcriptomic changes in UWO241

Next, we examined global metabolomic heat-induced changes in UWO241 cultures grown at 4, 10 and 15°C and subsequently

exposed to heat stress at 24°C for 6 hr. PCA analysis of all 771 detected metabolites demonstrated a separation along both principal components between the metabolome of the UWO241 cultures grown at 4°C and the metabolome of the same cultures exposed to 24°C (Figure 5). We also observed a separation between the metabolomes of UWO241 grown at 10°C and those exposed to heat, albeit only along PC2. There was minimal separation along either component between the cultures acclimated to 15°C before the 6 hr heat stress (Figure 5). HCA (Figure 6a) revealed the strongest response when cultures grown at 4°C were exposed to 24°C for 6 hr (222 DAMs, 29%). This heat stress response was attenuated in cultures acclimated to 10°C, and even more so in the cultures acclimated to 15°C, with 71 (9%) and 26 (3%) DAMs after heat exposure, respectively.

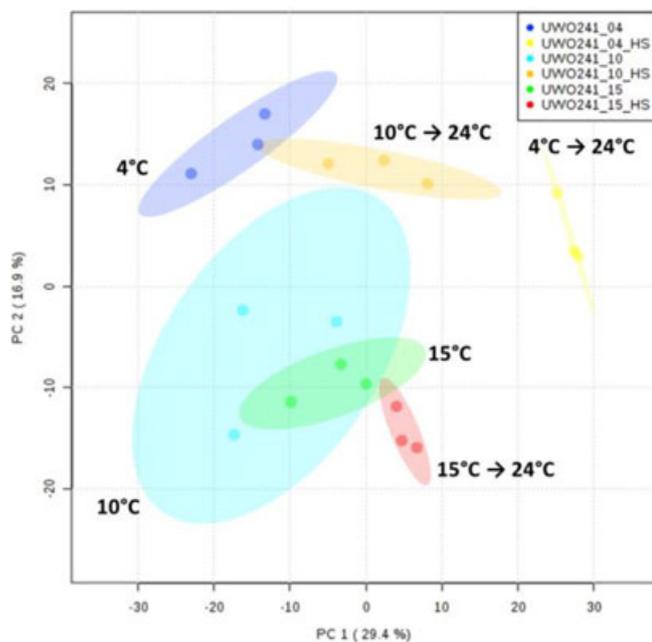
Next, we compared the accumulation of all 163 positively identified metabolites (Figure 6b, Data S2) and report the 20 metabolites



**FIGURE 4** Kinetics of cell death in UWO241 (a) and *C. reinhardtii* (b) acclimated to different growth temperatures and exposed to non-permissive conditions (24 and 42°C, respectively). Cell death was estimated as a percentage of algal cells stained with 0.5% Evans Blue that accumulates in cells with damaged membranes. Algal cells treated with 1% v/v chloroform were taken as a positive control and used to calculate 100% cell death. Data are means  $\pm$  SD of at least three independent experiments and analysed by two-way ANOVA followed by Bonferroni post-test comparing each treatment with 4°C (UWO241) and 28°C (*C. reinhardtii*). Statistical significance ( $p < 0.01$ ) is indicated as \* (c) Light microscope images of UWO241 acclimated to different steady-state temperatures (4, 10, 15°C) and exposed to non-permissive temperature (24°C) for 24, 48 and 72 hr. Algae are present as single cells or palmelloid colonies. Scale bar = 15  $\mu$ m ( $\times 400$  total magnification) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

that showed the largest abundance difference between steady state and heat stress (Table 2). Most major metabolite classes, including carbohydrates, sugar alcohols, amino acids, lipids, and antioxidants, increased in abundance in the cultures grown at 4°C and exposed to 24°C, except for carboxylic acids and sugar phosphates, which

increased significantly in the 10°C cultures (but not in those grown at 15°C). These metabolites are already present in high amounts in UWO241 acclimated to 4°C (Figure 3b). Ergosterol (FC 439.1) and  $\alpha$ -tocopherol (FC 308.1) increased at very high amounts in all cultures exposed to heat, regardless of the initial conditions (Table 2). These



**FIGURE 5** PCA of the primary metabolome of UWO241 acclimated to different steady-state temperatures and subsequently exposed to non-permissive temperature for 6 hr. UWO241 was grown at 4°C (blue, UWO241\_4) and exposed to 24°C (yellow, UWO241\_4\_HS); grown at 10°C (cyan; UWO241\_10) and exposed to 24°C (orange, UWO241\_10\_HS); and grown at 15°C (green; UWO241\_15) and exposed to 24°C (red, UWO241\_15\_HS). The analysis includes all quantified metabolites separated along the first two principal components and the 95% confidence interval for each treatment [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

increases follow a temperature-dependent pattern, with the highest FC seen in the cultures acclimated to 4°C prior to heat shock.

Using the same experimental design, we examined transcriptomic responses with RNA-Seq analysis based on the 16,325 gene models in the functionally annotated UWO241 genome (Zhang, Cvetkovska, et al., 2021). Differentially expressed genes (DEGs) were defined as those having an absolute FC >4 and a *p*-value <0.05. Similar to the metabolomic analyses (Figure 2; Figure 3), we found that steady-state culturing temperature had only a very minor effect on the overall transcriptome status in UWO241, with <3% of the total number of genes encoded in the UWO241 genome being identified as DEGs in the 10°C- and 15°C-grown cultures, when compared to those at 4°C (Figure 7a,b; Data S3). Exposure to 24°C induced a significant heat-induced response at the level of the transcriptome. The cultures acclimated to 10°C exhibited the strongest response with 1909 DEGs (11.7%; 939 up- and 970 down-regulated), followed by the cultures grown at 4°C with 1,610 DEGs (9.8%; 826 up- and 784 down-regulated). The cultures grown at 15°C were the least responsive to heat stress with 837 DEGs (5.1%; 456 up- and 381 down-regulated) (Figure 7c,d; Data S4). Regardless of the initial culturing temperature, pathways enriched for up-regulated DEGs included those in protein processing and endocytosis (Table 3; Data S5). Pathways involved in energy metabolism, nucleic acid metabolism, amino acid metabolism,

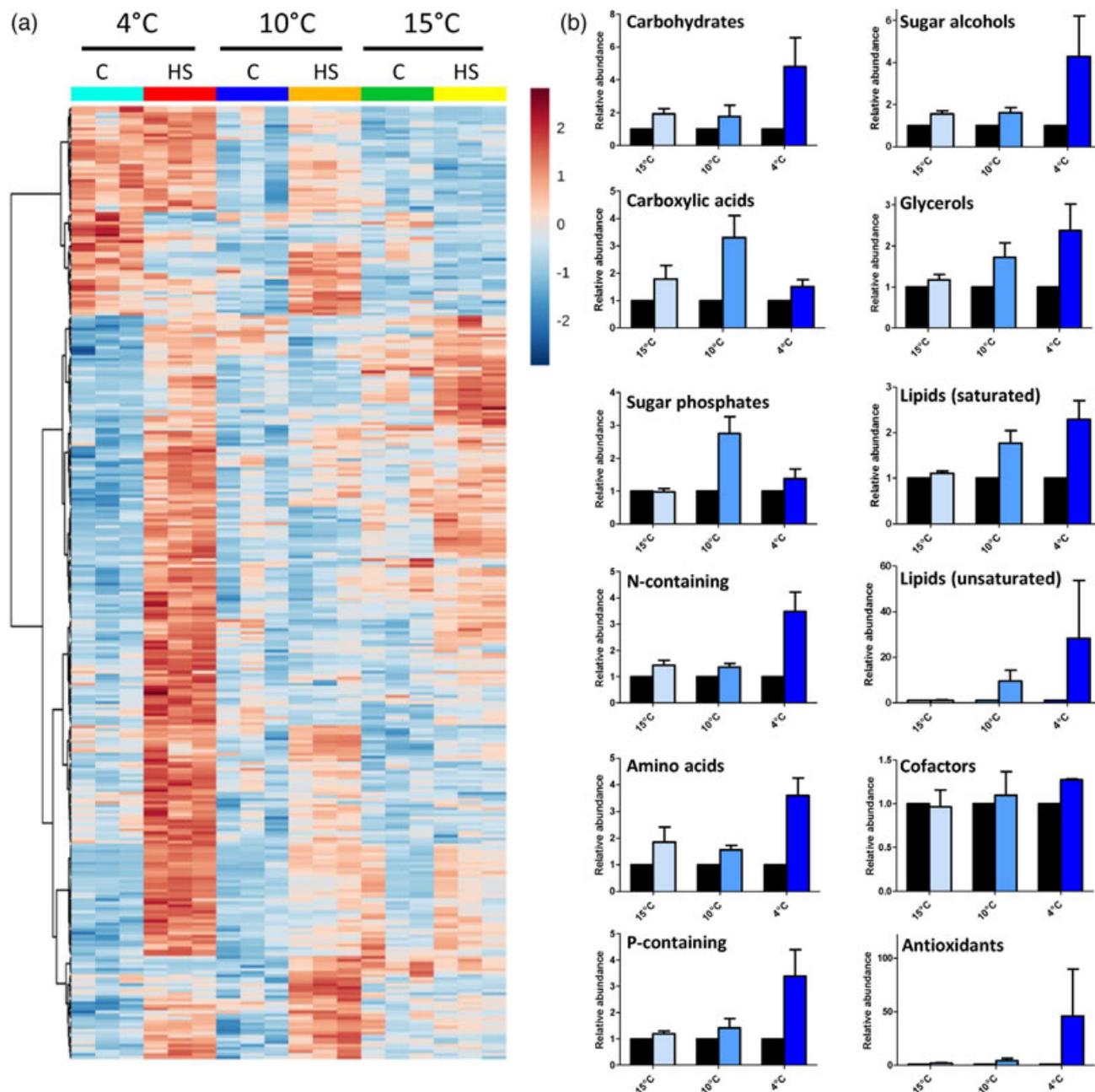
ribosome biogenesis, fatty acid biogenesis and cofactor synthesis were enriched in down-regulated DEGs (Table 3; Data S5).

### 3.4 | HSP gene family and expression profiling in UWO241 under heat stress

HSPs are highly conserved among evolutionary distant species and are often used as molecular heat-stress markers. We identified a total of 55 full-length, transcript-supported HSP genes in the UWO241 genome (Table 4, Data S6). This number was quite large compared to that reported for the *C. reinhardtii* genome (41 full-length HSP genes) (Schroda & Vallon, 2009) (Table 4). This expansion in the HSP gene family could be related to the extremophilic lifestyle of UWO241 or simply due to the more distant evolutionary relationship between UWO241 and *C. reinhardtii* (Possmayer et al., 2016). To distinguish between these possibilities, we screened the genomes of other green algae with publicly available draft nuclear genome sequences. We detected the highest number of HSP genes in the two psychrophiles: UWO241 (55) and ICE-L (51). The subfamilies containing HSP100s, HSP60s and small HSPs were significantly expanded in both species (Table 4; Data S6); however, UWO241 had more HSP70 genes and ICE-L had more HSP90 genes.

Previous work identified widespread duplication events in the UWO241 genome, including hundreds of highly similar full-length duplicate genes (HSDs; ≥90% pairwise identity) (Zhang, Cvetkovska, et al., 2021; Zhang, Hu, & Smith, 2021). Indeed, our analysis revealed that certain *C. reinhardtii* HSP genes had multiple homologs in the UWO241 genome (ClpB3, HSP70A, HSP70D, HSP70G, CPN60A, HSP22A). Not all of these qualify as duplicates according to the strict cut-off used by Zhang, Cvetkovska, et al. (2021); Zhang, Hu, and Smith (2021), but nevertheless they share a high % identity at the amino acid level (55–94%) and contain highly conserved domains important for HSP function (Data S6; Figure S4). The HSP70 gene subfamily was the most expanded one in UWO241, primarily due to a total of 6 cytosolic HSP70A isoforms. The other psychrophilic alga ICE-L encoded 3 cytosolic HSP70A genes, while the genomes of the other explored species encoded for only one or two cytosolic HSP70A genes (Figure S5).

To examine whether HSP gene expression is affected by temperature in UWO241, all full-length HSP genes were profiled using the RNA-Seq dataset described above. HSPs are induced by cold stress in *C. reinhardtii* (Maikova, Zalutskaya, Lapina, & Ermilova, 2016), and we asked whether the steady-state culturing temperature (4, 10, 15°C) affects HSP expression in UWO241. Most HSP genes were not significantly regulated in the cultures grown at different steady-state temperatures, except for ClpB1, HSP70B-2 and all HSP22A homologs that were either down-regulated or below the detection threshold in the 10°C cultures compared to those at 4°C (Figure 8). This suggests that steady-state temperature has only a minor effect on HSP regulation in UWO241. Some HSPs had high expression under steady-state conditions, in this case defined as FPKM>100 (Data S3). These include all members of the HSP90 and CPN60 families, one HSP100 gene



**FIGURE 6** Differences in the primary metabolome in UWO241 acclimated at different temperatures (4, 10, 15°C) and subsequently exposed to heat stress (24°C) for 6 hr. (a) Heat map showing the relative changes in metabolite abundances between control samples at each steady-state growth temperature (c) and heat-treated samples (HS). Only metabolites that are significantly different are shown (314 metabolites, ANOVA,  $p < 0.01$ ). Three biological replicates are represented using a colour-based metabolite profile as indicated (red – high abundance; blue – low abundance). Hierarchical clustering is based on Euclidean distances and Ward's linkage (b) Relative abundance of metabolites classified based on their chemical nature. Only metabolites that were positively identified based on their GC–MS spectra and retention times were taken into consideration. In this analysis, the metabolite abundance in algae grown at the three different steady-state temperatures were arbitrarily set to 1 (black bars) and all the heat-stress treatments were compared to the corresponding control sample (blue bars) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

(ClpB3–4) and some (but not all) members of the HSP70 family (HSP70A-1 and – 2, HSP70B-1, HSP70C, HSP70E and BIP1).

Exposure to heat stress for 6 hr induced the expression of some, but not all, HSP genes. (Figure 8, Table S5). Most highly expressed transcripts at steady-state conditions were not up-regulated under

heat stress, apart from HSP90A, which was up-regulated. The genes with largest expression differences between steady-state and heat stress were the HSP22A homologs (HSP22A-2, –3, –6 to –9;  $FC \approx 7$ –32,000), followed by several HSP70 (HSP70A-3 to A-5, HSP70B-2) and ClpB (ClpB3-1, ClpB1) homologs (Figure 8; Data S6).

**TABLE 2** The 20 metabolites that show the largest differences in abundance from UWO241 acclimated to different steady-state temperatures and exposed to non-permissive temperature for 6 hr. The metabolite abundance corresponding to cultures grown at steady-state conditions was arbitrarily set to 1, and all other treatments were compared to the control sample. The numbers represent FC as an average between three biological repeats, with (–) representing a decrease and a (+) representing an increase in abundance. The values that are significantly different in comparison with the control sample are marked with \* ( $p < 0.01$ , ANOVA, Tukey's post hoc test). The main pathways that each metabolite is involved in were designated using the KEGG database (not an exhaustive list)<sup>a</sup>

Metabolite	Class	KEGG pathway	15°C → 24°C	10°C → 24°C	4°C → 24°C
Sucrose	Carbohydrate	cre00500	+4.59*	+6.84*	+21.18*
Ribose	Carbohydrate	cre00030	–1.25	–1.06	+9.28*
α-Ketoglutarate	Carboxylic acid	cre00020; cre00053; cre01230	+10.38*	+11.37*	+1.93
Pyruvic acid	Carboxylic acid	cre00010; cre00020; cre00030; cre00710 cre00053; cre01230; cre00760	+2.85	+14.70*	+1.09
Fructose-1,6-bisphosphate	Sugar phosphate	cre00010; cre00030; cre00051	+1.03	+1.38	–4.26*
Glucose-6-phosphate	Sugar phosphate	cre00010; cre00030; cre00500	–2.66*	–1.69*	–1.69*
Mannitol	Sugar alcohol	cre00051	+1.67	–1.17	+12.32*
Glutamine	Amino acid	cre01230, cre00230; cre00240; cre00910	+1.08	–1.97	+17.16*
Lysine	Amino acid	cre01230	+1.67	–1.37	+14.58*
Histidine	Amino acid	cre01230	+4.16*	2.37	+12.98*
Aspartic acid	Amino acid	cre01230; cre00710; cre00760	–11.61*	+1.08	+1.18
Tryptophan	Amino acid	cre01230	+1.84	–1.06	+7.34*
Serotonin	Amino acid (derivative)	cre01230	+1.18	–2.49	+13.43*
Glycyl tyrosine	Amino acid (derivative)	cre01230	+2.15	+1.39	+18.65*
Adenosine	N-containing	cre00230	–1.05	+1.02	+13.22*
Inosine 5'-monophosphate	N/P-containing	cre00230	+3.39*	+2.56*	+9.04*
Oleic acid	Fatty acid	cre01040	–2.24	+11.34*	+5.43*
Linolenic acid	Fatty acid	cre01040	–1.53	+36.02*	+3.46*
Ergosterol	Lipid	cre00100	+1.98	+110.042*	+439.13*
α-Tocopherol	Antioxidant	cre01110	+2.24*	+16.70*	+308.12*

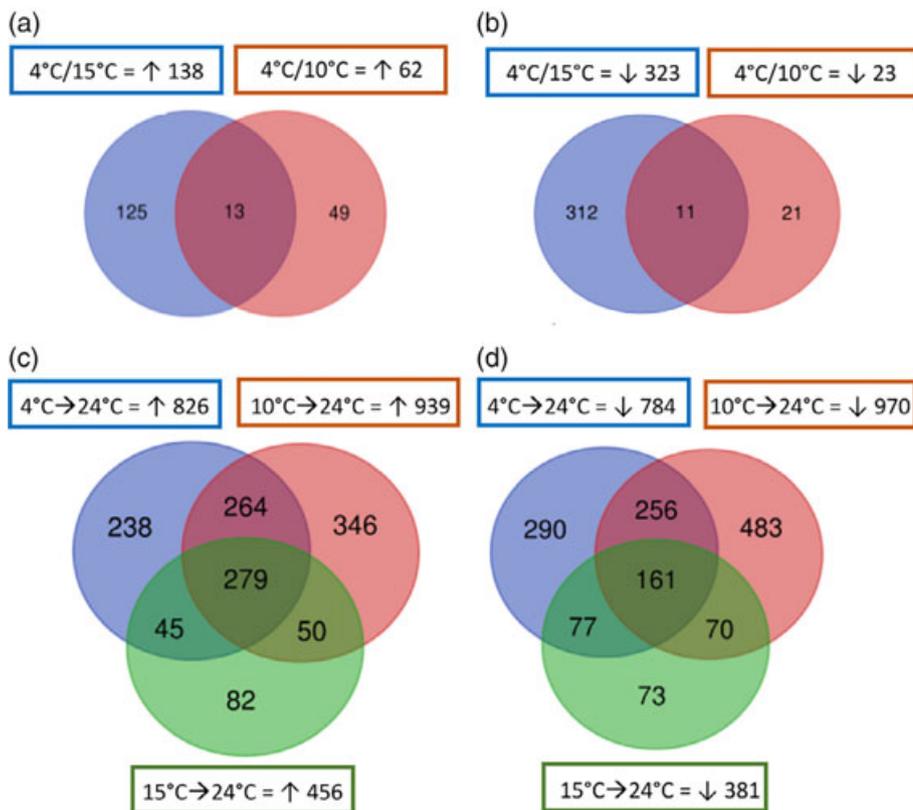
<sup>a</sup>KEGG Partways: cre00010 – Glycolysis/Glyconeogenesis; cre00020 – TCA cycle; cre00030 – Pentose phosphate pathway; cre00051 – Fructose and mannose metabolism; cre00053 – Ascorbate and alderate metabolism; cre00100 – Steroid biosynthesis; cre00230 – Purine metabolism; cre00240 – Pyrimidine metabolism; cre00500 – Starch and sucrose metabolism; cre00710 – Carbon fixation in photosynthetic organisms; cre00760 – Nicotinate and nicotinamide metabolism; cre00910 – Nitrogen metabolism; cre01040 – Biosynthesis of unsaturated fatty acids; cre01110 – Biosynthesis of secondary metabolites; cre01230 – Biosynthesis of amino acids.

These data imply that some HSPs may have homeostatic roles and are constitutively expressed at high levels under steady-state conditions, whereas others may play stress-related roles.

### 3.5 | HSP protein accumulation in UWO241 and *C. reinhardtii*

Next, we investigated whether HSP expression trends are mirrored at the protein level using antibodies for several major HSPs in UWO241 and *C. reinhardtii*. In the first set of experiments, we cultured both species at a range of temperatures, including the highest temperature that can support growth (4–17°C for UWO241; 22–37°C for *C. reinhardtii*). The cytosolic HSP70A, HSP90A, and the chloroplast

CPN60A exhibited higher protein levels in UWO241 when compared to *C. reinhardtii* at most culturing temperatures (Figure 9). HSP70B accumulated at comparable levels in both species, especially at higher growth temperatures, whereas HSP90C was lower in UWO241 in comparison with *C. reinhardtii* (Figure 9, Figure S6). The steady-state culturing temperature had only a minor effect on HSP accumulation, with four of the tested HSPs (CPN60A, HSP70A, HSP90A, HSP90C) accumulating at slightly higher levels at 4°C when compared to higher growth temperatures in UWO241 (Figure 9, Figure S6). To test whether the high accumulation of HSPs is due to growth at low temperatures regardless of the species, we also cultured *C. reinhardtii* at 15 and 10°C. We compared low-temperature HSP accumulation to that at optimal growth conditions (28°C) and to cultures exposed to heat shock (42°C) for 6 hr. Algal cultures that were acclimated to



**FIGURE 7** Venn diagram indicating the intersection of significantly up- (a) and down-regulated (b) differentially expressed genes (DEGs) in UWO241 grown at 4, 10 and 15°C. In this case, gene expression in the 4°C-grown UWO241 cultures were compared with 15°C (blue, on the left) and 10°C (red, on the right). The number above indicates the total number of up- or down-regulated DEGs in that treatment. Venn diagrams indicating the intersection of significantly (c) Up- and (d) Down-regulated transcripts in UWO241 grown at 4°C (blue), 10°C (red) and 15°C (green) and exposed to heat stress (24°C) for 6 hr. The number besides the diagram indicates the total number of differentially expressed DEGs in that treatment. In all cases, DEGs have a Fold Change >4,  $p$ -value <0.05 in three biological replicates [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

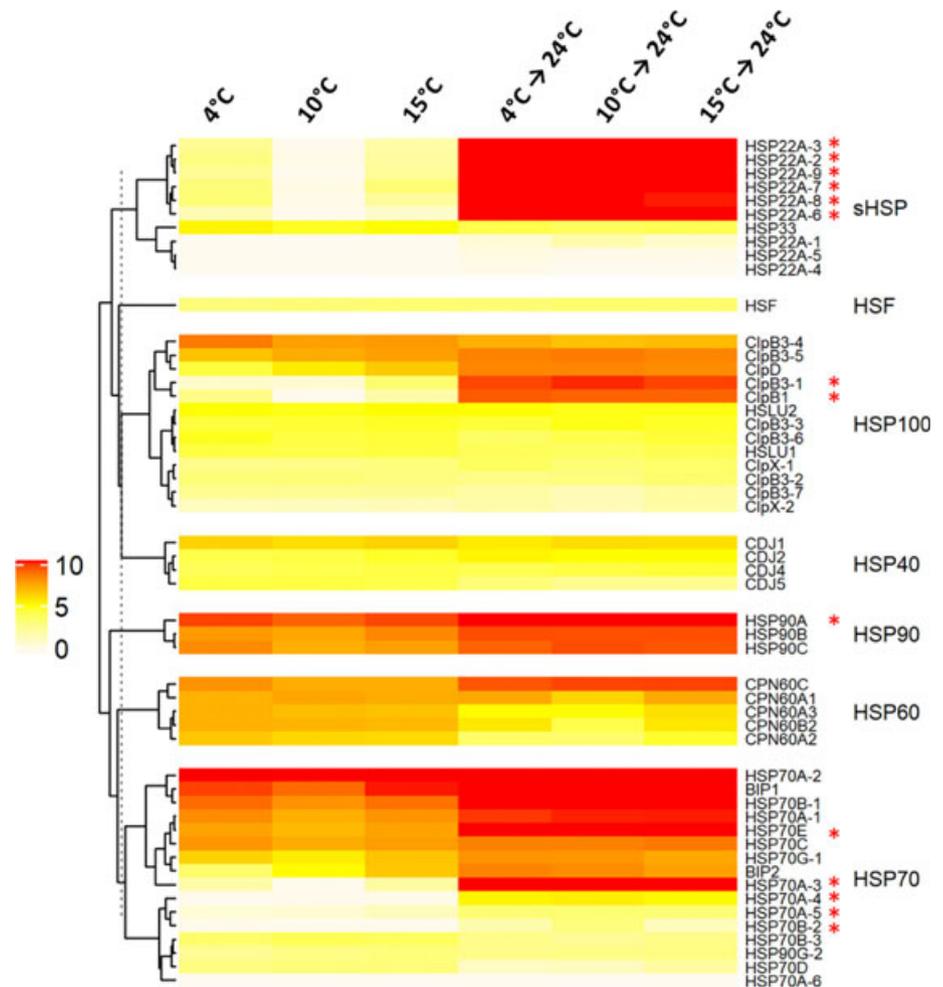
KEGG pathway	4-24°C	10-24°C	15-24°C
cre00020 citrate cycle (TCA cycle)	↓	↓	↓
cre00061 fatty acid biosynthesis		↓	
cre00190 oxidative phosphorylation	↓	↓	
cre00230 purine metabolism		↓	↓
cre00240 pyrimidine metabolism	↓	↓	↓
cre00270 cysteine and methionine metabolism	↓	↓	↓
cre00400 phenylalanine, tyrosine and tryptophan bios.	↓		↓
cre01100 metabolic pathways	↓	↓	↓
cre01110 biosynthesis of secondary metabolites	↓	↓	↓
cre01200 carbon metabolism		↓	↓
cre01230 biosynthesis of amino acids	↓	↓	↓
cre01240 biosynthesis of cofactors	↓	↓	
cre03008 ribosome biogenesis in eukaryotes	↓	↓	
cre03018 RNA degradation	↓		
cre03020 RNA polymerase	↓		
cre03030 DNA replication		↓	↓
cre04120 ubiquitin mediated proteolysis		↑	
cre04141 protein processing in endoplasmic reticulum	↑	↑	↑
cre04144 endocytosis	↑	↑	↑

**TABLE 3** KEGG analysis of the pathways significantly up-regulated (↑) or down-regulated (↓) in heat-shocked UWO241 (4-24°C; 10-24°C; 15-24°C) as analysed by Gage. Only pathways significantly enriched by DEGs are shown ( $p$ -value <0.05)

**TABLE 4** The number of heat shock proteins in various algal species. Species are ordered according to their evolutionary relationship to UWO241 (Possmayer et al., 2016; Zhang et al., 2020). The species with significantly more than the average number of HSPs in a particular class are highlighted with \*. Algal species: Ceu, *Chlamydomonas eustigma*; Dsa, *Dunaliella salina*; Cre, *Chlamydomonas reinhardtii*; Gpe, *Gonium pectorale*; Vca, *Volvox carteri*; Cso, *Chlorella sorokiniana*; Csu, *Coccomyxa subellipsoidea* C-169

	UWO241	Ceu	ICE-L	Dsa	Cre	Gpe	Vca	Cso	Csu
HSF TF	1	2	1	1	2	1	2	1	1
HSP100s/Clp	12*	9	12*	8	8	9	11	7	6
HSP90s	3	4	6*	4	3	3	3	3	3
HSP70s	16*	8	10	8	9	10	10	9	9
HSP60s	6*	4	6*	3	4	4	3	3	3
HSP40s (DnaJ-like)	5	3	4	5	6	5	5	3	5
sHSPs	11	1	12*	13*	9	6	6	2	4
Total	54	31	51	42	41	38	40	28	31

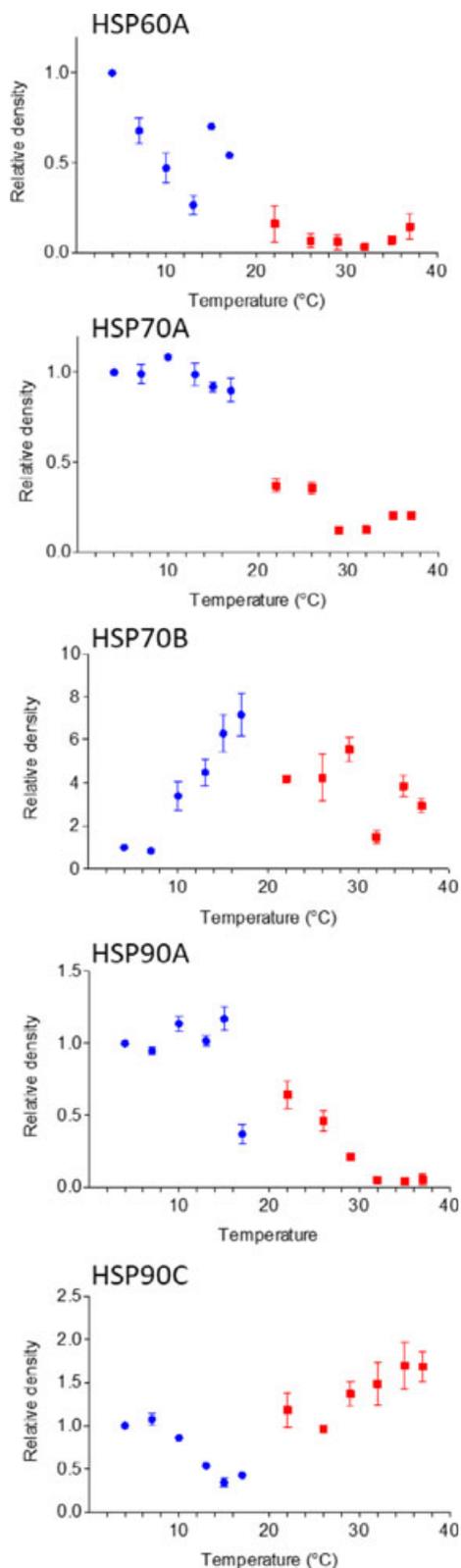
**FIGURE 8** Expression of Heat Shock Protein genes in UWO241 grown at three different temperatures (4, 10 and 15°C) and exposed to heat stress (24°C) for 6 hr. The average FPKM values of gene expression in three biological replicates are represented using a colour-based expression profile as indicated (white – low abundance; red – high abundance). All genes encoding putative HSP genes in the UWO241 genome are shown. The genes with a significantly higher expression ( $FC > 4$ ,  $p < 0.05$ ) when compared to the corresponding control treatment (e.g., 4°C control compared to 4°C→24°C heat-stressed sample) are highlighted with a red asterisk (\*). Only genes that are significantly regulated in all three heat shock treatments are highlighted [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



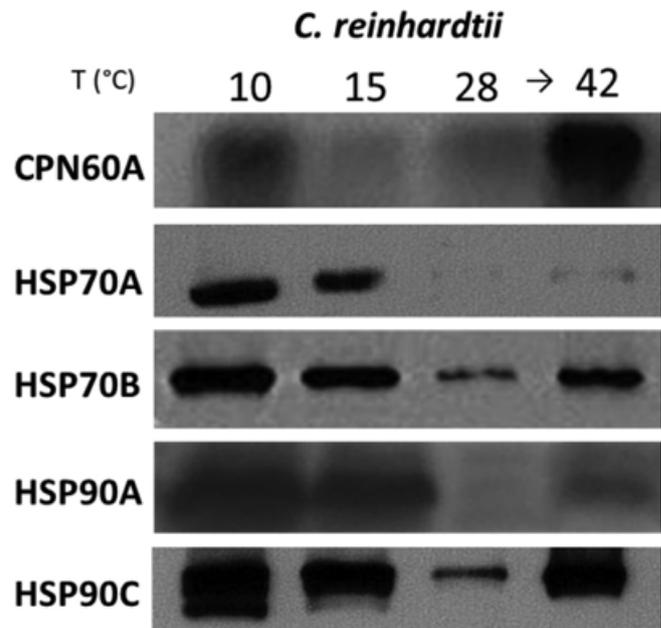
10 and 15°C accumulated HSPs at much higher levels than those acclimated to 28°C and at comparable levels to those exposed to heat shock at 42°C (Figure 10). These data suggest that the accumulation of high HSP levels could be a strategy for low temperature growth in green algae.

HSP gene expression and protein accumulation is strongly induced by heat stress in *C. reinhardtii* (Mühlhaus et al., 2011; Schmollinger et al., 2013). To test whether the same is true for an obligate psychrophile, we exposed UWO241 cultures grown at

4, 10 and 15°C to heat stress (24°C) for 6 hr. We applied an equivalent treatment to *C. reinhardtii* grown at low (10°C), optimal (28°C) and high (37°C) temperature by exposing the cultures to heat shock (42°C) for 6 hr. Most *C. reinhardtii* HSPs increased in abundance during heat shock, including the HSF1 transcription factor, regardless of the initial culturing temperature (Figure 11). HSP accumulation was induced even in cultures acclimated to 10°C, which accumulated high HSP amounts prior to the heat shock (Figure 6b, Figure 7). In contrast, heat shock had only a very minor effect on HSP accumulation in



**FIGURE 9** Densitometry analysis of the relative HSP abundance determined by Western blotting in cultures of UWO241 (blue) and *C. reinhardtii* (red) acclimated to different steady-state temperatures. The protein abundance in the 4°C-grown UWO241 was used as the basis of comparison for the relative abundance of HSPs in the other samples. In all cases, the results are the mean of three replicates ( $\pm$  SD) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



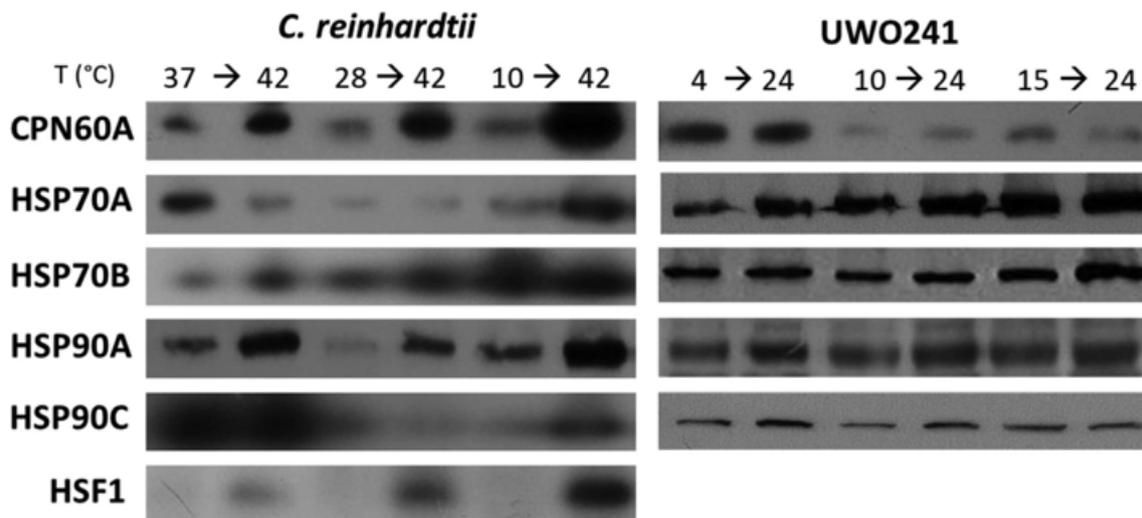
**FIGURE 10** HSPs in *C. reinhardtii* acclimated to a steady-state low temperatures (10 and 15°C) accumulate at comparable levels to those in cultures acclimated to 28°C and exposed to 42°C for 6 hr. The data shown reflect typical results of three biological replicates

UWO241, and HSP accumulation did not significantly increase with heat stress, regardless of the initial culturing temperature (Figure 7b, d). We could not detect HSF1 in UWO241, either due to low antibody specificity or very low protein levels. Thus, HSP accumulation is significantly induced by heat stress in the mesophile but not appreciably in the psychrophile (Figure 11).

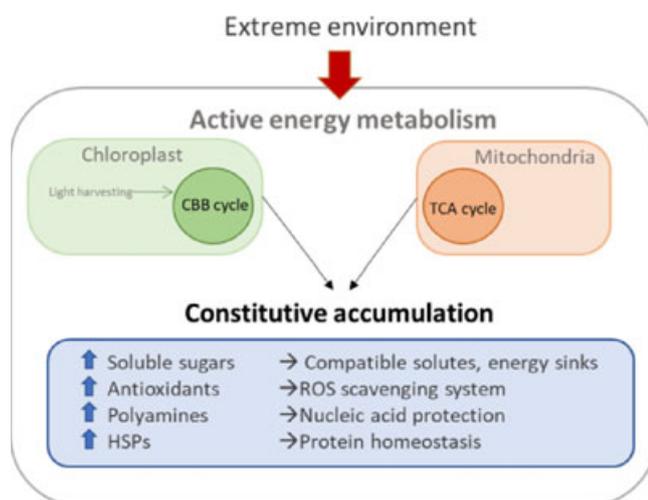
## 4 | DISCUSSION

### 4.1 | Metabolic signatures of growth at permanently low temperatures

Algae exhibit the fastest growth rates under optimal conditions, whereas stress can threaten their cellular homeostasis and lead to decreased growth rates and reduced fitness (Borowitzka, 2018). The Antarctic alga, UWO241, experiences low but very stable temperatures of 4–6°C year-round, but we found that its growth rate is fastest at 10 and 15°C (Figure 1). This indicates that the biochemical and metabolic processes operating in UWO241 are better adjusted to higher temperatures than what it experiences in nature (4°C). Nevertheless, we showed that UWO241 is, indeed, adapted for life at 4°C, which is reflected in the fact that light- and CO<sub>2</sub>-saturated rates of O<sub>2</sub> evolution for UWO241 at this temperature are comparable to the photosynthetic rate of *C. reinhardtii* grown at 28°C (Pocock, Vetterli, & Falk, 2011). The primary metabolome of 4°C-grown UWO241 did not differ significantly from that of cultures at their optimal growth temperatures of 10–15°C. In contrast, *C. reinhardtii* showed a strong temperature-dependent response at the level of the primary



**FIGURE 11** Accumulation of heat shock proteins in cultures of UWO241 and *C. reinhardtii* cultured under different temperature regimes until mid-log stage (37, 28 and 10°C for *C. reinhardtii*; 15, 10 and 4°C for UWO241) and exposed to 6 hr of heat stress at 42°C for *C. reinhardtii* and 24°C for UWO241. Due to differences in the initial HSP amount between the two species, the immunoblots were exposed and imaged at different intensities in order to capture the change in HSP accumulation between the control and heat-stressed samples. The data shown here reflect typical results of three biological replicates. It should be noted that HSF1 could not be detected at the protein level in UWO241



**FIGURE 12** The psychrophilic alga UWO241 is adapted to life at low but very stable temperatures of 4–6°C year-round in Lake Bonney, Antarctica. Its physiology at low temperature is characterized by efficient photosynthetic rates comparable to those of *C. reinhardtii* at 25–35°C. (Pocock et al., 2011) and an active energy metabolism characterized by increased accumulation of CBB and TCA cycle intermediates. This active central metabolism fuels a constitutive accumulation of metabolites and proteins important for life at low temperatures, including soluble sugars, antioxidants, polyamines and molecular chaperones that ensure efficient protein folding at low temperatures. We propose that the constitutive accumulation of proteins and metabolites with a known stress-related function provides UWO241 with the ability to cope with its extreme environment, but it also might impede its ability to finely manipulate this metabolic and molecular network in order to cope with additional environmental stressors [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

metabolome (Figures 2 and 3a). The 10°C-grown *C. reinhardtii* cultures accumulated increased levels of cryoprotectants and membrane stabilizers than cultures grown at their optimal temperature (28°C), a common response in photosynthetic organisms during cold stress (Fürtauer, Weiszmann, Weckwerth, & Nägele, 2019; Gray & Heath, 2005; Guy, Kaplan, Kopka, Selbig, & Hincha, 2007; Janská, Maršík, Zelenková, & Ovesná, 2010; Kaplan et al., 2007; Wanner & Juntila, 1999). We interpret this as evidence that the psychrophile UWO241 does not exhibit typical cold-stress responses when cultured at 4°C, despite its slow growth rates.

Our data reveal a steady-state re-routing of primary metabolism in UWO241 when compared to the mesophilic model *C. reinhardtii*. We detected the constitutive accumulation of several metabolites with known stress functions in UWO241, regardless of the growth temperature, while the same metabolites only accumulated at high levels in 10°C-grown *C. reinhardtii*. First, soluble sugars have known cryoprotectant roles in cold-adapted plants and algae (Leya, 2013; Su et al., 2016; Tulha et al., 2010). Most detected soluble sugars (trehalose, sucrose, ribose, maltose, fructose) accumulated at high levels in all UWO241 cultures but only in 10°C-grown *C. reinhardtii* (Table 1; Data S1). This suggests that the psychrophile has a re-wired central carbon metabolism and accumulates high amounts of carbohydrates at the expense of other photosynthetic intermediates, consistent with previous studies (Cook et al., 2019; Kalra et al., 2020).

Second, we observed similar patterns with ascorbic acid (AsA) and its oxidized form dehydroascorbic acid (DHA) (Table 1; Data S1). The ascorbate-glutathione (AsA-GSH) cycle is a fundamental metabolic pathway involved in the maintenance of cellular redox homeostasis during cold stress in many photosynthetic species (Dreyer & Dietz, 2018; Hasanuzzaman et al., 2019). Thus, UWO241 and other

organisms that live in perpetually cold environments may need a robust and constitutively active antioxidant system to cope with high ROS levels. Third, the non-proteinogenic amino acid ornithine also accumulated at high levels in UWO241 at all growth temperatures, but only at 10°C in *C. reinhardtii* (Table 1). Ornithine plays a pivotal role in polyamine, arginine and proline biosynthesis, and its accumulation has been linked to increased stress tolerance in plants (Ghahremani et al., 2014; Kalamaki et al., 2009; Kalamaki, Merkouropoulos, & Kanellis, 2009). We also detected the increased amount of the polyamine putrescine in UWO241 compared to *C. reinhardtii* (and no increases in arginine or proline, Data S1). High ornithine and polyamine levels may play a role in DNA and RNA protection and stabilization, protein synthesis and cell cycle progression at low temperatures (Chen, Shao, Yin, Younis, & Zheng, 2019; Gill & Tuteja, 2010; Minocha, Majumdar, & Minocha, 2014). Taken together, we suggest that in UWO241, the accumulation of stress-related compounds, such as soluble sugars, antioxidants and polyamines, represents a mechanism to ensure efficient metabolism, redox homeostasis and cell division at low temperatures (Figure 12). Unlike *C. reinhardtii*, where the accumulation of stress-metabolites is induced by cold, in UWO241 these protective compounds constitutively accumulate across a range of permissive growth temperatures.

## 4.2 | Can Antarctic algae mount a functional heat shock response?

A defining characteristic of psychrophiles is their inability to grow at moderate temperatures (Cvetkovska et al., 2017), but the underlying reasons for this sensitivity are unknown. In this work, we showed that despite living in a permanently cold environment, UWO241 can mount HSR at the level of the primary metabolome and transcriptome. We also showed, however, that the response to heat stress in UWO241 is dependent on the initial culturing temperature. UWO241 grown at 4°C and then exposed to 24°C resulted in the slowest cell death kinetics (Figure 4a,c) and a strong response at the level of its primary metabolome (Figure 6) and transcriptome (Figure 7c,d). UWO241 cultures grown at 10°C exhibit a strong response when exposed to heat for 6 hr but only at the level of the transcriptome (Figures 6 and 7). A response at the level of the metabolome is largely absent, except for increased accumulation of carboxylic acids and sugar phosphates (Figure 6b). Cultures initially grown at 15°C exhibited an attenuated response at both the metabolome (Figure 6) and the transcriptome (Figure 7c,d). Both 10°C-grown and 15°C-grown UWO241 cultures were less resistant to 24°C exposure and had faster cell death kinetics (Figure 4a,c). This study demonstrates that UWO241 cultured at a temperature closest to its natural environment (4°C) has the highest capacity to respond to heat stress, despite slow growth rates.

The stress metabolism in UWO241 appeared to be routed towards the accumulation of soluble sugars, antioxidants and cryoprotectants (Figure 6b). Many of these compounds are metabolic markers for cold stress in mesophiles and are already present in high amounts

in non-stressed UWO241 cells (Figure 3b). The metabolites ergosterol and  $\alpha$ -tocopherol showed the most dramatic increases in accumulation between steady-state and heat-stressed cultures, with the largest increases seen in the cultures initially grown at 4°C (Table 2, Table S2). The tocopherol antioxidant system has been studied during high light stress (Nowicka & Kruk, 2012; Sirikhachornkit, Shin, Baroli, & Niyogi, 2009; Szarka, Tomasskovics, & Bánhegyi, 2012; Trebst, Depka, & Holländer-Czytko, 2002), and to the best of our knowledge this is the first report of its involvement in heat stress in green algae. In yeast, increased ergosterol content and remodelling of the lipid composition of cellular membranes to has been shown to counteract the deleterious effects of several stressors, including heat (Caspeta et al., 2014; Godinho et al., 2018; Swan & Watson, 1998; Vanegas, Contreras, Faller, & Longo, 2012); however, a detailed lipidomic analysis is required to test whether a similar process is operational in UWO241.

In accordance with the metabolome data, a steady-state culturing temperature appeared to have only a minor effect on the UWO241 transcriptomic make-up (Figure 7a,b), but heat stress induced a stronger response (Figure 7b,c). In UWO241, up to 11.7% of all nuclear-encoded genes were differentially regulated by heat stress (10°C-grown cultures, Figure 4a,b). Broadly speaking, this response seems to be of a lesser intensity as compared to that of *C. reinhardtii* when exposed to 25 min of heat stress (25°C  $\rightarrow$  42°C), which results in approximately 19% DEGs (Légeret et al., 2016). When the green algal halophile *Dunaliella bardawil* was exposed to heat stress (25°C  $\rightarrow$  42°C) for 2 hr, it exhibited a similar transcriptomic response as we observed for UWO241 with 12% of all transcripts identified as DEGs (Liang, Jiang, Wang, & Zhu, 2020). Direct comparisons between studies involving different species, culturing conditions, and time points are difficult; however, we observed that in UWO241 (Table 3), *C. reinhardtii* (Légeret et al., 2016) and *D. bardawil* (Liang et al., 2020), the few pathways enriched in up-regulated genes are those involved in protein processing, which include HSPs. This suggests a conserved role for protein processing pathways between UWO241 and related mesophilic algae during HSR.

## 4.3 | High accumulation of HSPs in UWO241 could play a role at low temperature

HSPs are one of the first hallmarks of the HSR and key component of protein synthesis, folding, and the prevention of protein aggregates during heat stress (Schroda et al., 2015). Screening of the UWO241 genome revealed more putative HSP genes than any other green alga examined to date (Table 4, Figure S4). This expansion is mostly driven by multiple copies of a few genes, including six (6) ClpB3 and HSP70A genes, three (3) CPN60A genes and no less than ten (10) HSP22A genes homologous to those in *C. reinhardtii*. The genome of psychrophilic Antarctic sea-ice green alga ICE-L also encodes for a significantly more HSP genes as compared to non-psychrophilic species (Table 4). UWO241 belongs to the Moewusinia clade of the order Chlamydomonadales (Possmayer et al., 2016), whereas ICE-L is a

member of the closely affiliated *Monadina* clade (Zhang et al., 2020). This suggests that an expanded HSP gene family may be a consequence of life in the permanent cold, and not a feature common to a particular algal clade.

Sequencing of the UWO241 genome revealed an unusually large (212 Mb) nuclear genome with hundreds of highly similar duplicate (HSD) genes from diverse cellular pathways (particularly in protein translation, DNA packaging and photosynthesis), more than any other algal species with a sequenced genome (Zhang, Cvetkovska, et al., 2021; Zhang, Hu, & Smith, 2021). Its genome has been termed 'a genome in upheaval' due to the presence of multiple partial duplicates, gene fragments and pseudogenes (Zhang, Cvetkovska, et al., 2021). It was postulated that this rampant gene duplication, likely retrotransposon-mediated, is random and thus neutral or even maladaptive. But if the presence of a duplicated gene confers an increase in fitness, that gene will be fixed in the genome (Qian & Zhang, 2014). But why would multiple HSP gene copies be fixed in the UWO241 genome, and what would be the fitness advantage? HSPs in *C. reinhardtii* have been studied mostly for their role during stress responses (reviewed in Nordhues et al., 2010) but do they have an equivalent role in UWO241, an obligate psychrophile released from the challenges of a variable environment?

Detailed RNA-Seq analysis revealed that UWO241 induced the expression of some, but not all HSPs, when exposed to heat stress, regardless of the initial culturing temperature (Figure 8). This response was particularly strong for sHSP genes, and most of the HSP22A homologs were strongly up-regulated by exposure to heat. The strong heat induction of sHSP transcripts has been shown before in UWO241 under short-term heat stress (Possmayer et al., 2011), as well as in *C. reinhardtii* and other algae (Kobayashi et al., 2014; Liang et al., 2020; Mühlhaus et al., 2011; Uji, Gondaira, Fukuda, Mizuta, & Saga, 2019). Curiously, UWO241 failed to accumulate increased protein amounts when challenged with heat stress, despite increased HSP transcript levels (Figure 7).

In a direct comparison with *C. reinhardtii*, we showed that UWO241 accumulates significantly higher protein amounts of major HSPs (HSP70A, HSP90A, CPN60A) under a range of steady-state growth temperatures in the absence of heat stress (Figure 9). Furthermore, we also observed high HSP levels in *C. reinhardtii* acclimated to 10°C (Figure 10). Cold induction of HSP expression has been previously observed in *C. reinhardtii* (Maikova et al., 2016) and land plants (Renaut, Hausman, & Wisniewski, 2006; Timperio, Egidi, & Zolla, 2008), suggesting a role of chaperones during low-temperature growth. Unlike UWO241, *C. reinhardtii* cultured at 10°C retained the ability to induce further accumulation of most HSPs, including HSF1, when heat stressed despite having high levels of HSPs during steady-state growth (Figure 11). Thus, while high levels of HSPs are common between the two species at low temperature, only the mesophile could significantly increase HSP amounts when challenged with heat.

Whether constitutively high HSP levels confer resistance to heat is still a matter of debate. Thermotolerance in HSP over-expressors in plants, yeast and bacteria (Fragkostefanakis, Röth, Schleiff, & Scharf, 2015; Santhanagopalan, Basha, Ballard, Bopp, &

Vierling, 2015) is often limited to a narrow range of conditions or a specific developmental stage (Fragkostefanakis et al., 2015; Waters & Vierling, 2020). Considering the increased evidence for broader roles of chaperones in cellular protein homeostasis (Gupta et al., 2010; Lindquist & Craig, 1988; Park & Seo, 2015; Vierling, 2003; Wang et al., 2004), it appears that the ability of an organism to tightly regulate its central defence network plays a more prominent role in thermotolerance rather than the amount of chaperone proteins themselves. This certainly appears to be the case in UWO241, which cannot survive moderate temperatures (Figure 4a) despite constitutively accumulating high quantities of HSPs (Figure 9). Similarly, increased levels of HSPs in cold-grown *C. reinhardtii* did not confer greater survival under prolonged heat stress when compared to those grown at 28°C (Figure 4b). The significance of high HSP expression but not protein accumulation during heat stress is currently not clear. UWO241 does not experience temperature fluctuations in its natural environment, but inducible HSP expression could be a remnant of its distant past before its arrival in Lake Bonney. We do not know the evolutionary history of UWO241, but it is closely related to the marine species *Chlamydomonas parkeae* (Possmayer et al., 2016), indicating an ancestral lifestyle in a variable environment where stress-induced protective pathways would be beneficial.

We propose that the constitutively high accumulation of HSPs in UWO241 plays an important role in protein quality control and ensures a robust capacity for protein folding at low temperatures, rather than protection from heat stress. Protein synthesis and the folding of nascent proteins are temperature-sensitive cellular processes (Hebraud, Dubois, Potier, & Labadie, 1994; Phadtare, 2004; Piette, Struvay, & Feller, 2011; Rosa, Roberts, & Rodrigues, 2017), and it has been suggested that the involvement of molecular chaperones is a crucial component of cold adaptation in psychophilic bacteria (Feller, 2013; Piette et al., 2011). We propose a similar role for HSPs in UWO241. One of the main differences between the primary metabolomes of UWO241 and *C. reinhardtii* were that 10°C-grown *C. reinhardtii* showed high levels of amino acids, but UWO241 did not, at any growth temperature (Figure 3b, Table 1). Amino acid accumulation could be a protective cold-stress response, or the consequence of decreased efficiency of protein synthesis at low temperatures in *C. reinhardtii* (Valledor, Furuhashi, Hanak, & Weckwerth, 2013). The lack of a large free amino acid pool in UWO241 may indicate an efficient protein synthesis machinery that is not negatively affected by low temperatures.

Zhang, Cvetkovska, et al. (2021) and Zhang, Hu, and Smith (2021) proposed that HSDs in the UWO241 genome aid in survival at extreme environments by contributing increased protein amounts via gene dosage (Kondrashov, 2012; Qian & Zhang, 2014). Indeed, UWO241 was shown to have increased protein accumulation of photosynthetic ferredoxin (PETF) when compared to *C. reinhardtii*. Unlike other green algae, PETF in UWO241 is encoded by two near identical genes (Fd-1A and Fd-1B) (Cvetkovska et al., 2018). The increased PETF protein levels may contribute to the higher capacity for photosynthetic electron transport and maintenance of photostasis in UWO241 (Kalra et al., 2020; Szyszka, Ivanov, & Hüner, 2007;

Szyszkka-Mroz et al., 2019). We propose that the expansion and duplicate-gene retention of the HSP gene family in UWO241 is a result of life in the cold and it increases the capacity for protein folding at low temperatures.

#### 4.4 | A constitutive stress response is a key psychrophilic adaptation

This study reveals novel insights into psychrophilic lifestyle of the green alga UWO241 and potential roles of molecular chaperones and stress metabolism during cold adaptation and heat stress. In this work, we showed that many molecular and metabolic signatures commonly used as stress markers in *C. reinhardtii* (HSPs, carbohydrates, antioxidants) are constitutively accumulated at high levels in UWO241 grown at 4°C (Figure 12), a growth temperature, which also results in the more robust response to subsequent stress. We propose that this results from life at extreme conditions, where a permanent exposure to low temperature has geared psychrophilic metabolism towards a constitutive accumulation of proteins and metabolites with a known stress-related function (Figure 12). Further work is needed to determine whether this is an advantage for life on the edge. Nevertheless, what UWO241 appears to be lacking is the ability to fine-tune the regulation of this network, particularly the accumulation of HSPs, upon exposure to subsequent heat stress. For instance, in our work we did not detect up-regulation in HSF1 transcript, possibly due to the transient nature of its accumulation (Schmollinger et al., 2013), low protein levels, or low antibody specificity (data not shown). HSF1 has been shown to regulate HSP levels both during heat stress (Schmollinger et al., 2013) and cold stress (Maikova et al., 2016) in *C. reinhardtii*, but it is not clear whether a similar mechanism operates in UWO241. A more detailed analysis including expression and protein accumulation of HSF1 and other HSPs using UWO241-specific antibodies is needed. It should also be noted that *C. reinhardtii* and UWO241 are phylogenetically distant within the order Chlamydomonadales (Possmayer et al., 2016), and future work should examine these responses in species more closely related to UWO241.

Our work adds to a growing body of research on how heat stress affects psychrophilic algae (Barati, Lim, Gan, Poong, & Phang, 2018; Boo et al., 2013; Chong, Chu, Othman, & Phang, 2011; Hwang, Jung, & Jin, 2008; Poong et al., 2018; Possmayer et al., 2011; Su et al., 2016), a topic that is particularly relevant given recent trends in climate change. The Antarctic continent is one of the most rapidly warming locations on Earth (Chapman & Walsh, 2007). Studying the stress networks in psychrophilic algae will provide much needed insight into how these organisms adapt to their extreme environment and whether they are able to respond to temperature stress similarly to their mesophilic relatives.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHOR CONTRIBUTIONS

Marina Cvetkovska, David R. Smith and Norman P.A. Hüner conceptualized the work and designed the experiments. Marina Cvetkovska wrote the initial draft and performed cell death assays, microscopy, metabolomics experiments, UWO241 genomic screening and immunoblotting experiments in cooperation with Beth Szyszkka-Mroz and Nina Malczewski. Xi Zhang performed global transcriptomic analysis. Galyna Vakulenko and Samuel Benzaquen performed HSP expression analysis and genomic screening of selected species. All authors contributed towards manuscript preparation and editing.

#### DATA AVAILABILITY STATEMENT

The RNA-Seq data that support the findings of this study are openly available in NCBI Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/sra> under BioProject PRJNA701568. All other sequences are openly available at GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and Phytozome v113 (<https://phytozome-next.jgi.doe.gov/>) under accession numbers listed in Table S1. All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

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