Constitutively Up-regulated Carbon Metabolism is an Adaptation to Low Temperature in the Antarctic Psychrophile *Chlamydomonas* sp. UWO241

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Abstract

The Antarctic alga *Chlamydomonas* sp. UWO241 is an obligate psychrophile that thrives in the cold but is unable to survive at moderate, seemingly innocuous temperatures. We dissect the responses of UWO241 to temperature stress using global metabolomic approaches. UWO241 exhibits slow growth at 4°C, a temperature closest to its natural habitat, and faster growth at higher temperatures of 10-15°C. We demonstrate that the slower growth-rate characteristic of UWO241 at 40C is not necessarily a hallmark of stress. UWO241 constitutively accumulates high levels of protective metabolites including soluble sugars, polyamines and antioxidants at a range of steady-state temperatures. In contrast, the mesophile Chlamydomonas reinhardtii accumulates these metabolites only during cold stress. Despite low growth rates, 4°C-grown UWO241 cultures had a higher capacity to respond to heat stress (24°C) and accumulated increased amounts of antioxidants, lipids and soluble sugars, when compared to cultures grown at 10-15°C. We conclude that the slower growth rate and the unique psychrophilic physiological characteristic of UWO241 grown at 40C result in a permanently re-routed steady-state metabolism, which contributes to its increased resistance to heat stress. Our work adds to the growing body of research on temperature stress in psychrophiles, many of which are threatened by climate change.

INTRODUCTION

Green algae have a plastic metabolism and physiology, which allows them to cope with many environmental variables. Changes in temperature affect basic cellular functions by modifying the fluidity of biological membranes (Los & Murata 2004; Morgan-Kiss, Priscu, Pocock, Gudynaite-Savitch & Hüner 2006; Horváth *et al.* 2012; Los, Mironov & Allakhverdiev 2013) and the rate of enzymatic reactions (Feller 2013, 2018; Isaksen, Åqvist & Brandsdal 2016), and can influence a wide array of biological, chemical, and physiological processes. It is generally accepted that an optimal temperature results in highest rates of metabolism and growth of algal populations, whereas temperatures above and below this optimum inhibit metabolic processes, which leads to lower growth rates (Borowitzka 2018). At non-permissive temperatures (below the minimum or above the maximum for growth), these processes can fail and may ultimately cause cell death.

Perennially cold environments, such as polar and alpine regions, are among the world's largest ecosystems. Phototrophic microbes, including eukaryotic algae, are the dominant primary producers in polar habitats and are at the base of virtually all low temperature food webs (Morgan-Kiss *et al.* 2006; Margesin 2008; Lyon & Mock 2014; Chrismas, Anesio & Sánchez-Baracaldo 2015). Many of these organisms are obligate cold extremophiles (psychrophiles) which grow optimally at temperatures close to the freezing point of water but are unable to survive at moderate temperatures [?] 20oC (Casanueva, Tuffin, Cary & Cowan

2010; Cvetkovska, Huner & Smith 2017). The green alga *Chlamydomonas* sp. UWO241 is among the best-studied eukaryotic algal psychrophiles. Its close phylogenetic relationships with model green algae, including *Chlamydomonas reinhardtii* , make it an ideal candidate for comparative studies (Possmayer *et al.* 2016).

UWO241 was isolated 17 m below the surface of the perennially ice-covered Lake Bonney (McMudro Dry Valleys, Antarctica), where it thrives under many different extremes, including low but stable temperatures (~5degC), high salinity (0.7 M NaCl), low light (<10 μmol m^{-2} s^{-1}) enriched in blue-green wavelengths (450-550 nm), and extremes in photoperiod (e.g., long-term darkness during the polar night). Despite originating from such a severe habitat, UWO241 shows strong physiological plasticity, and can successfully grow under a variety of laboratory conditions (white light; intensity 10-250 μmol m^{-2} s^{-1}; salinity 0.01-1.2M) (Morgan-Kiss, Ivanov & Huner 2002; Morgan-Kiss *et al.* 2005; Pocock *et al.* 2004; Pocock; Szyszka, Ivanov & Hüner 2007; Takizawa, Takahashi, Hüner & Minagawa 2009; Szyszka-Mroz, Pittock, Ivanov, Lajoie & Hüner 2015). UWO241 is an obligate psyschrophile with an upper temperature limit of 18^{o}C, above which population growth ceases and cells eventually die (Possmayer *et al.* 2011).

Previous studies of UWO241 focused on understanding the photosynthetic mechanisms behind psychrophilic adaptations (reviewed in (Morgan-Kiss*et al.* 2006; Dolhi, Maxwell & Morgan-Kiss 2013). Studies on UWO241 revealed novel adaptations to extreme conditions, including a remodeled photosynthetic machinery. UWO241 is the only known natural algal variant that does not undergo typical photosynthetic state transitions, which balance the energy distribution between photosystems I and II (PSI and PSII) via a reversible LHCII phosphorylation and migration (Minagawa 2011; Rochaix 2014). Instead, UWO241 balances the energy budget of its electron transport chain by re-organizing the photosynthetic apparatus, resulting in direct energy spillover between the two photosystems and increased cyclic electron flow (CEF) around PSI (Szyszka *et al.* 2007; Szyszka-Mroz *et al.* 2015, 2019; Kalra *et al.* 2020). This unique mechanism of energy balance was recently linked to the presence of cold-adapted photosynthetic proteins. Ferredoxin (Fd-1) is involved in the transfer of electrons from PSI to various metabolic pathways, including carbon fixation and CEF (Hanke & Mulo 2013). The UWO241 genome, which was recently sequenced (Zhang, Cvetkovska, Morgan-Kiss, Hüner & Smith 2021), encodes two Fd-1 isoforms, which accumulate at higher amounts compared to mesophilic Fd-1 from *C. reinhardtii* . In UWO241, Fd-1 is most active at 10^{o}C but has increased structural sensitivity and lower activity when incubated at temperatures >40°C (Cvetkovska *et al* ., 2018). The chloroplast protein kinase Stt7 phosphorylates the major light-harvesting proteins associated with PSII (LHCII), facilitating their movement from PSII to PSI during state transitions in *C. reinhardtii* (Lemeille *et al* . 2010; Rochaix, 2014). In contrast to *C. reinhardtii* , Stt7 in UWO241 has higher LHCII-phosphorylation activity at 8^{o}C than at 23^{o}C, which appears to reflect the reorganization of the photosynthetic apparatus required for energy spillover (Szyszka-Mroz *et al.* 2019). Thus, these two key photosynthetic proteins appear to have evolved to optimize photosynthetic light harvesting, energy transfer, and carbon fixation at low temperatures at the expense of lower activities at moderate and high temperatures.

The underlying assumption of most studies on psychrophily is that cold-adapted organisms have inherent biochemical characteristics enabling them to thrive at low temperatures. However, the distinguishing feature of a psychrophile is not necessarily an exceptional ability to grow at low temperatures, but rather an inability to survive at moderate, seemingly innocuous temperatures. Indeed, many photosynthetic species, including crop plants, evergreen conifers, green algae and cyanobacteria, survive and grow at both cold and warm temperatures and are therefore not psychrophilic (Chang *et al.* 2020; Adams*et al* ., 1995; Hüner *et al.* , 1998; Tang and Vincent, 1999; Öquist and Hüner, 2003; Hüner *et al* ., 2012; Yamori *et al* ., 2014). UWO241 is one of the few psychrophilic chlamydomonadalean algae that have been studied in relation to heat stress. It has been shown that exposure to 24^{o}C can be lethal but cell death occurs slowly, and the effects are reversible in the first 12 hours (Possmayer *et al.* 2011). Moreover, short-term exposure to 24^{o}C resulted in a myriad of physiological changes, including cessation of cell growth, inhibition of PSII efficiency and accumulation of the molecular chaperone HSP22A (Possmayer *et al.* 2011). Long-term exposure of UWO241 to 24^{o}C leads to cell death.

Can UWO241, which likely never experiences temperatures above 5°C in its natural environment, mount a

heat stress response comparable to a mesophile? And can it acquire thermotolerance, whereby small, non-lethal increases in temperature prime the cell to respond more rapidly and strongly to subsequent heat stress? Indeed, exposure to higher but non-lethal temperatures can have a protective effect in mesophiles (Song, Jiang, Zhao & Hou 2012; Horváth *et al.* 2012; Yeh, Kaplinsky, Hu & Charng 2012; Kishimoto, Ariga, Itabashi & Mikami 2019) but this topic, to the best of our knowledge, has never been addressed in a psychrophile. Here, we examine the growth and metabolic profiles of UWO241 under steady-state low temperature and heat stress conditions. We analyze and describe its adaptive strategies to cold, as well as its heat stress response upon exposure to non-permissive temperatures. This work contributes towards the understanding environmental stress responses in a psychrophilic Antarctic alga — a contribution made more important by the fact that polar environments and organisms that thrive in the cold are particularly threatened by current patterns of global climate change (Kennicutt *et al.* 2015, 2019; Xavier *et al.*2016).

METHODS

Strains and Growth Conditions

UWO241 was grown axenically in Bold's Basal Medium (BBM) supplemented with 10 mM NaCl, whereas *C. reinhardtii* cc-1690 was grown in BBM with 0.43 mM NaCl. All cultures were aerated continuously with ambient air filtered by a 0.2 μm filter in 250 ml glass growth tubes suspended in thermo-regulated aquaria. A continuous growth irradiance of 100 μmol photons m^{-2} 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). UWO241 cultures were grown at 4, 10, 15, 17 and 20°C and exposed to heat stress by transfer to 24°C. *C. reinhardtii* cultures were grown at 5, 10, 15, 28, 37 and 40°C and exposed to heat stress by transfer to 42°C. Mid-log cultures were used in all experiments.

Growth and Cell Death Kinetics

Cell growth was estimated by determining chlorophyll concentration in algal cultures over time, as previously described (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). The maximal specific growth rate (μ_{max}) is the highest value of μ calculated according to the equation:

$\mu = \ln(\Delta[\varsigma\eta\lambda])/\Delta t_{hours}$

where $\Delta[\varsigma\eta\lambda]$ ις τηε ρατιο οφ τηε χηλοροπηψλλ βετωεεν τηε τωο σαμπλινγ τιμες, ανδ Δt_{hours} is the time elapsed.

The kinetics of cell death were determined in two ways. First, the loss of chlorophyll due to exposure to heat was measured as described above. Second, culture viability was assayed by resuspending pelleted algal cells in 0.5% (w/v) Evans Blue solution. Cells were incubated for 30 min and the unbound dye was removed by extensive washing with BBM medium. The 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 (16,000g, 3 min) to remove insoluble particles, and absorbance was measured spectrophotometrically at 600 nm. The absorbance of cells treated with 1% (v/v) chloroform (100% death) were 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. Images of algal cells were taken for observing cell morphology and chlorophyll loss due to heat stress. All images were processed using Image-Pro Premier 9.1 (Media Cybernetics, USA).

Gas Chromatography - Mass Spectrometry and Metabolomic Analysis

For determination of the primary metabolome, algae were grown in three biological replicate cultures as described above and sampled at a steady-state temperature or after 6h exposure to heat stress. Algal cells were harvested by centrifugation (6,000g, 5 min), washed once with fresh medium, flash frozen in liquid nitrogen and stored at -80°C. Metabolite extractions, chromatography and quality processing were done at the West Coast Metabolomics Center (UC Davis, CA, USA), following a previously established protocol (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, UC Davis, CA, USA). 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 (van den Berg, Hoefsloot, Westerhuis, Smilde & van der Werf 2006). 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.

RESULTS

Growth rates of UWO241 and *C. reinhardtii* at different steady-state temperatures

We compared the effects of temperature on growth in the psychrophile UWO241 and the mesophile *C. reinhardtii* . The growth rate of UWO241 cultured at 4°C (the approximate temperature in its natural environment) was 0.019 h^{-1}; however, maximal growth rates were achieved at 10°-15°C (0.025-0.030 h^{-1}; Figure 1A). We observed lower growth rates in UWO241 at 17°C (0.013 h^{-1}), and no growth was observed at 20°C. In accordance with previous reports (Schroda, Hemme & Mühlhaus 2015),*C. reinhardtii* exhibited maximal growth rates when cultured at 28°C (0.048 h^{-1}) and lower rates at growth temperatures both higher and lower than this range (Figure 1B). Despite a longer lag phase (not shown), *C. reinhardtii* cultures were able to grow at 10° and 15°C, albeit at a decreased rate (0.025 and 0.034 h^{-1}, respectively) compared to growth at 28°C (Figure 1B). This low temperature growth rate for *C. reinhardtii* was comparable to the maximum growth rates of UWO241 between 10°-15°C (Figure 1A). We did not observe growth of the mesophilic alga at 5°C or 40°C.

The primary metabolome of psychrophilic and mesophilic green algae at low temperature

To understand low temperature responses, we analyzed the primary metabolome of both algal species acclimated to low growth temperatures (4°C, 10°C, 15°C – UWO241; 10°C, 15°C, 28°C – *C. reinhardtii*). We 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 species-specific differences in the primary metabolomes along both components, regardless of the culturing temperature (Figure 2). 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 2-fold change in accumulation between at least two of the treatments ($p<0.01$, ANOVA, Tukey's post-hoc). Approximately 50% of the metabolites (392 out of 771) were identified as DAMs, with significant differences between species (Table 1). Hierarchical clustering analysis (HCA) revealed the following trends (Figure 3A): (1) The metabolome of*C. reinhardtii* responded strongly to growth temperature with 273 (35%) DAMs at 10°C compared to 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%) (Table 1; Figure 3A). (2) The metabolic profile of UWO241 was similar regardless of the culturing temperature. Cultures at 4°C had only 48 (6%) and 78 (10%) DAMs when compared to 10°C and 15°C, respectively (Table 1; Figure 3A). These findings indicate that the mesophile grown at low temperatures experiences cold stress and adjusts its metabolome accordingly. In contrast, the metabolome of the psychrophile is stable at temperatures between 4°C and 15°C.

Resolving the contribution of specific metabolites to cold acclimation in green algae

We analyzed the subset of 163 primary metabolites with positively identified chemical signatures (Supplemental Dataset S1) and identified 135 metabolites (83.6%) significantly different between at least two of the samples ($p<0.01$, ANOVA, Tukey's post hoc). We chose the metabolome of *C. reinhardtii* grown at 28°C as the control and compared all other samples to it (Figure 3B). Not all metabolites belonging to the same

class contributed equally to the observed differences, and we present the 20 metabolites that have the largest differences in abundance between the treatments (Table 2).

A subset of metabolites showed comparable accumulation patterns in both species. Carbohydrates and glycerol are well-known cryoprotectants in cold-adapted organisms (Roser, Melick, Ling & Seppelt 1992; Tulha, Lima, Lucas & Ferreira 2010; Su *et al.* 2016), including algae (Leya 2013). Here, we observed high accumulation of several carbohydrates (e.g. 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; Supplemental Dataset S1). Carboxylic acid accumulation showed a strong dependence on growth temperature and is increased at the lowest temperature for both algae. Notably, α-ketoglutarate (α-KG), 3-phosphoglycerate (3-PGA), and phosphoenolpuruvate (PEP) showed the highest increases in abundance, particularly in UWO241 grown at 4°C (FC 48.1, 39.8, and 18.0 respectively). Lactic acid is the exception, and its abundance is significantly increased in the mesophile (FC 70.2 at 10°C) but decreased in the psychrophile (FC 6.7 at 4°C). Fatty acid and lipid metabolism are important for algal cold adaptation (Lyon & Mock 2014; Jung *et al.* 2016; Suzuki, Hulatt, Wijffels & Kiron 2019). Ergosterol and linoleic acid exhibited a decreased accumulation in both species grown in the cold (Table 2), although overall lipid levels were not significantly affected by low temperatures (Figure 3B). Finally, we detected high accumulation of the antioxidant dehydroascorbic acid in both *C. reinhardtii* (FC 19.7) and UWO241 (FC 20.1) grown at their respective lowest temperatures (Table 2). Threonic acid, a product of ascorbate catabolism (Debolt *et al* ., 2007), also showed increased levels in both species, suggesting an important role for the ascorbate pathway during cold adaptation and acclimation.

We observed species-specific differences in the primary metabolomes. Glucose, a carbohydrate with known roles in osmotolerance, cold stress and freezing tolerance (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). This implies that there is a metabolic switch in the primary carbon metabolism of UWO241. Sugar alcohols are important molecules in cold-stress tolerance in plants and algae (Roser *et al* . 1992; Leya, 2013); the accumulation of metabolites from this compound class was increased in *C. reinhardtii* but not in UWO241 (Figure 3B), although both species showed a significant decrease in several sugar alcohols (e.g., mannitol and galactinol; Table 2) at low temperatures. Amino acid metabolism was significantly affected by low temperature in*C. reinhardtii* and nearly all detected amino acids increased in abundance (Figure 3A). Again, we did not observe this in UWO241, and amino acid abundance was largely unchanged or decreased (e.g., cysteine and aspartic acid; Table 2). An exception is the non-proteinogenic amino acid ornithine, which accumulated at low temperatures in both species. In *C. reinhardti* i, this accumulation was temperature dependent (higher at 10°C than at 28°C, FC 9.7), whereas in UWO241 its accumulation was constitutively high at all temperatures (FC 13.5 – 34.7; Table 2). We also observed a strong species-specific pattern in the accumulation of N-containing compounds, including those involved in purine and pyrimidine metabolism. These compounds 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 (e.g., thymidine, Figure 3A, Table 2). Altogether, we suggest that these data reflect metabolic adaptations in UWO241 to life in a perennially cold environment.

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°C 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 *et al.* 2014; Légeret *et al.* 2016), which showed that the response of *C. reinhardtii* to 42°C was comparable physiologically to the that of UWO241 to 24°C.

To quantify the effects of heat stress, we measured the progressive loss of chlorophyll and cell death using Evans Blue dye that accumulates in cells with damaged membranes (Supplemental Figure S1). UWO241 cultured at 10°C and 15°C (temperatures that lead to the fastest growth), completely lost their chlorophyll content and viability after 72h exposure to 24°C. In contrast, UWO241 cultures grown at 4°C (with slower

growth), were more resistant to 24°C exposure and suffered only 57% cell death and 72% chlorophyll loss at 72h (Figure 4A, C). Cell structures and chlorophyll were visible under the light microscope only in the 4°C-grown UWO241 after 72h exposure to 24°C, but not in the 10°C and 15°C ones (Figure 5).

C. reinhardtii was most resistant to 42°C when initially grown at of 28°C with fastest growth rates, showing a 62% chlorophyll and a 30% cell death at 48h. Cultures acclimated to lower (10°C) and higher (37°C) temperature were more sensitive to heat exposure and rapidly lost chlorophyll (82% and 98%, respectively) and viability (70% and 90%, respectively) by 48h. All cultures appeared to be completely dead after 72h exposure (Figure 4B, D). The loss of chlorophyll and cell structure was confirmed by light microscopy (Supplemental Figure S2).

In all experiments, we returned the cultures to their original growth temperature after 96h of heat exposure. All *C. reinhardtii*cultures recovered their growth, indicating that despite the apparent full loss of viability in our measurements not all cells were dead even after prolonged exposure to 42°C (Supplemental Figure 3A, B, C). In contrast, UWO241 cultures did not recover once exposed to 24°C for 96h, regardless of the initial culturing temperature, indicating a complete loss of viability (Supplemental Figure 3D, E, F).

The primary metabolome of UWO241 grown at 4°C responds strongly to heat stress

To understand the heat-induced changes to psychrophilic metabolism, we analyzed the primary metabolome of UWO241 exposed to the non-permissive temperature of 24°C for 6h. 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 6). 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 the highest growth temperature of 15°C before and after 6h heat stress (Figure 6). HCA (Figure 7A) and volcano plot analysis (Table 3) revealed a strong response at the level of the metabolome when cultures grown at 4°C were exposed to 24°C for 6 hrs (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.

Detailed analysis on the 161 positively identified metabolites revealed that 98 metabolites (54.8%) were significantly different between control and heat stress samples (ANOVA, $p<0.01$, Tukey's post hoc). We organized the positively identified metabolites by chemical categories and normalized all control samples to an arbitrary value of 1. We compared the metabolomes from cultures exposed to 24°C to the appropriate steady-state growth temperature (i.e., 4°C metabolome was compared to 4°C + 6h heat stress metabolome). We reported the differences between the metabolite classes (Figure 7B, Supplemental Dataset S2) and the 20 metabolites that showed the largest abundance difference between steady-state and heat stress treatments (Table 4). Most metabolite classes, including carbohydrates, sugar alcohols, amino acids, lipids and anti-oxidants, increased in abundance in the cultures grown at 4°C and exposed to 24°C, with the exception of carboxylic acids and sugar phosphates, which increased significantly in the 10°C cultures (but not in those grown at 15°C). Notably, these metabolites are already present in high amounts in UWO241 acclimated to 4°C (Figure 3B). We also analyzed the 20 metabolites that showed the most significant change in accumulation in heat stressed UWO241 (Table 4). Glucose-6-phosphate was the only compound that showed a small but significant decrease regardless of the initial culturing temperature (FC 1.7-2.6). Ergosterol (FC 439.1) and α-tocopherol (FC 308.1) increased at very high amounts in all cultures exposed to heat, regardless of the initial growth conditions (Table 5). These increases followed a temperature dependent pattern, with the highest FC seen in the cultures initially grown at 4°C.

DISCUSSION

Algae have the fastest growth and maximal reproductive potential under optimal conditions, while stress is viewed as a threat to cellular homeostasis and leads to decreased growth and fitness (Borowitzka 2018). The Antarctic alga UWO241 experiences low but very stable temperatures of 4-6°C year-round, but we found that the growth rate was faster at 10°C and 15°C (Figure 1). This indicates that the biochemical and metabolic

processes operating in UWO241 are better adjusted to optimal growth temperatures between 10-15°C than the temperature it experiences in nature (4°C), which would be considered a stress for UWO241 based on growth rates alone. Nevertheless, our metabolomic analyses showed that, despite the slower growth rates, UWO241 is adapted for life at 4°C. This was reflected in the fact that light- and CO_2-saturated rate of O_2 evolution for UWO241 grown at 4°C are comparable to the photosynthetic rate of *C. reinhardtii* grown at 28°C (Pocock, Koziak, Rosso, Falk & Hüner 2007).

Metabolic signatures of adaptation to permanently low temperatures

A key observation stemming from our work is that despite low growth rates, the primary metabolome of UWO241 cultures grown at 4°C did not differ significantly from those grown nearer their optimal growth temperatures of 10°C to 15°C (Figure 1). In contrast, *C. reinhardtii* showed a strong temperature dependent response at the level of the primary metabolome (Figure 2, Figure 3A), with 10°C-grown cultures accumulating increased levels of cryoprotectants and membrane stabilizers as compared to cultures grown near their optimal temperature (28°C). These compounds are typically present in photosynthetic organisms exposed to cold stress (Wanner & Junttila 1999; Gray & Heath 2005; Kaplan *et al.* 2007; Guy, Kaplan, Kopka, Selbig & Hincha 2007; Janská, Maršík, Zelenková & Ovesná 2010; Fürtauer, Weiszmann, Weckwerth & Nägele 2019). We interpret this as evidence that when the psychrophile UWO241 is cultured at 4°C it does not experience cold stress, despite this temperature being well below its growth optimum (10-15°C). *C. reinhardtii* , on the other hand, exhibits typical cold-stress responses at the level of the primary metabolome when cultured at temperatures that lead to slow growth rates.

We suggest that our metabolomic data reveal a constitutive re-routing of primary metabolism in UWO241 when compared to the mesophilic model *C. reinhardtii* . First, our study indicates that constitutively high accumulation of soluble sugars is a low-temperature adaptation in UWO241. It appears that this alga has a re-wired central carbon metabolism and accumulates high amounts of soluble sugars at the expense of other photosynthetic intermediates, consistent with previous results for UWO241 (Cook *et al.* 2019; Kalra *et al.* 2020).

Second, amino acids and their derivatives accumulated at high levels in low temperature-grown *C. reinhardtii,* but this response was absent in UWO241 (Figure 3B, Table 2). High amino acid levels could be a protective cold stress response, but it could also be the consequence of decreased efficiency of protein synthesis at low temperatures in *C. reinhardtii* (Valledor *et al* . 2013). The fact that UWO241 does not accumulate amino acids may indicate an efficient protein synthesis machinery that is not negatively affected by low temperatures. One exception was the increased amount of ornithine in UWO241 at all growth temperatures, but only at 10°C in *C. reinhardtii* (Table 2). Ornithine is a non-proteinogenic amino acid with 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* . 2009b, 2009a). We also detected increased amounts of the polyamine putrescine in UWO241 compared to *C. reinhardtii* (and no increases in arginine or proline; Supplemental Dataset S1). Polyamines play important roles in DNA and RNA protection and stabilization, protein synthesis and cell cycle progression (Gill & Tuteja 2010; Minocha, Majumdar & Minocha 2014; Chen, Shao, Yin, Younis & Zheng 2019). Our data suggest that in UWO241, constitutive accumulation of ornithine and polyamines is not a cold stress response, but a mechanism to ensure cell division and growth at low temperatures. Ensuring nucleic acid protection and efficient protein synthesis could be key psychrophilic adaptations to permanently cold environments.

Third, ascorbic acid (AsA) and its oxidized form dehydroascorbic acid (DHA) accumulate at high levels in UWO241 at all growth temperatures but only at 10°C in *C. reinhardtii* (Table 2; Supplemental Dataset S1). Photosynthesis creates an oxic intracellular environment, further exacerbated by reactive oxygen species (ROS) formation due to metabolic imbalances caused by low temperatures (Dreyer & Dietz 2018). The depth at which UWO241 is found in Lake Bonney (17 meters below the surface) is a hyperoxic environment (200% air saturation) due to oxygen having a higher solubility at low temperatures and poor diffusion in the presence of permanent ice cover (Morgan-Kiss *et al.* 2006). Thus, UWO241 and other organisms that live in such environments need a robust and constitutively active antioxidant system to cope with high intra- and

extracellular ROS. The ascorbate-glutathione (AsA-GSH) cycle is a fundamental metabolic pathway involved in maintenance of cellular redox homeostasis (Sakhno, Yemets & Blume 2019; Hasanuzzaman *et al.*2019). Constitutively high antioxidant levels and increased amounts of AsA-GSH enzymes have been reported previously in polar diatoms (Janknegt et al., 2008). We propose that the AsA-GSH cycle is constitutively active in the psychrophile UWO241 as an adaptation to low temperatures.

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). In other words, their physiology is very sensitive to increased temperatures, but the underlying reasons for this sensitivity are unknown. We showed that the response to heat stress in UWO241 is dependent on the initial culturing temperature. UWO241 grown at 4°C (with slowest growth rates), shows a strong response to exposure to a non-permissive growth temperature of 24°C at the level of its primary metabolome (Figure 7, Table 3 – 273 DAMs), and has the slowest cell death kinetics as compared to those grown at higher temperatures (Figure 4A, 4B; Figure 5). In UWO241 cultures grown at 10°C, a response at the level of the metabolome is largely absent, except for increased accumulation of carboxylic acids and sugar phosphates (Figure 7B, Table 3 – 71 DAMs). In accordance, 10°C-grown UWO241 cultures were less resistant to 24°C exposure and exhibited rapid cell death kinetics. Cultures initially grown at 15°C with the fastest growth rates, also had an attenuated metabolic response (Figure 7; Table 3 – 26 DAMs), and rapid cell death kinetics (Figure 4A, 4B; Figure 5). Exposure to higher (but not lethal) temperatures to generate a timely heat stress signal has been demonstrated to have a protective effect in mesophiles (Song *et al.* 2012; Horváth*et al.* 2012; Yeh *et al.* 2012; Kishimoto *et al.*2019), but this is clearly not the case for the psychrophile UWO241. This study demonstrates that when UWO241 is cultured at a temperature closest to its natural environment (4°C), it has a higher capacity to respond to high temperature stress than when cultured at temperatures that provide faster growth rate.

We suggest that UWO241 exhibits a constitutive routing of steady-state metabolism towards soluble sugars, antioxidant and cryoprotectant accumulation. Many of these metabolites are characteristic of low-temperature adaptation and are maintained or even increased during short-term heat stress, including high levels of carbohydrates (particularly sucrose) during heat stress (Figure 7B, Table 4). The antioxidants ascorbate and α-tocopherol (Table 4, Supplemental Table S2) were dramatically increased during exposure to 24°C. Tocopherol and ascorbate have been studied during high light stress (Trebst, Depka & Holländer-Czytko 2002; Sirikhachornkit, Shin, Baroli & Niyogi 2009; Nowicka & Kruk 2012; Szarka, Tomasskovics & Bánhegyi 2012), and to the best of our knowledge this is the first report of involvement of the tocopherol-ascorbate antioxidant system in heat stress in green algae. We also detected evidence for the re-modeling of lipid composition of cellular membranes due to heat stress and increased amounts of saturated palmitic (16:0), stearic (18:0) and monounsaturated oleic (18:1) FAs (Table 4, Supplemental Dataset S2). The most dramatic increase was for the lipid ergosterol (Table 4). Increased membrane rigidity and high ergosterol content has been shown to counteract the deleterious effects of several stressors, including heat, in yeast (Swan & Watson 1998; Vanegas, Contreras, Faller & Longo 2012; Caspeta *et al.* 2014; Godinho *et al.* 2018). Efficient photosynthesis and maintenance of energy metabolism could be the driving forces behind soluble sugar, fatty acid and antioxidant synthesis during short-term heat stress in UWO241. The constitutively up-regulated carbon metabolism due to cold adaptation could provide intermediates and energy for mounting a heat stress response in 4°C-grown UWO241.

Conclusion

The extreme and very stable conditions in the perennially ice-covered Antarctic Lake Bonney might appear to be stressful for organismal growth and cellular homeostasis; however, we demonstrate that a slow growth rate, as observed in 4°C-grown UWO241, is a result of a unique psychrophilic cell physiology that constitutively accumulates stress-related compounds as an adaptation to low temperature. We suggest that carbohydrate metabolism, efficient protein synthesis, polyamine accumulation and increased antioxidant capacity are important processes for cold adaptation since these pathways were constitutively operational in UWO241 at all steady-state temperatures (Figure 10). This is in contrast with *C. reinhardtii* , where these

metabolites accumulated only during low temperature stress. In addition, 4°C-grown UWO241 cultures had the highest capacity to respond to heat stress, compared to cultures grown at higher temperatures, and accumulated stress-related metabolites at high levels (Figure 10). Thus, temperatures resulting in a comparatively faster growth rate in this extremophile do not necessarily define the optimal conditions for responding to environmental stress. We suggest that the ability to regulate the partitioning of energy between fast growth rates versus cell maintenance and homeostasis may be a hallmark of photopsychrophily.

Our work adds to a growing body of research on how heat stress affects psychrophilic eukaryotes (Hwang, Jung & Jin 2008; Chong, Chu, Othman & Phang 2011; Possmayer *et al.* 2011; Boo *et al.* 2013; Su*et al.* 2016; Barati, Lim, Gan, Poong & Phang 2018; Poong*et al.* 2018), 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). Clearly, the responses to stressors in natural environments are much more complex due to the interplay of several conditions, but our study may serve as a baseline for understanding environmental stress responses in Antarctic algae in general.

Supplemental data

The following materials are available as supplemental data.

Supplemental dataset S1 : Relative abundance of all identified metabolites in *C. reinhardtii* and UWO241 grown at different steady-state temperatures

Supplemental dataset S2 : Relative abundance of all identified metabolites in UWO241 grown at different steady-state temperatures and exposed to non-permissive temperature (24°C) for 6 hours.

Supplemental Figure S1. Light microscopy images of*Chlamydomonas* cells stained with 0.5% Evans Blue

Supplemental Figure S2. Light microscope images of *C.reinhardtii* acclimated to different steady state temperatures (10°C, 28°C, 37°C) and exposed to non-permissive temperature (42°C)

Supplemental Figure S3. The kinetics of cell death and culture recovery in UWO241 after exposure to non-permissive temperatures

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CONFLICT OF INTEREST

The authors declare that they have no competing interests

AUTHOR CONTRIBUTIONS

MC, DRS and NPAH conceptualized the work and designed the experiments. MC performed all metabolomics experiments and analysis in cooperation with BSM and NM for microscopy and cell growth analysis. MC write the original draft and all authors contributed towards manuscript preparation and editing.

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TABLES

Table 1: Summary of differentially accumulated metabolites (DAMs) between steady-state culturing temperatures in *C. reinhardtii* and UWO241

Treatment comparison	**Total # DAMs**	**# Upregulated**	**# Downregulated**
C. reinhardtii 28°C vs 10°C	273	252	21
C. reinhardtii 28°C vs 15°C	72	59	13
UWO241 4°C vs 10°C	48	32	16
UWO241 4°C vs 15°C	78	32	46

Table 2: 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 to this 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 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 was designated using the KEGG database (not an exhaustive list)#

Metabolite	**Class**	**KEGG Pathway**	C. reinhardtii **15°C**	C. reinhardtii **10°C**
Glucose	Carbohydrate	cre00010; cre00030	+1.06	**+1.69***
α-ketoglutarate	Carboxylic acid	cre00020; cre00053; cre01230	-1.02	+2.25
3-phosphoglycerate	Carboxylic acid	cre00010; cre00030; cre01230; cre00523	-1.18	**+7.34***
Phosphoenolpyruvate	Carboxylic acid	cre00020; cre00010; cre00710; cre01230	+1.57	**+4.46***
Lactic acid	Carboxylic acid	cre00620; cre00010; cre00051	**+6.94***	**+70.16***
Galactinol	Sugar alcohol	cre00052	**-4.69***	**-2.11***
Mannitol	Sugar alcohol	cre00051	-2.94	+1.09
Ornithine	Amino acid	cre01230	+2.85	**+9.68***
Histidine	Amino acid	cre01230	**+6.76***	**+22.94***
Glutamine	Amino acid	cre01230; cre00230, cre00240; cre00910	+4.61	**+35.20***
Lysine	Amino acid	cre01230	**+7.25***	**+27.98***
Cysteine	Amino acid	cre01230; cre00920	-2.04	**+1.66***
Aspartic acid	Amino acid	cre01230; cre00710; cre00760	+1.71	**+2.29***

N-acetylglutamate	Amino acid (derivative)	cre00220	**+4.13***	**+20.62***
Thymidine	N-containing	cre00240	+1.21	+2.03
Xanthine	N-containing	cre00230	**+1.84***	**+13.59***
Ergosterol	Lipid	cre00100	+6.25	**-18.11***
Linoleic acid	Fatty acid	cre01040	**-1.89***	**-8.60***
Dehydroascorbic acid	Antioxidant	cre00053; cre00480	+3.25	**+19.68***
Threonic acid	Sugar acid	cre00053	**+7.34***	**+201.97***

#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 – Sulfur metabolism

Table 3: Summary of differentially accumulated metabolites (DAMs) between UWO241 cultures grown at different steady–state temperatures and cultures exposed to non-permissive temperature for 6h

Treatment comparison	**Total # DAMs**	**# Upregulated**	**#Downregulated**
4°C - 24degC	222	205	17
10°C - 24degC	71	68	3
15°C - 24degC	26	22	4

Table 4: 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 hours. 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 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 was designated using the KEGG database (not an exhaustive list)#

Metabolite	**Class**	**KEGG Pathway**
Sucrose	Carbohydrate	cre00500
Ribose	Carbohydrate	cre00030
α-ketoglutarate	Carboxylic acid	cre00020; cre00053; cre01230
Pyruvic acid	Carboxylic acid	cre00010; cre00020; cre00030; cre00710 cre00053; cre01230; cre007
Fructose-1,6-bisphosphate	Sugar phosphate	cre00010; cre00030; cre00051
Glucose-6-phosphate	Sugar phosphate	cre00010; cre00030; cre00500
Mannitol	Sugar alcohol	cre00051
Glutamine	Amino acid	cre01230, cre00230; cre00240; cre00910
Lysine	Amino acid	cre01230
Histidine	Amino acid	cre01230
Aspartic acid	Amino acid	cre01230; cre00710; cre00760
Tryptophan	Amino acid	cre01230
Serotonin	Amino acid (derivative)	cre01230
Glycyl tyrosine	Amino acid (derivative)	cre01230
Adenosine	N-containing	cre00230

Inosine 5'-monophosphate	N-containing P-containing	cre00230
Oleic acid	Fatty acid	cre01040
Linolenic acid	Fatty acid	cre01040
Ergosterol	Lipid	cre00100
α-tocopherol	Antioxidant	cre01110

#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

FIGURE LEGENDS

Figure 1. Maximum growth rate (μ_{MAX}) of exponentially growing algal cultures at various temperatures**. (a)** *Chlamydomonas* sp. UWO241 **(b)** *Chlamydomonasreinhardtii* . Data are the means ± SD of at least six biological replicates

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.

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 color based metabolite profile as indicated (red – increase in abundance; blue – decrease in abundance). Hierarchical clustering is based on Euclidean distances and Ward's linkage. A cluster of metabolites present in both species at the lowest temperature is highlighted by a *. (**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.

Figure 4. Kinetics of cell death in UWO241 **(a,b)** and*C. reinhardtii* **(c,d)** acclimated to different growth temperatures and exposed to non-permissive conditions (24°C and 42°C, respectively). Cell death was estimated as the loss of chlorophyll in cells exposed to heat **(a,c)** or as a percentage of algal cells stained with 0.5% Evans Blue that accumulates in cells with damaged membranes **(b,d)** . Algal cells treated with 1% v/v chloroform were taken as a positive control and used to calculate 100% cell death. Data are means ± SD of at least three independent experiments and analyzed 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 *

Figure 5. Light microscope images of UWO241 acclimated to different steady state temperatures (4°C, 10°C, 15°C) and exposed to non-permissive temperature (24°C) for 24h, 48h and 72h. Algae are present as single cells or palmelloid colonies. Scale bar = 15 μm (400x total magnification)

Figure 6. Principal component analysis (PCA) of the primary metabolome of UWO241 acclimated to different steady-state temperatures and subsequently exposed to non-permissive temperature for 6 hours.

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.

Figure 7. Differences in the primary metabolome in UWO241 acclimated at different temperatures (4°C, 10°C, 15°C) and subsequently exposed to heat stress (24°C) for 6 hours. (**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 color-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 which 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).

Figure 8: (a) UWO241 grown at temperatures closest to its natural environment in Lake Bonney has an active central metabolism and constitutively accumulates metabolites important for life at low temperatures, including soluble sugars, antioxidants and compounds involved in nucleic acid protection. This simplified pathway map shows key metabolites that are increased (red) or decreased (blue) in UWO241 at 4°C, when compared to *C. reinhardtii* at 28°C. Metabolites shows in black did not change significantly, and those in gray were not detected in this study. We propose that this metabolic state provides UWO241 with the ability to cope with environmental stress**. (b)**When UWO241 is exposed to short-term heat stress at 24°C, many metabolites characteristic for cold adaptation, including soluble sugars and antioxidants, are maintained or even increased. The maintenance of energy metabolism could provide the energy to drive the production of protective compounds during heat stress. We show key metabolites that are increased (red) or decreased (blue) in, when compared to UWO241 at 4°C. Metabolites shows in black did not change significantly, and those in gray were not detected in this study.

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