The role and regulation of heat shock proteins in the Antarctic alga *Chlamydomonas priscuii*

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Abstract

Chlamydomonas priscuii is a psychrophilic green alga found 17 m below the permanently ice-covered surface of the Antarctic Lake Bonney, where it experiences a myriad of extreme environmental conditions, including low temperature, low light, and high salinity. While this habitat is extreme, it is also very stable, and this alga rarely experiences changes in its environment. Heat shock proteins (HSPs) are a ubiquitous family of chaperone proteins that perform important housekeeping and stress-related roles. In most organisms, including the model green alga *Chlamydomonas reinhardtii*, HSP expression is induced during abiotic stress to regain protein homeostasis – a process regulated by heat shock transcription factors (HSFs). This work shows that *C. priscuii* constitutively accumulates high protein levels of HSPs in steady-state conditions but fails to induce additional HSP accumulation during heat and low temperature, high and low salt, high light, and with canavanine treatment. In this study, a single HSF was identified in the *C. priscuii* genome. Comparative sequence analysis revealed that most domains characteristic of a functional HSF are conserved, but the expression of a full length HSF1 transcript could not be detected in the cell. Furthermore, the promoters of many *C. priscuii* HSPs lack binding sites for HSF. This work has shown that *C. priscuii* has a diminished ability to regulate HSP expression under stressful conditions, which we hypothesize is a result of life in an extreme but very stable environment. This is the first demonstration of a loss of HSP accumulation in green algae, which carries implications on the ability of psychrophiles to survive in the face of climate change.

Acknowledgements and Preface

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Chapter 1: Introduction

Life can be found in the harshest environments. Much of the Earth, including the oceans, polar, and alpine regions, are very cold - permanently below 5°C (Feller & Gerday, 2003). Despite their seemingly inhospitable temperatures, these areas are home to a wide biodiversity of organisms, which have a plethora of unique adaptations that allow them to thrive at the extremes. Some of these organisms, which thrive in the permanent cold but cannot survive moderate temperatures, are called obligate psychrophiles.

A psychrophile is an organism that can only reproduce and grow optimally at temperatures <15°C (Morita, 1975). Many organisms can tolerate the cold but have a higher optimum growth rate temperature (>20°C) – these are called psychrotrophic or psychrotolerant species. Crucially, the difference between psychrophiles and psychrotrophs is that psychrophiles are sensitive to increased temperatures and cannot survive prolonged heating (Morita, 1975). By definition, psychrophiles suffer heat stress and lose viability at ≥20°C; however, many psychrophiles are sensitive to even very minor temperature increases above those in their natural cold habitat. Most psychrophiles are prokaryotes, but eukaryotic psychrophiles have also been described, spanning many clades of the tree of life (Cvetkovska *et al.,* 2017; Peck, 2018). Since they are sparsely distributed both phylogenetically and geographically, psychrophily has likely evolved independently multiple times, rather than descending from a single psychrophilic ancestor (Cvetkovska *et al.,* 2017).

Regardless of their phylogenetic position, psychrophiles employ many common strategies to survive constant cold (reviewed in Cvetkovska *et al.,* 2017). To combat the overly rigid membranes caused by low temperatures, psychrophiles have higher amounts of unsaturated fatty acids in their membranes. To prevent cytosol freezing and ensure proper membrane transport, many psychrophiles accumulate high quantities of compatible solutes (e.g., soluble sugars, neutral compounds). Many also accumulate ice-binding proteins – proteins that prevent the formation of harmful ice crystals within the cell. Finally, the proteins of psychrophilic species have amino acid substitutions in their peptide sequences that make them more active and stable at lower temperatures.

Photopsychrophiles are psychrophilic organisms that can perform photosynthesis. The cold temperatures in polar ecosystems limit the range of land plant growth (Walker *et al.,* 2005), so

most primary production is carried out by photopsychrophilic algae, including unicellular green algae. These primary producers are also important in the global sense, since polar regions are responsible for a portion of global oxygen production and carbon sequestration (Lyon & Mock, 2014). As such, it is important to study photopsychrophiles and their environmental adaptations since polar food webs rely on these creatures' growth and survival. In addition, studying photopsychrophiles can help identify candidate genes to be expressed in more temperate plants, which could create hardier and more cold-resistant crops.

Polar aquatic ecosystems are at increased risk of global warming due to climate change. Photopsychrophiles are highly specialized and well adapted to their cold environments, but they are particularly sensitive to increased temperatures. Since higher trophic levels (e.g., animals) are dependent on primary producers in these environments, the reduced survival of photopsychrophiles will also affect the entire food web (Steiner *et al.*, 2019). Furthermore, many photopsychrophiles live in marine waters, or in places covered in snow or ice for at least some of the year. In these habitats, even small changes can drastically shift the balance between liquid water and ice. Melting ice increases the amount of freshwater in the ecosystem, which leads to decreased salinity and increased light availability (Obryk *et al.*, 2019). How will hyperspecialized cold-loving organisms survive such drastic changes? Studying photopsychrophiles can help understand the impact of climate change on a large and vulnerable ecosystem.

1.1. Virid and varied: green algae and the model *C. reinhardtii*

Algae are defined as plastid-bearing protists, and they are a hard group to pin down. They are diverse and paraphyletic, with members belonging four of the five eukaryotic supergroups (Keeling, 2013). Green algae are a type of algae that all arise from a single clade: the Viridiplantae ("green plants") (Fang *et al.*, 2018). The Viridiplantae are split into three groups representing the three lineages of green algae: Prasinodermophytes, which are the most basal group in Viridiplantae (Li *et al.*, 2020); the Streptophytes, which includes plants and their closest algal relatives (Fürst-Jansen *et al.*, 2020); and the Chlorophytes, a morphologically diverse, specious group that contains most green algae (Turmel & Lemieux, 2018). Most Chlorophytes are unicellular, but some are multicellular (e.g., green seaweeds) or form symbiotic relationships with other organisms (e.g., lichens). Some Chlorophytes are also economically important, such

as the green alga *Monostroma kuroshiense*, which is edible and cultivated worldwide (Bast, 2015).

The model organism *Chlamydomonas reinhardtii* is a Chlorophyte and is the best-studied green alga within the order Chlamydomonadales. It is unicellular and motile, with two flagella, a carotenoid-rich light-sensing eyespot organelle, and a single cup-shaped chloroplast (Figure 1A). This alga thrives in a mesophilic (temperate) climate and grows in freshwater or damp soil around the world. Since it grows quickly and easily in lab conditions and can be easily transformed to create mutant strains (Tran & Kaldenhoff, 2020), it has been used as a model organism for over 70 years. Many important discoveries in the field of plant biology and beyond have been made using *C. reinhardtii* as a model system (reviewed in Salomé & Merchant, 2019). Since it is unicellular and has only one chloroplast, it has been viewed as a "simplified plant" and used to study photosynthesis. It also has unique features beyond plants: since it can detect light with its eyespot and its flagella allow it to be motile (unlike plants), it has been used to study phototaxis (movement towards or away from light), flagellar motion and optogenetics. Furthermore, *C. reinhardtii* has been identified as a candidate for biofuel production and bioproduct synthesis (reviewed in Adeniyi *et al.*, 2018). While it is a good model organism for studying photosynthesis and light sensitivity in a simple system, it is sensitive to changes in salinity and extreme (hot or cold) temperatures. To study how photosynthetic life adapts to various harsh environments, extremophilic green algae can be compared to *C. reinhardtii* to identify key adaptations.

1.2. Our main cast – Antarctic green algae

One of the best-studied photopsychrophiles is the unicellular green alga *Chlamydomonas priscuii* (renamed from *Chlamydomonas* sp. UWO241 (Stahl-Rommel *et al.*, 2022)). Morphologically, it is very similar to *C. reinhardtii*: it too is unicellular with two flagella and a cup-shaped chloroplast (Figure 1B), although the eyespot is much smaller in *C. priscuii*. This alga was isolated 17 m below the permanently ice-covered surface of Lake Bonney in the McMurdo Dry Valley of Antarctica. This lake is perennially covered with a 4 m-thick layer of ice, which prevents any wind-driven mixing of the water. As a result, the water column is highly stratified in its salinity, light and nutrient availability, oxygenation, and the composition of the microbial communities within it (Morgan-Kiss *et al.*, 2006). At 17 m, *C. priscuii* experiences a myriad of

harsh conditions: permanent cold (4°C), high salinity (0.7 M NaCl), extreme shading (~5 μmol photons m^{-2} s^{-1}), high amounts of dissolved oxygen, and low phosphorus levels. Furthermore, this alga experiences an unusual photoperiod, with months-long periods of continuous light during the polar day and continuous darkness during the polar night (Cvetkovska *et al.*, 2017).

In addition to the general psychrophilic adaptations described earlier, *C. priscuii* has numerous specific adaptations to its extreme environment. Considerable research has gone into studying its unusual photosynthetic apparatus, which has its optimum efficiency at 8°C (compared to 25-35°C in *C. reinhardtii*) (Cvetkovska *et al.,* 2017). Additionally, it is the only green alga that cannot undergo photosynthetic state transitions (Morgan-Kiss *et al.,* 2002), which is a mechanism of reorganizing light harvesting complexes from photosystem II to photosystem I that prevents photodamage and ensures efficient light harvesting in variable light conditions. *C. priscuii* also has a higher amount of unsaturated fatty acids in its chloroplast membranes at low temperatures when compared to *C. reinhardtii* (Morgan-Kiss *et al.,* 2006). Some cold-adapted proteins have been characterized in *C. priscuii,* including ferredoxin and the chloroplast kinase STT7 (crucial components of the photosynthetic electron transport chain), which have higher activity at 10°C than their mesophilic homologs in *C. reinhardtii* (Cvetkovska *et al.,* 2018, Szyszka-Mroz *et al.,* 2019). Recent metabolomic work has shown high amounts of compatible solutes when *C. priscuii* was grown at 4°C, which maintain the correct osmotic pressure in the cell at low temperatures (Cvetkovska *et al.,* 2022).

C. priscuii was isolated from Lake Bonney more than 20 years ago and has since become a model system for photosynthetic adaptations to the cold. The availability of an annotated genome (Zhang *et al.,* 2021) alongside transcriptomic and metabolomic resources (Cvetkovska *et al.,* 2022) makes *C. priscuii* an ideal candidate for this study.

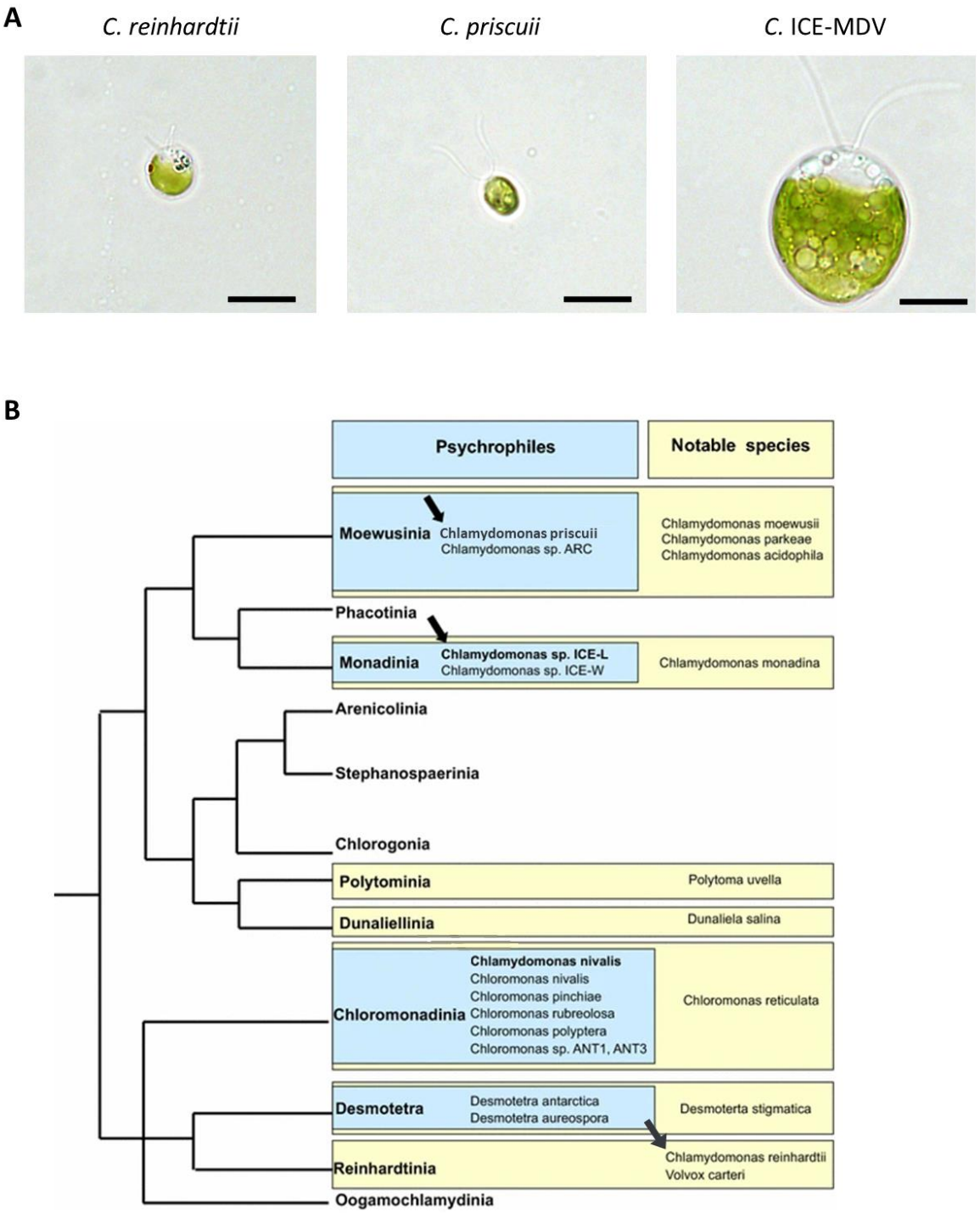

Figure 1. A) Brightfield photomicrographs of the algae used in this study, adapted from Poirier *et al.*, submitted. From left to right: *Chlamydomonas reinhardtii*, *Chlamydomonas priscuii*, *Chlamydomonas* sp. ICE-MDV. In all images, the scale bar represents 10 μm. B) Simplified Chlamydomonadalean phylogenetic tree highlighting known psychrophiles and other notable species of research interest. The arrows indicate the position of the algae in this study in relation to each other. Figure adapted from Cvetkovska *et al.*, 2017

Chlamydomonas sp. ICE-L is a related photopsychrophilic green alga that was originally isolated from sea ice off the Antarctic coast, but strains of this organism have been detected in various Antarctic habitats (Liu *et al.,* 2006; Li *et al.,* 2016). The two psychrophilic algae are phylogenetically closer to one another than to the model green alga *C. reinhardtii*: *C. priscuii* belongs to a unique lineage of the Moewusinia clade of Chlamydomonadales, and ICE-L belongs to the Monadinia clade (Figure 1B, Cvetkovska *et al.,* 2017). The genome of ICE-L was also recently sequenced and found to have duplications in genes encoding ice-binding proteins, among others that are important to cell survival (Zhang *et al.,* 2020). Investigations into this organism's physiology has revealed higher levels of unsaturated fatty acids, which allows for more flexible membranes and better cell function in the cold (An *et al.,* 2013). *Chlamydomonas* sp. ICE-MDV is a strain of ICE-L (Cook *et al.,* 2019) that has been isolated between 5-15 m in Lake Bonney's water column. This alga is an excellent system to study psychrophily in comparison to *C. priscuii*: these species are closely related and experience many of the same evolutionary pressures in their native Antarctic environment; however, ICE-MDV experiences a greater range of environmental conditions compared to *C. priscuii* (Li *et al.,* 2016). Thus, comparative analyses of these species may distinguish between features common to green algal psychrophiles in general and features specific to organisms from one highly isolated environment.

1.3. Algae stressed and genes expressed: the algal heat stress response

Every organism has a given set of optimal conditions: environmental factors at which they grow and develop the best. For microorganisms, this is often defined as the conditions when growth rate is highest (Borowitzka, 2018). The temperature at which an organism functions the best is commonly called the optimal temperature, while the highest temperature that still supports growth is the upper temperature limit. The optimal and upper temperature limits are species-specific and depend on the organismal physiology. *C. reinhardtii,* for instance, grows optimally at 24°C-28°C but stops growth and cellular division at temperatures >40°C, rapidly undergoes metabolic, transcriptomic, and proteomic changes as a result of exposure to heat, and finally, a prolonged exposure to this temperature leads to cell death (Hemme *et al.,* 2014). *C. priscuii* exhibits the highest growth rates at 10°C but stops growth at temperatures ≥18°C and prolonged exposure to a moderate temperature of 24°C led to cellular death (Possmayer *et al.,* 2011).

If an organism experiences a condition outside of its optimal conditions such that it disrupts homeostasis, this is considered a cellular stress (Borowitzka, 2018). Heat affects many processes in *C. reinhardtii*: (1) disrupting photosynthesis by damaging photosystems and impairing Rubisco's ability to fix CO_2 – this leads to higher rates of photorespiration and the production of toxic reactive oxygen species; (2) increasing membrane fluidity, which impacts cell structure and the ability to transport nutrients across membranes; (3) disturbing protein homeostasis through heat-induced protein denaturation, misfolding and aggregation (Schroda *et al.,* 2015). To combat these effects, the cell will mount a heat stress response (HSR) – a multifaceted response to the aforementioned effects. The first stage of the HSR in *C. reinhardtii* is the cessation of the cell cycle (Hemme *et al.,* 2014), wherein the cell shifts from normal growth to regaining homeostasis. Within a few hours of heat stress, the cell will synthesize saturated fatty acids to regain proper membrane fluidity.

Regaining protein homeostasis is a crucial part of the heat stress response: high temperatures decrease enzyme activity and causes proteins to denature, creating misfolded protein aggregates which are toxic in high quantities. To mitigate the damage and regain protein homeostasis, the cell accumulates higher levels of heat shock proteins (HSPs).

1.4. Protein fold in hot or cold: the heat shock proteins

HSPs are a highly conserved group of molecular chaperones found in every organism on Earth. They were first discovered when Ritossa (1962) noticed a spike in gene expression in *Drosophila hydrei* larvae after heat treatment. While they are best known for their presence during heat stress, we now know that many HSPs are constitutively expressed and have housekeeping roles as molecular chaperones, members of protein complexes, and proteases (Nadeem *et al.,* 2018). HSPs are split up into 5 main families based on their molecular weight - HSP100s, HSP90s, HSP70s, HSP60s, and small HSPs (sHSP). Their general functions and subcellular locations in *C. reinhardtii* are listed in Table 1.

In *C. reinhardtii*, HSP100s are heat stress inducible, but not detected in the cell in steady-state conditions (Schulz-Raffelt *et al.,* 2007) and found in the cytosol, mitochondria, and chloroplasts (Schroda, 2004). While studies of HSP100s in *C. reinhardtii* are limited, they have been found to confer thermotolerance by disassembling misfolded protein aggregates in plants (Mishra & Grover, 2019). HSP22A (an sHSP) was strongly induced during heat stress in *C. reinhardtii*

(Mühlhaus *et al.*, 2011), suggesting that it and HSP101 are important for thermotolerance in this green alga.

In *C. reinhardtii* the cytosolic HSP90A is responsible for folding newly-synthesized proteins (particularly signal transduction proteins; Richter & Buchner, 2001). The chemical inhibition of HSP90s induces the heat stress response, suggesting that HSP90A is an inhibitor of the HSR. HSP90C is chloroplast-localized and found to form a complex with HSP70B, which may assist in protein import into the chloroplast and the folding of chloroplast genes (Inoue *et al.*, 2013; Willmund & Schroda, 2005). The chaperonins (HSP60s) are plastid-localized HSPs that are constitutively expressed. In *C. reinhardtii*, CPN60 was found to have RNA-binding activity, suggesting it is responsible for RNA translational control (Balczun *et al.*, 2005). Its main role, however, is to fold Rubisco (Vitlin-Gruber & Feiz, 2018).

The HSP70s have a diverse number of functions in *C. reinhardtii*. Cytosolic HSP70A is both constitutively expressed and heat-shock inducible, and has been implicated both in the folding of new proteins and refolding of proteins during the heat stress response (Nordhues *et al.*, 2010; Schulz-Raffelt *et al.*, 2007). HSP70A is also found to be responsible for flagellar assembly (Nordhues *et al.*, 2010).

Table 1. General functions and cellular locations of the main HSP families in *Chlamydomonas reinhardtii* (adapted from Nadeem *et al.*, 2018).

HSP family	**Function**	**Subcellular compartment in *Chlamydomonas reinhardtii* (Schroda, 2004)**	**References**
HSP100	• disassembly of large protein aggregates	cytosol, mitochondria, chloroplasts	Mishra & Grover, 2019
HSP90	• maturation of signal transduction proteins • component of many protein complexes • inhibition of the heat stress response	cytosol, chloroplasts, ER	Zuehlke & Johnson, 2010; Nordhues *et al.*, 2010 Schroda *et al.*, 2015
HSP70	• chaperone for newly synthesized proteins • refolding denatured proteins • regulation of protein folding in non-stressful conditions	cytosol, chloroplasts, mitochondria, ER	Nadeem *et al.*, 2018

HSP60	• assisting with protein folding • regulation of the native configuration of the Rubisco large subunit	chloroplasts, mitochondria	Nadeem *et al.*, 2018
sHSP	• prevention of misfolded protein aggregates	cytosol, chloroplasts	Lee *et al.*, 1997

In photosynthetic organisms, the role of HSPs has been best studied in response to heat stress, but they have been found to be upregulated during many abiotic stresses. For example, high light causes photoinhibition, which induces the production of reactive oxygen species. These reactive oxygen species may damage proteins (Schieber & Chandel, 2014), which would then need to be repaired or resynthesized by HSPs. Proteomic analysis showed that HSPs were upregulated in response to high light in *Arabidopsis thaliana* (Phee *et al.*, 2004). HSP90s and HSP70s were also found to be upregulated in *C. reinhardtii* under high salt stress, another stressor that causes the production of reactive oxygen species (Yokthongwattana *et al.*, 2012; Kurusu *et al.*, 2015). Additionally, HSPs were induced in *C. reinhardtii* when exposed to cold stress (Maikova *et al.*, 2016) Since they may mitigate many forms of environmental stress, their regulation and quick induction is crucial to the cell's survival. The main regulators of HSPs are heat shock transcription factors (HSFs).

1.5. HSF1: the master regulator

A transcription factor is a protein that regulates a set of genes by binding a specific DNA sequence in a gene's promoter region and either inducing or repressing its transcription. HSF is the master regulator of HSPs and is also ubiquitously conserved in eukaryotes (Vihervaara & Sistonen, 2014). In prokaryotes, molecular chaperones are regulated by σ32 factors that perform a similar role (Grossman *et al.*, 1987). The structure and signal transduction mechanism of HSF varies slightly between animals, plants, and fungi, but it is generally activated in the presence of misfolded protein aggregates. This is called the unfolded protein response (illustrated in Figure 2A).

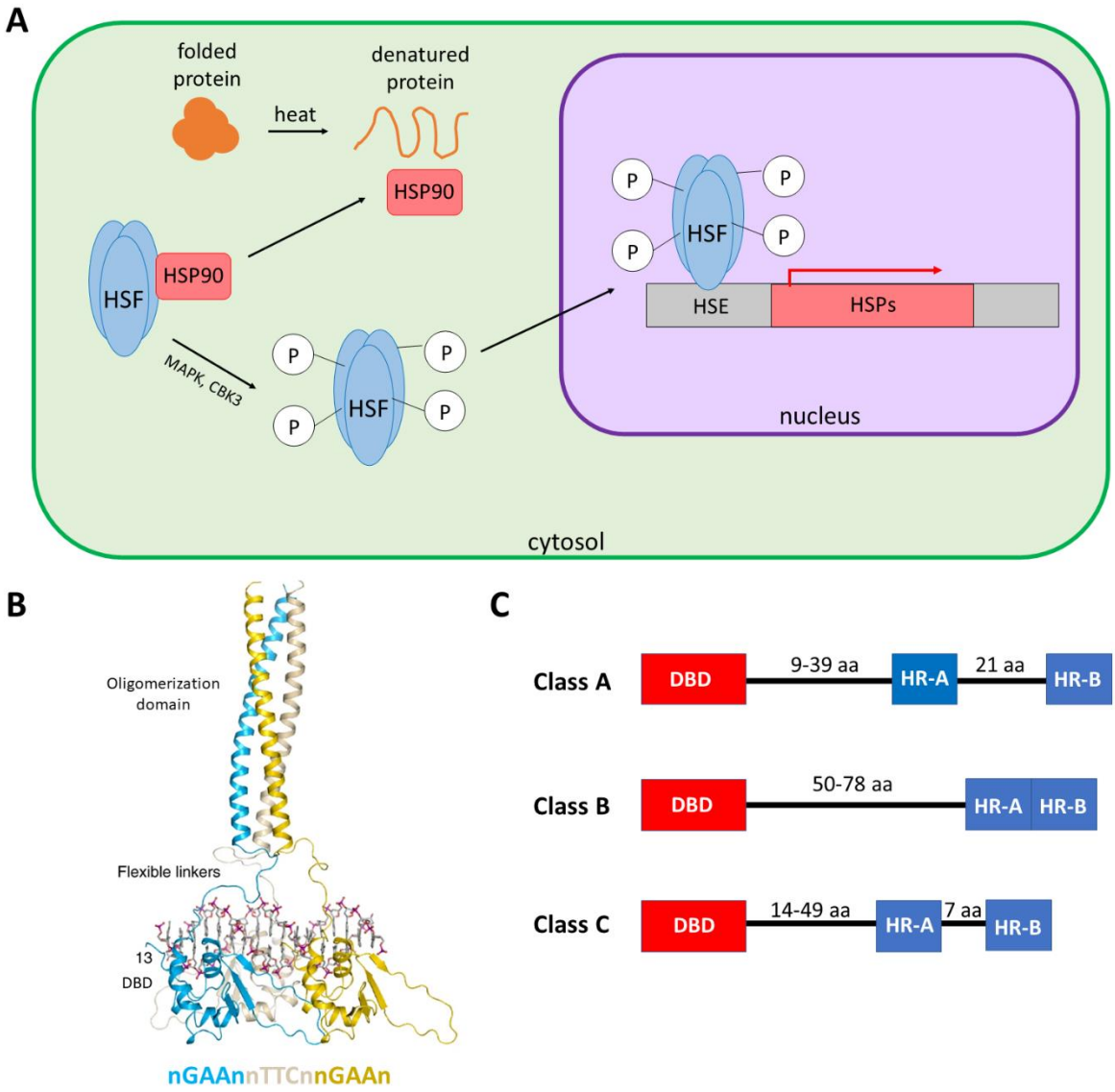

Figure 2. A) Schematic of HSF1 activation in *C. reinhardtii*, based on the mechanism proposed by Schroda *et al.*, 2015. In steady-state conditions, HSF is present as a trimer in the cytosol, inactivated by HSP90. In heat stress, when denatured proteins accumulate, HSF is released by HSP90, hyperphosphorylated by mitogen activated protein kinases (MAPK) and calmodulin binding kinase 3 (CBK3), then imported to the nucleus. There, it binds a heat shock element (HSE) in the promoter region of heat-responsive genes to induce their transcription. B) Ribbon diagram of an HSF trimer binding a heat shock element, consisting of inverted repeats of nGAAn. Functional HSF domains are labeled on the left. The three colours indicate three monomers of HSF, each binding an HSE subunit. Figure adapted from Neudegger *et al.*, 2016. C) Differences between the structure of the three classes of plant HSFs. Red bars represent the DNA-binding domain (DBD). The oligomerization domain consists of two hydrophobic repeats (HR-A and HR-B), with different lengths of inserts in different classes. The DBD and oligomerization domain are separated by a flexible linker of varying length.

The most important HSF functional domain is the N-terminal DNA-binding domain (DBD). The DBD features a helix-turn-helix motif that recognizes the heat shock element (HSE) – a repeating sequence of inverted 5-base pair units of 5'-nGAAn-3', where n represents any nucleotide (Vihervaara & Sistonen, 2014). Since HSF is active as a trimer, the optimal HSE features three units with the consensus sequence 5'-nGAAnnTTCnnGAAn-3' (Figure 2B). HSFs have an oligomerization domain downstream of the DBD, which consists of two hydrophobic heptad repeats (HR-A and HR-B). This domain folds into a coiled coil with other HSF monomers to form a trimer. The middle region of the gene is sometimes called the regulatory domain in animals, but its structure is highly disordered, and its function is poorly understood (Vihervaara & Sistonen, 2014).

All known eukaryotes have HSFs, but the size of the HSF gene family and the structure of the proteins they encode differ between taxa. Animals have 1-6 HSF genes, while plants have much greater numbers (between 16 in tea plants and 56 in wheat (Liu *et al.*, 2016; Xue *et al.*, 2014)). Screening the Chlorophytes with fully sequenced genomes has revealed between 1-3 HSFs (Grigoriev *et al.*, 2021). *C. reinhardtii* has two HSF genes (Schulz-Raffelt *et al.*, 2007). Out of these two genes, HSF1 is well-characterized and has the conserved structure and functions observed in other HSFs. HSF2 contains a conserved DBD, but otherwise does not resemble any other HSFs studied to date, and its role and expression is the cell is not yet understood (Schulz-Raffelt *et al.*, 2007). Thus, in green algae, as well as plants and animals, it appears that a single HSF is the master regulator of HSP expression. Other HSFs may modulate HSF1 activity or work synergistically with HSF1 to induce HSP transcription, but depend on its presence regardless (Schulz-Raffelt *et al.*, 2007; Yoshida *et al.*, 2011; Östling *et al.*, 2007; Mishra *et al.*, 2002).

Plant HSFs are classified into three types: class A, B, C, and they are differentiated by features in their oligomerization domain (Figure 2C). Class B HSFs have a compact oligomerization domain (as do animal HSFs), while Class A and C HSFs have their HR-A and HR-B cores separated by a 21 amino acid and 7 amino acid spacers, respectively (Nover *et al.*, 2001). Phylogenetic analysis has shown that the *C. reinhardtii* HSF1 qualifies as a plant class A HSF based on this classification (Schulz-Raffelt *et al.*, 2007). Additionally, the DBD and oligomerization domain

are separated by a flexible linker of varying length: 9-39 aa in class A, 50-78 aa in class B, and 14-29 aa in class C (Nover *et al.*, 2001).

The expression of some plant HSFs and all green algal HSFs are heat stress-inducible and precede the expression of the HSPs (von Koskull-Döring *et al.*, 2007, Schulz-Raffelt *et al.*, 2007). In *C. reinhardtii*, HSF1 is only weakly expressed under non-stress conditions but its expression is rapidly induced within the first 15-30 minutes of exposure to 40°C, then followed by the expression of HSPs within 1-3 hours of heat stress (Schulz-Raffelt *et al.*, 2007). The same study showed that cultures with silenced HSF1 by RNAi exhibited extreme sensitivity to heat and abolished expression and accumulation of most HSP classes, thus demonstrating that HSF1 is indeed a master regulator of HSP accumulation in this green alga. A similar effect has been observed in *C. reinhardtii* cells challenged with sudden cold shock (7°C), which rapidly induced the transcription of HSF1 and HSP70 (Maikova *et al.*, 2016).

The mechanism of HSF1 induction has been elucidated in *C. reinhardtii* (Figure 2A). During non-stress conditions, HSF1 is present in the cytosol as a homotrimer that associates with HSP90 and HSP70. While binding by HSP90 inactivates the HSF1, the role of HSP70A is less clear (Schulz-Raffelt *et al.*, 2007). It has been shown that in this alga HSF1 is activated not by the effects of the temperature stress directly, but rather through the associated accumulation of misfolded protein aggregates (Schroda *et al.*, 2015; Schmollinger *et al.*, 2013). In such conditions, HSP90 releases HSF1, which is then activated by hyperphosphorylation and imported into the nucleus. The active HSF1 binds to HSEs in the promoters of HSPs to induce their transcription (Schulz-Raffelt *et al.*, 2007). After HSPs are transcribed and the misfolded protein aggregates are disassembled, HSF1 is dephosphorylated, exported back into the cytosol where it once again associates with regulatory HSPs.

1.6. Heat shock proteins without heat: psychrophiles and the HSR

Antarctica is home to a wide diversity of psychrophilic taxa, ranging from algae and fungi to animals such as invertebrates and fish (Griffiths, 2010; Hassan *et al.*, 2016, Clark & Peck, 2009). The extreme sensitivity of these organisms to moderate, seemingly harmless, temperature increases (sometimes as little as 2-3°C above the temperature in their native cold niches) has brought forth the question whether these organisms lack the capability to induce the HSR and

whether they have HSPs. The heat stress response has been tested in a range of Antarctic psychrophilic organisms, but especially in Antarctic marine animals.

It has been shown that several Antarctic psychrophilic animals lack a classical HSR as described in mesophiles. Some examples include the Antarctic hydra *Hydra oligactis*, the Notothenioid fish *Trematomus bernacchii,* the shrimp *Paraceradocus miersi,* and two serpulid worms (Brennecke *et al.,* 1998; Buckley *et al.,* 2004; Collins *et al.,* 2021; Nieva *et al.,* 2021). All aforementioned species lacked an upregulation in the transcription of HSP70s upon exposure to heat stress. It is notable that this common lack of a response spans many taxa, even distantly related ones.

Some psychrophilic animals retain, at least in part, the ability to induce an HSR that involves upregulated HSP expression. The icefish *Chionodraco hamatus*, which lives at 0°C, did show an accumulation of HSP90 and HSP70 proteins in the gills in response to chronic heat stress (10 days at 4°C) (Garofalo *et al.,* 2019). The krill *Euphausia superba* lacks an acute HSR (Cascella *et al.*, 2015), but an induction of HSP70 was seen after chronic thermal stress (3 days at 3°C) and during stress recovery (Toullec *et al.,* 2020). The sea urchin *Sterechinus neumayeri* thrives at 1°C and it is exceptionally sensitive to very minor temperature increases. Exposure to 3°C significantly increased HSP90 transcript levels but only after 48 hours, illustrating a delayed HSR (González *et al.,* 2016). Furthermore, HSP upregulation was only observed during acute heat stress at 5°C, but not when the animal was acclimated by slow temperature increases (Collins *et al.,* 2021). The examples above demonstrate that many psychrophiles have an unusual heat stress response, and their HSP regulation is species-specific. The role of HSPs in psychrophiles is therefore nuanced and yet to be fully characterized. Many of these earlier heat stress studies focus on psychrophilic animals, but photopsychrophiles have received much less attention.

1.7. Pieces of the puzzle: *C. priscuii* and heat stress

The Antarctic *C. priscuii* is one of the best studied green algal psychrophiles. Its adaptation to low temperatures, low light and unusual photoperiods have been studied at length (recently reviewed in Hüner et al, 2022). It should be noted that *C. priscuii* rarely experiences any changes in its native environment due to the permanent ice-cover and intense water column stratification in Lake Bonney. It is likely that this chronic stability has affected the physiology of this

organism. Notably, *C. priscuii* is of one of the very few photopsychrophilic species that has been studied with regards to its responses to heat stress.

The first study on the temperature sensitivity of *C. priscuii* showed that this alga is unable to grow above 18°C, and exposure to 24°C causes algal growth to stop rapidly (Possmayer *et al.,* 2011). The effects of this heat stress were reversible within the first 12 hours of exposure, but lethal if the exposure was longer – thus confirming this alga's status as an obligate psychrophile. Short term (up to 12 hours) exposure to 24°C also induced physiological responses characteristic of heat stress in other algae, including a rapid drop in the rate of photosynthesis, decreased respiration capacity and upregulation of HSP90A and sHSP transcript levels increased upon exposure to heat stress (Possmayer et al., 2011).

Subsequent work with *C. priscuii* showed a global metabolomic and transcriptomic response upon short-term (6 hour) exposure to 24°C; however, this response was much attenuated when compared to that of mesophilic algae under equivalent heat stress (Cvetkovska *et al.,* 2022). For instance, exposing *C. reinhardtii* to 42°C for as little as 25 minutes resulted in similar physiological responses as described above (decreased photosynthesis and respiration) accompanied by ~20% of its genes in its transcriptome becoming differentially expressed (Hemme *et al.,* 2014; Légeret *et al.,* 2016). *C. priscuii* differentially expressed only ~11% of its genes after 6 hours of heat stress (Cvetkovska *et al.,* 2022). Furthermore, many molecular and metabolic compounds commonly used as stress markers in *C. reinhardtii* (e.g., antioxidants) were highly constitutively in *C. priscuii* at 4°C, but their accumulation was not further increased upon exposure to the lethal 24°C (Cvetkovska *et al.,* 2022). These results demonstrated that this alga has the capacity to respond to heat stress on a molecular level, but its response is unusual compared to the mesophilic *C. reinhardtii*.

The sequencing of the *C. priscuii* genome revealed important insights into its psychrophilic adaptations (Zhang *et al.,* 2021). *C. priscuii* has a relatively large genome (212 Mbp, twice the size of *C. reinhardtii*), with a high number of highly similar duplicated genes, particularly those involved in cell growth, photosynthesis, and protein translation. The *C. priscuii* genome also encodes for a significantly higher number of HSP genes (53 compared to ~40 in C. *reinhardtii* and other mesophiles), with duplications in the HSP70, HSP60, HSP100, and sHSP gene families (Cvetkovska *et al.,* 2022). The same study identified an enlarged HSP gene family in

ICE-L as well, suggesting that an increased number of HSP gene copies may be a psychrophilic trait in green algae. Furthermore, this study demonstrated that *C. priscuii* constitutively accumulates HSPs in steady-state conditions. In contrast, *C. reinhardtii* does not accumulate HSPs unless it is exposed to sudden heat stress (Cvetkovska *et al.,* 2022). Gene duplication has been associated with increased fitness in unfavourable conditions, due to the gene dosage effect (i.e., more genes lead to increased protein levels; Qian & Zhang, 2014). It was posited by Cvetkovska *et al.* 2022 that the expanded HSP gene family and their constitutive accumulation may give an advantage to life in the cold, but the role of the HSPs or their regulation was not studied.

C. priscuii is exposed to many extreme environmental conditions in addition to low temperature, and it has been studied with regards to other environmental stressors, including high light (Szyszka *et al.,* 2007) and high salinity (Pocock *et al.,* 2011). These studies have revealed a sensitivity to high light: *C. priscuii* cannot survive light intensities >250 µmol m^{-2} s^{-1}, while *C. reinhardtii* can withstand intensities up to 1000 µmol m^{-2} s^{-1}. *C. priscuii* is exceptionally halotolerant, and it has been shown to thrive at salinities between 10 mM and 1.2 M NaCl (Pocock *et al.,* 2011). In contrast, >200 mM NaCl is lethal to *C. reinhardtii.* Overall, previous research has shown an exceptional ability to survive a range of extreme conditions (permanently low temperatures, low light, high salinity) but accompanied by sensitivity to environmental change and conditions outside of this range. HSPs have been shown to be induced by various stressors in other algae and land plants (e.g., Yokthongwattana *et al.,* 2012; Kurusu *et al.,* 2015) but the involvement of HSPs and their regulation in *C. priscuii* under various abiotic stressors is currently unknown.

1.8. Thesis objectives and hypothesis

The objective of this thesis is two-fold:

1. Characterize HSF1 in the psychrophilic green alga *C. priscuii*.
2. Investigate the regulation of its HSPs in response to environmental stress.

It has been shown that the psychrophilic alga *C. priscuii* has an attenuated response to heat stress at the level of the global metabolome and transcriptome. I hypothesize that this alga has similarly lost the ability to regulate HSPs during environmental stress as a consequence of life in the extreme, but chronically stable, conditions in Lake Bonney. If this hypothesis is true, it would be

the first known alga that lacks the ability to induce HSP accumulation in response to environmental stress. This work will help elucidate the mechanisms underlying psychrophily and may provide insight on the vulnerability of this unique Antarctic species in the face of climate change.

Chapter 2: Materials and Methods

2.1. Genomic screening and HSF sequence analysis

The *C. priscuii* genome was recently sequenced and is publicly available (Zhang *et al.*, 2021). A single HSF gene was identified in the *C. priscuii* genome by a BLAST search using *C. reinhardtii* HSF1 as a query. The predicted HSF amino acid sequence was compared to HSFs from 19 other species, obtained from Phytozome v13 (https://phytozome-next.jgi.doe.gov/), Phycocosm (https://phycocosm.jgi.doe.gov/), and NCBI (https://www.ncbi.nlm.nih.gov/) (Table S1). For algal species, HSF genes were identified by searching Phycocosm for genes that contain the conserved HSF/DNA-binding PFAM domain (PF00447). Well-studied and annotated plant HSFs were selected from Phytozome, and well-studied animal HSFs were selected from NCBI. Sequences were aligned using ClustalW (Sievers *et al.* 2011) implemented in Geneious Prime v2020.2.3 (Biomatters Ltd, Auckland, New Zealand). Characteristic HSF domains were mapped onto the *C. priscuii* HSF1 gene as described by Schulz-Raffelt *et al.* (2007). The DBD secondary structure was annotated as described in Schultheiss *et al.*, (1996). Nuclear export signals were identified by searching all HSF sequences studied here for NES motifs according to the criteria described in La Cour *et al.*, 2014. A maximum-likelihood phylogenetic tree was created using a gamma-distributed LG model in MEGA X v10.2.2 (Kumar *et al.*, 2018).

2.2. Promoter analysis of HSPs

The *C. priscuii* genome encodes for 53 HSP genes, and ICE-L encodes for 50 HSP genes (Cvetkovska *et al.*, 2022). The HSP promoter regions of these two psychrophiles were compared to their homologs in *C. reinhardtii*. The accession numbers for all HSP genes that were screened here are listed in Table S2. To identify potential HSEs (HSF1 binding sites) in HSP promoters, the region 1000 bp upstream of the start codon was extracted from all three species' HSP gene sequences. Promoter regions for all species were analyzed using JASPAR (https://jaspar.genereg.net/), which searched for the presence of known HSE profiles from *Arabidopsis thaliana*: MA1664.1, MA1758.1, MA1759.1, MA1760.1, and MA2019.1, with a relative profile threshold score of 80%. HSEs were then extracted and aligned with ClustalW. A sequence logo of the HSE consensus sequence for each species was created using Weblogo (https://weblogo.berkeley.edu/logo.cgi).

2.3. Transcriptomic analysis of HSP expression

Previously, *C. priscuii* cultures were grown at 4°C and exposed to heat shock at 24°C for 6 hours (Cvetkovska *et al.,* 2022). RNA was sequenced with 100 base paired-end reads on an Illumina HiSeq4000 platform (Illumina, San Diego, USA), and the RNA-Seq reads were mapped to the *C. priscuii* assembled genome as described by Cvetkovska *et al.,* 2022. The total number of aligned reads was normalized by gene length and sequence depth, then expressed as fragments per kilobase per million (FPKM) (Cvetkovska *et al.*, 2022). To determine the expression level of HSP genes, transcripts corresponding to the predicted coding sequence of the *C. priscuii* HSPs were identified using MegaBLAST (E-value of $<1.0E^{-100}$; transcript spans at least 50% of the gene). Differentially expressed genes (DEGs), defined as a gene with a fold-change >4 and a p-value <0.05, were determined by Ballgown v2.22.0 (Pertea *et al.,* 2016). The expression data were normalized by $\log_2$ transforming the FPKM values, then compared between steady-state and heat shock conditions. HSP transcripts were organized as gene families and the heatmap expression profile of HSPs was generated using the ComplexHeatmap R package.

2.4. Algal growth and stress conditions

Cultures of *C. priscuii, C. reinhardtii* (cc-1690), and *C.* sp. ICE-MDV were grown axenically in Bold's Basal Medium (BBM) in glass tubes suspended in thermoregulated aquaria with continuous aeration with ambient air filtered by a 0.2 µm filter. A continuous growth irradiance was generated by full-spectrum LED lights (GrowLights, T5 LED 18W), and measured with a quantum sensor attached to a radiometer (Model LI-189; Li-Cor). To mimic natural conditions, *C. priscuii* growth media was supplemented with 700 mM NaCl, and cultures were grown at 4°C at a light intensity of 10 µmol photons m^{-2} s^{-1} (Morgan-Kiss *et al.,* 2016; Dolhi *et al.,* 2013). ICE-MDV media was supplemented with 70 mM NaCl, and cultures were grown at 4°C at a light intensity of 100 µmol photons m^{-2} s^{-1} (Li *et al*., 2016). For *C. reinhardtii,* media was supplemented with 0.43 mM NaCl and grown at 24°C at a light intensity of 100 µmol photons m^{-2} s^{-1}. The conditions used here are commonly used in laboratory experiments and considered optimal for *C. reinhardtii* (Harris, 2009).

All cultures were grown until mid-log phase before applying a stress treatment (summarized in Table 2). For temperature stress, algal cultures were transferred to aquaria maintained at 24°C (*C. priscuii* and ICE-MDV, high temperature), 42°C (*C. reinhardtii,* high temperature), -2°C (*C.*

priscuii, low temperature), or 10°C (*C. reinhardtii*, low temperature). For salinity stress, cultures were spun down at 5000 x g for 5 minutes, then resuspended in BBM supplemented with the corresponding NaCl concentration as described in Table 2. For high light stress, algal cultures were transferred to aquaria with higher intensity full spectrum LED lights maintained at the same temperature. Finally, algal cultures were treated with L-canavanine sulfate, an arginine analog that prevents proper protein folding if incorporated into a newly synthesized peptide (Rosenthal, 1977). Algal cultures were supplemented with 5 mg/ml L-canavanine sulfate, then mixed by inversion before returning the cultures into thermally regulated aquaria. As a control for amino acid supplementation, algal cultures were also supplemented with 5 mg/ml arginine in the same way as described previously.

Table 2. Environmental factors for each algal growth condition. For each stress treatment, one parameter from the control conditions was changed, and all other parameters were kept the same. A strikethrough indicates that the species was not exposed to that stress condition. Values were chosen based on previous literature that described a stress response in that condition, except for *C. priscuii* at low temperature (chosen instead as the lowest recorded temperature in Lake Bonney).

Stress condition	**C. reinhardtii**	***C. priscuii***	***C.* sp. ICE-MDV**
Control (in all stress experiments)	24°C 0.43 mM NaCl 100 µmol photons m^{-2} s^{-1}	4°C 700 mM NaCl 10 µmol photons m^{-2} s^{-1}	4°C 70 mM NaCl 100 µmol photons m^{-2} s^{-1}
High temperature	42°C (Hemme *et al.*, 2014)	24°C (Possmayer *et al.*, 2011)	24°C (Osmers, unpublished)
Low temperature	10°C (Maikova *et al.*, 2016)	-2°C (Spigel & Priscu, 1996)	~~———~~
High salt	100 mM NaCl (Khona *et al.*, 2016)	1.2 M NaCl (Pocock *et al.*, 2011)	~~———~~
Low salt	~~———~~	10 mM NaCl (Osmers, unpublished)	~~———~~
High light	500 µmol photons m^{-2} s^{-1} (Förster *et al.*, 2006)	250 µmol photons m^{-2} s^{-1} (Szyszka *et al.*, 2007)	~~———~~
Canavanine	5 mg/ml L-canavanine sulfate (Schmollinger *et al*, 2013)	5 mg/ml L-canavanine sulfate	~~———~~
Arginine	5 mg/ml L-arginine (Schmollinger *et al*, 2013)	5 mg/ml L-arginine	

For each stress treatment, cultures were sampled at 0 hours (i.e., before application of stress), then 1, 3, and 6 hours. These time points were chosen based on previous work showing global metabolomics and transcriptomic changes in *C. priscuii* exposed to heat stress for 6 hours (Cvetkovska et al., 2022), and mesophilic algal stress response times (Schroda *et al.*, 2015). At each time point, chlorophyll levels and cell death were measured to determine cell viability under stress. Chlorophyll was extracted in 90% acetone and measured spectrophotometrically at 647 and 664 nm according to Jeffrey & Humphrey (1975), using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies). Cell death was measured by treatment with the fluorescent dye SYTOX Green (Ex/Em = 504/523 nm), which indicates cell death. In all cases, 2 µM SYTOX Green was added to culture samples, which were then incubated in the dark for 15 minutes. The proportion of green-fluorescing cells was measured using a Countess II Automated Cell Counter using an EVOS™ GFP Light Cube (Ex/Em = 470/525 nm; ThermoFisher Scientific). Chlorophyll autofluoresces at 650-700 nm (Kodama, 2016), and cells that contain chlorophyll were measured with the Cy5 Light Cube (Ex/Em = 628/685 nm). After culture viability was assessed using the above two measurements, algal cells were harvested by centrifugation (5,000 x g, 5 min), and the cell pellets were flash frozen and stored at -80°C.

2.5. RNA and genomic DNA extraction

Total RNA from algal cultures were extracted using a modified CTAB protocol (Possmayer *et al.*, 2016). Flash-frozen cell pellets were thawed at 60°C in CTAB buffer [2% (w/v) CTAB, 1.4 M sodium chloride, 20 mM EDTA, 100 mM Tris, 2% (w/v) polyvinylpyrrolidone (M.W. 40000), 1% (v/v) β-mercaptoethanol, pH 8.0] (Doyle 1991). Samples were incubated at 60°C for 5 minutes, then extracted twice with and equal volume of chloroform. After the second extraction, an equal part isopropanol was added, the samples were centrifuged (16,000 g, 20 min, 4°C), and the supernatant was decanted. Pellets were washed in 70% ethanol, then again centrifuged (16,000 g, 2 min, 4°C) and the supernatant decanted. The pellets were resuspended in diethyl pyrocarbonate (DEPC)-treated water, then 1/3 volume of 8M lithium chloride was added. Samples were incubated at -20°C for 20 minutes to precipitate RNA. The samples were centrifuged (16,000 x g, 30 min, 4°C), the supernatant was decanted, and the pellet was washed with 70% ethanol again. Next, to purify the RNA, samples were resuspended in 250 µl DEPC-treated water, then amended with 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volume 99% ethanol. They were incubated at -20°C for an hour, then centrifuged (16,000 g, 30 min, 4°C), the

supernatant decanted, and the pellets washed with 70% ethanol again. After air-drying the pellets for 5 minutes on ice, RNA was resuspended in nuclease-free water and its concentration was assessed spectrophotometrically using a Nanodrop 2000 (ThermoFisher Scientific) at 260 and 280 nm. RNA quality was assessed by measuring the A260/280 ratio. All RNA samples were treated with DNase using the Turbo DNA-free kit (Thermo Fisher) and reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad), following the manufacturer's instructions.

To extract genomic DNA (gDNA), flash-frozen cell pellets were thawed in lysis buffer [50 mM Tris-HCl, 20 mM EDTA, 200 mM NaCl, 2% SDS, 1mg/ml Proteinase K (Thermo Fisher)], then an equal volume of CTAB buffer (described above) was added and the samples were incubated at 65°C for 30 minutes. After incubation, samples were centrifuged (14,000 g, 5 min, RT), keeping the supernatant. Samples were treated with 0.7 mg/ml RNAse A (Roche) to remove RNA and incubated at 37°C for 1 hour. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the samples were mixed by inversion, and centrifuged (16,000 g, 10 min, RT). The top aqueous phase was kept and an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed by inversion, and centrifuged again. An equal volume of isopropanol was added to the top aqueous phase and mixed by inversion. Following an incubation at room temperature for 30 minutes, the samples were centrifuged (16,000 g, 20 min, RT), decanted, and the pellets washed with 70% ethanol. The pellet was resuspended in 250 ul of 10 mM Tris-HCl (pH 7.8), then 2x volume 99% ethanol and 1/10 volume 3M sodium acetate (pH 5.2) were added. The samples were incubated at -20°C for 1 hour to precipitate DNA, then centrifuged (16,000 g, 20 min, RT), decanted, and the pellets were washed with 70% ethanol twice. The final gDNA pellets were resuspended in 10 mM Tris-HCl (pH 7.8), and the DNA concentration was assessed spectrophotometrically at 260 and 280 nm using a Nanodrop 2000. DNA sample quality was assessed by measuring the A260/280 ratio.

2.6 HSF1 detection using polymerase chain reaction

To experimentally detect the presence of HSF1 in the *C. priscuii* genome and transcriptome, HSF1-specific primers were designed using a modified version of Primer3 v.2.3.7 on Geneious Prime v.2020.2.3 (Table 3, Figure 3). PCR using cDNA and gDNA as templates was used to amplify the HSF transcript and gene to determine its presence in *C. priscuii* at steady-state and

heat stress conditions. PCR was performed using Taq DNA polymerase (FroggaBio) in a reaction mix containing 1X PCR buffer and 0.2 µM primers. A thermal cycler was programmed to the following conditions: 1 min at 95°C for initial denaturation, 30 cycles of 30 s at 95°C denaturation, 30 s at 56°C annealing, 2 min at 72°C extension, then a final extension of 72°C for 5 min. PCR amplicon size was determined by gel electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized under UV light.

Table 3. *C. priscuii* HSF1 primers designed to amplify overlapping segments of the gene and transcript.

Primer	Region of Binding	Sequence	Product size (gene / transcript)
14F	exon 1	CGCCCCCGTTTCTTACCAAA	718 / 197
210R	exon 4	CTTCCTGAAGCCGTACGTGT	
191F	exon 4	ACACGTACGGCTTCAGGAAG	445 / 253
443R	exon 5	CGGATGACCTCCTGCTTCAG	
421F	exon 5	CAGCTGAAGCAGGAGGTCAT	780 / 270
690R	exon 6	GTCGTACTCGTGGATCTCCG	
671F	exon 6	CGGAGATCCACGAGTACGAC	489 / 489
1159R	exon 6	CAAACGACGACAAGAACCCG	
1386F	exon 6	CGCCGACGAGCTACTTGC	255 / 255
1640R	exon 6	ATCGTGATGCCCCCGATG	
1822F	exon 6	CTGGAGCACCTTGAGTCTGG	209 / 209
2030R	exon 6	CTCTTGATGAGCGCCTGCAG	
169F	5'-UTR	CAACCACACCGCACCACC	3955 / 2735
2883R	3'-UTR	TACGCACACACTAGCTCAGC	
160F	5'-UTR	GTCAGCCCCCAACCACAC	3358 / 2138
2296R	3'-UTR	TAATGCACCGCCGCTACTAG	

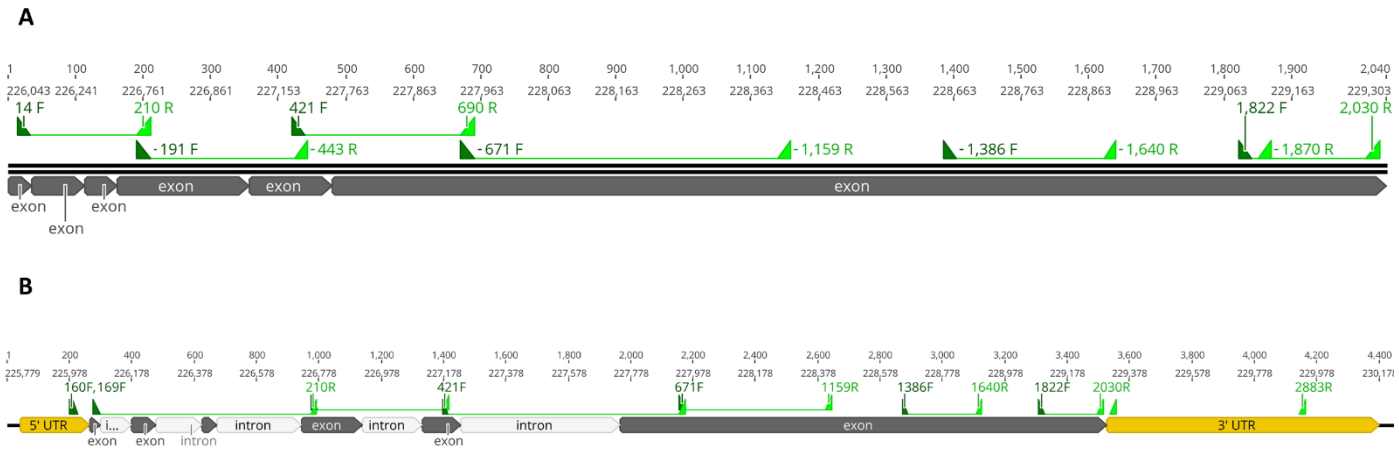

Figure 3. A) *C. priscuii* transcript with the annotated locations of the primers from Table 2. B) *C. priscuii* full gene with the annotated locations of the primers listed in Table 2.

2.7 Gene expression using droplet digital PCR

To measure HSP gene expression in *C. reinhardtii* and *C. priscuii* during environmental stress, gene-specific primers for HSF1, HSP70A, and HSP90A were designed for both species using Primer3 v2.3.7 (Table 4). These genes were selected for their roles and demonstrated expression during the heat stress response in *C. reinhardtii*, and both HSPs have a predicted HSE in their promoter region.

To determine primer specificity and the optimal target cDNA dilution (important factors for ddPCR experiment design), quantitative PCR (qPCR) was performed. For each primer set, a 10 µl reaction containing 0.4 µM primers, 2 ng/µl cDNA, and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was assembled. PCR was conducted using the following conditions: 3 min initial denaturation at 95°C, 40 cycles of 10 s at 95°C denaturation, 20 s at 56°C annealing, 20 s at 72°C extension, then 10 s at 95°C for inactivation. A melt curve was performed by incrementing the thermal cycler for 60-95°C at 0.5°C every 5 seconds. Finally, PCR product size was determined by gel electrophoresis as described previously. Primer specificity was determined by a singular peak on the melt curve combined with correct PCR product size via gel electrophoresis.

Droplet digital PCR was performed using the QX200 Droplet Digital PCR system (Bio-Rad) according to the manufacturer's instructions. In brief, a 23 ul reaction containing 2.3 µM primers, QX200 ddPCR EvaGreen Supermix, and cDNA template was assembled. 20 µl of the reaction was converted to droplets with the QX200 droplet generator (Bio-Rad). Droplet-partitioned samples were transferred to a 96-well plate and sealed with a PX1 PCR plate sealer (Bio-Rad). PCR was performed as follows: 5 min initial denaturation at 95°C, 50 cycles of 30 s at 95°C denaturation, 1 min at 56°C annealing, and 30 s at 72°C extension, then 5 min at 90°C. Droplets were read using absolute quantification in a QX200 Droplet Reader (Bio-Rad) and analyzed using QuantaSoft software (Bio-Rad). Sample gene expression was normalized to two reference genes (Actin and L32 in *C. priscuii*; RCK1 and L32 in *C. reinhardtii*). Statistical analysis was performed in R using a one-way ANOVA for each species and condition. Tukey's

HSD test was performed to identify significant differences between time points in each environmental stress condition.

Table 4. Primers used for measuring gene expression in this study.

Gene	Sequence	Product size
C. priscuii HSP70A	AAGAACCAGGTCGCGATGAA GTAGGGCCACAGCTTGATGT	111
C. priscuii HSP90A	GGGCCTCAAGTTTGACGAGA CATGATGCGCTCCATGTTGG	202
C. priscuii HSF1	ACACGTACGGCTTCAGGAAG CGAAGGTACGATCGACCCAG	152
C. priscuii actin	CAGTCCAAAAGGGGCATCCT CATCTTCTCGCGGTTTCCCT	182
C. priscuii L32	GCTCGCAGTGAAGGAGTCTT CATGATCAGCAGCTCCAGGT	186
C. reinhardtii HSP70A	CCACCGGCAACAAGAACAAG GATGGTGTTGCGCATGTTGT	179
C. reinhardtii HSP90A	GGAGATCAACCAGCTGCTGT TACAGCTCCGGGTTGTTGTC	159
C. reinhardtii HSF1	GTATGGAAGCCACCGGAGTT CCGCAACTGTTCCTTCTTGC	171
C. reinhardtii RCK1	CCCCGAGTTCAACATCACCA CCTCCTCCTAAACCCCTCCA	214
C. reinhardtii L32	TCGATCAAGACCTCATGGCG CGGTTGTGCATCATGAGCAG	191

2.8. Protein extraction, SDS-PAGE, and immunoblotting

To extract protein from the flash-frozen samples, algal cell pellets were resuspended in in 1.1 M Na_2CO_3 containing 0.1 M DTT, then incubated at -20°C for 1 hour. Protein samples were solubilized with an equal volume of 5% (w/v) SDS-30% (w/v) sucrose, and heated to 85°C for 5 min. The chlorophyll content (as a proxy for protein amount) was quantified by adding 10 µl of protein sample to 990 µl of 90% acetone, then measuring spectrophotometrically at 647 and 664 nm according to Jeffrey & Humphrey (1975), using a Cary 60 UV-Vis spectrophotometer.

The accumulation of HSPs in *C. priscuii*, *C. reinhardtii*, and *C.* sp. ICE-MDV was measured using SDS-PAGE and immunoblotting. Algal protein samples were loaded on equal chlorophyll basis. Electrophoresis was performed using the Mini-Protean II cell, using a 12.5% (w/v) bis-acrylamide resolving gel containing 5.6 M urea and 0.65 M Tris (pH 8.8), and a 5% (w/v) bis-acrylamide stacking gel containing 0.125 M Tris (pH 6.8). Proteins that were separated by SDS-

PAGE were either stained with Coomassie blue to confirm equal protein content per lane, or transferred to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) using the Trans-Blot Turbo transfer system (Bio-Rad) at 2.5A for 15 minutes.

Membranes were blocked with Tris-buffered saline (0.02M Tris, 0.15 M NaCl) containing 5% (w/v) skim milk powder and 0.1% (v/v) Tween 20. Membranes were then probed with primary antibodies raised against *C. reinhardtii* and specific for HSP70A (1:3,000), HSP70B (1:10,000), and HSP90A (1:3000) (Agrisera, Vännäs, Sweden). These antibodies were shown to bind specifically to their closest homolog in *C. priscuii* (Cvetkovska *et al.*, 2022), and were predicted to bind specifically to their closest homologs in ICE-MDV (Agrisera, personal communication). Anti-Rabbit IgG conjugated to horseradish peroxidase was used as a secondary antibody (1:10,000; Sigma-Aldrich), and chemiluminescence was visualized with ECL reagents (Bio-Rad). Protein amounts were quantified by densitometry using Image Lab 6.1 (Bio-Rad).

Chapter 3: Results

3.1. Characterization of the predicted HSF1 gene in *C. priscuii.*

The *C. priscuii* nuclear genome encodes for a single HSF1 gene (Zhang *et al.*,2021; Cvetkovska *et al.*, 2022) that has 6 exons and 5 introns (3261 bp in length) (Figure 4A). Aligning the predicted amino acid sequence with HSFs from closely related algae and a representative land plant revealed the presence of a highly conserved DNA-binding domain (DBD) and several motifs important for HSF function (Figure 4B, Figure 5). The DBD consists of three α-helices and four β-sheets, where the second two helices (α2 and α3) form the helix-turn-helix motif that allows for DNA binding (Vuister *et al.,* 1994). The entire DBD was 78.9% similar between all species examined here, and the helix-turn-helix motif was 91.5% similar across the alignment. In contrast, the percent identity of the entire predicted protein sequence across the alignment was 27.4%. This high degree of conservation in the DBD suggests that *C. priscuii* HSF1 can form the necessary structure to bind DNA and possibly function as a transcription factor.

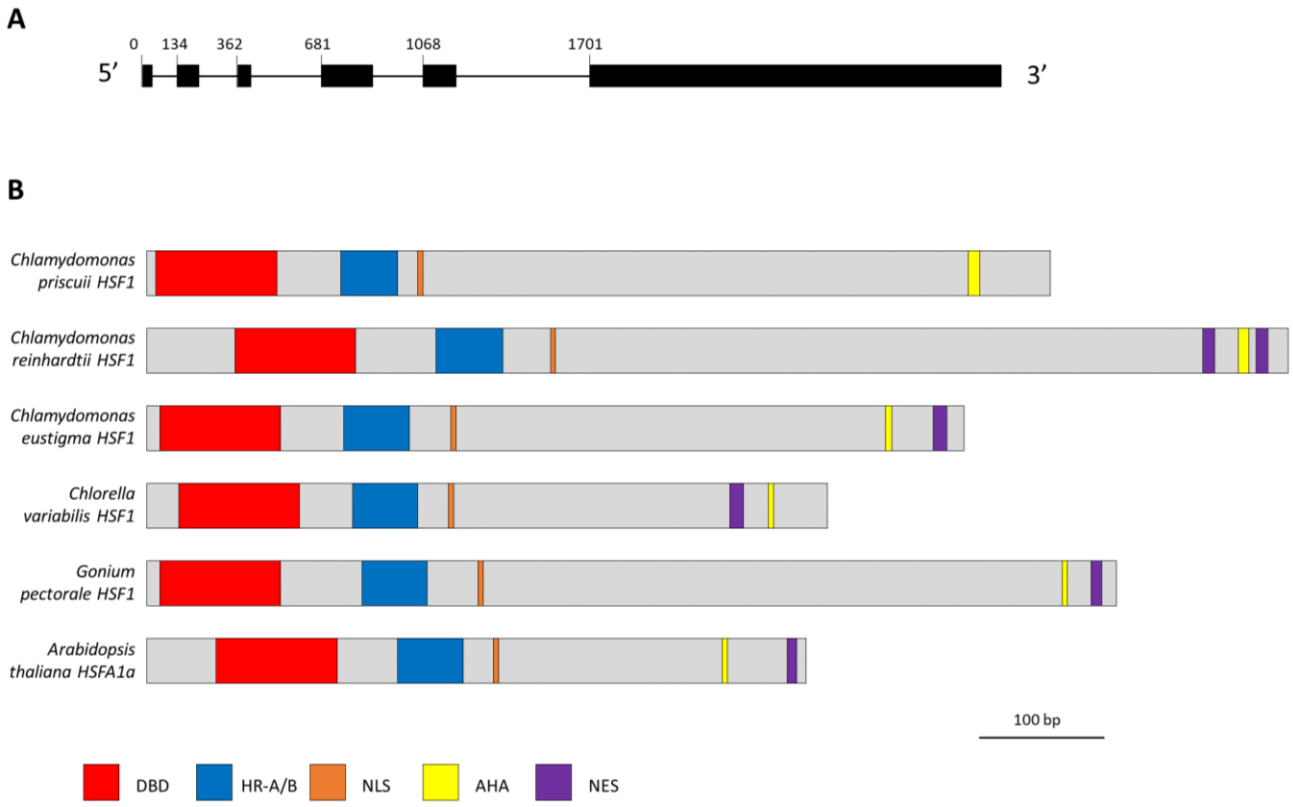

Figure 4. A) Gene structure of HSF1 from *C. priscuii*. Thick black bars represent the exons and thin bars represent introns. The numbers represent the first base position of each exon. B) Location of HSF1 functional domains in the coding region of HSF from *C. priscuii* and related green algae. The HSF1 sequence of *A. thaliana* is included as a representative of land plants. DBD = DNA-binding domain; HR-A/B = oligomerization domain cores; NLS = nuclear localization signal; AHA = aromatic, hydrophobic, acidic residue motif; NES = nuclear export signal.

The oligomerization domain is a coiled coil region that allows HSF1 to form a trimer (Nover *et al.*, 2001). It is located downstream of the DBD and the two are separated by a flexible linker of varying length (Figure 5). The linker in HSF1 from *C. priscuii* is 34 amino acids long, which is consistent with class A HSFs (Nover *et al.*, 2001). The oligomerization domain consists of two hydrophobic residue cores (HR-A and HR-B) separated by 5 amino acids in animal and plant class B HSFs (Takii & Fujimoto, 2016). In class A and C plant HSFs, in addition to the conserved 5 amino acids, there is a 21 and 7 amino acid insert, respectively (Figure 5, green bar). *C. priscuii* has a 14 amino acid insert, making it unlike any HSF characterized to date.

All HSF sequences examined in this study, including that of *C. priscuii*, had a highly conserved nuclear localization signal (NLS) consisting of basic amino acid residues with the consensus

sequence RKKRR. This NLS allows the transcription factor to be recognized by importins and transported into the nucleus (Lu *et al.,* 2021). The middle region of all HSFs is disordered and has very low conservation among all species in this study (only 10% identity). Finally, there are two main motifs in the C-terminal region of plant HSFs. The first is an aromatic, hydrophobic, and acidic (AHA) residue motif, which is involved in recruiting the transcription machinery (Kotak *et al.,* 2004). Like other algae in this study, *C. priscuii* has one AHA motif with the sequence DDLWGMF. The second C-terminal motif is the nuclear export signal (NES), which is present in class A HSFs and regulates the export of HSF back into the cytoplasm (Figure 4B). *C. reinhardtii* has two putative NES motifs, both up and downstream of the AHA motif (Schulz-Raffelt *et al.,* 2007). The other species examined here have one NES, either upstream (*D. salina, C. variabilis)* or downstream (*G. pectorale, C. eustigma, A. thaliana*) of the AHA motif. *C. priscuii* was the only HSF in this study that did not have a NES (Figure 4B, Figure 5). Based on these analyses, *C. priscuii* HSF1 has a very highly conserved DNA-binding and other important HSF motifs. Despite the missing NES and unusual length of the oligomerization domain, it is still possible that this gene encodes a functional transcription factor.

Figure 5. Pairwise alignment of HSF1 amino acid sequences from *C. priscuii* and its green algal relatives. The HSF1 sequence of *A. thaliana* is included as a representative of land plants. The color of the residue indicates the percent identity among the species (black = 100% similar, dark grey = 80-100% similar, light gray = 60-80% similar, white <60 % similar). The percent identity with *C. priscuii* is indicated in parentheses next to each species name in the first row. Important domains are highlighted: red bar = DNA-binding domain (DBD); pink bar = α-helix; yellow

arrow = β-sheet; grey bars = linker regions; blue bars = oligomerization domain hydrophobic cores; green bar = class A HSF insert in oligomerization domain; orange bar = nuclear localization signal (NLS); yellow bar = aromatic, hydrophobic, and acidic residue motif (AHA); purple highlighting = nuclear export signal (NES).

To confirm the similarity of *C. priscuii* HSF1 to homologs from other organisms, including distantly related ones, a phylogenetic tree of the DBD and oligomerization domain was constructed (Figure 6A). Here, we included HSFs from Chlamydomonadalean green algae with sequenced genomes (from Phycocosm; Grigoriev *et al.*, 2021), representative model green algae from the related Chlorellales and Sphaeropleales orders (both Chlorophytes), as well as more distantly related land plant and animal species. Despite the distant evolutionary relationship among these species, the DBD is extraordinarily conserved among these organisms, with 67 % similarity at the level of the amino acid sequence (Figure 6B). *C. priscuii* HSF1 is most similar to that of the acidophilic green alga *C. eustigma*, and HSF sequences group together according to the evolutionary relationships between the organisms (Figure 6A).

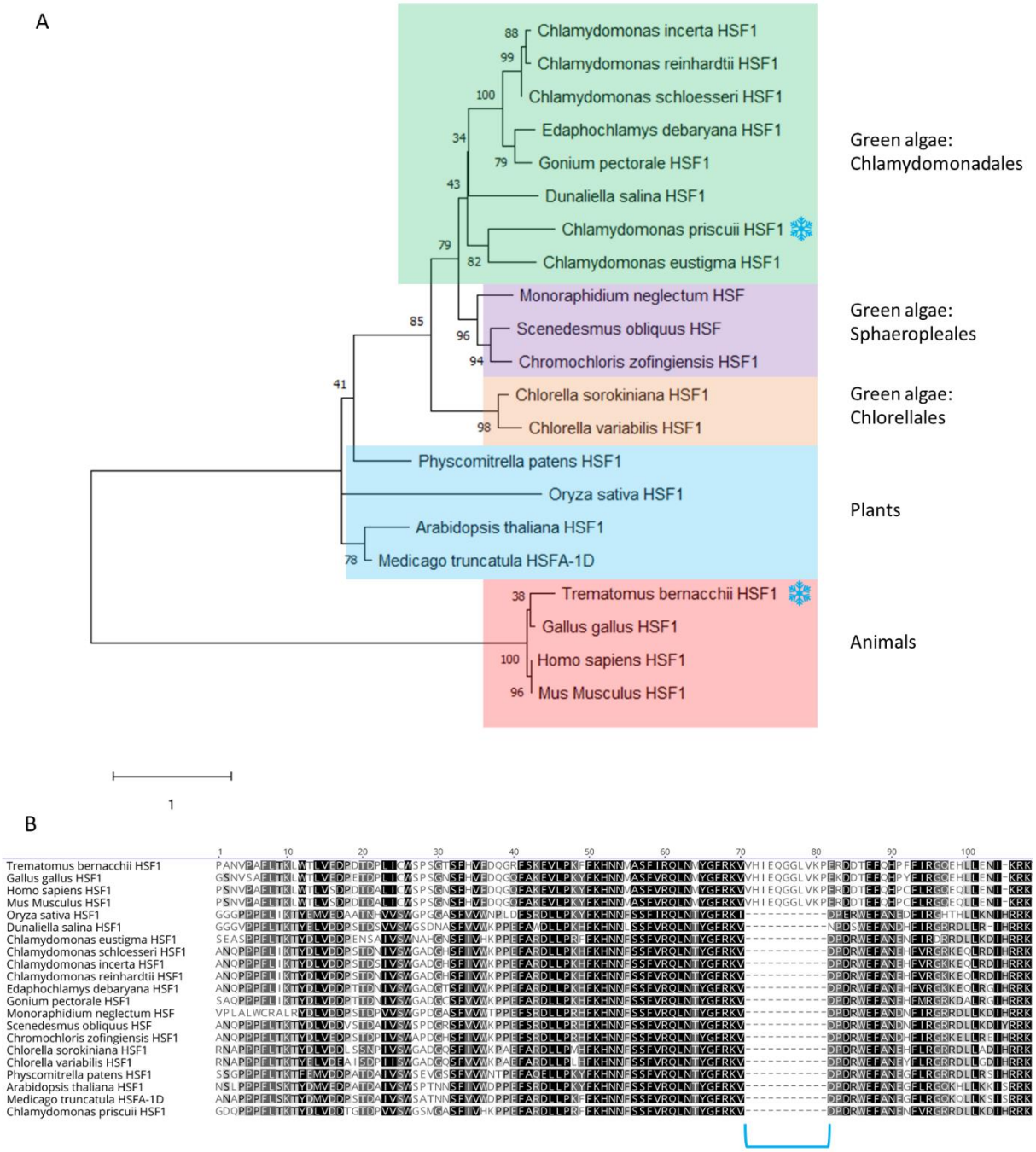

Figure 6. A) Phylogenetic tree of HSF1 DNA-binding domain sequences from representative algae, plants, and animals. Snowflakes indicate psychrophilic organisms. *C. priscuii* HSF1 forms a clade with other algal species in its order. B) Pairwise alignment of the HSF1 DNA-binding domain amino acid sequences showing high conservation in evolutionarily distant taxa. The color of the residue represents the percent identity among the species (black = 100% similar, dark grey = 80-100% similar, light gray = 60-80% similar, white <60 % similar). Blue bracket indicates the 11 base pair wing that is present in animal HSFs and absent in plant and algal HSFs.

3.2. Experimental detection of HSF1 in *C. priscuii*

To detect the presence of the *C. priscuii* HSF1 gene experimentally, primers were designed amplify fragments of the *C. priscuii* HSF1 coding domain sequence (Figure 7C, 7E, Table 3). The primers were designed to cover both highly conserved key motifs in the HSF1 sequence and the disordered, poorly conserved middle region (Figure 3, Figure 4B). These primers were used to amplify both *C. priscuii* genomic DNA (gDNA) and cDNA (synthesized from mRNA) from steady-state and heat-stressed cultures using PCR (Figure 7).

Using both gDNA and cDNA as templates, the regions of HSF1 that contain the conserved DBD, oligomerization domain and AHA motif could be successfully amplified (Figure 7A, 7D). The disordered region between these domains could not be amplified (Figure 7A, 7D). We were also not successful in amplifying the full-length HSF1 gene and transcript (results not shown).

Furthermore, when primer pairs were combined to amplify increasingly larger portions of the gene (Figure 7C), amplicons of the expected size could not be amplified, and we were only able to successfully detect the 5' region of the gene (Figure 7B). It is possible that *C. priscuii* HSF1 is truncated, with only the 5' region present in the cell; however more work is needed to conclusively show this.

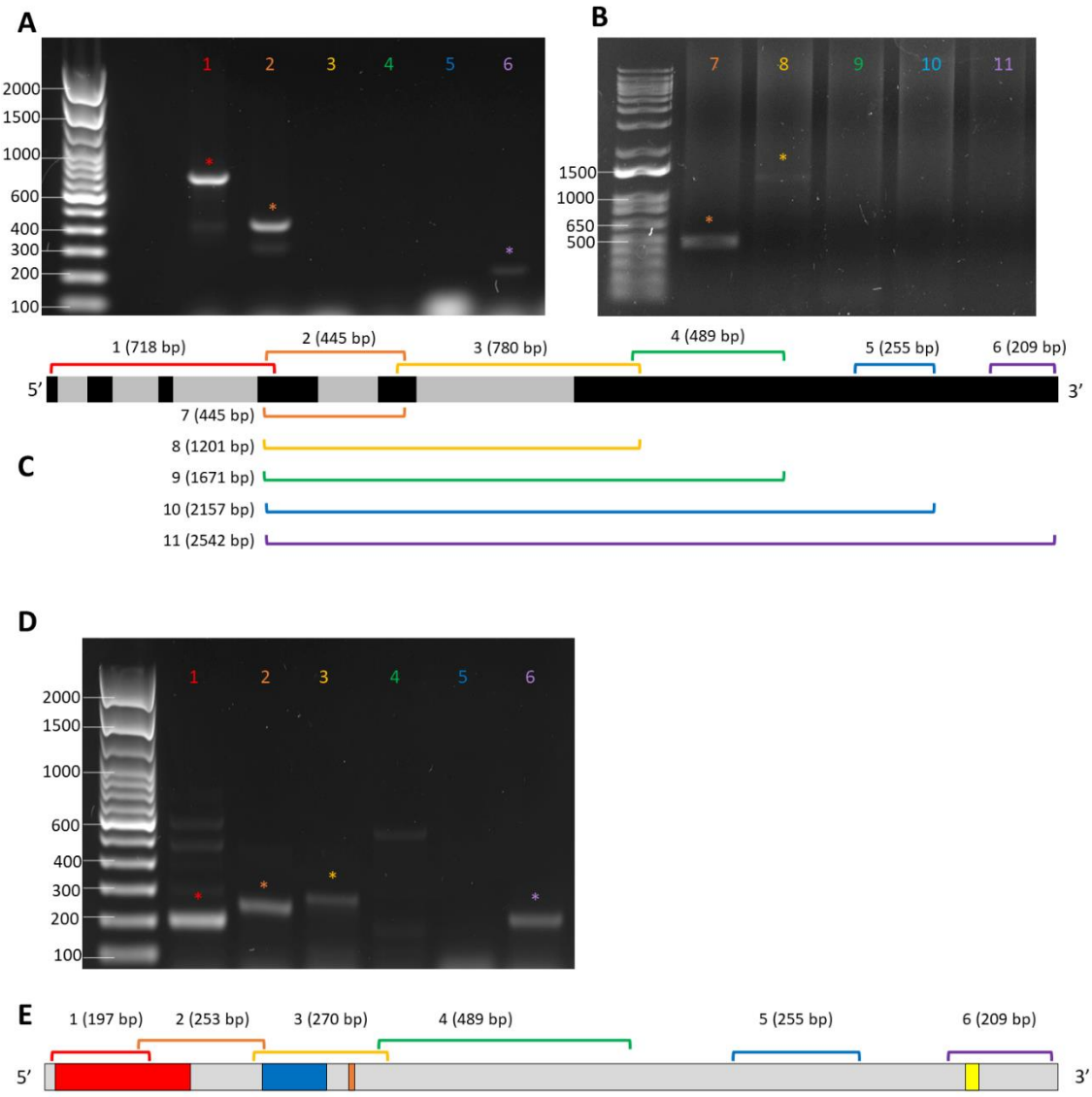

Figure 7. Gene fragments corresponding to the HSF1 gene (A, B) and transcript (D) in *C. priscuii*, as amplified by PCR. Also shown are diagrams of the full length HSF1 gene (C) and transcript (E), as predicted using bioinformatic analysis. The location of the primers and length of the expected amplicons are highlighted in colored lines, with the expected fragment size in brackets. Fragments corresponding to successfully amplified regions of the gene are marked with a colored asterisk. In C) exons are denoted with black bars, and introns are denoted with grey bars. In D) key functional domains are labeled: red bar = DNA-binding domain (DBD), blue bar = oligomerization domain, orange bar = nuclear localization signal (NLS); yellow bar = AHA motif. In all cases, only fragments within the conserved regions (DBD, oligomerization domain, and AHA) could be successfully and consistently amplified. In all cases, amplicons that span the sixth exon could not be amplified. Similar results were obtained using genetic material from heat stressed cells (not shown).

3.3. Many *C. priscuii* HSPs lack heat shock elements

The genomes of *C. priscuii* and its psychrophilic relative ICE-L encode for expanded HSP gene families (53 and 50 HSP genes, respectively) when compared to their mesophilic relatives such as *C. reinhardtii* that encode for 40 HSP genes (Cvetkovska *et al.*, 2022). To determine whether the expression of these HSPs can be induced by HSF1, their promoter regions were screened for the presence of HSEs (Table S3).

Only 42% (22/53) of *C. priscuii* HSPs had one or more HSE in their promoter region, compared to 77% (27/40) in *C. reinhardtii* (Figure 8A). The Antarctic sea-ice alga ICE-L is more closely related to *C. priscuii* than *C. reinhardtii* (Cvetkovska *et al.*, 2017), but HSEs were present in 74% (36/50) of ICE-L HSPs. In *C. priscuii*, only five HSP promoters contained multiple HSEs in their promoters, while ten and twelve HSPs contained more than one HSE in *C. reinhardtii* and ICE-L, respectively (Table S3). This suggests that having fewer HSE regulatory elements is not a common in psychrophilic algae in general, but rather is unique to *C. priscuii*.

In all organisms, a canonical HSE consists of two or three inverted repeats 5'-nGAAn-3', where n represents any base (Zhao et al., 2020). In *C. priscuii*, only three HSPs had canonical HSEs, compared to six in *C. reinhardtii* and fourteen in ICE-L. Other HSEs differed from the canonical sequence with one or two substitutions, often in the third or fourth position of 5'-nGAAn-3' (i.e., A → C,T,G) or the second and third position of 5'-nTTCn-3' (i.e., T → A,C,G). The G or C base, which is critical for HSF recognition (Zhao *et al.*, 2020), was more highly conserved. Overall, HSE sequences were highly conserved in all three species, with only minor deviations from the canonical sequence (Figure 8C).

Two tandem inverted repeats (5'-nGAAnnTTCn-3') are required for HSF-HSE binding (Perisic et al., 1989), but it has been shown that the presence of three repeats (5'-nGAAnnTTCnnGAAn-3') allows for higher affinity interaction between the DNA and DBD (Feng *et al.*, 2021). Approximately 60% of the HSEs in *C. priscuii* (17/22) contained three repeats, while ~50% of the HSEs detected in *C. reinhardtii* and ICE-L consisted of three tandem repeats (19/27 and 27/36, respectively). Taken together, these results suggest that *C. priscuii* has a decreased ability to regulate HSP expression via HSF1 due to having fewer HSEs in the promoter regions of these genes. The HSEs that are present, however, are highly conserved and resemble canonical HSEs.

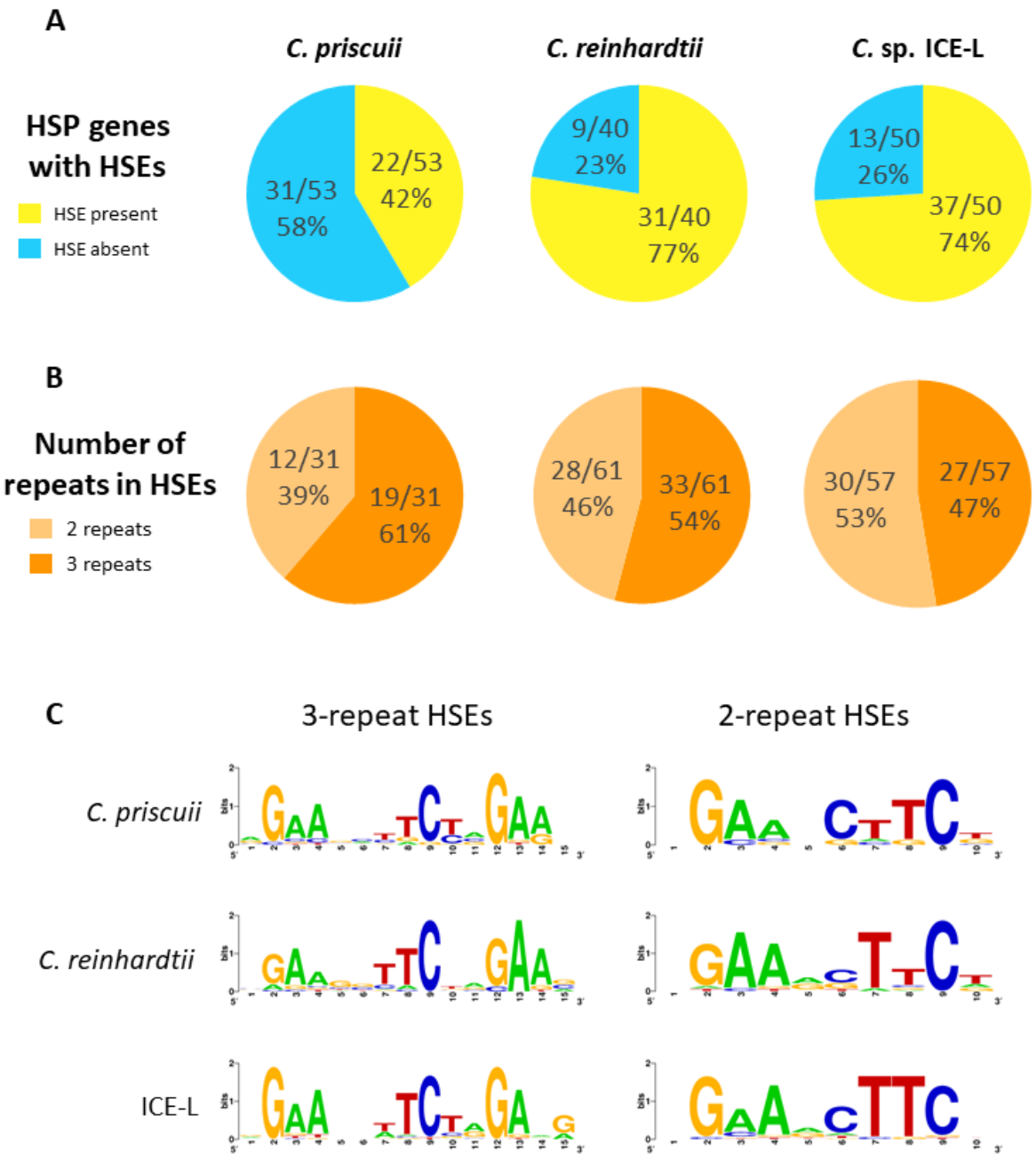

Figure 8. A) Percentage of heat shock protein genes that contain at least one heat shock element (HSE) in their promoters in *C. priscuii*, *C. reinhardtii*, and ICE-L. B) Percentage of heat shock elements that have 2 or 3 inverted repeats of 5'-nGAAn-3' in each species. C) Sequence logos of 3- and 2-repeat heat shock elements found in the promoters of *C. priscuii*, *C. reinhardtii*¸and ICE-L heat shock protein genes. The x-axis represents the base position of the sequence, and relative size of the letter represents the frequency of that base at a given position (measured in bits).

3.4. Transcriptome-wide analysis of HSP expression in *C. priscuii*

The ability of *C. priscuii* to regulate HSP expression during heat stress was examined using an RNA-Seq dataset from steady-state (4°C) and heat stressed algal cells (24°C for 6 hours) (Figure 9). Some HSPs had very high expression level under steady-state conditions, defined here as FPKM >100 (Table S4). These include HSPs with known homeostatic and stress-related functions: all genes in the HSP90s and CPN60s families, one HSP100 gene (ClpB3-4) and most (but not all) members of the HSP70 family (HSP70A-1, HSP70A-2, HSP70B-1, HSP70C, HSP70E, and BIP1).

Exposure to heat stress for 6 hours induced the expression of some, but not all, HSP genes. Overall, 14 HSPs (28% of all HSPs in the genome) were significantly differentially upregulated during heat stress. Most highly expressed transcripts at steady-state conditions were not up-regulated under heat stress, apart from HSP90A, which was modestly up-regulated (FC ≈ 7). The genes with largest expression differences between steady-state and heat stress were the small HSPs (HSP22A-2, -3, -6, -7, -8, -9) that increased more than 100-fold (Table S4), followed by several HSP70s (HSP70A-3 to A-5, HSP70B-2) and HSP100s (ClpB3-1, ClpB1) (Figure 9). Many homologs of these genes in other organisms (sHSPs, HSP90A, HSP70s) have well-documented responses to heat stress (e.g., Schroda *et al.*, 2015). Curiously, only a subset of the differentially expressed HSP genes also have an HSE in their promoter region (HSP22A-7, HSP22A-9, HSP90A, ClpB1, ClpB3-1), and some genes with an HSE are not significantly differentially upregulated (HSP22A-1, HSP70A-1, HSP70A-2, HSP70B-3, HSP70C, BIP1, HSP90C, ClpB3-2, ClpX-2) implying a mechanism of transcriptional regulation different than HSF1 (Table S4).

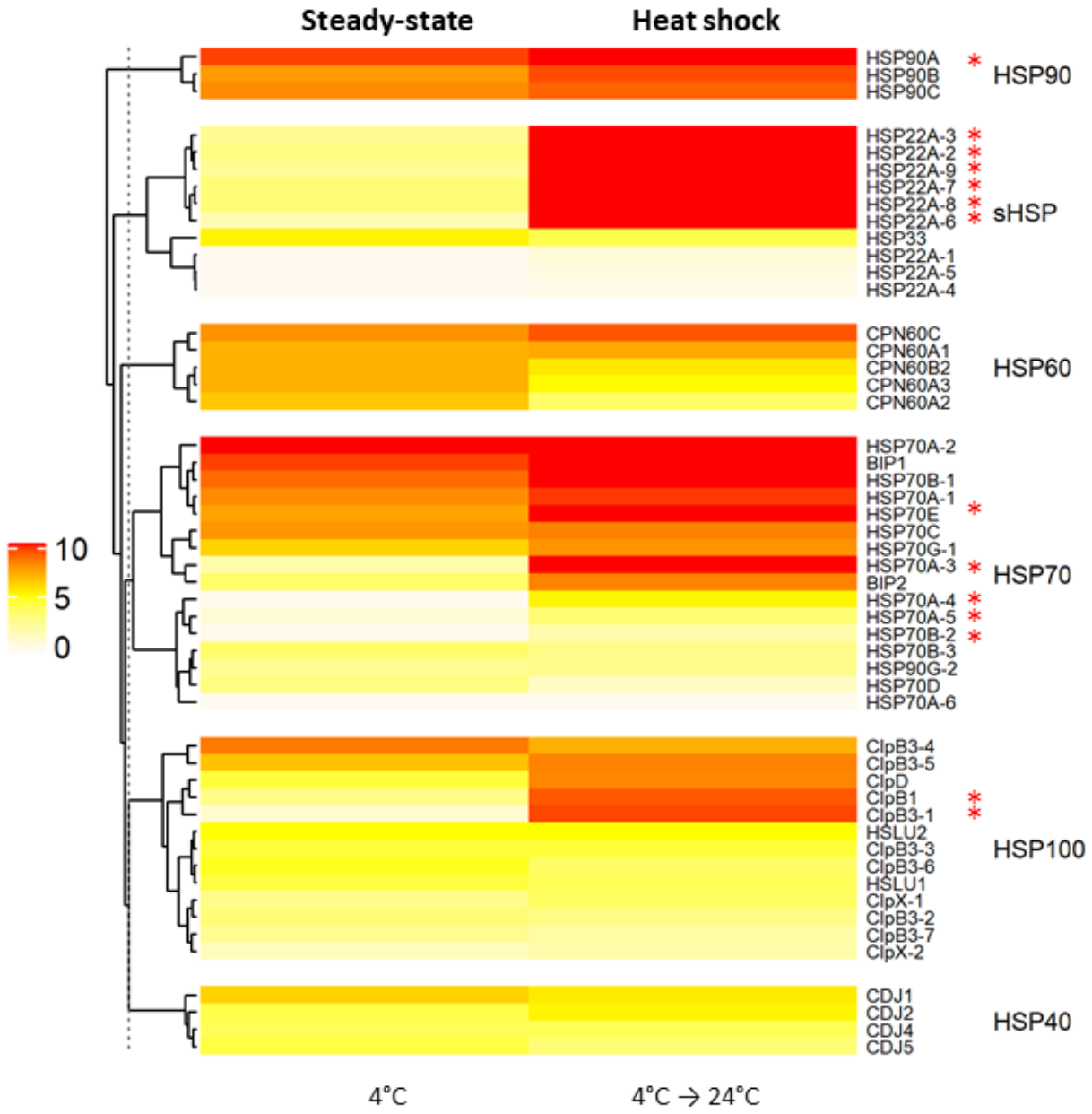

Figure 9. Heat map showing changes of expression of HSP genes in *C. priscuii* grown at 4°C and exposed to 6 hours of heat shock (24°C). The mean $\log_2$-transformed FPKM values of three biological replicates are represented using a color-based expression profile as indicated (white – low abundance; red – high abundance). All genes encoding putative HSP genes in the *C. priscuii* genome are shown. DEGs (defined as FC > 4, $p < 0.05$ between steady state and heat shock) are denoted with a red asterisk.

Careful analyses of this dataset revealed that HSF1 had a low expression during steady-state conditions (FKPM = 8.34) and transcript levels did not increase in response to heat stress (FKPM = 9.30; FC = 1.1; Table S4). This in contrast with *C. reinhardtii*, where HSF1 is nearly

undetectable during steady-state conditions but is rapidly and robustly induced during heat stress (Schmollinger *et al.*, 2013). To further examine the expression of this gene, RNA-Seq transcriptomic reads were mapped to the predicted HSF1 coding sequence from the *C. priscuii* genome (Figure 10A). These analyses confirmed the very low transcript levels detected in the transcriptome: in steady-state and heat stress conditions, HSF1 had a mean read coverage of 12.0 and 34.8 reads, respectively. Furthermore, the read coverage was inconsistent across the coding sequence: the 5' region, which contains the highly conserved DBD, had a mean coverage of 17.6 and 50.1 reads, under steady-state and heat stress respectively (Figure 10A). The remaining sequence had lower coverage, including a region with 0 reads (Figure 10A, highlighted by an arrow). In comparison, mapping the reads to HSP90A (a gene with high expression during both steady state and heat stress; Figure 9) yielded consistent and high coverage in steady-state and heat stress (average coverage = 970.1 and 17963 during steady-state and heat stress, respectively) throughout the entire coding sequence (Figure 10B). Given the low expression and coverage of the HSF1 transcript, it is possible that HSF1 is not transcribed in the cell.

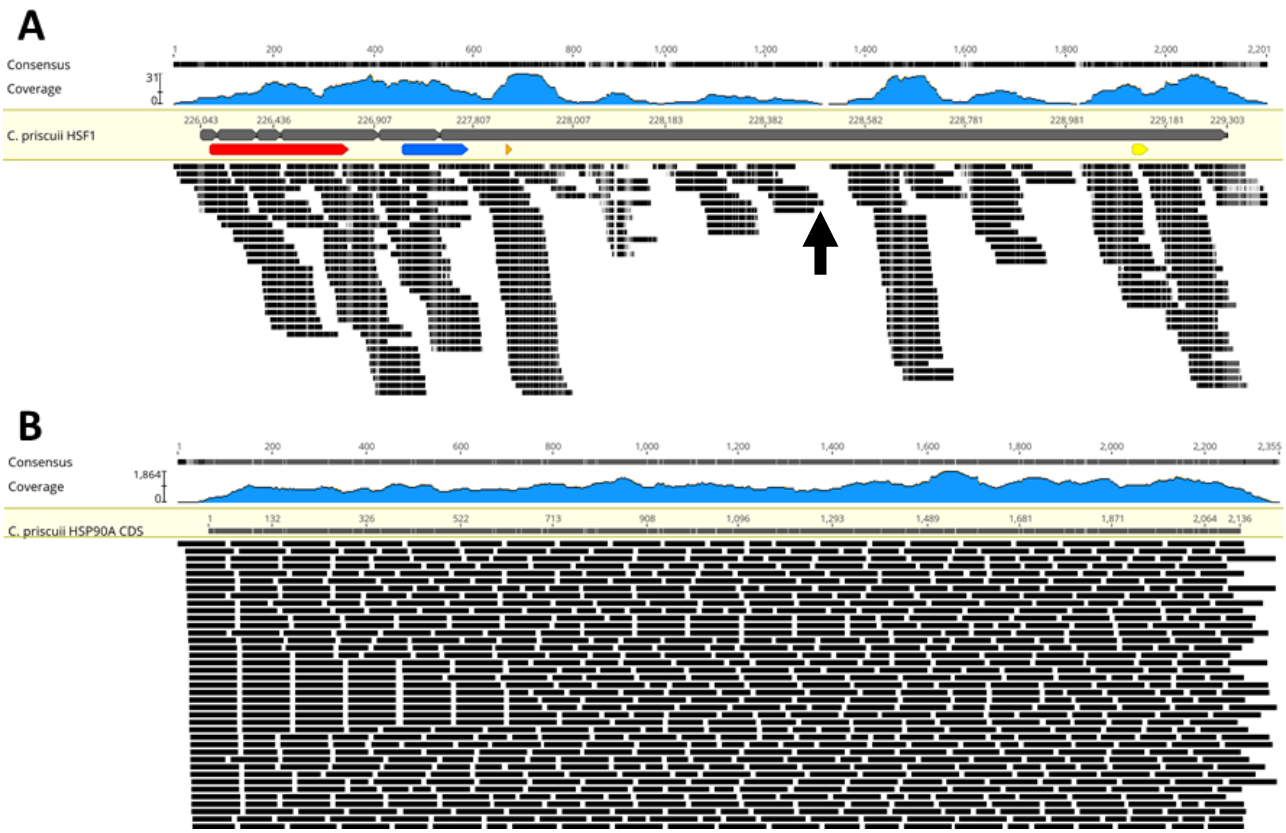

Figure 10. A) Mapping all transcriptome reads to the HSF1 coding domain sequence in *C. priscuii* yields inconsistent and low coverage in steady-state conditions. Similar results were obtained with the heat shock transcriptome (not shown). Exons are denoted with grey bars, and important HSF domains are indicated: red bar = DNA-binding domain (DBD); blue bar = oligomerization domain; orange triangle = nuclear localization signal (NLS); yellow bar = aromatic, hydrophobic, acidic residue (AHA) motif. Areas with high coverage coincide with functional domains. Black arrow indicates a region (position 1300) with 0 read coverage. B) Mapping all transcriptome reads to the HSP90A coding domain sequence yields a mean read coverage of 1019. Steady-state results are shown; heat stress results show similar consistent coverage (not shown).

3.5. HSP transcription and protein accumulation during temperature stress

Previous work has shown that *C. priscuii* has constitutively high levels of several key HSPs under steady-state conditions but fails to induce HSP accumulation at the protein level when exposed to heat for 6 hours (Cvetkovska et al, 2022). In mesophiles, HSP expression and accumulation are one of the earliest responses to heat (Schroda 2015), but early time points had not been examined in C. priscuii previously. To further examine the response to temperature stress at the protein and transcript level, algal cultures of *C. priscuii* and the model mesophile *C. reinhardtii* were grown at steady-state conditions and exposed to temperature stress for up to 6

hours. For this set of experiments, we chose two cytosolic HSPs (HSP70A, HSP90A) that have an HSE in their promoters (Table S3), available antibodies and demonstrated responsiveness to heat stress in *C. reinhardtii* (Schmollinger *et al.*, 2013). We also measured the expression of HSF1 in both species, but HSF1 protein levels could not be detected in *C. priscuii* with an antibody raised against *C. reinhardtii* (data not shown).

Exposure to heat stress (24°C) for 6 hours did not induce HSP70A expression (Figure 11A) or protein accumulation at any time point (Figure 11B; Figure S1). A modest (but significant; $p<0.05$) upregulation was detected in HSP90A after 1 hour of heat stress, but this response was very transient and expression levels dropped back to steady-state levels at 6 hours. This modest upregulation at the transcript level was not reflected at the protein level: no increased HSP90 accumulation was observed (Figure 11B; Figure S1). A 3-fold increase was seen in HSF1 expression, but only after 6 hours (Figure 11A). In contrast, *C. reinhardtii* exhibited a classic heat stress response, and a significant increase in the expression of all three genes was observed after 1 hour at high temperature (42°C; Figure 11A). A corresponding increase in the accumulation of HSP70A and HSP90A was seen 1 hour after temperature stress that peaked after 3 hours of heat stress (Figure 11B; Figure S1).

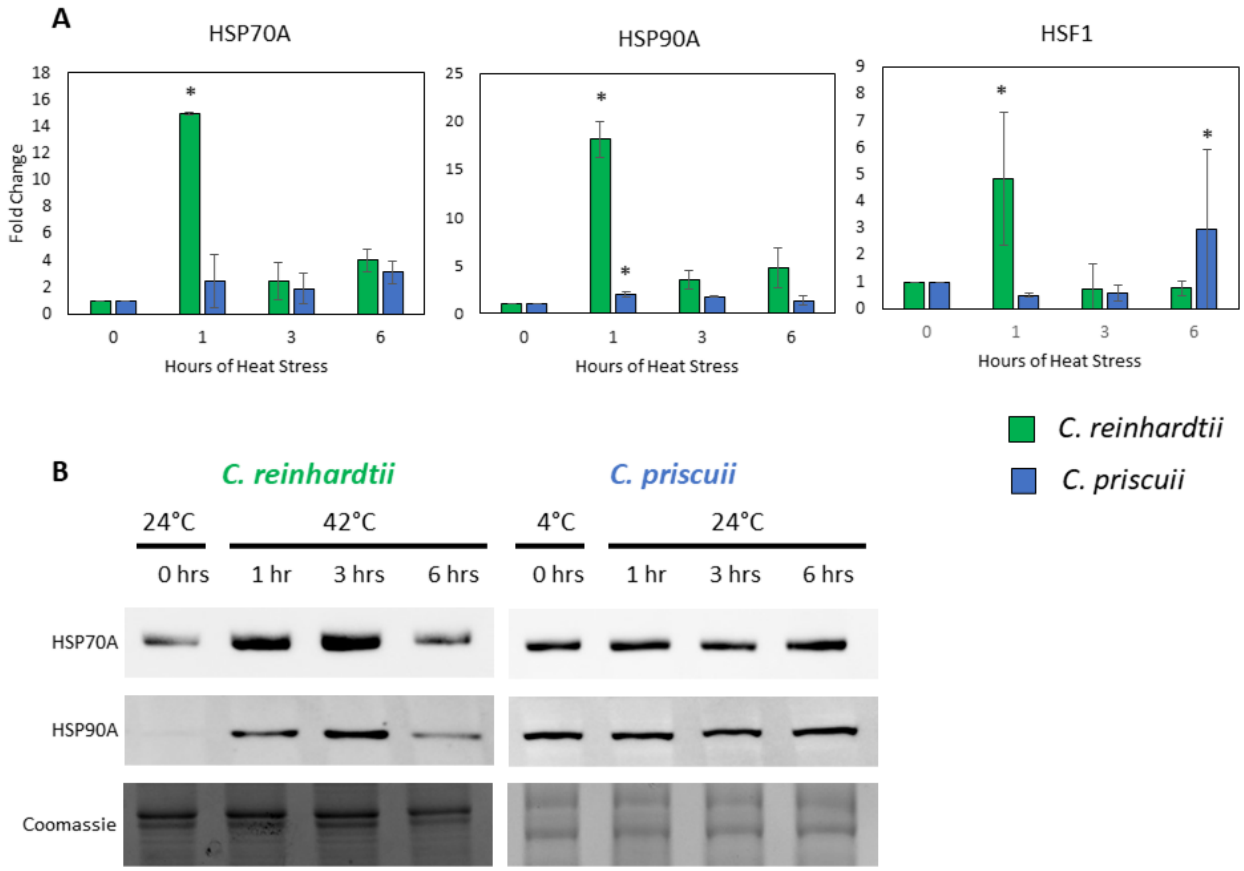

Figure 11. A) Changes in gene expression for HSF1, HSP70A and HSP90A in *C. reinhardtii* and *C. priscuii* after exposure to heat stress (42°C and 24°C, respectively). Asterisk indicates significant change in expression ($p < 0.05$) from control (0 hours) within the species. Error bars represent standard deviation (n=3). B) Representative western blots (n=3) of HSP70A and HSP90A in *C. reinhardtii* and *C. priscuii* when exposed to heat stress. Coomassie-stained gels are shown to demonstrate equal protein loading.

HSPs have been shown to respond to cold stress in green algae as well (Maikova *et al.*, 2016). To observe the effects of low temperature on HSP amounts, both *C. reinhardtii* and *C. priscuii* were exposed to 6 hours of low temperature (10°C and -2°C, respectively). No change in HSP expression (Figure 12A) or protein accumulation (Figure 12B, Figure S1) was observed in *C. priscuii*. In *C. reinhardtii*, a 15-fold increase was observed in HSP70A transcript levels after 1 hour of cold (Figure 12A), but this increase was not reflected on a protein level (Figure 12B, Figure S1). Additionally, no significant increase was observed in HSP90A or HSF1 at both transcript (Figure 12A) or protein (Figure 12B, Figure S1) levels. It is possible that misfolded protein accumulation is not an acute consequence of low temperature stress.

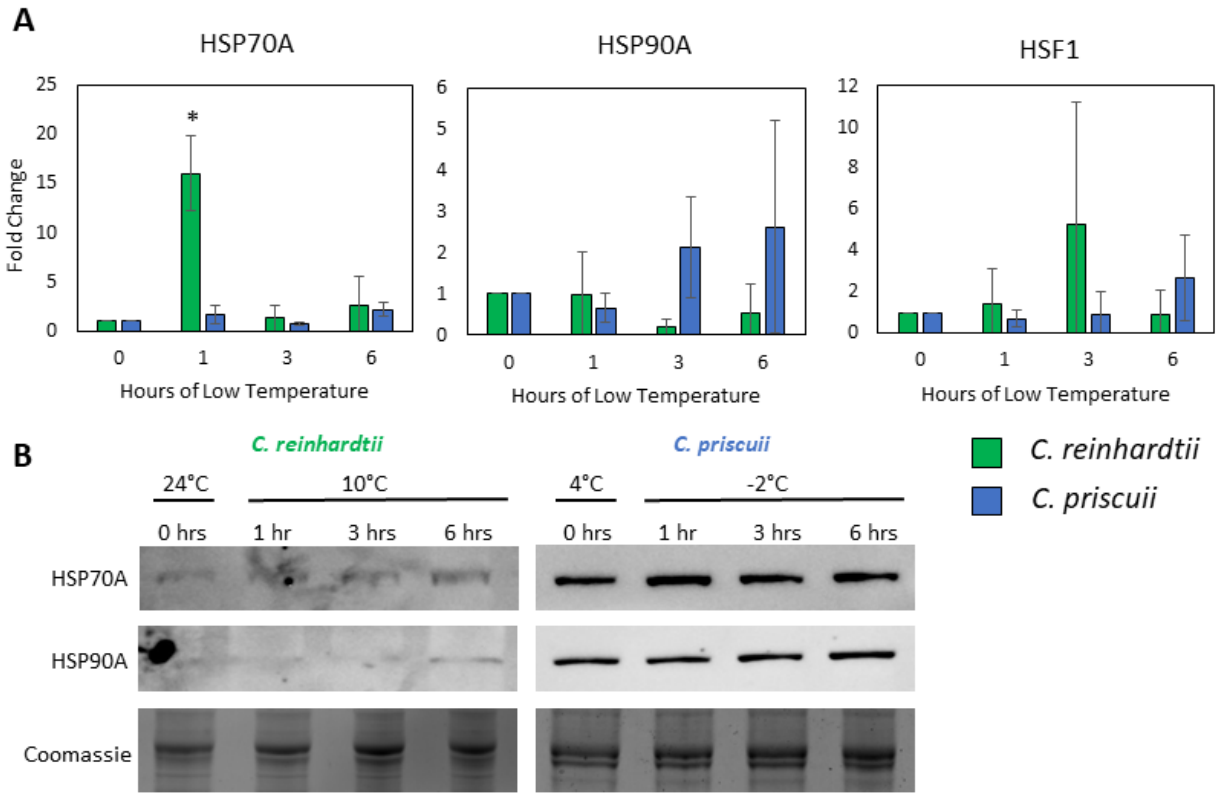

Figure 12. HSF1 gene expression, HSP gene expression and protein accumulation in low temperature. A) Changes in gene expression for HSF1, HSP70A and HSP90A in *C. reinhardtii* and *C. priscuii* after exposure to low temperatures (10°C for *C. reinhardtii;* -2°C for *C. priscuii*). Asterisk indicates significant change in expression ($p < 0.05$) from control (0 hours) in that species. Error bars represent standard deviation (n=3). B) Representative western blots showing accumulation of HSP70A and HSP90A in *C. reinhardtii* and *C. priscuii* in cold treatment. Coomassie-stained gels are shown to demonstrate equal protein loading.

It has been shown that the heat stress response is triggered by the accumulation of unfolded proteins, rather than the heat stress directly (Schroda *et al.*, 2015). The unfolded protein response was chemically induced in both species by supplementing their media with canavanine, an arginine analog that prevents correct protein folding if incorporated into a new peptide. As a control, the media of both algal species was supplemented with arginine. As expected, arginine supplementation did not lead to changes in HSP accumulation in either species (Figure 10B, Figure S1).Once again, no changes in gene expression or protein accumulation were observed in *C. priscuii* (Figure 13A, 13B, Figure S1) under canavanine treatment. In contrast, canavanine treatment led to a moderate increase in HSP70A (FC = 2, $p<0.05$) transcript levels, but only small changes in HSP90A or HSF1 (Figure 13A). On a protein level, a clear accumulation of

HSP70A and HSP90A was observed after 6 hours of canavanine treatment (Figure 13B, Figure S1).

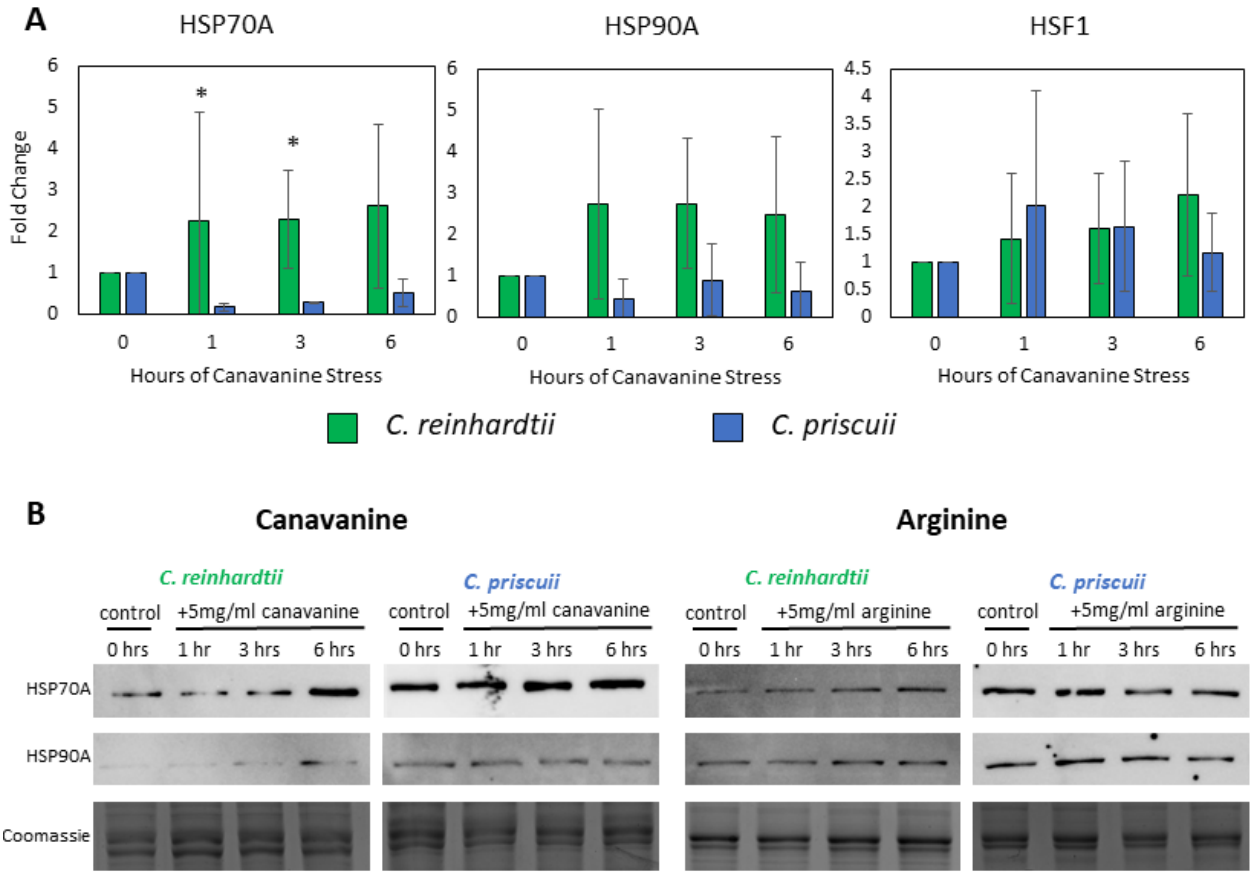

Figure 13. A) Changes in gene expression for HSP70A, HSP90A, and HSF1 in *C. reinhardtii* and *C. priscuii* after canavanine treatment. Asterisks indicate significant change in expression ($p < 0.05$) from control (0 hours) within the species. Error bars represent standard deviation (n=3). B) Representative western blots (n=3 for canavanine treatment; n = 2 for arginine treatment) of HSP70A and HSP90A in the mesophile *C. reinhardtii* and psychrophile *C. priscuii* when supplemented with amino acids canavanine (left) and arginine (right) for up to 6 hours. Coomassie-stained gels are shown to demonstrate equal protein loading.

3.6. HSPs transcription and protein accumulation during osmotic stresses

The water column of Lake Bonney has a steep salinity gradient, that ranges from freshwater near the surface to a hypersaline brine (1.7 M NaCl) near the lake's bottom (Patriarche *et al.*, 2021). As such, organisms that live in the lake may be exposed to a range of salinities. *C. priscuii* thrives at 0.7M NaCl (the salinity reported at 17M below the surface of Lake Bonney, where it was isolated), but it has been shown to successfully acclimate and grow at a wide range of salinities (0.01 M to 1.2M; Pocock et al. 2011). *C. priscuii* exhibited a very high sensitivity to

decreased salinity, with cell death (~50% of the culture) evident only 1 hour after transfer to media supplemented with 0.01M NaCl (Figure S3). This is unlike any other stressor tested here (including transfer to high salinity at 1.2M NaCl), where short term exposure did not result in significant increases in cell death or decrease in chlorophyll levels (Figure S3). Thus, we asked whether this sensitivity to osmotic stress is reflected at the level of HSP expression and accumulation.

Despite the rapid increase in cell death, no change was observed in HSP and HSF1 levels on a transcript (Figure 14A) or protein level (Figure 14B, Figure S1). Similarly, high salt stress (1.2M) did not result in significant upregulation in gene expression and increased protein accumulation, although a moderate downregulation of HSP70A was observed (Figure 14A). *C. reinhardtii* is a freshwater alga and therefore could not be exposed to low salinity stress. However, high salinity stress induced a moderate increase in HSP70A and HSP90A accumulation (Figure S2) and an increase in gene expression was also demonstrated elsewhere (e.g., Khona *et al.*, 2016). Altogether, these results reinforce the idea that *C. priscuii* is unable to rapidly regulate HSPs in response to environmental stress.

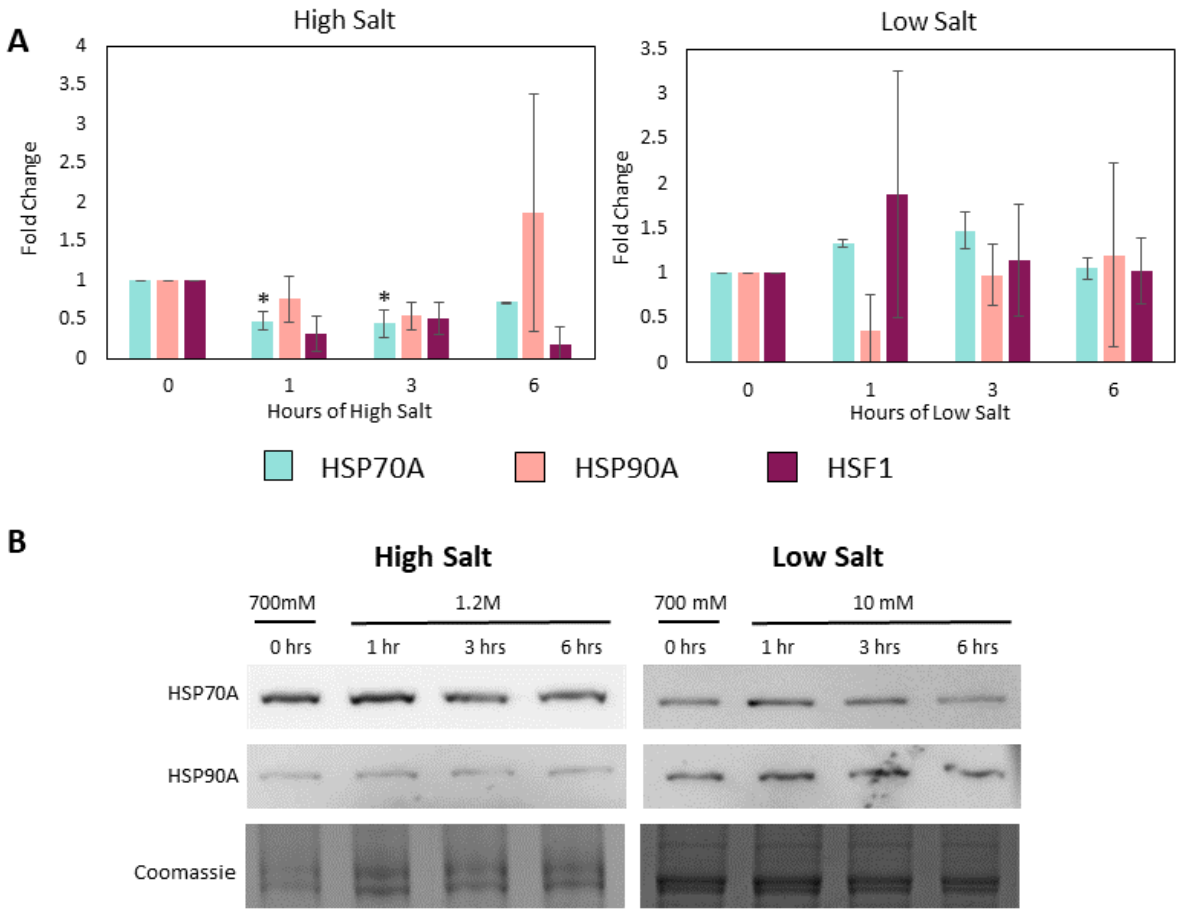

Figure 14. A) Changes in gene expression for HSP70A, HSP90A, and HSF1 in *C. priscuii* after exposure to 1.2 M salt (left) and 0.01 M salt (right) for 6 hours. Asterisks indicate significant change in expression ($p < 0.05$) from control (0 hours). Error bars represent standard deviation (n=3). B) Representative western blots (n=3) of HSP70A and HSP90A in *C. priscuii* exposed to high salt (left) and low salt (right) stress for 6 hours. Coomassie-stained gels are shown to demonstrate equal protein loading.

3.6. HSPs transcription and protein accumulation during high light stress with emphasis on chloroplast HSP accumulation

In its natural environment, *C. priscuii* is exposed to perpetually low light conditions (~10 µmol photons m^{-2} s^{-1}), so we tested its responses to high light stress. As with other stress conditions, no protein increase was observed in the cytosolic HSP70A and HSP90A in *C. priscuii* exposed to high light (250 µmol photons m^{-2} s^{-1}), while a gradual and large increase was evident in *C. reinhardtii* exposed to high light (500 µmol photons m^{-2} s^{-1}) (Figure 15; Figure S1).

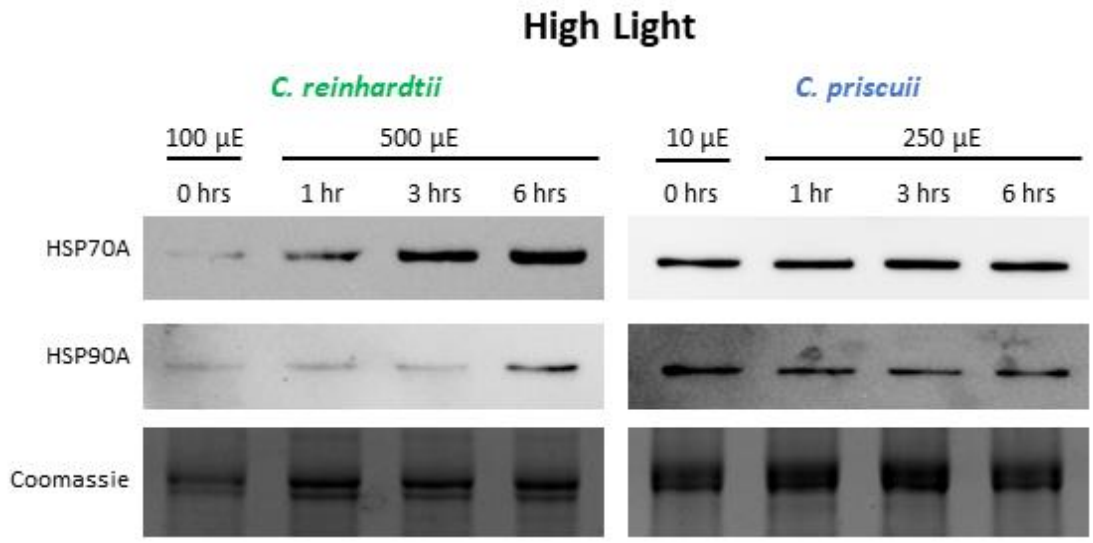

Figure 15. Representative western blots (n=3) of HSP70A, and HSP90A in *C. reinhardtii* and *C. priscuii* when exposed to high light conditions (500 µmol photons m^{-2} s^{-1} and 250 µmol photons m^{-2} s^{-1}, respective) for 6 hours. Coomassie-stained gels are shown to demonstrate equal protein loading.

Finally, we also tested the accumulation of the chloroplast chaperone HSP70B during all the environmental conditions discussed previously. As a shade-adapted alga, *C. priscuii* responds to a variety of stressors at the level of its photosynthetic apparatus (Szyszka-Mroz *et al.,* 2015), and we tested whether this is reflected in chloroplast-targeted HSPs. In *C. reinhardtii*, HSP70B is both heat- and light-inducible (Drzymalla *et al.,* 1996), with known functions in photosystem II repair (Schroda *et al.,* 1999). In *C. reinhardtii*, HSP70B increased in high light, high temperature, and canavanine (Figure 16; Figure S1), confirming previous literature findings. In *C. priscuii*, no increases were seen in HSP70B levels in any condition.

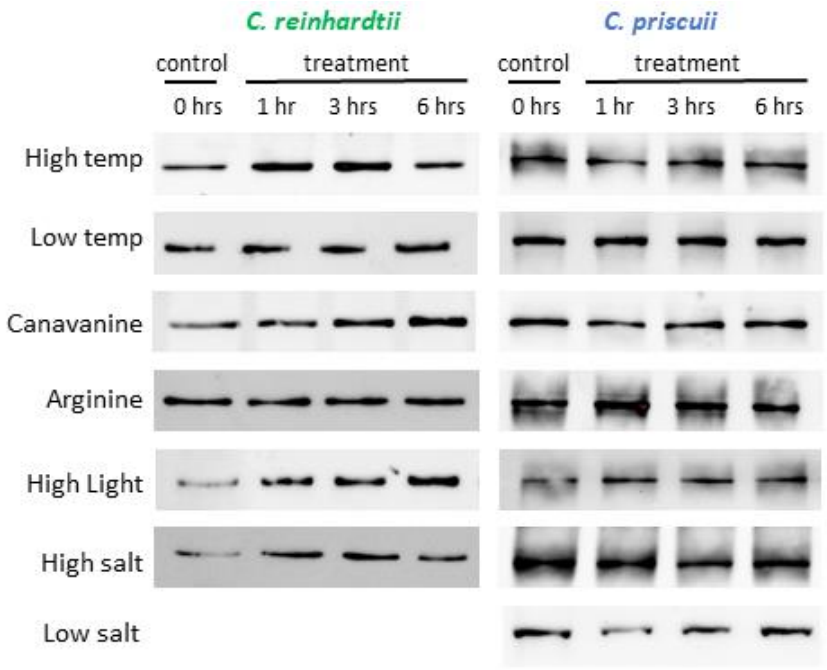

Figure 16. Representative western blots (n=3; n=2 for arginine treatment) of HSP70B in *C. reinhardtii* (left) and *C. priscuii* (right) under 6 hours of treatments (conditions summarized in Table 2).

3.7. Heat stress in other psychrophilic algae

The results shown above suggest that *C. priscuii* lacks the ability to increase the accumulation of HSPs under various stressful conditions. To test whether this aberrant HSP response is a common feature in psychrophiles, we examined another psychrophilic green alga. ICE-MDV is another Lake Bonney native (likely a strain of ICE-L (Cook *et al.*, 2019; Smith *et al.*, 2018) that is subjected to similar environmental conditions as *C. priscuii* (Li *et al.*, 2016). HSP accumulation was measured in ICE-MDV exposed to high temperature stress (24°C for 6 hours). Unlike *C. priscuii*, moderate increases in HSP70A, HSP70B, and HSP90A protein levels were observed in ICE-MDV (Figure 17). The heat stress response of ICE-MDV is gradual and peaks at 6 hours, unlike the rapid 1-hour response seen in *C. reinhardtii* (Figure 17B-D). These results suggests that ICE-MDV has an attenuated HSR compared to mesophiles, but a more robust one when compared to *C. priscuii*. Thus, the absence of HSP accumulation in *C. priscuii* is specific to this alga and not a common psychrophilic feature.

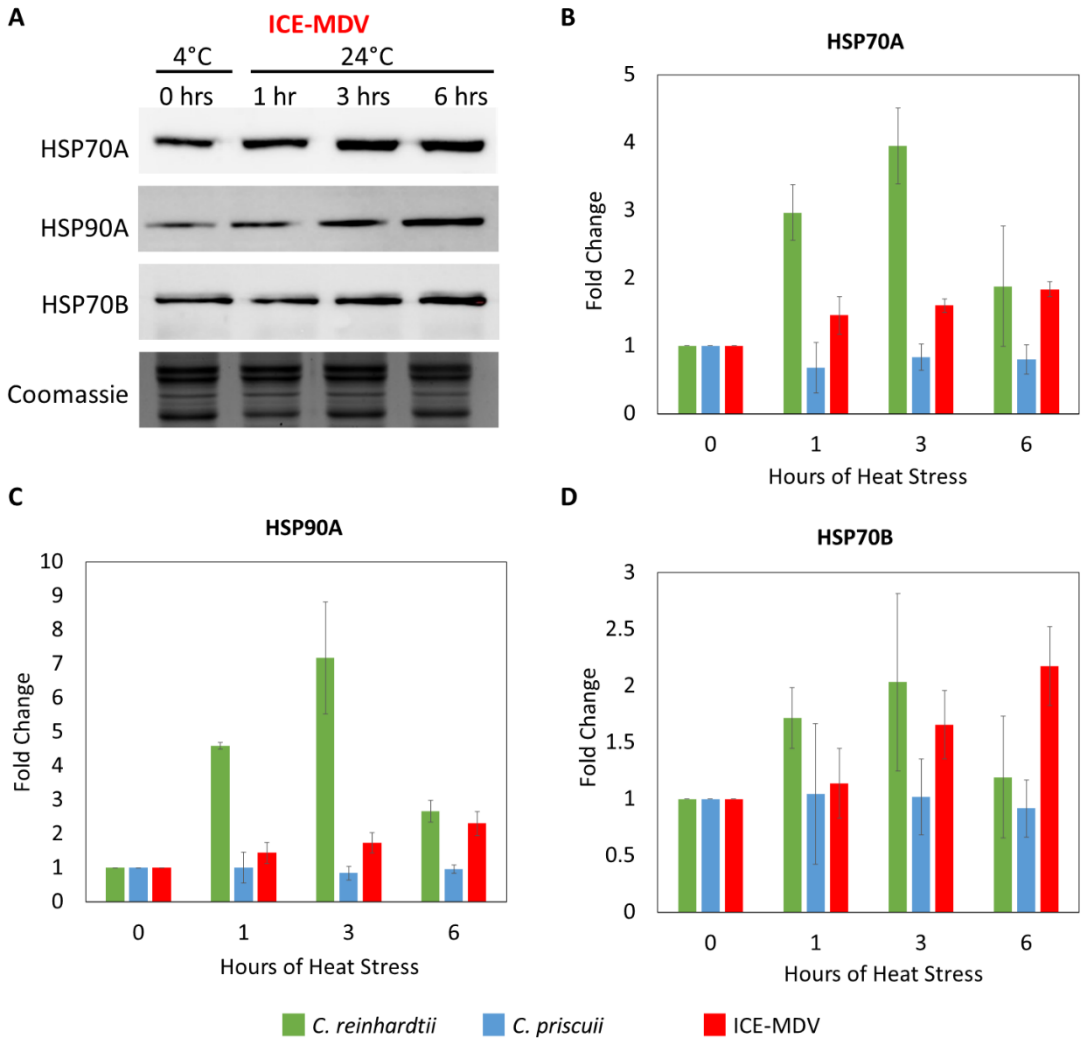

Figure 17. A) Representative western blots (n=3) showing of HSP70A, HSP90A, and HSP70B in ICE-MDV after 6 hours of heat stress. Coomassie-stained gels are shown to demonstrate equal protein loading amounts. B) Densitometry analysis of the relative HSP abundance determined by western blotting in cultures of *C. priscuii*, *C. reinhardtii* and ICE-MDV after exposure to 6 hours of heat stress. Values represent the mean of 3 biological replicates, and the error bars represent standard deviation. The protein abundance at steady-state conditions (time point 0) was set as 1 and used as the basis of comparison for the relative abundance of HSPs within each species. ICE-MDV exhibits an intermediate heat stress response between *C. reinhardtii* (classic) and *C. priscuii* (absent).

Chapter 4: Discussion

4.1 *C. priscuii* lacks the ability to rapidly regulate HSP transcription and accumulation

C. priscuii has an unusual heat stress response compared to that of mesophiles. In the mesophilic alga *C. reinhardtii*, exposure to heat stress resulted in a rapid accumulation of HSP70A and HSP90A transcripts and proteins (Figure 11). However, in *C. priscuii*, HSP70A and HSP90A did not increase after 6 hours when measured using droplet digital PCR. HSP70A, HSP70B, and HSP90A protein accumulation was absent in *C. priscuii* as well, and this is in stark contrast to the sharp and significant increase in both transcript and protein levels in the mesophilic *C. reinhardtii* (Figure 11, Figure 16).

C. priscuii did not increase HSP amounts on a transcript or protein level in any stress conditions tested here: low temperature, high salt, low salt, or high light. These conditions (except for low temperature, which has not been tested in this alga to date) have been shown to be stressful in *C. priscuii*: the light levels used here caused photoinhibition in previous work (Szyska *et al.,* 2007), and the high salt treatment chosen was the upper salinity limit of *C. priscuii* growth (Pocock *et al.,* 2011). At low salt, a rapid spike in cell death was observed (Figure S1). *C. reinhardtii* showed an HSP increase in both high salt and high light in this study and in previous research, particularly the chloroplastic HSP70B (Hounslow *et al.,* 2021; Yokthongwattana *et al.,* 2012; Drzymalla *et al.,* 1996). Low temperature was also seen to increase HSP expression in *C. reinhardtii* (Maikova *et al.,* 2016), although there was no significant difference on a transcript or protein level in this study. Based on these results, it seems that *C. priscuii* does not respond strongly to any environmental stress at the level of HSP accumulation. This alga has retained a limited capability to modulate the level of HSP transcripts (Figure 9, Table S4), but this does not translate to increased protein amounts.

Finally, *C. priscuii* did not induce HSPs on a transcript or protein level (Figure 13) when supplemented with canavanine, an arginine analog that prevents proper protein folding (Rosenthal, 1977). In contrast, supplementing *C. reinhardtii* with canavanine yielded a modest increase in HSP70A, HSP90A, and HSP70B protein levels after 6 hours in this study (Figure 13, Figure 16). In previous work, a sharp increase in HSPs and HSF1 was observed in *C. reinhardtii* mutants that could not synthesize arginine after 1 hour of canavanine treatment (Schmollinger *et al.,* 2013). These results suggested that HSF1-mediated HSP transcription was induced in

response to the accumulation of misfolded proteins. However, this response was not seen in *C. priscuii.* Previous work showed that *C. priscuii* does mount a global transcriptomic and metabolomic response to heat stress, albeit less robustly than other green algae (Cvetkovska *et al.,* 2022). Thus, we conclude that *C. priscuii* can respond to environmental stress, but it cannot respond to the unfolded protein response. To investigate the breakdown of this response, we characterized the master regulator of HSPs: the transcription factor HSF.

4.2 *C. priscuii* has an unusual HSF

The *C. priscuii* genome encodes for a single HSF gene, which contains most of the domains necessary to be a functional transcription factor. Unlike all other green algae examined here, *C. priscuii* HSF1 lacks a C-terminal nuclear export signal (NES). Based on their peptide sequences, *C. priscuii* and *C. reinhardtii* HSF1 are both plant class A HSFs (Schulz-Raffelt *et al.,* 2007; Nover *et al.,* 2001), that exist as inactivated trimers in the cytosol and get imported into the nucleus when misfolded proteins accumulate into the cell (Schroda *et al.,* 2015). Upon cessation of the stress signal, HSF1 it is exported back into the cytosol and inactivated once again. Without an NES, *C. priscuii* HSF1 can likely enter the nucleus but is effectively trapped within it upon cessation of the stress signal.

Could it still be a functional HSF despite this? In land plants, class B and C HSFs lack NES motifs, meaning that they are permanently localized to the nucleus (Scharf *et al.,* 2012). However, the subcellular localization of these proteins has not been experimentally proven, and these HSFs are not the primary inducers of the heat stress response (Scharf *et al.,* 2021; Liu *et al.,* 2011). Yeast (*Saccharomyces cerevisiae)* have only one HSF which is exclusively present in the nucleus and is responsible for HSP transcription in both steady state and heat shock conditions (Solis *et al.,* 2016). While the N-terminal activation domain seen in *S. cerevisiae* HSFs is not present in *C. priscuii*, it is still possible for primary HSFs to be functional while localizing permanently in the nucleus without an NES.

The oligomerization domain, which is necessary to form a functional trimer, is also unusual in *C. priscuii*. The hydrophobic repeats (HR-A and HR-B, Figure 5) are conserved, but there is a 14 amino acid insert between them that does not match any plant HSF classes (Nover *et al.,* 2001) and has not been described in any other HSF to date. The other functional domains of *C. priscuii* HSF1 (DNA-binding domain, nuclear localization signal, AHA motif) are well conserved and

most similar to class A HSFs in plants, which have a 21-aa insert (Nover *et al.,* 2001). While this 14-aa insert may not prevent it from being a functional transcription factor, it could be a sign of HSF sequence degeneration. Further work is needed to characterize the three-dimensional structure and oligomerization capabilities of *C. priscuii* HSF1.

C. priscuii HSF1 has very low and inconsistent expression as determined by aligning the RNA-Seq reads to the predicted coding sequence of the gene (Figure 10), and a full-length transcript could not be detected using RNA-Seq data or experimental methods (Figure 11). The DBD and oligomerization domain are the only regions that can be reliably experimentally detected. Taken together, these findings could mean that HSF1 is truncated, with only the 5' region of the gene being expressed. Since the DBD has not lost any of its highly conserved features, it is possible that this HSF still retains DNA-binding activity even if it is truncated. Truncated HSFs have been described in some eudicot plants. HSFB5 is one such HSF whose sequence terminates after the NLS – matching the region that was experimentally isolated in *C. priscuii* (Scharf *et al.,* 2012). It should be noted that this is a class B HSF, which is not the main activator of the HSR in plants (Yoshida *et al.,* 2011), and its role in HSP regulation in plants is unknown. Unlike green algae (and other organisms) that have a single functional HSF, land plants are characterized by large HSF gene families (e.g., 21 genes in *A. thaliana*; Scharf *et al.,* 2012) that respond differently to various environmental and developmental signals and display a large degree of functional redundancy within their respective classes (Scharf *et al.,* 2012). The effect of a truncated HSF gene in an organism with only one HSF gene has not been reported.

4.3 Evidence of degeneracy in the Heat Shock Elements in the promoters of HSPs

The promoter regions of HSP genes in *C. priscuii* contain fewer HSEs compared to the mesophilic *C. reinhardtii* and even the sea-ice alga ICE-L (Figure 8; Table S3). However, the HSEs that were nonetheless present in *C. priscuii* were similar to the canonical HSE sequence, and most had 3-unit repeats (necessary for optimal HSF binding). Thus, since the HSF1 DBD is highly conserved, it is possible for the transcription factor to bind these HSEs and induce the transcription of their corresponding genes. In future work, HSF-HSE binding assays should be done to validate HSF1 functionality.

Curiously, the presence of an HSE within the promoters of individual HSP genes did not correlate with that gene's induction during heat stress according to RNA-Seq data. Not all

upregulated HSPs had an HSE, and not all HSPs with an HSE were upregulated (Table S3). These data suggest a higher level of nuance between HSF, HSEs, and transcriptional induction of HSPs. Similar phenomena have been reported in animals: in *Caenorhabditis elegans*, some HSF1 target genes lacked a canonical HSE (Brunquell *et al.*, 2016). In humans, some HSEs are used as HSF2 binding sites for constitutive, non-stress-induced transcription (Stephanou & Latchman, 2011). Our work is the first report of HSE analyses in the HSP promoters in green algae, and more research is needed to fully elucidate the intricacies of HSF-HSE interactions in these organisms in general and psychrophiles specifically.

The discrepancy in HSE presence and transcribed HSPs during heat stress could also imply the existence of an additional HSP regulation mechanism other than HSF. Many plant HSPs have stress response elements (STREs) in their promoter regions, with the consensus sequence AGGGG or CTTTT (e.g., Montero-Barrientos *et al.*, 2008). These are recognized by C_2H_2 zinc finger proteins, which are part of the general abiotic stress response (Han *et al.*, 2020). In yeast, these STREs work redundantly with HSEs or additively in heat stress, depending on HSE-STRE promoter proximity and nucleosome formation (Estruch, 2000). Additionally, recent work on the Chlorophyte *Ulva prolifera* showed that low temperature resistance (LTR) elements worked synergistically with HSEs to confer cold tolerance (Wu *et al.*, 2019). All these examples suggest that HSEs could help recruit other transcription factors to mount a general response.

Finally, it is possible that due to lesser frequency of HSEs in the HSP promoters and the lack of a full-length HSF transcript, *C. priscuii* HSF1 has lost its functionality entirely. In *C. reinhardtii*, which has two HSF genes, knocking out HSF1 causes a drastic reduction of HSP induction ability, and the phenotype cannot be rescued by HSF2 (Schulz-Raffelt *et al.*, 2007). Knockout studies in plants are difficult due to the high number of HSF genes providing functional redundancy and partial phenotype rescue (e.g., Liu *et al.*, 2011), but gene knockout has been performed in organisms with fewer HSF genes. *Drosophila melanogaster* contains only one HSF1 gene, and interrupting its expression yields adult flies that are phenotypically normal in steady-state conditions, but very sensitive to heat stress (Jedlicka *et al.*, 1997). Although the phylogenetic distance between algae and flies prevents direct comparison, this shows that an organism lacking a functional HSF can exist and survive. Thus, missing a fully functional HSF1 could lie behind the inability of this algae to regulate HSP accumulation during stress.

4.4. Is an absent HSR a signature of psychrophiles?

C. priscuii is the first known photopsychrophile with an absent HSR at the level of HSP accumulation. We also examined temperature-induced HSP accumulation in ICE-MDV, another Lake Bonney native. We showed that it accumulates HSP70 and HSP90s on a protein level, but only with moderate intensity (Figure 17). In ICE-MDV, this response is an intermediate between that of *C. priscuii* (no response) and *C. reinhardtii* (robust response). ICE-L is a sea-ice strain of ICE-MDV (Cook *et al.,* 2019), and examination of its genome showed a similar proportion of conserved HSEs in its promoter regions as *C. reinhardtii* (Figure 8). These results suggest that ICE strains have an attenuated, but nonetheless present HSR. Strains of ICE have been found throughout the Antarctic coast, including at various depths in the water column of Lake Bonney. Many of these environments are prone to drastic changes in temperature, salinity, light availability, and UV light (Morgan-Kiss *et al.,* 2006). In contrast, *C. priscuii* is found within a very narrow region of the Lake Bonney water column. The extraordinarily isolated and stable conditions of Lake Bonney (Morgan-Kiss *et al.,* 2006) release this alga from the selective pressure of a variable environment. This isolation is likely the reason behind its absent HSP accumulation.

Our work is the first to provide a detailed and comprehensive examination of HSP responses in a psychrophilic green alga. Very few polar photosynthetic species have been characterized regarding their HSR. The best-studied psychrophilic plant is the Antarctic hairgrass *Deschampsia antarctica*, which was shown to accumulate HSP70s in its leaves after heat stress (Reyes *et al.,* 2003). There have been very few other studies in other psychrophilic algae, such as the Antarctic diatom *Chaetoceros neogracile*, which showed a modest increase in HSP transcripts during heat stress (Hwang *et al.,* 2008).

It should be noted that both species are exposed to a larger range of environmental conditions compared to *C. priscuii*. *D. antarctica* is native to the maritime Antarctic, which experiences surface air temperature ranges from -7-2°C during the Antarctic summer growing season, high intensities of UV light, and a mean annual precipitation of 75 cm in some locations (Strauss *et al.,* 2009). *C. neogracile* (and other polar diatoms) are found in open sea and at the ice-water interface, where they experience a range of temperatures between -2-5°C and extreme photoperiods annually (Gwak *et al.,* 2010).

More detailed studies characterizing psychrophilic HSRs have been done with psychrophilic marine animals. One of the earliest cases of the absent HSR was described in the Antarctic hydra, *Hydra oligactis.* In response to heat, there was no increase in HSP70 transcript levels (Gellner *et al.,* 1992), although there was an activation of transcription in response to heat (Brennecke *et al.,* 1998). A functional heat shock transcription factor (HSF) was present in the organism, but the half-life of HSP70 transcripts was found to be much shorter in *H. oligactis* than its temperate relatives (Brennecke *et al.,* 1998). *H. oligactis* is therefore believed to have lost its HSR due to decreased mRNA stability.

Another Antarctic psychrophile with an absent HSR is the ciliate *Euplotes focardii*. This organism does not induce additional HSP accumulation on a transcript or protein level during heat stress (La Terza *et al.*, 2001; La Terza *et al.,* 2000). A recent study showed that none of the seven HSP70 genes identified in *E. focardii* responded to thermal stress (Mozzicafredo *et al.,* 2021). A characterization of an HSP70 showed that it has conserved heat shock elements, and an HSF transcript was isolated (La Terza *et al.,* 2007). In this same study, it was posited that HSF had degraded and lacked HSE binding capability, but this work was unpublished and not investigated further (La Terza *et al.,* 2007).

Many Antarctic notothenioid fish have been found to lack an HSR, starting with *Trematomus bernacchii* (Buckley *et al.,* 2004). This fish has an extremely limited range of permissible growth temperatures (-2 to 4°C) and experiences heat shock at only 8°C (Weinstein & Somero, 1998). Both temperature stress and cadmium treatment, which causes protein misfolding, failed to increase HSP accumulation in these fish (Buckley *et al.,* 2004). An HSF with promoter-binding activity was successfully isolated in the same study, suggesting that it is present and functional despite the absent HSR. Moreover, HSFs isolated from multiple notothenioid fish had highly conserved sequences compared to their mesophilic relatives, and the HSFs of the Antarctic fish contained specific amino acid substitutions in key post-translational modification sites, which are known to affect HSF activity and regulation in mesophiles (Bilyk *et al.,* 2021). Despite this apparent high selective pressure on HSF, several studies have found that *T. bernacchii* HSPs have fewer HSEs, and that these HSEs had a high rate of mutation (Tercero & Place, 2020; Bogan & Place, 2019). It is believed that notothenioid fish have lost their HSR due to HSE degeneration in HSP promoter regions. These results closely resemble what was seen in *C.*

priscuii here, despite the immense phylogenetic distance between the two. The function of HSF in these fish remains unclear, although HSF has been shown to have important roles in aging and development of multicellular organisms aside from inducing HSPs (Barna *et al.*, 2018).

Overall, many psychrophiles across different taxa seem to have a delayed, attenuated, or fully absent heat stress response, although the mechanism underlying the abnormal HSR is species-specific. One pattern does seem to emerge, however: species who live in stable environments and do not experience environmental change (e.g., *C. priscuii*, notothenioid fish) seem to lose their HSP regulation, while organisms that still experience a range of different environmental conditions (e.g., ICE-MDV, Antarctic plants) retain the ability to induce HSP accumulation. This suggests that an absent heat stress response is a consequence of a life in an isolated and stable environment, rather than a hallmark of psychrophily.

4.5. What is the role of HSPs in psychrophiles?

Aside from their role during environmental stress, HSPs play an important role in new protein synthesis and maintaining protein homeostasis. At colder temperatures, enzyme activity and reaction rates decrease, and overexpressing a protein so that it accumulates at higher levels within the cell is one method of increasing the overall reaction rate (Somero, 2004). Additionally, proteins can denature in the cold as well as hot temperatures (Graziano, 2014), and some polar marine species have higher RNA:protein ratios than temperate species, indicating that proteins are being synthesized but not retained (Fraser *et al.*, 2002). Considering these lines of evidence, it has been proposed that higher HSP concentrations are necessary in psychrophilic organisms to maintain proper protein synthesis and stability in steady-state conditions (Peck, 2015). To investigate these potential solutions, HSPs have been studied in a plethora of Antarctic psychrophilic species.

C. priscuii has duplications in members of the HSP70, HSP100, and sHSP gene families (Cvetkovska *et al.*, 2022). Of note, *C. priscuii* has 7 copies of the cytosolic HSP70A. *C. reinhardtii* has only one HSP70A gene which is involved in many processes outside of heat shock, including flagellar assembly, cell division and microtubule stability (Schroda and Vallon 2009). Additionally, *C. priscuii* has 6 chaperonin CPN60 genes (compared to 3-4 in *C. reinhardtii*), which are chloroplast-localized HSP60s that play a prominent role in the folding of the Rubisco large subunit in photosynthetic organisms (Hartl and Hayer-Hartl 2009). Notably,

high levels of Rubsico have been detected in C. priscuii (Dolhi et al. 2013), possibly enabled by increased amount of CPN60s. HSP gene family duplications were also seen in the sea-ice green alga ICE-L, particularly in the HSP100, HSP90, HSP60, and sHSP gene families (Cvetkovska *et al.,* 2022).

Beyond algae, HSP gene family duplications have been reported in other Antarctic psychrophiles. Two species of Antarctic krill (*Euphausia. superba* and *Euphausia crystallorphias*) have more than 4 isoforms of HSP70 (Cascella *et al.,* 2015). HSP70 and HSP40 gene duplications have been reported in the Antarctic fish *T. bernacchii* (Tercero & Place, 2020). The fixation of duplicate genes may be driven by positive selection for higher protein accumulation as an adaptive mechanism (Kondrashov, 2012). These gene duplications in psychrophiles of distantly related taxa suggest an important role for HSPs in the psychrophilic lifestyle.

Many HSPs in *C. priscuii* are constitutively expressed at high levels based on RNA-Seq data (Figure 9, Table S4), and accumulate at high levels during non-stress conditions. In comparison, HSP levels were low in *C. reinhardtii* in the absence of heat stress (Cvetkovska *et al.* 2022). While HSP accumulation does not respond to environmental stressors in *C. priscuii*, they are nonetheless present in the cell in high amounts. This is mirrored in other aspects of the stress responses in this alga. The primary metabolome of *C. priscuii* grown at its native temperature (4°C) is geared towards the constitutive accumulation of many compounds with stress related functions, including antioxidants, polyamines, glycerol, and soluble sugars (Cvetkovska *et al.,* 2022). These compounds are present at high amounts at low temperatures, but do not accumulate further when this alga is exposed to heat. It has been postulated that this feature enables growth at extreme conditions. The presence of high amounts of HSPs and their lack of regulation during stress implies an important role during homeostatic growth conditions at extreme conditions, rather than a response to stress.

Important insights on the function of HSPs in the cold can be gleaned from studies with other photosynthetic organisms. When *C. reinhardtii* is exposed to a sudden cold shock (7°C), there is an increase in transcript and protein levels of three HSP70 isoforms and the HSF1 transcription factor (Maikova *et al.,* 2016). The Trebouxiophyte green alga *Auxenochlorella protothecoides* induced the expression of the HSFA1d and several HSP genes during prolonged cold stress

(Xing *et al.,* 2022). Cold stress (5°C) induced rapid upregulation of pathways related to protein processing and transcript accumulation of several small HSPs in the rhodophytes *Pyropia yezoensis* and *Gracilariopsis lemaneiformis* (Uji *et al.,* 2019; Qin *et al.,* 2021). HSPs are also prominent players in cold and freezing stress in land plants (Timperio *et al.,* 2008), suggesting a broad role in low temperature resilience.

As with HSP gene duplications, constitutive HSP expression has been reported in evolutionarily distant taxa. HSPs are constitutively expressed on a transcript level in the well-studied notothenioid fishes, including *T. bernacchii, Harpagifer antarcticus, and Chionodraco hamatus* (Hofmann *et al.,* 2000; Clark *et al.,* 2008; Garofalo *et al.,* 2019; Bilyk *et al.,* 2021). This was also seen the phylogenetically distant Antarctic fish *Lycodichthys dearborni*, belonging to the sub-order Zoarcoidei (Place & Hofmann, 2005). Apart from fish, high constitutive HSP70 expression was also found in a diverse group of other animals: the ciliate *Euplotes focardii,* the sea urchin *Sterechinus neumayeri,* and the krill *Euphausia superba*, among many others (La Terza *et al.,* 2000; Gonzàlez-Aravena *et al.,* 2018; Toullec *et al.,* 2020). Interestingly, the adults of the Antarctic midge *Belgica antarctica* have HSP expression akin to their temperate relatives; however, their larvae, which freeze in ice experience chronically stable, cold temperatures, have high constitutive expression of HSP70s, HSP90s, and sHSPs, and lack an HSR (Lopez-Martinez *et al.,* 2008). These numerous incidents of constitutive HSP expression further support the hypothesis that HSPs are important to the psychrophilic lifestyle.

C. priscuii may have lost the ability to tightly regulate its HSR as a consequence of life in a very stable environment, however, this environment is still very extreme. The need to maintain effective protein folding and cellular function in the permanent cold may have caused the HSP gene family expansion in *C. priscuii*, their high constitutive expression, as well as their apparent lack of regulation in response to changing abiotic stressors.

Chapter 5: Conclusion and Future Work

The psychrophilic *C. priscuii* is the first known green alga that cannot regulate its HSPs in response to environmental stresses. Additionally, this is the first detailed characterization of HSF in photopsychrophilic algae, and the first comprehensive characterization of their molecular chaperones. It is possible that the mechanism behind this loss is due to a degeneration of HSEs the promoter regions of HSPs and peculiarities in the HSF sequence (Figure 18).

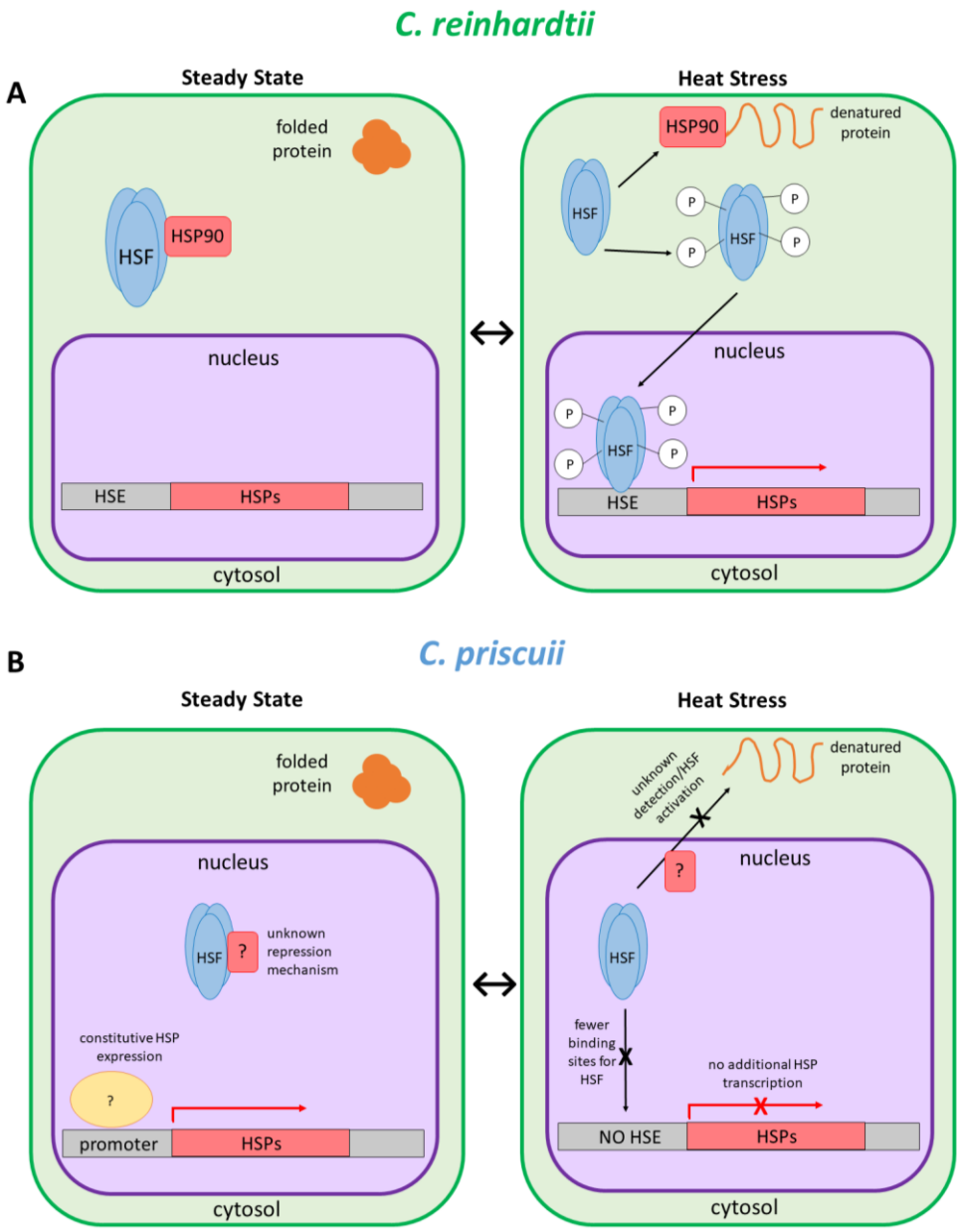

Figure 18. Proposed mechanism behind the loss of HSR in *C. priscuii*. A) Regulation of HSF1 in *C. reinhardtii* (based on Schroda *et al.*, 2015). In steady state conditions, HSF1 is present as a trimer in the cytosol, inactivated by HSP90. During heat stress, when proteins get denatured, HSP90 becomes titrated away from HSF1, and HSF1 becomes phosphorylated and localized to the nucleus. There it binds HSEs in the promoter regions of heat-responsive genes. B) Proposed regulation of HSF1 in *C. priscuii*. In steady-state conditions, HSF1 is present as a trimer in the nucleus, where HSPs are being transcribed at a high constitutive level. In heat stress, degenerated HSEs prevent HSF's ability to bind to heat responsive genes, preventing their additional transcription. The detection of unfolded proteins and HSF regulation (if any) is currently unknown.

To fully characterize the heat stress response, the future work begins with isolating the full-length HSF1 transcript as it appears in the cell. Sequencing it will confirm its length and functional domains, providing insight on how this transcription factor works. Next, creating antibodies to probe for the HSF1 protein will help understand its accumulation in steady-state and abiotic conditions on a protein level. Genetic manipulation of the *C. priscuii* genome and HSF1 expression, such as HSF1 knock-out and RNAi, can determine the effect of a missing HSF1 on *C. priscuii*'s physiology. Finally, an electrophoretic mobility shift assay (EMSA) (as described in Buckley *et al.,* 2004), which tests if a protein interacts with nucleic acids, can be performed to determine whether *C. priscuii* HSF1 has HSE-binding capabilities.

Next, the unique response of this alga should be placed in a wider context. Current detailed studies of the HSR in psychrophiles span very phylogenetically distant taxa, such as fish and marine invertebrates. While finding similar responses across a breadth of organisms is interesting, it is difficult to draw a single conclusion from these comparisons due to the distant relationships between these organisms and green algae. It has been suggested that psychrophily has emerged independently many times within different cold-temperature taxa and thus overall conclusions of psychrophilic traits may be difficult to make. The order Chlamydomonadales has been studied for decades, and sampling algae from different cold habitats from within this order will allow for detailed comparative analysis of psychrophilic traits among related organisms. Several candidate psychrophilic green algae have emerged: *Chlamydomonas malina*, an Arctic marine alga from the Beaufort Sea and *C. priscuii*'s closest phylogenetic relative (Morales-Sànchez *et al.,* 2020; Possmayer *et al.,* 2016); *Chlamydomonas nivalis*, a snow alga responsible for "watermelon snow" (Bidigare *et al.,* 1993); and *Chlamydomonas klinobasis*, an uncharacterized freshwater alga from the Svalbard Archipelago (Hulatt *et al.,* 2017). Characterizing their HSP gene families, HSP accumulation, and abiotic stress responses can help elucidate the role of HSPs and the HSR in psychrophilic green algae that thrive in varied cold environments.

Since *C. priscuii* is adapted to a myriad of conditions in addition to the cold (high salinity and low light), it is an excellent organism to identify candidate genes to improve other species. The constitutive accumulation of HSPs in *C. priscuii* suggests that these proteins are important for

cold acclimation. Overexpressing these proteins in economically relevant crop plants may help confer cold tolerance and allow for their cultivation in a wider region around the world.

It seems that *C. priscuii* has lost this ability to regulate HSPs due to its extreme, stable, and isolated environment. These results illustrate just how vulnerable *C. priscuii* and other psychrophiles like it are in the face of climate change. The melting ice cover in Lake Bonney would increase the amount of freshwater and irradiance in the ecosystem (Obryk *et al.*, 2019). If this alga is adapted to only one set of conditions and cannot fine-tune its HSP amounts in response to changing abiotic factors, it may not survive when its habitat inevitably changes due to warming temperatures. Furthermore, this impacts not just the one species of algae: since green algae are important primary producers in polar ecosystems, having their survival be threatened will in turn threaten the entire ecosystem. Beyond the other denizens of Lake Bonney, the Earth is full of isolated ecosystems populated by highly specialized species. Characterizing their environmental stress responses is one step towards mitigating a climate disaster and saving polar biodiversity.

Appendix

Table S1. HSF sequences used for *C. priscuii* HSF alignment and phylogeny.

	Order	Accession ID	Source
Arabidopsis thaliana	Brassicales	AT4G17750.1	Phytozome v13
Chlamydomonas eustigma	Chlamydomonadales	g8032.t1	Phycocosm
Chlamydomonas incerta	Chlamydomonadales	g3063.t1	Phycocosm
Chlamydomonas priscuii	Chlamydomonadales	rna-FOA52_010560	Phycocosm
Chlamydomonas reinhardtii	Chlamydomonadales	Cre09.g387150	Phytozome v13
Chlamydomonas schloesseri	Chlamydomonadales	g3500.t1	Phycocosm
Chlorella sorokiniana	Chlorellales	CSJ00010868-RA	Phycocosm
Chlorella variabilis	Chlorellales	gw1.24.267.1	Phycocosm
Chromochloris zofingiensis	Sphaeropleales	Cz04g32130.t1	Phycocosm
Dunaliella salina	Chlamydomonadales	Dusal.0228s00017.1	Phycocosm
Edaphochlamys debaryana	Chlamydomonadales	g2392.t2	Phycocosm
Gallus gallus	Galliformes	NP_001292185.1	NCBI
Gonium pectorale	Chlamydomonadales	rna2986	Phycocosm
Homo sapiens	Primates	NP_005517.1	NCBI
Medicago truncatula	Fabales	AES95803.1	NCBI
Mus Musculus	Rodentia	NP_001318083.1	NCBI
Oryza sativa	Poales	LOC_Os05g45410.1	Phytozome v13
Physcomitrium patens	Funariales	Pp3c11_20430V3.1	Phytozome v13
Scenedesmus obliquus	Sphaeropleales	maker-scaffold_80-augustus-gene-2.105-mRNA-1	Phycocosm
Trematomus bernacchii	Perciformes	XP_033981220.1	NCBI

Table S2. HSP accession IDs in Phycocosm (*C. priscuii* v1.0), Phytozome v13 (*C. reinhardtii* v5.6), and GenBank (*C.* sp. ICE-L (PRJNA636631)).

	C. priscuii		*C. reinhardtii*		*C.* sp. ICE-MDV	
sHSPs	HSP22A-1	rna-FOA52_007552	HSP22A	Cre07.g318800	HSP22A-1	JACBWV010000626: 29,447,384 (partial)
	HSP22A-2	rna-FOA52_003312	HSP22B	Cre07.g318850	HSP22A-2	JACBWV010000099: 13,183,087 (partial)
	HSP22A-3	rna-FOA52_014211	HSP22C	Cre03.g145787	HSP22A-3	JACBWV010000099: 13,263,365
	HSP22A-4	rna-FOA52_012758	HSP22D	Cre01.g020575	HSP22A-4	JACBWV010000099: 13,269,513
	HSP22A-5	rna-FOA52_008985	HSP22E	Cre14.g617450	HSP22A-5	JACBWV010000099: 13,264,189
	HSP22A-6	rna-FOA52_005447	HSP22F	Cre14.g617400	HSP22A-6	JACBWV010000610: 21,353,691
	HSP22A-7	rna-FOA52_012768	HSP22G	Cre13.g572350	HSP22A-7	JACBWV010000610: 17, 248,498
	HSP22A-8	rna-FOA52_005448	HSP22H	Cre07.g318600	HSP22A-8	JACBWV010000610: 17,558,407
	HSP22A-9	rna-FOA52_012759			HSP22A-9	JACBWV010000610: 17,607,970
					HSP22A-10	JACBWV010000220: 12,621
					HSP22A-11	JACBWV010000220: 13,769
	HSP33	rna-	HSP33	Cre17.g715000	HSP33	JACBWV010000059:137,050

		FOA52_000435				
HSP40s	CDJ1	rna-FOA52_002555	CDJ1	Cre12.g507650	CDJ1	JACBWV010000730: 1,025,848
	CDJ2	rna-FOA52_000252	CDJ2	Cre07.g316050		
	CDJ3	rna-FOA52_011741	CDJ3	Cre01.g009900	CDJ3	JACBWV010000524: 3,605,489 (partial)
	CDJ4	rna-FOA52_011741	CDJ4	Cre02.g104500	CDJ4	JACBWV010000610: 62,460,754
	CDJ5	rna-FOA52_000042	CDJ5	Cre07.g320350	CDJ5	JACBWV010000610: 43,537,304
			CDJ6	Cre12.g560700		
HSP60s	CPN60A-1	rna-FOA52_009247	CPN60A	Cre04.g231222	CPN60A1	JACBWV010000610: 55,297,042
	CPN60A-2	rna-FOA52_010786			CPN60A2	JACBWV010000476: 2,911,829
	CPN60A-3	rna-FOA52_011129			CPN60A3	JACBWV010000552: 6,137,028 (partial)
	CPN60B-1	rna-FOA52_014026	CPN60B-1	Cre17.g741450	CPN60B1	JACBWV010000626: 21,868,884
	CPN60B-2	rna-FOA52_014240	CPN60B-2	Cre07.g339150	CPN60B2	JACBWV010000591: 78,471
	CPN60C	rna-FOA52_013222	CPN60C	Cre06.g309100	CPN60C	JACBWV010000825: 9,108,740
HSP70s	HSP70A-1	rna-FOA52_011714	HSP70A	Cre08.g372100	HSP70A1	JACBWV010000223:16,681,483
	HSP70A-2	rna-FOA52_014816			HSP70A2	JACBWV010000476:1,655,364
	HSP70A-3	rna-FOA52_012811			HSP70A3	JACBWV010000610:25,013,404
	HSP70A-4	rna-FOA52_008984				
	HSP70A-5	rna-FOA52_008993				
	HSP70A-6	rna-FOA52_013887				
	HSP70B-1	rna-FOA52_011375	HSP70B	Cre06.g250100	HSP70B1	JACBWV010000660:1,227,449
	HSP70B-2	rna-FOA52_011379			HSP70B2	JACBWV010000610:159,354,451
	HSP70B-3	rna-FOA52_015447				
	HSP70C	rna-FOA52_000226	HSP70C	Cre09.g393200	HSP70C	JACBWV010000610:113,557,794
	HSP70D	rna-FOA52_013510	HSP70D	Cre12.g535700	HSP70D	JACBWV010000821:115,188
	HSP70E	rna-FOA52_003223	HSP70E	Cre16.g677000	HSP70E	JACBWV010000839:1,277449
	HSP70G-1	rna-FOA52_003181	HSP70F	Cre09.g412880		
	HSP70G-2	rna-FOA52_001792	HSP70G	Cre10.g439900	HSP70G	JACBWV010000364:17,127,077
	BIP1	rna-FOA52_016081	BIP1	Cre02.g080700	BIP1	JACBWV010000610: 23,713,042

	BIP2	rna-FOA52_016081	BIP2	Cre02.g080600		
HSP90s	HSP90A	rna-FOA52_007700	HSP90A	Cre09.g386750	HSP90A1	JACBWV010000364: 6,770011
					HSP90A2	JACBWV010000610: 101,256,406
	HSP90B	rna-FOA52_016082	HSP90B	Cre02.g080650	HSP90B1	JACBWV010000610: 52,079,131 (partial)
					HSP90B2	JACBWV010000688: 1,356,877
	HSP90C	rna-FOA52_005954	HSP90C	Cre12.g514850	HSP90C1	JACBWV010000626: 5,311,990
					HSP90C2	JACBWV010000626: 24,742,627
HSP100s	ClpB1	rna-FOA52_000032	ClpB1	Cre11.g467644	CLPB1	JACBWV010000610: 69,071,851
	ClpB3-1	rna-FOA52_012992	ClpB3	Cre02.g090850		
	ClpB3-2	rna-FOA52_008212	ClpB4	Cre11.g467575	CLPB3	JACBWV010000730: 13,079,709
	ClpB3-3	rna-FOA52_012183	ClpB5	Cre12.g533351	CLPB4	JACBWV010000610: 3,145,419
	ClpB3-4	rna-FOA52_012017			CLPB5	JACBWV010000099: 12,628,935 (partial)
	ClpB3-5	rna-FOA52_015182			CLPB6	JACBWV010000223: 4,992,409
	ClpB3-6	rna-FOA52_000763			CLPB7	JACBWV010000610: 22,000,032
	ClpB3-7	rna-FOA52_003161			CLPB8	JACBWV010000610: 73,910,048
	ClpD	rna-FOA52_013900	ClpD	Cre10.g465550	CLPD1	JACBWV010000476: 2,930,825
					CLPD2	JACBWV010000610:119,018,665
	ClpX-1	rna-FOA52_012727	ClpX	Cre16.g663350	CLPX	JACBWV010000610: 36,957,236 (partial)
	ClpX-2	rna-FOA52_003593				
	HSLU1	rna-FOA52_001522	HSLU1	Cre12.g510900	HSLU1	JACBWV010000626: 6,847,734
	HSLU2	rna-FOA52_006344	HSLU2	Cre02.g147850	HSLU1	JACBWV010000243: 1,222,068

Table S3. Sequences of heat shock elements (HSEs) detected in the promoter regions of HSP genes in *C. priscuii, C. reinhardtii*, and ICE-L. Underlined bases indicate deviations from the canonical HSE. Empty cells indicate that an HSE was not detected in the promoter of that gene. All sequences are in the 5' → 3' direction. The genomes of all three organisms were previously screened for the presence of HSPs (Cvetkovska et al, 2022).

	C. priscuii		***C. reinhardtii***		**ICE-L**	
	Gene	**HSE Sequence**	**HSP**	**HSE Sequence**	**HSP**	**HSE Sequence**
small HSPs	HSP22A-1	CGAAAAGTCTGGAAC	HSP22A	AGAAACTGCA AGAAGCGACCGGAA GGAGTTTCTACAA	HSP22A-1	
	HSP22A-2		HSP22B	CGACGCTTCGAGAAG GGATCCTTCCAGAACC	HSP22A-2	TGTAACTTCT
	HSP22A-3		HSP22C	GGATTCTTCT	HSP22A-3	GGAACGTTCGAGAGC AGAAGCTTCC
	HSP22A-4		HSP22D	CAAATCTTCCAGAACG	HSP22A-4	
	HSP22A-5	CGCAGTTTCTGGAGG	HSP22E	GGAAGGTTCG CACACGTTCTAGAAG CCAAGGTTCTAGAAGC	HSP22A-5	CGAAACTTCTCGAAC GGAACAATCCAGACG GGAAGCTTCT
	HSP22A-6		HSP22F		HSP22A-6	GAAGTATCTGGAT
	HSP22A-7	GGAAGAGTCTCGAGG	HSP22G	GGAGGGTTCGGGAAA	HSP22A-7	GGAAACTTAG CGAAGTATCTGGATA CGAAACTTCG
	HSP22A-8		HSP22H	GGAAGCTCCAAGAAG GGAATGCTCTCGAAC	HSP22A-8	
	HSP22A-9	GGAACCCTGTGGAAC AGACTCTTCCAGAGC			HSP22A-9	CGAAGTATCTGGATA
					HSP22A-10	CGAAACTTCTCGAAC
					HSP22A-11	CGAACATTCT AGAATGTTCGAGAGC
	HSP33	GGAAGCTGCTT	HSP33		HSP33	GGATGCCTCTTGAAC
HSP40s	CDJ1		CDJ1	GGAAAATTCGCGAGA	CDJ1	GGAAACCTCG
	CDJ2		CDJ2			
	CDJ3		CDJ3		CDJ3	AGAAAGTTCGAGAGA
	CDJ4		CDJ4		CDJ4	
	CDJ5		CDJ5	GAAGGCACTAGAG	CDJ5	GAACTATTTGGAA
			CDJ6	TGATGATTCTGGAT		
HSP60s	CPN60A1		CPN60A		CPN60A1	AGAAAATTCA AGAACATTCTGGTGT GGGAGTTTCTCGAAG
	CPN60A2	TGACACTTCC			CPN60A2	CCCAAACTTCCA
	CPN60A3				CPN60A3	
	CPN60B1		CPN60B1		CPN60B1	GGAAGTGACCAGAAG
	CPN60B2		CPN60B2	CGAAACTTCTC	CPN60B2	AGAAGCTGCT
	CPN60C	GGCACCTTCTCGAAA	CPN60C		CPN60C	
	HSP70A-1	AGAACCTACGAGAAT	HSP70A	AGCAGCTTCA	HSP70A1	GGTACGTTCTAGAAC
P70	HSP70A-2	CGAAAGTTCT CGAAAGTTCG			HSP70A2	AGAATGTTCC
	HSP70A-3				HSP70A3	AAAAGTATCTAGATC

						AGATCCTTCC GGTACGTTCTGGAAG
	HSP70A-4	TGATGCCTCCAGAAA				
	HSP70A-5					
	HSP70A-6					
	HSP70A-7	AGAATGTTCCAGGAA				
	HSP70B-1	AGAACAGCCTAGAAG	HSP70B	GGAAGGTTCG GGAAGTTACA	HSP70B1	GGATCCTTCTAGAGA
	HSP70B-2				HSP70B2	AGAATGATCTAGAGG
	HSP70B-3	AGCAGCTTCT				
	HSP70C	CGAGGCTTCT	HSP70C		HSP70C	
	HSP70D	TGAAGGTGCTGGATC	HSP70D	TGAAAGTTCA	HSP70D	CGCAGCTTCT
	HSP70E		HSP70E	CGAACCATCT CGAAGGTTCG	HSP70E	
	HSP70G-1		HSP70F			
	HSP70G-2		HSP70G	GAAATTTTCTGGAAC	HSP70G	GGAAGCTTCT
	BIP1	CGAACCTTCG	BIP1	GGAAATTTCGAGAAC GGAATGCTCC	BIP1	TGCAGCTTCT
	BIP2		BIP2			
HSP90s	HSP90A	AGAAGTCACTCGAAG ACAAGACTCCAGAAG	HSP90A	AGAAGTTTCT	HSP90A1	AGATGCTTCC CGAAACTTCC TGAAACTTCC AGGAACTTCT AGGATCTTCC
					HSP90A2	TGAACCTTCTAGGCG
	HSP90B		HSP90B	AGAGGGTTCTCGAAA	HSP90B1	AGAAACTTTGA GGAAATTTCGC
					HSP90B2	GAATGTTCTAGGC CGAGACTTCT TGGGGGTTCTAGAAG
	HSP90C	GGTAGCTTCTGGTAA	HSP90C	GGAGACTTCT CGACGGTTCGAGAAC	HSP90C1	AGAAACGTCTAGAAA CGAATGATCTGGAAA
					HSP90C2	
HSP100s	ClpB1	AGAATACGCCAGAAA CGAACTTTCTGGAAGT	ClpB1	AGACGCTTCA	ClpB1	GGAAGGTTCCGGAAA
	ClpB3-1	AGACGATTCTAGAAT	ClpB2			
	ClpB3-2	AGAAGCATCA AGAAGCATCA	ClpB3	CGAAGGTTCTCGAGG TTAAACTTCTC	ClpB3	AGCAGCTTCT AGATACTTCA
	ClpB3-3					
	ClpB3-4		ClpB4		ClpB4	
	ClpB3-5		ClpB5	AGAACCTTCATGAAA TGAAACTTTA	ClpB5	AGGATCTTCA
	ClpB3-6				ClpB6	GGAAGCTTCA AGCAGCTTCT
	ClpB3-7				ClpB7	TGAATTTTCT
					ClpB8	
	ClpD		ClpD	CGAAACTGCT	ClpD1	
					ClpD2	
	ClpX-1		ClpX	CGAAACTGCT	ClpX	

	ClpX-2	CGAATCATCG				
	HSLU1		HSLU1	AGAAGCTCCT	HSLU1	TGAAACTTGAA
	HSLU2		HSLU2	AGAAGCATCGGCAAG	HSLU2	AGAAGAAGCCAGATG

Table S4. Fold-changes of HSP transcript levels in *C. priscuii* when grown at 4°C and exposed to heat shock (24°C) for 6 hours. Values of N/A designate cases where the expression is below the detection threshold at 4°C. Bolded values represent a fold-change >4, and highlighted rows represent HSPs with at least one HSE in its promoter region.

Gene Family	Gene ID	Gene	FPKM at 4°C	FPKM at 24°C	4 → 24°C (FC)
HSF	scf7180000014819_CUWO241_v1.0-g10710	HSF1	8.34	9.30	1.11
sHSP	scf7180000015005_CUWO241_v1.0-g14937	HSP22A-1	0	0.71	**N/A**
	scf7180000011828_CUWO241_v1.0-g5467	HSP22A-2	7.26	3737.44	**514.48**
	scf7180000011327_CUWO241_v1.0-g436	HSP22A-3	5.11	7083.10	**1385.57**
	scf7180000012186_CUWO241_v1.0-g7581	HSP22A-4	0	0.18	**N/A**
	scf7180000011555_CUWO241_v1.0-g3322	HSP22A-5	0.05	0.32	**6.19**
	scf7180000012632_CUWO241_v1.0-g9028	HSP22A-6	2.24	1469.61	**656.12**
	scf7180000014937_CUWO241_v1.0-g12922	HSP22A-7	8.63	1645.50	**190.57**
	scf7180000011828_CUWO241_v1.0-g5468	HSP22A-8	9.47	1156.19	**122.08**
	scf7180000014937_CUWO241_v1.0-g12912	HSP22A-9	4.69	2972.65	**633.36**
	scf7180000014986_CUWO241_v1.0-g14370	HSP33	38.47	17.86	0.46
HSP40	scf7180000011484_CUWO241_v1.0-g2562	CDJ1	74.37	45.77	0.62
	scf7180000011314_CUWO241_v1.0-g252	CDJ2	18.28	37.48	2.05
	scf7180000014876_CUWO241_v1.0-g11891	CDJ4	14.57	15.99	1.10
	scf7180000011308_CUWO241_v1.0-g42	CDJ5	19.86	8.96	0.45
HSP60	scf7180000012988_CUWO241_v1.0-g9306	CPN60A1	133.33	160.30	1.20
	scf7180000014828_CUWO241_v1.0-g10936	CPN60A2	89.17	11.35	**0.13**
	scf7180000014842_CUWO241_v1.0-g11279	CPN60A3	135.87	33.15	**0.24**
	scf7180000014987_CUWO241_v1.0-g14400	CPN60B2	137.58	48.64	0.35
	scf7180000014951_CUWO241_v1.0-g13378	CPN60C	235.57	581.94	2.47
HSP70	scf7180000014874_CUWO241_v1.0-g11864	HSP70A-1	260.91	790.92	3.03
	scf7180000015006_CUWO241_v1.0-g14978	HSP70A-2	1924.96	5403.09	2.81
	scf7180000012632_CUWO241_v1.0-g9027	HSP70A-3	2.95	1521.00	**515.80**
	scf7180000012632_CUWO241_v1.0-g9036	HSP70A-4	0.07	38.36	**521.96**
	scf7180000014981_CUWO241_v1.0-g14046	HSP70A-5	0.65	9.22	**14.27**
	scf7180000014938_CUWO241_v1.0-g12965	HSP70A-6	0	0.02	**N/A**
	scf7180000014855_CUWO241_v1.0-g11525	HSP70B-1	431.19	1678.28	3.89
	scf7180000014855_CUWO241_v1.0-g11529	HSP70B-2	0.14	2.76	**19.58**
	scf7180000015023_CUWO241_v1.0-g15609	HSP70B-3	10.96	6.07	0.55
	scf7180000011314_CUWO241_v1.0-g226	HSP70C	224.86	312.82	1.39
	scf7180000014963_CUWO241_v1.0-g13667	HSP70D	7.35	1.43	**0.19**

	scf7180000011549_CUWO241_v1.0-g3232	HSP70E	174.69	1028.00	**5.88**
	scf7180000011547_CUWO241_v1.0-g3190	HSP70G-1	73.95	232.49	3.14
	scf7180000011407_CUWO241_v1.0-g1794	HSP90G-2	4.99	5.83	1.17
	scf7180000011611_CUWO241_v1.0-g3811	BIP1	723.54	2119.84	2.93
	scf7180000015050_CUWO241_v1.0-g16243	BIP2	11.22	299.12	**26.67**
HSP90	scf7180000012203_CUWO241_v1.0-g7729	HSP90A	715.95	5164.20	**7.21**
	scf7180000015050_CUWO241_v1.0-g16244	HSP90B	203.83	641.97	3.15
	scf7180000011907_CUWO241_v1.0-g5977	HSP90C	261.16	479.99	1.84
HSP100	scf7180000011308_CUWO241_v1.0-g32	ClpB1	6.38	543.31	**85.23**
	scf7180000014944_CUWO241_v1.0-g13147	ClpB3-1	1.02	680.95	**665.31**
	scf7180000012319_CUWO241_v1.0-g8248	ClpB3-2	8.76	6.48	0.74
	scf7180000014897_CUWO241_v1.0-g12168	ClpB3-3	21.43	22.34	1.04
	scf7180000015013_CUWO241_v1.0-g15344	ClpB3-4	348.58	138.16	0.40
	scf7180000015028_CUWO241_v1.0-g15924	ClpB3-5	97.92	298.90	3.05
	scf7180000011344_CUWO241_v1.0-g764	ClpB3-6	27.30	12.37	0.45
	scf7180000011543_CUWO241_v1.0-g3170	ClpB3-7	5.21	3.27	0.63
	scf7180000014981_CUWO241_v1.0-g14059	ClpD	21.49	285.62	**13.29**
	scf7180000014937_CUWO241_v1.0-g12881	ClpX-1	6.01	12.78	2.13
	scf7180000011584_CUWO241_v1.0-g3603	ClpX-2	1.72	3.00	1.74
	scf7180000011401_CUWO241_v1.0-g1523	HSLU1	20.12	14.50	0.72
	scf7180000011989_CUWO241_v1.0-g6369	HSLU2	30.69	31.29	1.02

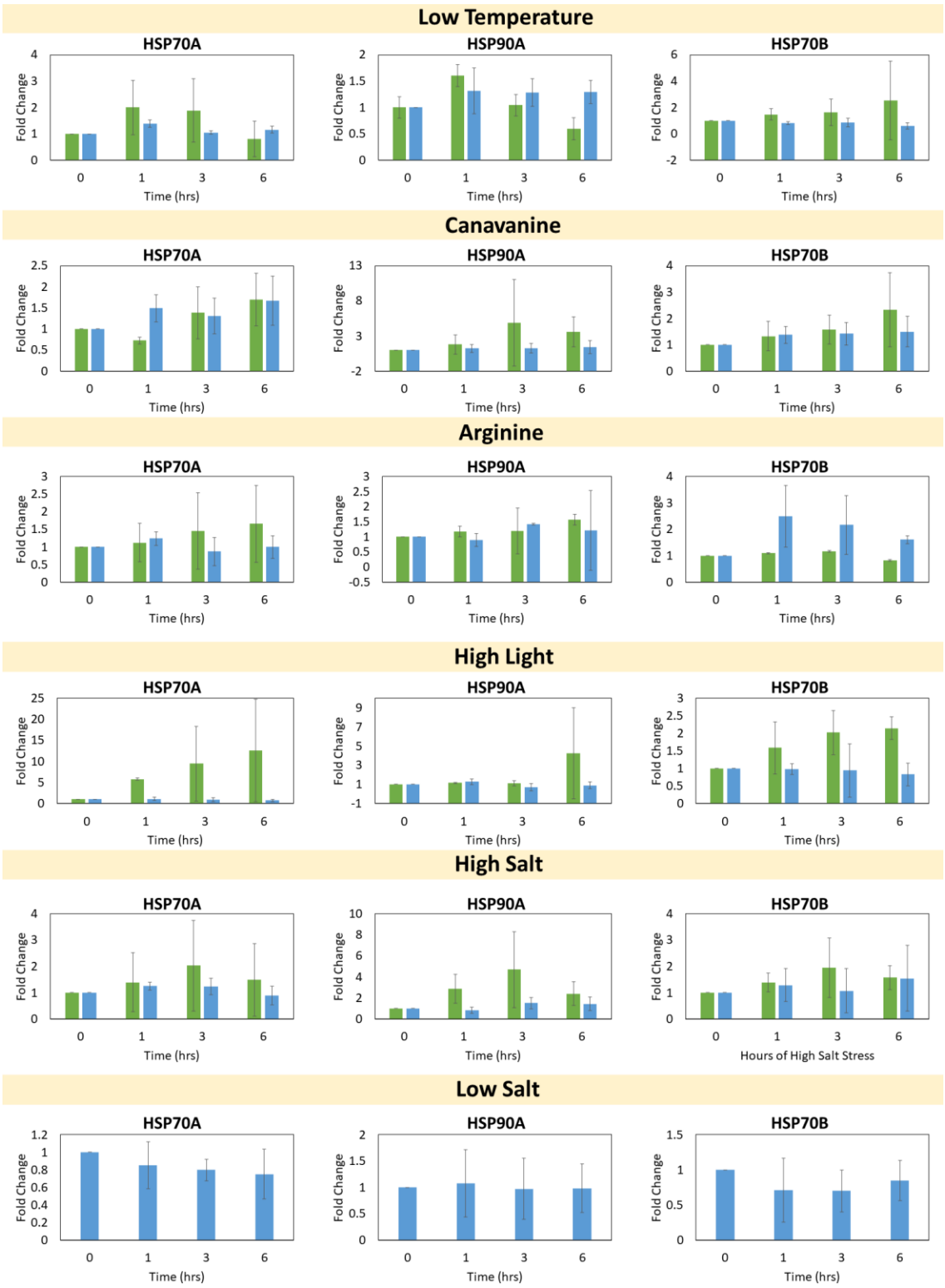

Figure S1. Densitometry analysis of the relative HSP abundance determined by western blotting in *C. priscuii* (blue) and *C. reinhardtii* (green). All blots were normalized to a Coomassie gel (total soluble protein) and represented as a mean fold change (n = 3; n = 2 for arginine treatment). The protein abundance at steady-state conditions (time point 0) was set as 1 and used

as the basis of comparison for the relative abundance of HSPs within each species. For each condition, the x-axis represents the number of hours that each treatment was applied. Error bars represent standard deviation.

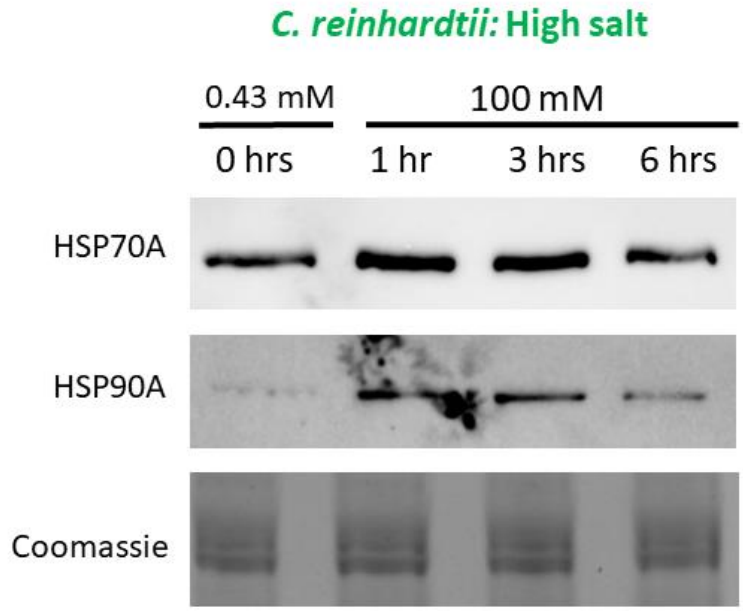

Figure S2. Representative western blots (n=3) of *C. reinhardtii* of HSP70A and HSP90A after exposure to high salt (100 mM NaCl) for 6 hours. Coomassie-stained gels are shown to demonstrate equal protein loading.

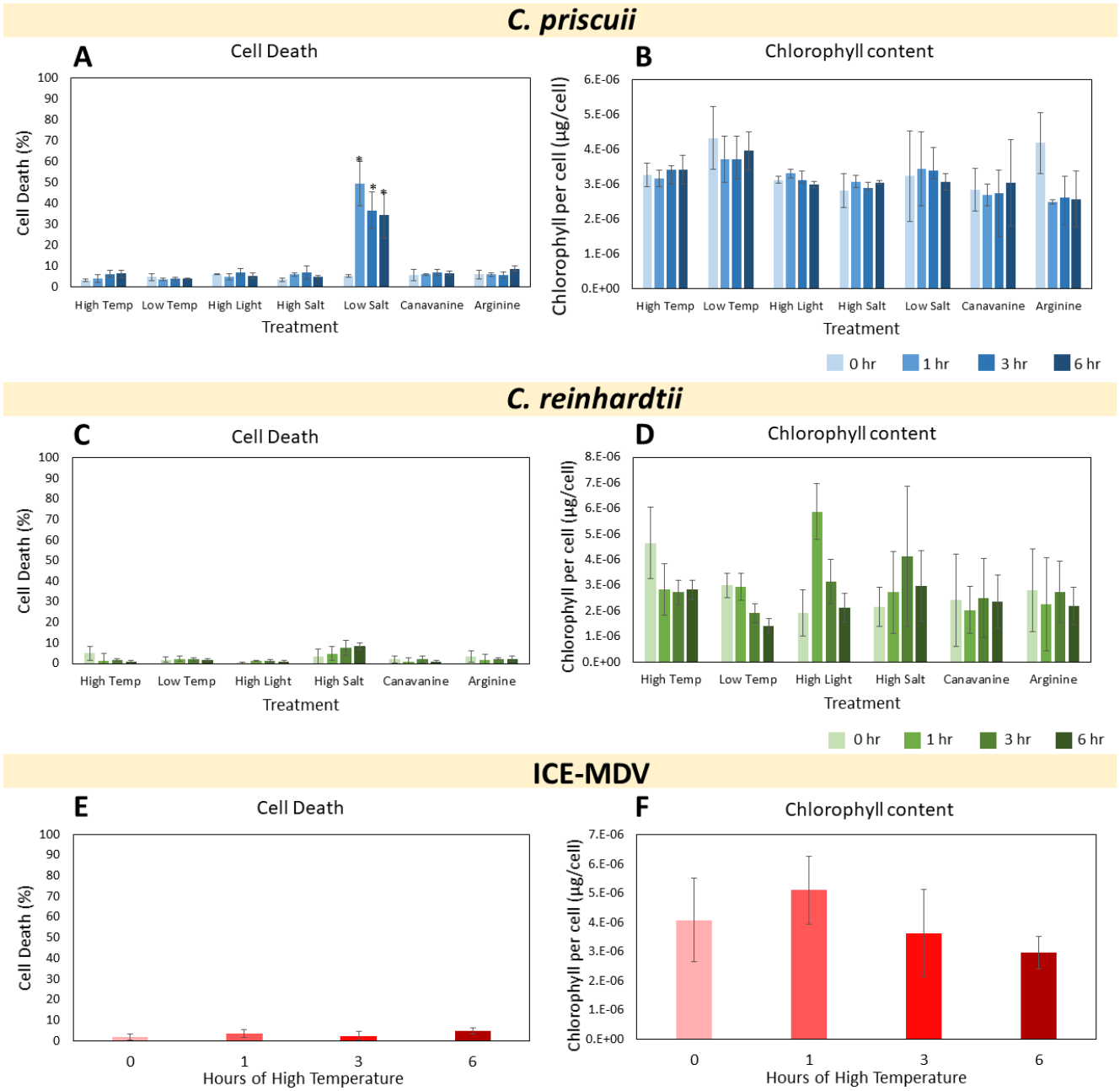

Figure S3. Chlorophyll content and cell death were measured as proxies for cellular stress during different stress treatments *C. priscuii*, *C. reinhardtii*, and ICE-MDV. A) Cell death does not significantly change in *C. priscuii* in all conditions except for low salt, where it causes a large increase. B) Chlorophyll content does not significantly change in any conditions in *C. priscuii*. C) Cell death does not change in any conditions in *C. reinhardtii*. D) Chlorophyll content does not change in any conditions in *C. reinhardtii*. E) Cell death does not change when exposing ICE-MDV to 6 hours of high temperature (24°C). F) Chlorophyll content does not change when exposing ICE-MDV to 6 hours of high temperature (24°C). Error bars represent standard deviation (n=3 in all species and conditions). An asterisk represents significant change from 0 hours for that treatment ($p<0.05$)

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