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Expression analysis of Hsp70 genes in the psychrophilic yeast, *Glaciozyma antarctica* PI12 as biomarkers for thermal stress during heat waves

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Abstract: Heat shock proteins 70 (Hsp70) are potential thermal stress markers as they play a pivotal role in safeguarding cells against heat shock-induced damage. The Hsp70s are present in several variants with each containing its peculiar importance due to their specific functions such as cell protection during elevated thermal stress. The present investigation evaluated the gene expression profiles of all Hsp70 genes in *Glaciozyma antarctica* PI12 during a heat wave condition. In this study, we exposed *G. antarctica* PI12 cells to a realistic heat wave to understand the impacts of the extraordinary, unprecedented heat waves that hit Antarctica at nearly 40°C above the average in 2022. The experiment was carried out through eight days where cells were exposed gradually at 0, 2, 4, 8, 12, 16, 20, 25 and 30°C. The gene expression profiles were obtained during the simulated heat wave along with non-stressed control treatments by real-time PCR. Out of the six Hsp70 genes in *G. antarctica* PI12, five were expressed under the conditions tested. Among the expressed genes, gahsp70-1, gahsp70-5, and gahsp70-6 showed significant upregulation. Specifically, their expression levels increased by five- to eightfold after exposure to heat shock at 4°C. Gene expression patterns at 20°C and 30°C also showed induction with the highest at 3.6 folds and 5.8 folds, respectively. These results indicate that the expression of Hsp70 genes in *G. antarctica* PI12 was inducible under thermal stress, indicating their

importance in cells during the heat waves. These results conclude that the gene expression patterns of Hsp70 during heat waves contribute vital information on thermal adaptation in the Antarctic marine ecosystem under climate stress.

Keywords: Antarctic, Casey Station, Southern Ocean, chaperone, global warming, biomarkers

Introduction

Global warming has been impacting Antarctica more immensely than before. The Antarctic Peninsula region experienced a pronounced rise in air temperatures between the 1950s and 2016, surpassing the global average warming trend. These heightened temperatures have had discernible effects on Antarctica's ecosystems, highlighting the region's vulnerability to climate change (Cannone *et al.* 2022). Between 1999 and 2016, a brief yet substantial cooling period was observed from the Antarctic Peninsula to the South Orkney Islands. However, recent heat waves, notably in March 2022, have led to a remarkable global warming effect. During this time, temperatures over the eastern Antarctic ice sheet surged to levels 10 to 32°C above normal, signaling a significant and concerning shift in regional climate dynamics (Blanchard-Wrigglesworth *et al.* 2023). In February 2020, the Antarctic Peninsula region experienced an unprecedented heat wave, marked by a new Antarctic maximum temperature of 18.4°C. This event stands as one of the most extreme heat waves ever documented in Antarctica, underscoring the profound impact of climate change on the region's environmental conditions (González-Herrero *et al.* 2022). These phenomena are alarming to environmentalists, especially global warming scientists. Up until today, global warming has been reported to alter the ecosystem distribution in the Antarctic (McBride *et al.* 2021). Antarctica is experiencing some of the most rapid changes due to global warming, making it a critical area for study in understanding the impacts of climate change on ecosystems. Studying the resistance and resilience of biological organization in Antarctica's terrestrial, freshwater and marine environments is essential for understanding how these ecosystems respond to climate change and for informing conservation and management strategies to mitigate its impacts (Convey and Peck 2019; Gutt *et al.* 2021; McGaughran *et al.* 2021; Yadav *et al.* 2023). Observing different responses of the Antarctic's living organisms towards global warming will determine their evolution or extinction. The Scientific Committee on Antarctic Research (SCAR) AnT-ERA initiative highlights the crucial need to comprehensively understand the effects of environmental changes on biodiversity in Antarctica. This initiative recognizes the importance of identifying biomarkers and understanding

ecosystem tolerance limits to assess the impacts of global warming and to enhance our understanding of adaptation and resilience.

One of the main effects of heat waves towards Antarctica's ecosystem is thermal stress (Yadav *et al.* 2023a). *Glaciozyma antarctica* PI12 is a psychrophilic yeast with strict cold-adaptation characteristics. It was discovered in the Antarctic marine waters close to Casey Station in the Southern Ocean, Antarctica. This organism thrives optimally at 12°C but demonstrates remarkable resilience, capable of surviving temperatures below freezing (<0°C). It exhibits its maximum growth potential at a temperature of 20°C (Boo *et al.* 2013). The genome analysis has unveiled a total of 7857 protein-coding genes (Firdaus-Raih *et al.* 2018). This yeast was found to have remarkable cold adaptation features such as cold-adapted and cold-active enzymes, antifreeze proteins and molecular chaperones that collectively enable the yeast to thrive in cold environments by ensuring proper enzyme function, preventing ice formation, and maintaining protein stability, thereby enhancing its survival and growth in cold conditions (Yusof *et al.* 2021). Additionally, *G. antarctica* has been documented to exhibit the expression of FAD genes crucial for enhancing membrane fluidity, particularly in freezing conditions, alongside antifreeze proteins, chaperones, and antioxidant enzymes such as SOD and exopolyphosphatase. Moreover, it expresses genes involved in the glycolytic pathway (Bharudin *et al.* 2018). Previously, Boo *et al.* (2013) conducted an analysis of gene expression levels in *G. antarctica* PI12 subjected to cold shocks and heat shocks utilizing quantitative real-time PCR. Various related studies have contributed to understanding the adaptation mechanisms of *G. antarctica* PI12 to thermal stress. Notably, among the genes exhibiting differential expression in *G. antarctica* PI12 under elevated temperatures are the 70 kDa heat-shock protein (Hsp70) genes (Yusof *et al.* 2021b).

Hsp70s represent molecular chaperones found abundantly across all living organisms. They serve critical roles in various cellular processes including protein folding, assembly, transport across biological membranes, and degradation (Horwich and Fenton 2020). Hsp70s are additionally implicated in stress response mechanisms owing to their capacity to be induced by various forms of stress (Bakhos-Douaihy *et al.* 2021; Mitra *et al.* 2021; Rathor *et al.* 2023). Hsp70s participate in protein refolding under stress conditions and regulate key proteins within signal transduction pathways (Mayer and Bukau 2005). In heat stress conditions, for instance, Hsp70s will be activated and start shielding the cell by preventing the denaturation of proteins in the cell (Kabani and Martineau 2008). The importance of Hsp70 ranging from bacteria to eukarya was reported in several studies (Moss *et al.* 2019; Rosenzweig *et al.* 2019; Karunanayake and Page 2021; Yusof *et al.* 2022). One study showed that Hsp70 was abundantly expressed in *Shewanella frigidimarina*, which is a psychrophilic bacterium when exposed to heat stress at

28°C (García-Descalzo *et al.* 2014). Another study on the *Antarctic haloarchaea* showed that *Halohasta litchfieldiae* and *Halorubrum lacusprofundi* had higher expression levels of Hsp70 at high temperatures (Williams *et al.* 2017). In addition, studies in *Drasophila melanogaster*, *Caenorhabditis elegans*, rodents, insects, and humans have also indicated that the higher the expression level of Hsp70, the better the thermal stress tolerance of cells (Gong and Golic 2006; Kuennen *et al.* 2011; Cho and Park 2019; Jin *et al.* 2020).

The mechanism of action of Hsp70s involves binding to affected proteins, thereby preventing their aggregation within cells (Ambrose and Chapman 2021). Upon cellular stress induction, a cascade of post-translational and transcriptional mechanisms unfolds. Initially, heat shock factors remain inactive within cells. However, under heat-stress conditions, they undergo post-translational modifications, transitioning into an active state. This activation leads to heightened transcription of Hsp70. Consequently, the cell efficiently translates heat shock messages to shield against stress-induced denaturation. To ensure optimal translation efficiency, pre-existing messenger RNA (mRNA) is preserved for translation, minimizing competition with new mRNA. Notably, during heat shock, previously unstable Hsp70 mRNA stabilizes, further bolstering the cellular stress response (Young 2010). Due to their critical roles, Hsp70s are highly conserved across various organisms, rendering them excellent candidates for biomarkers to indicate thermal stress (Daugaard *et al.* 2007).

In this study, the gene expression of Hsp70 from *G. antarctica* PI12 was quantified using qPCR upon induction with different heat stress ranging from 4°C to 30°C. Cells were acclimatized at 0°C for a week to measure the cell's thermal response to gradual temperature increase. Thermal stress was done until 20°C since the highest temperature ever recorded in Antarctica was 18.4°C during the heat wave. It is possible with the current global warming; the air is hotter and could cause an increment in temperature higher than 18.4°C. Since Hsp70s gene expression in *G. antarctica* PI12 has shown differential expression at 30°C, we decided to test the resistance of the Antarctic yeast until 30°C (Yusof *et al.* 2021b). Upon exposure to different temperatures respectively, the cells were harvested, and their RNA was extracted for qPCR quantification. The expression trends were observed for both strains upon induction with thermal stress. The discoveries from this study are poised to lay the groundwork for the development of biomarkers that can reliably predict the impacts of heat waves on Antarctic marine organisms.

Material and methods

Culturing *Glaciozyma antarctica* PI12. — The isolated culture of *G. antarctica* PI12 was generously provided by the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. Initially, *G. antarctica* PI12 was isolated from the Antarctic marine water near Casey Station (66° 21′ 25″ S; 110° 37′ 09″ E) in the Southern Ocean, Antarctica. The cell cultures were grown in Yeast Peptone Dextrose broth (10% (w/v) yeast extract (Sigma-Aldrich, St. Louis, Missouri, USA), 20% (w/v) peptone (Sigma-Aldrich, USA), and 20% (w/v) dextrose (Sigma-Aldrich, USA)) supplemented with 50 µg/mL ampicillin and 50 µg/mL kanamycin in shake flasks at 12°C, shaking at 180 rpm. The cultures were collected after 4–6 days or until the OD_{600} reached approximately 0.6–0.8. Subsequently, the cultures were then exposed to different temperatures of −20, 0, 12, 20, and 30°C. The cells were harvested via centrifugation at 10 000 x g at 4°C for ten minutes, then immediately snap-frozen in liquid nitrogen and stored at −80°C until further use.

Experimental set-up. — A single colony *G. antarctica* PI12 isolate was cultivated in YPD broth supplemented with 50 µg/mL ampicillin and 50 µg/mL kanamycin under 80% aeration. The cultures were maintained at their optimal growth temperature which was 12°C until reaching an optical density at 600 nm (OD_{600}) of approximately 0.6–0.8. Subsequently, the cultures were acclimatized at 0°C for seven days before thermal stress experiments. For thermal resistance analysis, *G. antarctica* PI12 cultures were exposed to a series of temperature increases that were similar to the Antarctic condition (Table 1). On Day 1, the *G. antarctica* PI12 cultures were exposed to 2°C for 24 h incubation. On Day 2, the cultures were exposed to 4°C for another 24 h. The step was repeated until the cultures were incubated at 30°C on Day 8. Sample cultures were collected on Day 0, 2, 4, 6, and 8. The samples collected on Day 0, stored at 0°C, were utilized as the control for our relative gene expression analysis. The control served on the need for a baseline reference point to establish a comparative standard against which to measure changes in gene expression over time and at different temperatures. Samples were harvested and stored at −80°C until total RNA extraction. A total of three sets of biological replicates were set up for this thermal resistance experiments analysis.

Obtaining *Hsp70* Genes from *Glaciozyma antarctica* PI12 Genome. — The nucleotide sequences of the *Hsp70* genes from *G. antarctica* PI12 have been designated as follows: gahsp70-1, gahsp70-2, gahsp70-3, gahsp70-4, gahsp70-5, and gahsp70-6. These Hsp70 sequences were extracted from the genome data of *G. antarctica* PI12 (GenBank assembly accession GCA_002917775.1). The accession numbers for the Hsp70 genes were MZ313862.1, JF412505.1, OQ625425, OQ625426, OQ625427, and OQ625428, respectively (Table 2). The

Hsp70 amino acid sequences were aligned using a multiple sequence performed with the ClustalW program (https://www.genome.jp/tools-bin/clustalw; accessed on 15 March 2023) to analyze conserved regions of the proteins, specifically focusing on the substrate binding and ATP binding domains. Following the alignment of Hsp70 amino acid sequences, primers were designed towards the 3' end of the DNA sequence Hsp70, optimizing amplification performance, avoiding non-specific binding and secondary structures using the Primer 3.0 software.

RNA Extraction, Quantification and Quality Analysis. — The total RNA of *G. antarctica* PI12 after exposure to thermal stress was extracted using the GeneJET RNA Purification kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol. Following extraction, the concentration and purity of the total RNA were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc, Carlsbad, CA, USA) using the absorbance at 230, 260, and 280 nm. The RNA integrity was assessed using 1% (w/v) agarose (Nacalai, Japan) supplemented with GelRed Nucleic Acid stain (Biotium, San Francisco, CA, USA). Gel electrophoresis was conducted at 100 V for 20 minutes, and the presence of distinct bands corresponding to 28S and 18S ribosomal RNAs was examined to verify RNA integrity.

First-strand cDNA synthesis and subsequent qPCR amplification in a single tube. — Before the cDNA synthesis and qPCR analysis, the extracted RNA underwent DNase I treatment (Thermo Scientific, USA) to eliminate any residual genomic DNA contamination, following the manufacturer's instructions. The first-strand cDNA synthesis and subsequent real-time PCR (qPCR) for each target gene were performed in a single tube using SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline, London, UK). The RT-qPCR master mix was prepared as shown in Table 3. Specific primers targeting *G. antarctica* PI12 Hsp70 genes were utilized to amplify the cDNA during the qPCR analysis (Table 4). PCRs were performed as follows: Reverse transcription at 95°C for 10 min, polymerase activation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and elongation at 72°C for 10 s. The RT-qPCR reactions were carried out using a real-time thermal cycler, CFX96 (Bio-Rad, USA). Primer pair efficiencies (E) were calculated using standard curves which were constructed using tenfold serial dilutions (100, 10, 1, 0.1, and 0.01 ng) of RNA for each primer pair. The analysis was conducted in triplicate. A one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$ was utilized to compare the expression levels of all *G. antarctica* PI12 Hsp70 genes at different temperatures. To compensate for any variation in the amount of starting material among the samples, the *G. antarctica* PI12 Hsp70 (gahsp70) expression profiles were normalized using the 18S ribosomal RNA. After the reaction had reached completion, the instrument was instructed for the option of melt-profile analysis. An amplification reaction without a cDNA

template was utilised as a blank control. Following qPCR amplification, the specificity of the PCR products was assessed through melting curve analysis and agarose gel electrophoresis (1% w/v) conducted at 100 V for 40 minutes. Subsequently, the relative expression levels of gahsp70 genes were calculated using the delta-delta Ct method, also known as the $2^{-\Delta\Delta Ct}$ method. The average expression of gahsp70 and 18S genes served as the reference for normalization (González *et al.* 2016).

Results

Primers' efficiency and specificity. — The efficiency of the Hsp70 primers had values between 100 and 110, corresponding to slopes between −3.1 and −3.3, acceptable for gene expression application (Table 5). The annealing temperature for all primers was optimal at 60°C. The coefficient of determination (R^2) values were between 0.968 and 0.999. Since the efficiency of the primers was over 100% hence, they were acceptable for qPCR applications (González *et al.* 2016). However, several attempts to develop primers for gahsp70-4 with good efficiency were unsuccessful. Hence, gahsp70-4 was excluded from gene expression analysis.

Relative expression of target genes. — The gene expression profiles of *G. antarctica* PI12 under the control condition which was at 0°C revealed varying levels of Hsp70 gene expression upon exposure to different temperatures. Overall, the response of Hsp70 genes exhibited inducible expression patterns, with pronounced induction observed in gahsp70-1, gahsp70-5, and gahsp70-6. There was a significant difference in the expression of Hsp70 genes across the four tested temperatures ($p > 0.05$) (Figure 1). The analysis of the Hsp70 expression level showed a significant increase in gahsp70-5, gahsp70-1 and gahsp70-6 at 8.1 folds, 6.9 folds and 5.4 folds, respectively, when cells were exposed at 4°C on day-2 of thermal stress treatment. On the other hand, the expression levels of gahsp70-3 and gahsp70-2 showed low increments at 2 folds and 0.8 folds, respectively. Moreover, the expression of Hsp70 genes was significantly increased when cells were exposed to temperatures higher than 12°C. The Hsp70 gene expression during thermal stress at 20°C showed the highest increase in gahsp70-1 at 3.6 folds, followed by gahsp70-6 at 1.9 folds, gahsp70-5 at 1.2 folds, gahsp70-2 at 1 fold and gahsp70-3 at 0.5-folds. Similarly to the exposure at 20°C, the expression levels of Hsp70 at 30°C exhibited the highest induction in gahsp70-1, followed by gahsp70-5, gahsp70-6, gahsp70-2, and gahsp70-3, with fold changes of 5.8, 4.1, 2.5, 1.5, and 1.1, respectively. Interestingly, the expression of Hsp70 genes in *G. antarctica* PI12 did not show a significant increase at 12°C. The expression levels were the

highest in gahsp70-3, followed by gahsp70-5, gahsp70-2, gahsp70-1, and gahsp70-6, with fold changes of 1.1, 0.8, 0.77, 0.6, and 0.57, respectively.

Discussion

This study represents the first comprehensive characterization of Hsp70 mRNA expression under heat wave thermal stress conditions in the Antarctic psychrophilic yeast *G. antarctica* PI12. To date, only a few studies explored Hsp70 gene expression profiles in Antarctic marine organisms, focusing on the heat wave thermal stress response. For instance, studies the effects of thermal stress response in organisms have been reported for *e.g.* the sea urchin *Sterechinus neumayeri* (González-Aravena *et al.* 2018), notothenioid fishes (Bilyk *et al.* 2021), clam *Laternula elliptica* (Park *et al.* 2007) and krill *Euphausia superba* (Toullec *et al.* 2020). These studies emphasized the important role of Hsp70 in mediating thermal stress and providing tolerance in the studied organisms. While studies have been done in a higher level of eukaryotes, the detailed analysis of the expression of Hsp70 in an Antarctic yeast has not been evaluated. In addition, all living organisms are known to have different variants of Hsp70 where some are associated with thermotolerance while others have their functions such as folding of polypeptides, protein three-dimensional structure and disassociation of protein complexes in biological systems (Hassan *et al.* 2019). Therefore, elucidating the physiological mechanism of Hsp70 thermal stress response, resistance, and adaptation at a cellular level is crucial for comprehending the response of *G. antarctica* PI12 to environmental challenges. Furthermore, investigating the suitability of Hsp70 as biomarkers to gauge the impact of climate change in the Antarctic regions is essential for monitoring and assessing ecosystem health and resilience. The experimental temperatures used in this study were designed to correspond to the heat wave situations that happened in Antarctica. Since some parts of the Antarctic Peninsula have reached almost 3°C over the last 50 years and heat waves in the Antarctica regions hit as high as 18.4°C (UN 2022), therefore, the information attained by us at 2°C to 30°C is relevant to study the impact of global warming to polar environment and may reveal the suitability of Hsp70 to be used as a bioindicator to measure the resistance capacity of this species in responding to thermal stress. This study contributed to a good understanding of the Hsp70 gene family in *G. antarctica* PI12 and provided an important foundation for further studies on the functional characteristics of Hsp70 genes during exposure to heat stress amidst global warming.

The heat shock protein 70s (Hsp70) are indeed highly conserved and ubiquitous molecular chaperones present across diverse life forms, spanning from microorganisms to plants and

humans. Research on Hsp70 genes has revealed the existence of multiple forms of Hsp70 family members encoded in the genome of every living organism (Rehman *et al.* 2020; Yu *et al.* 2021). In *Escherichia coli*, three genes encode Hsp70 have been identified (Zhu *et al.* 1996), 14 genes of Hsp70 in *Saccharomyces cerevisiae* (Kominek *et al.* 2013), seven genes encode Hsp70s in *Chlamydomonas reinhardtii*, nine cytosolic Hsp70 in the moss *Physcomitrella patens* (Rensing *et al*. 2008), 32 Hsp70 genes in *Oryza sativa* (Sarkar *et al*. 2013), 18 Hsp70 genes in *Arabidopsis thaliana* (Lin *et al*. 2001), seven Hsp70 genes in *D. melanogaster* (Nikolaidis and Nei 2004) and at least 13 Hsp70 homologs are present in human (Boswell-Casteel et al. 2015). The different types and numbers of Hsp70 homologs in all living organisms reflect the long evolutionary and environmental selection pressure that causes genome and gene duplications (Yu *et al*. 2015; Hu et al. 2022). In this study, a total of five Hsp70 genes in the *G. antarctica* PI12 were characterized by their gene expression profiles in the scenario of global warming in Antarctica. Indeed, studies have demonstrated that Hsp70 homologs within the same organism can exhibit considerable variation in their gene expression levels in response to environmental stressors, particularly fluctuations in temperature. The hard clam, *Mercenaria mercenaria*, showed expression patterns of some Hsp70 genes that are highly tissue-specific in response to heat and hypoxia stressors (Hu *et al.* 2022). Furthermore, the Pacific oyster *Crassostrea gigas* showed different Hsp70 gene expressions under high and low-temperature stresses (Zhu *et al.* 2016). Moreover, in the plant *Chenopodium quinoa*, gene expression exhibited compelling variations of Hsp70 homologs in response to drought stress (Liu *et al.* 2018).

In the present study, the gene expression profiles of Hsp70 genes in *G. antarctica* PI12 under thermal stress revealed significant expression of three Hsp70 genes, namely gahsp70-1, gahsp70-5, and gahsp70-6, when cells were exposed to temperatures above 0°C (control group). When cells were exposed to 4°C of thermal stress treatment, the expression levels of the mRNA Hsp70 gene were the highest in gahsp70-5 at 8.1 folds, followed by gahsp70-1 at 6.9 folds and gahsp70-6 at 5.4 folds. Interestingly the mRNA levels of Hsp70 in gahsp70-3 and gahsp70-2 were also induced at 2 folds and 0.8 folds during the 4°C stress treatment, but the increment was lower and less compelling compared to gahsp70-1, gahsp70-5 and gahsp70-6. This observation suggests that all Hsp70 genes in *G. antarctica* PI12 are activated during thermal stress treatment, but only gahsp70-1, gahsp70-5, and gahsp70-6 appear to play a critical role in defending against thermal stress. In silico structural analysis reveals that *G. antarctica* Hsp70 proteins generally exhibit fewer π-interactions at buried regions compared to their mesophilic homologs, except for gahsp70-2. π-interactions involve the stacking of aromatic rings, and their presence or absence can influence protein stability and function. Gahsp70-1 and gahsp70-6 from *G. antarctica* show

fewer aromatic-sulfur interactions compared to mesophilic Hsp70 proteins (Yusof *et al.* 2021b). Aromatic-sulfur interactions involve the interaction between aromatic rings and sulfur-containing amino acids like cysteine or methionine. Gahsp70-5 from *G. antarctica* lacks cation-π interactions that are typically found in mesophilic Hsp70 proteins at buried regions. Cation-π interactions involve the interaction between positively charged ions (cation) and aromatic rings (Mahadi *et al.* 2023). The absence of certain aromatic interactions in *G. antarctica* Hsp70 proteins, which are associated with structural flexibility, may enable these proteins to adapt to heat stress while maintaining catalytic activity at lower temperatures. This characteristic may explain the high expression of these proteins at the gene level, indicating their importance in thermal adaptation mechanisms. Moreover, the significant induction of these three Hsp70 genes in *G. antarctica* PI12 likely correlates strongly with the yeast's stress response mechanism, providing potential protection and facilitating thermal adaptation during periods of thermal stress. Earlier, an investigation into the transcriptome of *G. antarctica* revealed that cells subjected to thermal, cold, and freeze shocks synthesized signal transduction proteins, enabling them to respond effectively for survival and adaptation. Conversely, cells significantly impacted by thermal shocks exhibited increased expression of genes associated with apoptosis, indicating preparation for cell death. These findings imply that *G. antarctica* PI12, a psychrophile, possesses sophisticated cellular structures and adaptation mechanisms, enabling it to endure and overcome thermal stress (Koh *et al*. 2019). In a study on the local breed of chickens, the high expression of Hsp70 genes during thermal stress demonstrated that the gene played a protective role and allowed the breeds to be very resistant to high temperatures (Cedraz *et al*. 2017). Moreover, in Tharparkar cattle, the Hsp70 expression was found to be higher than the control corresponding to heat stress treatment which indicates that Hsp70 is possibly involved in heat stress adaptive response and protection during chronic heat stress (Bharati *et al*. 2017). Studies investigating Hsp70 gene expression in both prokaryotes (bacteria and archaea) and eukaryotes (ciliates, algae, plants, and animals) have demonstrated that Hsp70 plays a crucial role in regulating gene expression in Antarctic marine ecosystems during thermal stress (Yusof *et al*. 2022).

In contrast with the Hsp70 gene expression profile that showed induction at 4°C, the expression level at 12°C expression showed a tremendous decline to 0.63 folds, 0.77 folds, 1.17 folds, 0.8 folds and 0.57 folds in gahsp70-1, gahsp70-2, gahsp70-3, gahsp70-4, gahsp70-5 and gahsp70-6, respectively. This finding is very intriguing as theoretically it was expected to show induction in the Hsp70 gene expression similar to expression profiles at 4°C stress treatment. However, the contrast finding may reflect the response of the cells at 12°C, in which at the cell's optimal growth temperature, the over-expression of Hsp70 was probably inessential for thermal

protection (Boo *et al*. 2013). It was likely after acclimatizing at 0°C for 7 days, the cells reacted to increase the Hsp70 gene expression when exposed at 4°C to prevent protein damage, unfolding and aggregation that could happen as the temperature increases. A gradual increase in temperature up to 12°C, which coincides with the optimal growth temperature for *G. antarctica* PI12, may facilitate cellular adaptation to the temperature shift without necessitating the induction of high expression levels of Hsp70 genes in the cells. In the Antarctic fish *Notothenia rossii*, the effect of acclimation and gradual increase in temperature on the levels of stress response showed opposite responses to the thermal shock model (Guillen *et al*. 2022). Additionally, evolutionary adaptation has been reported in *Kluyveromyces marxianus*, where a gradual increase in temperature facilitated the acquisition of multi-stress tolerance (Pattanakittivorakul *et al*. 2022).

Our previous work on short-term, simultaneous thermal exposure has demonstrated that the *G. antarctica* Hsp70 gene expression levels referring to gahsp70-1 and gahsp70-2 were highly induced at 20°C followed by 30°C (Yusof *et al*. 2021b). Gradual exposure at 20°C and 30°C with longer exposure time showed both genes demonstrated induction patterns but with expression level at 30°C was higher than 20°C. The difference in the expression profiles reflects the process of an organism's adaptation change to thermal stress by phenotypic plasticity that allows survival during heat stress and acquisition of adaptation (Price *et al*. 2003). The ability of organisms to respond to environmental variations is associated with their phenotypic plasticity; phenotypic variations in morphology, physiology, and behavior aspects of an organism’s phenotype, which is acquired during evolution (Sommer 2020). A study of Hsp70 in *Leptinotarsa decemlineata* has reported that phenotypic plasticity plays a significant role in climatic adaptation (Lyytinen *et al*. 2012).

Thermal stress can disrupt cellular homeostasis and cause cell damage. The significant induction of gahsp70-1, gahsp70-5, and gahsp70-6 underscores the critical role of Hsp70 gene expression in promoting thermotolerance as a strategy for coping with thermal stress in the environment. When faced with sudden changes in environmental conditions, cells can adapt their transcriptional regulatory networks to control gene expression levels accordingly (Kim *et al*. 2020). Meanwhile, the expression of the gahsp70-2 and gahsp70-3 genes in *G. antarctica* PI12 showed mild induction probably because these genes are the constitutive members that function to lessen the effects of thermal damage such as accumulation of misfolded and non-native proteins in the cells (Kozeko 2021). Our study demonstrated that Hsp70s in *G. antarctica* PI12 respond to thermal stress either simultaneously or gradually through differential expression, reflecting the dynamic cellular response to stress and potential adaptation under conditions mimicking natural

environments. This suggests the significant potential of Hsp70s in *G. antarctica* PI12 as biomarkers for assessing thermal stress effects in Antarctic marine animals (Yusof *et al*. 2021b).

Conclusions

In conclusion, our study proposes the capability and suitability of Hsp70 in *G. antarctica* PI12 as potential biomarkers for measuring the effects of climate change in the Antarctic regions. Our results demonstrate that the expression of three Hsp70 genes (gahsp70-1, gahsp70-5, and gahsp70-6) in *G. antarctica* PI12 significantly increased, indicating their strong inducibility during thermal stress. The discoveries from this research endeavor will offer enhanced insights into the repercussions of heat waves on Antarctic microorganisms, fostering a deeper comprehension of the challenges they face in an evolving environment and further exploring the suitability of Hsp70 response in *G. antarctica* PI12 as biomarkers to measure thermal impacts in the global warming scenario. Further investigations are warranted to explore the effects of temperature fluctuations on Hsp70 expression, thereby enhancing our understanding of the resistance and resilience of polar organisms in the context of global warming.

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Table 1.

Thermal stress parameters.

Day	Temperature (°C)
0	0
1	2
2	4
3	8
4	12
5	16
6	20
7	25
8	30

Table 2.

Glaciozyma antarctica Hsp70 accession numbers and protein size.

HSP70 Genes	Genbank Accession Num.	Amino Acid Length	Molecular Weight (kDa)
gahsp70-1	MZ313862.1	672	72
gahsp70-2	JF412505.1	627	68
gahsp70-3	OQ625425	782	85
gahsp70-4	OQ625426	603	64
gahsp70-5	OQ625427	637	70
gahsp70-6	OQ625428	635	69

Table 3.

RT-qPCR master mix components.

Component	Volume (µL)	Final concentration
2x SensiFAST™ SYBR® No-ROX One-Step Mix	10	1x
10 µM Forward Primer	1	100 nM
10 µM Reverse Primer	1	100 nM
Reverse transcriptase	0.5	-
RiboSafe RNase Inhibitor	0.5	-
RNA template, 100 ng	5	25 ng
DEPC treated Water	2	-
Final volume	20	

Table 4.

List of Hsp70 primers used in qPCR.

Gene Target	Direction	Primer DNA Sequence
gahsp70-1	Forward Reverse	5′- ATCATCGCCAACGACCAGGG-3′ 5′- CTTGGCGTCGAAGACGGTGTTG-3′
gahsp70-2	Forward Reverse	5′- AGGCTCATGTCCGCCACAAC-3′ 5′- TTGGCGGGAAGTCCAACTAATCG-3′
gahsp70-3	Forward Reverse	5′- TAC TCG CAT GAT TGG AGA GAC -3′ 5′- TCT TAA GGG AAC CGA CAG TG -3′
gahsp70-4	Forward Reverse	5′- CAT TGC GAA CGA TGA TGG AG-3′ 5′- TTC AGC TTG TGC GAA ACG-3′
gahsp70-5	Forward Reverse	5′- CCA GAA GGA TCT CAA GCA CTA C-3′ 5′- TCC TTC ATC TTT CCG AGC AC-3′
gahsp70-6	Forward Reverse	5′- GAT CAT TGC CAA CGA CCA AG-3′ 5′- TGG ACC ATG TCC TTC TTG AC-3′
18S	Forward Reverse	5′- ACC AGG TCC AGA CAC AAT -3′ 5′- TAA CCA GAC AAA TCA CTC C -3′

Table 5.

Analysis of primer efficiency.

Gene	Efficiency (%)	R^2	Slope
gahsp70-1	100	0.998	−3.3
gahsp70-2	100	0.975	−3.3
gahsp70-3	105	0.999	−3.19
gahsp70-4	n/a	n/a	n/a
gahsp70-5	109	0.968	−3.11
gahsp70-6	110	0.994	−3.1

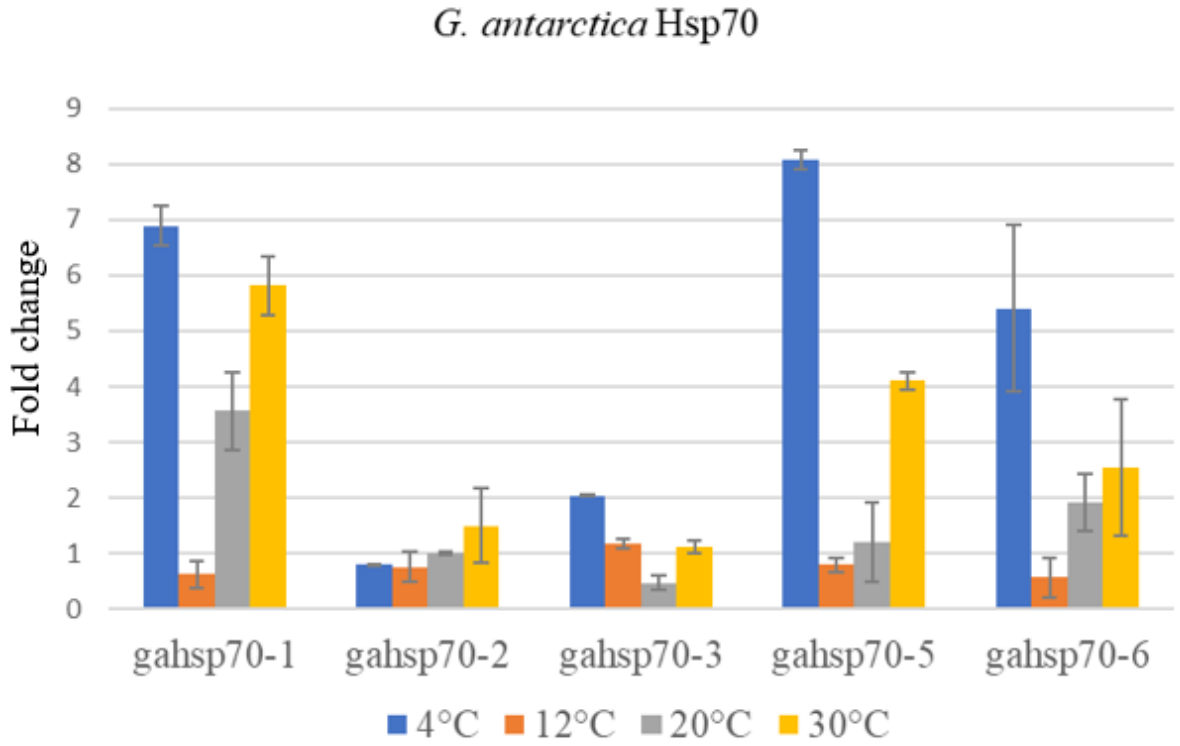

Fig. 1. Variation of gene expression during thermal stress in Hsp70 genes of *Glaciozyma antarctica*. After 7 days acclimatized at 0°C, the Antarctic yeast cells were gradually exposed to 4°C, 12°C, 20°C and 30°C for 8 days. The gene expression levels were measured in cells exposed at different temperatures of 4°C, 12°C, 20°C, and 30°C and normalized to 18S (reference gene) levels. The fold changes of the Hsp70 genes were calculated relative to the control (0°C). Error bars represented the standard deviation with $p<0.05$.