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Resilience in Greenland intertidal *Mytilus*: The hidden stress defense

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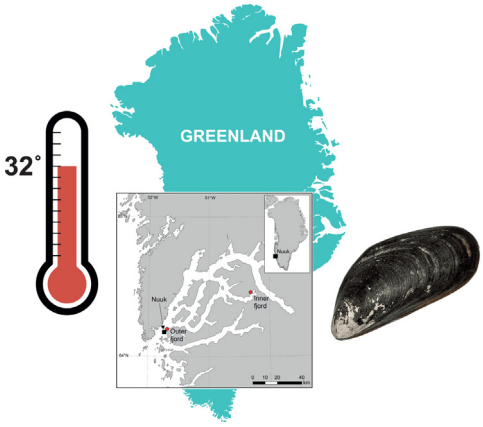
HIGHLIGHTS

- Greenland *Mytilus edulis* survive warming to 27 °C with little cellular response.
- Different transcriptomic responses in experimentally warmed and *in situ* sampled *Mytilus edulis*
- No strong stress response identified even at 32 °C.
- Acclimation and predictability of stress are key factors in cellular resilience.
- Large expansion of HSPA12 gene family, which act as intertidal cellular regulators.

GRAPHICAL ABSTRACT



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ABSTRACT

The Arctic is experiencing particularly rapid rates of warming, consequently invasive boreal species are now able to survive the less extreme Arctic winter temperatures. Whilst persistence of intertidal and terrestrial species in the Arctic is primarily determined by their ability to tolerate the freezing winters, air temperatures in the Arctic summer can reach 36 °C in the intertidal, which is beyond the upper thermal limits of many marine species. This is normally lethal for the conspicuous ecosystem engineer *Mytilus edulis*. Transcriptomic analyses were undertaken on both *in situ* collected and experimentally warmed animals to understand whether *M. edulis* is able to tolerate these very high summer temperatures. Surprisingly there was no significant enrichment for Gene Ontology terms (GO) when comparing the inner and outer fjord intertidal animals with outer fjord subtidal (control) animals, representing animals collected at 27 °C, 19 °C and 3 °C respectively. This lack of differentiation indicated a wide acclimation ability in this species. Conversely, significant enrichment for processes such as signal transduction, cytoskeleton and cellular protein modification was identified in the expression profiles of the 22 °C and 32 °C experimentally heated animals. This difference in gene expression between *in situ* collected and experimentally warmed animals was almost certainly due to the former being acclimated to a fluctuating, but predictable, temperature regime, which has increased their thermal tolerances. Interestingly, there was no evidence for enrichment of the classical cellular stress response in any of the animals sampled. Identification of a massive expansion of the HSPA12 heat shock protein 70 kDa gene family presented the possibility of these genes acting as intertidal regulators underpinning thermal resilience. This expansion has resulted in a modified cellular stress response, as an evolutionary adaptation to the rigour of the invasive intertidal life style. Thus, *M. edulis* appear to have considerable capacity to withstand the current rates of Arctic warming, and the very large attendant thermal variation.

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1. Introduction

Global warming has accelerated in recent decades, redistributing biodiversity, as species are shifting poleward (Burrows et al., 2019; Antao et al., 2020). In the northern hemisphere, the Arctic is warming 2–3 times faster than the global average and, marine boreal species are expanding into the Arctic, at a pace reflecting environmental warming (Sunday et al., 2012). Arctic intrusions of marine species are widely documented, especially in regions where major north-flowing ocean currents transport propagules of boreal taxa (Drinkwater, 2006; Bluhm et al., 2011; Renaud et al., 2015). The establishment of boreal species results in regime shifts, assemblage compositional changes, and altered food web structures (Kortsch et al., 2012, 2015; Molinos et al., 2016). For example, the fish assemblage has changed around Svalbard where Arctic species have retracted northwards to cooler areas while the abundance of boreal species has increased (Fossheim et al., 2015).

Nevertheless, the poleward expansion of boreal marine species is restricted by their ability to tolerate low water temperatures, and in the case of intertidal species, sub-zero air temperatures during winter (Sunday et al., 2012; Thyrring et al., 2020). In Greenland, however, the number of days with extreme sub-zero air temperatures has decreased by more than 50% since the 1950's, suggesting that the low air temperature constraint is weakening (Thyrring et al., 2017a). Thus, although boreal intertidal species could potentially benefit from this warmer climate to establish populations in the Arctic, recent work has shown that local intertidal summer air temperatures in Greenland can exceed 36 °C (Thyrring et al., 2017a). This temperature is above the thermoregulatory capacity of many boreal intertidal species, including the highly abundant blue mussel *Mytilus edulis*, which do not survive at 32 °C following repeated exposures to air (Jones et al., 2009). Intertidal organisms have poor capacities for acclimating to increasing temperatures (Stillman, 2003). Thus, summer conditions in Greenland may be too warm to provide a thermal refuge for intertidal species shifting northwards in response to severe heat at lower latitudes (Sanford et al., 2019), and the very large temperature variation in the Arctic intertidal may be a barrier for many species. However, the effects of heat stress in the Arctic intertidal zone are largely unexplored.

Predicting heat stress in intertidal organisms is complicated, as the body temperature during low tides is modified by a suite of physiological and ecological conditions (Gilman et al., 2006; Helmuth et al., 2006). For instance, microhabitats and surface rugosity modify local temperatures (Mota et al., 2015; Thyrring et al., 2017a), and small-scale variation in the position of an individual can result in a 14 °C difference in body temperature among neighboring animals (Miller and Dowd, 2017). Mobile species move between microhabitats for thermal refuge during emersion (Dong et al., 2017), and various rapid gene expression responses shape thermal resilience (Gracey et al., 2008; Clark et al., 2008, 2018). In addition, some intertidal species reduce their heart rate (Tagliarolo and McQuaid, 2016), or enter a stage of reversible metabolic depression to balance the detrimental temperature-induced rise in energy demand (Guppy and Withers, 2007; Dong et al., 2011). Thus, remotely sensed atmospheric air temperatures are not suitable to capture physiological conditions at the level of microhabitats (Sears et al., 2016). Additionally, numerous biotic and abiotic factors and their interactions can significantly influence *Mytilus* physiology and morphology at both the micro- and macro-scales (Telesca et al., 2018, 2019). Laboratory experiments on thermal stress can, for similar reasons, overestimate actual impacts of global warming on the physiology of intertidal organisms, and field based physiological and molecular evaluations are needed for a deeper understanding of the role of thermal stress on natural populations (Roberts et al., 1997; Buckley et al., 2001; Gracey et al., 2008; Clark and Peck, 2009; Connor and Gracey, 2011, 2020; Clark et al., 2019; Martino et al., 2019). Studies on thermal responses in intertidal *Mytilus* species have often used laboratory experimentation and largely focused on upper lethal limits and the analysis of heat shock genes and proteins, particularly the inducible 70 kDa family members (Hsp70) (*e.g.* Roberts et al., 1997; Buckley et al., 2001; Halpin et al., 2004; Dutton and Hofmann, 2009; Ioannou et al., 2009). There is much less knowledge on the subtle molecular changes associated with microhabitats in the natural environment, particularly sub-lethal effects, and the potential responses of organisms to chronic warming regimes.

To advance knowledge on intertidal heat stress in the Arctic, we conducted a series of experiments to identify if current Arctic summer temperatures induce any *in situ* cellular stress responses in Greenland *M. edulis*. Responses were compared across a natural temperature gradient, with samples taken on the first warm days of the year alongside equivalent experimentally manipulated temperatures. Specifically, intertidal *M. edulis* were sampled from the inner (warmer) and outer (cooler) regions of the Godthåbsfjorden around Nuuk (64°N) to examine the fjord temperature gradient effect. Furthermore, subtidal *M. edulis* were also collected and subjected to two acute temperature shocks of 22 and 32 °C, which represented common and extreme summer air temperatures for intertidal habitats near Nuuk.

2. Materials and methods

2.1. Specimen collection

Mytilus edulis were collected from the Godthåbsfjorden near Nuuk, Greenland (64°27′20″ 51°08′39″) at the following locations and dates: Inner fjord (64°27′33.90″ 50°18′37.10”W) on 11/06/2018; outer fjord (64°11′48″ 51°41′24″) on 13/06/2018, and sub-tidal (64°11′48″ 51°41′24″) on 13/06/2018 (outer fjord at 20-40 cm below the lowest low water mark) (Fig. 1). Ten animals from each of the inner and outer fjord locations were sampled *in situ* and gill tissue placed directly into RNA*later*™ for preservation. These samples were returned to the laboratory where they were kept at 4 °C overnight, before storage at −80 °C.

2.2. Heat shock experiment

Sub-tidal animals from the outer fjord (collected as detailed above) were kept submerged in water and returned to the laboratory, where they were placed in aquaria prior to experimentation. Heat shock experiments were conducted in programmable waterbaths, which maintained a water temperature within ±0.1 °C. Twenty *M. edulis* were placed in individual 50 ml plastic vials in the preheated waterbaths at both 22 °C and 32 °C for 2 h, to mimic air conditions. The method of placing animals in submerged tubes has been used before, and is an effective way to match the air temperature to the water temperature (Kuo and Sanford, 2009, Thyrring et al., 2017a, 2017b). The vials were preheated in the water and kept submerged (only the opening was above water) when the mussels were placed inside. A HOBO temperature logger was placed in an empty vial and recorded the temperature throughout the experiment. The air temperature was constantly within 0.4 °C of the water temperature. Two hours exposure was used as this is the approximate air exposure time mussels experience in the low intertidal around Nuuk, Greenland. After each heat shock treatment, animals were sampled and either added to RNA*later*™ using the protocol above, or freeze-clamped. All tissue samples were stored at −80 °C until the date of departure, before being transferred to the UK using ice packs cooled to −80 °C to keep the samples cold during transit. All samples were subsequently stored at −80 °C in the UK until required for RNA extractions. Robomussels incorporating Hobo™ Tidbit loggers (Helmuth and Hofmann, 2001) were deployed in both the inner and outer fjord sites between 04/06/2018 and 29/08/2018 to monitor the intertidal temperatures every 30 min. Subtidal temperatures were also monitored at the same sites over the same period, but on an hourly basis.

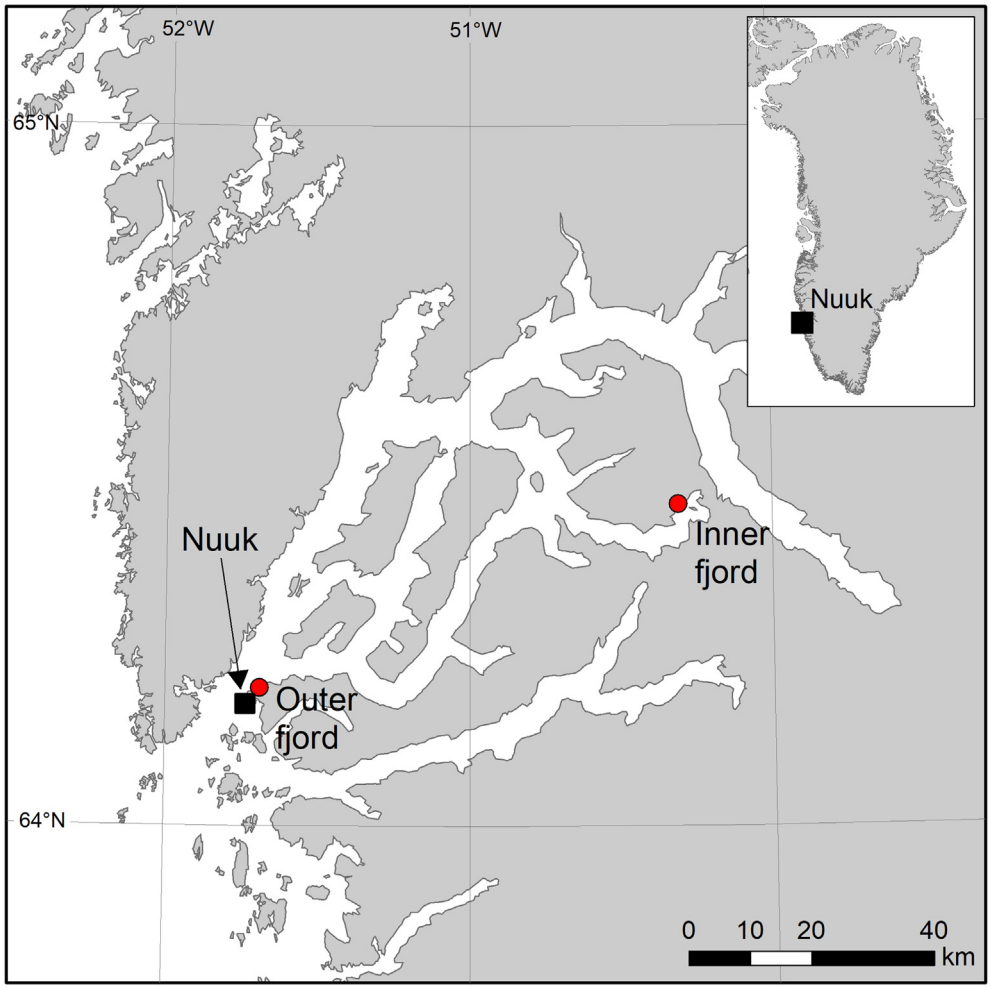

Fig. 1. Map showing sampling locations in the Godthåbsfjorden around Nuuk with insert showing position of Nuuk in Greenland.

2.3. RNA extraction and sequencin

RNA was extracted from 5 sets ($n = 5$ for each set) of samples comprising: subtidal control animals (MC), *in situ* sampling from the inner fjord on a warm day (MI), *in situ* sampling from the outer fjord on a warm day (MO), experimental samples (subtidal) warmed to 22 °C in the laboratory (M22) and experimental samples (subtidal) warmed to 32 °C in the laboratory (M32). For each sample approximately 30 mg of *M. edulis* tissue was extracted using the SV total RNA system (Promega), which included a DNAse treatment, according to manufacturers' instructions. All samples were quantified and QC'ed. on a NanoDrop™ spectrophotometer and an Agilent TapeStation™ (Agilent). Twenty-five individual samples with a RIN ≥ 8 were sent for library preparation and Illumina PE150 sequencing at Novogene (China).

2.4. Bioinformatic analyses

In total 25 individuals were sequenced from the 5 sets of sampling/experimental work detailed above (n = 5 for each treatment). All analyses were performed by Novogene and comprised the following stages and application of software packages. Raw reads were quality controlled for error rate using Phred and GC content using the Illumina CASAVA v1.8 software. Reads were cleaned with the removal of Illumina kit adapter sequences and adapter contamination, where the level of uncertain nucleotides $N > 10\%$ and where low quality bases as defined by the Phred Q20 score constituted more than 50% of a read. *De novo* transcriptome assembly was performed using Trinity version r20140413p1 with parameters min_kmer_cov = 2, min_glue = 2, others were set to default, using the modules Inchworm, Chrysalis and Butterfly (Grabherr et al., 2011). Hierarchical clustering was performed using the Corset program in Trinity to remove read redundancy. The longest transcripts from each cluster were selected as unigenes. Annotation of the unigenes was performed using seven databases (NR, NT, KO, SwissProt, Pfam, Go and KOG) (detailed in Table 1). Blast searching against NT was performed using NBCI blast 2.2.28+ with an e-value threshold of $1e^{-5}$ (Altschul et al., 1997). Diamond v0.8.22 (Buchfink et al., 2015) was used to blast search the unigenes against NR, SwissProt and KOG. The e-value threshold for NR and SwissProt was $1e^{-5}$ and $1e^{-3}$ for KOG. Pfam (Finn et al., 2008) was screened using the hmmscan package in HMMER v3.1b1 with an e-value threshold of 0.01. GO annotation was based on the protein annotation results from NR and Pfam using Blast2GO vb2g4pipe_2.5 (Götz et al., 2008; Young et al., 2010) with an e-value threshold of $1e^{-6}$. KEGG mapping was performed using KAAS (KEGG Automated Annotation Server) v.r140224 with an e-value threshold of $1e^{-8}$ (Mao et al., 2005; Moriya et al., 2007; Kanehisa et al., 2008). GO enrichment was performed using GOSeqtopGO vGOSeq 1.32.0, topGO-2.32.0) with a corrected *p* value of <0.05. KEGG enrichment was performed using KOBAS v3.0 with a corrected p value of <0.05.

The *de novo* transcriptome was used as a reference assembly and the reads from each library mapped back to the transcriptome and quantified using Bowtie2 vbowtie2–2.2.2.2 and RSEM vRSEM-v1.3.0 (Li and Dewey, 2011) with output referenced as FPKM (Fragments Per Kilobase of transcript sequence per Million base pairs sequenced). The threshold

Table 1
Transcriptome assembly statistics and gene annotation rates.

Assembly statistics	
Number of nucleotides (nt)	345,804,392
Number of unigenes	402,060
Unigene minimum length (bp)	201
Unigene mean length (bp)	860
Unigene median length (bp)	554
Unigene maximum length (bp)	30,590
N50	1208
N90	387
Number of unigenes between 200 and 500 bp	177,946
Number of unigenes between 500 bp - 1kbp	124,474
Number of unigenes between 1kbp - 2kbp	67,214
Number of unigenes between >2kbp	32,426
Annotation rates	
Database	% annotation
NR: NCBI non-redundant protein sequences	32.65
NT: NCBI nucleotide sequences	10.01
KO: Kyoto Encyclopaedia of Genes and Genomes Orthologues	8.27
SwissProt: Curated Protein sequences	18.58
Pfam: Protein domains and families	12.19
GO: Gene Ontology	9.35
KOG: euKaryotic Orthologous Groups	9.81
Annotated in all databases	1.15
Annotated in at least 1 database	36.58

for expression was set at FPKM >3.0. Differential expression between the different sets of samples was calculated using DEGseq v1.12.0 (Wang et al., 2010) with normalization *via* TMM and FDR calculated using BH (Benjamini and Hochberg, 1995) with output threshold of $\log_2$fold change >1 and adjusted p value <0.005.

Protein gene identifiers were extracted from the SwissProt annotations for the MI v. MO differential expression analysis and a set of unique identifiers were entered into the STRING v11 program (https://string-db.org/) to visualize protein-protein interactions (Szklarczyk et al., 2019). Transcripts showing sequence similarity to HSPA12A and HSPA12B were blast searched against the SwissProt database (Bateman et al., 2017) and regions of amino acid alignment against human orthologues noted. To estimate a minimum number of paralogues in *Mytilus*, the transcripts showing substantial overlap (greater than 500 amino acids) were translated into proteins and aligned using ClustalW (Thompson et al., 1994) and percentage identities calculated using NEEDLE within the EMBOSS suite of programs in BioLinux (Field et al., 2006). Alignment data were visualized and annotated in BoxShade version 3.21 https://embnet.vitalit.ch/software/BOX_form.html).

3. Results

3.1. Temperature data

2018 was a cold and wet summer in Greenland, yet temperatures in the inner fjord intertidal region still reached 31 °C (Fig. 2A), illustrating the relevance of the 32 °C laboratory experiment. The outer site was considerably cooler with maximum temperatures of 21 °C (Fig. 2B), similar to the 22 °C laboratory experiment. Over the summer season, not only were the average temperatures experienced by the animals in the inner fjord intertidal region 6 °C warmer than those in the outer fjord, but they also experienced a much more variable thermal regime, with temperatures ranging from 1 °C – 31 °C compared with 1 °C – 21 °C respectively (Fig. 2, Table 2). There was a similar 6 °C difference in the subtidal temperatures at the two sites, albeit with a smaller seasonal temperature range and maximum temperatures (Fig. 2C and D, Table 2). Intertidal animals were sampled from both sites in the Godthåbsfjorden on warm days (27 °C and 19 °C respectively, as measured by Tidbit loggers in the same location and tide height) (Figs. 2E(i), E(ii)). The subtidal animals were sampled at 3 °C (Fig. 2 E (iii)).

3.2. Transcriptome statistics

Gill tissue samples from 25 individual *M. edulis* were sequenced to produce a reference transcriptome, and for the analysis of gene expression differences between the five different conditions sampled. Quality control resulted in the removal of one library from the 32 °C set due to excessive adapter contamination; hence, the reference transcriptome of 345,804,392 nucleotides comprising 402,060 unigenes was generated from 24 individuals (Table 1). Figures for the number of Trinity transcripts and metrics were very similar (99.95%) to those for Trinity unigenes, so only the unigene data are reported (Table 1). Library coverage varied from 19,044,267–36,074,420 raw reads with a Phred Q20 score of between 96.27%–97.65% (Supplementary Table S1). Maximum annotation rates were achieved using the NR database with a 32.65% annotation rate (18.58% against the SwissProt database) (Table 1). The majority of annotations (approximately 70%) were achieved against four bivalve species, the Mediterranean blue mussel *Mytilus galloprovincialis* (23.1%), the Japanese scallop *Mizuhopecten yessoensis* (19.9%) and the oysters *Crassostrea gigas* (13.9%) and *Crassostrea virginica* (12.8%).

3.3. Differential expression and GO enrichment

RNA-Seq analysis revealed distinct patterns in gene expression using FPKM clustering. The expression patterns of subtidal control and *in situ* sampled intertidal animals were much more similar to each other, than those of the experimentally treated animals (Fig. 3). The magnitude of those differences was very similar in the differential expression analysis when the expression levels associated with particular treatments were compared with control (outer fjord subtidal) animals. *Circa* 3000 sequences were up-regulated in inner and outer fjord animals, whilst much higher numbers of around 9000 genes were up-regulated in the 22 °C and 32 °C experimentally manipulated animals (Table 3, for gene lists see https://doi.org/10.5285/26DDB511-3050-4D87-9E13-D034262CA566).

These relative expression levels were reflected in the GO enrichment results, with significant gene enrichment identified in the experimental animals, but none in the naturally sampled animals (Table 4). The enriched GO categories in the two sets of experimentally treated animals (22 °C and 32 °C) were very similar in content with the top GO categories in each case being Signal Transduction (Biological Process), Cytoskeleton (Cellular Component) and Cellular Protein Modification Process (Biological Function), closely followed by the Molecular Function of Phosphatase Activity (Table 4). When the genes underpinning these enriched categories from each treatment were examined in more detail, approximately 40% were shared, indicating that similar pathways were being invoked in response to warming, irrespective of temperature (either 22 °C or 32 °C) (Supplementary Table S2). When GO enrichment was performed directly between the 32 °C and 22 °C animals, there was a similar emphasis on signaling and the cytoskeleton *via* the Molecular Functions of Structural Molecule Activity and GTPase Activity (Supplementary Table S3). Identification of the genes underpinning the Molecular Function GO enrichment terms, specifically for GTPase Activity revealed that most were involved in diverse Ras signaling pathways, often linked to the cytoskeleton, but also cell adhesion and migration and cell proliferation (*e.g.* Rab21, MRAS, NKIRAS2, RHOU and CDC42) (Supplementary Table S3). Those allocated to Structural Component of the Ribosome were largely ribosomal proteins. When a minimum set of SwissProt gene identifiers from the genes assigned to GTPase Activity were added into the STRING program, no single network was produced using moderate to high confidence levels indicating the diversity of Ras signaling pathways invoked (data not shown, Supplementary Table S3).

Although there was no GO enrichment between the inner and outer fjord samples when compared with control subtidal animals or with each other, an insight into the general processes upregulated in the

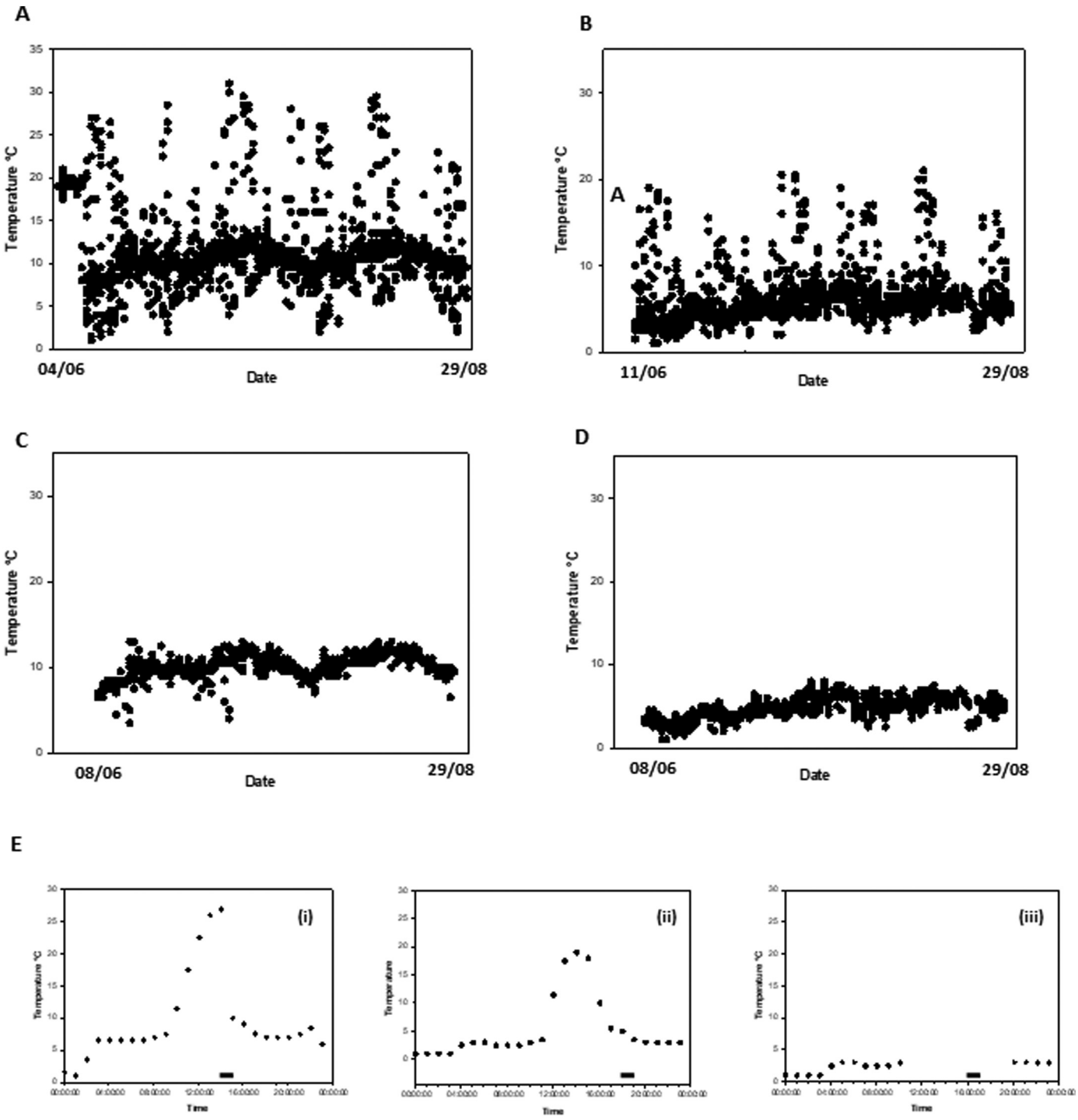

Fig. 2. Graphs showing temperatures logged in the Godthåbsfjorden during the summer of 2018. A: Inner fjord intertidal; B: Outer fjord intertidal; C: Subtidal inner fjord; D: Subtidal Outer fjord; E: Temperatures logged on the day of sampling (i) Inner fjord intertidal (11/06/2018); (ii): Outer fjord intertidal (13/06/2018); (iii): Outer fjord Subtidal (13/06/2018). Bar near X-axis denotes approximate collection time.

warmer inner fjord animals compared to the cooler outer fjord animals could be visualized using STRING program generated protein-protein interactions. Of the 1467 sequences differentially expressed between inner and outer fjord animals, 456 had associated SwissProt identifiers. The gene names were extracted from these identifiers and duplicates removed, leaving 383 unique names, only 259, of which were associated with human proteins (See https://doi.org/10.5285/26DDB511-3050-4D87-9E13-D034262CA566). When entered into the STRING program, application of medium confidence for protein-protein interactions revealed a highly connected network with five main clusters. These clusters were retained and more clearly defined when the highest confidence levels were applied in the STRING program. Of particular note was the cluster involving numerous heat shock proteins (chaperones), but other clusters also comprised proteins involved in apoptosis, cell proliferation, migration and adhesion, transcription of RNA and RNA processing and the cytoskeleton (Supplementary Fig. S1). However, since gene enrichment was not identified for these biochemical pathways, these genes are clearly also expressed in the other conditions.

Table 2
Variation in water temperatures at the sampling sites during the summer of 2018 (04/06/18–29/08/18).

	Inner fjord intertidal	Outer fjord intertidal	Inner fjord subtidal	Outer fjord subtidal
Mean	11.7 °C	5.8 °C	10.3 °C	4.8 °C
SE mean	0.10	0.06	0.04	0.04
Minimum temperature	1 °C	1 °C	3.5 °C	1 °C
Maximum temperature	31 °C	21 °C	13 °C	8 °C
Median temperature	10.5 °C	5.5 °C	10.5 °C	5 °C

3.4. Identification of the classical stress response genes

In spite of the different temperature treatments (experimental manipulation and *in situ* sampling) and their differing severities, no enrichment was found in any of the bioinformatic comparisons for the classical stress response genes, such as the enzymes involved in antioxidant activities and detoxification of reactive oxygen species (catalase, superoxide dismutase, thioredoxin and the glutathione family), hypoxia (hypoxia inducible factor), apoptosis (caspase genes) or the heat shock response (heat shock proteins) (Table 4). Datamining of the differentially expressed gene lists using these as keyword searches showed little activation of catalase, superoxide dismutase or hypoxia inducible factor. There were higher numbers of differentially expressed transcripts identified using thioredoxin, glutathione and caspase as keywords. However, these keywords identify both domains (as in thioredoxin peroxidase, thioredoxin 1) and also gene family members. In some cases, such as glutathione transferase, these families can comprise many different closely related genes with subtly different functions (Table 5, Supplementary Table S4). What was particularly surprising was the number of heat shock protein transcripts identified in the transcriptome (180 in total) and the fact that the vast majority (155 transcripts or 86%) showed highest sequence similarity to the HSP70 family members HSPA12A and HSPA12B (Table 5, Supplementary Table S5).

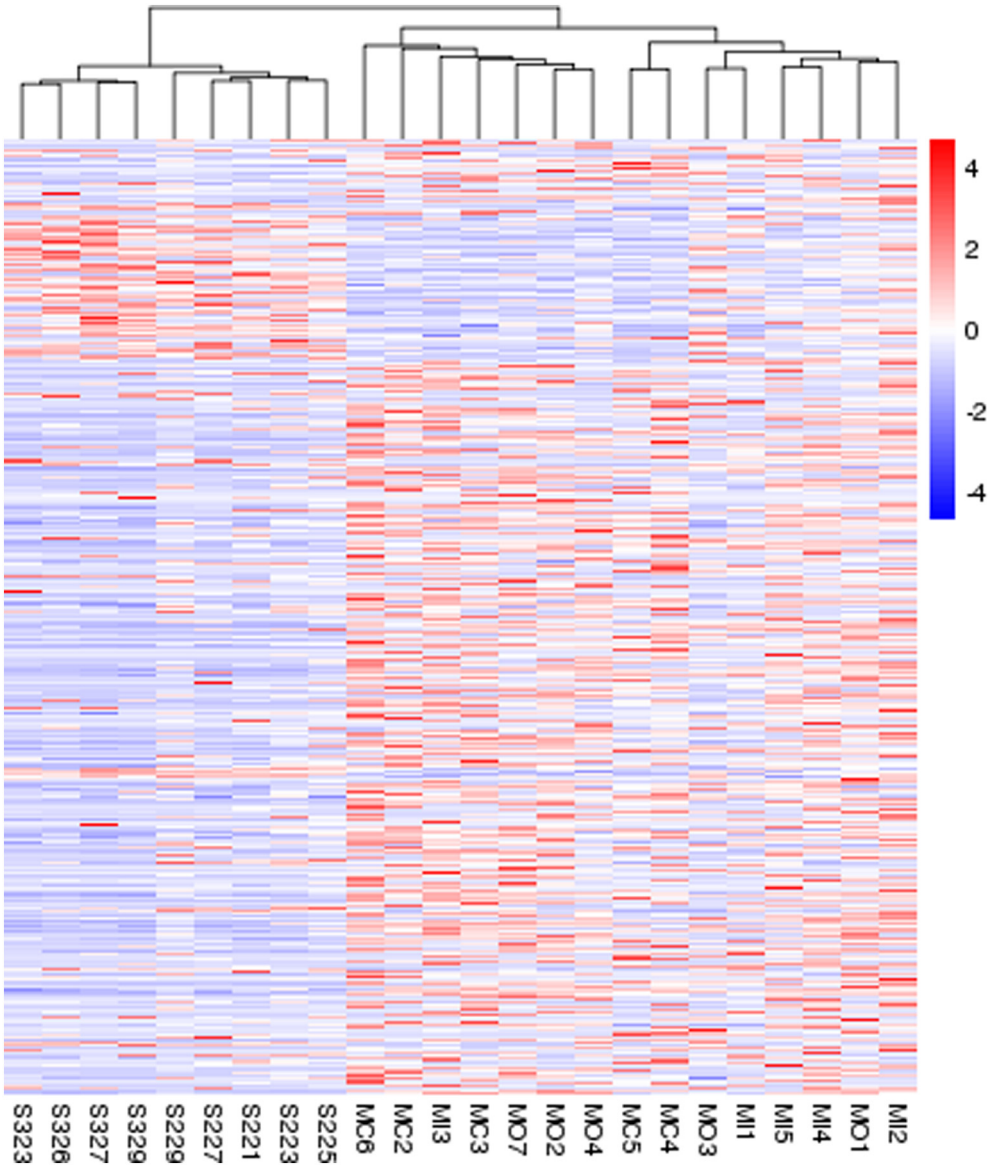

Fig. 3. FPKM hierarchical clustering heat map. The heat map represents the overall results of the FPKM cluster analysis using the $\log_{10}$(FPKM+1) values. Red denotes genes with high expression levels, and blue denotes genes with low expression levels. The colour range from red to blue represents the $\log_{10}$(FPKM+1) value from large to small. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Differential gene expression counts for the different sample comparisons.

Comparison	Up-regulated	Down-regulated	Total
Inner fjord v. control	3106	2461	5567
Outer fjord v. control	2929	2410	5339
Inner v. outer fjord	1467	1200	2667
22 °C v. control	8810	24,214	33,024
32 °C v. control	9611	32,650	42,261
32 °C v. 22 °C	1039	4041	5080

3.5. The HSPA12 heat shock proteins

There was relatively little redundancy in the HSPA12A and HSPA12B transcripts expressed in each treatment with 133 unique transcripts identified. Of these, 92 and 41 transcripts were identified respectively for HSPA12A and HSPA12B. When these unique transcripts were translated, 43 matched in excess of 500 amino acids of the full-length human proteins on Blast sequence similarity searching (30 and 13 transcripts respectively for HSPA12A and HSPA12B) (Supplementary Table S6). Alignment of the 30 HSPA12A translated proteins identified 23 unique sequences at the protein level (Supplementary Information S1). However, when the nucleotide sequences of the transcripts which showed 100% identity at the amino acid level (unigenes17285 and 17286; 212835 and 212836 and 212837; 217586 and 217587; 227366 and 227368) were examined in more detail, all showed differences of between 73.9% and 99.5% outside of the putative coding region, indicating variant 5′ and/or 3′ non-coding regions. These differences included single nucleotide changes, deletions of 11-300 bp and different length 5′ and 3' UTRs. Therefore, it is most likely that these 30 transcripts represent either unique sequences or alternative splice forms. This was a minimum estimate as only 30/92 transcripts were analysed in depth and the closest human match to all of these 30 sequences was O43301_HSP12A, whilst shorter sequences also matched other HSPA12A human variants (*e.g.* accession numbers B7Z2M8, B7Z2F7) (Supplementary Table S6). A similar level of diversity was identified in the 13 HSPA12B translated proteins examined in detail. Of these, 11 transcripts were unique at the amino acid level (Supplementary Information S2) with further non-coding variation identified between those transcripts showing identical amino acid sequences (71405 and 84541; 221672 and 47867). Thus, the 13 sequences represent a minimum number of unique HSPA12B transcripts in *M. edulis*. These data indicate massive numerical expansion of these two genes in *M. edulis*.

Table 4
GO enrichment results for the different sample comparisons. Abbreviations: BP: Biological process; CC: Cellular Component; MF: Molecular Function.

Category	ID	Description	Adjusted *p*-value
22 °C v. control			
BP	GO:0007165	Signal transduction	8.75E-18
CC	GO:0005856	Cytoskeleton	6.84E-08
BP	GO:0006464	Cellular protein modification process	1.99E-06
MF	GO:0016301	Kinase activity	0.00016079
MF	GO:0016791	Phosphatase activity	0.00017394
MF	GO:0022857	Transmembrane transporter activity	0.00053667
MF	GO:0030234	Enzyme regulator activity	0.0028471
MF	GO:0008092	Cytoskeletal protein binding	0.021221
32 °C v. control			
BP	GO:0007165	Signal transduction	2.73E-14
CC	GO:0005856	Cytoskeleton	1.21E-06
BP	GO:0006464	Cellular protein modification process	1.21E-06
MF	GO:0016791	Phosphatase activity	5.20E-06
MF	GO:0030234	Enzyme regulator activity	0.0013982
MF	GO:0016301	Kinase activity	0.0016688
MF	GO:0022857	Transmembrane transporter activity	0.025377
22 °C v. 32 °C			
CC	GO:0005840	Ribosome	0.0045489
MF	GO:0005198	Structural molecule activity	0.0045489
MF	GO:0003924	GTPase activity	0.011977
CC	GO:0005856	cytoskeleton	0.022669
MF	GO:0003735	Structural constituent of ribosome	0.028507
CC	GO:0005576	Extracellular region	0.031775
No enrichment			
Inner fjord v. control			
Outer fjord v. control			
Inner fjord v. outer fjord			

Table 5
Stress response genes identified in the differentially expressed profiles from the environmental and experimental samplings of *M. edulis*. Abbreviations: MC: *Mytilus* control; MI: *Mytilus* inner fjord; MO: *Mytilus* outer fjord; M22: *Mytilus* subjected to 22 °C experimental warming; M32: *Mytilus* subjected to 32 °C experimental warming.

Gene family	Family member	Comparisons			
		M22 v. MC	M32 v. MC	MI v. MC	MO v. MC
Catalase		0	1	0	0
Hypoxia inducible factor		2	1	0	0
Superoxide dismutase		0	0	2	0
Thioredoxin		2	8	1	0
Glutathione		1	12	2	1
Caspase		21	25	8	7
Heat shock proteins	Small HSP	4	1	3	6
	HSP68	3	0	0	3
	HSP70B2	2	0	0	1
	HSP70 family	0	0	0	2
	HSC70	0	1	0	0
	HSPA12A	33	32	19	15
	HSPA12B	16	24	6	10
	Total	58	57	28	37

4. Discussion

Our data clearly demonstrate the molecular resilience of *M. edulis* to warming in the Greenland intertidal region. This is extraordinary given the inner fjord intertidal animals were sampled at 27 °C in the field, with temperature data obtained from robomussel dataloggers. These devices have been repeatedly shown to provide very accurate body temperature measurements (accurate to within 0.75 °C of living mussels), and are widely acknowledged to produce information on the thermal characteristics of the habitat/body temperature, which can be used to estimate cellular damage and thermal stress (Hofmann and Somero, 1995; Helmuth, 1998; Helmuth and Hofmann, 2001; Helmuth et al., 2002, 2016; Harley and Helmuth, 2003; Fitzhenry et al., 2004). The initial molecular data depicted in the FPKM heat map (Fig. 3) showed highly similar expression profiles between the subtidal controls from the outer fjord (MC) and the intertidal samples from both the outer fjord (MO) and the inner fjord (MI). This was an unexpected result given the 24 °C difference in sampling temperatures from 3 °C in the subtidal outer fjord, through 19 °C in the outer fjord intertidal to 27 °C in the inner fjord intertidal (Fig. 2E (i), (ii), (iii)). Furthermore, these raw FPKM data were substantiated by the GO analyses, which showed no functional enrichment between the subtidal controls and either the outer or inner fjord expression profiles (Table 4) and also, the paucity in differential expression between the same samples (approximately 1.3% of all transcripts, with only 0.7% up-regulated) (Table 3). Thus, responses to the quite different environmental temperatures were neither particularly marked, nor involved the strong induction of identifiable gene pathways.

4.1. Molecular acclimation

In the vicinity of Nuuk, intertidal *M. edulis* are generally found in the mid to low intertidal zone (Blicher et al., 2013), and low intertidal animals are only emersed for a relatively short period a day (Fig. 2). Hence, the average summer temperatures experienced by intertidal

animals in the inner (11.7 °C) and outer fjord (5.8 °C) are very similar to their subtidal cohorts (at 10.3 °C and 4.8 °C respectively). Thus, on average, the difference in temperature between these two intertidal habitats is much less, at 6 °C, not the 23 °C experienced on emersion. Previous studies of mussels from the *Mytilus* species complex have shown considerable capacity for seasonal and long-term acclimation (Roberts et al., 1997; Chapple et al., 1998; Buckley et al., 2001; Ioannou et al., 2009). Therefore, it is perhaps not as surprising to see no molecular acclimation of the inner fjord animals when viewed solely in terms of the average temperatures these animals experienced compared with those in the cooler outer fjord. Even so, previous experiments have demonstrated upper lethal temperatures for *M. edulis* between 25 °C to 38 °C (Bayne, 1976; Widdows and Bayne, 1971; Jones et al., 2010; Thyrring et al., 2015, 2020), and some of the animals within this study were clearly experiencing temperatures towards the upper end of this range (Fig. 2A and 32 °C experiment), but there were no mortalities. Obviously, acclimation and thermal tolerance are to a certain extent dictated by population source and thermal history. Although, behavioural responses, such as valve closure and gaping, may play a role in short-term thermal tolerance, such behaviours in the different *Mytilus* species are complex and highly dependent on environmental conditions (Connor and Gracey, 2020). However, another important factor is predictability of the stress.

4.2. Preparative defense and predictability

At the molecular level, a regular stress can induce a constitutive preparative defense or front-loading of genes that enable the organisms to deal more efficiently with the stress. Such an effect has been shown previous in species such as limpets, corals and the marine snail *Chlorostoma funebralis* (Dong et al., 2008; Clark et al., 2008; Barshis et al., 2013; Barshis, 2015; Gleason and Burton, 2015; Drake et al., 2017). Indeed, fluctuating temperature regimes were shown to increase thermal tolerance in the limpet *Lottia digitalis* (Drake et al., 2017); Repeated aerial exposure, regardless of the magnitude of the temperature ramp, had the largest effect on maintaining a high upper thermal limit in L. *digitalis*, thus enabling rapid recovery from emersion during low tides (Drake et al., 2017). Furthermore, experiments on limpets have shown that they do less well in more benign, but unpredictable environments (Wang et al., 2020). These observations from previous studies potentially explain the resilience shown by the Greenland *M. edulis* to elevated temperatures at low tides. They also provide an explanation as to the difference in response between the *in situ* collected samples and the experimentally manipulated animals, as the latter were sampled from the subtidal region of the outer fjord, which had not been pre-subjected to a regular fluctuating temperature regime. Particularly, these results emphasize the importance of sampling from the natural environment under a variety of different conditions, in tandem with laboratory experiments (Roberts et al., 1997; Buckley et al., 2001; Gracey et al., 2008; Clark and Peck, 2009; Connor and Gracey, 2011, 2020). Thus, it is entirely feasible that whilst *M. edulis* can experience very high temperatures, which under normal circumstances would be lethal, if the exposures are short-lived with plenty of time for recovery and predictable in timing, then they can survive in this environment without invoking their cellular stress response. A process, which can also be referred to as "heat hardening" (Connor and Gracey, 2020). Furthermore, the phenotypic flexibility of this species may be further enhanced by hybridization with closely related species (Gosling, 1992). This is particularly relevant in Greenland where *M. edulis* is found in the waters around Nuuk while *M. trossulus* dominates northern Greenland populations (Wenne et al., 2016, 2020; Mathiesen et al., 2017). To date, pure *M. galloprovincalis* (the most heat resilient of the three *Mytilus* species) has not been found in either the Arctic or sub-Arctic and only a small contribution of *M. galloprovincialis* genes has been identified in the genetic composition of *M. edulis* populations found in Iceland, Spitsbergen, Norway and Northern Russia (Simon et al., 2020; Wenne et al., 2020). However, the physiological and ecological consequences of this competitive mussel and ongoing hybridization in Arctic regions remains poorly understood and needs to be studied further, particularly with reference to long-range transport *via* shipping (Simon et al., 2020).

4.3. The complexity of the Mytilus stress response

Previous analyses of thermal stress in *Mytilus* species have revealed the involvement of HSPs, ubiquitination and lipid peroxidation (Hofmann and Somero, 1995; Helmuth and Hofmann, 2001; Gracey et al., 2008; Jimenez et al., 2016). Therefore, a particularly surprising aspect of this study was the lack of enrichment for these classical stress response genes in any of the animal treatments (Table 4). Molecular analyses of warming responses in *Mytilus* species have largely concentrated on *M. trossulus*, *M. galloprovincialis* and *M. californianus* along the coast of the USA using either candidate genes such as HSP70s or microarrays. These have demonstrated the complexity of the environmental stress response in *Mytilus* species. Analyses of data generated using microarray hybridizations performed across tidal cycles in *M. californianus* for both low and high intertidal populations demonstrated regular fluctuating gene expression profiles (Gracey et al., 2008). Profiling of up-regulated gene clusters indicated the metabolic cost of heat exposure, including the up-regulation of heat shock proteins (Gracey et al., 2008). Using *M. californianus* from the same study area allied to laboratory experimentation, more detailed subsequent studies identified a set of unique daytime stress genes (as distinct from circadian and nighttime recovery genes) (Connor and Gracey, 2011, 2020). Interestingly, when the 100 most significantly up-regulated genes from this 2020 study were compared to results of one, which finished with an acute heat shock (Connor and Gracey, 2011), only two genes were in common, indicating significant differences between gene expression patterns associated with repeated intertidal exposure and acute heat stress (Connor and Gracey, 2011, 2020). Similarly, some of the 369 unique daytime stress genes identified in Connor and Gracey (2020), and also candidates from the RNA-Seq climate change manipulation study of Martino et al. (2019) were identified in the differentially expressed gene lists in this study (https://doi.org/10.5285/26DDB511-3050-4D87-9E13-D034262CA566), especially in the 32 °C experiment. However, there were very large differences between differentially expressed sequences in these studies indicating the wide variation in responses. Much of which will almost certainly be due to local conditions and levels of aerial exposure (20 h per day (Connor and Gracey, 2020) compared with four hours per day in this study). This variation in response was identified in an extensive analysis of *M. californianus* populations along a latitudinal gradient, which revealed distinct gene signatures across the different spatial scales (Place et al., 2012). Differences were identified in the primary metabolic pathways, even within a single site, thus indicating the very complex interactions between physiological responses and environmental variables (Place et al., 2012). Highly individualistic responses were also demonstrated in an experiment examining the effects of decreased salinity in *M. galloprovincialis* and *M. trossulus* (Lockwood and Somero, 2011). Interesting Lockwood and Somero (2011) particularly highlighted the lack of transcriptional differences between *M. galloprovincialis* and *M. trossulus* in spite of different physiological tolerances. It was suggested that differences between the two species were probably not solely regulated at the transcriptional level, with cell cycle control identified as a key mechanism and the action of MAP kinases and GTPases important in mediating downstream transcriptional cascades (Malagoli et al., 2004; Anestis et al., 2007; Lockwood and Somero, 2011). Their data reflect the homogeneity of GO enrichment results and lack of a significant cellular stress response identified in this study. It is clear that subtle differences in gene expression profiles among *Mytilus* species, for example in transcriptional factors, small heat shock proteins and the cytoskeleton (differentially expressed

transcripts that have been identified in this study *e.g.* Table 4 and Supplementary Fig. S1 and others) can significantly affect physiological outcomes (Lockwood et al., 2010; Lockwood and Somero, 2011; Tomanek, 2014). Indeed, the complexity of the potential heat shock response in bivalve molluscs, due to gene family expansions and multiple gene isoforms, was noted in Connor and Gracey (2020) although the example they used was of *Crassostrea gigas* (Zhang et al., 2012).

4.4. Expansion of the HSPA12 heat shock protein family

One constraint of the previous *Mytilus* studies was that the majority used either candidate genes or microarrays. Development of gene probes for the latter often involves the removal of highly repetitious sequences, so one of the major results of this current study, the expansion of the HSPA12A and HSPA12B genes remained undetected. Our data indicate that this gene family expansion is potentially critical in the environmental stress response of *M. edulis.* HSPA12A and HSPA12B were first described in humans and were associated with atherosclerosis susceptibility (Han et al., 2003). Later experiments in zebrafish demonstrated that HSPA12B was required for normal development and was involved in endothelial functions, cell migration and wound healing (Hu et al., 2006). Both belong to the HSP70 family, but are quite distinct from the other classical HSP70 members, with no conservation of splice sites and few regions of homology. They have an atypical ATPase domain, no substrate binding and TPRI domains (which interact with HSP90) or the ubiquitin-binding peptide (Brocchieri et al., 2008). Thus, at the sequence level and structurally these two HSP70s are very different to the classical members, which are closely associated with the cellular stress response. Currently, relatively little is known about the function of these proteins, even in humans (Radons, 2016).

Phylogenetic analysis suggests that HSPA12A and HSPA12B evolved early in vertebrates, sharing more sequence similarities with prokaryote and primitive eukaryote HSP70 genes than the more classical members of this family (Brocchieri et al., 2008). In general, the A and B paralogues only share 61–65% amino acid similarity with each other within the same species, whilst each paralogue is relatively conserved between species (at 82–83% amino acid similarity) (Brocchieri et al., 2008). Whilst previous analyses have shown a unique duplication event early in the tetrapod lineage before the radiation of amphibia and mammals, the recent sequencing of mollusc genomes has revealed a massive expansion of both HSPA12A and HSPA12B in bivalves (Cheng et al., 2016). To date 73 copies of the HSPA12 family have been identified in the Pacific oyster (*Crassostrea gigas*), 97 in the pearl oyster (*Pinctada fucata*), 55 in the invasive golden mussel (*Limnoperna fortunei*) and 57 in the scallop (*Patinopecten yessoensis*) (Zhang et al., 2012; Uliano-Silva et al., 2014; Takeuchi et al., 2016; Cheng et al., 2016). These large numbers of duplicates are the result of a bivalve-specific expansion, which was followed by species-specific tandem duplications (Cheng et al., 2016). It has been suggested that the bivalve expansion of this divergent HSP70 family was strongly selected for, and maintained by, adaptation to the sessile lifestyle in the dynamically changing marine environment with its complex biotic and abiotic stresses (Cheng et al., 2016). Indeed, evaluation of expression levels of this gene family in different bivalves has demonstrated that specific sub-sets of the HSPA12 family are associated with responses to particular stresses, such as temperature and heavy metals in *C. gigas* and toxic dinoflagellates in *P. yessoensis* (Zhang et al., 2012; Cheng et al., 2016). To date only a single HSPA12A gene has been cloned in *Mytilus* with up-regulation associated with response to cadmium in *M. galloprovincialis* (You et al., 2013). Nonetheless, data presented here, demonstrate for the first time that this HSPA12 species-specific gene family expansion is also present in *M. edulis.* This expansion of HSPA12A and HSP12B can also be retrospectively identified in the gene lists associated with a 454 pyrosequencing study in *Mytilus* species (Malachowicz and Wenne, 2019). Although only 8333 transcripts were produced for *M. edulis,* 24 HSPA12A and 3 HSPA12B genes are present in the listings in the appendices. This expansion was not identified at the time, as the focus of the study was biomineralization (Malachowicz and Wenne, 2019).

4.5. HSPA12 as intertidal stress regulators

It has long been demonstrated that HSP70 proteins have roles far beyond that of responses to heat stress, acting as multifunctional gene hubs (Csermely, 2004; Korcsmáros et al., 2007), as demonstrated in the data in this study by the STRING analysis, where the cluster of HSP genes shows multiple interactions with other proteins with different functions (Supplementary Fig. S1). However, the STRING analysis cannot accurately reflect the expansion and protein interactions of the HSPA12 family in *M. edulis* due to the limited number of such proteins in humans. Duplicated genes are often retained within an organism due to processes such as sub-functionalization, whereby the duplicated genes acquire subtly different functions and interactions (Force et al., 1999). Such a process has recently been demonstrated in another bivalve species, in the Antarctic clam *Laternula elliptica, via* gene network interactions (Ramsoe et al., 2020). This example examined the duplicated forms of the inducible HSP70 genes and HSP70A interacts with ribosomal genes, whereas the paralogue HSP70B interacts with a range of signaling pathways including transcription factors and the cytoskeleton (Ramsoe et al., 2020). This expansion of the HSPA12 family potentially explains, not only the high molecular and physiological plasticity of this species and therefore its invasive success, but also the lack of the classical heat stress response, with the multitude of HSPA12 genes acting as important regulators in the intertidal to the numerous biotic and abiotic stressors associated with that habitat. These studies are supported by the transcription profiles obtained in this experiment, whereby the classical stress response genes are not significantly enriched in response to warming (either in inner fjord animals or the 22 °C and 32 °C-treated animals). Gene enrichment was identified, but for a diverse set of conserved processes, including signal transduction, the cytoskeleton, phosphatase activity and Ras signaling (Table 4), which can lead to multiple outcomes depending on the particular gene cascade activated. These data agree with previous discussions suggesting that evolution and modification of the traditional conserved cellular stress response may be an important factor in invasiveness (Evans and Hofmann, 2012; Han and Dong, 2020). Identifying how cellular stress responses are modified, particularly in ecosystem engineers such as bivalves from the *Mytilus* species complex, is key to predicting competition success and ecosystem change under future climate change (Menge et al., 2008).

5. Conclusions

Overall, our data indicate the resilience of intertidal *M. edulis* in Greenland to warming. The combination of regular fluctuating exposure to different environmental variables and a cellular preparative defense ensure that *M. edulis* can survive short exposures to very high temperatures. We hypothesize that this resilience is facilitated by the expansion of the HSPA12 gene family, which function as important intertidal cellular regulators. Clearly more extensive experiments involving gene network analyses are required to validate these ideas and determine the precise gene and protein interactions of this extensive gene family. Although, survival of *Mytilus* species in the Arctic is ultimately defined by the ability to withstand low temperatures and ice scouring (Blicher et al., 2013; Fly and Hilbish, 2013; Thyrring et al., 2017b; Thyrring et al., 2020), conditions are changing and warming has led, and will continue, to facilitate the expansion of *Mytilus* species into the Arctic (Berge et al., 2005; Thyrring et al., 2017a). As ecosystem engineers, these organisms can significantly influence future Arctic nearshore and intertidal biodiversity. This current study also emphasizes the complexity of the *Mytilus* genome (Figueras et al., 2019) and responses to environmental perturbation, which are potentially further complicated by multiple and numerous hybridization events within this species complex. It

also clearly demonstrates the importance of conducting environmental sampling, alongside mechanistic laboratory experiments to understand and predict future responses to climate change.

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CRediT authorship contribution statement

Melody S Clark: Conceptualization, Investigation, Analyses, Data curation, Writing – original draft; **Lloyd S. Peck**: Conceptualization, Funding acquisition, Writing – review & editing; **Jakob Thyrring**: Conceptualization, Investigation, Resources, Analyses, Funding acquisition, Writing – original draft.

Data availability

RNA-Seq data have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-9786 and the assembled transcriptome has been deposited in the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/browser/home) under ASSEMBLY_NAME | STUDY_ID | SAMPLE_ID | SEQUENCE_ACC Greenland Mytilus | PRJEB41447 | ERS5419259 | HBDQ01000001-HBDQ01402060. Gene lists and gene annotations are available from the UK Polar Data Centre, Natural Environment Research Council, UK Research & Innovation: https://doi.org/10.5285/26DDB511-3050-4D87-9E13-D034262CA566

Declaration of competing interest

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

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