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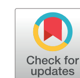
Large within, and between, species differences in marine cellular responses: Unpredictability in a changing environment



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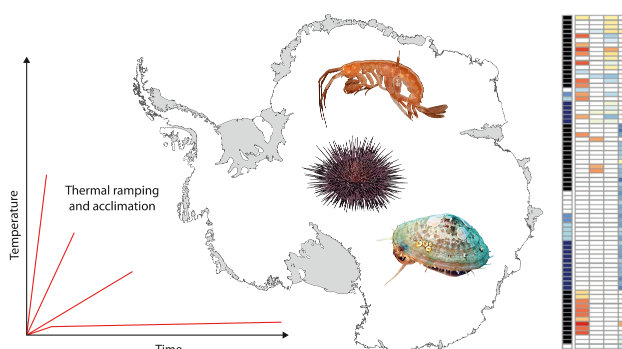
HIGHLIGHTS

- Highly individualistic cellular responses to warming
- Classical stress response genes rarely activated in response to warming
- Heat shock proteins activated at different warming rates depending on species
- Very few transcripts in common between warming treatments within each species
- No shared transcripts between species irrespective of warming treatments

GRAPHICAL ABSTRACT



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ABSTRACT

Predicting the impacts of altered environments on future biodiversity requires a detailed understanding of organism responses to change. To date, studies evaluating mechanisms underlying marine organism stress responses have largely concentrated on oxygen limitation and the use of heat shock proteins as biomarkers. However, whether these biomarkers represent responses that are consistent across species and different environmental stressors remains open to question. Here we show that responses to four different thermal stresses (three rates of thermal ramping (1 °C h^{-1}, 1 °C day^{-1} or 1 °C 3 day^{-1}) and a three-month acclimation to warming of 2 °C) applied to three species of Antarctic marine invertebrate produced highly individual responses in gene expression profiles, both within and between species. Mapping the gene expression profiles from each treatment for each of the three species, identified considerable difference in numbers of differentially regulated transcripts ranging from 10 to 3011. When these data were correlated across the different temperature treatments, there was no evidence for a common response with only 0–2 transcripts shared between all four treatments within any one species. There were also no shared differentially expressed genes across species, even at the same thermal ramping rates. The classical cellular stress response (CSR) i.e. up-regulation of heat shock proteins, was only strongly present in two species at the fastest ramping rate of 1 °C h^{-1}, albeit with different sets of stress genes expressed in each species. These data demonstrate the wide variability in response to warming at the molecular level in marine species. Therefore, identification of biodiversity stress responses engendered by changing conditions will require evaluation at the species level using targeted key members of the ecosystem, strongly correlated to the local biotic and abiotic factors.
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1. Introduction

Understanding the mechanisms underpinning marine invertebrate responses to environmental change is key to predicting future impacts on food web interactions and ecosystem functioning. To date, several

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diverse approaches have been taken including the use of theoretical analyses to identify suitable future habitat using climate and landscape models (Gillingham et al., 2012; Mendenhall et al., 2016) and mechanistic analyses of relationships between animal performance, and altered environmental conditions (Peck et al., 2014; Pörtner et al., 2017). However, the processes that confer resistance to chronic and incremental environmental change (and therefore processes that can be used to assess species resilience) are poorly understood. In the marine field, there has been much debate around universal mechanisms, particularly the emphasis on the oxygen- and capacity-limitation of thermal tolerance (OCLTT) paradigm and heat shock proteins as universal biomarkers (Iwama et al., 2004; Pörtner et al., 2017; Jutfelt et al., 2018). Whilst the existence of any overarching mechanism, would greatly facilitate the development of models predicting future biodiversity responses and monitoring strategies, there is increasing evidence that the diversity of the marine stress response is more complex.

Heat shock proteins have long been at the core of our evaluations of marine stress responses (Feder and Hofmann, 1999). This is due, not only to their well-known critical roles in maintaining cellular homeostasis under changing conditions, but also the fact that they are highly conserved and therefore relatively easy to survey in a wide range of non-model species. Hence their preferred status for stress biomarkers. The stress molecular repertoire was extended in 2005, with the identification of an evolutionary conserved set of genes (the minimal stress proteome), hypothesized to be at the core of the cellular stress response (CSR) (Kültz, 2005). Since then, numerous molecular studies have been conducted examining marine organisms' responses to changing conditions, most recently using Next Generation Sequencing in discovery-led approaches (e.g. Traylor-Knowles et al., 2017; Clark et al., 2017; Martino et al., 2019). These have confirmed the importance of heat shock proteins in the marine stress response, but also highlighted the variability in responses and additional pathways that can be invoked, such as the cytoskeleton, redox proteins, DNA damage and transcription factors (Tomanek, 2014; Traylor-Knowles et al., 2017; Aguilar et al., 2019; Martino et al., 2019). Hence the question arises as to whether *a priori* assumptions can be made as to which of these conserved stress response genes are invoked under different conditions within the same species or between different species subjected to the same environmental challenge?

Dissecting out critical elements of the stress response (and therefore the mechanisms underlying that response) is fraught with difficulties. The same is true when investigating the extent of variability in response. In laboratory experiments, small changes in technique or experimental design and sampling can significantly influence results, which detracts from between species comparisons (Clark et al., 2016). In addition, care is needed when sampling directly from natural conditions to either minimise or accommodate the effect of natural cycles, such as diurnal gene expression patterns and seasonality (Buckley et al., 2001; Gracey et al., 2008). Therefore to interrogate the extent of common or variable responses to different environmental stresses even within the same species (irrespective of the CSR hard-wired into the genome) standardised methodologies are required.

In this study, we tested the hypothesis that responses to different rates of thermal ramping and a three month acclimation would produce highly divergent cellular responses, even within the same species. Three species of Antarctic marine invertebrate: *Nacella concinna* (limpet), *Paraceradocus miersi* (amphipod) and *Sterechinus neumayeri* (urchin) were obtained from Ryder bay, at the same time. Therefore all animals had experienced the same environmental conditions, such as food, light and critically, thermal history, all of which affect the heat shock response (Feder and Hofmann, 1999). Antarctic species also have the advantage of living in a relatively pristine environment and not subject to the potential effects of pollution, which may confound the response to warming. All three species were subjected to four different thermal stressors: three different temperature ramping experiments (1 °C h^{-1}, 1 °C day^{-1}, 1 °C 3 day^{-1}) and a three month acclimation at 2 °C. Animals were sampled close to their thermal limits in the ramping experiments and at the end of the acclimation period. Upper thermal limit (UTL) experiments were performed at the beginning and end of the acclimation period, in both control and treated individuals, to identify if whole animal acclimation to 2 °C had occurred within the three month period (Schmidt-Nielsen and Schmidt-Nielsen, 1997; Peck et al., 2014). RNA-Seq was used to identify transcriptional responses to the different treatments. These data were correlated within species and across species to determine whether there was any commonality of transcriptional responses to heat stress.

2. Materials and methods

2.1. Experimental design

Three species of Antarctic marine invertebrate (*N. concinna*, *P. miersi* and *S. neumayeri*) were subjected to a three-month acclimation at 2 °C (a temperature, which these species were known to tolerate) and a series of three thermal ramping experiments (1 °C h^{-1}, 1 °C day^{-1} or 1 °C 3 day^{-1}) with their cellular responses evaluated via RNA-Seq. Animals were sampled at the beginning and end of the acclimation experiment and below their lethal limits in the ramping experiments (Table 1) with full sets of control animals taken at each stage, at the same time as the treated animals, to avoid any effects on gene expression due to circadian rhythms. For each treatment, 10 animals were sampled. Furthermore, to verify if the animals had acclimated to 2 °C at the whole animal level, upper thermal limits were carried out at the beginning of the acclimation and at the end of the acclimation. For each UTL experiment, at least 20 individuals were used.

2.2. Animal collection

The field studies did not involve endangered or protected species and the experimental organisms were non-regulated so ethical approval was not required. *N. concinna*, *P. miersi* and *S. neumayeri* were collected by SCUBA divers at 8-15 m depth from South Cove near Rothera Research Station, Adelaide Island, Antarctic Peninsula (67° 4′ 07″ S, 68° 07′ 30″ W) in the austral summer of 2011–2012. The temperature of the sea water in the Antarctic is very stable, usually varying from −1.86 °C in winter up to +1 °C for brief periods in the summer (Clarke et al., 2008). After collection the animals were held in a through-flow aquarium system at ambient temperature (0.56 °C ± 0.04 SE mean) and a 12:12 h light:dark lighting regime for at least seven days prior to use in the experiments to ensure recovery from collection. They were then transferred to the experimental systems.

2.3. Acclimation experiment

Animals were transferred to 300 L volume flow-through aquaria. One set of animals was maintained in control conditions for the three–month experiment with separate tanks for each species. A second set of animals was transferred to flow-through tanks, which were maintained at 2 °C by balancing a constant slow flow (10 l min^{-1}) with heating from 0.5 kW fluoropolymer coated immersion heaters with a PT100 probe (Dryden Aquaculture). Mixing and aeration were provided by two airlines attached to standard aquarium airstones. Animals were initially placed in these tanks at ambient temperature with the temperature raised 0.5 °C day^{-1} until the final target of 2 °C was reached and maintained for three months (average tank temperatures for *P. miersi* 2.09 ± 0.02 SE; *N. concinna* 2.31 °C ± 0.05 SE; *S. neumayeri* 2.08 ± 0.03 SE with the control tank at 0.56 ± 0.04 SE). Tank temperatures were recorded daily (Supplementary file S1). UTLs (used as a measure of whole animal acclimation (Schmidt-Nielsen and Schmidt-Nielsen, 1997; Peck et al., 2014)) were measured at the start of the acclimation experiment (28/10/11) and then in controls and treated animals at the end of the experiment (02/02/12).

Table 1
Sampling temperatures of ramping experiments and ranges of UTL of the three study species in other years. Data from Peck et al. (2009, 2014) and Peck (pers comm).

Species	Temperature ramp	Year	Sample temperature in this study
P. meirsi	1 °C h^{-1}	2010: 15.3–17.6	13.4
	1 °C day^{-1}	2006: 10.0–16.0	9.6
		2012: 11.3–16.4	
		2013: 12.0–14.8	
	1 °C 3 day^{-1}	2010: 8.5–12.6	7.6
N. concinna	1 °C h^{-1}	2010: 15.1–26.2	13.4
	1 °C day^{-1}	2006: 10.0–13.0	8.4
		2012: 14.3–17.4	
		2013: 14.3–17.4	
	1 °C 3 day^{-1}	2012: 10.6–14.9	9.7
S. neumayeri	1 °C h^{-1}	2010: 15.1–21.6	13.4
		2012: 24.0–25.3	
	1 °C day^{-1}	2006: 7.5–14.0	6.7
		2010: 8.0–14.0	
		2012: 13.7–17.2	
	1 °C 3 day^{-1}	2010: 5.0–12.6	4.3

2.4. UTL experiments

Animals were transferred to a 60 L jacketed tank with aerated sea water at the same temperature as the ambient sea water (either 0 °C or 2 °C, if in the treated acclimation group) and connected to a thermocirculator (Grant Instruments Ltd., Cambridge, UK). The temperature was raised at 1 °C h^{-1} with the temperature limit of each animal noted when they no longer responded to appropriate external stimuli (e.g. touching or prodding with a seeker and movement of antennae or tube feet) (Peck et al., 2009). A minimum sample size of n = 21 for each species was used in each UTL test. UTL data were non-normal, even after transformations, so Mann-Whitney non-parametric statistical tests test (Minitab v. 19) were used to identify any significant difference in UTL between control and 2 °C animals (https://doi.org/10.5285/19C70CF4-6972-42E6-8474-1322B220104F).

2.5. Thermal ramping experiments

Animals were transferred to a 60 L jacketed tank with aerated sea water at the same temperature as the ambient sea water (0.56 °C) and connected to a thermocirculator (Grant Instruments Ltd., Cambridge, UK). The temperature was raised at either 1 °C h^{-1}, 1 °C day^{-1} or 1 °C 3 day^{-1}. Animals were sampled at temperatures before they became comatose. These temperatures were identified from previous research and were approximately 10% lower than their previously evaluated UTLs at that particular ramping rate (Table 1), although regular checks were maintained as these CT_{max} limits can vary annually (Table 1) and sampling temperatures adjusted accordingly if any individuals started to become markedly less responsive to stimuli. The latter happened very rarely. For the longer term experiments (1 °C day^{-1} or 1 °C 3 day^{-1}), control animals were sampled at both the start and end of the experiment to identify and allow for any seasonal or tank effects. For the 1 °C h^{-1}, only one set of controls was needed due to the very short time-scale of the experiment. For all other trials initial controls and final controls were run.

2.6. Animal sampling

For the RNA extractions, tissue was taken from 10 animals for each of the control, thermal ramping and acclimation experiments and snap frozen in liquid nitrogen with subsequent storage at −80 °C until needed. Animal metrics were recorded for each specimen (length, height and width (mm) of shell for *N. concinna*, wet weight (g) for *P. miersi* and test diameter (mm) for *S. neumayeri*). Animal size between the different experiments was compared using standard *t*-tests (MiniTab v. 19) (Supplementary file S1 and https://doi.org/10.5285/19C70CF4-6972-42E6-8474-1322B220104F).

2.7. RNA extraction and sequencing

RNA was extracted using TRI reagent (Bioline, London, UK) and purified on RNeasy mini columns (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was quantified using an Agilent Technologies Tape Station 2200. The highest quality extractions (n = 5) were chosen from each experiment for sequencing by the Earlham Centre, Norwich. The RNAs were subjected to RNA-Seq on an Illumina Hi-Seq 2000/2500 (125PE) across three full lanes, with a small sub-set of 9 samples run on a MiSeq due to issues with adapter spikes (these comprised one *N. concinna* sample and 8 *P. miersi* samples across three different treatments).

2.8. Transcriptome analysis

Read quality was assessed using FastQC v0.11.8 (Babraham Bioinformatics) and MultiQC v1.7 (Ewels et al., 2016). Adapter and quality trimming was then performed using Trimmomatic v0.39 (Bolger et al., 2014) (mean PHRED score < 5 across a 4-bp sliding window). Transcriptomes were assembled for each species using Trinity v2.8.4 (Haas et al., 2013) using default parameters. Assemblies were screened against the NCBI UniVec database to remove contigs containing technical sequences. Assembly quality was assessed by quantifying read support for the transcriptome using Bowtie2 v.2.3.5.1 (Langmead and Salzberg, 2012) and Samtools v1.9 (Li et al., 2009). Transcriptome completeness was assessed using BUSCO v3 (Waterhouse et al., 2018) and the metazoan ortholog database ("Metazoa odb9"). Transcriptomes were annotated using Trinotate v3.1.1 (Bryant et al., 2017). Transcript and translated protein sequences, produced by Transdecoder v5.5.0, were searched (blastx or blastp) against the Swissprot/Uniprot database with an E-value cut-off of 1×10^{-5}. GO term annotations were obtained from Trinotate's Uniprot-GO term mappings. For gene expression analyses, read mapping was performed using Salmon 0.13.1 (Patro et al., 2017). Counts data were imported into R v3.5.1 using tximport v1.10.1 (Soneson et al., 2015). Differential gene expression analysis was performed using DESeq2 v1.22.2 (Love et al., 2014) and significantly affected genes (Padj < 0.05) were identified from pairwise comparisons. Functional enrichment analysis (GO terms) was performed using goseq v.1.34.1 (Young et al., 2010) on up/downregulated DEGs (log2-FC > 1/<−1, Padj < 0.05). Enriched GO categories were compared both within species and between species to identify common genes and functions up- and downregulated across the different thermal ramping and acclimation experiments. GO terms are not species-specific. Therefore comparing GO terms between species (and relating those terms to back to annotated transcripts within the particular species) avoids any potential issues associated with defining a set of conserved orthologues and paralogues across highly divergent species.

3. Results

There were no mortalities in any of the thermal stress experiments and there were no significant size differences between any of the control and treated animal groups, with one exception. This was *P. miersi* in the acclimation experiment where controls sampled at the start were significantly larger than the animals maintained as controls throughout the 3-month period (Supplementary file S2). Whilst size (as a proxy for age) can be a factor in the stress response (Clark et al., 2013), this was not considered serious issue as these animals were in the initial control groups and did not affect comparisons of thermally manipulated specimens with relevant controls. Although the thermal tolerance of Antarctic marine invertebrates varies annually (Peck et al., 2014), the animals in this experiment were all sampled below the lower UTL established in experiments conducted in different years (Table 1).

Transcriptome assemblies produced 361,735 transcripts (*P. miersi*), 475,160 transcripts (*N. concinna*), and 562,112 transcripts (*S. neumayeri*)

(Supplementary file S2). The assemblies were of a high quality as revealed by the proportion of paired reads aligning concordantly back to each of the reference transcriptomes (91.55%, 89.78% and 90.42% for *P. miersi*, *N. concinna*, and *S. neumayeri* respectively). These values are substantially higher than the Trinity guidelines of 70–80% for a good quality assemblies. In addition, BUSCO assessment results were 96.3%, 99.3% and 99.0% for *P. miersi*, *N. concinna*, and *S. neumayeri* respectively (Supplementary file S3). When each treatment was mapped back to each of the three species, there was considerable difference in the number of differentially regulated transcripts ranging from 10 to 3011 (Fig. 1). When these data were correlated across the different temperature treatments within each species, there were only 0, 2 and 0 transcripts shared between all four treatments, for *P. miersi*, *N. concinna* and *S. neumayeri* respectively (Fig. 1). The response of each species was also highly individual.

3.1. Responses in the amphipod P. miersi

There was a poor transcriptional response at 1 °C h^{-1} in this species (Fig. 1A). The few transcripts that were up-regulated, included a single *hsp70* gene and genes involved in neurons and neurotransmission (e.g. *st1c4*, *vcx3b*, *ogt1* and *nfh* (Supplementary file S4)). More transcripts were up-regulated at 1 °C day^{-1} which included traditional elements of the conserved cellular stress response (CSR) such as heat shock proteins, chaperones and protein production (Fig. 2). At 1 °C 3 day^{-1} again the up-regulated response was poor and similarly in the acclimation treatment, the majority of transcripts were down-regulated. The unique down-regulated transcripts in the 1 °C 3 day^{-1} experiment mainly comprised cuticle proteins and some immune genes e.g. phenoloxidase. During acclimation the putative function of the down-regulated transcripts covered most cellular categories (Supplementary file S4). These differential expression data were validated by GO enrichment analyses with relatively little enrichment for both the up-regulated and down-regulated ontologies. The exceptions were the up-regulation of categories associated with translation at 1 °C day^{-1} correlating with the need to produce the CSR as identified in the differential expression analyses. In the acclimated animals there was very strong GO enrichment across many ontologies (201 categories) in the down-regulated transcripts (classes for ribosome and protein synthesis, TCA cycle, ion transport etc.) (Supplementary file S5). There were very few shared transcripts between the different rates of warming (Fig. 1A). *P. miersi* had not acclimated to 2 °C within the 3 month experiment, as there was no significant difference in UTL between control and treated animals (medians of 13.7 °C v. 13.8 °C, Mann Whitney adjusted P value = 0.433).

3.2. Responses of the limpet N. concinna

N. concinna produced a much stronger response compared with *P. miersi* in terms of differential regulation for all the ramping experiments. There was also much more overlap between the different responses at 1 °C h^{-1}, 1 °C day^{-1} and 1 °C 3 day^{-1} than identified in the amphipod (Fig. 1B). There was a pronounced CSR at 1 °C h^{-1}, with apoptosis functions appearing in the 1 °C day^{-1}, which became more apparent at 1 °C 3 day^{-1}, data and were validated by GO enrichment (Supplementary file S5). Shared transcripts between 1 °C h^{-1} and 1 °C day^{-1} included transcription factors and the map kinase, *mapk*. The functions of shared up-regulated transcripts between 1 °C day^{-1} and 1 °C 3 day^{-1} included protein degradation, peptidase, GTP-binding and autophagy (Supplementary files S4 and S5). There were no significant GO enrichment terms for up-regulated transcripts at 1 °C day^{-1}, but ontologies at 1 °C 3 day^{-1} were dominated by necrosis and apoptosis. Interestingly, shared down-regulated genes (between 1 °C day^{-1} and 1 °C 3 day^{-1}) included caspases, which are key regulators of apoptosis. There was little response identified in the differential expression analysis at the end of the acclimation treatment. After 3 months at 2 °C, the UTL of *N. concinna* was not significantly different to animals kept at 0 °C, indicating no physiological acclimation at the whole animal level (medians of 14.8 °C v. 14.8 °C, Mann Whitney adjusted P value = 0.339 respectively). However, the UTLs of *N. concinna* controls were significantly lower at the start of the acclimation in October compared with control animals at the end of the experiment in February (median of 14.5 °C v. 14.8 °C, Mann Whitney adjusted P value = 0.002). Whilst the highest UTL temperature was the same in both the October and February experiments, the spread was greater with the October animals failing at 14 °C whilst after three months under control conditions the first animals succumbed at 14.8 °C (https://doi.org/10.5285/19C70CF4-6972-42E6-8474-1322B220104F). Control tank temperatures were not static as the system used was flow-through and the control temperature was effectively the ambient temperature, which increased over the summer period to slightly above 1 °C (Supplementary file S1).

3.3. Responses in the sea urchin S. neumayeri

S. neumayeri showed a mixed response compared to the two other species (Fig. 1C). Similar to *N. concinna* there was overlap of shared genes between warming of 1 °C h^{-1} and 1 °C day^{-1} (Supplementary file S4) and the induction of the CSR at 1 °C h^{-1} (Fig. 2). There was very little response at 1 °C 3 day^{-1}. Several 100's of transcripts were differentially expressed in the 2 °C acclimation trial. There was very little GO enrichment within the treatments for the urchin (Supplementary file S5). The exception was at the fastest ramping rate with up-regulated enrichment for ontologies involved in translation and signalling pathways. In contrast to the other species, the UTL of *S. neumayeri* at 2 °C after 3 months was significantly lower than that of the control animals (median of 13.7 °C compared with 15.9 °C, Mann Whitney adjusted P value = 0.043), indicating that chronic exposure to 2 °C had a detrimental effect. However, these physiological data were not reflected in the categories of GO enrichment associated with up-regulated transcripts in the animals in the acclimation trial, which included signalling pathways and the immune responses.

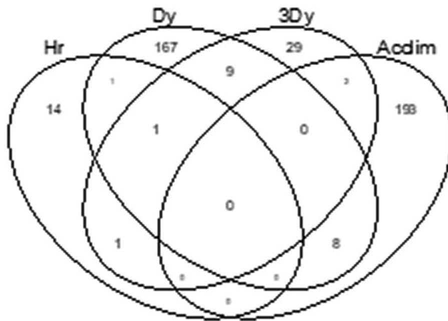
As there were very few shared transcripts between the different treatments within a single species and the levels of differential expression varied markedly between species (Fig. 1), it was perhaps not surprising to find little commonality between the species, as evaluated by comparison of GO enrichment categories in both up- and down-regulated genes (Supplementary file S5). There was no overlap in GO enrichment categories at either 1 °C day^{-1} or in the acclimation trial. At 1 °C h^{-1} there was only overlap for GO terms involved in the up-regulation of protein folding between *N. concinna* and *S. neumayeri*, which correlated with their classical CSR at this ramping rate (Fig. 2). At 1 °C 3 day^{-1} *P. miersi* and *N. concinna* shared GO enrichment for structural molecule activity.

4. Discussion

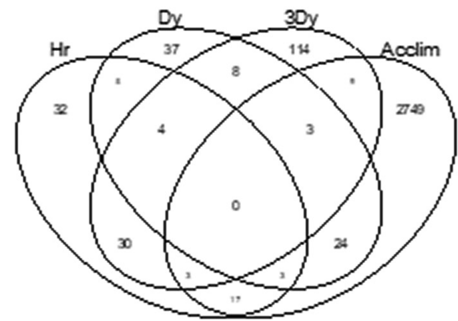
Transcriptional evaluation of the three species across four different temperature treatments emphasised how individual the responses were, with very little commonality either within or between species. The crustacean *P. miersi* showed a poor response at 1 °C h^{-1}, which matched closely with a previous similar 1 °C h^{-1} experiment that combined transcriptional and metabolomics profiling (Clark et al., 2017). Similar to those previous data, the few transcripts that were up-regulated, included genes involved in neurotransmission (Supplementary file S4). Although there was more of a response at 1 °C day^{-1} with the induction of the classical CSR including heat shock proteins and the translation machinery required to underpin this, generally this species showed a low level of transcriptomic response, which likely reflected physiological coping mechanisms. During acclimation, most of the differentially expressed genes were downregulated and comprised a whole range of functional categories, with the obvious conclusion that the animals were shutting down many elements of their

A: *P. miersi* (111; 270; 213; 3,011)

Up-regulated transcripts

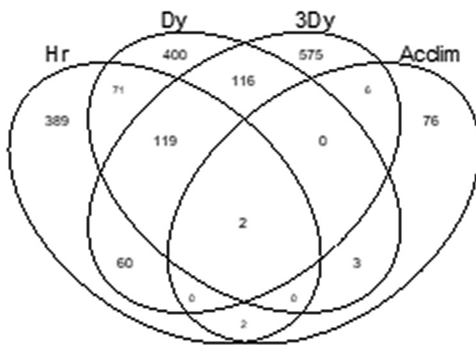


down-regulated transcripts

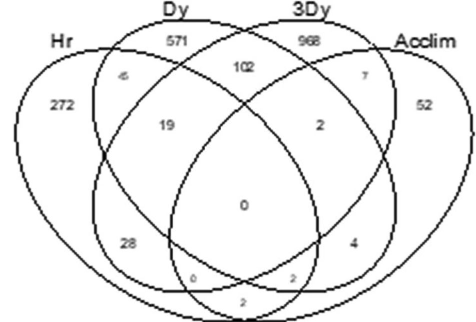


B: *N. concinna* (1,011; 1,456; 2,004; 158)

Up-regulated transcripts

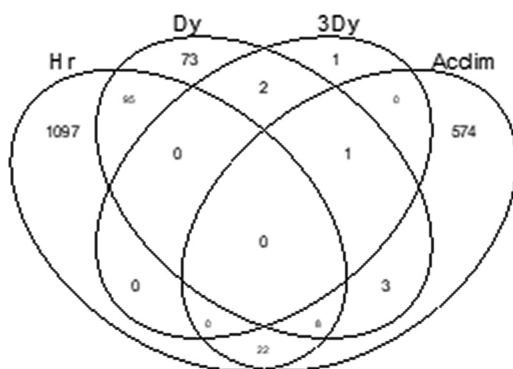


down-regulated transcripts



C: *S. neumayeri* (1,845; 428; 10; 851)

Up-regulated transcripts



down-regulated transcripts

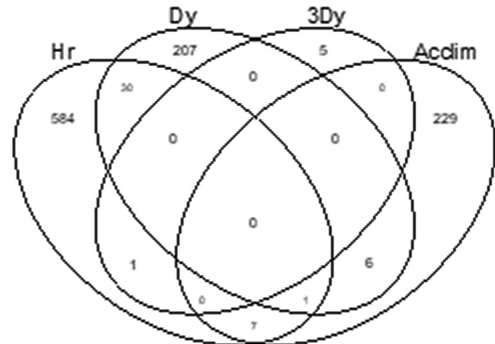
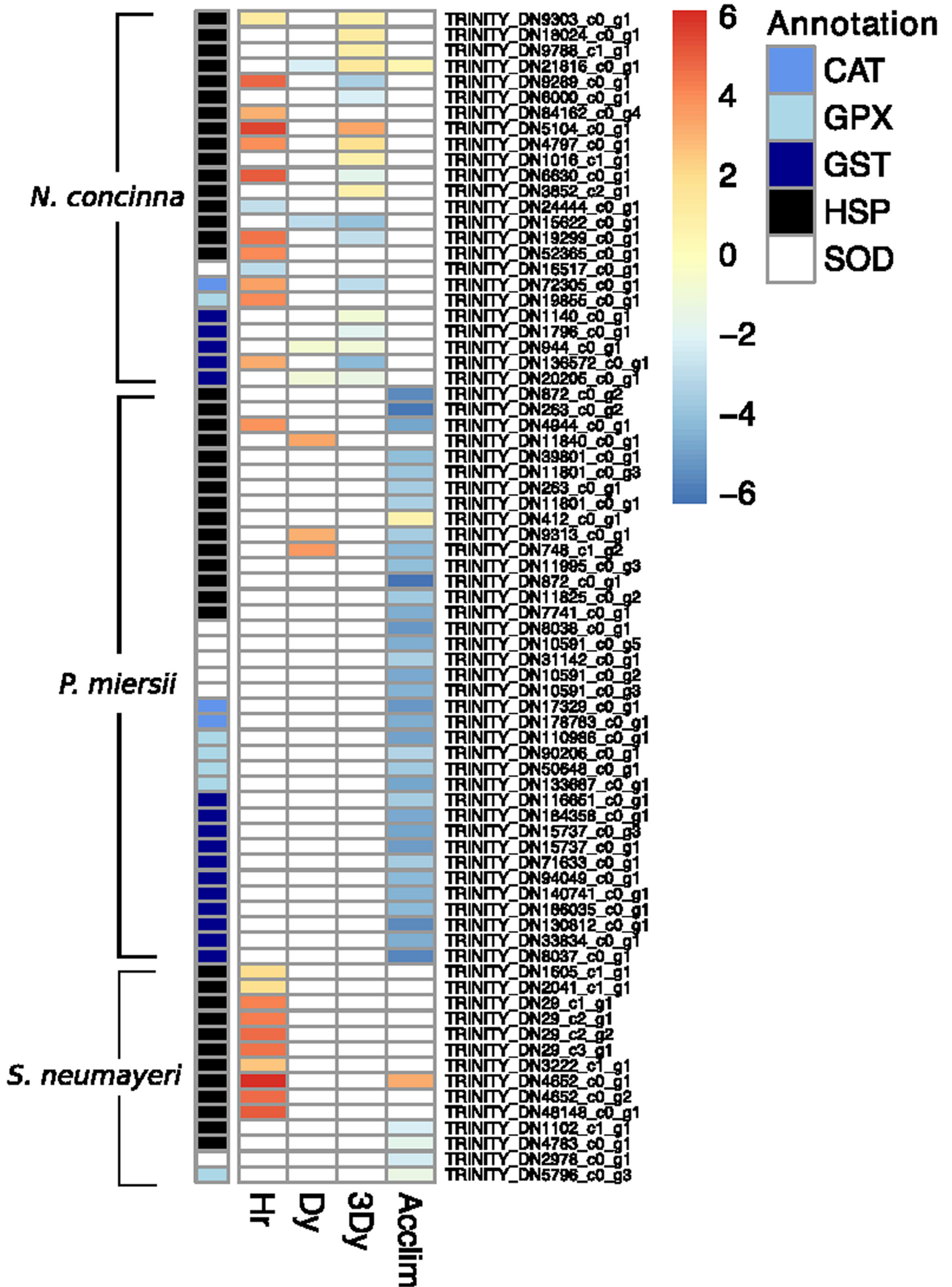


Fig. 1. Venn diagrams showing number of up- and down-regulated transcripts between treatments for each species. Numbers in brackets after the species name denote the total number of differentially expressed transcripts at 1 °C h^{-1}, 1 °C day^{-1}, 1 °C 3 day^{-1} and 3 months at 2 °C respectively.

metabolism to compensate for the prolonged exposure to the higher temperature (Supplementary file S4). This recourse to hypometabolism in stressful conditions is a well-documented strategy in Crustacea, most recently reported at the molecular level in the response of the amphipods *Gammerus chevreuxi* and *Echinogammarus marinus* to severe hypoxia (Collins et al., 2019, 2020). To date, the acclimation ability of this species is unknown (as in this experiment acclimation was not achieved to 2 °C within three months), with the only previous acclimation experiment


N. concinna
P. miersii
S. neumayeri
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TRINITY_DN1140_c0_g1
TRINITY_DN1796_c0_g1
TRINITY_DN944_c0_g1
TRINITY_DN136572_c0_g1
TRINITY_DN20206_c0_g1
TRINITY_DN872_c0_g2
TRINITY_DN263_c0_g2
TRINITY_DN4944_c0_g1
TRINITY_DN11840_c0_g1
TRINITY_DN39801_c0_g1
TRINITY_DN11801_c0_g3
TRINITY_DN263_c0_g1
TRINITY_DN11801_c0_g1
TRINITY_DN412_c0_g1
TRINITY_DN9313_c0_g1
TRINITY_DN748_c1_g2
TRINITY_DN11995_c0_g3
TRINITY_DN872_c0_g1
TRINITY_DN11825_c0_g2
TRINITY_DN7741_c0_g1
TRINITY_DN8038_c0_g1
TRINITY_DN10591_c0_g5
TRINITY_DN31142_c0_g1
TRINITY_DN10591_c0_g2
TRINITY_DN10591_c0_g3
TRINITY_DN17329_c0_g1
TRINITY_DN178783_c0_g1
TRINITY_DN110986_c0_g1
TRINITY_DN90206_c0_g1
TRINITY_DN50648_c0_g1
TRINITY_DN133687_c0_g1
TRINITY_DN116851_c0_g1
TRINITY_DN184358_c0_g1
TRINITY_DN15737_c0_g3
TRINITY_DN15737_c0_g1
TRINITY_DN71633_c0_g1
TRINITY_DN94049_c0_g1
TRINITY_DN140741_c0_g1
TRINITY_DN186035_c0_g1
TRINITY_DN130812_c0_g1
TRINITY_DN33834_c0_g1
TRINITY_DN8037_c0_g1
TRINITY_DN1605_c1_g1
TRINITY_DN2041_c1_g1
TRINITY_DN29_c1_g1
TRINITY_DN29_c2_g1
TRINITY_DN29_c2_g2
TRINITY_DN29_c3_g1
TRINITY_DN3222_c1_g1
TRINITY_DN4652_c0_g1
TRINITY_DN4652_c0_g2
TRINITY_DN48148_c0_g1
TRINITY_DN1102_c1_g1
TRINITY_DN4783_c0_g1
TRINITY_DN2978_c0_g1
TRINITY_DN5796_c0_g3
Hr
Dy
3Dy
Acclim
6
4
2
0
−2
−4
−6
Annotation
CAT
GPX
GST
HSP
SOD

performed on this species showing a failure to acclimate to the slightly warmer temperature of 3 °C after 60 days (Peck et al., 2010).

N. concinna responded to the warming treatments much more strongly at the cellular level than *P. miersi*. In this species, the CSR was produced at 1 °C h^{-1}. Interestingly, genes with putative functions in apoptosis were evident at 1 °C day^{-1} and 1 °C 3 day^{-1}. Furthermore shared transcripts between these two ramping rates included *mapk*, a key indicator of the environmental stress response (Cowan and Storey, 2003) and genes indicating a decline in robustness (protein degradation, peptidase, GTP-binding and autophagy). Similarly ontologies at 1 °C 3 day^{-1} were dominated by necrosis and apoptosis. Specimens from the 1 °C 3 day^{-1} treatment were sampled at a slightly higher temperature compared with 1 °C day^{-1} (8.4 °C and 9.7 °C), when a lower sampling temperature would be expected based on previous and subsequent experiments (Peck et al., 2009, 2014). Sampling temperatures in this experiment were based on previous observations of species' thermal tolerance and individual responses during the course of this experiment. Therefore, it would appear that the limpets ramped at 1 °C 3 day^{-1} may have exhibited a greater thermal tolerance in this experiment. Curiously, shared down-regulated genes (between 1 °C day^{-1} and 1 °C 3 day^{-1}) included caspases, which are key regulators of apoptosis. This may indicate that autophagy is a part of an, at least temporary, coping mechanism to elevated temperature in *N. concinna*, as has previously been demonstrated in other species (Rabinowitz and White, 2010). Whilst *N. concinna* did not show any level of whole animal acclimation after 3 months at 2 °C, it was interesting to note was that the UTLs of *N. concinna* controls were significantly lower at the start of the acclimation in October compared with control animals at the end of the experiment in February. This may be due to the fact that the temperature of the flow through control tanks increased over the summer months to slightly above 1 °C and the controls were themselves being gradually warmed (Supplementary file S1). Thus, there were indications from the UTL experiments that some level of acclimation to warmer temperatures occurred in the control animals, which may have masked the physiological response in the treated cohort. It also suggests that acclimation in this species to temperatures above experienced summer maxima is more difficult than for temperature variation within the experienced range. A similar situation has been previously demonstrated with elevated UTLs in control animals, where ambient tank temperatures had increased from −1 °C to 0 °C during a 5 month 3 °C acclimation experiment, which led to a much reduced differentiation between control and treated animals at the end of the experiment (Peck et al., 2010). Furthermore, a certain level of acclimation to chronic warming was indicated at the molecular level, as evidenced by the low levels of differential expression between treated and control animals after 3 months at 2 °C (Fig. 1). In addition, GO enrichment for metabolic processes during acclimation involved categories associated with lipids, suggesting mobilization of energy reserves to fuel responses to warming and energy store limitation has been suggested as one of the potential mechanisms dictating survival in slow, chronic warming scenarios (Peck, 2018). Previous studies using *N. concinna* have indicated that this species requires at least 2 months, but less than 5 months to acclimate to warmer conditions (Peck et al., 2014), which aligns with the partial results of this study after 3 months treatment. This was the only species to show any level of acclimation and interestingly this species also showed the highest levels of differential gene expression across the different ramping temperatures, indicating a more active response to changing conditions. In the previously published 1 °C h^{-1} experiment (which did not include *N. concinna*), the ability to respond to changing conditions at the gene expression level was one of the hallmarks of the more thermally tolerant species (Clark et al., 2017).

The transcriptomic response of *S. neumayeri* was intermediate between the two other species. Similar to *N. concinna* there was overlap of shared genes between warming of 1 °C h^{-1} and 1 °C day^{-1}, including those involved in translation, which potentially underpinned the classical CSR at the fastest ramping rate (Fig. 2). This is the first time that expression of *hsp70* genes in response to thermal ramping has been demonstrated in adult *S. neumayeri* (Fig. 2). There was very little response at 1 °C 3 day^{-1}, but this may have been due to a large variation between individuals in that treatment. Several 100's of transcripts were differentially expressed in the 2 °C acclimation trial, which was perhaps not surprising as the UTL of *S. neumayeri* at 2 °C after 3 months was significantly lower than that of the control animals indicating that chronic exposure to 2 °C had a detrimental effect. In contrast, categories of GO enrichment associated with up-regulated transcripts in the animals in the acclimation trial included signalling pathways and the immune responses. These indicated an active defence rather than the expected apoptotic pathways and energy depletion as would be expected with failing animals and the shutting down of cellular metabolism. Certainly, other studies indicate that *S. neumayeri* can successfully acclimate to $^{+}$2–3 °C, although this may take up to 8 months, particularly if multiple stressors are involved (Suckling et al., 2015). Therefore, this reduction in UTL may reflect temporarily increased energy costs for the cells whist they undergo reprogramming to cope with the new conditions.

Given the wide variation in response between different treatments in the same species, it was not surprising that no commonality was found between the species. Although the CSR (as represented by CAT, GPX, GST, HSP and SOD transcripts in Fig. 2) was expressed in all three species (although less strongly in *P. miersi*), it was invoked at different thermal ramping rates, potentially indicating different thresholds (e.g. Kenkel et al., 2014). One potential common element (GO enrichment for structural molecule activity) was identified at 1 °C 3 day^{-1} in both *P. miersi* and *N. concinna*. However, this category can encompass many different functions. In *P. miersi* it was almost certainly related to the down-regulation of cuticle genes and repression of moulting, whilst in *N. concinna* it was likely related to autophagy, as suggested by the gene expression analyses.

Overall, these data, considerably expand a previous metabolomics and transcriptomic evaluation of six Antarctic marine invertebrates warmed at 1 °C h^{-1} and emphasise the complexity of the environmental stress response across a wide range of temperatures (Clark et al., 2017) and the mechanisms underpinning those responses. Heat shock proteins are highly conserved throughout evolution, both in structure and function and play a key role in the acute stress response and maintenance of protein homeostasis in the majority of organisms studied to date (Feder and Hofmann, 1999). However, even these highly conserved genes vary between species. For example members of the *hsp70* family (HSPA12) are massively duplicated in some bivalve molluscs, such as the Pacific and pearl oysters, the invasive golden mussel, scallops and the blue mussel (73, 97, 55, 57 and a minimum of 34 copies respectively), all of which have subtly different functions (Cheng et al., 2016; Clark et al., 2021). These extra *hsp70* genes are thought to have evolved to enable these sessile animals to cope in a highly dynamic changing marine environment and certainly appear to have enhanced their invasive potential (Zhang et al., 2012; Cheng et al., 2016). Furthermore, studies in model organisms encompassing prokaryotes through to multicellular eukaryotes, such as *C. elegans*, *A. thaliana* and *H. sapiens* indicate that personalised species-specific sets of damage

Fig. 2. Heat map of the expression of candidate stress response genes with treatment and species. Scale bar indicates log2-FC of significantly differentially expressed genes (Padj < 0.05) between treatments (1 °C h^{-1}, 1 °C day^{-1}, 1 °C 3 day^{-1}, acclimation) and their respective controls, for three Antarctic invertebrates: *N. concinna*, *P. miersi* and *S. neumayeri*. For annotation information and expression data of candidate genes, see Supplementary file S4. White boxes indicate that a gene was not significantly differentially expressed for a given pairwise comparison (Padj > 0.05). Different patterns of expression of candidate genes can be seen between and within a species across different rates of warming. Intraspecific variation reflected by different magnitudes of log2-FC for each treatment versus controls (n = 5 individuals per treatment) within a given species.

control proteins have evolved outside of the highly conserved HSP system (Richter et al., 2010). These stress inducible proteins comprise seven functional classes including molecular chaperones (e.g. Hsps), elements of the proteolytic system, DNA/RNA repair, metabolic enzymes which are needed to reorganise and stabilise the energy supply to the cell, regulatory proteins (e.g. transcription factors to initiate expression cascades such as stress response pathways), proteins involved in sustaining cellular structures, such as the cytoskeleton and finally transport, detoxification and membrane modulating enzymes (Richter et al., 2010). Furthermore, the kinetic expression of these different functional classes varies with the response and the biochemistry associated with a particular lifestyle (activity levels, metabolic status, age and environmentally programmed methylation) of the organism (Richter et al., 2010; de Nadal et al., 2011; Gidalevitz et al., 2011). Elements of these different functional groupings, such as the cytoskeleton, redox proteins and DNA damage have also been identified previously as critical in the marine environmental stress response along with heat shock proteins (Somero, 2020). Furthermore, several examples exist describing how cellular biochemistry at a particular life history stage impacts marine invertebrate responses to stress (Buckley et al., 2001; Peck et al., 2009; Clark et al., 2018; Aguilar et al., 2019; Martino et al., 2019). Whilst a core of evolutionary conserved stress response genes has been identified in all species examined to date (Kültz, 2005) and in this study as evidenced by the CSR in Fig. 2, there is clearly great diversity in how these have evolved to produce specialised damage control networks in each species relevant to their particular environment. In Antarctic species examples of this include the unexpected duplication of *hsp70* genes in different species of Antarctic krill, which live in very cold environments (Cascella et al., 2015). It is thought these duplicated *hsp70*s evolved in response to their active pelagic lifestyle rather than temperature (Cascella et al., 2015), and active pelagic species have very different metabolic and energy storage profiles to benthic or low activity pelagic species, such as gelatinous zooplankton (Clarke and Peck, 1991; Cavallo & Peck, 2020). Another example is the atypical HSR in some Antarctic species (Clark and Peck, 2009).

The resources and technologies available for model organisms provide unprecedented detail currently unachievable in the vast number of non-model environmental species. The ability to conduct genome wide studies has moved the field of stress responses in model species from gene-centric analyses to the characterisation of regulatory mechanisms across the genome (Vihervaara et al., 2018). These have demonstrated that stress triggers a global reprogramming of transcription at genes and enhancers, a process that also involves chromatin modification, including 5′ UTR methylation (de Nadal et al., 2011; Liu and Qian, 2014; Vihervaara et al., 2018). These detailed analyses in model organisms and their evaluation of the diversity of the cellular stress response correlate with the data described here, which is highly specific to species and level, and type of, thermal stress. However, even for model organisms, there is still much to learn about the internal cellular co-ordination of the stress response and how these integrate across diverse cell types over the life history and metabolic state of an organism (Gidalevitz et al., 2011; Vihervaara et al., 2018). For example, experiments in *C. elegans* suggest the potential for neurons as an environmental sensing system (Gidalevitz et al., 2011). How this finding may translate through to different organism architectures remains to be elucidated. This is especially relevant when considering translation of results to marine invertebrates with their huge variation in structure, physiology and biochemistry.

To date, the limited number of genome-wide studies in marine invertebrates has evaluated SNPs across latitudinal gradients in species such as urchins and scallops. These have indicated specific adaptations to local conditions, including temperature (Pespeni et al., 2012; Van Wyngaarden et al., 2018; Vendrami et al., 2019) and provide valuable starting points on which to base more mechanistic laboratory experiments. In this, the application of a more systems biology approach with analyses of protein and gene network analyses is key (Kültz, 2020). Such experiments are increasingly achievable in non-model species. They are particularly informative as they incorporate the extensive number of "unknown" genes or proteins (often 70–80% in some non-model species) within functionally annotated clusters (Sleight et al., 2020; Ramsøe et al., 2020). Detailed genome characterisations invariably underpin the application of new technologies to non-model species, such as analysis of epigenetic effects and generation of gene editing resources for functional studies (Chen et al., 2018; Cleves et al., 2020). Indeed, recent experiments using CRISPR-induced mutation of heat shock transcription factor (*HSF1*) has demonstrated reduced thermal tolerance in the reef-forming coral *Acropora millepora* (Cleves et al., 2020). As the application of technologies developed in model species are increasingly applied to marine organisms, the environmental stress field will move away from a gene-centric focus, particularly the highly conserved *hsps*, to genome-wide studies. These will enable the examination of the fine detail of the environmental stress response and expand understanding the subtleties of the organism-specific response. These will ultimately be more informative about how stress networks operate near capacity and how the individual response is dictated by client proteins in the cell and that "one size does not fit all" (Somero, 2020).

5. Conclusions

Whilst a highly conserved set of genes has been identified across taxa as a conserved evolutionary strategy to deal with environmental stress, the CSR invoked by an organism is highly individualistic. This reflects the complexity of the mechanisms underpinning the response to stress, including factors such as the type and severity of the stress, the local habitat conditions, food availability and the condition of the animal etc. alongside any evolutionary changes to particular proteins at the genome level. The CSR is a graded response, finely tuned to the complexity of individual animals and circumstances (Somero, 2020).

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Data and materials availability

Sequencing data are freely available at ArrayExpress (E-MTAB-8241) and ENA (ERP116807). The source data for the animal metric, acclimation tank temperatures and UTL experiments are available from the UK Polar Data Centre, Natural Environment Research Council, UK Research & Innovation: https://doi.org/10.5285/19C70CF4-6972-42E6-8474-1322B220104F.

CRediT authorship contribution statement

Michael Collins: Bioinformatic analyses, Data curation, Generation of tables, figures and supplementary files, Writing – review and editing; Lloyd S. Peck: Conceptualization, Investigation, Funding Acquisition, Writing – review and editing; Melody S Clark: Conceptualization, Investigation, Funding Acquisition, Analyses, Writing – original draft.

Declaration of competing interest

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

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