Variable heat shock response in Antarctic biofouling serpulid worms

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Introduction

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Abstract:

The classical heat shock response (HSR) with up-regulation of *hsp70* in response to warming is often absent in Antarctic marine species. Whilst in Antarctic fish, this is due to a mutation in the gene promotor region resulting in permanent constitutive expression of the inducible form of *hsp70*, there are further questions as to whether evolution to life below 0°C has resulted in a generalised alteration to the HSR in Antarctic marine invertebrates. However, the number of species investigated to date is limited. In the first evaluation of the HSR in two spirorbid polychaetes *Romanchella perrieri* and *Protolaeospira stalagmia,* we show highly variable results of HSR induction depending on warming regimes. These animals were subjected to *in situ* warming (+1°C and +2°C above ambient conditions) using heated settlement panels for 18 months and then the HSR was tested in *R. perrieri* using acute and chronic temperature elevation trials. The classic HSR was not induced in response to acute thermal challenge in this species (2 hours at 15°C) and significant down-regulation of Hsp90 occurred during chronic warming at 4°C for 30 days. Analysis of heat shock protein (HSP) genes in a transcriptome study of *P. stalagmia,* which had been warmed *in situ* for 18 months, showed upregulation of HSP70 and HSP90 family members, thus further emphasizing the complexity of the response in Antarctic marine species. It is increasingly apparent that the Antarctic HSR has evolved in a species-specific manner to life in the cold.

Introduction

The up-regulation of production of heat shock proteins is generally considered as a universal component of the environmental cellular stress response and a key factor in organisms' abilities to tolerate warmer conditions (Feder and Hofmann, 1999). To date, very a few exceptions to this phenomenon have been recorded, and these have almost entirely been in Antarctic fish and marine invertebrates (Clark and Peck, 2009a). It is thought that an altered HSR in Antarctic species is potentially due to the problems of folding proteins in the cold, resulting in an enhanced requirement for the constitutive expression of chaperone proteins in Antarctic species compared with temperate relatives (Peck, 2016). In fact the lack of up-regulation of the inducible form of *hsp70* in Antarctic Notothenioid fish is due to a mutation in the promoter region of the gene, which means this gene is permanently turned on (Hofmann et al. 2000; Buckley et al. 2004). In other Antarctic species, constitutive expression of *hsp70* family members is relatively high compared to levels in temperate animals and the inducible form of *hsp70* is not always produced in response to laboratory heat shocks (Clark and Peck, 2009a). However, the situation is more complex, and evaluations of environmentally sampled animals revealed up-regulation of *hsp70* genes, triggered by abiotic factors

other than temperature (Clark and Peck, 2009b). RNA-Seq discovery-led approaches have shown the HSR in Antarctic species, which previously tested negative for such sequences using candidate gene approaches, albeit under different warming regimes (e.g. Clark et al. 2017; Collins et al. 2021). Although these RNA-Seq analyses clearly demonstrate the potential benefits of using non-targeted approaches, recent data have shown that HSPs are not universally upregulated in response to thermal stress in Antarctic species (Collins et al. 2021). Upregulation of HSPs varies considerably between species in response to the same thermal stress under standardised conditions and also within the same species when subjected to different thermal stresses (Collins et al. 2021). Furthermore, multiple copies of *hsp70* genes with highly variable responses to temperature have been identified in species of Antarctic krill (*Euphausia superba* and *E. crystallorophias,* each with five copies of *hsp70*) (Cascella et al. 2015), and duplicated copies of the inducible form of *hsp70* in the limpet *Nacella concinna* and the clam *Laternula elliptica* (Clark et al. 2008a). In the case of the clam, gene network analyses revealed sub-functionalisation of each duplicated *hsp70* gene via their association with different sub-modules, the components of which differed in their gene-gene interactions and functions (Ramsøe et al. 2020). Thus, heat shock proteins clearly have pivotal roles in the Antarctic cellular stress response.

To further investigate the HSR of Antarctic species, the current study used heated settlement panels deployed in the sea near Rothera Research Station on the Antarctic Peninsula to study the long-term effect of *in situ* warming on encrusting biofouling species (Ashton et al. 2017; Clark et al. 2019). The settlement panels heated the surface and a film of seawater 3-5 mm thick above each panel to +1°C and +2°C above ambient conditions (Ashton et al. 2017). Given the very slow growth rates of Antarctic species, the colonising communities developed and remained within this warmed layer for over 18 months, enabling a unique study of long-term chronic warming on cellular functioning (Clark et al. 2019). Two of the main benthic colonisers of the panels were spirorbid worms from the family *Serpulidae, Romanchella perrieri* and *Protolaeospira stalagmia.* Both exhibited faster growth and higher settlement success under the warmer regimes, with individuals of *R. perrieri* on average 70% larger in +1°C warmed conditions compared with those on control panels (Ashton et al. 2017).

This study evaluated the HSR of *R. perrieri* and *P. stalagmia* subjected to long term *in situ* warming in order to identify whether the spirorbids possessed the classic HSR and whether the different levels of warming had affected their HSR. Samples from both species were collected from control and heated (+1C, +2C) settlement panels deployed in the field for 18 months, and HSP expression analysed either by qPCR (*R. perreiri*) or datamining of previously generated RNA-seq data (*P.*

stalagmia). In addition, in order to assess the contribution of HSPs to acute and chronic warming following long term acclimation to elevated temperatures, warming experiments of 15°C for 2 hours and +4°C for 30 days were performed on *R. perrieri* from both control and heated settlement panels using qPCR.

Methods

The methods comprise three main sections:

1) The generation of a transcriptome for *R. perrieri.* This was based on acute and chronic heat treatments, with the identification of candidate heat shock proteins.

2) The assessment of HSP expression via candidate gene qPCR in *R. perrieri.* These analyses were performed after acute and chronic heat shocks on long term field acclimated *R. perrieri.*

3) Datamining of HSP transcriptome data from long-term field acclimated *R. stalagmia.*

Due to limited species numbers and the very small amounts of nucleic acid obtained from each individual, it was not possible to perform all analyses on the same species of spirorbid.

The generation of a transcriptome for *R. perrieri*

Animal collection for identification of heat shock proteins in R. perrieri: Rocks colonised by *R. perrieri* were collected by SCUBA divers between 18 and 25m water depth from South Cove in January 2014 near Rothera Research Station, Adelaide Island, Antarctic Peninsula (67° 4' 07" S, 68° 07' 30" W). Animals were immediately transferred, underwater at all times to the laboratory and maintained in a flow-through aquarium at a temperature of ambient temperature, under a 12:12 simulated natural light: dark cycle. Animals were transferred to the British Antarctic Survey aquarium facilities in Cambridge, UK and were habituated to aquarium conditions for 6 months (closed water system at water temperature and salinity of 0 ± 0.5°C and 35 ± 3.8ppt respectively, 12:12 h light: dark) prior to experimentation.

Heat shock experiments to generate and identify HSP transcripts: To maximise the identification of HSP transcripts in *R. perrieri*, two different temperatures were used: acute (15°C for 2 hours) and chronic (4°C for 30 days). To induce an acute heat shock response, rocks (n=3) colonised by *R. perrieri* were exposed to a thermal shock by immediate transfer to seawater at 15°C for 2 hours. Animals kept at 0°C were used as controls. To induce a chronic heat shock response, 60 *R. perrieri* on rock substratum were transferred to a 60 L jacketed acrylic tank (Engineering Design and Plastics) attached to a LTD20G thermocirculator (Grant instruments Ltd) and exposed to 4°C seawater for 30

days. As a control, another 50 *R. perrieri* were kept in a 60 L jacketed tank with aerated seawater at 0°C for 30 days, the same temperature as the main stock aquarium and field conditions when collected. Water temperature was checked twice a day for the duration of the experiment. In each experiment (treatments and controls), individuals (n=12) were dissected from their outer calcified skeleton under low power microscopy, snap frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

RNA extraction and sequencing: Total RNA was extracted from the whole organism using ReliaPrep™ RNA Miniprep System (Promega) according to manufacturer's instructions. RNA samples were assessed for concentration and quality using a NanoDrop ND-100 Spectrometer (NanoDrop Technologies) and an Agilent 2200 Tapestation (Agilent Technologies). RNAs were used with Tapestation readings of between 7.2 and 9.5. RNAs of equal quantities from six individuals were pooled for each experimental treatment to make a total of 4 libraries (0°C for 2 hours and 15°C for 2 hours, 0°C and 4°C for 30 days). Library preparation and sequencing was carried out by the Department of Biochemistry at the University of Cambridge. For each pool, RNA was converted to a sequencing library using the Illumina TruSeq stranded mRNA-seq library Prep kit (RNA input 1ug, fragmentation time 8 min, 10 PCR cycles), and barcoded libraries were pooled and sequenced on an Illumina MiSeq using 300 base paired-end reads, to generate 25 million raw reads per pool.

Generation of transcriptome and identification of HSP transcripts: All analyses were carried out using default parameters unless otherwise specified. Adapters were trimmed from the raw reads using Trimmomatic v.0.33 (Bolger et al. 2014). The reads were further trimmed based on quality and length using Fastq-mcf v.1.04.636 (Aronestry, 2011, (http://code.google.com/p/ea-utils/wiki/FastqMcf)). The Phred quality score was set to 30 and minimum read length to 80 bp. The reads were normalised *in silico* with different coverage values and contigs were assembled using Trinity v.2.0.6 (Grabherr et al. 2011), with the SS_lib_type_parameter set to RF to match the stranded library construction. Contigs were assembled using *de novo* mode. The read alignment bam file for input to the Trinity genome guided mode was generated using TopHat v.2.0.13 (Kim et al. 2013) and sorted using SAMtools v.1.1 (Li et al. 2009). All sequence similarity searches were carried out using BLAST (blastx or blastp) v.2.2.30 (Altschul et al. 1990) with an E-value cutoff less than l e^{-10} against SwissProt and putative heat shock proteins identified from the BLAST annotation. The sequences were sequence similarity searched again using blastx against SwissProt (28/07/21) to update the annotations for this publication. Transcriptome data are available from the European

Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/<ACCESSION NUMBERS>) with the assigned accessions: ASSEMBLY_NAME | STUDY_ID | SAMPLE_ID | SEQUENCE_ACC Acclimation of Romanchella perrieri | PRJEB30561 | ERS6578606 | HBVW01000001-HBVW01085472

The assessment of HSP expression via candidate gene qPCR in *R. perrieri*

Thermal shocks on in situ heated settlement panels : An acute heat shock and a chronic heat shock were performed on *R. perrieri,* which had colonised the heated settlement panels from South Cove near Rothera Research Station. Animals on panels (1 panel each of control, +1°C and +2°C) were either incubated at 15°C for 2 hours, or 4°C for 30 days. Unfortunately, due to limited material availability (i.e. one panel per treatment), the individuals sampled are effectively pseudoreplicates, which may have influenced the results. Time point controls (animals on panels maintained at 0°C) were performed for both of these treatments (2 hours and 30 days). Each panel was colonized by >50 *R. perrieri*. All experiments were performed in 60 L jacketed acrylic tanks (Engineering Design and Plastics) attached to LTD20G thermocirculator (Grant instruments Ltd), which maintained the animals at the required temperatures. After the experimental treatment period, *R. perrieri* (n=12) were selected at random and dissected from each panel and snap frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

qPCR analyses of HSP expression in R. perrieri: Total RNA was extracted and quantified from the whole organism as described above. 250ng of total RNA was DNAse treated using gDNA Wipeout Buffer and reverse transcribed using a first strand synthesis kit, Quantitect Reverse Transcription kit (Qiagen) according to manufacturer's instructions. Gene-specific primers were designed from HSP transcripts identified from the assembled transcriptome, using the Primer 3 software (http://frodo.wi.mit.edu). Amplicons with a size of approximately 200–400bp, an annealing temperature of 58-62°C and a GC content between 55–60% were designed and tested (Supplementary File S1). Three HSP genes (*hsp70, hsp90* and *hsp60*) were successfully amplified and the elongation factor transcript (*efa1*) was used as a positive control and reference housekeeping gene. Testing of *efa1* amplification on control and heated samples demonstrated that amplification levels did not change between treatments and that it was a suitable control. Sequences were amplified using Brilliant SYBR Green Master Mix (Agilent) according to manufacturer's instructions on an Eco Real-Time PCR System (Illumina). Samples (n = 3 per panel, per treatment) were run in triplicate, with a no template control. Amplification efficiencies for each assay were calculated using the EcoStudy Software (Illumina) from a standard curve produced through serial dilutions of cDNA template pools. PCR conditions were as follows: 95°C, 3 min, 50 cycles of 95°C, 5 s and 61°C, 20s

followed by a melt curve analysis (95°C, 15s, 55°C, 15s and 95°C at a ramping rate of 0.25°C/s). Relative gene expression of the target genes (*hsp90, hsp70, hsp60* and *efa1*) was analysed using the Relative Expression Software Tool (REST). The mathematical model used is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample groups and control groups. Subsequently the expression ratio results of the investigated transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomisation Test and plotted using standard error estimation via a complex Taylor algorithm (Pfaffl et al. 2002).

Datamining of HSP transcriptome data from long-term field acclimated *R. stalagmia.*

The results of RNA-Seq analyses on a second species of spirorbid *P. stalagmia* from heated settlement panels exposed *in situ* for 18 months from a previous publication (Clark et al. 2019) were data-mined for very long term chronic exposure data of HSP expression (without heat shocks). The processed gene listings of up-regulated sequences from this study were searched for HSP transcripts using the original SwissProt annotations and the sequences sequence similarity searched using blastx against NCBI (28/07/21) to improve annotations. The *P. stalagmia* data are available from the European Nucleotide Archive with the ENA accession number: PRJEB27537.

Results

Identification of HSP transcripts in *R. perrieri*

In spite of using two different temperatures (15°C for 2 hours and 4°C for 30 days) to induce the expression of heat shock transcripts in *R. perrieri,* identification of transcripts for heat shock proteins was very limited. Only three contigs of almost full-length were identified in the reference transcriptome, which shared sequence similarity with heat shock proteins (as defined by BLAST sequence similarity searching and motif scanning). These three sequences had sufficient overlap to enable them to be identified as distinct family members and to allow comparative analyses with other species (Figure 1). The transcripts were putatively identified as *hsp70*, *hsp90* and *hsp60* using phylogenies and sequence similarity searching (Figure 1, Table 1).

Of the three sequences analysed in more detail at the amino acid level, one showed highest sequence similarity to *hsp90* (contig ID DN61228) (Table 1). The translated contig DN61228 contained three motifs characteristic of the Hsp90 family (NKEIFLRELISNSSDALDKIR IGQFGVGFYSAYLVAD; IKLYVRRVFI) and the C-terminus with the cellular localisation motif (Gupta, 1995). The translated contig DN38410

showed highest sequence similarity to *hsp60*. It contained a pre-peptide of 26 amino acids at the N terminus that is required for importation into the mitochondria (Yang et al. 2014) and had a conserved ATP-binding/Mg^{2+} binding site (Marchler-Bauer et al. 2007). It also contained a conserved GGM repeat at the C-terminal end (Brocchieri & Karling, 2000). The remaining contig (DN61199) was putatively identified as *hsp70*. The translated contig DN61199 contained three conserved signature motifs characteristic of the Hsp70 family (IDLGTTYS; FDLGGGTFDV and VVLVGGSTRIPKIQ) (Cottin et al. 2008, Song et al. 2014). A putative ATP binding site (AEAYLGK) was also identified (Song et al. 2014).

11 different *hsp60* sequences, 11 different *hsp90* sequences and 12 different *hsp70* sequences were downloaded from GenBank to determine their similarity with the deduced *Romanchella perrieri* transcripts. Phylogenetic analysis positioned the *R. perrieri hsp60* transcript in a cluster with the annelids *Capitella teleta*, *Dimorphilus gyrociliatus* and *Helobdella robusta* (Figure 1A). The *R. perrieri hsp60* transcript shared greater identity with *Capitella teleta* than with the other species under this cluster. Phylogenetic analysis of the *R. perrieri hsp90* transcript showed that the proteins were mainly in one cluster (Figure 1B). The *R. perrieri hsp90* transcript and the polychaete *Dimorphilus gyrociliatus* did not cluster with the other species used in the analysis. Only one other suitable annelid hsp90 protein was identified and used in the phylogenetic analysis for hsp90: *Platynereis dumerillii*. This species clustered with the other species used in the analysis and shared greater identity with the cluster containing the molluscs *Pinctada imbricata*, *Crassostrea ariakensis*, *Laternula elliptica* and *Corbicula fluminea*. Phylogenetic analysis positioned the *R. perrieri* h*sp70* transcript in a cluster containing the molluscs *Crassostrea virginica*, *Ostrea edulis*, the polychaete *Alvinella pompejana* and the Antarctic krill *Euphausia superba* (Figure 1C). The *hsp70* transcript shared greater identity with the Antarctic krill *Euphausia superba* than with the other species under this cluster.

qPCR analyses of *R. perrieri in response to heat shock*

Treatment of the panels for 2 hours at 15°C produced no significant up- or down-regulation of *hsp90*, *hsp70* or *hsp60* in any of the animals in the different panel treatments (+1°C, +2°C and control) (P = 0.1) (Figure 2). The longer-term heat shock at 4°C for 30 days showed a significant down-regulation of *hsp90* in the animals on the +1°C and +2°C heated panels ($P < 0.05$). For *hsp70* and *hsp60* genes there was no significant up-regulation or down-regulation of the genes in animals in either of the panel treatments (+1°C, +2°C and control) ($P > 0.9$) (Figure 3).

Data-mining in *P. stalagmia*

RNA-Seq analyses of *P. stalagmia* obtained from *in situ* heated settlement panels at the same site as for the *R. perrieri* samples revealed that 14,631 transcripts were significantly differentially expressed between animals on control panels and panels heated to +1°C. The majority of these transcripts (13,034) were up-regulated, with annotations for 1,549 transcripts. Ten non-redundant annotations for HSPs were retrieved from the up-regulated gene lists, mainly comprising representatives of the HSP90 family and HSP70 families (Table 2). Fewer genes were up-regulated in the +2°C animals compared to those on the control panels (1,020 in total) of which 1,013 were up-regulated and annotations associated with 312 transcripts. Only four of the transcripts identified in the +1°C animals were still up-regulated in the +2°C animals with representatives from the HSP70 and the HSP90 families (Table 2). Most of the upregulated HSP transcripts identified (Table 2) were relatively short (less than 200 amino acids) and their sequences matched to different parts of the hsp70 and hsp90 genes. With very little overlap between the different transcripts it was not possible to reliably align these sequences to identify whether they represented different parts of the same gene or duplicated genes. Two of the *P. stalagmia* sequences for *hsp70* (698135 and 649584 (Table 2)) overlapped with each other and the *R. perrieri hsp70* sequence by 400 amino acids. There was considerable divergence between the two *P. stalagmia hsp70*s (64.8% identity) and also between each of these and the *R. perrieri hsp70* (62.3-71.1% identity) indicating that there was at least one duplication of *hsp70* in *P. stalagmia* and that neither of these were likely orthologues of the *R. perrieri hsp70.* Only one *P. stalagmia hsp90* trinity transcript (670310) was of sufficient length to reliably compare with the *R. perrieri hsp90.* These two sequences shared only 74.2% identity and therefore were unlikely to be orthologues.

Discussion:

Our results show that the HSR is highly variable in both spirorbids, *R. perrieri* and *P. stalagmia,* with the response dependant on the thermal stress applied and the species tested. These analyses support earlier studies (Clark et al., 2008a; b; c; 2017; Clark and Peck, 2009b) in demonstrating the complexity of the heat shock response in Antarctic species. Whilst the classical HSR may not be activated in response to acute temperature experiments seen universally in temperate and tropical species, such a response can still be activated, at least in some, if not the majority of Antarctic species under different circumstances. There was no significant upregulation of HSP activity in *R. perrieri* in the acute temperature experiment (15°C for 2 hours) for any of the family members tested using qPCR of candidate genes on any of the settlement plates, including the controls. In some evaluations of HSP activity, the error bars are quite large (e.g. *hsp70* in Figures 2 and 3)

284 indicating uncertainty in the estimate from the mean and a variable response between individuals.
285 This is to be expected, as wild collected animals invariably show wide differences in response (e.g.
286 Clark et al. 2008a), when compared with cultivated or model organisms.
287
288 Previous laboratory-based experiments on other Antarctic marine invertebrates have failed to
289 demonstrate the classical HSR to acute exposures at 15°C (Clark and Peck, 2009a), but as seen in the
290 *P. stalagmia* results described below, this expression is almost certainly contexual. For example, the
291 Antarctic crustacean *Paraceradocus miersi* was originally thought to lack a HSR after an acute heat
292 shock at 10°C using a candidate gene approach (Clark et al. 2008b) but was found to up-regulate
293 *hsp70* after an NGS transcriptome-led approach at a different thermal ramping rate (Clark et al.
294 2017). In contrast, the results here from the chronic heat shock at 4°C for 30 days showed a
295 significant down regulation of *hsp90* in *R. perrieri* on the heated panels (+1°C and +2°C) in
296 comparison to controls, but no significant difference in the other candidate HSP genes. Conversely,
297 members of the HSP70 and HSP90 gene families were all significantly upregulated in the related
298 spirorbid, *P. stalagmia* on similar panels at +1°C and +2°C in response to exposures of 18 months,
299 with all other HSP transcripts down-regulated. Comparison of the longer *P. stalagmia* Trinity
300 transcripts to the *R. perrieri* genes revealed only 62.3-74.2% identity, indicating that they were
301 unlikely to be orthologues and therefore may not respond to the same environmental signals.
302 Furthermore, although many of the Trinity transcripts were quite short, limited analysis of those
303 transcripts coding for more than 400 amino acids indicated likely duplications of *hsp70* genes in *P.*
304 *stalagmia.* The acute HSR in *R. perrieri* was in contrast to that previously found in other Antarctic
305 invertebrates. *hsp90* and *grp78* were both induced in response to acute heat stress, with *hsp70*
306 constitutively expressed and not responsive to thermal stress in the sea urchin *Sterechinus*
307 *neumayeri* (González et al. 2016; González-Aravena et al. 2018). *hsp70b* (an isoform of hsp70) and
308 *hsc70* were upregulated in chronic 30 day warming in the limpet *N. concinna* (Clark and Peck,
309 2009b). Furthermore, an evaluation of the HSR of three Antarctic marine invertebrates (the limpet
310 *N. concinna,* the sea urchin *S. neumayeri* and the crustacean *P. miersi*) warmed under standardised
311 conditions at three different thermal ramping rates (1°C hr^{-1}, 1°C dy^{-1}, 1°C 3 day^{-1}) and also subjected
312 to a three month acclimation at 2°C showed highly variable HSR results (Collins et al. 2021). A strong
313 HSR was only identified in two species, *N. concinna* and *S. neumayeri,* at the fastest ramping rate of
314 1°C per hour (1°C hr^{-1}). Whilst heat shock proteins were occasionally upregulated under the different
315 thermal regimes in the three species, the HSR was highly individual according to species and heat
316 shock.
317

Chaperone proteins of the HSP70 family are routinely constitutively expressed at high levels in Antarctic species, a situation not often found in temperate species. For example, in the clam *L. elliptica*, *grp78* was constitutively expressed, whilst *hsc70* was permanently highly expressed in *N. concinna* (Clark et al. 2008a). Unfortunately, it was not possible to survey *hsc70* in the qPCR experiments here, as a sequence of suitable length was not identified in the transcriptome. It is thought that this "extra" constitutive expression of *hsp70* gene family members is due to the problems of protein folding in the cold (Peck, 2016). Evidence also suggests that this high constitutive expression underpins the altered *hsp70* response in Antarctic species, as this readily available pool of HSPs is used first in a heat shock response, rather than the induction of new proteins, often resulting in a delayed HSR (Toullec et al. 2020).

Thus, although the number of examples is limited with regard to the production of an HSR in response to thermal stress in Antarctic species, the distribution across taxa is vast: from a microbial eukaryote, *Euplotes focardii* (LaTerza et al. 2001) to fish (Hofmann et al. 2000; Clark et al. 2008c). There is no strict rule for the expression or loss of the HSR in Antarctic organisms. As more species are studied, the more complex the picture becomes. Certainly, evolution to life in the Southern Ocean has produced diversifying selection on members of the *hsp70* gene family, with selective relaxation potentially resulting in loss of function (Cascella et al. 2015; Papot et al. 2016). As such, sequence similarity of *hsp70* genes between species does not necessarily correlate with shared functions, particularly the capacity for induction under the same conditions. Therefore, the use of the terms "inducible" and "cognate", which are applied to gene names based on sequence similarity results, can be misleading when applied to *hsp70* genes in Antarctic marine invertebrates and fish (Cascella et al. 2015; Papot et al. 2016). The fact that *hsp70* genes are often duplicated in Antarctic species (including *P. stalagmia* in this study) with retention of duplicates due to subfunctionalisation (Cascella et al. 2015; Papot et al. 2016; Ramsøe et al. 2020) attests to their critical role in Antarctic cellular metabolism. As sequencing costs decrease and gene network methodologies become routine, it will be increasing possible to dissect, in detail, the heat shock response of Antarctic species (e.g. Ramsøe et al. 2020) and determine the currently intractable question of how evolution to life in the cold has affected their HSR.

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Figure Legends

Figure 1: Phylogeny

Phylogenetic analysis of *Romanchella perieri* sequences *hsp60* (A), *hsp90* (B) and *hsp70* (C). Alignment of amino acid sequences were produced by Clustal W and the bootstrap maximum-likelihood phylogeny tree was constructed by MEGA X. Numbers at nodes are bootstrap values (based on 1000 replicates). Sequence accession numbers encoding *hsp60* are as follows: *Helobdella robusta* (XP_009028388.1), *Capitella teleta* (ELU10444.1), *Dimorphilus gyrociliatus* (A0A718VKH4), *Drosophila melanogaster* (NP_511115.2), *Eriocheir sinensis* (AKN44272.1), *Penaeus monodon* (ALS05377.1), *Caenorhabditis elegans* (NP_497429.1), *Dissostichus mawsoni* (KAF3843626.1), *Gallus gallus* (NP_001012934.1), *Anemonia viridis* (AAR88509.1) and *Bos taurus* (XP_027378769.1); Sequence accession numbers encoding *hsp90* are as follows: *Rattus norvegicus* (NP_786937.1), *Homo sapiens* (NP_005339.3), *Gallus gallus* (NP_001103255.1), *Eriocheir sinensis* (ADE60732.1), *Euphausia superba* (Clark et al. 2011; SRA023520), *Platynereis dumerilii* (ABB29612.1), *Pinctada*

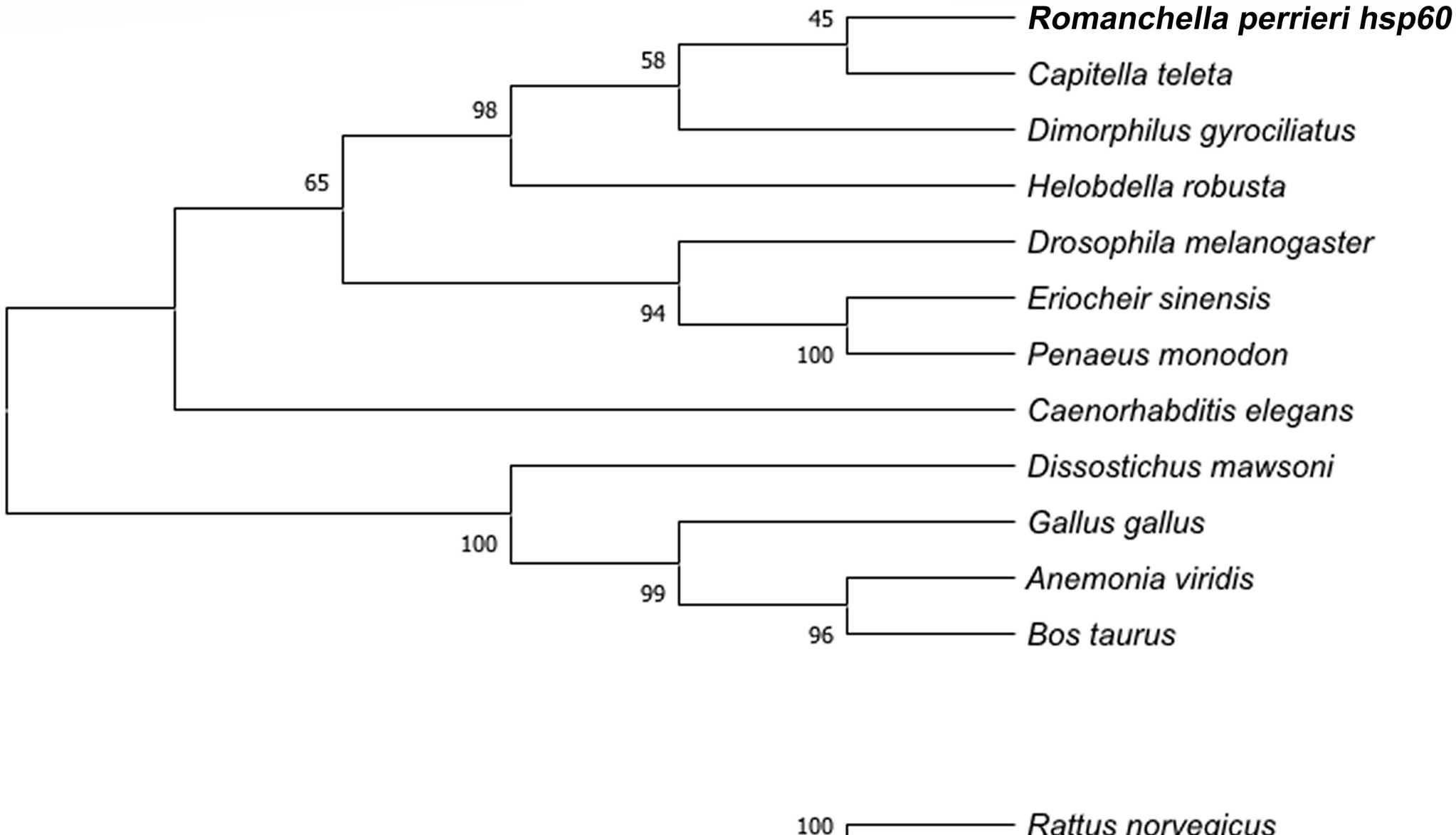
imbricata (AHM22922.1), *Crassostrea ariakensis* (ADT63790.1), *Laternula elliptica* (ACF35426.1), *Corbicula fluminea* (AMM04544.1) *and Dimorphilus gyrociliatus* (CAD5118349.1); Sequence accession numbers encoding *hsp70* are as follows: *Crassostrea virginica* (CAB89802.1), *Ostrea edulis* (AAM46634.1), *Alvinella pompejana* (AGH18393.1), *Euphausia superba* (AIR72273.1), *Homo sapiens* (NP_001374860.1), *Mus musculus* (NP_001002012.1), *Riftia pachyptila* (CBM42050.1), *Tevnia jerichonana* (CBM42052.1), *Eisenia fetida* (ADV57677.1), *Helobdella robusta* (XP_009029445.1), *Arenicola marina* (CBM42047.1) and *Modiolus modiolus* (QES86443).

Figure 2:

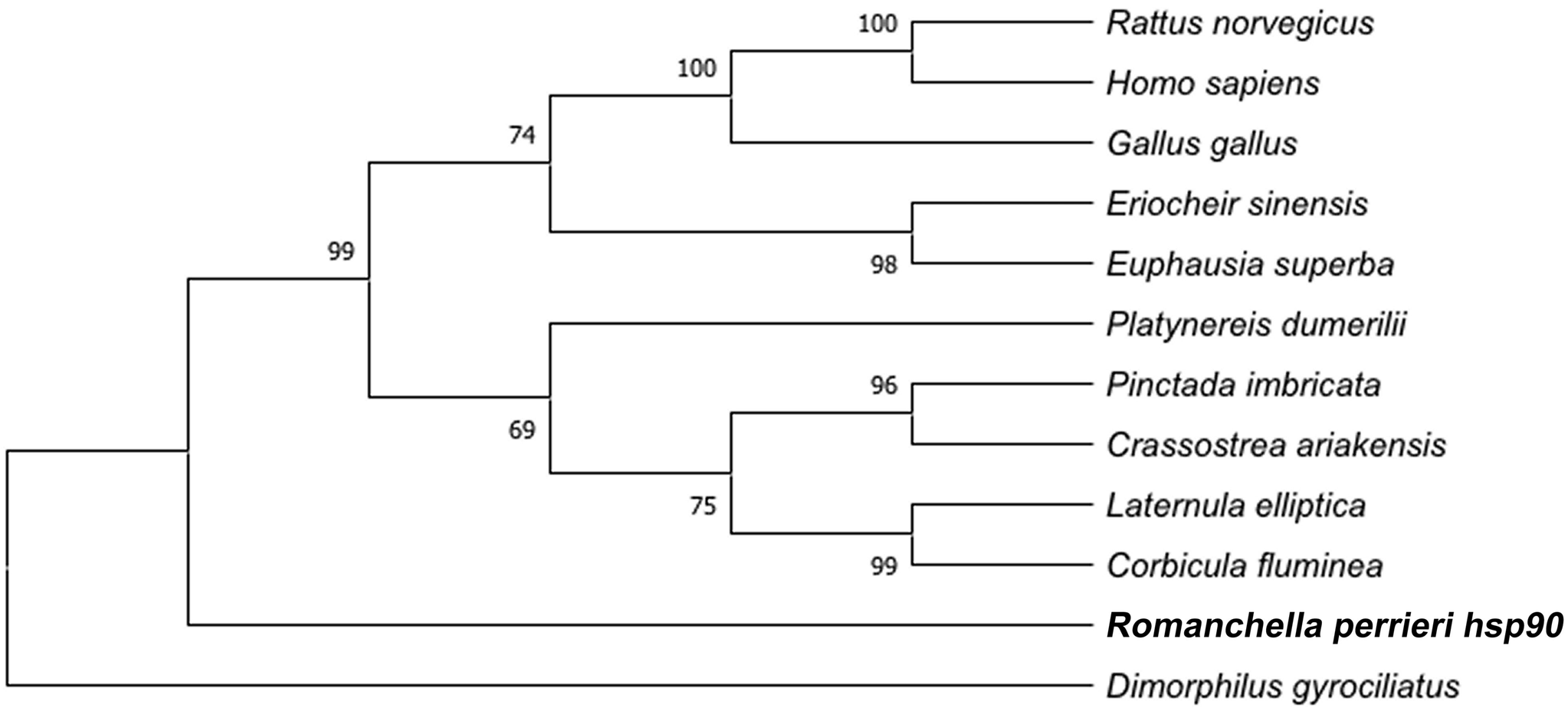
Figure 2: Fold change in expression levels in *R. perrieri* (n=3) after an acute heat shock at 15°C for 2 hours for the genes A) *hsp90* B) *hsp70* and C) *hsp60* on panels at 0°C (control), +1°C and +2°C (*in situ*) above ambient temperature

Figure 3: Fold change in expression levels in *R. perrieri* (n=3) after a chronic heat shock at 4°C for 30 days for the genes A) *hsp90* B) *hsp70* and C) *hsp60* on panels at 0°C (control), +1°C and +2°C (*in situ*) above ambient temperature

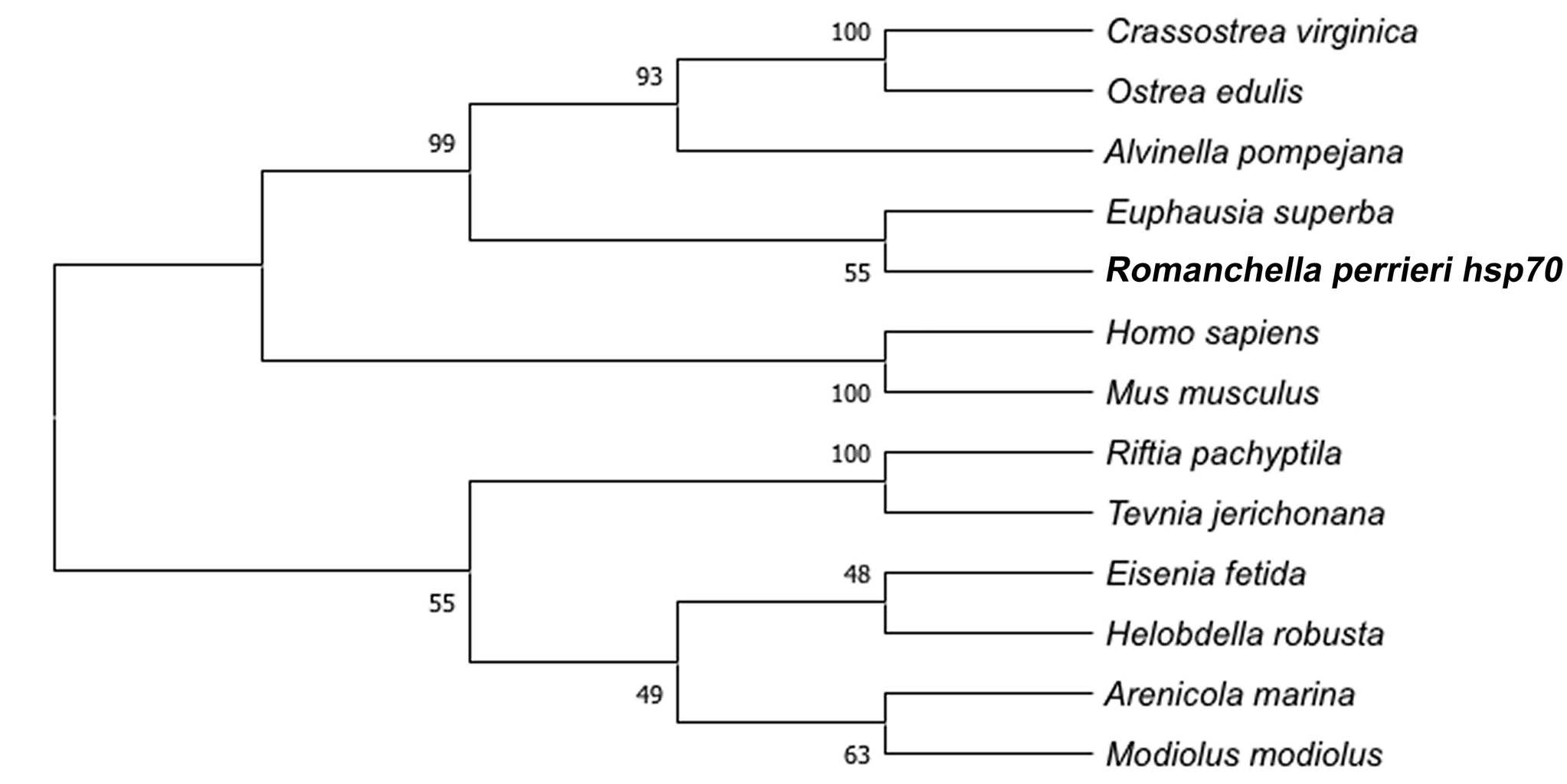
A

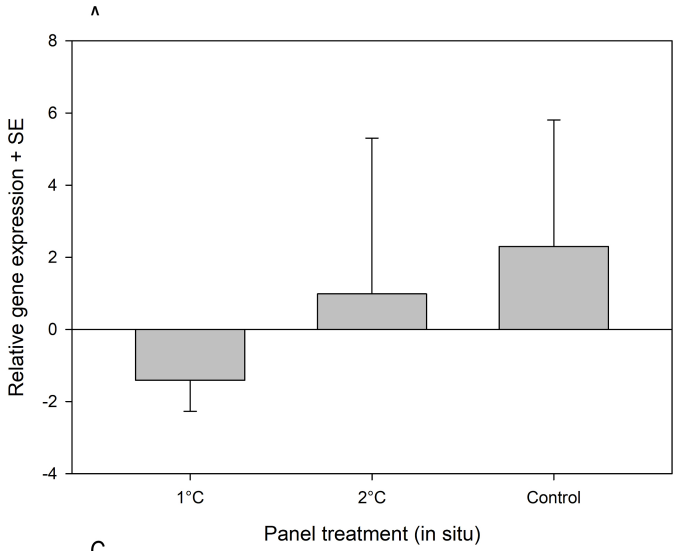

45
58
98
65
94
100
100
99
96
Romanchella perrieri hsp60
Capitella teleta
Dimorphilus gyrociliatus
Helobdella robusta
Drosophila melanogaster
Eriocheir sinensis
Penaeus monodon
Caenorhabditis elegans
Dissostichus mawsoni
Gallus gallus
Anemonia viridis
Bos taurus

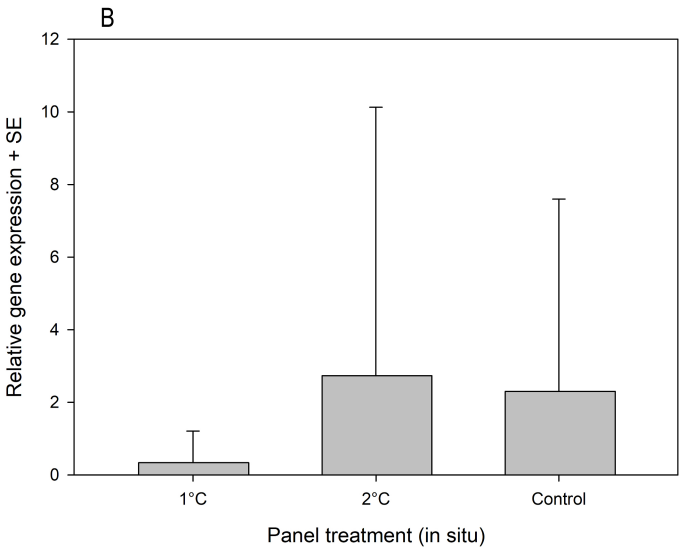
B

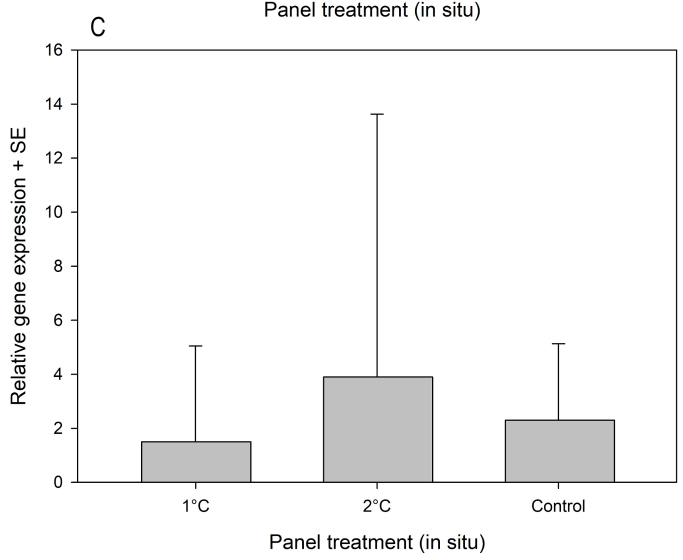

100
100
74
99
98
69
96
75
99
Rattus norvegicus
Homo sapiens
Gallus gallus
Eriocheir sinensis
Euphausia superba
Platynereis dumerilii
Pinctada imbricata
Crassostrea ariakensis
Laternula elliptica
Corbicula fluminea
Romanchella perrieri hsp90
Dimorphilus gyrociliatus

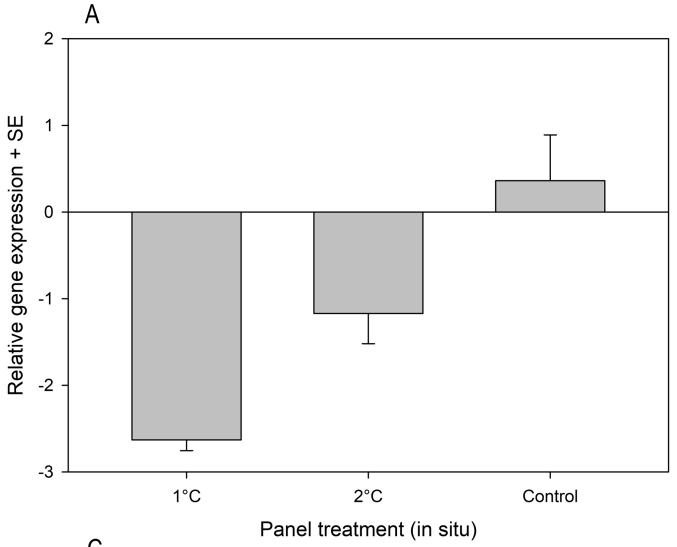
C

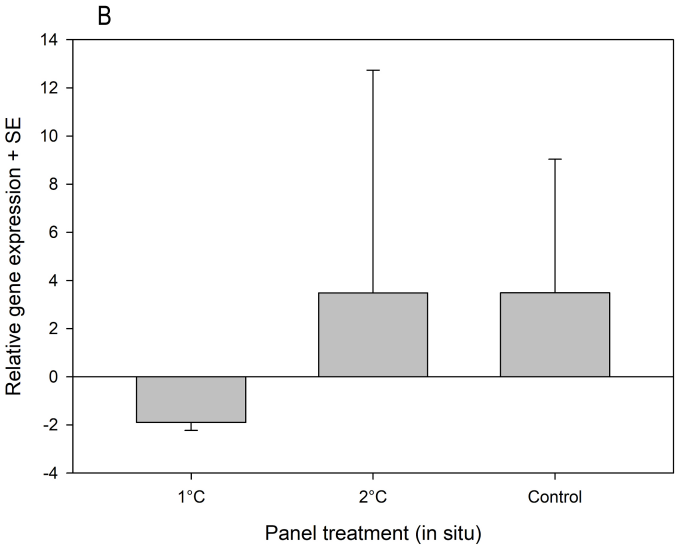

100
93
99
55
100
100
48
49
63
Crassostrea virginica
Ostrea edulis
Alvinella pompejana
Euphausia superba
Romanchella perrieri hsp70
Homo sapiens
Mus musculus
Riftia pachyptila
Tevnia jerichonana
Eisenia fetida
Helobdella robusta
Arenicola marina
Modiolus modiolus


^
Relative gene expression + SE
8
6
4
2
0
-2
-4
1°C
2°C
Control
Panel treatment (in situ)


B
Relative gene expression + SE
12
10
8
6
4
2
0
1°C
2°C
Control
Panel treatment (in situ)


C
Relative gene expression + SE
16
14
12
10
8
6
4
2
0
1°C
2°C
Control
Panel treatment (in situ)


A
Relative gene expression + SE
2
1
0
-1
-2
-3
1°C
2°C
Control
Panel treatment (in situ)


B
Relative gene expression + SE
14
12
10
8
6
4
2
0
-2
-4
1°C
2°C
Control
Panel treatment (in situ)

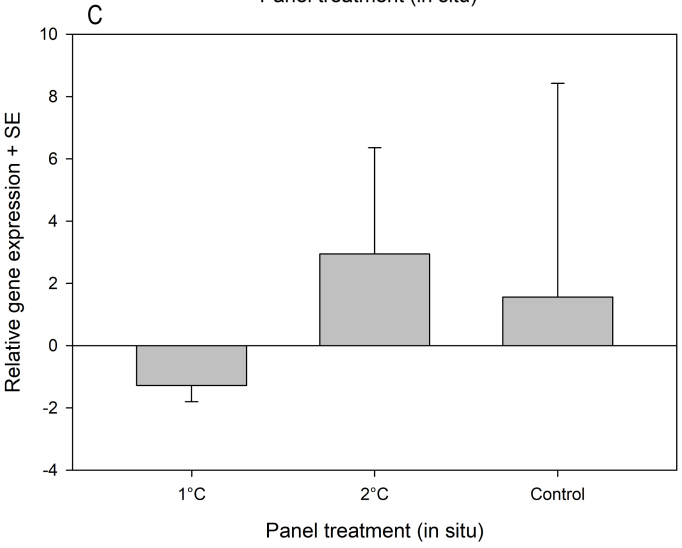

C
Relative gene expression + SE
10
8
6
4
2
0
-2
-4
1°C
2°C
Control
Panel treatment (in situ)

Table 1: Designation of *hsp* gene family member status based on BLAST results from database sequence similarity searches, including the closest annelid match.

Transcript	Organism	Accession number	Score	E value	Closest annelid	Accession number	% Identity	Score	E value
*hsp*70	*Hydra oligactis*	Q05945	482	$2.5e^{-056}$	*Alvinella pompejana*	D7GL47	72.12%	956	0.0
hsp60	*Gallus gallus*	Q5ZL72	1819	0.0	*Capitella teleta*	R7UVT7	75.67%	862	0.0
hsp90	*Homo sapiens*	Q14568	652	$3.0e^{-079}$	*Dimorphilus gyrociliatus*	A0A718VRJ2	73.23%	488	$1.0e^{-160}$

Table 2: *Hsp* transcript data extracted from long-term heated settlement panel acclimation study (Clark et al., 2019). In each case, the longest isoform of each transcript is listed with the maximum logFC recorded for that transcript. Length of transcript is shown in amino acids (aa)

Transcript	Longest isoform	length (aa)	Gene	Organism	Accession number	score	E value	Up-regulated 1°C		Up-regulated 2°C	
								logFC	FDR	logFC	FDR
TRINITY_DN698135	c1_g1_i4	480	HSP70	*Laternula elliptica*	ABM9234	853	0.0	13.2637	0.0100	12.554	0.0142
TRINITY_DN649584	c2_g3_i2	406	HSP70	*Sterkiella histromuscorum*	AED86994	649	0.0	12.9359	0.0092	12.0393	0.0287
TRINITY_DN665271	c1_g1_i10	160	HSP90	*Crassostrea ariakensis*	AEF33377	275	$3e^{-89}$	12.5178	0.0319	X	X
TRINITY_DN679451	c1_g2_i2	208	HSP90	*Laternula elliptica*	ACF35426	430	$3e^{-144}$	11.8073	0.0087	11.643	0.0321
TRINITY_DN670310	c4_g3_i4	446	HSP90	*Laternula elliptica*	ACF35426	828	0.0	11.7525	0.0087	11.077	0.0216
TRINITY_DN693482	c0_g1_i14	87	*HSP70*	*Meloidogyne enterolobii*	CAD2182874	167	$1e^{-49}$	11.0882	0.0087	X	X
TRINITY_DN667506	c0_g1_i2	114	*HSP70*	*Capitella teleta*	ELU05805	142	$4e^{-37}$	10.8664	0.0087	X	X
TRINITY_DN712943	c3_g7_i1	87	HSP90	*Laternula elliptica*	ACF35426	187	$2e^{-52}$	9.9050	0.0087	X	X
TRINITY_DN683946	c2_g1_i6	168	HSP83L	*Diaphorina citri*	XP008482889	228	$8e^{-70}$	6.8788	0.0380	X	X
TRINITY_DN693482	c0_g1_i8	131	HSP70B	*Euphasia superba*	AMB66738	243	$4e^{-76}$	5.5956	0.0368	X	X

Supplementary file S1: PCR primers used for Q-PCR analysis of four genes. Primer sequence, RSq and PCR efficiency values are included for each gene, as calculated using the EcoStudy Software v 5.0 from the Eco Real-Time PCR System software (Ilumina).

Gene	Primer name	Sequence	RSq	PCR efficiency
hsp70	HSP70Rev HSP70F	CCTATGCCACACCAGAAACG TGGCTACGTACTGTGTGTGT	0.97	97
hsp60	HSP60Rev HSP60F	CAATAACATTCCCTCGCGCA GAGGAGCCGGGTGATGATAA	0.95	94
hsp90	HSP90Rev HSP90F	AGGTACTCTCACGTCCCTCT AGCTTCTTCAGAGCCCACAT	0.99	98
efa1	EFA1Rev EFA1F	GCAGGTGCCTCTACCTCAAG CAATGCTATGGCCACCTTTT	0.96	91