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Contrasting transcriptome response to thermal stress in two key zooplankton species, *Calanus finmarchicus* and *C. glacialis*

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ABSTRACT: Climate change has already led to the range expansion of warm-water plankton assemblages in the northeast Atlantic and the corresponding range contraction of colder-water species. The temperate copepod *Calanus finmarchicus* is predicted to shift farther northward into polar waters traditionally dominated by the arctic copepod *C. glacialis*. To identify temperature-mediated changes in gene expression that may be critical for the thermal acclimation and resilience of the 2 *Calanus* spp., we conducted a whole transcriptome profiling using RNA-seq on an Ion Torrent platform. Transcriptome responses of *C. finmarchicus* and *C. glacialis* from Disko Bay, west Greenland, were investigated under realistic thermal stresses (at +5, +10 and +15°C) for 4 h and 6 d. *C. finmarchicus* showed a strong response to temperature and duration of stress, involving up-regulation of genes related to protein folding, transcription, translation and metabolism. In sharp contrast, *C. glacialis* displayed only low-magnitude changes in gene expression in response to temperature and duration of stress. Differences in the thermal responses of the 2 species, particularly the lack of thermal stress response in *C. glacialis*, are in line with laboratory and field observations and suggest a vulnerability of *C. glacialis* to climate change.

KEY WORDS: RNA-seq · Copepods · Temperature stress response · Heat shock protein · Climate change

INTRODUCTION

Climate change profoundly impacts both marine and terrestrial ecosystems, ranging from biogeographical and phenological changes to abrupt ecosystem shifts (Thackeray et al. 2008, Beaugrand et al. 2009). Its effects are particularly intense in the Arctic, where temperatures have increased 2 to 4 times faster in the past few decades compared to the global average (Hansen et al. 2006). A striking consequence of climate change is the range expansion of warm-water plankton assemblages in the northeast Atlantic, and the corresponding range contraction of cold-water species (Beaugrand et al. 2002, 2009). The increase in water temperature and different thermal tolerances of copepods appear to be the major drivers of such shifts in zooplankton communities (Beaugrand et al. 2002, 2009, Helaouët & Beaugrand 2007).

Copepods of the genus *Calanus* are predominant in the zooplankton biomass of the high-latitude oceanic regions and play a significant role in energy transfer in marine food webs (Falk-Petersen et al. 2009) and in the biological pump of biogenic carbon into the deep sea (Pasternak et al. 2002). The temper-

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ate species *C. finmarchicus* (Gunnerus, 1770), with a temperature range in nature between −1.8 and 15ºC, and the Arctic species *C. glacialis* Jaschnov, 1955, with a temperature range in nature from −1.8 to 7ºC, can comprise up to 70 to 80% of the zooplankton biomass in the North Atlantic and Arctic shelf seas, respectively (Conover 1988, Bonnet et al. 2005, Blachowiak-Samolyk et al. 2008, Carstensen et al. 2012). Although there are only very subtle morphological differences between these 2 closely related species, they are adapted to different thermal environments, differ in phenology and energy-rich lipid content, and are suggested to support different Arctic food webs (Conover 1988, Bucklin et al. 1995, Falk-Petersen et al. 2009). A northward shift (ca. 8 km decade^{-1}) of the temperate species *C. finmarchicus* has been detected, with temperature being one of the main explanatory factors (Beaugrand et al. 2002, Chust et al. 2014). With ongoing climate change, the northward shift of *C. finmarchicus* is predicted to continue into Arctic waters currently dominated by *C. glacialis* and *C. hyperboreus* (Helaouët et al. 2011, Wassmann et al. 2011). In addition, warming of the Arctic will likely benefit *C. finmarchicus* more than *C. glacialis* (Kjellerup et al. 2012), resulting in changes in food-web dynamics and secondary production (Falk-Petersen et al. 2007). Despite an extensive knowledge of the ecology and phenology of these 2 species, the molecular basis of physiological responses (particularly changes in gene expression) to increased water temperature remains largely unexplored. This impairs our understanding of species performance, abundance and distribution in a changing climate, and therefore, reduces our power to predict climate-related shifts in ecosystem structure and function.

Temperature is a crucial factor for an organism's performance, particularly for ectotherms, as their physiological functions are strongly temperature-dependent. Therefore, the biogeographic range and abundance of a species is highly dependent on the thermal tolerance of an organism (e.g. Hofmann & Todgham 2010). In response to environmental conditions that fluctuate beyond an organism's tolerance limits, an immediate uniform cellular stress response is activated; however, prolonged stress exposure initiates a stressor-specific secondary response (cellular homeostasis response) to re-establish homeostasis under the new environmental conditions (Kültz 2005). One of the components of the cellular stress response is heat shock response, a universal molecular stress response that is particularly well-studied in response to stressful temperatures and mainly involves up-regulation of heat shock proteins (HSPs), which act as molecular chaperones (Hofmann & Todgham 2010). HSPs protect damaged proteins from aggregation, unfold aggregated proteins and refold damaged proteins or target them for efficient degradation (Verghese et al. 2012), thereby stabilizing the functioning of the organism under elevated temperature and increasing its thermal tolerance range (Feder & Hofmann 1999). Despite the universality of the heat shock response, species adapted to extremely stable and cold environments, such as several Antarctic invertebrates and notothenioid fishes, appear to lack the ability of HSP up-regulation (Bilyk & Cheng 2014).

The rapid advance in high-throughput sequencing technologies and whole transcriptome profiling (RNA-seq) enables investigation of species that are not yet established genetic models (Wang et al. 2009), but that display thermal tolerance mechanisms that are of high ecological and evolutionary interest (Smith et al. 2013). Studies on transcriptional regulation of gene expression under thermal stress in closely related species have started to emerge only recently, covering a wide range of organisms from amphipods (Bedulina et al. 2013) and mussels (Lockwood et al. 2010) to endosymbiotic dinoflagellates of reef corals (Barshis et al. 2014). However, few studies have targeted *Calanus* or even copepods.

Here we examined thermal stress responses in the temperate *C. finmarchicus* and the Arctic *C. glacialis* simultaneously sampled from the same location and, therefore, sharing thermal histories. Using realistic temperatures and 2 durations of stress, we explored the global transcriptomic patterns of gene expression changes and focused on key differences between the 2 species.

MATERIALS AND METHODS

Sampling and experimental set-up

Copepods were collected in Disko Bay, Greenland (69° 14′ N, 53° 23′ W), on 20 April 2012 by vertical hauls with a 200 µm net in the upper 100 m, where water temperature ranged between −1 and 1ºC. The sampling site was monitored every week from mid-March. During the whole period, the surface water temperature was below 0ºC, whereas it slowly increased towards the bottom and reached 3ºC at 200 m (E. F. Møller & T. G. Nielsen unpubl. data). The phytoplankton bloom started at the end of April, with a value of 8 µg chl *a* l^{-1} in the surface water. Neither

Calanus finmarchicus nor *C. glacialis* were producing eggs 2 wk before sampling, whereas on the day of sampling egg production was 6 and 12 eggs female^{-1} d^{-1} in *C. finmarchicus* and *C. glacialis*, respectively (E.F. Møller & T.G. Nielsen unpubl. data).

Shortly after collection the copepods were transferred to a laboratory in 25 l thermo boxes. Owing to difficulties of species identification between live *C. finmarchicus* and *C. glacialis*, females were selected for experiments and sorted according to the red pigmentation of the antenna and somites (Nielsen et al. 2014). To overcome stress associated with collection, copepods were incubated in 600 ml bottles with an *in situ* water temperature of 0ºC for 48 h. During the acclimation and experimental periods, copepods were fed the diatom *Thalassiosira weissflogii* in accordance with Kjellerup et al. (2012), the density of copepods was 5 individuals per bottle, and the bottles were capped and rotated once a day. The selected copepod density was slightly lower than in previous experiments (e.g. Hjorth & Nielsen 2011); nevertheless, even at 20 times higher density, the oxygen saturation does not fall lower than 80% after 21 h (Hildebrandt et al. 2014). Acclimated copepods were transferred to 600 ml Nunc bottles containing filtered seawater with ~5 µg chl *a* l^{-1} of *T. weissflogii* and incubated at 0, 5, 10 and 15°C with 8 replicates (bottles) per temperature in each species (Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m534p079_supp.pdf). Half of the replicates of each species were terminated after 4 h to evaluate a short-term thermal stress (STS) response. Remaining replicates were incubated for 6 d to evaluate the chronic response to long-term thermal stress (LTS), thus resulting in 20 individuals per treatment (Fig. S1 in Supplement 1). Every second day, copepods were transferred to new bottles with fresh *T. weissflogii* to ensure an excess supply of prey. Mortality was noted and the grazing response to temperature was evaluated by measuring the fecal pellet production during the experiments. At the termination of the experiments the copepods were anaesthetised by bubbling with CO_2 and were immediately preserved in RNAlater (Qiagen).

RNA/DNA extraction and genetic species identification

RNA and DNA were extracted simultaneously from each individual with the E.Z.N.A. DNA/RNA Isolation Kit (Omega Bio-Tek) in accordance with the manufacturer's instructions. To ensure correct species identification, *C. finmarchicus* and *C. glacialis* were genotyped for 3 microsatellite loci (EL696609, EL585922 and EH666870) (Provan et al. 2007) or 6 insertion/deletion (InDel) nuclear markers (Smolina et al. 2014). Individuals were assigned to species (Table S1 in Supplement 2 at www.int-res.com/articles/suppl/m534p079_supp.xls) in accordance with Smolina et al. (2014).

Transcriptome Ion Torrent PGM sequencing

C. finmarchicus and *C. glacialis*, following the 4 h (STS) and 6 d (LTS) exposures to 0, 5 and 10°C, were used for transcriptome sequencing. An equal amount of total RNA was pooled from 5 individuals per library to build 12 libraries in total. Pooled RNAs were treated with Baseline-ZERO DNase (Epicentre) and subsequently cleaned using the RNA Clean & Concentrator kit (Zymo Research). To control for technical variation during library preparation and sequencing, ERCC ExFold RNA Spike-In Mix 1 or 2 (Ambion) was added to each library of 1.6 mg total RNA in a random assessment, following the manufacturer's guidelines. Isolation of mRNA from total RNA was performed using 2 rounds of Mag-Bind mRNA Enrichment (Omega Bio-Tek) with subsequent cleaning using the RNA Clean & Concentrator kit. Libraries were constructed using 50 ng poly(A) RNA and the Ion Total RNA-Seq Kit for AB Library Builder System (Life Technologies) with individual barcodes from Ion Xpress RNA-Seq Barcode kit (Life Technologies). Emulsion PCR was done with the Ion One Touch 2 System, using the Ion PGM Template OT2 200 Kit (Life Technologies), followed by enrichment and quality control according to the manufacturer's protocol. The 6 libraries of each species were pooled and sequenced with the Ion PGM System using the Ion PGM 200 Sequencing Kit and one Ion 318 chip per species.

RNA-seq analysis

The transcriptomes from both species were analyzed separately following the same protocol. To check the quality of the libraries, ERCC RNA Spike-In reads were processed with ERCC_Analysis plugin v.4.0-r72040 implemented in Torrent Suite 4.0.2. ERCC RNA Spike-In reads were then filtered out by mapping to ERCC RNA reference sequences using bowtie2 v.2.2.1 (Langmead & Salzberg 2012) with '--very-sensitive' parameters. Remaining reads were

quality trimmed using the fastq_quality_trimmer tool from the FASTX Toolkit v.0.013 with a threshold of a Phred quality score 17, and a length threshold of 35 bp (Ion RNA-Seq 2012). Identical sequences among the reads were collapsed into a single sequence using fastx_collapser from FASTX Toolkit v.0.013 with default parameters. Resulting reads from 6 libraries per species were concatenated for subsequent species-specific de novo assembly and normalization using Trinity v.r2013_08_14 (Haas et al. 2013). Reads were normalized using normalize_by_kmer_coverage.pl tool with the targeted maximum coverage value of 30 and assembled with default parameters. The assemblies were annotated using local BLASTX (NCBI BLAST 2.2.28+) against the UniProtKB/Swiss-Prot protein database (downloaded on 19 February 2014) with the following parameters: –outfmt 6 std qcovs –evalue 0.00001 –max_target_seqs 1.

Cleaned reads were mapped to their respective species assemblies using Subread package v.1.4.3-p1 (Liao et al. 2013) with default parameters. Counts of mapped reads per Trinity transcript were extracted for each library using featureCounts (Liao et al. 2014). Differential gene expression analysis was performed for the 2 species separately using the BioConductor package DESeq2 v.1.4.5 (Love et al. 2014) in R v.3.1.0 (R Development Core Team 2011). Expression profiles of each library were normalized with a size factor calculated from ERCC RNA Spike-In reads belonging to the group B with functions estimateSizeFactorsForMatrix and sizeFactors. Gene expression data were analyzed with a statistical design that included 2 factors: duration (levels STS and LTS) and temperature (levels 0, 5 and 10°C). Using the function contrast, in total 4 comparisons per species were analyzed: STS versus LTS, 0 vs. 5°C, 0 vs. 10°C and 5 vs. 10°C. To remove potential sequencing errors, Trinity transcripts with *baseMean* < 5 counts were filtered out prior to calling significant differentially expressed transcripts (Barshis et al. 2014). Transcripts were recognized as differently expressed at $q < 0.05$ after a multiple comparison correction with false discovery rate (FDR) (Benjamini & Hochberg 1995) implemented in the DESeq2 package. The heat map of differentially expressed transcripts was generated using the heatmap.2 function of the R package gplots (Warnes et al. 2014), where expression counts of each transcript were normalized by dividing counts in each sample by the average expression of that transcript across all samples. Functional enrichment of differentially expressed transcripts was identified using R package topGO (Alexa & Rahnenfuhrer 2010). The analysis was done for 3 gene ontology (GO) categories (biological process, molecular function and cellular component) using Fisher's exact test and correction for FDR at $q < 0.05$.

Quantitative real-time PCR of selected genes

Gene selection and design of primers

Validation of the RNA-seq results and sensitivity assessment of our medium throughput approach was performed by quantitative real-time PCR (qPCR) of 11 genes. The genes were chosen either because they were identified from differential expression analysis in the present study (*hsp60*, *hsp70_2*, *gdh*, *rpl14*) or because of their known function in stress response (*dnaja1*, *hsp10*, *hsp70_3*, *hsp70_5*, *nap1l1*, *rps11*, *znf207*) (Table S2 in Supplement 2 at www.int-res.com/articles/suppl/m534p079_supp.xls). In addition, as reference genes for *Calanus* during temperature stress have not been reported, 5 genes were tested as putative reference genes for *C. finmarchicus* and *C. glacialis* during STS and LTS. Genes *cdc42*, *eif1ax* and *trx-2* were selected for their stable transcript counts in all libraries, and *efa1α* and 16S rRNA were selected from the literature on gene expression in *C. finmarchicus* (Tarrant et al. 2008, Hansen et al. 2013). Reciprocal BLAST searches (MegaBLAST in Geneious 7.1.0 [Biomatters], default settings) were performed between the 2 species to identify homologous genes. Aligned regions were visually inspected and searched for conserved regions to design primers resulting in a product length of between 70 and 150 bp using Primer3 (Koressaar & Remm 2007) in Geneious 7.1.0 (Table S2 in Supplement 2).

Synthesis of cDNA and qPCR

qPCR of selected genes was performed for individuals of *C. finmarchicus* (n = 6 to 10) and *C. glacialis* (n = 8 to 10) exposed to 0, 5, 10 and 15°C for 4 h and 6 d. RNA was quantified using the Qubit RNA Assay kit (Life Technologies) and a Qubit 2.0 Fluorometer (Life Technologies); integrity of selected RNA from each extraction was checked on a 1% agarose gel. Total RNA from 6 to 10 samples for each treatment and species (5 of which were also used for RNA-seq), as well as 2 controls (positive control and no-reverse-transcriptase control) were individually reverse-transcribed to cDNA in 10 µl reactions using the

QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions, with a starting amount of 49 ng.

The qPCR reactions were carried out in a StepOnePlus Real-Time PCR System (Life Technologies) in a total volume of 5 µl containing 2.5 µl PerfeCta SYBR Green FastMix (Quanta BioSciences), 2 µl cDNA (1:15) and 0.5 µl of primer mix with 3 µM of forward and reverse primers each. All reactions, including controls and dilution series, were run in duplicate with the following amplification protocol: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 20 s (for all primers). At the end of each qPCR, melting curve analysis of amplified products was performed to verify amplification specificity. The PCR amplification efficiency (E) and the regression coefficient (R^2) were calculated from a dilution series (1:3 dilution/step, from 1:1 to 1:81) of the cDNA pools using the regression slope of the threshold cycle (Ct) versus cDNA quantity plot following Pfaffl et al. (2002). The best reference genes (most stable in expression) were identified separately for *C. finmarchicus* and *C. glacialis* using geNorm 3.5 and were used to calculate the corresponding normalization factors (Vandesompele et al. 2002).

Statistical analysis

Data were analysed in R v.3.1.0 (R Development Core Team 2011). Normal distribution and homogenous variance of data were assessed visually by frequency histograms and Q-Q plots. As not all the data satisfied parametric assumptions, nonparametric methods were applied. Effects of the temperature stress duration (STS vs. LTS) and stress temperatures (0, 5, 10 and 15°C) on expression of selected genes were analysed separately. The duration effect was assessed with a Mann-Whitney U-test, first for a combination of all stress temperatures, then at each temperature separately with p-value correction for multiple comparisons using the FDR method (Benjamini & Hochberg 1995). The temperature effect on gene expression and fecal pellet production was assessed with a Kruskal Wallis test separately for STS and LTS. In the case of significant temperature effects, values were compared pairwise between all temperatures with a nonparametric analog of Tukey test in the R package nparcomp (Konietschke 2012).

Results of RNA-seq and qPCR were compared using the Spearman correlation of $\log_2$ fold change (FC) of expression values in all comparisons (STS vs. LTS, 0 vs. 5°C, 0 vs. 10°C, 5 vs. 10°C) for 16 genes (except *znf207* for *C. finmarchicus* that had too few reads in RNA-seq to obtain any $\log_2$ FC values). Correlation analysis and tests of significance were performed in Hmisc R package (Harrell 2014). The strength of correlation was interpreted in accordance with Mukaka (2012).

RESULTS

Physiological response

During the thermal experiments aimed at comparing the transcriptome responses of *Calanus finmarchicus* and *C. glacialis* to elevated temperatures, maximum mortality of 20 and 35% was observed for the 2 species, respectively, at 15°C during LTS. Mortality during STS did not exceed 5% for either species at any temperature. Fecal pellet production, as an indicator for grazing activity, was measured during STS but not during LTS, due to an unnoticed tear in the mesh filter. Fecal pellet production during STS significantly increased with temperature for *C. finmarchicus* until 10°C and plateaued at 15°C (Fig. S2 in Supplement 1 at www.int-res.com/articles/suppl/m534p079_supp.pdf), while for *C. glacialis* it was significantly higher at 5 and 10°C compared to 0°C and decreased at 15°C (Fig. S2 in Supplement 1).

Transcriptome sequencing

Sequencing of *C. finmarchicus* and *C. glacialis* transcriptomes yielded 4 837 616 and 3 307 190 reads, respectively, with approximately equal numbers of reads among libraries within each species (Fig. S3 in Supplement 1). Overall, 1.2 to 2.9% of the reads were mapped to ERCC RNA Spike-In reference sequences, and the good correlation between known concentration and count of mapped reads for each library ($R^2 > 0.85$) indicated that all libraries were of good quality. After ERCC RNA Spike-In filtering and quality trimming, cleaned reads represented 87.6 to 93% of the original raw reads (Fig. S3 in Supplement 1) with mean lengths of 115 and 119 bp for *C. finmarchicus* and *C. glacialis*, respectively. Trinity assemblies for *C. finmarchicus* and *C. glacialis* resulted in 28 954 and 36 880 transcripts, respectively (Table 1), with mean transcript lengths of 353 and 427 bp. Transcriptome assemblies of the 2 species displayed a similar guanine and cytosine (GC) content (ca. 48%) and annotation success (ca. 50%) against UniProtKB/Swiss-Prot protein database

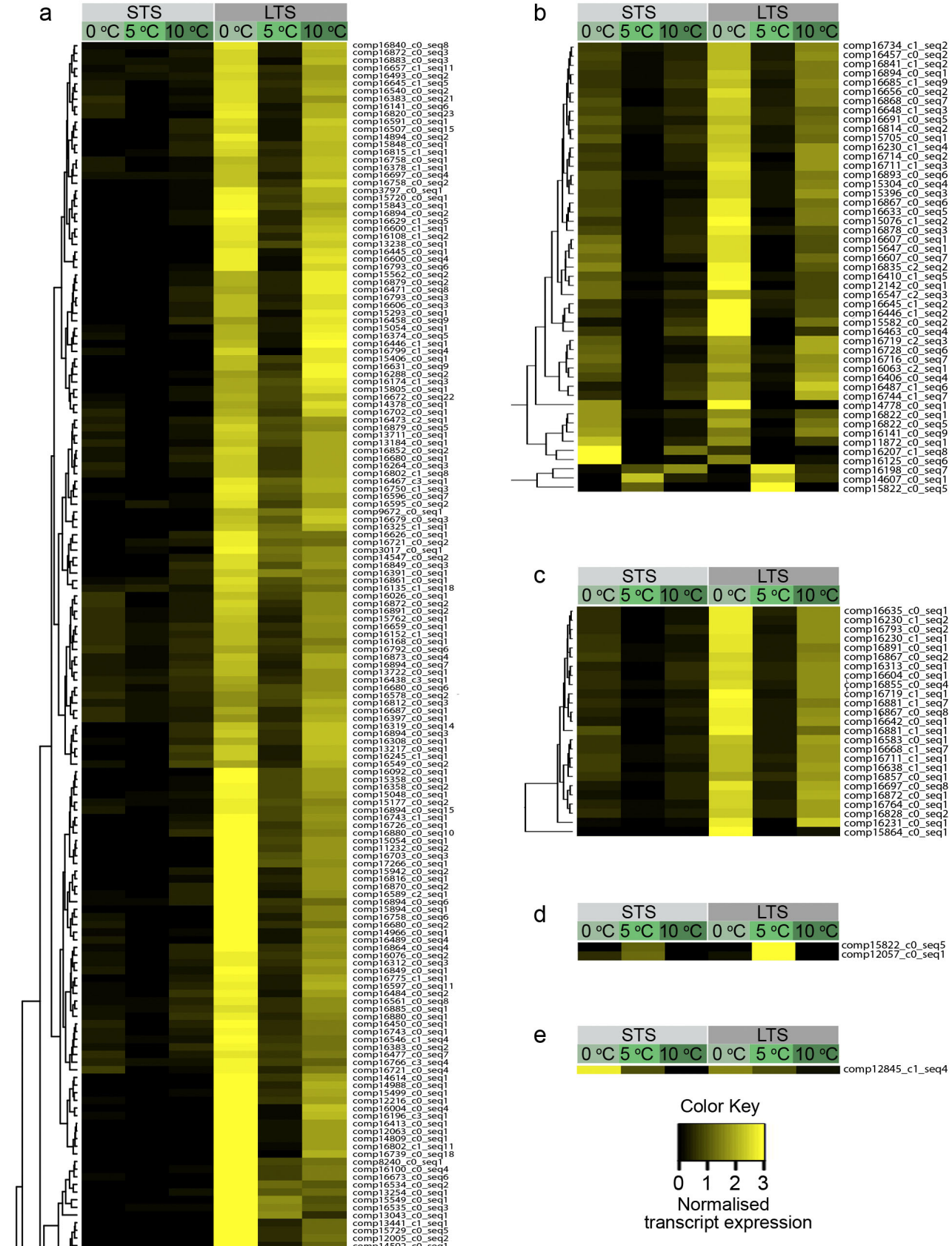

Fig. 1. Heat map of differentially expressed transcripts in (a–d) *Calanus finmarchicus* and (e) *C. glacialis*, detected in the following comparisons: (a) STS vs. LTS; (b) 0 vs. 5°C; (c) common between STS vs. LTS and 0 vs. 5°C comparisons; (d) 5 vs. 10°C; and (e) 0 vs. 10°C. Colour values represent normalized transcript expression: black: no expression; yellow: high expression. STS = short-term (4 h), and LTS = long-term (6 d) thermal stress response

(Table 1, Tables S3 & S4 in Supplement 2 at www.int-res.com/articles/suppl/m534p079_supp.xls). Functional classifications of annotated transcripts in *C. finmarchicus* and *C. glacialis* represented similar fractions of transcripts in GO categories for biological processes (Fig. S4 in Supplement 1 at www.int-res.com/articles/suppl/m534p079_supp.pdf).

Differential expression

Whole transcriptome profiling

Differential expression analysis was performed on uniquely mapped reads from each library (Fig. S3 in Supplement 1). Filtering of Trinity transcripts with baseMean <5 counts (to minimize the influence of sequencing errors) reduced the number of transcripts to 5566 for *C. finmarchicus* and 8083 for *C. glacialis*. The range of $\log_2$ FC in response to treatments was different for the 2 species: wider for *C. finmarchicus* (10.08 ± 1.15 $\log_2$ FC) and narrower for *C. glacialis* (7.30 ± 0.23 $\log_2$ FC). Comparison between STS and LTS experiments revealed 212 differently expressed transcripts in *C. finmarchicus* but none in *C. glacialis* (Fig. 1). In *C. finmarchicus,* among these 212 transcripts, 206 were up-regulated in LTS with a mean $\log_2$ FC of 3.42, and 6 transcripts were down-regulated with a mean $\log_2$ FC of −4.2 (Table S5 in Supplement 2). Enrichment analysis of genes responsive to stress duration indicated 5 over-represented and 1 under-represented GO terms that included genes involved in molecular chaperone and protein folding activity, protein biosynthesis, DNA replication and metabolic processes (Table 2).

In *C. glacialis*, differential analysis of gene expression at different temperatures revealed that comparisons 0 vs. 5°C and 0 vs. 10°C had no significant differences in expressed transcripts, while one transcript (*rpl14*) was differentially expressed in the 0 vs. 10°C comparison (Table S6 in Supplement 2, Fig. 1). For *C. finmarchicus*, 74 and 2 differentially expressed transcripts were detected in the 0 vs. 5°C and 5 vs. 10°C comparisons, respectively, and none in the 0 vs. 10°C comparison (Fig. 1, Tables S7 & S8 in Supplement 2). In the 5 vs. 10°C comparison, both transcripts were down-regulated at 10°C. Of 74 differently expressed transcripts in the 0 vs. 5°C comparison, 71 were down-regulated at 5°C with a mean $\log_2$ FC of −3.3. These down-regulated transcripts included molecular chaperones and heat shock proteins, proteins involved in reactive oxygen species (ROS) detoxication, proteolysis and energy metabolism (Table S7 in Supplement 2). In addition, up-regulated transcripts were functionally enriched in structural con-

Table 1. Summary statistics and annotation success for the de novo assemblies of *Calanus finmarchicus* and *C. glacialis* transcriptomes. GC: guanine and cytosine

Statistic	*C. finmarchicus*	*C. glacialis*
Total number of cleaned reads	4 406 567	2 962 408
Total Trinity transcripts	28 954	36 880
Minimum transcript length (bp)	201	201
Mean transcript length (bp)	353	427
Maximum transcript length (bp)	2945	4021
Transcript N50 length (bp)	354	471
Total number of assembled bases	10 223 122	15 748 460
GC content for assembly (%)	47	49
Total number of annotated transcripts	13 057	18 387
Annotation success (%)	45	50

Table 2. Enriched gene ontology (GO) terms among differently expressed transcripts in *Calanus finmarchicus*. P: biological process; F: molecular function; C: cellular component; MCM: mini-chromosome maintenance

GO	Category: term	Significant genes	Expected genes	Corrected p-value
STS vs. LTS				
GO:0006457	P: protein folding	16	6.84	0.02
GO:0003746	F: translation elongation factor activity	8	1.12	0.002
GO:0005524	F: ATP binding	47	43.46	0.005
GO:0031681	F: G-protein beta-subunit binding	4	0.24	0.03
GO:0005737	C: cytoplasm	116	140.59	6.12×10^{-7}
GO:0042555	C: MCM complex	8	1.79	0.04
0 vs. 5°C				
GO:0003735	F: structural constituent of ribosome	9	2.52	0.03
GO:0022627	C: cytosolic small ribosomal subunit	5	0.41	0.02

Table 3. Differentially expressed transcripts in response to duration of the stress (STS [4 h] versus LTS [6 d]) and thermal stress at 0 vs. 5°C in *Calanus finmarchicus*. Log_2 FC: log_2 fold change of expression values; GO: gene ontology; UniProt accession: accession number to the UniProt protein sequence database

Trinity transcript	Gene description	UniProt accession	log_2 FC STS vs. LTS	log_2 FC 0 vs. 5°C	GO annotation for biological processes
comp16711_c1_seq1	40S ribosomal protein SA	B5FXT6	1.84	−2.22	Translation; cell adhesion; ribosomal small subunit assembly
comp16857_c0_seq1	60S ribosomal protein L4-1	Q9SF40	1.62	−1.84	Translation
comp16828_c0_seq2	Adenosylhomocysteinase	P50247	2.18	−2.26	One-carbon metabolic process
comp16697_c0_seq8	Elongation factor 1-gamma	P12261	2.69	−2.92	
comp16891_c0_seq1	Elongation factor 2	Q3SYU2	1.93	−2.36	
comp16583_c0_seq1	G2/mitotic-specific cyclin-B1	P14635	2.06	−2.97	Response to mechanical stimulus; cellular response to fatty acid; cellular response to hypoxia; protein phosphorylation; protein complex assembly; mitotic spindle checkpoint; mitotic spindle stabilization; tissue regeneration; oocyte maturation; spermatogenesis
comp16668_c1_seq7	Guanine nucleotide-binding protein subunit beta-like protein	O18640	1.60	−2.57	Oogenesis; oviposition; locomotory behavior; cuticle development; wing disc development; mRNA splicing, via spliceosome; muscle cell cellular homeostasis; regulation of autophagic vacuole size; positive regulation of glycogen biosynthetic process
comp16764_c0_seq1	Heat shock protein 83	O02192	2.27	−1.98	Response to stress; protein folding
comp16638_c1_seq1	Heat shock-related 70 protein 2	Q9TUG3	2.21	−2.71	Response to stress
comp16231_c0_seq1	Importin subunit alpha-1	P52293	3.27	−3.47	Protein import into nucleus
comp16855_c0_seq4	Probable aconitate hydratase, mitochondrial	P34455	1.86	−2.47	Tricarboxylic acid cycle
comp16642_c0_seq1	Proliferating cell nuclear antigen	O16852	2.45	−2.56	DNA replication; regulation of DNA replication; regulation of catalytic activity
comp16230_c1_seq1	Proliferation-associated protein 2G4	P50580	2.71	−3.86	rRNA processing; regulation of translation; transcription, DNA-templated; negative regulation of transcription
comp16230_c1_seq2	Proliferation-associated protein 2G4	P50580	2.74	−3.42	rRNA processing; regulation of translation; transcription, DNA-templated; negative regulation of transcription
comp16793_c0_seq2	T-complex protein 1 subunit epsilon	Q5RF02	2.51	−3.73	Protein folding
comp16635_c0_seq1	Transitional endoplasmic reticulum ATPase TER94	Q7KN62	2.73	−3.06	Transport; oogenesis; Golgi organization; ATP catabolic process; dendrite morphogenesis; ER organization; microtubule cytoskeleton organization; ER membrane fusion; regulation of neuron apoptotic process; regulation of pole plasm oskar mRNA localization; ER-associated ubiquitin-dependent protein catabolic process; positive regulation of proteasomal ubiquitin-dependent protein catabolic process
comp16313_c0_seq1	Tubulin alpha-3 chain	P06605	2.32	−2.69	Mitotic cytokinesis; protein polymerization; microtubule-based process
comp15864_c0_seq1	Unknown		5.94	−4.48	
comp16719_c1_seq1	Unknown		2.82	−3.51	
comp16867_c0_seq2	Unknown		1.73	−2.38	
comp16867_c0_seq8	Unknown		2.88	−3.30	
comp16872_c0_seq1	Unknown		2.73	−2.36	
comp16881_c1_seq1	Unknown		2.34	−3.86	
comp16881_c1_seq7	Unknown		1.92	−2.28	
comp16604_c0_seq1	Voltage-dependent anion-selective channel	Q94920	2.45	−2.91	Phototransduction; sperm individualization; sperm mitochondrion organization

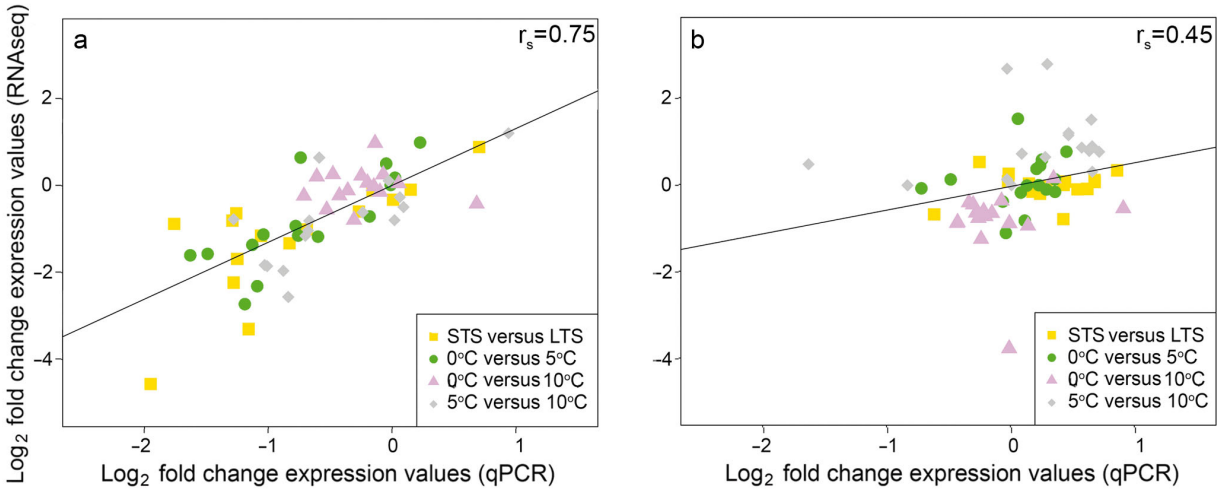

Fig. 2. Correlation between $\log_2$ FC expression values in all comparisons obtained with RNA-seq and qPCR for (a) *Calanus finmarchicus* and (b) *C. glacialis*

stituents of ribosome and cytosolic small ribosomal subunit (various 40S and 60S ribosomal proteins) (Table 2). In both STS vs. LTS and 0 vs. 5°C comparisons, 25 transcripts were detected (Table 3). The majority of the transcripts were related to protein biosynthesis (elongation factors and ribosomal proteins) and protein folding (HSPs and other chaperones). Others were involved in metabolic processes, transport, oogenesis, cell cycle and replication (Table 3).

qPCR profiling of selected genes

Expression values obtained by RNA-seq and qPCR were significantly correlated in *C. finmarchicus* (r_S = 0.75, $p < 0.001$) and *C. glacialis* (r_S = 0.45, $p < 0.001$) (Fig. 2). The correlation improved for *C. glacialis* (r_S = 0.51, $p < 0.001$) after filtering out 2 genes that had correlation coefficients below average: *rpl14* and *znf207* (Fig. S5 in Supplement 1 at www.int-res.com/articles/suppl/m534p079_supp.pdf). FC values obtained by RNA-seq and qPCR matched for most genes and $\log_2$ FC values ranged from –2 to 2 (Fig. S5 in Supplement 1). Out of 5 potential reference genes, *cdc42* and *eif1ax* were the 2 most stable in *C. finmarchicus* and *C. glacialis* under elevated temperatures (Fig. S6 in Supplement 1), and were therefore used.

In *C. finmarchicus*, all 11 genes of interest were significantly up-regulated in LTS compared to STS at 0, 10 and/or 15°C (Figs. 3 & 4). In *C. glacialis*, only 5 of 11 genes (*hsp60*, *hsp70_3*, *hsp70_5*, *gdh* and *nap1l1*) had significant differential expression (down-regulation) in LTS compared to STS at 0 and/or 10°C (Figs. 3 & 4). For both species, none of the selected genes were differentially expressed during STS at any temperature. During LTS in *C. finmarchicus*, 5 genes (*dnaja1*, *gdh*, *nap1l1*, *znf207* and *rps11*) were significantly down-regulated at 5°C compared to 0 and/or 15°C. During LTS, *C. glacialis* down-regulated 4 genes (*dnaja1*, *hsp60*, *hsp70_3* and *gdh*) at 10°C compared to 5 and 15°C, but showed no differential expression compared to 0°C (Figs. 3 & 4).

DISCUSSION

Contrasting response to thermal stress

Thermal stress responses between the temperate *Calanus finmarchicus* and the Arctic *C. glacialis* differed substantially. While over 200 transcripts were differentially expressed in response to temperature and duration of stress in *C. finmarchicus*, *C. glacialis* showed no changes in gene expression based on RNA-seq, and only a few genes displayed differential expression using qPCR. The difference in transcriptome responses to thermal stress between the 2 *Calanus* spp. exceeds differences in global gene expression between sister species of mussels *Mytlius galloprovincialis* and *M. trossulus*, where the majority of genes have highly similar changes in expression (Lockwood et al. 2010).

Furthermore, closely related species pairs inhabiting cold and warm habitats often express *hsps* differentially. Typically, the species from a cold habitat activates overexpression of *hsps* at lower temperatures than the species from a warmer habitat, as shown in the congener amphipods *Eulimnogamma-*

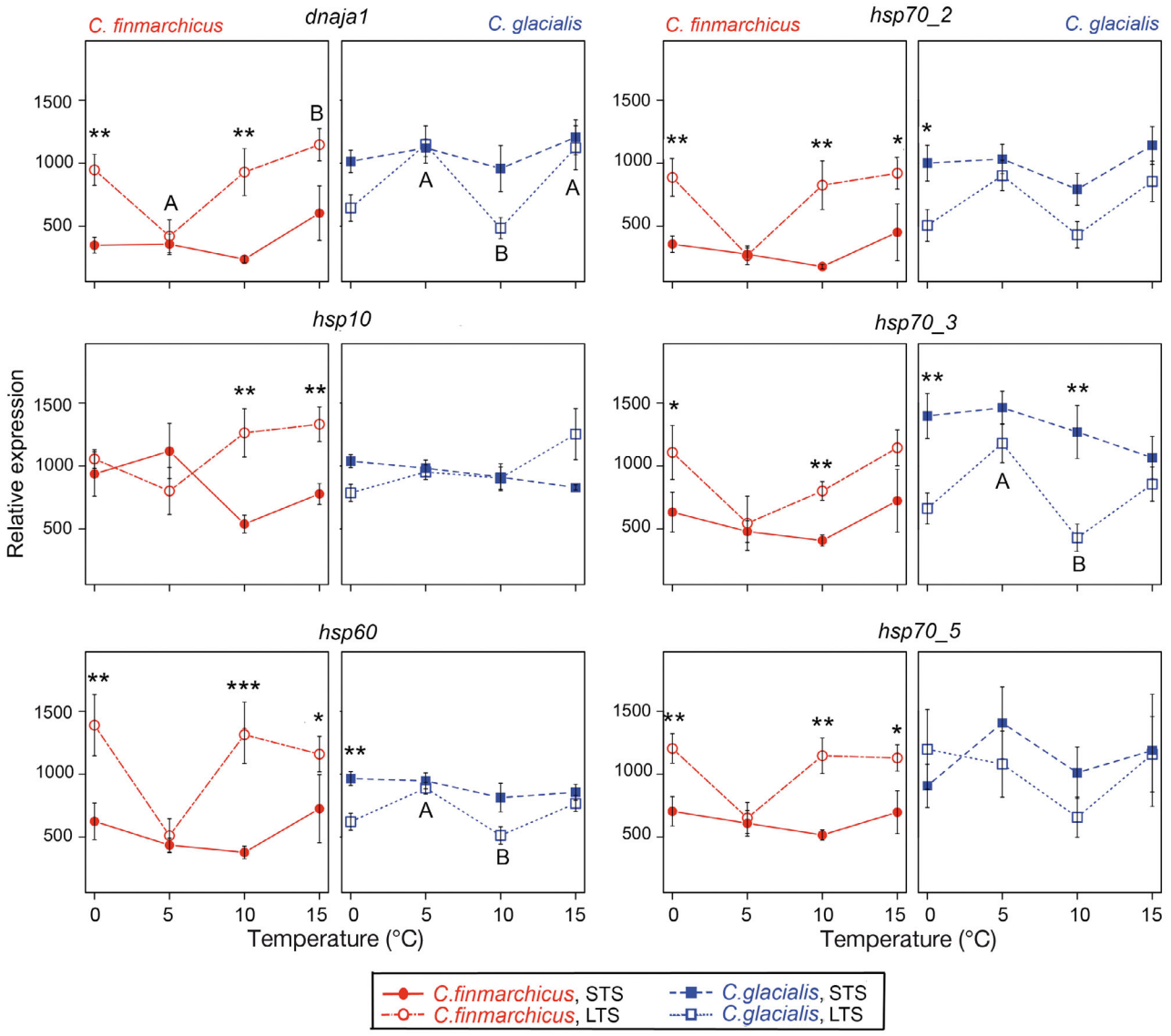

Fig. 3. Relative transcript levels of heat shock protein genes for *Calanus finmarchicus* and *C. glacialis* obtained with qPCR. STS = short-term (4 h) and LTS = long-term (6 d) thermal stress response. Expression values are shown as mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Different letters indicate significantly different expression values between temperatures during LTS

rus: the cold-water *E. verrucosus* shows a lower basal level of HSP70 and a stronger induction after thermal stress compared to *E. cyaneus* from warmer waters (Bedulina et al. 2013). In the present study, however, the cold-water *C. glacialis* showed no up-regulation of *hsps* and no significant changes in global gene expression with increasing temperature, whereas the temperate *C. finmarchicus* up-regulated *dnaja1* (*hsp40*) and changed expression of 74 genes. The lack of thermal stress response in *C. glacialis* compared to *C. finmarchicus* is similar to divergent responses in the Antarctic ciliates *Euplotes*: in contrast to psychrotrophic (cold-tolerant) *E. nobilii*, psychrophilic (cold-loving) *E. focardii* lacks HSP70 up-regulation in response to thermal stress (La Terza et al. 2001).

Genes that were monitored with qPCR did not change their expression in *C. finmarchicus* or *C. glacialis* in response to increased temperatures after STS (4 h), but were differentially expressed in response to LTS (6 d). In addition, RNA-seq showed up-regulation of over 200 transcripts in LTS compared to STS in *C. finmarchicus*, supporting the contention that LTS generally requires a significant change of expression for many genes to ensure cellular homeostasis (e.g. Meistertzheim et al. 2007), while *C. glacialis* did not reveal to keep homeostasis. Similarly to *C. finmarchicus*, a higher number and little

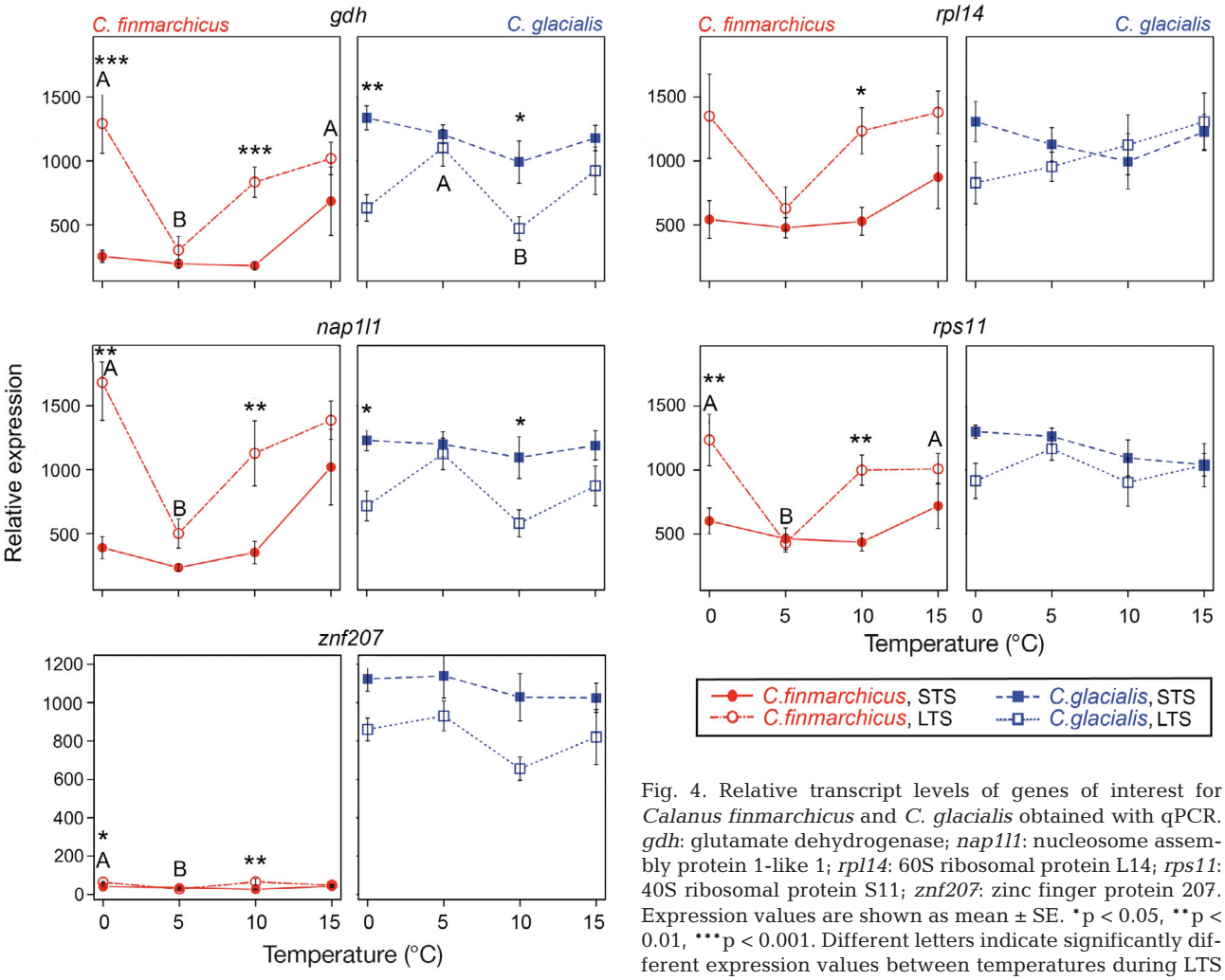

Fig. 4. Relative transcript levels of genes of interest for *Calanus finmarchicus* and *C. glacialis* obtained with qPCR. *gdh*: glutamate dehydrogenase; *nap1l1*: nucleosome assembly protein 1-like 1; *rpl14*: 60S ribosomal protein L14; *rps11*: 40S ribosomal protein S11; *znf207*: zinc finger protein 207. Expression values are shown as mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001. Different letters indicate significantly different expression values between temperatures during LTS

overlap between differentially expressed genes after LTS compared to STS is observed in the coral *Acropora millepora* under exposure to elevated temperatures (Meyer et al. 2011). Consequently, functional divergence in response to STS and LTS may exist, and highlights the importance of stress duration for experiments that aim to investigate resilience-potential of species under climate change.

Lack of thermal stress response in *C. glacialis*

The absence of strong induction of stress-responsive genes in *C. glacialis* suggests a lack of thermal stress response. Two hypotheses explaining the absence are (1) *C. glacialis* was not stressed during the experiments and is more resilient than *C. finmarchicus*; and (2) *C. glacialis* was thermally stressed but, as a cold-water Arctic species, it may lack the molecular mechanisms to respond to thermal stress. The second hypothesis is more likely, given that presence and abundance of *C. glacialis* in the natural environment is strongly linked to temperatures <7°C (Carstensen et al. 2012), while the copepods become torpid and motionless >15°C (Hirche 1987). Accordingly, physiological data from the present experiment showed decreased grazing and elevated mortality at 15°C, supporting earlier studies at the same location showing *C. glacialis* sensitivity to temperatures >7°C (Hjorth & Nielsen 2011, Kjellerup et al. 2012).

A similar lack of or low-magnitude response to thermal stress was observed in several Antarctic cold-specialized species, ranging from notothenid fishes (e.g. *Trematomus bernacchii*) (Buckley & Somero 2009) to invertebrates (La Terza et al. 2001, Clark et al. 2008). In most cases only heat shock response, particularly expression of HSP70, was in-

vestigated and absence of HSP up-regulation was detected. However, recent full transcriptome profiling in the nototheniid fish *Pagothenia borchgrevinki* showed that the stress response even to a small temperature increase includes a low-magnitude down-regulation of many genes including *hsps* (Bilyk & Cheng 2014). Similar down-regulation of several genes (*dnaja1*, *hsp60*, *hsp70_3* and *gdh*) at 10 compared to 5°C after LTS was detected in *C. glacialis* using qPCR. The lack of cellular stress response in Antarctic species is due to adaptation and specialization to stably cold Antarctic waters (Clark et al. 2008, Bilyk & Cheng 2014). Although environmental conditions in the Arctic Ocean are more variable then in the Antarctic (Clarke & Peck 1991), and the lack of cellular stress response has not been reported in Arctic species, the lack of thermal stress response in *C. glacialis* may be explained by cold-water specialization. More studies and locations are needed to confirm this hypothesis and to further explore cold-water specialization of *Calanus* spp. by examining another Arctic species *C. hyperboreus* and the Antarctic species pair *C. propinquus* and *C. simillimus*.

Transcriptome-wide response to thermal stress in *C. finmarchicus*

Overall, transcriptome-wide changes in *C. finmarchicus* involved macromolecular stabilization and repair, protein biosynthesis and proteolysis, and energy metabolism, mirroring the classical cellular stress response aiming to increase tolerance and survival of an organism (Kültz 2005). Such a response has previously been detected in various species exposed to stress, such as mussels (Lockwood et al. 2010) and copepods (Schoville et al. 2012), and includes up- and down-regulation of many genes. Although sub-lethal stress at 18°C for 48 h induces up-regulation of *hsp70* in *C. finmarchicus* (Voznesensky et al. 2004), the change of temperature from 0 to 5°C during LTS in the present study resulted in down-regulation of several *hsps* and other stress-responsive genes. The 3 *hsp70* genes that were used in qPCR in our study are distinct from those previously examined in *C. finmarchicus* (Voznesensky et al. 2004, Aruda et al. 2011), suggesting an even larger complexity of multiple homologues within *hsp* families in *Calanus* spp. and their differential regulation under various stresses. The genes of *dnaja1*, *gdh* and *rps11* were up-regulated at 15 compared to 5°C. Glutamate dehydrogenase (*gdh*), a mitochondrial enzyme that plays a key role in the metabolism of free amino acids, responds to acute salinity stress in the Chinese mitten crab (Wang et al. 2012) but not in the euryhaline copepod *T. californicus* (Willett & Burton 2003), and may have an important role in the thermal stress response in *C. finmarchicus*. The down-regulation of ribosomal protein genes, particularly *rpl14*, has been found in the copepod *T. californicus* in response to heat stress (Schoville et al. 2012), while large-scale up-regulation of ribosomal proteins in response to heat shock or chronic stress has been found in the Pacific oyster (Meistertzheim et al. 2007), suggesting an effort to increase translation capacity or protect ribosomal function through the addition or replacement of ribosomal proteins (Kültz 2005).

The last group of stress-responsive genes that our study targeted is involved in the cell cycle: nucleosome assembly protein 1l1 (*nap1l1*), which is involved in modulation of chromatin formation and regulation of cell proliferation; and zinc finger protein 207 (*znf207*), which can regulate chromosome alignment (Toledo et al. 2014). These genes were down-regulated at 5 compared to 0°C and up-regulated under LTS, and may be important for cell cycle regulation under stressful conditions. An overexpression of *nap1l1* was found in response to hyperosmotic stress in European whitefish (Papakostas et al. 2012), while there is no reported evidence about the involvement of *znf207* in stress response. Additionally, RNA-seq analysis in *C. finmarchicus* revealed more differentially expressed genes connected to cell cycle, meiosis and oogenesis.

The large-scale down-regulation of genes at 5 compared to 0 (RNA-seq) and 15°C (several genes targeted with qPCR including several *hsps*), together with the fact that many of *hsps* are stress-inducible genes (Feder & Hofmann 1999), suggests 5°C to be the more optimal temperature for *C. finmarchicus* from Disko Bay. This is supported by physiological experiments on *C. finmarchicus* from the same area during the spring bloom showing an increase in grazing and egg production from 0.5 to 10°C (Hjorth & Nielsen 2011, Kjellerup et al. 2012) and the fact that *C. finmarchicus* has an annual temperature optimum of approximately 5°C (Wilson et al. 2015). Nevertheless, in the Norwegian Sea, 10°C is regarded as optimal for *C. finmarchicus* (Harris et al. 2000), while populations from the North Sea show the highest population growth rate at 12°C (Møller et al. 2012). Thus, physiological and gene expression plasticity of *C. finmarchicus* may be of particular importance for mitigating climate change, as *C. finmarchicus* might cross the limit of its thermal niche for several months

per year in the North Sea (Helaouët et al. 2011) by 2100. Even if examples of locally adapted populations with different thermotolerance are known for copepods, such as *Tigriopus californicus* (Schoville et al. 2012), more detailed studies are needed to investigate a contribution of both genetic adaptation and phenotypic plasticity into the thermal tolerance of *C. finmarchicus*.

Reliability of approach

The overall good agreement between RNA-seq and qPCR (Fig. 1, Fig. S5 in Supplement 1 at www.int-res.com/articles/suppl/m534p079_supp.pdf) demonstrates the reliability of our RNA-seq results, particularly in *C. finmarchicus*, where the correlation is similar to other studies on non-model species (e.g. Meyer et al. 2011). The reduced strength of correlation between qPCR and RNA-seq in *C. glacialis* is likely due to the limited sequencing depth and small FC in gene expression under stress conditions. Despite the close match of $\log_2$ FC of qPCR and RNA-seq, statistical analysis of qPCR data resulted in better resolution of differently expressed genes, indicating weaker discriminative power of RNA-seq analysis, which could originate from pooling replicates into one library per treatment. We followed Biswas et al. (in press) as most differentially expressed genes can be called with a biologically averaged (pooled) design. Nonetheless, replicates of pools may add power to the statistical test to identify subtle changes in gene expression (Kendziorski et al. 2005) observed in *C. glacialis*.

In the experiments, we tried to minimize the influence of factors other than temperature. Nevertheless, maturation of gonads and oocytes in used females, as well as egg production, may result in some of the observed changes during LTS, particularly those connected to cell cycle, meiosis and oogenesis. In addition, phytoplankton grazed by *Calanus* spp. could be utilized for growth, maturation of gonads and egg production (Falk-Petersen et al. 2007), and thus may influence gene expression. The diatom *Thalassiosira weissflogii* diet is widely used in long-term experiments with live *Calanus* spp. (e.g. Hjorth & Nielsen 2011, Kjellerup et al. 2012) without apparent detrimental effects on physiology. Nonetheless, this diatom diet has been shown to affect egg production and hatching success in the long term (14 d) in another copepod species (*Temora stylifera*, Ceballos & Ianora 2003), and feeding of *C. helgolandicus* on toxic diatom *Skeletonema marinoi* resulted in up-regulation of genes involved in protein folding or degradation, and re-organization of actin and tubulin filaments (Carotenuto et al. 2014).

Conclusion and future perspectives

The low-magnitude transcriptional response in *C. glacialis* to thermal stress suggests that this species has limited potential to respond to novel thermal conditions via a change in gene expression. Integration of these results with laboratory and field observations suggests that, under climate change, *C. glacialis* might be a vulnerable element in the Arctic ecosystem. New regulatory mechanisms involved in stress response, however, have been recently highlighted (e.g. microRNAs, long non-coding RNAs and epigenetics). Consequently, it may be possible for species (e.g. *C. glacialis*) that do not show a strong transcriptomic response to thermal stress to have alternative mechanisms to cope with temperature changes. Additionally, knowledge about population genetic structure of the 2 species in space and time, as well as the role of thermal history in thermal tolerance of the species, will lead to a better understanding of their resilience potential to climate change.

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Data accessibility. Sequence data and the de novo assemblies have been submitted to the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov) under bioproject numbers PRJNA236983 (*C. finmarchicus*) and PRJNA 237014 (*C. glacialis*).

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