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Complementary Roles for Differential Gene Expression and Differential Exon Use in the Heat Shock Response of an Intertidal Copepod

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Synopsis Understanding the mechanisms by which organisms adapt to variation in temperature is key to explaining their distribution across environments and to predicting their persistence to changing climate. The cellular response to heat shock, heat shock response (HSR), is a highly conserved mechanism for coping with elevated temperatures which functions through the upregulation of molecular chaperones like heat shock proteins (HSPs). Recent studies have also shown cellular response to heat shock can be quantitative (changing the magnitude of expression) or qualitative (differential usage of exons originating from the same gene). However, few studies have explored the time course of these two mechanisms in response to heat shock. We conducted a time-course experiment to examine the gene expression and exon usage changes in response to heat shock at four post-stress timepoints (30 min, 1 h, 2 h, 24 h) in a splash pool copepod, *Tigriopus californicus*. We detected signatures of both gene expression and exon usage changes across all timepoints. The magnitude of this response was higher at timepoints closer to heat shock and decreased with time post-heat shock. We observed that heat shock predominantly induced changes in gene expression in genes coding for chitin, HSPs, cellular growth, and differentiation. In contrast, we found that genes coding for peptidases showed both altered expression levels and exon usage. Genes associated with cellular metabolism and cytoskeletal elements primarily showed changes in exon usage. These ontology-specific response mechanisms provide new insights into the temporal landscape of HSR in *Tigriopus* and highlight the need to integrate qualitative and quantitative changes in gene expression to fully understand organismal responses to heat shock.

Introduction

An organism's ability to acclimate and adapt to temperature changes is crucial in determining its survival and, ultimately, the spatial distributions of species and populations, especially in a rapidly warming world. For many organisms, temperatures slightly above their optimum thermal range may present a challenge to survival (Richter et al. 2010; Kontopoulos et al. 2024). Everything from rates of reactions in the cell to the behavior of the organism is affected by temperature (Hochachka & Somero 2002; Schmidt-Nielsen 1997). Inside the cell, an increase in temperature causes protein misfolding and the aggregation of misfolded proteins. These aggregations eventually disrupt protein homeostasis, damage the cytoskeleton, affect the number and integrity of cellular organelles like mitochondria, and negatively affect rates of transcription and translation as well as cell growth and development (Richter et al. 2010). Therefore, it is essential for an organism to sense and rapidly respond to this cellular challenge in order to survive changes in temperature.

The mechanisms by which cells stabilize proteins, repair the damage to protein structures, and restore protein homeostasis are collectively called the heat shock response (HSR), discussed in detail in Richter et al. 2010 and de Nadal et al. 2011. Inside the cell, one of the major challenges of heat stress is the damage to proteins: increasing heat stress causes proteins to lose their

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native structure and their function, resulting in their denaturation. Accumulation of denatured proteins in the cell can be toxic (Hochachka and Somero 2002; Stefani and Dobson 2003) and therefore requires their removal from the cell. One of the primary cellular responses to heat stress is increased expression of molecular chaperones such as heat shock proteins (HSPs) (Hochachka and Somero 2002), which help to prevent and repair the damage to the proteins caused by heat stress. HSPs are a family of proteins that work as molecular chaperones to ensure correct folding of nascent proteins and prevent their aggregation (Hartl et al. 2011). In non-stressed conditions, HSPs exist in a complex with a transcription factor (HSF1). Heat stress triggers the release of chaperones from this complex and frees them to bind with the denatured protein aggregates formed in the cells while HSF1 localizes to the nucleus and initiates expression of more HSPs. Under heat stress, HSPs help to maintain protein homeostasis by preventing aggregate formation and facilitating the correct folding of the heat damaged proteins to their native conformation. Additionally, HSPs work with the members of the proteasome system to mark misfolded proteins for degradation (Freilich et al. 2018; Genest et al. 2019; Haselbeck et al. 2019). The HSR is broadly conserved across the tree of life (Alagar Boopathy et al. 2022), but there is also variation in this trait both within (Dutton and Hofmann 2009; Henkel et al. 2009; Schoville et al. 2012; Barreto et al. 2018; Li et al. 2018; Eshel Gil et al. 2022) and across species (Ritossa 1962; Kelley and Schlesinger 1978; Lemaux et al. 1978; Ashburner and Bonner 1979; McAlister and Finkelstein 1980; Earhart et al. 2022). However, comparisons of HSR across species have also revealed that the timing and duration of the response varies across species and even within populations of the same species (Snutch and Baillie 1983; Peter and Candido 2002; Han and Lee 2006; Velichko et al. 2013; Jayaraj et al. 2020).

Historically, most studies of HSRs have focused on changes in gene expression, but increasing evidence suggests changes in gene isoforms are also an important component of animal (Steward et al. 2022) and plant (Dikaya et al. 2021; John et al. 2021) responses to heat stress. Differential exon usage could allow organisms to achieve different combinations of exons in stress vs. non-stressed conditions (Healy and Schulte 2019) and can arise from several mechanisms such as alternative splicing, exon skipping, using alternative transcription start sites, and incorrect transcription (Sameth et al. 2008). This combination of qualitative and quantitative responses to stress has been reported in several organisms (De Nadal et al. 2011; Kornblihtt et al. 2013; Laloum et al. 2018; Xia et al. 2018; Chaudhary et al. 2019; Healy and Schulte 2019; Tan et al. 2019; Zhang et al. 2019; Salisbury et al. 2021; Thorstensen et al. 2021). Heat shock may shift expression towards more thermally stable isoforms of key enzymes, contributing to inter and intra-specific variation in heat tolerance (Scafaro et al. 2016; Nagarajan et al. 2024). More broadly, mounting evidence for the role of alternative splicing in HSRs underscores the need to include analyses that consider changes in gene expression as well as changes in exon usage (Ling et al. 2021). Previous studies on plants, vertebrates and invertebrates have observed that genes involved in HSR such as HSPs, heat shock factor (HSF), as well as genes enriched for metabolic and energy synthesis processes show differential exon usage in response to heat shock (Takechi et al. 1994; Fujikake et al. 2005; Liu et al. 2013; Thorstensen et al. 2022; Hook et al. 2025; Stankiewicz et al. 2025). Therefore, an integrative approach examining both differential gene expression and differential exon usage is necessary to fully explore acclimation potential and responses to heat stress over time.

Here, we investigate the relative importance of differential exon usage and differential gene expression during HSRs, using the common splash pool copepod, *Tigriopus californicus* as our study system. With a habitat that spans >27° of latitude from Baja, California to Alaska, *T. californicus* tolerates a wide range of latitudinal and seasonal thermal variation (Burton and Lee 1994). Within a site, the splash pools that *T. californicus* inhabit also experience a wide range of temperature variation over short periods of time—with temperatures fluctuating by as much as 17°C over the course of a day (Scheffler et al. 2019; Bogan et al. 2024). *Tigriopus californicus* also has a short generation time (3–4 weeks), is easy to maintain in the lab, and shows population specific variation in heat tolerance (Kelly et al. 2012; Barreto et al. 2018; Foley et al. 2019). Moreover, the mechanistic basis of heat tolerance as a trait has been widely studied in *Tigriopus* (Schoville et al. 2012; Kelly et al. 2013; Kelly et al. 2017; Tangwancharoen et al. 2014; Graham and Barreto 2019; Harada et al. 2019), but very few studies have focused on the changes in exon use in response to heat shock or the time course of gene expression after experiencing heat shock. To address this gap in knowledge, we performed a time course experiment consisting of four time points post-heat shock (30 min, 1 h, 2 h, 24 h) to identify the how and when exon usage and gene expression change over time following heat shock. The 30 min, 1 h, and 2 h timepoints post-heat shock were chosen to observe the time course of transcriptomic response of *T. californicus* to heat shock, as the time course of response to heat stress varies among species (Alagar Boopathy et al. 2022). *Tigriopus californicus* also shows the ability to heat harden, where prior exposure to sublethal heat stress

increases thermotolerance when tested within 24–48 h. Although the heat hardening phenotype is well documented (Kelly et al. 2016; Kelly et al. 2017; Pereira et al. 2017; Healy et al. 2019), the molecular mechanisms conferring the increased thermotolerance remain largely unexplored. We chose the 24 h post-heat shock time point to observe the changes in gene expression and exon usage to test if either of these two mechanisms contribute to the heat hardening phenotype. As previous studies in vertebrate and invertebrate study systems have shown that there is very little overlap in genes showing both DEU and DEG in response to environmental stress (Jakšić and Schlötterer 2016; Jacobs and Elmer 2021; Ren et al. 2022; Liu et al. 2024), we hypothesized that gene expression and differential exon usage contribute independently to the HSR in *T. californicus*, with little overlap between the two response mechanisms broadly and within biological processes known be associated with HSR. We also hypothesized that this pattern would hold true for all of the experimental time points tested in our study.

Methods

Animal collection and maintenance

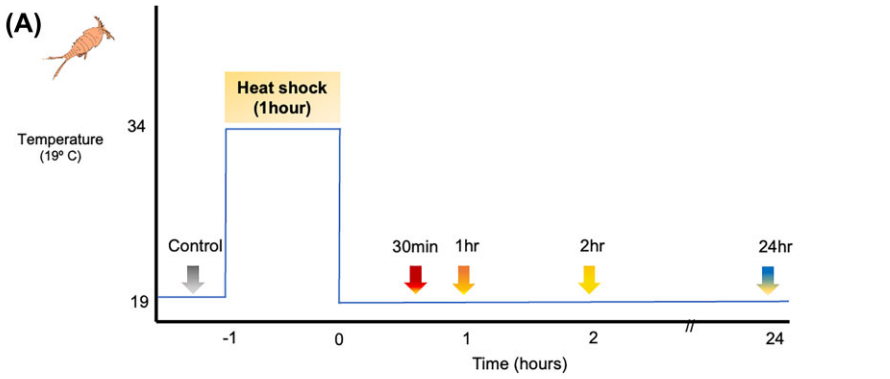
We collected *T. californicus* copepods from Strawberry Hill, Oregon, USA (44°15′ N, 124°06′ W) in December of 2023 and established lab cultures from these wild-caught individuals following protocols described in Kelly et al. (2012). Briefly, copepods were maintained at 19°C and at 34 ppt salinity under a 12-h light/12-h dark cycle and fed a diet of ground spirulina fish food *ad libitum* until beginning the experiment in February of 2024.

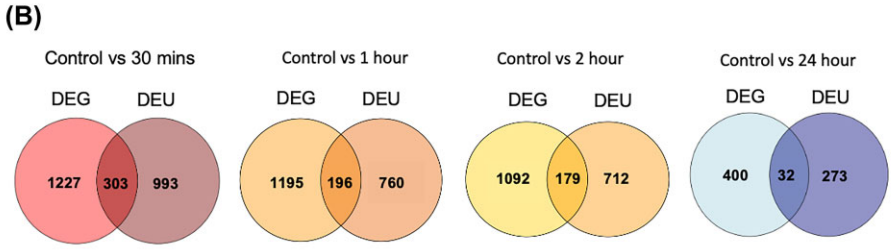
Experimental design

Our experimental design consisted of four time points post-heat shock to mirror the course of immediate response to heat shock and heat hardening (30 min, 1 h, 2 h, and 24 h), with three replicates for each time period and for one non-heat shocked control treatment. Briefly, for each treatment, 50–60 male and female adult copepods (copepods past their final molting stage) were haphazardly chosen from the established lab cultures and transferred into a 50 mL falcon tube containing 50 mL of sea water at 34 ppt salinity. Each time point (and the non-shocked control) had three falcon tubes each full of 50–60 copepods, which served as three biological replicates for that particular treatment (3 falcon tubes for non-shocked controls, 3 falcon tubes for each of the four heat shocked timepoints: $3 + 3 \times 4 = 15$ falcon tubes in total). For heat shock treatments, tubes were exposed to a sub-lethal temperature of 34°C for 1 h (following Kelly et al. 2012) in a water bath (VWR WBE20 General Purpose Water Bath) that was set to 34°C, and then immediately removed and returned to room temperature where they were maintained until they were flash frozen in liquid nitrogen at their respective time points post-heat shock (30 min, 1 h, 2 h, and 24 h) (Fig. 1A). The control tubes were kept at room temperature for the duration of the heat shock and were all flash frozen by the end of the 2 h time point. Our study only included controls at one time point, which we felt would reasonably represent the other heat shocked time points as they were all taken at a similar time of day and thus unlikely to be confounding HSR with diurnal changes in expression, but our single time point for the control is a potential limitation to the study. The flash frozen samples were stored in −80°C for RNA extractions.

RNA extraction and sequencing

We extracted total RNA from flash frozen copepods using a combination of TRIzol reagent (Invitrogen; catalog no. 15,596,026) and the Qiagen RNAeasy Plus kit (Qiagen, catalog no. 74,134). Briefly, copepods were homogenized using a TissueRuptor II (Qiagen, catalog no. 9,002,755) before we followed steps 1–7 of the TRIzol protocol, followed by steps 4–11 of RNAeasy Plus kit protocol. Total RNA extracted from a total of 15 ((4 timepoints + 1 control) x 3) samples were sent to Novogene Corporation Inc. at Sacramento, California, where RNA quality control was confirmed using a 2100 Agilent Bioanalyzer on a Eukaryote Total RNA Nano chip and non-directional libraries were produced using poly-A tail selection. The resulting 15 libraries were sequenced on NovaSeq X Plus, with 150-bp paired-end reads. We removed adapter sequences using Trimmomatic (Bolger et al. 2014), and we used the program FASTQC (Andrews 2010) to ensure that all of the reads considered for downstream analysis had quality scores of at least 35 (Table S1). The reads were then mapped to the *T. californicus* reference genome (Barreto et al. 2018) using STAR RNA-seq aligner (version 2.6.0a) (Dobin et al. 2013). Reads were mapped to gene features with the options (quantMode GeneCounts—outFilterScoreMinOverLread 0.50—outFilterMatchNminOverLread 0.50) to adjust for poly-A tail contamination (Sirovy et al. 2021), which generates a count matrix using ReadsPerGene.out.tab output. Transcripts per million (TPM) values used for visualizing our RNASeq dataset were generated using the RSEM package (version 1.3.3) (Li and Dewey 2011). All downstream analyses were performed in R version 4.0.3 software (R Core Team 2021).





(C)

	# DEG	# DEU	# Both	DEG = DEU? (p-value)
Heat shock proteins	19*	9	7	0.365
Cell differentiation/growth	72*	35	9	0.083
Cytoskeleton	15	73*	9	**<0.00001**
Metabolism	99	239*	61*	**<0.00001**
Peptidases	144*	139*	65*	0.056
Chitin	69*	4	18*	**<0.00001**

Fig. 1 (A) Schematic of the experimental design showing when *T. californicus* were sampled after the 1-h heat shock at 34°C. Control samples were taken without exposure to heat stress around the 2-h time point. (B) Venn diagrams showing overlap between differentially expressed genes (DEGs) and differential exon usage genes (DEUs) at each experimental timepoint. (C) A table showing the number of DEGs, DEU genes, and genes that showed both differential expression and exon usage across all time points in six gene categories of interest. An asterisk indicates that this category of gene was found to be significantly enriched within genes that displayed only DEG, only DEU, or both when examined across all time points. The final column shows *P*-values from a chi-square test asking whether each functional category contained a significant bias towards the use of DEGs or DEUs across all responding genes within that category (bold indicates *P*-values < 0.05) *Tigriopus* illustration by Xochitl Gonzalez .

Differential gene expression analysis

We removed lowly expressed genes, which were defined as genes that had less than 10 counts in 25% of our samples (Brown et al. 2025) from our dataset. This retained 15,052 genes out of 16,820 genes that were input to DESeq2 (v 1.24.0) (Love et al. 2014) to perform pairwise comparisons of each time point relative to controls, obtain a list of differentially expressed genes (DEGs) for each time point, and calculate false discovery rates (FDRs) using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). Our DEG analysis included pairwise comparisons of non-heat shocked controls (3 replicates) to heat shocked treatments (3 replicates) for all timepoints. Genes with an adjusted *P*-value cutoff of < 0.01 and a log2foldchange of >1 (for upregulated genes) or < -1 (for downregulated genes) were considered to be significantly differentially expressed. Gene ontology enrichment was performed using GO_MWU (Wright et al. 2015), using the Fisher's Exact Test ($P < 0.05$) and the scripts available at: https://github.com/z0on/GO_MWU/blob/master/GO_MWU.R.

Differential exon usage analysis

We used DEXSeq v1.22.0 (Anders et al. 2012) to calculate differential exon usage (DEU), following the protocols described in the package manual (available at http://bioconductor.org/packages/release/bioc/html/DEXSeq.html). As changes in exon usage arising from alternative splicing and usage of alternative transcription sites are counted as exon changes by DEXSeq, we refer to all DEXSeq results as differential exon usage to account for both of these possibilities, as stated in the DEXSeq manual. Briefly, we first created a flattened GFF (general feature format file) for *T. californicus* using ENSEMBL annotations (Tcal_SD_v2.1, accession number: GCA_007,210,705.1). The file collapses all transcripts produced from a gene into a single, "flattened" gene model with non-overlapping exons (counting bins). Reads were mapped to these counting bins, and DEU was calculated as difference between control vs. experimental treatments in the ratios of number of reads mapping to an individual exon and the number of genes mapping to all other exons of the same gene. We calculated DEU for each timepoint using pairwise comparisons with control samples and calculated false discovery rates using Benjamini–Hochberg method (Benjamini and Hochberg 1995). Genes with an adjusted P-value of < 0.05 were considered to be genes showing DEU.

Before performing GO enrichment analyses, the gene names in DEXSeq output files were first converted to NCBI gene name format, because our GO analysis pipeline (GO_MWU (Wright et al. 2015)) uses annotations in NCBI format. We merged the NCBI (https://ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/6832/GCF_007210705.1-RS_2023_08/) and ENSEMBL (https://ftp.ebi.ac.uk/ensemblgenomes/pub/release-60/metazoa/gff3/tigriopus_californicus_gca007210705/) (Barreto et al. 2018) GFF files for *T. californicus* based on genomic coordinates to produce a consensus file containing gene names for each gene in both formatting styles. This also allowed us to compare DEXSeq results to DEG results. Gene ontology enrichment for genes showing DEU was performed using GO_MWU (Wright et al. 2015), using the Fisher's Exact Test ($P < 0.05$) and GO annotations files for *T. californicus* were downloaded from NCBI (https://ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/6832/GCF_007210705.1-RS_2023_08/) (Barreto et al. 2018). The R package ggvenn (version 1.5.2) was used to identify the overlap between genes showing DEG and DEU.

Based on the categories of genes we found significantly enriched in our GO enrichment analysis across DEG and DEU genes, we identified six categories of genes that were consistently enriched across multiple timepoints and warranted further exploration. To explore patterns within each category, we created representational graphs of a subset of genes from each category (see Results and Discussion). For all genes responding to heat stress, we tested for overrepresentation of DEG vs. DEU responses within a functional category using a chi-squared test. For this test, we began by identifying all responding genes within each functional category. Across all responding genes, we observed 48% of genes were DEG, 39% were DEU and 12% were both DEG and DEU. Therefore, our null expectation, if a particular category was not enriched for one of these responses, was that the percentages genes that were DEU, DEG or "both" would follow the same distribution for each category. Our chi-squared tests estimated the probability that the observed number of DEG, DEU and "both" genes within a functional category could have been observed by chance if the distribution of DEG vs. DEU responses were in fact the same across all categories.

Results

Overall, across timepoints, the response to heat shock consisted of both DEG and DEU, and the number of DEG and DEU genes decreased with time post heat shock (Fig. 1B; Table S2; Fig. S1). We observed the highest number of DEGs (1530) and DEU genes (1296) at 30 min post-heat shock and the lowest number of DEGs (432) and DEU genes (305) at 24 h post-heat shock. Among DEGs, more genes increased their expression in response to heat shock: 84%, 82%, 64%, and 53% at 30 min, 1 h, 2 h, and 24 h post-heat shock, respectively. Our Venn diagram comparisons revealed that a total of 303,196, 179, and 32 genes responded to heat shock by changing their expression and their exon usage (hereafter referred as DEG + DEU, Fig. 1B) at 30 min, 1 h, 2 h, and 24 h post- heat shock. In comparison, a total of 1227,1195, 1092, and 400 genes showed changes only in their gene expression levels compared to the total of 993,760, 712, and 213 genes that showed changes only in their exon usage at 30 min, 1 h, 2 h, and 24 h post heat shock.

Gene Ontology (GO) enrichment analysis of DEGs upregulated at 30 min, 1 h, 2 h, and 24 h post-heat shock revealed enrichment of GO terms involved in de novo protein folding, protein refolding, HSP binding, chaperone mediated protein folding, proteolysis, peptidases, chitinase; while the downregulated DEGs at these time point were enriched for GO terms involved in developmental process, cell differentiation, cell fate commitment, regionalization, pattern specification process, and

regulation of transcription. GO analysis of DEU genes at 30 min, 1 h, and 2 h post-heat shock showed enrichment of GO terms involved in cytoskeleton organization, peptidase, metabolic processes, and generation of precursor metabolites and energy, while the 24 h post heat shock time point did not yield any GO terms. Genes related to chitin synthesis and breakdown responded to heat shock predominantly by changing their expression ($\chi^2(2, N = 91) = 32.82, P < 0.0001$; Fig. 1C; Fig. 2A), while genes related to metabolism and cytoskeletal proteins responded primarily by changing their exon usage (χ^2 $(2, N = 399) = 47.79, P < 0.0001$; $X(2, N = 97) = 27.98, P < 0.0001$; Fig. 2B and C). Genes coding for peptidases responded to heat shock by changing their expression as well as exon usage and were significantly enriched in both our DEG and DEU analysis, although this was marginally not significantly different than what we would expect by chance (χ^2 $(2, N = 348) = 5.76, P = 0.056$; Fig. 2D). Finally, while the response of HSPs and genes related to cell differentiation and growth were predominantly through DEGs, they were not significantly higher in this category than expected (χ^2 $(2, N = 35) = 2.02, P = 0.36$; χ^2 $(2, N = 116) = 5.0, P = 0.083$; Fig. 2E and F).

Discussion

Our data highlight the importance of differential exon usage (DEU) and differential gene expression (DEG) as two important, and largely complementary, cellular mechanisms involved in response to thermal stress. Differential exon usage offers the opportunity to diversify the available transcriptome and proteome independent of changes in the expression levels of a gene. By including DEU analyses in our study, we were able to identify several hundred additional genes that responded to thermal stress solely through changing their exon usage and these would not be captured by DEG analysis alone, as is the norm in previous transcriptomic studies. Our hypothesis that there would be limited overlap between DEU and DEG genes was true for genes associated with chitin, which significantly favored DEG and genes associated with metabolism and cytoskeleton which were significantly more enriched for DEU (Fig. 1C). We also observed limited overlap in DEU and DEG genes across all of our experimental timepoints (Fig 1B), which agrees with our hypothesis. However, genes associated with peptidases, HSPs, and cell growth and differentiation did not show any significant preference for DEU or DEG and in the case of peptidases had a strong pattern of overlap between DEG and DEU in contrast to our predictions. Our results underscore the importance of considering differential exon usage in addition to gene expression in order to gain a comprehensive understanding of the changes occurring in the cellular landscape in response to thermal stress.

Genes coding for peptidases (i.e., enzymatic degradation of proteins) responded to thermal stress through changes in both exon usage and gene expression levels (Fig. 2D). Expression levels of carboxypeptidases, which can break down proteins by cleaving the peptide bond at carboxy terminus (Lipscomb 1980), increased at 30 min and 1 h post-heat shock, followed by a decrease at 2 h and 24 h post heat shock. On the other hand, aminopeptidases, which break down proteins at the amino-terminus, and metallopeptidases, which require a metal ion for their peptidase activity, were upregulated from 30 min to 2 h post-heat shock. Expression levels of genes coding for dipeptidases, which break down pairs of amino acids (Rawlings and Bateman 2019), showed a decrease in response to heat shock across all timepoints. Taken together, these expression patterns suggest that peptidases are an important component of HSR in *T. californicus*. Their presence across several time points indicates that, at the cellular level, thermal stress negatively affects protein folding in *T. californicus*, leading to aggregation of mis-folded proteins in cells, which are then presumably degraded by these peptidases. As the metabolic cost of breaking down misfolded proteins and remaking them is higher than repairing them (Richter et al. 2010), the presence of peptidases in the HSR could mean that after experiencing thermal stress, *T. californicus* has large quantities of potentially irreversibly damaged proteins that need to be degraded. Similarly, the decrease in expression levels of peptidases after 2 h post-heat shock also suggests how long these potentially irreversibly damaged proteins were present in the cell. Once these proteins are no longer present in the cell, it makes sense that the cells would cease to upregulate peptidases. Differential exon usage of peptidases also showed similar variation based on their mode of action and time post-heat shock.

On the other hand, genes associated with chitin showed changes in DEG responses with little change in DEU (Fig. 2A). Chitin-related genes, such as chitin deacetylase, have been reported to be upregulated in response to temperature and hypoxia in *Tigriopus*, although their function remains unexplored in *T. californicus* (Schoville et al. 2012; Graham and Barreto 2019; Healy and Burton 2023; Harada et al. 2019; Andriot 2024). Because chitin is an essential component of the copepod exoskeleton, microscopic examination of stressed copepods may prove helpful to identify cuticle remodeling that could be occurring in response to temperature (Graham and Barreto 2019; Harada et al. 2019; Andriot 2024).

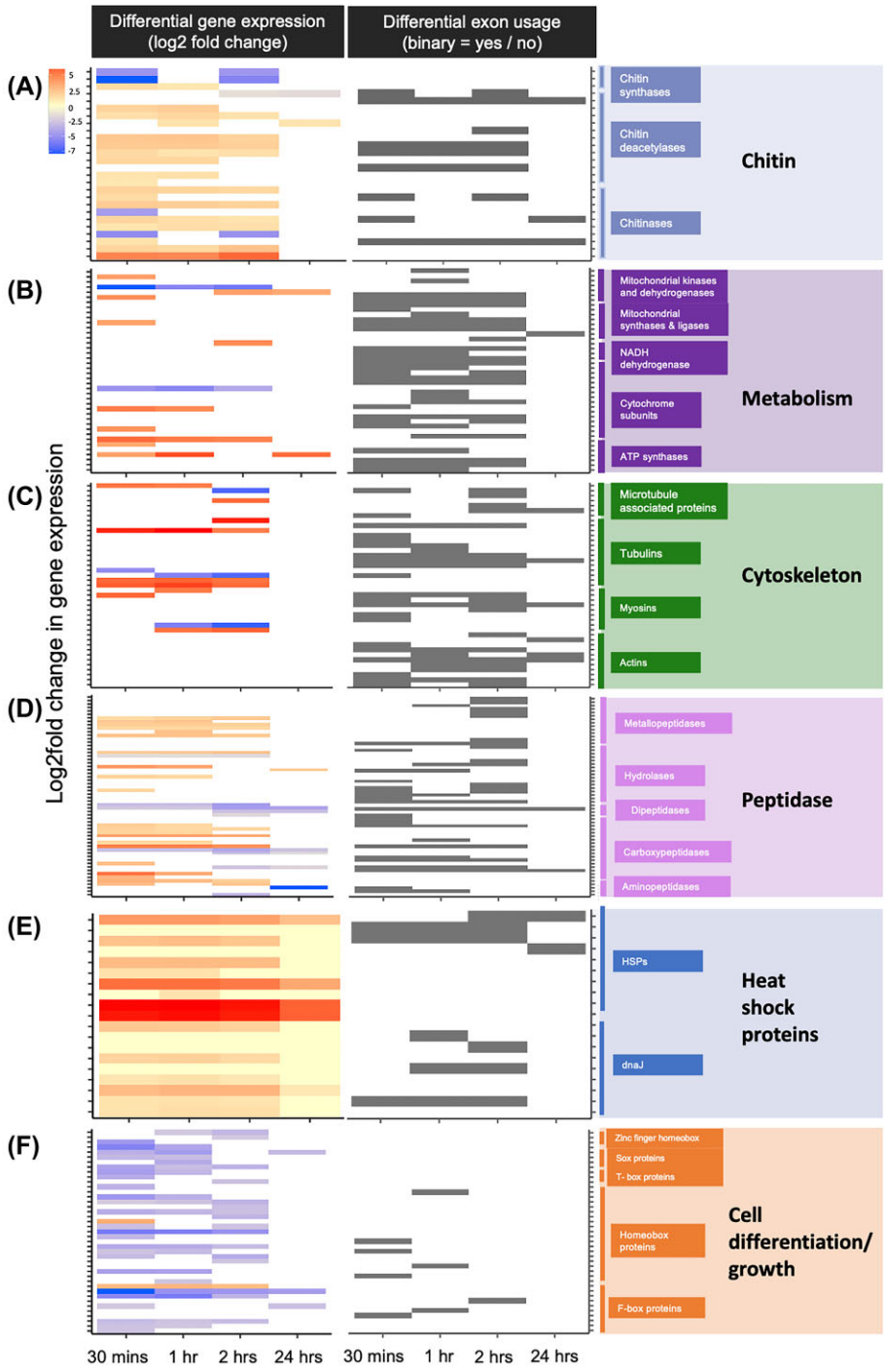


Fig. 2 Graphs of genes that show significant (false discovery rate adjusted *P*-value cutoff of 0.01) differential gene expression (DEG) and differential exon usage (DEU) in response to heat shock across four experimental time points (*x*-axis) when compared to controls. The *y*-axes of the graphs correspond with individual genes that are grouped within categories of interest (A) Chitin, (B) Metabolism, (C) Cytoskeleton, (D) Peptidase, (E) Heat shock proteins, (F) Cell differentiation/growth), and with additional gene details shown in the colored boxes to the right of the graphs. The left half of the graph consists of a heatmap showing log2fold change values for DEGs obtained from DESeq2. The right half of the graphs show whether a given gene at a given time point has significant DEU (shown with gray squares) or not (white squares) based on results from DEXSeq.

Genes coding for HSPs and cell differentiation and growth also responded to thermal stress predominantly through changing their expression levels, although this was not a significant pattern in our chi-square tests (Fig. 1C; Fig. 2E and F). Several members of the HSP family including hsp70, hsp16, and dnaJ, showed increased expression levels at all timepoints post heat-shock. We also observed paralog specific differences in expression levels in HSPs. Out of six genes coding for hsp70, three showed increased expression at all timepoints, whereas the other three were upregulated at a specific time point post-heat shock. Only a small subset of HSPs (hsp83, hspb1, and hsp16) showed DEG and DEU responses. Interestingly, paralogs of dnaJ that showed DEU activity were not upregulated in response to heat shock. The role of HSPs in HSR as molecular chaperones that prevent protein aggregation is a widely conserved response to temperature stress across the kingdom of life (Morimoto 1998; Feder and Hofmann 1999; Sørensen et al. 2003). Previous studies in the *Tigriopus* system have also identified an upregulation in HSPs an hour after heat shock (Schoville et al. 2012; Harada and Burton 2020; Tangwancharoen et al. 2020). Our time point data provide additional temporal context to this upregulation—we now know that HSPs are upregulated as early as 30 min after heat shock and continue to be upregulated as late as 24 h post-heat shock. Results from our DEU analysis for HSPs reveal isoform-specific responses which were not detected in our DEG analysis and are an excellent example of where DEU and DEG complement each other in the global response to thermal stress in *Tigriopus*.

Along with upregulation of HSPs, the reduction of cell growth and proliferation immediately after heat stress is another hallmark of the HSR (Richter et al. 2010). Thermal stress negatively affects several key processes occurring in the nucleus and arrests the cell cycle, leading to a pause in cell growth and proliferation (Hochachka & Somero 2002). We see similar trends in our DEG results, which show downregulation of transcription factors like HOX, SOX, and FOX, which are integral to cell growth and differentiation processes (Kamachi and Kondoh 2013). Interestingly, we observed this downregulation only until 2 h post-heat shock. By 24 h after shock, many of these genes were not downregulated, indicating a possible recovery to non-stressed cellular state by that time. Similar to HSPs, we observe very few instances of DEU activity in these genes, and there was no overlap between genes showing DEG and DEU activity.

Finally, we observed a subset of genes that responded to heat shock predominantly through DEU. These included genes involved in cytoskeletal elements (e.g., actin, tubulin) and metabolism (e.g., ATP synthase, pyruvate kinase, Acetyl CoA) (Fig. 2B and C). Few genes from either of these functional categories were differentially expressed, and there was little overlap between differential expression and differential exon usage in these genes. Thermal stress is known to cause disruption of actin and tubulin networks in the cell (Mounier and Arrigo 2002; Toivola et al. 2010; Wettstein et al. 2012). Thermal stress also adversely affects energy production in *T. californicus* (Harada et al. 2019). We detected several genes related to ATP synthesis and metabolism in our exon usage analysis, and a few in our DEG analysis. While a surprising result, the presence of cytoskeleton and metabolic genes in the DEU category may point toward a potential mechanism wherein exons leading to heat-stable isoforms are preferentially used to maintain the integrity of the cytoskeleton and help sustain energy production during heat shock, a potential benefit of DEU which warrants further investigation.

Our observations of both DEG and DEU responses in several genes present an intriguing puzzle regarding the mechanistic decisions made in *T. californicus* in response to thermal stress. Exon switching offers the advantage of generating multiple protein isoforms with distinct functions from a single gene, which could be particularly beneficial under stress conditions. As the molecular mechanisms involved in exon switching are different than those involved in gene expression (Bush et al. 2017; Lynch and Marinov 2015), future investigations comparing the metabolic costs of these two mechanisms would provide valuable mechanistic insights into how *T. californicus* adapts to thermal stress. While the time course of transcriptomic response to hypoxia has been reported in *T. californicus* (Graham and Barrreto 2019), our study is the first to report the time course of transcriptomic response to heat shock in *T. californicus*. Understanding the chronology of these cellular biological responses is essential for understanding how an organism recovers from stress, and findings from a non-model study can contribute to expanding our knowledge of HSRs across diverse taxa.

Incorporating DEU analyses alongside DEG analyses revealed several genes that respond to heat shock through either DEU alone or a combination of DEU and DEG. Importantly, while some argue that a signal of DEU could be explained by transcriptional noise under stress, the fact that we found significant enrichment in gene categories that are known to be linked to heat stress responses indicates that this is an important and impactful mechanism in the HSR of *T. californicus*. Identities of these novel target genes could be valuable for future studies aimed at understanding the molecular mechanisms of heat tolerance in cope-

pods and other species. DEG and DEU provide two possible, distinct mechanisms underlying the heat stress response with important implications for how thermal acclimation and adaptive tolerance may be operating in this species. By understanding the mechanisms underlying thermal acclimation responses, we can gain a better understanding of how organisms are evolving and adapting to changing environments.

Author contributions

Rujuta V. Vaidya: Writing—review & editing, Writing—original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation. Isabelle P. Neylan: Writing—review & editing, Writing—original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis. Morgan W. Kelly: Writing—review & editing, Writing—original draft, Visualization, Supervision, Resources, Methodology, Conceptualization.

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Supplementary data

Supplementary data available at *ICB* online.

Supplementary data from this study is available at https://doi.org/10.5281/zenodo.15352774.

Conflict of interest

The authors declare no conflict of interests.

Data availability

Raw sequencing reads are available at NCBI BioProject ID PRJNA1259036.

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