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RESEARCH ARTICLE

Acute thermal stress elicits interactions between gene expression and alternative splicing in a fish of conservation concern

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ABSTRACT

Transcriptomic research provides a mechanistic understanding of an organism's response to environmental challenges such as increasing temperatures, which can provide key insights into the threats posed by thermal challenges associated with urbanization and climate change. Differential gene expression and alternative splicing are two elements of the transcriptomic stress response that may work in tandem, but relatively few studies have investigated these interactions in fishes of conservation concern. We studied the imperilled redside dace (*Clinostomus elongatus*) as thermal stress is hypothesized to be an important cause of population declines. We tested the hypothesis that gene expression–splicing interactions contribute to the thermal stress response. Wild fish exposed to acute thermal stress were compared with both handling controls and fish sampled directly from a river. Liver tissue was sampled to study the transcriptomic stress response. With a gene set enrichment analysis, we found that thermally stressed fish showed a transcriptional response related to transcription regulation and responses to unfolded proteins, and alternatively spliced genes related to gene expression regulation and metabolism. One splicing factor, *prpf38b*, was upregulated in the thermally stressed group compared with the other treatments. This splicing factor may have a role in the Jun/AP-1 cellular stress response, a pathway with wide-ranging and context-dependent effects. Given large gene interaction networks and the context-dependent nature of transcriptional responses, our results highlight the importance of understanding interactions between gene expression and splicing for understanding transcriptomic responses to thermal stress. Our results also reveal transcriptional pathways that can inform conservation breeding, translocation and reintroduction programs for redside dace and other imperilled species by identifying appropriate source populations.

KEY WORDS: Differential exon usage, Redside dace, *Clinostomus elongatus*, Transcriptomics, mRNA transcription, CT_{max}, Species at risk

INTRODUCTION

Environmental temperature influences many aspects of the physiology and behaviour of ectothermic animals (Fry, 1947; Schulte, 2015). The thermal environment, especially maximum temperatures, is therefore one of the most important factors that determines the fundamental niche, and thus geographic distribution, of many ectotherms (Bennett et al., 2021; Bozinovic et al., 2011; Day et al., 2018). Aquatic systems are especially vulnerable to increasing temperatures in conjunction with other factors, which can threaten resource availability and biodiversity (Dudgeon, 2019). Anthropogenic disturbances, ranging in scope from global climate change to local land use changes, have increased maximum water temperatures in many aquatic systems, and these temperature extremes are predicted to become more severe (O'Reilly et al., 2015). This warming is hypothesized to be a threat to the distribution and even long-term persistence of many aquatic species (Heino et al., 2009; Myers et al., 2017). However, there is a high degree of interspecific variation in thermal sensitivity even among species that share a common habitat, and the underlying physiological mechanisms are poorly understood (Komoroske et al., 2021; Pörtner et al., 2017). An improved mechanistic understanding of responses to high temperatures is important for predicting population responses to thermal challenges and for guiding recovery actions, particularly for species at risk (e.g. Eliason et al., 2011; Lefevre et al., 2021; McDonnell et al., 2021; Wenger et al., 2011).

Transcriptomic research has emerged as a powerful tool for characterizing the mechanisms of organismal response to stressors such as high temperature, which can then be applied to conservation management (Connon et al., 2018). Comparisons of molecular responses among populations can reveal the mechanisms underlying vulnerable and resistant populations in response to a stressor, with implications for managing habitat and guiding reintroduction (e.g. Jeffries et al., 2019). In addition to tests of mRNA abundance, RNA sequencing also enables tests of alternative splicing (Salisbury et al., 2021). Instead of mRNA abundance changing in response to a stressor as in differential gene expression (Conesa et al., 2016; Jeffries et al., 2021), exons within genes are differentially assembled in post-transcriptional modifications of RNA.

Although mRNA abundance is better understood than alternative splicing in the eukaryotic stress response (Salisbury et al., 2021), splicing also contributes to controlling stress responses in plants, yeast, fruit flies, shrimp and humans (Chaudhary et al., 2019; De Nadal et al., 2011; Kornblihtt et al., 2013; Laloum et al., 2018; Zhang et al., 2019). Furthermore, recent studies indicate that alternative splicing is a key element of the response to environmental change in fishes, such as salinity changes (Thorstensen et al., 2021), acute hypoxia (Xia et al., 2018), cold acclimation (Healy and Schulte, 2019), cold stress (Li et al., 2020) and heat stress (Tan et al., 2019). Differential splicing also contributes to evolutionary change including local adaptation of ecotypes (Jacobs and Elmer, 2021; Salisbury et al., 2021) and speciation (Singh et al., 2017; Terai et al., 2003). However, relatively little is known about changes in splicing following an acute thermal stress event (Tan et al., 2019).

Gene expression and alternative splicing have often been studied separately, but recent work suggests these mechanisms should be

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considered in tandem (e.g. Healy and Schulte, 2019; Jacobs and Elmer, 2021; Singh and Ahi, 2022). This combined approach can reveal interactions between splicing and gene expression, such as by differentially expressed splicing factors that contribute to downstream splicing. For instance, transcription cofactor binding genes were alternatively spliced, while genes involved in the spliceosome were differentially expressed in a cold stress experiment in Nile tilapia (*Oreochromis niloticus*) (Li et al., 2020). Different splicing factors have been found to affect gene expression in contexts outside of the thermal stress response, sometimes referred to as cross-talk between gene expression and the spliceosome (Änkö, 2014; Kim et al., 2018; Smith et al., 1989). Therefore, we hypothesized that gene expression–splicing interactions may also contribute to the acute heat stress response.

To understand the transcriptome-level interactions between differential gene expression and alternative splicing, we studied the regionally imperilled redside dace (*Clinostomus elongatus*). This cyprinid inhabits cool-water streams in northeastern North America, but population sizes and range areas have declined dramatically (COSEWIC, 2017). Redside dace are considered endangered in Canada (COSEWIC, 2017; Redside Dace Recovery Team, 2010), and many populations are considered imperilled in the United States (Serrao et al., 2018). Several studies suggest that redside dace population declines may be linked to thermal stress resulting from the combined effects of urbanization and climate change, and that thermal tolerance varies among genetically distinct redside dace populations (Leclair et al., 2020; Turko et al., 2020, 2021). Understanding the mechanistic basis of these differences is important for predicting thermal responses for different populations and for guiding potential conservation programs such as translocation or reintroduction based on captively bred individuals. Thus, in addition to our main goal of testing the overarching 'expression–splicing interaction' hypothesis, we also aimed to identify thermally responsive genes of redside dace that can be applied to future conservation reintroduction programs for this imperilled species.

Using individuals directly from their natal stream, we experimentally investigated the molecular mechanisms underlying the acute thermal stress response in a wild population of redside dace (see Fig. 1). We sampled livers from adult redside dace exposed to acute thermal stress [following a standard critical thermal maximum (CT_{max}) protocol; Turko et al., 2020] and two control groups: 'wild', fish sampled immediately after capture from the stream, and 'handling control', fish that were treated the same as thermally stressed fish but kept at ambient temperatures. We used RNA sequencing to profile differentially expressed and alternatively spliced genes unique to thermal stress to understand the molecular mechanisms that redside dace use to respond to acute thermal stress. Differential gene expression was tested with mRNA abundance, whereas alternative splicing was assessed with differentially used exons from within mRNA transcripts. Our hypothesis was that the thermal response involves interactions between splicing and gene expression. This hypothesis predicts that splicing factors that show differential expression in response to a thermal challenge are among the specific genes that enable interactions between splicing and gene expression. Therefore, splicing factors upregulated in the thermally stressed fish compared with both other groups were analyzed for possible connections with stress and thermal response genes.

MATERIALS AND METHODS

Fieldwork and thermal stress

Adult redside dace [*Clinostomus elongatus* (Kirtland 1840), *N*=30] were collected using a seine net from a single pool in the Kokosing River, OH, USA (40°32′43.1″N 82°39′15.2″W), over 2 days in February 2019. Fish were then randomly assigned to one of three treatments (each *n*=10): 'wild' fish, thermal stress or handling control (Fig. 1). Wild fish were euthanized via blunt force trauma to the head and spinal severance within 1 min of capture, the body cavity was opened with a ventral incision, and fish were submerged in a high salt solution (700 g l^{-1} ammonium sulfate, 25 mmol l^{-1} sodium citrate, 20 mmol l^{-1} ethylenediaminetetraacetic acid, pH 5.2; Wellband and Heath, 2017) to preserve tissues for transcriptomic analysis. Tissues were stored first at 4°C for 48 h to facilitate preservation, and were subsequently stored at −20°C until RNA extraction. For the thermally stressed group, fish were subjected to a standard CT_{max} protocol as described in detail elsewhere (Turko et al., 2020). Briefly, fish were quickly (within 10 min of capture) transferred to individual mesh-walled plastic containers submerged in an aerated, thermostatically controlled water bath (VWR model 1203) filled with river water. After a 15 min acclimation period, water temperature was raised by 0.33°C min^{-1} until fish could not maintain equilibrium (upright position) in the water column for 3 s. Temperature and dissolved oxygen (always >80%) were monitored throughout each experiment (YSI Pro Plus multi-parameter instrument, Yellow Springs, OH, USA). Once fish lost equilibrium (75–85 min), temperature was recorded and fish were immediately euthanized and preserved as described above. Although transcript levels often continue to rise after an acute stressor and a recovery period of 1 h may have captured this increase (Wiseman et al., 2007), the protocol was chosen to identify early and acute transcriptional changes, as opposed to longer-term patterns. Fish in the handling control group were treated identically to thermally stressed fish except they did not experience increased water temperatures. Instead, these fish were sampled after the average length of a thermal stress experiment

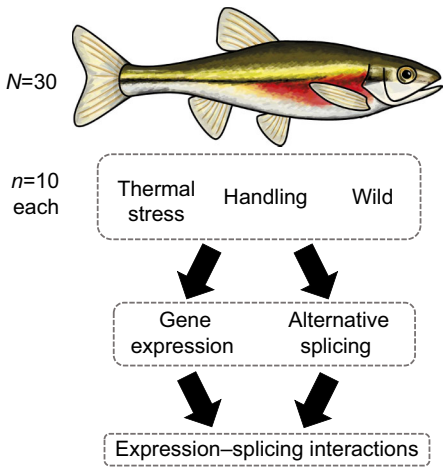

Fig. 1. Conceptual diagram of experimental design and analysis approaches. Redside dace (*Clinostomus elongatus*) were divided into three experimental treatments of *n*=10 individuals each (*N*=30 total): CT_{max} as a thermal stressor, a handling treatment where fish were handled as in the CT_{max} protocol but not heated, and a wild control. Messenger RNA sequencing was performed on liver tissue of all individuals. Genes showing differential expression and alternative splicing were analyzed, with particular emphasis on the thermal stress treatment compared with both others. We hypothesized that gene expression–splicing interactions may contribute to the thermal stress response, and analyzed differentially expressed splicing factors in the thermal stress treatment. Spliced genes associated with the splicing factors were also analyzed, with implications for expression–splicing interactions in the context of thermal stress.

(~80 min). Hereafter, fish in the wild group are referred to as 'wild', fish that underwent CT_{max} as the 'thermal stress' group, and fish that were in the handling control as 'handled'.

RNA extraction and sequencing

RNA was extracted from fish liver using RNeasy Plus Mini Prep Kits (QIAGEN) following the manufacturer's protocol. Liver was chosen for its transcriptional plasticity (Jeffries et al., 2021) and key roles in energy mobilization (Sheridan, 1988). Total RNA was sent to the Génome Québec Innovation Centre sequencing facility (http://gqinnovationcenter.com), where 250 ng of total RNA per fish was used with the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). RNA integrity number (RIN) scores assessed with a Bioanalyzer (Agilent) were >7 for all fish (8.83±0.67, mean±s.d.). Stranded cDNA libraries were created with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). The 30 fish were sequenced for paired-end 100 bp reads on one lane of a NovaSeq 6000 (Illumina). A mean of 50.5 million reads per sample were sequenced (±9.2 million s.d.) (Table S1). Raw sequencing reads are available at the National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA692568).

Transcriptome assembly and annotation

Raw reads were trimmed with Trimmomatic version 0.36, where reads under 36 bp long were discarded, leading and trailing base pairs were discarded with Phred scores lower than 5, and a sliding window of 4 bp was used where the window was removed if the average read quality fell below 5 (Bolger et al., 2014). Read quality metrics before and after trimming were checked with FastQC version 0.11.8 and multiQC version 1.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; Ewels et al., 2016). Following trimming, a mean of 49.9 million reads per sample were retained (±9.2 million s.d.) (Table S1). Trinity version 2.9 was used to assemble the transcriptome with trimmed reads with default options, followed by BUSCO version 3.0.2 with the ray-finned fish lineage (actinopterygii_odb10) to assess transcriptome completeness (Grabherr et al., 2011; Seppey et al., 2019). Trinotate version 3.2.0 was used for transcriptome annotation following software guidelines, except RNAMMER was not used with these data (https://github.com/Trinotate/Trinotate.github.io/wiki/Software-installation-and-data-required) (Bryant et al., 2017). In short, the NCBI blastx and blastp databases were used to search transcripts and predicted proteins, respectively, HMMER version 3.3 was used to identify protein families, signalP version 4.1 was used to identify signal peptides, and tmhmm version 2 was used to identify transmembrane helices (Altschul et al., 1990; Krogh et al., 2001; Petersen et al., 2011; Wheeler and Eddy, 2013). All results were collected in Trinotate for transcriptome annotations. Following Pearson (2013), annotations were filtered for those with E-values $<1\times10^{-6}$ and bit scores >50.

The SuperTranscripts pipeline was used because of its potential for describing differential exon usage; SuperTranscript results were thus also used for gene expression (Davidson et al., 2017). Here, Salmon version 1.1.0 with the –dumpEq option was used for initial transcript quantification against the reference transcriptome (Patro et al., 2017). Equivalence classes from Salmon were used in Corset version 1.07 to generate super-clusters with five nucleotides minimum required to overlap between transcripts, and super-clusters were discarded if over 1000 contigs aligned to them (Davidson and Oshlack, 2014). A linear representation of the transcriptome was generated with the Corset outputs and the Trinity transcriptome using Lace version 1.14.1 (https://github.com/Oshlack/Lace).

Differential gene expression

Corset counts for super-clusters were used for differential gene expression (DGE) using edgeR version 3.30.3 (Robinson et al., 2010). Although the counts from Corset super-clusters reflect gene transcription (Buccitelli and Selbach, 2020; Jeffries et al., 2021), the resulting statistical tests are referred to here as DGE in a manner consistent with similar literature (e.g. Conesa et al., 2016). Count data were first filtered for genes showing any expression. Then, a genewise negative binomial generalized linear model with quasi-likelihood test (glmQLFit) was used after data normalization and robust dispersion estimation. The design formula for the model included experimental group (wild, handled, thermally stressed) and RIN to explicitly model differences in RNA quality between samples (Table S1) (Gallego Romero et al., 2014). Pairwise comparisons were drawn between each experimental treatment using genewise negative binomial generalized linear models with quasi-likelihood tests (glmQLFtest). Only clusters significant at a Benjamini–Hochberg adjusted false discovery rate (q) <0.05 were retained for downstream analyses (Benjamini and Hochberg, 1995). In addition, clusters with higher or lower expression in the thermal stress treatment compared with both the wild and handled treatments (i.e. $|\log_2\text{-fold change}| >0$ compared with both wild and handled) were retained as exhibiting 'thermal stress-specific' expression. Multidimensional scaling as implemented in edgeR and a heatmap were used to visualize broad patterns of differential gene expression among all clusters and those specific to thermal stress, respectively.

To find summary gene ontology (GO) terms represented by differentially expressed and spliced super-clusters, we used the EnrichR version 2.1 databases Biological Process 2018, Molecular Function 2018 and Cellular Component 2018 (Kuleshov et al., 2016). GO terms were analyzed in pairwise comparisons between each experimental treatment in both the DGE and differential exon usage (DEU) results. Because we were interested in patterns of splicing and expression with respect to thermal tolerance, results unique to the thermal stress experimental treatment were given special attention. For DGE, statistically significant clusters (q<0.05) that were either upregulated ($\log_2$-fold changes >0) or downregulated ($\log_2$-fold changes <0) in the thermally stressed treatment with respect to both the handled control and wild group were retained for these thermal stress-specific results, in addition to overall thermal stress-specific genes ($|\log_2\text{-fold change}| >0$). Gene set enrichment analysis was conducted with overall thermal stress-specific genes, because the upregulation of certain genes may downregulate given pathways, and downregulation of other genes may upregulate pathways (Reynolds et al., 2013). For visualization, non-redundant GO terms for genes that showed thermal stress-specific expression were explored with Revigo, where significant GO terms and adjusted P-values were used with an allowed semantic similarity of 0.7, and terms were searched against the whole UniProt database (Supek et al., 2011).

Early response genes

To investigate the possibility that thermally stressed or handled fish exhibited gene expression changes indicative of an acute stress response, several early response genes were explored in the DGE data. These were clusters annotated to the genes transcription factor Jun/AP-1 (*jun*), transcription factor jun-B (*jun-B*), transcription factor jun-D (*jun-D*), immediate early response gene 2 (*ier2*), myc proto-oncogene (*myc*), proto-oncogene c-Fos (*c-Fos*) and

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metallothiol transferase FosB (*fosB*). The panel of early response genes represents a positive control of genes we expected would change in expression if the thermal and handling stressors were reflected in a transcriptomic response (Bahrami and Drabløs, 2016; Fowler et al., 2011; Jeffries et al., 2018; Sopinka et al., 2016). Therefore, if differential expression was observed in these genes in both the handling and CT_{max} groups compared with the wild group, then genes that show differential expression between the handling and CT_{max} groups are likely specific to the thermal stress response.

Differential exon usage

Differential exon usage (DEU), or the relative usage of exons within genes, was estimated using STAR version 2.7.3a to create splice junction files for each individual (SJ.out.tab), which were concatenated into one splice junction file (Dobin et al., 2013). STAR was run in two-pass mode with all reads mapped on the first pass. The Mobius.py script in Lace version 1.14.1 was used to create a .gtf file from the Lace-clustered transcriptome and the splice junction file from STAR. Then, the featureCounts function in Subread version 2.01 was used with fractional counts (–fraction), where input files were the new splice junction-specific .gtf file, the super-clusters count file from Corset and the aligned .bam files from STAR to generate exon counts (Liao et al., 2013). DEU was tested for with DEXseq version 1.34.1 (Anders et al., 2012). As with tests for DGE, RIN scores were used but with a centered and scaled mean around 0 for generalized linear model convergence. The design formula for DEU included the individual fish, scaled RIN, exon expression and experimental treatment in interaction with exon expression. After estimating size factors and dispersions, exon usage coefficients were estimated by being fit to experimental treatments. Only exons with differential expression significant at a $q<0.05$ were retained for downstream analyses. Similar to DGE analyses, exons with higher or lower expression in the thermal stress treatment compared with both the wild and handled treatments (i.e. $|\log_2\text{-fold change}| >0$ compared with both wild and handled) were retained as exhibiting 'thermal stress-unique' expression. These steps were performed following guidelines in the Lace GitHub repository (https://github.com/Oshlack/Lace/wiki/Example:-Differential-Transcript-Usage-on-a-non-model-organism).

Only GO terms from the Biological Process 2018, Molecular Function 2018 and Cellular Component 2018 databases with $q<0.05$ were retained for further analyses. As with GO terms represented by DGE, Revigo was used to explore non-redundant GO terms for exons that showed thermal stress-specific expression (Supek et al., 2011).

Gene expression–splicing interactions

To explicitly evaluate our hypothesis that alternative splicing is an important mechanism used by redside dace responding to a thermal challenge, we focused on splicing factors uniquely upregulated (DGE $\log_2$-fold changes >0 when compared with both other groups) in fish in the thermal stress treatment. Using the STRING version 11.0 database (Szklarczyk et al., 2019), we analyzed genes in molecular pathways with the splicing factors identified previously using the *Danio rerio* database. Here, genes with significant DEU were identified as possibly important for the thermal stress response.

RESULTS

Transcriptome assembly and annotation

Trinity assembled unaligned reads into a transcriptome of 714,933 unique transcripts in 429,016 unique genes with a BUSCO score for transcriptome completeness of 89.8%. Of these putative transcripts and genes, 155,547 transcripts representing 59,755 genes were annotated using Trinotate and associated programs after filtering for E-values $<1\times10^{-6}$ and bit scores >50. Corset clustered transcripts from Trinity into 83,217 super-clusters representing 143,841 clusters.

Differential gene expression

Of the 143,841 clusters from Corset irrespective of available annotations, count data were observed for 46,140 clusters in any single individual. Between the thermal stress group and handled control, 1531 clusters showed significant DGE, 786 with relatively higher expression in thermal stress and 745 with relatively lower expression in thermal stress compared with the handled control (Table 1). Between the thermal stress and wild groups, 6770 clusters showed significant DGE, 3992 with relatively higher expression in thermal stress and 2778 with lower expression in thermal stress compared with the wild group. For clusters with expression unique to thermal stress, 579 showed higher expression compared with the two other groups, while 559 showed lower expression compared with the two other groups (Fig. 2). Multidimensional scaling with all clusters and a heatmap of counts per million for each of 1138 clusters showing significant DGE unique to the thermal stress treatment (579 positive, 559 negative; $q<0.05$) revealed a gradient in expression response from the wild group to the handled control, and the thermal stress group (Fig. 2). Although the fish 'wild 3' was an outlier in mRNA abundance profile and had the lowest RIN score out of all individuals of 7.1 (Fig. 2; Table S1), its removal from DGE

Table 1. Summary table of pairwise results for differential gene expression among three experimental treatments

	Wild versus Handled	Wild versus CT_{max}	CT_{max} versus Handled
Number of significant DGE clusters overall	2362	6770	1531
Number of significant positive DGE clusters	578	2778	786
Number of significant negative DGE clusters	1784	3992	745
Positive DGE genes	263	1478	328
Negative DGE genes	670	1682	218
Positive DGE Biological Process GO terms	0	98	27
Positive DGE Molecular Component GO terms	0	5	12
Positive DGE Cellular Component GO terms	0	40	0
Negative DGE Biological Process GO terms	60	211	0
Negative DGE Molecular Component GO terms	32	43	0
Negative DGE Cellular Component GO terms	1	1	0

Clusters (~transcripts) were identified and quantified with Corset, and differential gene expression (DGE) was analyzed with edgeR. EnrichR was used to summarize annotated clusters under different pairwise comparisons into gene ontology (GO) terms, among three databases: Biological Process 2018, Molecular Function 2018 and Cellular Component 2018. Counts of clusters associated with known genes are reported as genes. Positive and negative expression for clusters and GO terms are relative to the pairwise comparison used; positive expression represents clusters higher in the first treatment of a comparison, whereas negative expression represents clusters higher in the second treatment of a comparison. The thermal stress treatment is abbreviated as CT_{max}. A total of N=30 individuals were used for this experiment (n=10 per treatment).

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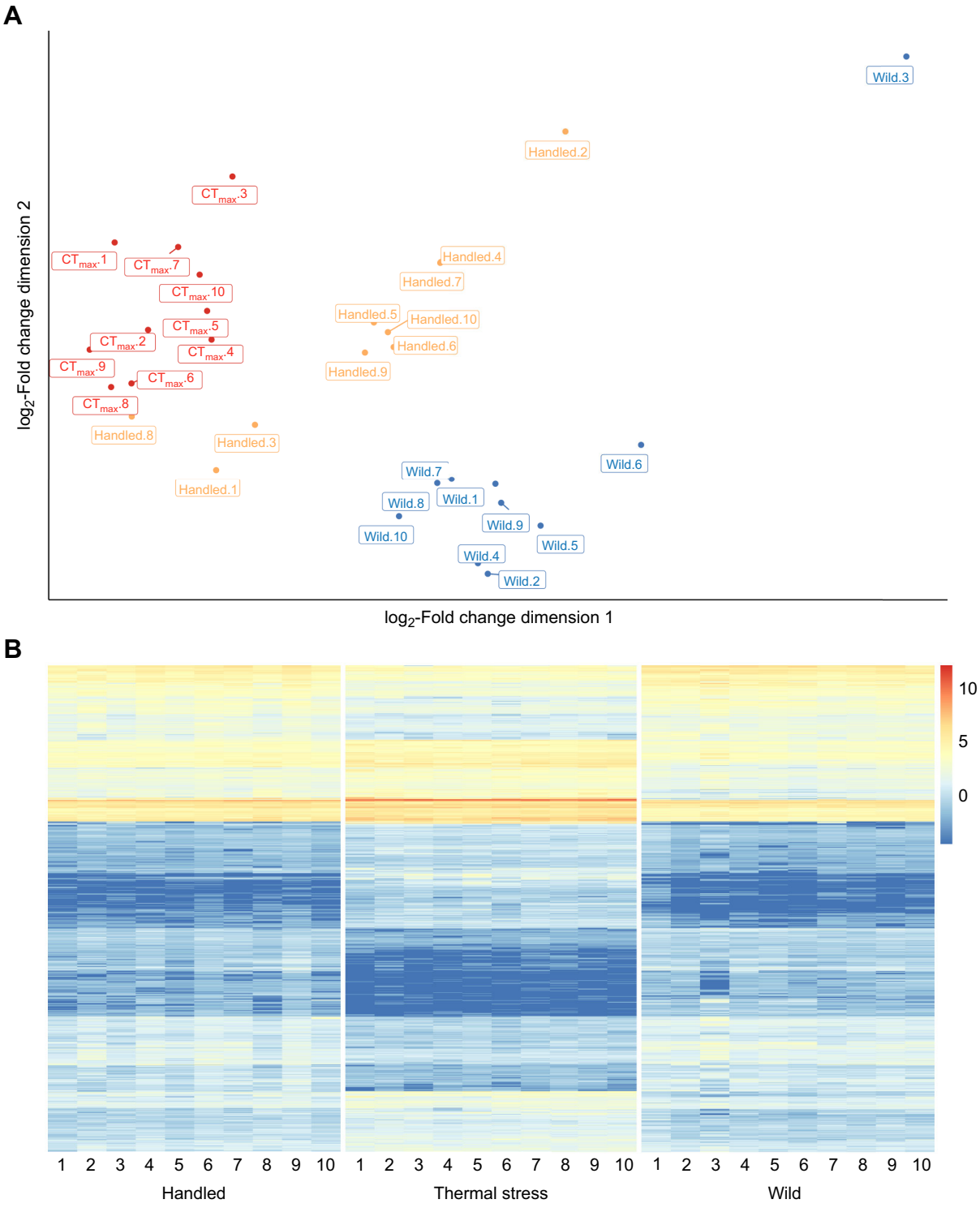

Fig. 2. Differential gene expression in response to thermal stress. (A) Visualization of cluster expression as implemented by a multidimensional scaling (MDS) plot using edgeR, where distances between plots are approximations of $\log_2$-fold changes between samples. Input data are cluster (~transcript) expression counts filtered for any expression among any of the *N*=30 individuals in the experiment. Individual labels are composed of the experimental treatment or control [thermally stressed (abbreviated as CT_{max}), handled or wild, *n*=10 each] and the individual's identifying number. (B) Heatmap of counts per million for each of 1138 clusters showing significant differential gene expression unique to the thermal stress treatment (579 positive, 559 negative; $q<0.05$). Each cluster included in this plot either shows higher expression in the thermal stress treatment compared with both the wild and handled controls, or lower expression in the thermal stress treatment compared with both controls. Individuals are grouped by experimental treatment, and numbers identifying individuals within each treatment are on the *x*-axis.

analyses did not qualitatively affect downstream results. Rather than introduce bias by removing this outlier individual, it was retained for all analyses.

Among annotated clusters showing significant DGE, 328 genes were identified as showing relatively higher expression in thermal stress compared with the handled control, and 218 genes lower for

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the thermal stress group (Table 1). Between the thermal stress treatment and handled control, 39 GO terms were identified from genes showing higher expression for thermal stress (no GO terms were found for genes with higher expression in the handled control). Between the thermal stress and wild groups of fish, 1682 genes were higher for thermal stress (1478 higher for wild group) (Table 1). Between the thermal stress treatment and wild group, 256 GO terms were identified from genes showing higher expression in thermal stress, while 143 genes had higher expression in wild fish.

For genes that showed thermal stress-specific expression (i.e. $|\log_2\text{-fold changes}| > 0$ compared with both other groups for thermal stress-specific expression, respectively), 579 were identified as showing higher expression in thermal stress compared with both controls (216 annotated clusters), and 559 showed lower expression in thermal stress compared with both controls (103 annotated clusters). For GO terms related to genes specific to thermal stress, 32 GO terms were identified among genes with positive expression (21 Biological Process terms, 11 Molecular Function terms), whereas no GO terms were identified for genes with negative expression (Fig. S1). Using Revigo with the thermal stress-specific GO terms, 12 Biological Process GO terms and eight Molecular Function GO terms were retained for visualization (Fig. S1). With the 1138 total clusters identified as unique to thermal stress (775 annotated), we identified 37 Biological Process terms, 30 Molecular Function terms and one Cellular Component GO term. With Revigo, 25 Biological Process terms, 21 Molecular Function terms and one Cellular Component non-redundant GO term were retained for visualization (Fig. 3). The GO terms regulation of transcription, DNA-templated (GO:0006355), RNA binding (GO:0003723) and RNA polymerase II transcription regulator complex (GO:0090575) were the terms with the greatest number of clusters in each of the enrichment databases searched (Kuleshov et al., 2016). Also prominent were terms related to unfolded proteins and protein turnover, such as response to unfolded protein (GO:0006986), regulation of protein ubiquitination (GO:0031396), chaperone cofactor-dependent protein refolding (GO:0051085) and ubiquitin protein ligase binding (GO:0031625).

Early response genes

Each of *jun*, *jun-B*, *jun-D*, *ier2*, *myc*, *c-Fos* and *fosB* showed higher expression in the thermal stress treatment than in the wild group, while only *jun* showed higher expression in the thermal stress treatment compared with the handled control (Fig. 4).

Differential exon usage

Among 143,841 clusters in the data, 31,042 had detectable exons and 4943 of these clusters (~16%) had at least one exon that showed significant DEU between any two experimental treatments

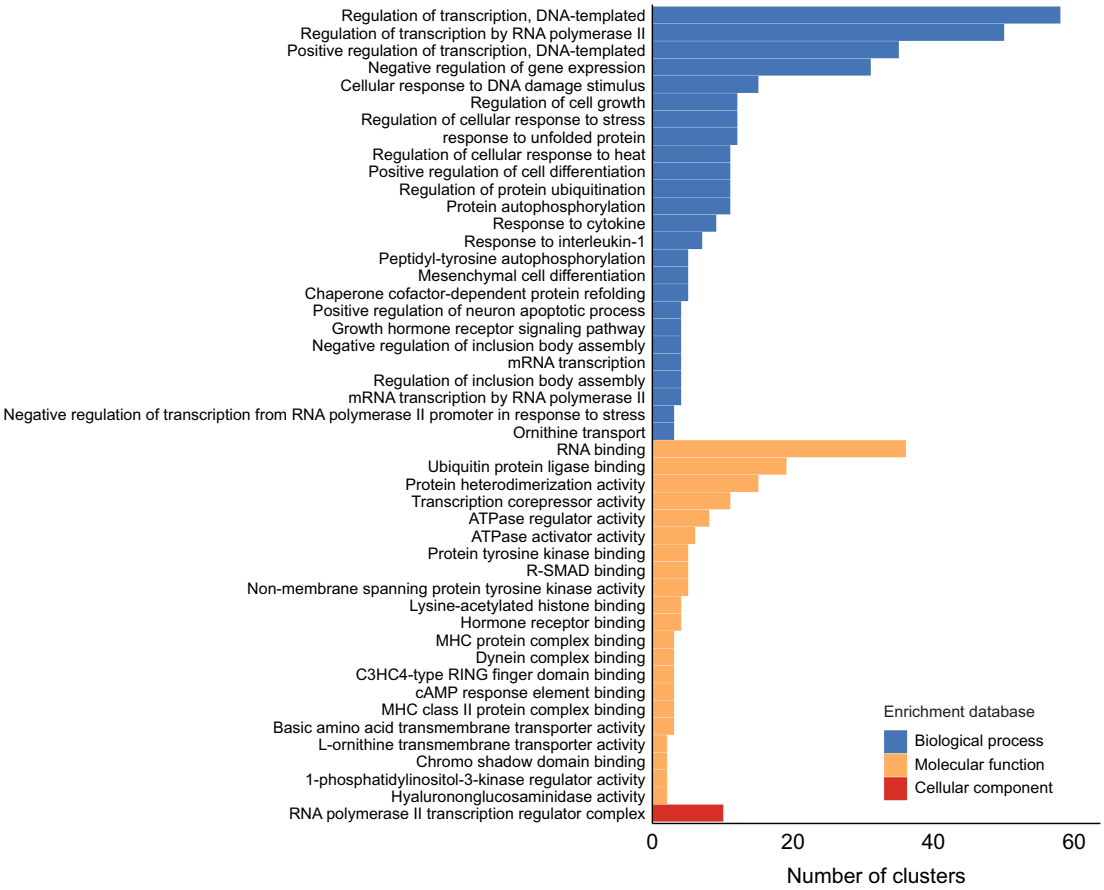

Fig. 3. Non-redundant gene ontology (GO) terms representing clusters (~transcripts) that showed differential expression ($|\log_2$-fold change$|>0$) in the thermal stress treatment compared with both the handled and wild groups. Clusters were first identified as showing differential expression with edgeR, then these GO terms were called using a list of annotated genes input into enrichR. Non-redundant terms were identified with Revigo and visualized here. All terms are significant at a $q<0.05$. Enrichment databases searched were the Biological Process 2018 (blue), Molecular Function 2018 (yellow) and Cellular Component 2018 (red). Number of clusters represents the number of genes annotated to clusters summarized within GO terms. A total of $N=30$ individuals were used for this experiment ($n=10$ per treatment).

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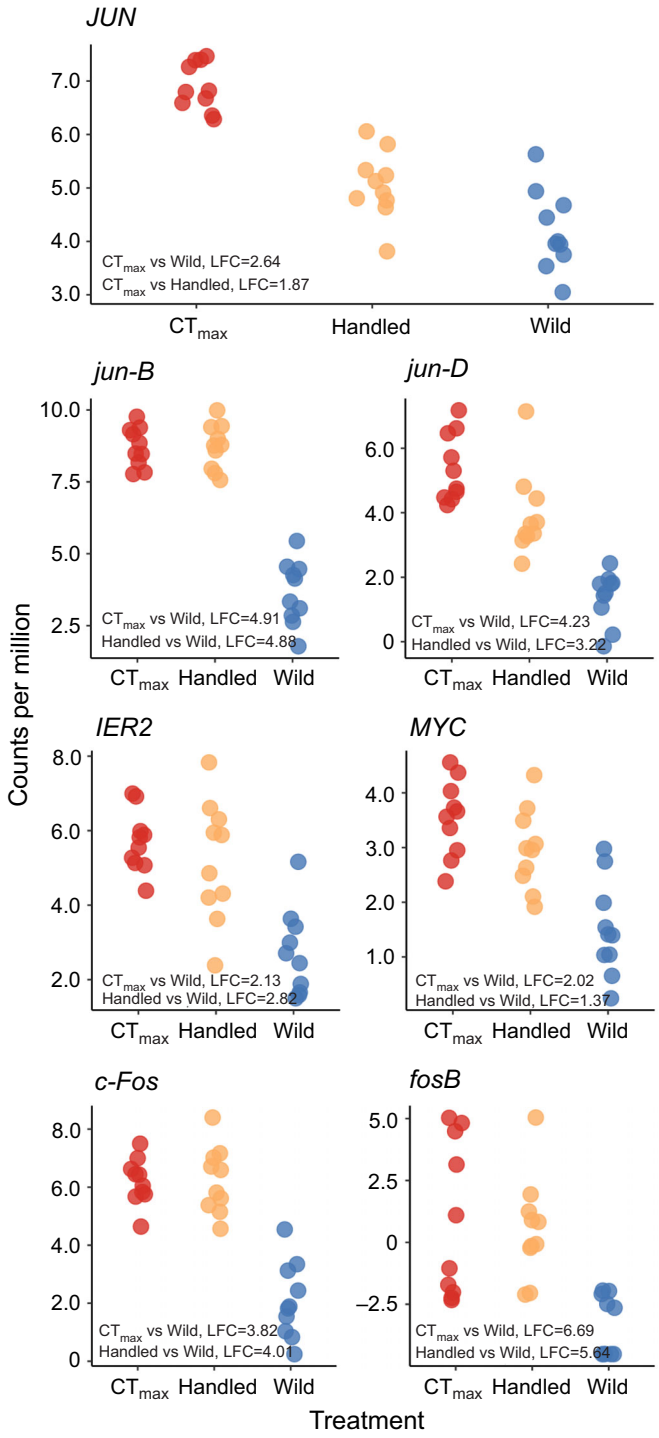

Fig. 4. Expression of seven early response genes that are generally associated with an acute stress response. Log_2-fold changes (LFCs) are provided for significant (q<0.05) comparisons within each plot; non-significant comparisons are not shown. Individual points represent individual fish within each experimental treatment. The thermal stress treatment is abbreviated as CT_{max}. A total of N=30 individuals were used for this experiment (n=10 per treatment). *JUN* is associated with *transcription factor AP-1*, *jun-B* is transcription factor *jun-B*, *jun-D* is transcription factor *jun-D*, *IER2* is *immediate early response gene 2*, *MYC* is *proto-oncogene (myc)*, *c-Fos* is *proto-oncogene c-Fos*, and *fosB* is *metallothiol transferase FosB*.

(Table 2). These clusters with significant DEU were composed of 284,631 exons total, of which 10,314 exons showed significant DEU between any two experimental treatments. In the thermal stress experimental treatment with respect to both the handled and wild treatments, 88,031 exons in 3230 clusters had higher expression (exon base mean±s.d. 34.6±132.21 counts across samples in each exon normalized by sequencing depth), while 76,307 exons in 2530 clusters had lower expression (exon base mean 70.84±208.98 counts).

Exons that showed higher expression in the thermal stress treatment compared with both controls were represented by 1688 annotated genes, summarized in 72 GO terms (46 Biological Process, 13 Molecular Function and 12 Cellular Component GO terms). Using Revigo with annotated clusters containing exons showing higher thermal stress-specific expression, 25 Biological Process GO terms, nine Molecular Function GO terms and 11 Cellular Component GO terms were retained for visualization (Fig. S2). For exons with lower expression in the thermal stress compared with both controls, 1170 genes were annotated, summarized by six GO terms (one Biological Process and five Molecular Function GO terms) (Fig. S2). Using Revigo, all six GO terms representing exons with lower expression in thermal stress were retained for visualization (Fig. S2). Among GO terms represented for overall thermal stress-specific differential exon usage (Fig. 5), gene expression (GO:0010467), transcription from RNA polymerase II promoter (GO:0006366) and transcription, DNA templated (GO:0006351) were present. Terms directly related to metabolism were: rRNA metabolic process (GO:0016072), RNA metabolic process (GO:0016070), regulation of primary metabolic process (GO:0080090), carboxylic acid metabolic process (GO:0019752) and creatine metabolic process (GO:0006600). Mitochondria-related enrichment terms of mitochondrion organization (GO:0007005), mitochondrion (GO:0005739) and mitochondrial inner membrane (GO:0005743) were also prominent.

Gene expression–splicing dynamics

One splicing factor, *pre-mRNA-splicing factor 38B* (*prpf38b*), was upregulated in the thermal stress treatment compared with both the wild and handled groups (0.58 log_2-fold change compared with wild, 0.70 log_2-fold change compared with handled) (Fig. S3). The gene *prpf38b* is associated with several genes that showed DEU between treatments: *splicing regulatory glutamine/lysine-rich protein 1* (*srek1*), *regulator of chromosome condensation* (*rcc1*), *pinin* (*pnn*), *RNA-binding protein 25* (*rbm25*) and *RNA-binding protein 39* (*rbm39*) (Fig. 6; Fig. S3). The last gene, *rbm39*, is a transcriptional coactivator of *transcription factor AP-1* (*jun*), which showed higher expression in the thermal stress treatment compared with both other treatments (2.64 log_2-fold change compared with wild, 1.87 log_2-fold change compared with handled) (Fig. 4).

DISCUSSION

Our data show that alternative splicing and gene expression may be complementary and interacting mechanisms used to mount a cellular response to thermal stress. We identified several hundred differentially transcribed genes unique to thermal stress, and these presumably represent the molecular mechanisms that redside dace use to respond to acute thermal stress. We also identified alternative splicing-based responses to thermal stress that may provide a complementary mechanism for an acute thermal stress response. Consistent with the hypothesis that DGE influences DEU, we identified differentially transcribed splicing factors unique to thermal stress. A total of 1138 clusters (~transcripts) showed significant DGE unique to the thermal stress treatment (579 positive, 559 negative). Moreover, 88,031 exons in 3230 clusters

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Table 2. Summary table of pairwise results for differential exon usage among three experimental treatments, with a focus on exons unique to the CT_{max} treatment

	Overall DEU	Positive CT_{max} DEU	Negative CT_{max} DEU
Total number of clusters	4943	3230	2530
Number of exons	10,314	88,031	76,307
Number of genes	3136	2125	1471
Number of Biological Process GO terms	56	46	1
Number of Molecular Function GO terms	5	13	5
Number of Cellular Component GO terms	23	12	0

Clusters (~transcripts) were identified and quantified with Corset, and differential exon usage (DEU) was analyzed with DEXSeq. DEXSeq was used to summarize annotated clusters under different pairwise comparisons into gene ontology (GO) terms, among three databases: Biological Process 2018, Molecular Function 2018 and Cellular Component 2018. Counts of clusters associated with known genes are reported as genes. Positive and negative expression for clusters and GO terms are with respect to both controls; positive CT_{max} DEU represents clusters with exons showing higher expression in the CT_{max} treatment compared with both controls, while negative CT_{max} DEU represents exons showing lower expression in the CT_{max} treatment compared with both controls. A total of N=30 individuals were used for this experiment (n=10 per treatment).

had higher expression in the thermal stress treatment compared with both other groups, while 76,307 exons in 2530 clusters had lower expression. One splicing factor (*prpf38b*) that was upregulated in the thermal stress-challenged fish, and its increased expression was concurrent with DEU in downstream genes, representing a possible stress response pathway that incorporates both alternative splicing and gene expression.

Gene expression

By comparing thermal stress-challenged redside dace with handled and wild groups, we were able to identify gene expression unique to thermal stress. An observed gradient of expression responses was consistent with the handled control representing an intermediate, general stress response between the thermal stress and wild fish groups. Meanwhile, the thermal stress treatment represented a

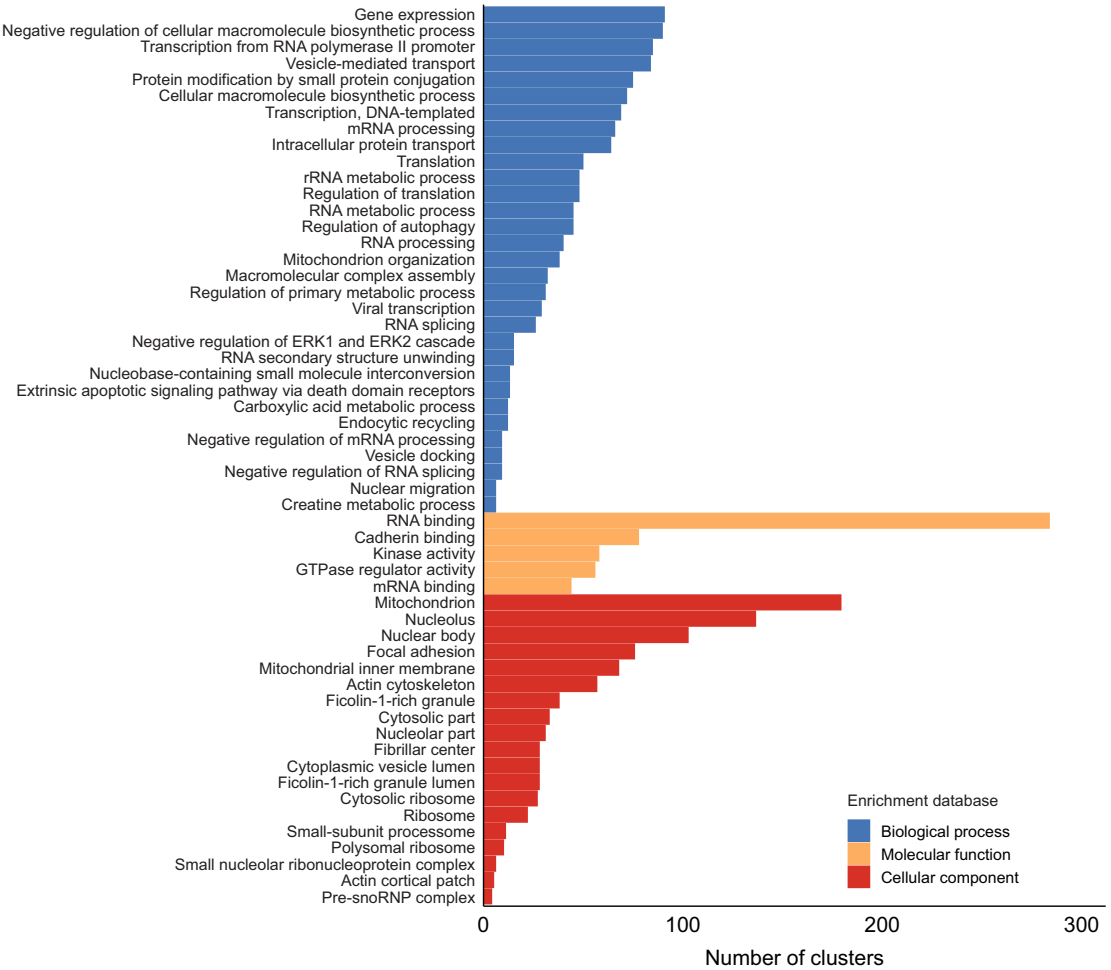

Fig. 5. Non-redundant GO terms representing exons in clusters (~transcripts) that showed differential exon usage (|log_2-fold change|>0) in the thermal stress treatment compared with both the handled and wild groups. Clusters were first identified as showing differential exon usage with DEXSeq, then these GO terms were called using a list of annotated genes input into enrichR. Non-redundant terms were identified with Revigo and visualized here. All terms are significant at a q<0.05. Enrichment databases searched were the Biological Process 2018 (blue), Molecular Function 2018 (yellow) and Cellular Component 2018 (red). Number of clusters represents the number of genes annotated to clusters summarized within GO terms. A total of N=30 individuals were used for this experiment (n=10 per treatment).

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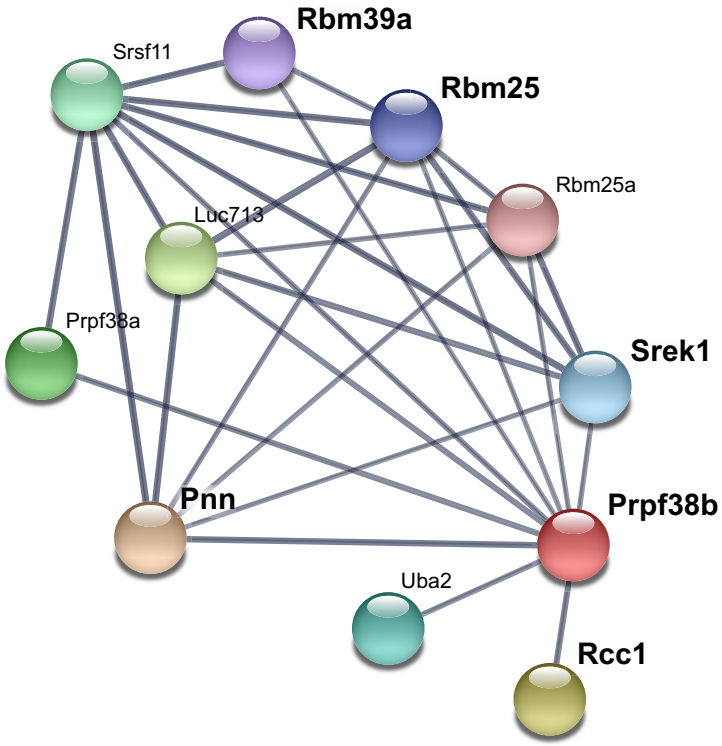

Fig. 6. Predicted associations for Pfpf38b using the String v11.0 database. The width of lines between proteins represents confidence in the interaction, and only proteins of high confidence (>0.700) are included in this figure. The red node, Prpf38b, is the query protein against the *Danio rerio* database. In bold are proteins associated with Prpf38b that showed evidence of alternative splicing via differential exon usage. A total of *N*=30 individuals were used for this experiment (*n*=10 per treatment).

combined thermal and handling stress response, while the wild treatment represented a transcriptional state as close to baseline as was feasible for an experiment conducted in the field.

As a positive control, we used a set of seven early response genes (*jun*, *jun-B*, *jun-D*, *ier2*, *myc*, *c-Fos* and *fosB*) that would be expected to show a stress response to verify that whole-organism acute stress was reflected in transcriptomic responses. This panel of genes was more highly expressed in the thermal stress treatment relative to the wild group. None of the genes in this panel except *jun* showed differential expression between fish in the thermal stress treatment and handled control, indicating their role in a general stress response associated with the experimental treatments as opposed to a temperature-specific stress response. Nevertheless, their higher expression in thermal stress compared with the wild group (in addition to handled compared with wild) confirms that a stress response associated with handling, transport and confinement was reflected in gene expression. Because this panel of genes establishes that thermal and handling stressors were reflected in the transcriptomic response, thermal stress-specific genes likely represent a temperature-specific stress response when compared with both other groups.

Transcription regulation was prominent among genes differentially expressed in the thermal stress fish compared with both other groups, indicating that these genes likely play a role in coping with an acute thermal challenge. While the rate-limiting step for protein synthesis is often the initiation of translation (Sonenberg and Hinnebusch, 2009; Spriggs et al., 2010), transcription regulation is another key element of the stress response (De Nadal et al., 2011). An accumulation of unfolded proteins is thought to induce a heat shock protein response (reviewed in Richter et al., 2010), and the observed enrichment terms response to unfolded protein (GO:0006986), regulation of protein ubiquitination (GO:0031396), chaperone cofactor-dependent protein refolding (GO:0051085) and ubiquitin protein ligase binding (GO:0031625) were consistent with this model. Therefore, the redside dace challenged by an acute thermal stressor exhibited a 'classic' acute heat shock response as demonstrated by the multiple enrichment terms consistent with acute stress responses in the literature.

One concern with CT_{max} methodology is that it is based on rapid warming, which may not induce the same molecular responses that slower warming would in wild fish (Åsheim et al., 2020). However, in zebrafish (*D. rerio*) slow warming was found to share underlying physiological mechanisms with rapid warming, evidence that CT_{max} induces molecular responses with consistencies across short and ecologically relevant longer time scales (Åsheim et al., 2020). With a foundation in the conserved heat shock response among eukaryotes (Richter et al., 2010), consistency between slow and rapid warming responses in fish (Åsheim et al., 2020), and the empirical data presented in this study, the thermal stress-specific genes identified here are one mechanism of the transcriptomic response to acute thermal stress in the redside dace.

Alternative splicing

Given the broad importance of splicing in fishes and other organisms (Chaudhary et al., 2019; De Nadal et al., 2011; Healy and Schulte, 2019; Kornblihtt et al., 2013; Laloum et al., 2018; Li et al., 2020; Salisbury et al., 2021; Tan et al., 2019; Thorstensen et al., 2021; Xia et al., 2018; Zhang et al., 2019), we hypothesized that alternative splicing is an important component of the transcriptome response to thermal stress in redside dace. Therefore, we analyzed alternative splicing (measured by differential exon usage) for its possible roles in the acute stress response and interactions with gene expression. Although splicing may expand the proteome far beyond the number of genes otherwise present in eukaryotic genomes (Nilsen and Graveley, 2010), an important caveat is that alternative splicing does not necessarily change protein diversity in a cell (Chaudhary et al., 2019; Tress et al., 2017). The functional effects of splicing patterns identified with mRNA sequencing, as in the present study, cannot be directly inferred. Instead, we identified enrichment terms in spliced genes at the transcriptome level.

Regulation of gene expression was a prominent function among enrichment terms identified in genes showing alternative splicing in response to thermal stress. These enrichment terms are consistent with the roles of both gene expression regulation in response to stress, such as heat (De Nadal et al., 2011), and splicing in transcription regulation more generally (Smith et al., 1989). Also prominent were metabolism-related enrichment terms among genes showing DEU. Alternative splicing is one mechanism that regulates cellular metabolism, such as by splicing factors being targets of metabolic stress (Biamonti et al., 2018). Energy utilization was found to change in response to warming acclimation in fish, with decreased aerobic scope but increased energy utilization efficiency (Nyboer and Chapman, 2017; Zeng et al., 2010). Therefore, alternative splicing may represent a mechanism underlying energy use responses to environmental changes in redside dace by changing the transcribed mRNA isoforms and, therefore, proteomic diversity (Singh and Ahi, 2022). Consistent with this role of splicing in energy use, several mitochondria enrichment terms were significant among genes responding to thermal stress. Cellular mitochondrial content has been linked to gene expression and splicing variability (Guantes et al., 2015), and nucleus-encoded splicing machinery may splice mtRNA in humans (Herai et al., 2017). Although connections between splicing, metabolism and mitochondria are less well characterized in fishes, these processes may play important roles in the response to increasing temperatures.

Gene expression and alternative splicing

One of our main goals was to test the hypothesis that there are direct and interacting links between patterns of alternative splicing and DGE in response to thermal stress. To do this, we carefully searched for splicing factors among the genes that were found to be differentially expressed in thermally stressed fish relative to both other groups. One splicing factor, *prpf38b*, fitted those criteria. Because protein abundance and mRNA levels are often correlated (Buccitelli and Selbach, 2020), and even small differences in pathway intermediates can lead to large changes in pathway flux (e.g. Hochachka and Somero, 2002), the small $\log_2$-fold change values we measured may be biologically important.

The splicing factor *prpf38b* may influence two important genes that are part of the thermal stress response. The gene *rbm39* was associated with *prpf38b* by co-expression in the STRING v11 database (Szklarczyk et al., 2019), and showed DEU in response to thermal stress in our experiment. Furthermore, *rbm39* was differentially expressed in one of two thermally distinct populations of tambaqui (*Colossoma macropomum*) and is thought to play a role in local adaptation to thermal conditions (Fé-Gonçalves et al., 2020). In spotted seabass (*Lateolabrax maculatus*), *rbm39* was identified as a differentially expressed transcript in salt water versus fresh water (Tian et al., 2019), consistent with the DEU identified in the present study. Among other roles, *rbm39* is a transcriptional coactivator for Jun/AP-1 (Jung et al., 2002). This role may be significant for the redside dace thermal stress response because *jun* was more abundant in the thermal stress treatment relative to both other groups. Activation of c-Jun/AP-1 has been implicated in numerous, sometimes opposing, context-dependent cellular stress responses (e.g. both inhibition and activation of apoptotic responses; Leppä and Bohmann, 1999). More broadly, our data linking *prpf38b*, *rbm39* and *jun* illustrate how the interplay between splicing and gene expression may be an essential element of the redside dace thermal stress response.

Beyond *rbm39* and *jun* specifically, *prpf38b* has been linked to the co-expression and direct regulation of numerous other genes (Ouyang et al., 2021). Therefore, although *jun* may be one regulatory element with far-reaching effects for cellular stress responses, *prpf38b* may have effects beyond *jun* as well. As a splicing factor that was uniquely differentially transcribed in the thermally stressed group compared with all other splicing factors, *prpf38b* may be a key connection between the transcriptional mechanisms of DGE and alternative splicing. In the present data, the separate gene expression and splicing analyses present enrichment term results that are presented in isolation. However, large interaction networks among genes indicate that splicing and gene expression rarely operate in isolation (e.g. Ouyang et al., 2021 for *prpf38b*; see also Boyle et al., 2017; Davidson, 2010). Therefore, further connections between the mechanisms likely exist but remain largely unexplored, possibly because of context-specificity in which splicing–gene expression interactions occur. Connections between splicing and gene expression may contribute to whole-organism stress responses, highlighting a need to study these two mechanisms in tandem.

Conclusions

Understanding the mechanisms of thermal tolerance, and how these vary among populations and species, is critical for predicting the effects of environmental change and of conservation breeding, translocation and reintroduction programs. Although these mechanisms are complex and remain poorly understood (Gangloff and Telemeco, 2018), cellular stress responses have deeply conserved elements across all organisms (Horne et al., 2014; Kültz and Somero, 2020). Therefore, elements of the transcriptional response to thermal stress as studied in redside dace here may be applied to understanding transcriptional mechanisms in many fishes. Our data show widespread changes in both gene expression and alternative splicing related to metabolic and mitochondrial processes. Consistent with the present transcriptomic data that implicated a connection between energy use and the thermal stress response, improved nutrition has previously been shown to increase thermal tolerance of redside dace (Turko et al., 2020). Several studies have demonstrated similar patterns of nutrition and thermal tolerance in other species (Hardison et al., 2021; Lee et al., 2016; Robinson et al., 2008). We therefore speculate that the energetic status of fishes may be an important factor that determines their ability to cope with thermal stress.

There is widespread interest in understanding patterns of inter-individual and inter-population differences for many imperilled economically and ecologically important species. For example, genetically distinct redside dace populations are known to vary in both thermal tolerance and scope for thermal acclimation (Turko et al., 2021), but the mechanisms underlying these differences are unknown. The present study represents the first investigation of the redside dace transcriptome, and lays the groundwork for future inter-population studies in the redside dace and other imperilled species. Following an acute thermal stress, gene expression revealed a ‘classic’ heat shock response, while alternative splicing revealed the potential underpinnings of changes in transcriptional regulation and cellular metabolism. Moreover, one splicing factor (*prpf38b*) was found to be uniquely upregulated in the thermally stressed group compared with both others here, which itself has been associated with elements of the cellular stress response (via *jun*). Alternative splicing and gene expression may thus operate in tandem in the transcriptional response to thermal stress. Therefore, the responses identified here may be among many context-dependent, biologically important interactions between alternative splicing and gene expression.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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Data availability
All scripts used for bioinformatics and analyses are available on GitHub (https://github.com/BioMatt/redside_dace_RNA). Results from DGE and DEU analyses for both transcripts and GO terms are available at figshare (https://doi.org/10.6084/m9.figshare.19699744.v1). Raw sequencing reads are available from the National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA692568).

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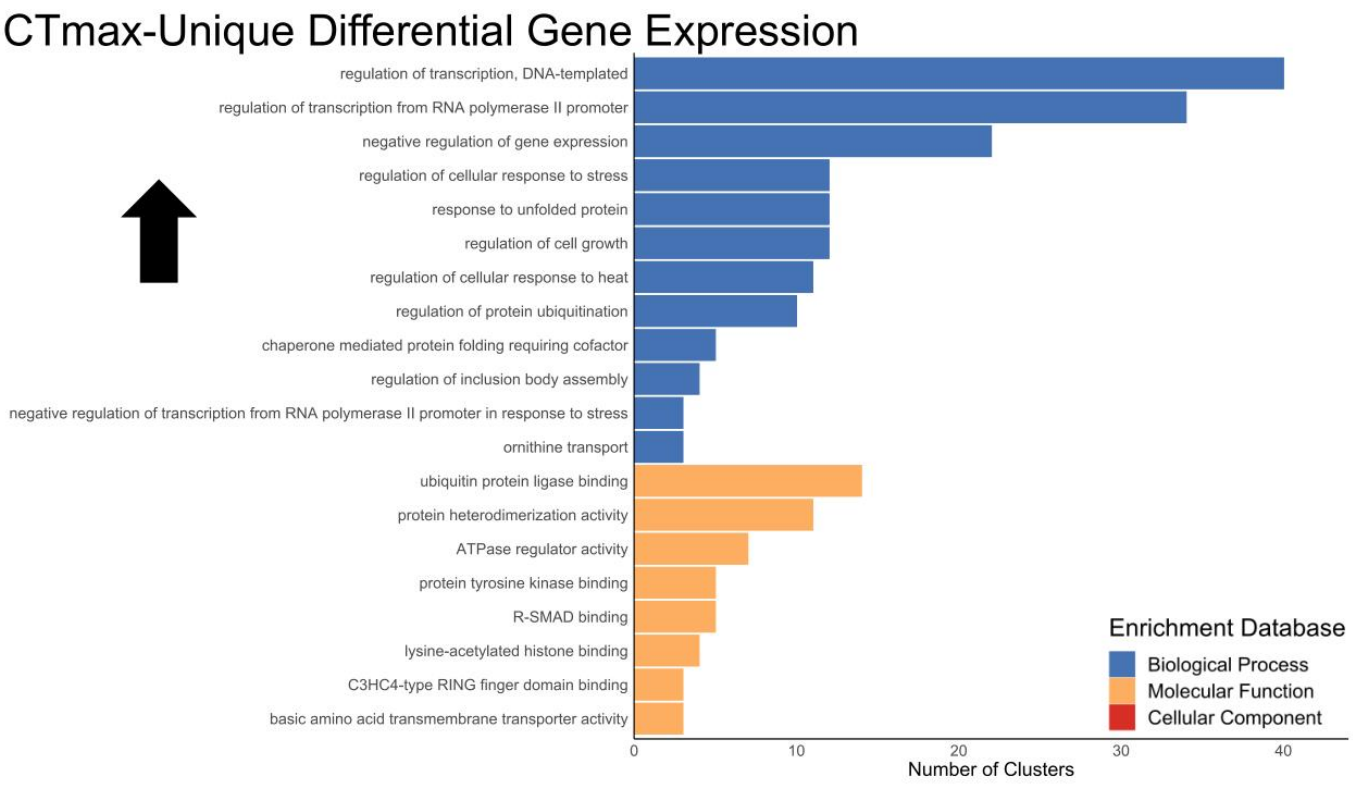

Fig. S1. Non-redundant gene ontology (GO) terms representing clusters (~transcripts) that showed higher expression (log_2-fold change > 0) in the CTmax experimental treatment compared to both the Handle and Wild controls. Clusters were first identified as showing differential expression with edgeR, then these GO terms were called using a list of annotated genes input into enrichR. Non-redundant terms were identified with Revigo and visualized here. All terms are significant at a false discovery rate < 0.05. Enrichment databases searched were the Biological Process 2018 (blue), Molecular Function 2018 (yellow), and Cellular Component 2018 (red). Number of clusters represents the number of genes annotated to clusters summarized within GO terms.

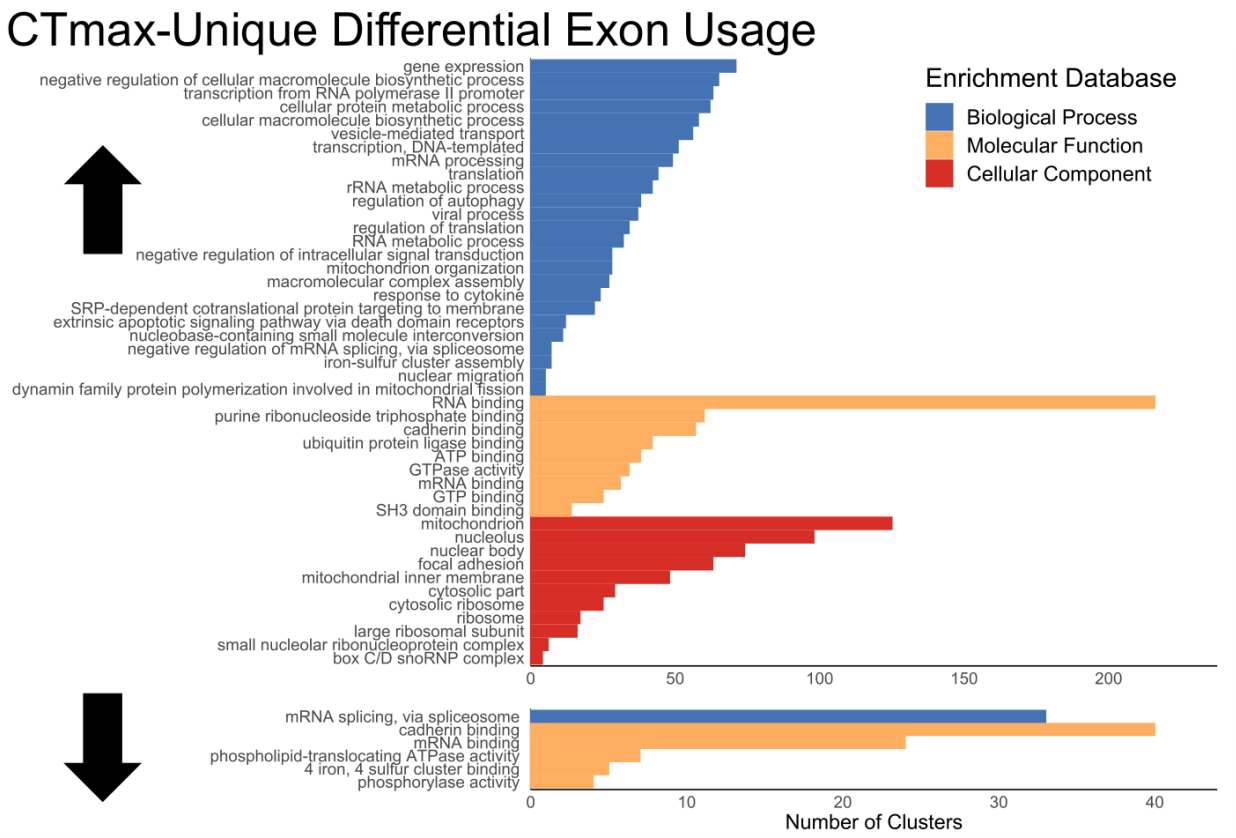

Fig. S2. Non-redundant gene ontology (GO) terms representing exons in clusters (~transcripts) that showed higher (log_2-fold change > 0) or lower (log_2-fold change > 0) expression in the CTmax experimental treatment compared to both the Handle and Wild controls. Clusters were first identified as showing differential exon usage with DEXSeq, then these GO terms were called using a list of annotated genes input into enrichR. Non-redundant terms were identified with Revigo and visualized here. All terms are significant at a false discovery rate < 0.05. Enrichment databases searched were the Biological Process 2018 (blue), Molecular Function 2018 (yellow), and Cellular Component 2018 (red). Number of clusters represents the number of genes annotated to clusters summarized within GO terms. The black arrows represent GO terms containing exons with higher expression in the CTmax treatment (up arrow) or lower expression in the CTmax treatment (down arrow).

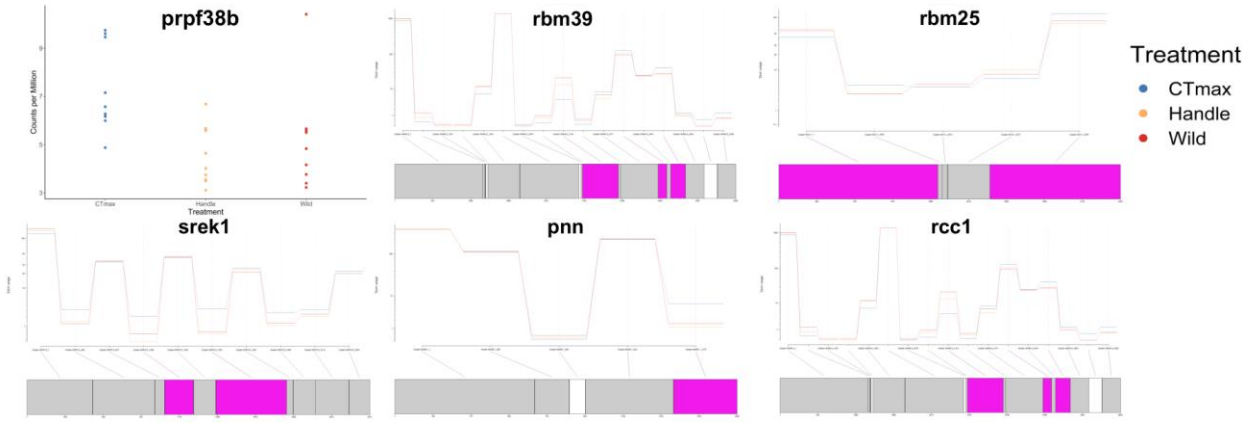

Fig. S3. Counts per million for *pre-mRNA-splicing factor 38B* (*prpf38b*) and differential exon usage for *RNA-binding protein 39* (*rbm39*), *RNA-binding protein 25* (*rbm25*), *splicing regulatory glutamine/lysine-rich protein 1* (*srek1*), *pinin* (*PNN*), and *regulator of chromosome condensation* (*rcc1*). The gene *prpf38b* showed differential expression higher in the CTmax treatment compared to both controls (0.58 $\log_2$-fold change (LFC) higher than Wild, $q = 1.95\text{x}10^{-2}$, 0.70 LFC higher than Handle, $q = 1.71\text{x}10^{-2}$). Each of gene in this plot had exons with differential expression among the three experimental groups, possibly because of regulatory action by *prpf38b* (see Figure 6). Of note is *rbm39*, which acts as a transcriptional coactivator for JUN/AP-1, among other genes (see Figure 4).

Table S1. CTmax, number of raw reads sequenced, number of reads remaining after trimming, RNA integrity scores, mapping rates with Salmon, and uniquely mapped reads with STAR for redside dace (*Clinostomus elongatus*).

Sample Identification	CTmax °C	Number of Raw Reads	Number of Trimmed Reads	RNA Integrity Score	Salmon Mapping Rate %	STAR Uniquely Mapped Reads %
CTmax1	33.1	39,256,958	39,070,816	8.7	97.26	63.29
CTmax2	34.7	48,152,261	47,912,254	8.5	97.36	62.86
CTmax3	34.8	49,820,723	49,463,131	7.7	97.28	60.29
CTmax4	34.8	46,370,991	45,959,602	8.8	97.32	61.97
CTmax5	35.1	54,438,330	53,790,909	9.4	97.51	59.76
CTmax6	33.7	55,036,633	54,559,223	10.0	97.56	60.43
CTmax7	33.7	67,635,235	67,190,887	8.7	97.41	60.70
CTmax8	33.9	53,400,964	53,020,828	9.3	97.47	60.90
CTmax9	34	35,217,633	34,931,096	9.6	97.49	63.79
CTmax10	34.2	55,010,043	54,531,683	8.8	97.41	61.50
Handle1	-	47,249,235	47,051,990	9.8	97.70	59.34
Handle2	-	48,555,398	47,486,283	7.5	97.29	62.49
Handle3	-	57,165,012	56,807,704	9.7	97.80	63.06
Handle4	-	41,006,689	39,038,901	7.9	96.86	60.18
Handle5	-	53,097,480	52,142,889	8.6	97.67	59.64
Handle6	-	69,005,168	68,107,886	8.6	97.27	63.17
Handle7	-	38,172,117	37,495,080	8.6	97.38	61.07
Handle8	-	42,283,580	41,881,594	9.4	97.47	62.32
Handle9	-	58,386,510	57,540,607	8.9	97.24	63.39
Handle10	-	50,177,982	49,723,799	9.3	97.55	59.62
Wild1	-	52,089,483	51,605,698	8.8	97.31	59.46
Wild2	-	55,994,211	55,530,501	9.0	97.51	58.45
Wild3	-	40,560,761	38,784,153	7.1	95.81	61.33
Wild4	-	49,197,975	48,774,973	9.1	97.54	60.80
Wild5	-	36,636,257	35,981,121	8.7	97.43	61.01
Wild6	-	56,898,603	56,260,878	8.0	97.47	58.35
Wild7	-	47,397,177	47,112,774	8.9	97.51	62.12
Wild8	-	66,069,975	65,401,726	8.7	97.48	58.53
Wild9	-	36,825,441	36,460,064	9.4	97.57	61.96
Wild10	-	65,275,729	64,667,339	9.4	97.60	59.89