Transcriptomic Insights into the Impact of Cold Stress on Zebrafish (Danio rerio) Larvae: Impaired Biological Pathways and Adaptive Responses

Abstract

Water temperature significantly influences the immune system of teleost, affecting numerous physiological and biochemical activities. Consequently, many studies have been conducted to understand the mechanisms underlying cold stress in fish. However, the impact of temperature on zebrafish larvae remains poorly studied and largely unknown. In this study, zebrafish larvae were exposed to lethal cold stress (10 °C) for 24 hours and then allowed to recover at 28 °C for six hours. Whole larvae were used for RNA extraction, followed by RNA-seq library preparation and sequencing with 150 bp paired-end reads. The analysis identified 416 differentially expressed genes, with the majority (301 genes) being downregulated. Gene ontology enrichment analysis revealed that most upregulated genes are associated with transcription corepressor binding, regulation of circadian rhythm, locomotor rhythm, and entrainment of the circadian clock. In contrast, most of the downregulated genes are involved in peptidase regulator activity, activation of immune responses, stress responses, inflammatory responses, response to bacterium, Toll-like receptor 5 signaling, wound response, and innate immune responses. Furthermore, steroid biosynthesis and metabolic pathways were upregulated under cold stress conditions, while downregulated genes were linked to processes such as Herpes simplex virus 1 infection, arachidonic acid metabolism, and the Toll-like receptor signaling pathway. These findings demonstrate that zebrafish larvae develop cold tolerance mechanisms in response to cold stress.

Keywords: Lethal cold stress, Transcriptomic analysis, Zebrafish, Larvae, Gene expression

1. Introduction

The influence of water temperature is one of the key regulators of most fish, controlling metabolism, growth, development, and behavior [1]. If water temperature exceeds the tolerance level, most fish face deleterious consequences, and low temperatures may kill the fish, as reported in studies [2, 3]. Many studies have reported that species such as tilapia (Oreochromis niloticus) and milkfish (*Chanos chanos*) are vulnerable to low temperatures. Therefore, many scientific studies have been conducted to uncover the mechanisms underlying cold tolerance in teleosts [4, 5]. Multicellular organisms, such as vertebrates, have a defense system known as the immune system to protect themselves from viruses, bacteria, microorganisms, and other non-self-particles [6]. The immune system of vertebrates is a very complex process involving many cells and molecules [7]. The immune response can be divided into two main branches: the innate and adaptive immune responses. Generally, the innate immune response is a quick and less specific defense system against pathogens, while the adaptive immune response is an antibody-mediated, specific, and slower process that may take a few days to weeks to become fully activated [7, 8]. The innate immune system plays a crucial role in host defense in teleosts [9] . External barriers such as the skin, gut, gills, olfactory organs, and mucosal surfaces provide fish with the first line of defense against pathogens [10, 11]. The mucus of teleosts contains various substances, such as proteases, mucins, lysozymes, and natural antibodies, that play a role in defending against bacteria. Pattern recognition receptors (PRRs) are involved in the recognition of pathogen-associated molecular patterns (PAMPs) in the innate immune response. Toll-like receptors (TLRs) belong to the PRR families and are involved in the innate immune response by recognizing conserved molecular patterns [11].

RNA sequencing has emerged as a transformative high-throughput sequencing technology in recent years [12]. It is widely utilized for identifying regulatory elements, such as non-coding RNAs, biomarkers, and for analyzing differential gene expression [13]. RNA sequencing offers valuable insights into key biological features, including regulatory genes, circular RNAs, and alternative splicing. Transcriptome analysis through the Illumina NGS platform provides reliable detection and quantification of active gene transcripts [14]. Most fish, being ectothermic, are highly influenced by temperature during the early stages of development, which can have lasting effects on their phenotype, genotype, and adaptive evolution [15]. Temperature also impacts metabolic activity, phagocytic recruitment, and susceptibility to infectious diseases in teleosts [16]. For instance, temperature significantly affects the percentage of leukocytes in peripheral blood and serum lysozyme activity. A study in zebrafish (Danio rerio) revealed that larvae incubated at low temperatures (24 °C) experience increased oxidative stress and upregulation of hypoxia-related pathways [16] . Additionally, temperature-stressed juvenile sea bass and rainbow trout during early development exhibit reduced cortisol production. Fluctuating water temperatures also affect IgM levels, body weight, and osmolality in sea bass [15].

Zebrafish *(Danio rerio),* a freshwater species native to South Asia, was utilized as the model organism in this study [17]. It is widely recognized as an exceptional experimental model for aquaculture research, providing valuable insights into fish nutrition, growth, and welfare in various farmed fish species. The short life cycle of zebrafish, combined with the availability of its fully sequenced genome, makes it an ideal model for studying the effects of diets at the transcriptomic level [17]. Based on the evidence discussed, it can be concluded that low water temperature has a detrimental impact on teleost fish. Therefore, we hypothesize that water temperature may influence the transcriptome of zebrafish larvae. This study aims to investigate the effects of low-temperature stress on zebrafish larvae at the transcriptomic level.

2. Materials and Methods

2.1: Bioinformatics analysis

I utilized RNA-Seq data from a previous study and conducted a comprehensive bioinformatics analysis using various tools tailored for next-generation sequencing data. First, fastq-dump was employed to convert SRA files into FASTQ format, which contains the sequencing data. Since raw sequencing reads often include adapters and low-quality reads, the FASTQ files underwent a quality check (Figure 1). For this, FastQC, a widely used quality control tool, was applied to assess the sequencing quality.

After quality assessment, Cutadapt was used to remove adapters and filter out low-quality reads. The cleaned and trimmed reads were then aligned to a reference genome obtained from the National Center for Biotechnology Information (NCBI). This alignment served as the foundation for subsequent analyses.

To prepare for alignment, genome indexing was performed using HISAT2, a powerful tool for mapping reads to a reference genome. Once the index files were generated, paired-end alignment was conducted for the RNA-Seq data, matching one forward read, and one reverse read for each of the six samples. After aligning the RNA-Seq reads to the genome, FeatureCounts was used to quantify the number of reads mapped to each feature (e.g., genes) in each sample.

The output of the alignment was in SAM (Sequence Alignment Map) format, which served as input for FeatureCounts to generate gene-level read counts for each sample. The resulting feature count data was combined into a single CSV file using R programming for further analysis. Finally, differential gene expression analysis was performed using DESeq2 and limma, while ggplot2 was utilized to visualize the differentially expressed genes.

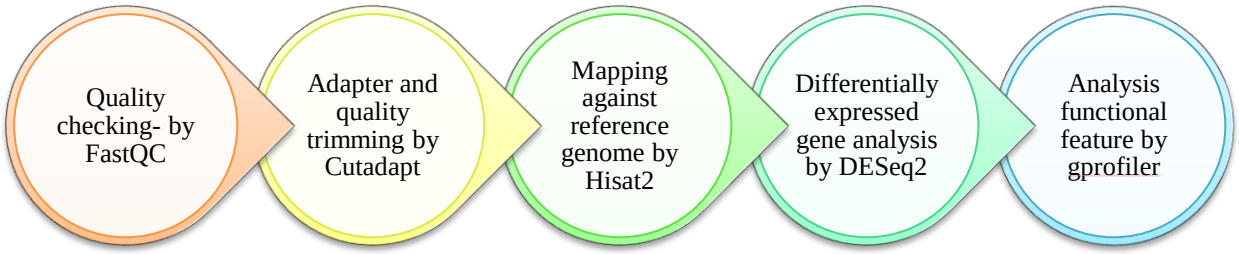


Figure 1: An overview of bioinformatics analysis of mRNA analysis

2.2 Gene ontology and phylogenetic tree analysis

gProfiler was utilized to investigate the functional features (Figure 1) of the top differentially expressed genes. To identify these genes, the dataset was filtered based on the following criteria: genes with a logFC value greater than 1 and a p-value less than 0.05 were categorized as upregulated, while those with a logFC value less than -1 and a p-value less than 0.05 were classified as downregulated. Subsequently, one of the most significantly differentially expressed genes was selected for further analysis, including phylogenetic investigation.

3. Results

3.1 Quality control and trimming of the RNA-seq data

The quality control report of the sequencing data from two different group of the zebrafish kooks fine and no difference were observed before and after trimming. There was no difference between the total GC% before and after trimming.

Figure 2: The figure shows the fastqc report before trimming and read counts before, after trimming and after mapping with reference genome (middle), difference of GC% before and after trimming

3.2 Differentially expressed genes

Multidimensional Scaling (MDS) is a statistical technique used to visualize dissimilarity data by representing samples in a two-dimensional space. An MDS plot highlights the clustering of genes between different groups. For instance, in the MDS plot, the analyzed data shows clear clustering, with control recovery samples positioned on the left and lethal cold stress samples on the right. The volcano plot, a type of scatterplot, displays fold change versus p-value to highlight statistically significant genes with substantial logFC values. In the zebrafish dataset, a total of 20,687 genes were identified, of which 416 were significantly differentially expressed. Among these, 115 genes were upregulated, while 301 were downregulated.

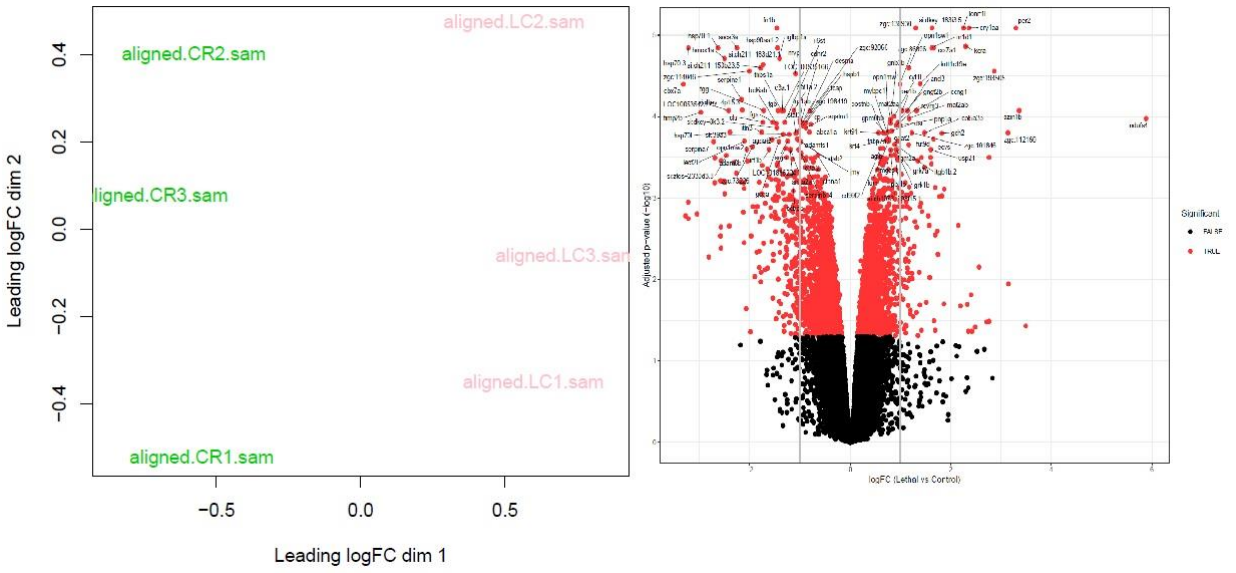


Figure 3: left | Figure shows the clustering the genes between the groups. right| Differentially expressed genes red dots indicated that significantly differential expressed gene and Black dots indicated no significant difference between the groups of genes. The right-hand side (Red dots) whose logFC values are positive they are upregulated gene, other hand red dot in the left side of the graph whose logFC value are negative they are differentially expressed down regulated gene.

3.3. Gene ontology of differentially expressed genes

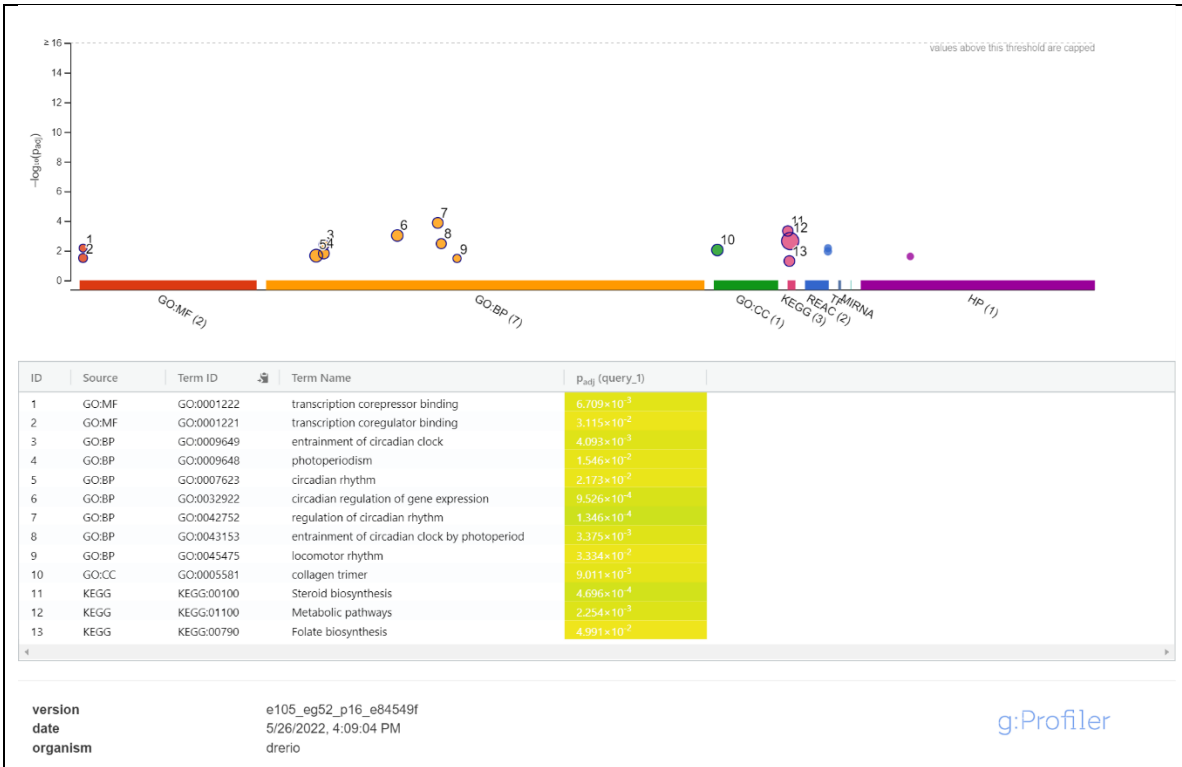
Gprofiler was used to identify the functional features of the differentially expressed genes. Among the 416 differentially expressed genes, the gene ontology analysis revealed several significant biological pathways and processes. For the upregulated genes, the analysis showed that many are involved in metabolic pathways, with 11 genes contributing to this category. Other key processes include transcription coregulator binding, regulation of circadian rhythm, circadian regulation of gene expression, and steroid biosynthesis. In contrast, the downregulated genes were prominently associated with processes such as herpes simplex virus 1 infection, arachidonic acid metabolism, and toll-like receptor signaling pathways. Additionally, 20 genes were linked to responses to external biotic stimuli, 33 to stress response, and 29 to responses to external stimuli. These findings highlight the diverse functional roles of the differentially expressed genes.

Table 1: Table shown the top 5 up regulated genes and their functions

Gene Names/id	Adj.P.Value	Description	Functions
per2	8.86E-06	Period Circadian Regulator 2	Transcription factor binding and transcription coactivator activity
cry1aa	8.86E-06	cryptochrome circadian regulator 1a	Cryptochrome 1 (CRY1) is a transcriptional coregulator associated with the circadian clock.
kera	1.75E-05	Keratocan	Involved in visual perception.
col7a1	1.75E-05	collagen, type VII, alpha 1	It functions as an anchoring fibril between the external epithelia and the underlying stroma
lonrf1l	9.66E-06	LON peptidase N-terminal domain and ring finger 1, like	metal ion binding activity

Table2: Table shown the top 4 up regulated genes and their functions

Gene Names/id	P.Value	Description	Functions
fn1b	8.86E-06	fibronectin 1b	enable integrin binding activity and proteoglycan binding activity
hsp70.3	9.66E-06	heat shock cognate 70-kd protein, tandem duplicate 3	ATP binding activity; ATP hydrolysis activity; and misfolded protein binding activity.
hsp70.1	1.01E-05	heat shock cognate 70-kd protein, tandem duplicate 1	ATP binding activity, camera-type eye morphogenesis
hsp90aa1.2	1.45E-05	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	ATP binding activity, unfolded protein binding activity, protein stabilization



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0001222	transcription corepressor binding	6.709×10^{-3}
2	GO:MF	GO:0001221	transcription coregulator binding	3.115×10^{-2}
3	GO:BP	GO:0009649	entrainment of circadian clock	4.093×10^{-3}
4	GO:BP	GO:0009648	photoperiodism	1.546×10^{-2}
5	GO:BP	GO:0007623	circadian rhythm	2.173×10^{-2}
6	GO:BP	GO:0032922	circadian regulation of gene expression	9.526×10^{-4}
7	GO:BP	GO:0042752	regulation of circadian rhythm	1.346×10^{-4}
8	GO:BP	GO:0043153	entrainment of circadian clock by photoperiod	3.375×10^{-3}
9	GO:BP	GO:0045475	locomotor rhythm	3.334×10^{-2}
10	GO:CC	GO:0005581	collagen trimer	9.011×10^{-3}
11	KEGG	KEGG:00100	Steroid biosynthesis	4.696×10^{-4}
12	KEGG	KEGG:01100	Metabolic pathways	2.254×10^{-3}
13	KEGG	KEGG:00790	Folate biosynthesis	4.991×10^{-2}

Figure 3: The figure shows the groups of gene among the upregulated gene are involved in the transcription coregulator binding, metabolic pathways and other biological and cellular functions.

4. Discussion:

The FastQC report before and after trimming revealed no significant difference in read counts, indicating that the overall base quality of the sequence reads was consistently high. This study found that the number of reads per sample ranged between 80 million and 130 million. After mapping the reference genome, the mapped reads ranged from 60 million to 80 million, demonstrating efficient read alignment. The overall GC content across samples was between 48% and 49%, with no significant differences observed before and after trimming, suggesting uniformity in GC composition. Gene ontology analysis identified 416 differentially expressed genes (DEGs), with the majority being downregulated. These downregulated genes were associated with molecular functions such as misfolded protein binding, peptidase regulator activity, and protein folding chaperone activity. Numerous downregulated biological processes were identified, including response to stress, defense response, activation of immune response, humoral immune response, response to stimuli, MyD88-dependent toll-like receptor signaling, innate immune response, response to cytokines, and toll-like receptor 5 signaling pathways. These processes are critical for maintaining cellular homeostasis and responding to environmental challenges, highlighting the substantial impact of cold stress on zebrafish larvae.

Additionally, several KEGG pathways were downregulated, including arachidonic acid metabolism, herpes simplex virus 1 infection, and Toll-like receptor signaling pathways. Among the most significantly downregulated genes were fn1b, hsp70.3, hsp70.1, and hsp90aa1.2, which are crucial for integrin binding activity, proteoglycan binding activity, ATP binding and hydrolysis, misfolded protein binding, and camera-type eye morphogenesis. These genes play vital roles in protein stability, stress responses, and cellular signaling. Conversely, the upregulated genes were associated with processes such as circadian rhythm regulation, entrainment of the circadian clock, and metabolic pathways. Key upregulated genes included cry1aa, lonrf1l, kera, and col7a1, which are involved in transcription factor binding, transcriptional coregulation of the circadian clock, and visual perception. Gene enrichment analysis underscores the profound negative effects of low-temperature stress on the overall health of zebrafish larvae. However, the observed gene expression changes may also suggest the larvae's attempt to develop cold tolerance mechanisms.

In conclusion, this study demonstrates that cold stress adversely affects zebrafish larvae, impairing crucial biological and molecular processes. At the same time, the findings provide insights into the larvae's ability to initiate cold tolerance strategies, which may be essential for their survival in low-temperature environments.

5. Reference

1. Long, Y., et al., Transcriptomic characterization of cold acclimation in larval zebrafish. *BMC genomics*, 2013. **14**(1): p. 1-16.
2. López-Olmeda, J. and F. Sánchez-Vázquez, Thermal biology of zebrafish (Danio rerio). *Journal of Thermal Biology*, 2011. **36**(2): p. 91-104.
3. Donaldson, M., et al., Cold shock and fish. *Journal of Fish Biology*, 2008. **73**(7): p. 1491-1530.
4. Hwang, H., et al., Physiological response of juvenile red sea bream pagrus major exposed to low temperature shock. *B Eur Assoc Fish Pat*, 2012. **32**(1): p. 34-40.

5. Kuo, C. and S. Hsieh, Comparisons of physiological and biochemical responses between milkfish (Chanos chanos) and grass carp (Ctenopharyngodon idella) to cold shock. *Aquaculture,* 2006. **251**(2-4): p. 525-536.
6. Du Pasquier, L., The immune system of invertebrates and vertebrates. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology,* 2001. **129**(1): p. 1-15.
7. Zimmerman, L., L. Vogel, and R. Bowden, Understanding the vertebrate immune system: insights from the reptilian perspective. *Journal of Experimental Biology,* 2010. **213**(5): p. 661-671.
8. Mashoof, S. and M.F. Criscitiello, Fish immunoglobulins. *Biology,* 2016. **5**(4): p. 45.
9. Ellis, A., Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology,* 2001. **25**(8-9): p. 827-839.
10. Cabillon, N.A.R. and C.C. Lazado, Mucosal barrier functions of fish under changing environmental conditions. *Fishes,* 2019. **4**(1): p. 2.
11. Zhang, Q., et al., Low incubation temperature during early development negatively affects survival and related innate immune processes in zebrafish larvae exposed to lipopolysaccharide. *Scientific reports,* 2018. **8**(1): p. 1-14.
12. Bloom, J.S., et al., Measuring differential gene expression by short read sequencing: quantitative comparison to 2-channel gene expression microarrays. *BMC genomics,* 2009. **10**(1): p. 1-10.
13. Spies, D., et al., Comparative analysis of differential gene expression tools for RNA sequencing time course data. *Brief Bioinform,* 2019. **20**(1): p. 288-298.
14. Patnaik, B.B., et al., Transcriptome Profile of the Asian Giant Hornet (Vespa mandarinia) Using Illumina HiSeq 4000 Sequencing: De Novo Assembly, Functional Annotation, and Discovery of SSR Markers. *Int J Genomics,* 2016. **2016**: p. 4169587.
15. Mateus, A.P., et al., Thermal imprinting modifies adult stress and innate immune responsiveness in the teleost sea bream. *J Endocrinol,* 2017. **233**(3): p. 381-394.
16. Langston, A.L., et al., The effect of temperature on non-specific defence parameters of three strains of juvenile Atlantic halibut (Hippoglossus hippoglossus L.). *Fish & Shellfish Immunology,* 2002. **12**(1): p. 61-76.
17. Adam, A.-C., et al., Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (Danio rerio) progeny. *PloS one,* 2018. **13**(8): p. e0201278.