IJBPAS, June, 2021, 10(6): 1812-1840

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

CO-EXPRESSION NETWORK ANALYSIS IDENTIFIES GENE MODULES ASSOCIATED WITH MULTIPLE ABIOTIC STRESS RESPONSES IN ZEBRAFISH

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Received 10th July 2020; Revised 10th Aug. 2020; Accepted 16th Sept. 2020; Available online 1st June 2021

https://doi.org/10.31032/IJBPAS/2021/10.6.5501

ABSTRACT

Stress affects health thereby its production in aquaculture, and fish are simultaneously exposed to multiple stresses. Here, we performed network-based gene clustering to characterize regulatory gene networks and metabolic pathways which unveil the relationships between multiple abiotic stress responsive genes.To analyze multiple responses, we performed metaanalysis and identified 1214 genes were upregulated, and 670 genes were down-regulated in zebrafish.The database for annotation, visualization, and integrated discovery was employed for functional analysis of the differentially expressed genes, PPI modules and WGCNA co-expression modules. WGCNA identified 12 modules enriched significantly in multiple stress response.These modules are enriched for genes involved in controlling the immune response, endocrine response, carbohydrate metabolism, antioxidant response, stimulus response, homeostasis,and apoptosis. These results extend our knowledge of the multiple stress responses of fish at the molecular level and indicate that fish have evolved common mechanisms to cope with multiple stresses.

. **Keywords: Multiple stressors; Meta-analysis; WGCNA; PPI modules; Zebrafish**

1. INTRODUCTION

Aquatic environments are characterized by the presence of multiple interacting abiotic stressors **[1]**. Aquatic organisms regularly encounter various related or unrelated stressors which significantly influence the level of stress. These multi-level stress conditions canresult highly-complexed biological responses. The fitness of the aquatic organisms deteriorates when their habitats are continuously exposed to stressors **[2]**. The interactions between stressors and stress responses are complex. The prolonged exposure of fish species to these stressors can induce adaptive homeostasis- affecting the naturally-developed physiological traits during evolution. The sudden drift may bring down natural breeding and physiological habits of the fish species **[3]**. If the exposure continues even after the threshold adaptive patterns, the fish species ceases to maintain the homeostasis leading to a decline in its population growth in that particular environment **[4]**. The United States Environmental Protection Agency Report (US EPA, 2004) expressed the difficulty in developing a 'Risk Assessment Guide' for multiple interacting stressors (EPA, 2004).

It is essential to identify differentially expressed genes (DEGs), interaction among DEGS, and pathways under stress conditions to understand the stress response of an organism. There have been single gene or multiple gene analysis studies reported molecular responses of organisms to stress; however, the advent of cDNA microarray technology in the field of functional genomics allowed profiling and unveiling of global gene expression changes. The blend of both microarray technology and analysis of candidate genes facilitates an in-depth study of stress responses in fish **[5]**. Substantial research has been conducted to study the responses of fish to a single stressor **[6]** and different functions of many genes have been noted to be related to stress response, but the relationships between these genes are unknown.Meta-analysis and network analysis of similar microarray stress studies could help to characterizethe global transcriptomic of same or related stress conditions; however, these studies have not available for most of the fish species unlike the case of plant species which are extensively analyzed **[7]**.

Studies on genome-wide transcriptome data in fish species reported global co-expression networks **[8]**. Various methods and algorithms are used to build gene-metabolite gene regulatory, transcriptional regulatory, protein-protein

interaction, and coexpression biological networks **[9]**. Coexpressionnework of genes relate them to similar biological processes, and the individual modules of genes can be referred to specific processes. In biological networks, coexpressedgenes are known to participate in similarsignaling or metabolic pathways **[10]**. Weighted Gene Co-expression Network Analysis (WGCNA) is modernapproach to decipher correlation patterns across microarray samples **[11]**. Implemented in R as a package, WGCNA is used to detect, analyze and export individual and conserved modules from various microarray studies which are related.

Zebrafish is chosen as a model organism in this study because it is a highly-celebrated model for scientific research on a variety of biological disciplines including-Genetics, Developmental Biology, Biochemistry, Physiology, and Environmental Genomics. In this study, we performed the meta-analysis of microarray data to identify the transcriptome responses to multiple abiotic stresses, during which we could study the modular organization of transcription networks, using protein-protein interaction (PPI) network and weighted gene expression network analysis. We could finally identify the stress-responsive modules and critical regulatory genes to understand responses to multiple abiotic stressorsfurther.

2. METHODS

2.1.Collection of gene expression data and Identification of DEGs

Microarray studies involving zebrafish challenged with multiple abiotic stresses is collected from NCBI GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo **[12]**.

We manually selected the samples and categorizedsix stress conditions (Hypoxia, Cold, Metal, Pesticide, Oil emulsion, and Radiation), as shown in **Sup Tab 1**. In total, we analyzed 64 samples belonging to 7 series records from GPL1315Affymetrix Array platform. The raw intensity values of CEL files of samples were extracted using the Bioconductor package Affy in R computing environment. R is, an open-source language, highly used tool for biological data mining and transcriptome data analysis, and it is free software and runs on almost all computing platforms **[13]**. Array Quality Metrics package was used to asses the quality of samples **[14]** and to remove the samples thathave bad quality. RMA method was followed to normalize the intensity values of samples **[15]**. Meta-analysis was performed for normalized samples to identify multiple stress responsive DEGs with the rank product

method **[16]**. The number of permutation testsand falsepositives cutoff value was set to 100 and 0.05, respectively. Annotations of differentially expressed genes were retrived fromhttps.://biodbnetabcc.ncifcrf.gov/db/db2dbRes.php.

2.2. PPI network and module analysis

PPI network can identify the important genes and key gene modules of multiple stress responses using interactive relationships among DEGs. PPI data of DEGs was retrieved using online tool Search Tool for the Retrieval of Interacting Genes (STRING) which is a database contains protein-protein interactions and functional association data from several sources such as literature, high-throughput experiments, and estimates taken from genomic context analysis **[17]**. Cytoscape software was employed to construct PPI network graphs that facilitates the analysis of data and the observation of relationships between proteins. Molecular Complex Detection (MCODE) plug-in for Cytoscapewas used to detect three modules from PPI network. MCODE plug-in identifies networks by mining (modules) clusters that are very common than the basic motifs observed with Cytoscape package. MCODE can identify highly interconnected clusters because it is not limited by the number of nodes or edges.

2.3. Weighed Gene Co-expression network analysis

We used the normalized, log-transformed gene expression values of each stress condition to construct the coexpression network and identify co-expression modules from MSRGs. In WGCNA, nodes represent gene module, and edges are the degree of their co-expression **[18]**. A module indicates a group of genes with similar expression trends in different samples. WGCNA builds gene modules based on pairwise correlations between gene expression levels of gene expression data. We removed outlier samples because they can have ahigh impact on co-expression values. Pearson's correlations were calculated for all genes across all stress treatments. WGCNA transforms coexpression similarities into connection strengths by raising all value to a power ß, and a threshold of 14 was chosen based on a fit to scale-free topology in this study **(Sup Fig S1))**. This power function weights the network by calculating the topological overlap matrix (TOM) from the transformed correlation matrix, and it also converts the topological overlap matrix into a dissimilarity matrix. TO measures connection strength between genes, and

genes with high TO are clustered into co-expression modules. Hierarchical cluster tree was created based on the dissimilarity matrix to detect network modulesusing a dynamic tree cut method **(Sup Fig S2)**. The minimum size of the modules was set at 30 and cut height at 1.Eigengenes were calculated for each gene co-expression module and visualized the gene expression patterns for each module, andheat mapsfor eigengeneswere constructed to describe the expression levels of all genes in a module and to relate external gene or sample information to network modules.

2.4.Functional and pathway enrichment analysis of modules

Functional annotation was performed for the gene modules expressed in common with responses every stress.Gene ontology biological process terms were carried out using Database for Annotation, Visualization and Integrated Discovery (DAVID, release 6.8) online tools https://david.ncifcrf.gov/home.jsp **[19]** at default settings of thresholds of *P*-value <0.05 and fold enrichment gene count >2 using Benjamini p-value correction.

3. RESULTS

3.1.Identification of commonstress responsive genes to multiple abiotic stress

A total of 2202 DEGs were identified as multiple stress responsive genes (MSRGs) in zebrafish and the number of MSRGs are shown in **Figure 1**. The complete list of genes and their fold change values are given in Sup Tab2. The number of MSRGs with fold change (FC) value ≥1.0 was 1% of upregulated genes, and 100% of down-regulated genes, respectively. Notably, four genes showed >5-fold downregulation in MSRGs, parvalbumin 2, myosin, heavy chain a, apolipoprotein B and apolipoprotein C2.

3.2. GO term enrichment analysis of MSRGs

GO analysis results showed that up-regulated MSRGs were significantly enriched in biological processes (BP), including antigen processing and presentation, glycolytic process, and microtubule-based process **(Sup Tab3)**; the down-regulated MSRGs were significantly enriched in biological processes, including cellular response to estrogen stimulus, oxidation-reduction process, gluconeogenesis, and immune response **(Sup Tab 4)**. In the cellular component, the up-regulated MSRGs were significantly enriched in the microtubule cytoskeleton, neuron part, cell projection, and the downregulatedMSRGs were significantly enriched inthe extracellular region,

extracellular region part and extracellular matrix. In molecular function, the up-regulated DEGs were significantly enriched in ATPase coupled ion transmembrane transporter activity, cation-transporting ATPase activity, and ATPase activity, coupled to transmembrane movement of ions binding,and the down-regulated DEGs significantly enriched instructural molecule activity, extracellular matrix structural constituent and peptidase inhibitor activity. These significantly enriched pathways and terms of MSRGs could be helpful to understandtheir role in multiple abiotic stress response.

3.3. KEGG pathway analysis of MSRGs

The up-regulated DEGs were enriched in the phagosome, gap junction, oocyte meiosis,an intestinal immune network for IgA production and lysosome, and the down-regulated DEGs were enriched in the biosynthesis of antibiotics, ECM-receptor interaction, glycolysis/ gluconeogenesis, glycine, serine and threonine metabolism, and carbon metabolism.Sup Table 5shows the most significantly enriched pathways of upregulated and downregulated DEGs.

3.4. PPI network and module analysis

PPI network of MSRGs with 1292 nodes and 11889 edges, was built using Cytoscape from STRING database. The top 10 MSRGs with ahigh degree of connectivity were selected as the hub genes of multiple abiotic stress response. These hub genes were separately actin alpha 1a, actin, alpha 2, skeletal muscle actin alpha, cardiac muscle 1b, smooth muscle, aorta, heat shock protein 90, cell division cycle 42, mitogen-activated protein kinase 1, glyceraldehyde-3-phosphate dehydrogenase, cyclin-dependent kinase 1, SWI/SNF related matrix associated actin dependent regulator of chromatin, subfamily a, member 4, troponin C type 2 (fast), phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazolesuccinocarboxami desynthetase **(Sup Fig. S3)**. MCODE plug-in was employed to perform module analysis. The top three gene modules were significantly enriched in immune response with 88 nodes and 975 edges, organ development with 66 nodes, and 540 edges, and response to other organism with 51 nodes and 241 edges, respectively **(Fig. 2& Sup Tab 6)**.

3.5. Weighted correlation network analysis of DEGs and module analysis

WGCNA approach was used to find modules of functionally related co-expressed genes that are differentially expressed in response to multiple abiotic stress.

Construction of transcriptional networks of multiple abiotic stress may helpful to understand the core and critical responses to these stressors at the molecular levels. The datasets used in this study included transcriptional measurements of a total of 64 samples from 6 abiotic stress conditions, representing cold, hypoxia, oil emulsion, metal, pesticide and copper toxicity, respectively **(Sup Tab 1)**. First, we performed a WGCNA on the 2,202 most differentially regulated transcripts to group the multiple abiotic stress responding transcripts into stress regulatory modules. The analysis of the transcriptional network of multiple abiotic stresses showed that a total of 12 distinct transcriptional modules, designated black, brown, greenyellow, grey, lightcyan, midnightblue, pink, red, salmon, tan, and turquoise with each containing at least 30 genes (Figure 3).The largest module ("pink") contained 651 genes; the least module ("lightcyan") contained 43genes. About 14 probe sets were not grouped into any above modules; therefore, they were included in the "gray" module which represented poorly connected genes **(Table 1)**. Next, the modules of MSRG indicated by module color, k Total (whole network connectivity) was used to identify highly connected transcripts inside in the wholenetwork, kWithin, kOut, and kDiff(intramodular connectivity) were used toidentify highly connected transcripts inside the modules **(Sup Tab7)**. Then, we identified that turquoise, midnightblue, lightcyan, greenyellow and brown modules with similar expression patterns for hypoxia, metal, pesticide and cold microarray samples. The module and the module eigengene (ME) expression showed that the midnight blue module was upregulated while remaining four modules were downregulated **(Figure 4)**. Finally, we performed DAVID gene ontology functional enrichmenton these modules to asses the key modules and biological processes involved in multiple stress responses and the results are presented in **(Sup Tab 8)**.

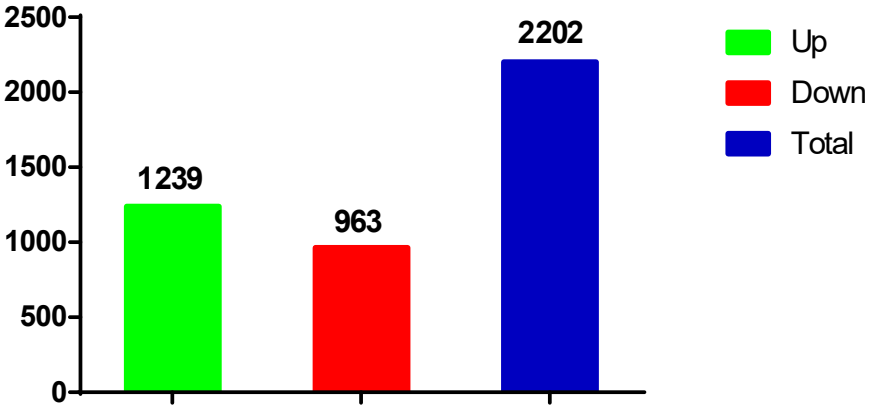


Figure 1: Number of up-regulated and down-regulated DEGs in all abiotic stresses

Figure 2 A

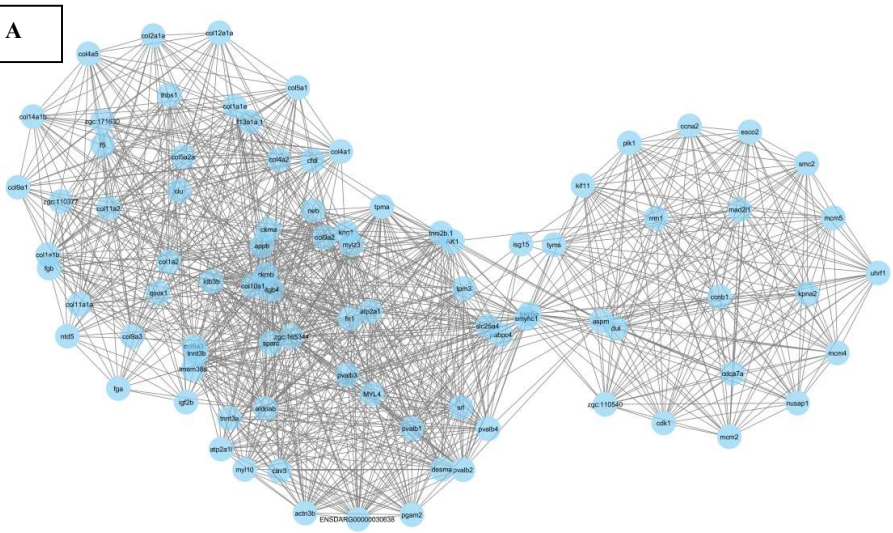
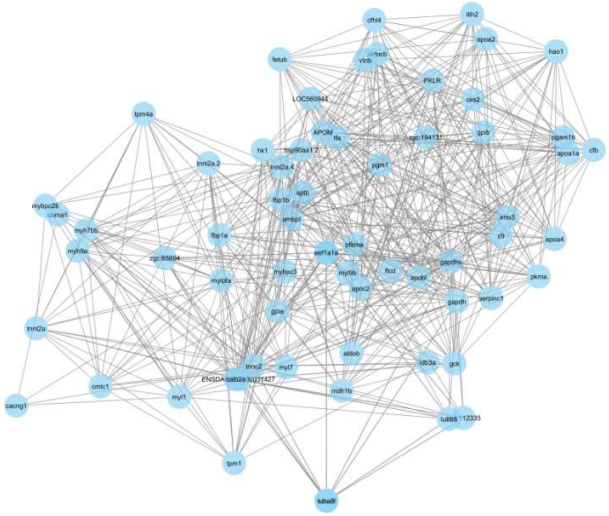


Figure 2 B



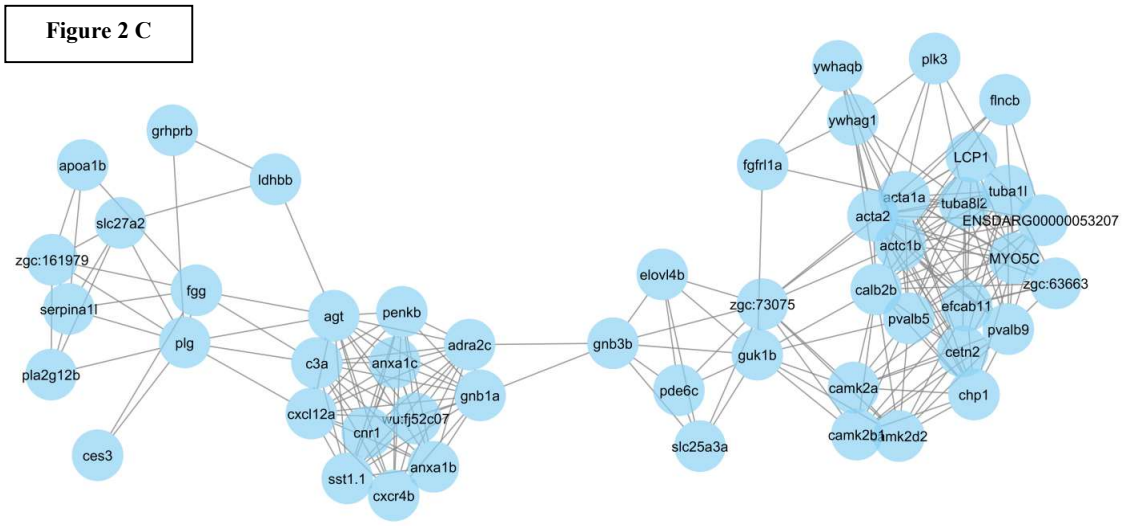


Figure 2: The significant modules in the protein–protein interaction network of common DEGs . A module 1; B module 2; C module 3

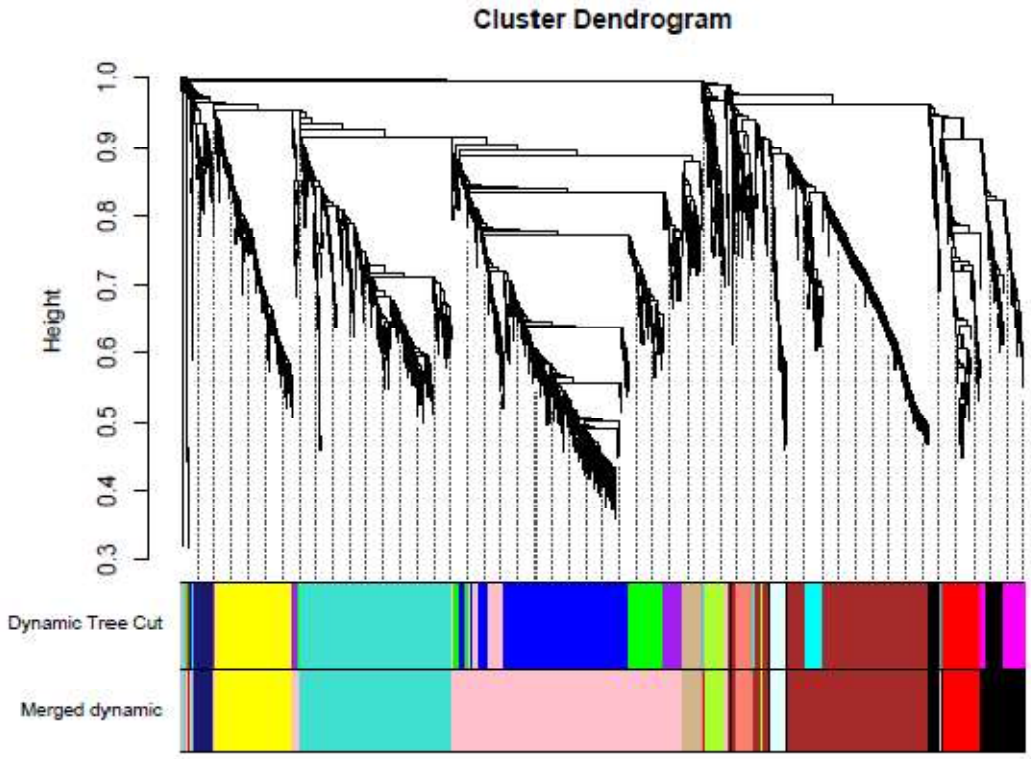
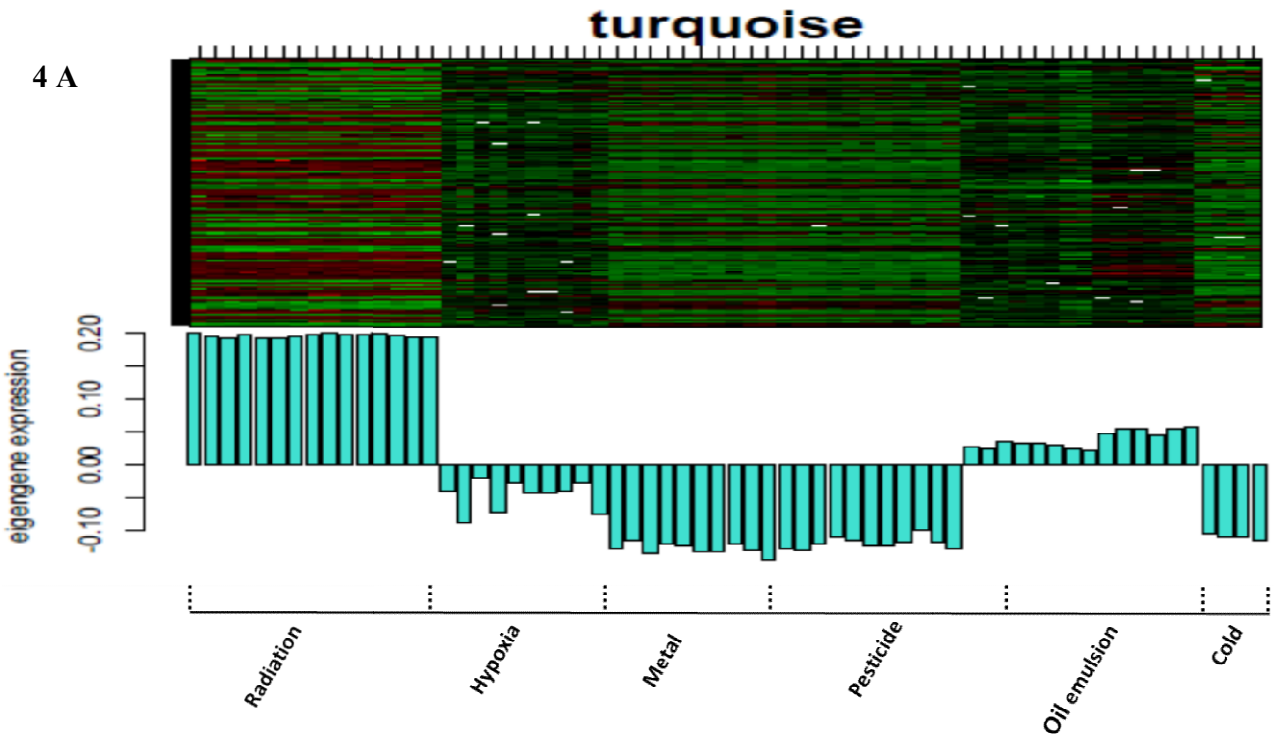
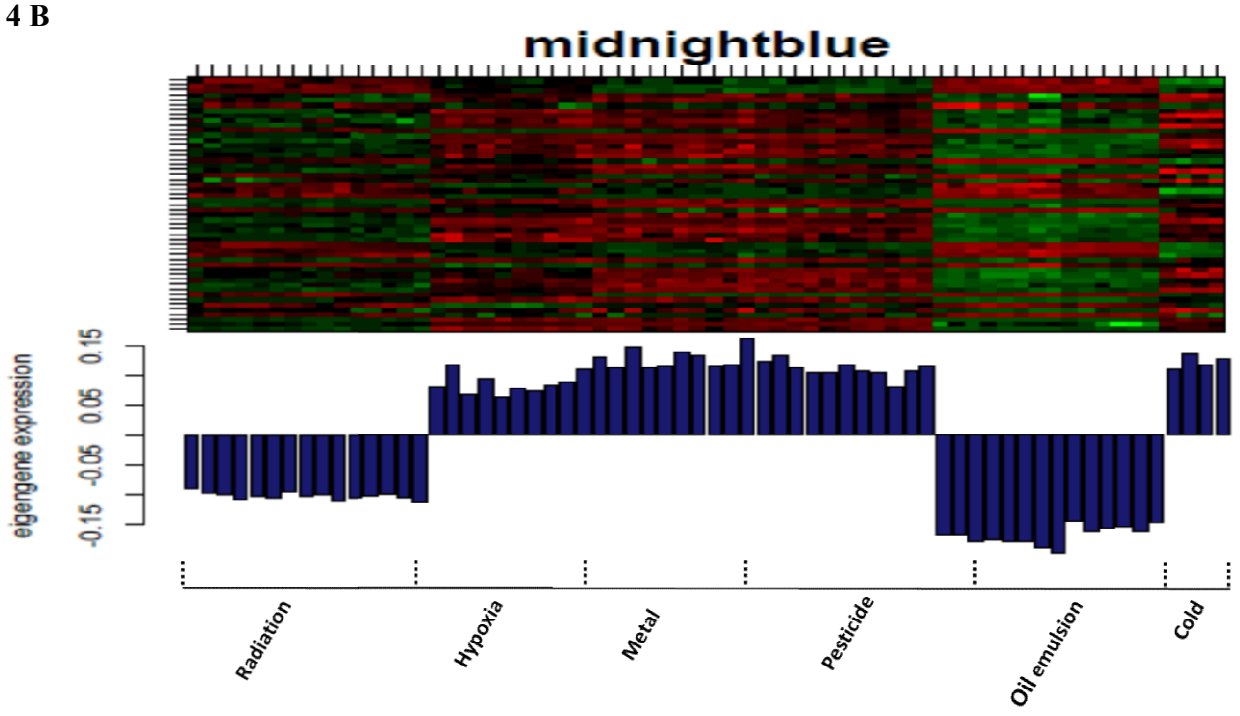


Figure 3: Dendrogram for co-expressed modules. Dendrogram depicting co-expressed modules before and after merging using the WGCNA package. Colors in the horizontal bar depicts the different modules

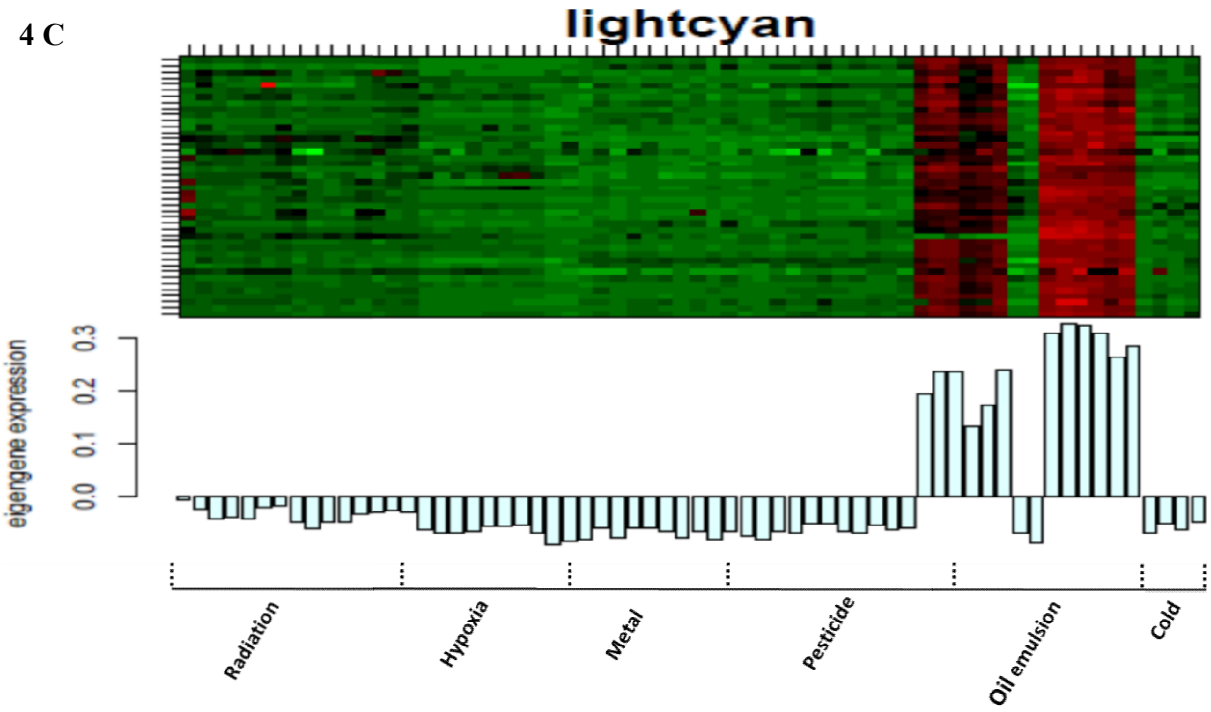
4 A


turquoise
eigengene expression
0.20
0.10
0.00
-0.10
Radiation
Hypoxia
Metal
Pesticide
Oil emulsion
Cold

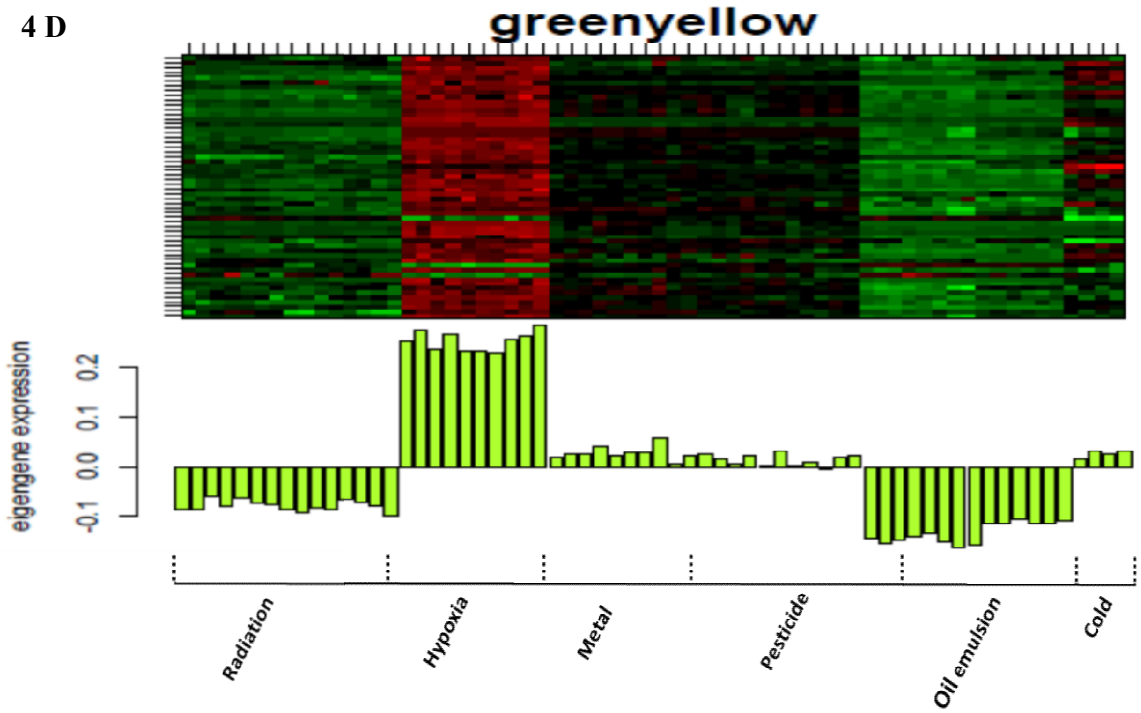
4 B


midnightblue
eigengene expression
0.15
0.05
-0.05
-0.15
Radiation
Hypoxia
Metal
Pesticide
Oil emulsion
Cold

4 C


lightcyan
eigengene expression
0.0
0.1
0.2
0.3
Radiation
Hypoxia
Metal
Pesticide
Oil emulsion
Cold

4 D


greenyellow
eigengene expression
-0.1
0.0
0.1
0.2
Radiation
Hypoxia
Metal
Pesticide
Oil emulsion
Cold

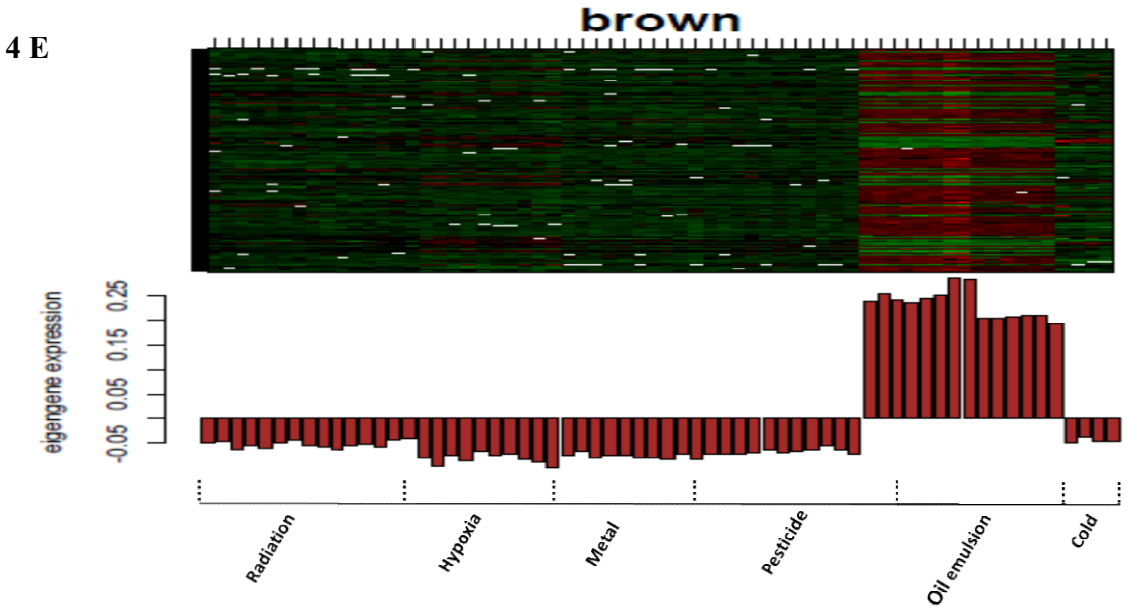


Figure 4 (A-E): Heatmaps of turquoise, midnightblue, lightcyan, greenyellow and brown modules in zebrafish under multiple abiotic stresses.Inx-axis, the columns represent microarray samples from multiple abiotic stresses and y-axis rows depict genes found in the module. The expression is scaled for each gene, where red denotes that the gene is highly expressed in that sample and green denotes low expression Below the heatmapis the ME that represents the average gene expression throughout the module, and it demonstrates that the genes in the module are mostly whether upregulated or down regulated.

.

Table 1: List of consensus co-expression modules found in multiple stress response gene set.

S.No	mergedColors	Freq
1	Black	155
2	Brown	425
3	Greenyellow	56
4	Grey	14
5	Lightcyan	43
6	Midnightblue	51
7	Pink	651
8	Red	98
9	Salmon	52
10	Tan	54
11	Turquoise	399
12	Yellow	203

4. DISCUSSION

Natural or anthropogenic stressors are introduced into the aquatic environment, so organisms in these environments are continuously subjected to various stressors simultaneously. In this study, significant DEGs were foundbetween multiple stress samples and normal samples with metaanalysis to unveil key genes and pathways that are closely associated with multiple stress responses in zebrafish. By meta-analysisof microarray data using R/ Bioconductor program, we found 1239 upregulated DEGs, and 963 downregulated

DEGs. We identified important biological processes and key genes involved in multiple stress responses. The major aimof this study was to apply the network-based approach and to provide an insight into the organization of functional modulesof MSRGs under abiotic stress.

PPI network analysis and WGCNA were employed to link the expression patterns to the abiotic stress and to find the abiotic stress-specific modules, which increase our knowledge of multiple abiotic stresses responsive mechanisms from different perspectives. Also, we conducted gene ontology term enrichment analysis and KEGG pathway analysis to attribute a biological significance of the constructed PPI and gene co-expression networks and identified turquoise, midnightblue, lightcyan, greenyellow and brown modules of co-expressed genes were significantly associated with abiotic stress.

PPI network analysis of the DEGs was performed to disclose the relations between the differentially expressed genes under multiple abiotic stress. It was inferred from the top three PPI network modules that the essential biological processes are functionally connected. These implicated in the pathways of multicellular organismal process, homeostatic process, carbohydrate catabolic process, response to stress, and etcthat are crucial to multiple abiotic stress responses. This is an indication that the genes in three PPI modules act together to encounter abiotic stress conditions.

WGCNA analysis of the DEGs were performed to unveil the relationships between multiple abiotic stress responsive genes and found modules turquoise, midnightblue, lightcyan, greenyellow and brown related to immune responses, endocrine regulation, adjustments in energy metabolism, antioxidant responses, homeostasis processes, and apoptotic processes under multiple stress conditions. Thus, genes within these modules underwent dramatic changes under multiple stress conditions.

Immune response of fish may undergo obvious changes under stress. Like mammals, teleosts have both the innate immune system and an adaptive immune system. An aggressive immune response is a common characteristic of fish when they are stressed bymanynumbers of cellular perturbations **[20]**. Handling and confinement stresshave been shown to alter Immune responses in fish **[21]**. In GO term enrichment analysis, 14 up-regulated DEGs significantly were found to be enriched in antigen processing and presentation, 14 up-

regulated DEGs significantly were found to be enriched inthe regulation of immune system process. Similarly, 51 up-regulated DEGs were enriched in the immune system process, and 7 up-regulated DEGs significantly were found to be enriched negative regulation of immune system process. KEGG pathway analysis showed that 7 upregulated DEGs were found to be enriched in an intestinal immune network for IgA production, antigen processing and presentation, 21 upregulated DEGs were found to beenriched in MAPK signaling pathway, and 8 upregulated DEGs were enriched in proteasome pathway. In module analysis of PPInetwork, third genemodule significantly was found to beenriched in the immune response. WGCNA suggested that Lightcyan, Midnightblue (51MSRGs) and brown modules (425MSRGs) were enriched in the immune response. In the present study, several genes involved in immune response including cd74, ctss(cathepsin S), mhc1, CUB and zona pellucida-like domains1-tandem duplicate1(cuzd1.1), beta-2-microglobulin(b2m), chemokine (C-C motif) ligand 19a-tandem duplicate1(ccl19a.1), interleukin-1 receptor-associated kinase1-binding protein 1(irak1bp1), TIMP metallopeptidase inhibitor 2a(timp2a), jun B proto-oncogene a(junba), jun B proto-oncogene b(junbb), complement factor B (cfb)were identified as common differentially expressed genes under multiple abiotic stressors. Cathepsinis a protease that performs proteolysis, and its expression is greater in animals under stress.As the stress increasesthe susceptibility of fish to bacterial infections and diseases, they produce cathepsin to lyse aquatic bacteria **[22]**. Antigen processing and presentation genes such as major histocompatibility complex class I UBA(mhc1uba), major histocompatibility complex class I ZBA(mhc1zba)and major histocompatibility complex class II DAB gene(mhc2dab) have been found to be associated with stress to enhance immune defense to a wide array of pathogens. IL-1R–associated kinase 1 binding protein 1 (irak1bp1) is overexpressed under multiple abiotic stressors, and itdown-modulates toll-like receptor-mediated transcription of several pro-inflammatory cytokines.Stress enhances innate immunity by increasing components such as lysozyme, C3 proteins andmyeloid-type leukocytes(23). Also, stress enhances immune responses that decrease the pathogen infections there by alters the immune regulatory system **[24]**. However, prolonged stress exposure has been shown to suppress immune-competence in fish **[25]**. The gut mucosal-associated

lymphoid tissue (MALT) of teleost fish is a leading site for immunoglobulins (Igs) there by it plays a key role in the immune response. It has been described that synthesis of immunoglobulinsis decreased in the MALT of the gut after exposed to stressors **[26]**. In contrast, we found that 7 upregulated DEGs were enriched in the intestinal immune network for IgA production. The present study suggests that elevated expressions of immune-related genes seem to be helpful for fish to prevent diseases those arising due to high infection under stress. Also, it suggests that increase production of these factors would keep the fish in a hyper-alert and stressed state. Hence it is inferred that under multiple abiotic stress these genes play a vital role.

Steroid hormones are considered to be key regulators of homeostasis in the organism due to their action on metabolism and immune function **[27]**. Researchers have reported impaired steroid hormone levels after exposure to stressors in fish **[28]**. In this study, we identified several enriched gene ontology terms which include response to estradiol, steroid hormones, the responses to estrogen stimuli, and sterol and cholesterol biological processes. KEGG pathway analysis identified pathways are associated with steroid hormone biosynthesis. Inmodule analysis of PPI network, second gene module was found to be significantly enriched in endocrine response. WGCNA indicated that greenyellow module with56 MSRGs was enriched in response to estradiol. Several genes involved in endocrine response including cytochrome P450 (cyp19), vitellogenin 2(vtg2) and chemokine (C-X-C motif), receptor 4b were identified as common differentially expressed genes under multiple abiotic stressors. Cytochrome P450 enzyme plays a critical role in the oxidative metabolism of different endogenous and exogenous compounds, and its overexpression has been identified in fish after exposed to stressors. Therefore, it suggests that these genes are part of the endocrine response to abiotic stress response and play a crucial role in fish when challenged by multiple stressors.

Glucose is a critical energy fuel that is oxidized to liberate energy to cope with the energy demand linked to stress response. The increased activation or expression of the carbohydrate metabolic enzymes have been identified after exposed to stressors **[29]**. In GO term enrichment analysis. 7 up-regulated DEGs were found to be significantly enriched in the glycolytic process, 8 up-regulated DEGs were found to be significantly enriched in carbohydrate

catabolic process, 7 up-regulated DEGs were found to be significantly enriched in pyruvate metabolic process, 18 up-regulated DEGs were found to be significantly enriched in ribose phosphate metabolic process and 7 up-regulated DEGs were found to be significantly enriched in hexose metabolic process, and 7 up-regulated DEGs were significantly enriched in monosaccharide metabolic process. KEGG pathway analysis identified that 14 upregulated DEGs were enriched inglycolysis / gluconeogenesis analysis. Also, KEGG pathway analysis identified that 10 down-regulated DEGs were enriched in pyruvate metabolism, 7 down-regulated DEGs were enriched in pentose phosphate pathway, and 6 down-regulated DEGswere enriched in citrate cycle (TCA cycle) pathway. In module analysis of PPI network, the second gene module was found to be significantly enriched in carbohydrate metabolism. WGCNA indicated that turquoise module with 399MSRGs was enriched in the glycolytic process, carbohydrate metabolic process, gluconeogenesis, and glucose metabolic process. The identified biological processes and pathways are mainly involved in blood glucose raise and glucose utilization. In this study, several genes related to both gluconeogenesis and glycolysis including-galdolase C, glucose-6-phosphate isomerase a, glyceraldehyde-3-phosphate dehydrogenase, hexokinase 1, phosphor fructokinase, phosphogly-ceratemutase, pyruvate kinase, UDP-glucose 6-dehydrogenase, amylase, fructose-1,6-bisphosphatase, lactate dehydrogenase, phosphoglucomutase 1, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase 1, phosphoenolpyruvate carboxykinase 2 were differentially regulated in response to stress. UDP-glucose dehydrogenase (UGDH) transforms UDP-glucose (UDP-Glc) to UDP-glucuronic acid (UDP-GlcUA), and UDP-GlcUA) is a key precursor for the biosynthesis of exopolysaccharides such as UDP-xylose, UDP-arabinose, and UDP-galacturonic acid which perform detoxification of lipophilic hormones and xenobiotics by glucuronidation **[30]**. Glucose-6-phosphatase and fructose-1,6-bisphosphatase enzymes havea vital role in glycolysis and gluconeogenesis pathways, and it has been shown that their activity is increased under stress **[31**. Phosphoenolpyruvate carboxy-kinase links TCA cycle intermediates and glycolytic pools, and oxidative stress has been shown to induce phosphoenolpyruvate carboxykinase expression **[32]**. Hexokinase (HK), a rate-limiting enzyme in glycolysis, converts glucose to glucose-6-phosphate, and

it has been found that HK activity is increased under stress **[33]**. Lactate dehydrogenase (LDH) is an oxidoreductase and converts pyruvic acid into lactic acid, and vice versa. The expression of LDH enzyme has been found to be increased in oxidative stress **[34]**. Glycogen is a stored glucose polysaccharide in cells and is vital for energy supply and glucose homeostasis. Glycogen phosphorylase breakdown glycogen andreleases glucose residues. Pyruvate kinase (PK) catalyzes the reaction of transforming phosphoenolpyruvate (PEP) to pyruvate, and it plays a pivotal role in balancing oxidative stress, and its activity has been shown to be increased under stress **[35]**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme which plays a pivotal role in cell death, and it has been found to be triggered by oxidative stress **[36]**. These carbohydrate metabolic enzymes are mainly involved in gluconeogenesis and glucose utilization. It can conclude that the identified genesdisplay an important role while the fish adapt to stressors. The present results are consistentwith studies those have shown elevated activities of glycolytic enzymes after exposure to a stressor. It implies that these enzymes are crucial to coping up with energy demand to restore homeostasis by organism **[37]**. Activation of these biological processes in response to stress has also been identified in several previous studies **[38]**. Generally, carbohydrate metabolism and endocrine system are very intimately associated with organisms **[39]**. Therefore, thesegenes are crucial to fish under multiple abiotic stress asthey are enriched in biological processes and pathways of carbohydrate metabolism and endocrine system to meet the high energy demand to re-establish homeostasis after exposure to stressors.

Antioxidant response and MSR. Stress induces the generation of reactive oxygen species (ROS) which in turn activates the oxidative stress response in organisms. Genes involved inthe antioxidant system and oxidative stress are altered when fish exposed to stressors **[40]**. In GO term enrichment analysis, 7 up-regulated DEGs were found to beenriched significantly in oxidoreduction coenzyme metabolic process. 51 down-regulated DEGs wereidentified to be enriched significantly in the oxidation-reduction process. KEGG pathway analysis identified that 7 downregulated DEGs were enriched in glutathione metabolism. Inmodule analysis of PPI network, the second gene module was found to beenriched significantly in the antioxidant system.

WGCNA indicated that turquoise module including 399 MSRGs, was mainly enriched in the oxidation-reduction process. This kind of biological responses have been noticed in fish upon exposure to stress; moreover, they are mainly associated with the removal of ROS **[41]**. We identified that 3-hydroxyisobutyrate dehydrogenase, 4-hydroxyphenylpyruvate dioxygenase, D-amino-acid oxidase, UDP-glucose 6-dehydrogenase, aldehyde dehydrogenase, glutamate dehydrogenase, glutathione peroxidase,homogentisate 1,2-dioxygenase, hydroxyacyl-CoA dehydrogenase, phosphoglycerate dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, 4-hydroxyphenylpyruvate dioxygenase a, NADH dehydrogenase, homogentisate 1,2-dioxygenase, hydroxyacidoxidase1(hao1), hydroxysteroid dehydrogenase, methylenete-trahydrofolate dehydrogenase, phenylalanine hydroxylase, phytanoyl-CoA dioxygenase, sarcosine dehydrogenaseand urate oxidase were differentially regulated in response to multiple stressors.Cytochrome P450 enzyme plays a critical role in the oxidative metabolism of different endogenous and exogenous compounds, and overexpression of cytochrome P450 has been identified in fish after exposed to stressors **[42]**. Cells are usually protected from from oxidative damage by Glutathione peroxidase 1b that catalyzes the reduction of lipid hydroperoxides and hydrogen peroxide (H_2O_2) to alcihols and water. Over expression of glutathione peroxidase in Atlantic salmon fish isfound in oxidative stress **[43]**. The antioxidant enzyme glutathione S-transferase (GST) inactivates high-capacity metabolic electrophilic compounds and toxic substrates, and it has been noticed to be activated by stress to protect the organisms against oxidative damage. The expression of glutathione S-transferase gene has been found to be induced in tissues of fish exposed to stressors **[44]**. Malate dehydrogenase converts malate and oxaloacetate using nicotinamide adenine dinucleotide (NAD) as a cofactor to generate reducing equivalents, and its expression has been found to be induced by oxidative stress in stress **[45]**. Glutamate dehydrogenase (GDH) converts glutamate to α-ketoglutarate, and vice versa. The reduced levels of GDH have been identified under stress in fish **[46]**. D-amino acid oxidase (DAO) triggers production of hydrogen peroxide upon acting on its substrates there by induces oxidative stress. Its activity has also been identified to be decreased upon exposure to stress.Uric acid is an antioxidant protein which is degraded

by urate oxidase (UOX). Knockdown of urate oxidase has been shown to increases oxidative stress in organisms **[47]**.

Methylenetetrahydrofolate dehydrogenase and sarcosine dehydrogenase synthesizesglycine which is a precursor of glutathione biosynthesis. Glutathione is a key reductant of endogenous peroxides, and its activity has been identified to be decreased in oxidative stress **[48]**. The hydroxysteroid dehydrogenase (HSD)converts cortisol to cortisone, and vice versa. The activity of HSD has been shown to beenhanced duringadaptation to stressin animals **[49]**. Cortisol activates and prepares the organism for an appropriate stress response by stimulating gluconeogenesis, proteolysis, lipolysis, and etc, and its levels have been found to be increased under stress **[50]**. The hydroxyacid oxidase is a peroxisomal enzyme that oxidizes glycolate to glyoxylate and produces H2O2, and it has been shown that hydroxyacid oxidase is down regulated in oxidative stress **[51]**. Aldehyde dehydrogenase involves in a variety of biological processes, and its expression is up-regulated in response to stress **[52]**. 3-hydroxybutyrate dehydrogenase is a short-chain dehydrogenase which catalyzes a rate-limiting step in the biogenesis of the siderophore, and inhibition of siderophore expression has been shown toresults oxidative stress and abnormal accumulation of intracellular iron **[53]**. Therefore, this suggests that these antioxidant response genes play a crucial role in fish under abiotic stress to clear ROS and to provide antioxidant defense against stress.

Homeostasis and MSR. Stress is a process of altered biochemical homeostasis, and it is caused by various physiological, or environmental stressors. Homeostasis is crucial for the general functioning of the body, and it is known to adversely affect health outcomes in organisms **[54]**. Besides, It has been shown that environmental stress can induce a quick, temporary response termed the cellular stress response and a more permanent response known as the cellular homeostasis response **[55]**. In GO term enrichment analysis, 7 up-regulated DEGs were found to be enriched significantly in cellular homeostasis, 35 up-regulated DEGs were identified to be enriched significantly in the homeostatic process, 17 up-regulated DEGs were found to be enriched significantly in cellular ion homeostasis, 5 up-regulated DEGs were found to be enriched significantly in tissue homeostasis, 22 up-regulated DEGs were found to be enriched significantly in chemical homeostasis. In module analysis

ofPPI network, the second gene module were found to be significantly enriched in hemostasis, also; it was identified that heat shock protein (HSP) was a top 10 hub protein of the network. It suggests that HSP is an important factor to maintain homeostasis in organisms. Besides, WGCNA indicated that brown module with 425 MSRGs was mainly enriched in homeostasis. Therefore, the enrichment of biological processes and pathways of the homeostasis suggest that they are necessary to respond and to re-establish homeostasis under multiple stress factors exposure. HSPs, an evolutionarily conserved proteins, are constitutively expressed and function asthe molecular chaperones in protein biogenesis and homeostasis **[56]**. Moreover, HSPs involves in cellular protection against different kinds of stress factors, and the transcription level of HSPsare altered byvarious environmental stressors **[57]**. Carbonic anhydrase plays an important role in systemic acid-base regulation and also it links the physiological responses to stress in fish. Increased gene expression of carbonic anhydrase has been observed upon stress **[58]**. In this study, we observed that exposure to multiple stressors caused the dysregulation of genes related to cellular homeostasis. Therefore, this suggests that these cellular homeostasis responsive genes play a crucial role in fish under abiotic stress to maintain homeostasis which is dysregulated upon exposure to a stressor.

. Cells have various protective mechanisms against stress, but acute stress may cause extensive DNA damage and cell apoptosis **[59]**. Generally, stressors like UV radiation, cold and high temperature are often involved in activating the apoptosis in organisms, and cell death has been reported in fish after exposure to stressors such as **[60]**. Apoptosis is a normal physiological process which removes old, excess, damaged and necrotic cells **[61]**. InGO term enrichment analysis, 34up-regulated DEGs are foundsignificantly enriched in programmed cell death. In KEGG pathway analysis, 8upregulated DEGs are identified to beenriched in the proteasome pathway, 8 upregulated DEGs are identified to be enriched inthe MAPK signaling pathway,and 9 downregulated DEGsare identified to be enriched in the PPAR signaling pathway. In the present study, several genes involved in apoptosis and cell survival including cd74, caspase 3, cathepsin, proteasome, Bcl2, interferon regulatory factor 1b(irf1b), protein kinase C, beta b(prkcbb), protein phosphatase 2A, serine/threonine kinase 3 were differentially regulated under multiple stress conditions The pro- and anti-apoptotic

proteins such as Bcl-2, Bcl-xL, Bak, and Bax are the Bcl-2 family members, and release cytochrome c from the mitochondria there by activate caspases **[62]**. Protein kinase C (PKC) family, which contains ten structurally related serine/threonine proteinkinases, is a key regulator of cell proliferation and survival. Protein kinase C expression has been identified to be altered under oxidative stress **[63]**. PP2A is a protein serine/threonine phosphatase that regulates various cellular processes such as cell-cycle progression, transcription, signal trans-duction, and cell differentiation, and ithas been found to be involved in oxidative stress mediated apoptosis **[64]**. The proteasomeis a multi-catalytic proteinase complex, and it involves in ATP/ubiquitin-dependent non-lysosomal pathway and cleaves peptides. psme1 (Proteasome activator subunit 1) gene encodes a protein that inhibits the activation of the proteasome. Mitogen activating phospho kinases (MAPK's) are important components of signal transduction pathways that transduce a variety of extracellular stress signals, and cellular stress induces MAPK that in turn regulates cell survival **[65, 66]**. Peroxisome proliferator-activated receptor (PPAR) activation has been noted to inhibit cell growth and induces cell apoptosis **[67]**. Hsp90, an abundant molecular chaperone, stabilizes and functionally regulates a variety of cellular proteins in response to stress has been found to be overexpressed under stress **[68]**. Repair or elimination of damaged cells or molecules regulates the survival of an organism under stress **[69]**. It has been found that cathepsin D mediates apoptosis induced by oxidative stress. **[70]**. It indicates that proteolysis is important to remove damaged cells when exposed to stress and it was observed to be altered in nearly all abiotic stresses. Therefore, it suggests that these genes play a crucial role to provoke apoptosis and cell survival biological processes under multiple stress conditions.

Involvement of the genes in contolling the immune response, endocrine response, carbohydrate metabolism, antioxidant response, stimulus response, homeostasis, and apoptosis when exposed tostress response, although not known previously, were clearly understood in this study.As fish exposed to multiple stressors simultaneously in their habitat due to natural causes or anthropogenic causes, meta-analysis of transcriptome and network analysis of co-expression modules of genes reveals a genetic network involving in multiple stress response mechanisms. Also, this study could be beneficial for future studies to focus on the identified core genes

and their biological systems and pathways for a better understanding of multiple stress responses in aquaculture fish species.

5. CONCLUSION

In this study, a novel approach has been adopted to delineate the intricasies of fish responses to abiotic stressors at the transcriptional level by usinga large amount of expression data of zebrafish. In the present study, investigation of transcription profiles from multiple stress samples by GO enrichment, KEGG pathway, PPI network, and WGCNA network approaches revealed the co-expression networks, biological processes,and pathways ofmultipleabiotic stresses. Our results showed the stress-responsive modules that have a similar expression pattern in almost all stresses. Besides, this study has explored multiple biological processes, intracellular signaling pathways and key genes that are potentially operating in the regulation of multiple abiotic stress in zebrafish. We are encouraged by the results of this study to apply these approaches to understanding furtherspecies and population-level adaptation to multiple environmental stresses.Considered together, the data furnish the information regarding novel and common genes to multiple abiotic stress factors, and it also provides information of mechanisms by which multiple abiticstressors impair the health of fish.

Competing interests:

No competing interests

Acknowledgments.

This research did not receive any specific grant from funding agencies in the public,commercial, or not-for-profit sectors.

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