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Comparative Transcriptomics-based Analysis of Common Carp (*Cyprinus carpio*) Kidney Tissues Reveals Differential Immune Responses at Two Temperatures

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ABSTRACT

Background: Common carp is the third most widely farmed fish, globally. It is tolerant to cold stress and resistant to EUS pathogenic infections, when it faces lower temperatures. In the present study, common carp was used as the research model to examine the interplay of temperature and immunity.

Methods: High-throughput transcriptome sequencing was used to study the differential gene expression profiles of kidney tissues, reared at two different temperatures; 30°C (control) and 20°C (experimental).

Result: Out of 588 differential expressed genes (DEGs), genes under GO terms for abiotic stress were categorised under oxidative stress, response to heat, mechanical stimulus and cold. Fifteen DEGs were found under the immune category, which out of which twelve were under the innate immune category, included 5 lectin family members and 1 tumor necrosis factor. The information generated in common carp would form a transcriptome resource for tolerance to abiotic stress. At the lower temperature studied, modulatory immune response, through the cytokine-cytokine interaction pathway, seems to be a major player, besides higher energy production, up-regulated stress responses, cell growth and survival. These results have the potential to be developed into bio-markers for temperature stress tolerance and modulatory immune responses in aquaculture pond production during the lower temperature conditions.

Key words: Abiotic stress, Cytokine-cytokine interaction, Differential gene expression, Low temperature, RNA-seq.

INTRODUCTION

Fishes frequently encounter changes in environmental conditions, which disrupt the balance between fish physiology and their surroundings and trigger a stress response. Temperature has a critical role in biological processes of fish (Gonen *et al.,* 2024) and due to the stress of regular and/or seasonal temperature changes, modulatory immune responses have evolved in fish occupying specific temperature niches (Goldspink *et al.,* 1995).

Common carp (*Cyprinus carpio*) is cultured across the globe in Asian and some European countries, ranging from temperate to tropical regions. And unlike other cyprinids carps, the common carp exhibits optimal growth between 20 and 30°C, making it well-suited for aquaculture due to their adaptable characteristics (Billard, 1995). It was observed that during the winter season, when the water temperature falls between 18 and 22°C, Epizootic Ulcerative Syndrome (EUS) infection is common in many fish species, as the host immunity gets suppressed in the susceptible hosts (Kumar *et al.,* 2020). However, common carp is recognised as a species naturally resistant to this disease (OIE, 2019). Pradhan *et al.* (2014) also reported that EUS pathogen was not found in common carp fry and larvae, even in the presence of other fish species getting infected. To study the innate immunity mechanism in common carp at this range of temperature, 20°C was chosen as one of the temperatures for the present study. The kidney, particularly the head kidney/anterior kidney, acts as the principal immune organ, involved in various immune functions (Deivasigamani, 2006; Sivasanker *et al.,* 2020)

Advancements in high-throughput transcriptome sequencing technology have led to the studies of global gene expression profiles in various fish species, *e.g.* in cold susceptible species (Tilapia) for immune and metabolic responses (OuYang *et al.,* 2023) and immunity related gene expression and in cold resistant species for the role of cold-adaptive responses (common carp) (Long

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et al., 2020). Since the ability of change in mRNA expression levels determines the immunological response to environmental stress, RNA sequencing has been frequently used for relevant investigations in fish (Wang *et al.,* 2020, Xu *et al.,* 2023) and other animals (Tianjiao *et al.,* 2023).

The present study aimed to identify comparative large-scale transcriptome responses, especially those involved in immune system in *C. carpio*, during low temperature acclimation (20°C) as compared to that at 30°C, which would be useful to reveal the mechanism of disease avoidance/ resistance, at the varying temperature conditions.

MATERIALS AND METHODS

Experiment design

C. carpio specimens (16-19 cm, 91-133 g) were collected from the fish germplasm resource centre of ICAR-NBFGR, Lucknow, India from the progeny of commercial breeding of the same brood stocks, in July month. Fish were randomly divided into two groups in circulatory water fibre-reinforced plastic tanks, each with 500 L of freshwater, with continuous aeration and photoperiod of ten hours' light, pH: 7.0±1, DO 4.5 to 5 mg/L. After the acclimation period at room temperature, step-cooling/heating (1±0.2°C/day) was carried out to 20±1°C (experimental)/30±1°C (control) and maintained for 45 days, in three replicates of 10 fishes each, with the same feeding regime and husbandry conditions. One day before sample collection, feeding was stopped.

Sample collection and RNA isolation

After the temperature exposures, fishes were euthanized with MS222 (Sigma Aldrich, USA) and kidney samples of five random biological replicates of each group were collected in RNAlater (Ambion, USA), snapped frozen in LN2 and stored at -80°C. Total RNA from kidney tissues were extracted based on the TRI-Reagent method (Rio *et al.,* 2010). RNA Integrity Number (RIN) of purified RNA was determined by Agilent Tape station (Agilent Technologies, USA) and libraries were prepared from RNA from five biological replicates of each group, using an Illumina Paired-end library sequencing kit (Illumina Inc., USA).

Transcriptome sequencing and assembly

Libraries were sequenced, through Illumina HiSeq2500 (Illumina Inc., USA) and the raw fastq reads were preprocessed and quality assessed using FastQC and MultiQC (Ewels *et al.,* 2016). The trimmed fastp reads were filtered for rRNA reads using bowtie2 v2.4.2 (Langmead *and* Salzberg, 2012) and *de-novo* assembled by Trinity v2.13.2 (Grabherr *et al.,* 2011). The assembled transcripts were further clustered using CD-HIT-EST (Huang *et al.,* 2010) and filtered by CPAT (Wang *et al.,* 2013) for protein-coding sequences extraction and long non-coding RNAs (lncRNAs) removal. Completeness of final transcriptome was assessed against Actinopterygii, Vertebrate and Eukaryote core genes databases, through BUSCO Orthology pipeline, embedded in gVolante (https://gvolante.riken.jp/).

Differential gene expression (DGE) estimation and their functional annotations

The final set of transcripts was quantified using RSEM v1.3.3.4 (Li and Dewey, 2011). The resulting abundance tables were imported into R using the bioconductor package 'tximport'. The 'regularized log' transformation in DESeq2 (Bray *et al.,* 2016) was used for principal component and clustering analysis. Differential expression analysis and normalizations were performed using DESeq2, p-value<0.05 with Log2FoldChange (Log2FC) >2.

The DEGs identified were analyzed for gene ontology (GO) terms associated with the annotated genes and molecular pathways for the mapped genes using DAVID v6.8 (https://david.ncifcrf.gov/). The genes under GO terms responses to abiotic stress (GO:0009628), metabolic process (GO:0008152) and signal transduction (GO:0007165) were identified using QuickGO: Annotation List (https://www.ebi.ac.uk/QuickGO/ annotations). The deciphering of network of immune genes and visualization of the functional grouping was according to the GO term and KEGG database (http://www.genome.jp/kegg/kaas/) with reference to zebrafish (p<0.05).

Validation of immune genes expression through quantitative real time PCR (qRT-PCR) analysis

The gene-specific PCR primers of housekeeping and target genes were designed for qRT-PCR analysis from the present study (Table 1). For cDNA preparation from the RNA samples, first strand cDNA was synthesized using Revert Aid H Minus First strand cDNA Synthesis Kit (Thermo Scientific, USA) using 1 µg of RNA, following standard protocols and qPCR analysis according to Mohindra *et al.* (2022). Primer efficiency (E) for these genes ranges from 1.9-2.5 and the specificities of the PCR amplifications were confirmed by melting curve analysis. The Ct values were derived using Light Cycler (TM)480 (Roche Diagnostic International Holding AG, Switzerland) software. Reference genes expression was evaluated with RefFinder (Xie *et al.,* 2023) and fold changes of gene expression at 20°C in comparison to control (30°C) were calculated using the $2^{-\Delta\Delta Ct}$ method, through Excel.

RESULTS AND DISCUSSION

Transcriptome assembly and functional annotation

In the present study, kidney transcriptome generated from two different temperature conditions represented a complete complement of expressed genes, including the major immune genes as well as the important pathways. A total of 84.47 Gb reads were generated and after trimming and removal of contaminants, high-quality paired-end reads and clustering resulted in 1.275 million transcripts, which confirmed 98.60% completeness of the transcriptome.

DEGs and analysis for functional enrichment

Differential gene expression analysis of common carp kidney tissues from 20°C experimental temperature versus

30°C water temperature (control) conditions revealed 588 DEGs and 253 and 335 genes were found upregulated and downregulated, respectively. Under biological processes, most significant GO terms (FDR<0.05) were DNA integration (GO:0015074), followed by transmembrane transport (GO:0055085) and intracellular signal transduction (GO:0035556), while under molecular functions, GO:0003676-nucleic acid binding followed by GO:0005509-calcium ion binding and GO:0005085-guanyl-nucleotide exchange factor activity and under Cellular component, GO:0016020-membranes and GO:0005886 plasma membrane.

Molecular pathway analysis of these DEGs identified 118 genes in Organismal Systems, with a maximum number of 41 genes under immune system; 76 in environmental information processing with 57 genes under signal transduction, 29 in genetic information processing with 9 genes under folding, sorting and degradation and 59 genes under metabolism with 16 genes under amino acid metabolism. A total of 56 genes were classified under cellular processes, with 26 genes under transport and catabolism.

Under pathway analysis, maximum DEGs were observed in signal transduction (57) and signalling molecules and interaction (28) under environmental information processing, followed by endocrine system (47) and immune system (41) under organismal systems. Under signal transduction and signaling molecules and interaction, maximum number of DEGs was observed in Calcium signaling pathway [PATH: ko04020], MAPK signaling pathway [PATH: ko04010], Apelin signaling pathway [PATH: ko04371], cAMP signaling pathway [PATH: ko04024], PI3K-Akt signaling pathway [PATH:ko04151] with DEGs 13, 13, 8, 8 and 8, respectively. Under metabolism,

Table 1: Primers list of target and housekeeping genes validated through qRT-PCR identified in differentially expressed genes of *Cyprinus carpio* kidney tissue exposed to 20°C and 30°C temperatures.

Gene	Primer id	5′→3′
Myb2	Cyca_ *Myb2*F	AGCACTTAAAGGGGCGGATT
	Cyca_ *Myb2*R	AAGTTTCTCATGCGCCTGGT
18s Rrna	Cyca_*18s Rrna*F	AAACGGCTACCACATCCAA
	Cyca_*18s Rrna*R	TTACAGGGCCTCGAAAGAGA
B-Actin	Cyca_B-*Actin*F	AGACATCAGGGTGTCATGGTTGGT
	Cyca_B-*Actin*R	CTCAAACATGATCTGTGTCAT
Kat2B	Cyca_*Kat2b*F	TCATTCTGCGCGTGATGAAG
	Cyca_*Kat2b*R	CAAGCCAACAAGCCACATGA
Anxa1a	Cyca_*Anxa1a*F	TGGATTCGGTCTTGGTTGAG
	Cyca_*Anxa1a*R	CCCCTGTGTTGTCTTGCATT
Errfi1a	Cyca_*Errfi1a*F	CGCTGGCTGCTTTAACAAAC
	Cyca_*Errfi1a*R	GATTGGTCGTGGTGGAATTG
Ep300a	Cyca_ *Ep300a*F	GCCATGAGCACAAGATGGAT
	Cyca_ *Ep300a*R	ATGCAGCGTTGGATGCTAAG
Rps	Cyca_*Rps*F	TTTATTACCCCGGCCATG
	Cyca_*Rps*R	TGTGGACCATTCCTTCAGCA
Hprt2	Cyca_*Hprt2*F	GCTCCTGTGTCGTGATCAAT
	Cyca_*Hprt2*R	CAGTTCGGTCCATGATGAGT
Eif5a	Cyca_*Eif5a*F	AGAGGAATGGAAGCTGGACA
	Cyca_*Eif5a*R	GGCATGCATCTTCTCTGTGA
Gapdh	Cyca_*Gapdh*F	TGTGACCTGATGCGACACAT
	Cyca_*Gapdh*R	GGTGCAAACATCCAACCAGA
Rpl7	Cyca_ *Rpl7*F	GCATGGGGTTATCCCAACTT
	Cyca_ *Rpl7*R	TCCCATATTGTCCGAGATGC
Immune genes	**Primer id**	**Sequence**
ccr7	DN101490-F	GTTGCAATCCGTTGTGTCCT
	DN101490-R	GCCTTCAAGGTCATCATTGC
ifi44	DN14921-F	GAGCAGGGAAGTCCAGCTTT
	DN14921-R	GATGCGGTATGCCATCAGTC
si:dkey-241l7.3	DN196-F	GACCTTGGAGGAAACCTTGC
	DN196-R	TTGCATCATGAGCACCAATC
ccr2	DN98467-F	GCAGCGTCTTCTTCATGGTC
	DN98467-R	AAACAAGGCCAGAGCCATTT

pathways of Arginine and proline metabolism [PATH: ko00330] and Purine metabolism [PATH: ko00230] contained 5 and 4 DEGs, respectively; and under cell growth and death category, apoptosis [path: ko04210] 6 DEGs. Thus, the present study revealed an increase in purine, arginine and proline metabolisms, which may play a vital role in countering cold stress. These pathways were reported to be involved in energy production and mitigating the effects of cold stress (Melis *et al.,* 2017, Xu *et al.,* 2018) and the results are in line with the earlier studies that fish tolerate cold temperature stress by regulating its metabolism in response to increased energy demand (Schleger *et al.,* 2021). Since the current study found that the cold temperature had no effect on the glycolysis or glycogenesis pathways, the genes involved in the purine, arginine and proline metabolism pathways appear to be important for energy production and to combat cold stress in common carp.

Molecular pathways identified under immune system category of organismal systems identified 40 DEGs, out of which 7 genes were under complement and coagulation cascades pathway, Chemokine signalling pathway and Platelet activation followed by 5 genes each under B cell receptor signaling, C-type lectin receptor signaling and T cell receptor signaling pathways and 5 genes under IL-17 signaling pathway (Table 2).

DEGs classification under abiotic stress

With reference to *Danio rerio* database, 5 GO terms with 22 DEGs were identified, with 10 DEGs under response to oxidative stress (GO:0001666, GO:0055093, GO:0071456), followed by 4 under response to heat (GO:0009408, GO:0034605), 5 under response to mechanical stimulus (GO:0009612, GO:0071260) and 2 under response to cold (GO:0009409, GO:0070417) (Fig 1). The most significant pathways identified were MAPK signalling, Focal adhesion and necroptosis for these genes. The s*tress*-regulated MAPK signaling pathway mainly involves kinases, which are reported to be mainly the mitogen-activated protein kinase (MAPK) family members (Cross *et al.,* 2000), that is involved in numerous significant cellular functions, including proliferation, differentiation and migration of cells (https://www.kegg.jp/pathway/hsa04010).. Interesting points of concern are significant upregulation of genes: Mitogen-activated protein kinase 4 (MAP4K4), cytosolic phospholipase A2 (cPLA$_{2\alpha}$) and Thrombospondin-1 (tsp-1). Up-regulation of MAP4K4 gene is indicative of positive stress response (Singh *et al.,* 2023) andcPLA$_{2\alpha}$ activation in renal epithelium has been suggested to promote renal repair by initiating partial de-differentiation, proliferation and migration (Montford *et al.,* 2016). and Third up-regulated gene under abiotic stress under the present study, that is *tsp-1* under colder conditions, which is a component of the PI3K-Akt signaling pathway, has been reported with a role in the immune system regulation (Bao *et al.,* 2022) and in adaptation to varying temperatures (Zhang *et al.,* 2021).

Differentially expressed immune and related genes

Molecular pathways identified under immune system category of organismal systems identified 40 DEGs, out of which 7 genes were under complement and coagulation cascades pathway, Chemokine signaling pathway and Platelet activation followed by 5 genes each under B cell receptor signaling, C-type lectin receptor signaling and T

Table 2: Molecular pathways identified under Immune system category of total differentially expressed genes in kidney tissue transcriptome of *Cyprinus carpio* during exposure to two temperatures, 20°C vs 30°C ($p<0.05$).

Level I	Level II	Level III	Up regulated genes	Down regulated genes	Total DEG count	Gene count in transcriptome
Organismal systems	Immune system	Chemokine signaling pathway	3	4	7	128
		Complement and coagulation cascades	4	3	7	66
		Platelet activation	3	4	7	88
		B cell receptor signaling pathway	2	3	5	53
		C-type lectin receptor signaling pathway	1	4	5	69
		T cell receptor signaling pathway	2	3	5	66
		IL-17 signaling pathway	1	3	4	58
		Fc gamma R-mediated phagocytosis	2	1	3	58
		Neutrophil extracellular trap formation	2	1	3	76
		NOD-like receptor signaling pathway	1	2	3	110
		Th17 cell differentiation	1	2	3	64
		Toll and Imd signaling pathway	-	3	3	27
		Toll-like receptor signaling pathway	-	3	3	60
		Antigen processing and presentation	1	1	2	30
		Fc epsilon RI signaling pathway	1	1	2	38
		Natural killer cell mediated cytotoxicity	1	1	2	57
		Th1 and Th2 cell differentiation	-	2	2	48

cell receptor signaling pathways and 5 genes under IL-17 signaling pathway (Table 2). Out of 40 DEGs, 15 were immune genes, majority (12) under innate immune categorywhile only 3 adaptive immune DEG were identified (Table 3). These genes fall under 9 GO terms, of which under biological process, immune response (GO:0006955); under cellular component under membrane (GO:0016020); under molecular function signaling receptor activity (GO:0038023), carbohydrate binding (GO:0030246) and GTP binding (GO:0005525) (Fig 2). Three DEGs were found under under cytokine-cytokine receptor interaction (CCRI) pathway and 2 each under Chemokine signaling pathway and Viral-protein interaction with CCR (Fig 3). In the current study, these results involving DE immune genes point to highly significant groups, with the largest number of genes are under Response to stimulus and CCRI pathways (Fig 4),

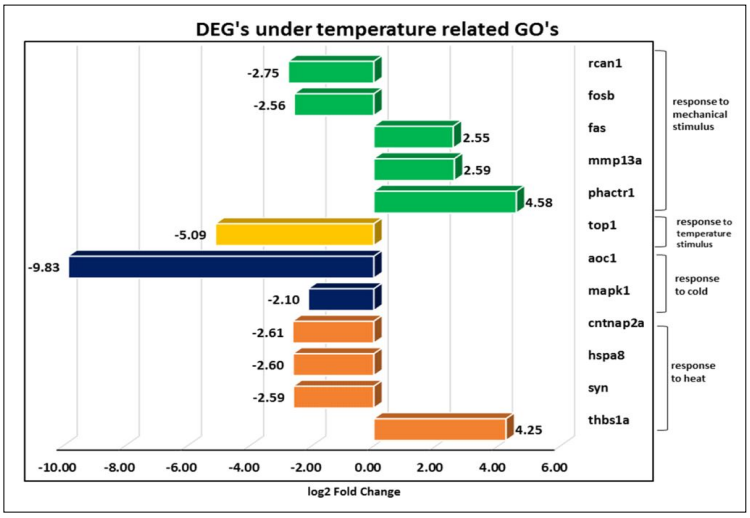

Fig 1: Temperature related DEG's identified under Gene Ontology term (GO:0009628) responses to abiotic stress of *Cyprinus carpio* kidney tissue exposed to 20°C and 30°C temperatures.

Table 3: Significant Immune differentially expressed genes list in kidney tissue of *Cyprinus carpio* during exposure to two temperatures, 20°C vs 30° (p<0.05).

Innate immune	Description	log2FC	P adj (FDR)
Chemokine			
loc109070792	C-C chemokine receptor type 1-like	3.58	0.03
ccr2	C-C chemokine receptor type 2-like	2.98	0.03
Complement system			
c7b	Complement component 7b	4.51	0.03
Interferon			
mxd	Interferon-induced GTP-binding protein Mx2-like	6.66	1.00E-02
loc795887	Interferon-induced protein 44-like	5.19	1.20E-02
loc100007727	Interferon-induced very large GTPase 1-like	2.16	0.05
Lectin family member			
loc110438235	Galactose-specific lectin nattectin-like	21.89	6.07E-11
si: dkey-241l7.3	Ladderlectin-like	2.68	0.04
lgals2a	Galectin-2-like	-2.22	0.03
lman2la	Lectin, mannose-binding 2-like a isoform X1	-2.61	0.03
loc110438301	L-rhamnose-binding lectin CSL2-like	-4.18	0.03
Tumour necrosis factor			
loc109053146	Tumor necrosis factor receptor superfamily member 17-like	2.55	0.05
Adaptive immune			
Immunoglobulin			
igsf9bb	Immunoglobulin superfamily, member 9Bb	-2.49	0.01
igsf10	immunoglobulin superfamily member 10-like	-4.24	0.04
MHC class I			
Cyca-UA	MHC class I antigen	7.44	0.03

which are interlinked and share most of the associated DEGs. CCRI, one of the vital immunity pathways, has a crucial role in the inflammatory and defense process of the host (Cheng *et al.,* 2017). In an earlier study in transgenic zebrafish under cold stress (Wang *et al.,* 2014), where CCRI genes were mostly down-regulated. However, in the current study, up-regulated CCRI genes (*ccr1, 2*) at 20°C might be providing the innate immune response in

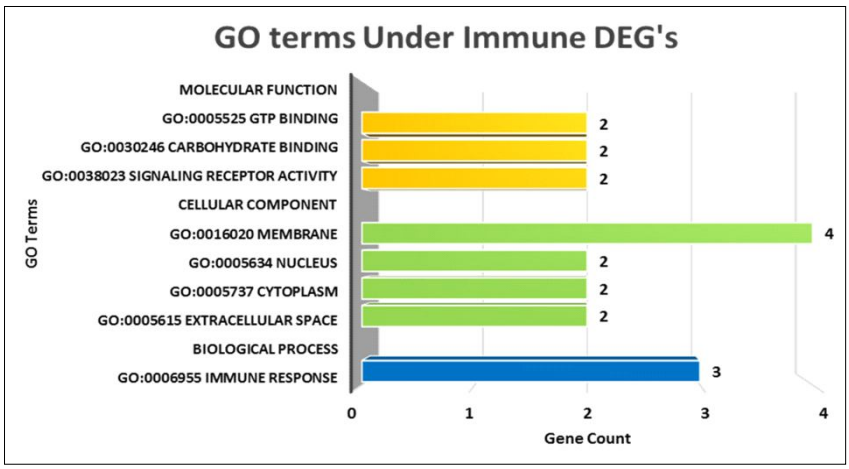

Fig 2: Gene Ontology terms identified in differentially expressed immune related genes in kidney tissue of *Cyprinus carpio* during two temperatures, 20°C and 30°C.

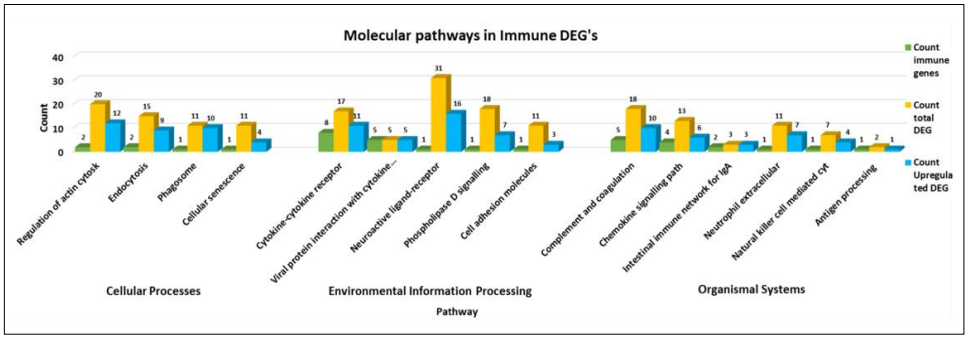

Fig 3: Significant molecular pathways identified in immune and immune related differentially expressed genes of *Cyprinus carpio* kidney tissue exposed to 20°C and 30°C temperatures.

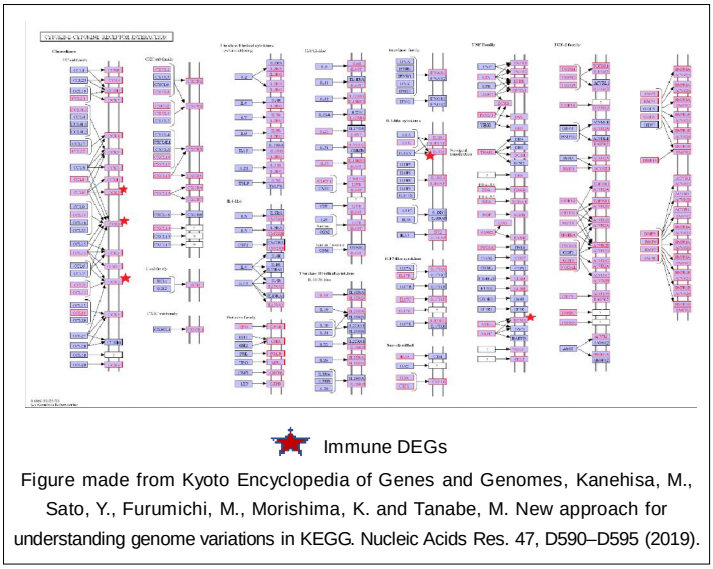

Fig 4: Molecular pathway Cytokine-cytokine receptor interaction identified in differentially expressed immune related genes of *Cyprinus carpio* kidney tissue exposed to 20°C and 30°C temperatures [20±1°C (experimental group)/30±1°C (control group)].

common carp. Verma *et al.,* (2021) also reported the chemokine signaling pathway to be a critical pathway, with the innate immune gene *ccr2* up-regulation in the early and middle stage infection of oomycete, *A. invadans* in common carp. It is fascinating to find in the present study, a very high expression of another innate immune gene, complement component 7b, identified under C-C chemokine binding, which performs function in host defence against pathogens and promotion of inflammation through complement and coagulation pathway (Bossi *et al.,* 2009). Thus, the relative higher expression of the immune genes indicates enhanced pathogen defense (Philominal *et al.,* 2025).

Validation of expression pattern of immune genes

Out of the 11 genes*, rps20, hprt, eif5a, rlp7, b-actin, 18s-1, myb-2, anxa1a, errif1a, kat2b-1* and *ep300a-1* tested for their stability in expression, *eif5a* was found to show most stable expression, so it was utilized as the house keeping gene, for normalization of Ct values of target genes. The target genes, *ccr2, si:dkey-241l7.3, cc7* and *ifi44/loc795887* were tested and all these genes showed similar trend in the expression patterns for transcriptome and qRT-PCR experiments, except *ccr7* at 20°C.

Thus, the present transcriptomic study points out that cold resistant common carp can modulate the cold stress positively and also its immune responses, which may facilitate the resistant responses to the disease, to which cold susceptible species succumbed to. However, deep studies are required for the combinations of these factors and processes, to reveal overall modulatory effects.

CONCLUSION

The information generated in common carp, a cold resistant species, in the present study, would form a transcriptome resource for tolerance to abiotic stress, along with information on possible modulatory mechanisms, when it encounters lower temperature conditions. These strategies include arginine and proline metabolism essential for energy production and thus mitigating the effects of cold stress. Other important mechanisms to overcome temperature stress response were by MAP4K4 signal transduction and PI3K-Akt signaling pathways. At a low temperature, upregulation of most genes of innate immune response in the cytokine-cytokine interaction seem to be a major player. These results have the potential to be developed into biomarkers for temperature stress tolerance and modulatory immune responses in common carp in aquaculture pond production during the lower temperature conditions. However, in-depth protein studies are needed to support the results obtained through present transcriptome studies.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All the protocols followed were approved by the Institute Animal Ethics Committee (IAEC), ICAR-NBFGR, Lucknow, India, vide G/IAEC/2022/6, Dated 25-05-2022. All the methods were performed in accordance with the relevant guidelines and regulations.

Conflict of interest

The authors declare that there is no conflict of interest.

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