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Integrated bioinformatics and qRT-PCR analyses identify candidate genes associated with seasonal thermal variation in *Rutilus frisii* (Pisces: Leuciscidae)

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ABSTRACT

Heat stress adversely affects fish physiology and survival, underscoring the need to identify heat-stress candidate biomarkers for conservation and aquaculture management. This study aimed to identify such biomarkers and characterize gene regulatory networks associated with thermal adaptation in *Rutilus frisii*. Since no transcriptomic datasets were available for this non-model species, a comparative transcriptomic approach was used, drawing on publicly available datasets from zebrafish, *Danio rerio* and common carp, *Cyprinus carpio*. Differential expression analysis, functional enrichment, and protein interaction network analyses were conducted to identify key heat-responsive genes and hub regulators. Candidate genes were validated by quantitative real-time PCR (qRT-PCR) in liver tissues from male and female broodstock *R. frisii* collected during the spawning season. Functional enrichment analysis indicated significant involvement of biological processes related to stress response, protein folding, immune regulation, and cellular homeostasis. Experimental validation confirmed significant differential expression of *hsp90ba, grp94, hsp90aa1*, and *Trap1*, aligning with bioinformatic predictions. These results indicate that these genes serve as candidate heat-stress-responsive markers and provide a preliminary molecular framework for broodstock monitoring and conservation of *R. frisii* populations under climate change scenarios.

Keywords: *Rutilus frisii*, Heat shock proteins, Hub genes; Gene regulatory network, Thermal stress.

INTRODUCTION

Previously, *Rutilus frisii kutum* was recognized as a subspecies belonging to the carp family (Cyprinidae). *Rutilus kutum* has been reported as a species inhabiting the Black and Caspian Seas. In recent years, *Rutilus frisii*, belonging to the minnow family (Leuciscidae), has been classified as a species in the National Center for Biotechnology Information (NCBI), one of the world's most important biological databases.

In addition to its revised taxonomic status, *R. frisii* is the most commercially significant bony fish species in the Southern Caspian Sea basin. It constitutes over 70% of the annual commercial catch along the Iranian coastline, making it a vital component of the regional fisheries sector (Farasati *et al*. 2025b). The high market demand for *R. frisii* is attributed to its meat quality and its central role in local culinary traditions (Moezzi & Eagderi 2024).

R. frisii spawns during late winter and spring, mainly from April to May (Freyhof *et al*. 2025). Although *R. frisii* is considered a flagship species, its natural populations are increasingly threatened by habitat degradation, overfishing, pollution, reduced genetic diversity, and unstable ecological conditions. The autumn strain (AS) is particularly endangered, comprising less than 2% of total stocks, largely due to degradation of Anzali Wetland

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and phytophilous spawning grounds, while spring populations are also declining. Among these threats, climate change and rising water temperatures are of particular concern since they can alter migration and spawning phenology and impair metabolism, biochemical processes, and physiological homeostasis, thereby threatening reproductive success and increasing the risk of irreversible loss of genetic resources (Hossain Aunkor *et al*. 2025). Ecological modeling has further indicated significant shifts in the environmental niche of *R. frisii* in response to changing thermal regimes in the southern Caspian basin (Moëzzi *et al*. 2024). More broadly, anthropogenic climate change has substantially altered thermal regimes in the Southern Caspian Sea and poses increasing challenges to aquatic ecosystems (Reid *et al*. 2019). As an ectothermic species, *R. frisii* is particularly vulnerable to such thermal fluctuations, which can impose chronic physiological stress and compromise cellular integrity and population persistence (Hossain Aunkor *et al*. 2025).

At the cellular level, the primary and evolutionarily conserved defense mechanism against thermal stress is the induction of Heat Shock Proteins (HSPs; Akbarzadeh *et al*. 2018). These proteins act as essential molecular chaperones, maintaining cellular proteostasis by facilitating proper protein folding, refolding denatured polypeptides, and preventing the accumulation of cytotoxic protein aggregates under environmental stress (Swirplies *et al*. 2019; Hossain Aunkor *et al*. 2025). In teleosts, major HSP families such as HSP70 and HSP90 are especially important, orchestrating a multi-layered defense system that includes managing signal transduction and preserving the integrity of the endoplasmic reticulum and mitochondria during severe thermal stress (Hassan *et al*. 2019; Malik & Kim 2021). Although individual expression of these chaperones is a recognized marker of thermal adaptation, their function as interconnected master regulators within complex gene regulatory networks (GRNs) remains insufficiently characterized in many non-model species. Elucidating the synergistic interactions among these proteins is crucial to understanding how species such as *R. frisii* maintain physiological stability amid rising temperatures in the Caspian Sea.

A key biological factor influencing the thermal response of *R. frisii* is the presence of two distinct seasonal populations: the spring strain (SS) and the autumn strain (AS). These populations employ different spatiotemporal spawning strategies and encounter markedly different thermal gradients during migration to Anzali Wetland and regional rivers (Farasati *et al*. 2025b). Although the role of individual HSPs in thermal stress is well established, it remains unclear whether these seasonal strains have developed divergent molecular strategies at the network level to adapt to their respective thermal environments. Previous studies have primarily examined isolated gene expression, resulting in a significant knowledge gap regarding the complex gene regulatory networks (GRNs) and master regulators (hub genes) that govern proteostasis in this species. Addressing this regulatory landscape is particularly urgent, as AR stocks are now highly endangered, comprising less than 2% of total stocks (Valipour & Khanipour 2009).

To resolve the biological enigma of seasonal strains and their divergent resilience, the present study employs an integrated transcriptomic and systems-biology pipeline. Recognizing that the *R. frisii* is a non-model species with limited genomic infrastructure, we first adopted a comparative genomics strategy, mining high-throughput datasets from related model cyprinids, including zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*), to identify the core components of the teleostean thermal response (Farasati *et al.* 2025b). Moving beyond traditional single-gene analysis, we reconstructed complex Gene Regulatory Networks (GRNs) using advanced computational algorithms, including Bayesian networks (BNArray) and Structural Equation Modeling (SEM), to pinpoint the master regulators—or hub genes—that drive cellular proteostasis under thermal load (Malik & Kim 2021). By implementing an advanced pipeline that combines transcriptomic data mining with Structural Equation Modeling (SEM), we identified a core set of four hub genes—*hsp90ba*, *Grp94*, *hsp90aa1*, and *Trap1*—that act as master regulators of cellular proteostasis. A significant contribution of this study is the comparative molecular analysis of seasonal strains (spring and autumn), providing the first evidence of divergent expressional plasticity between these two populations during their critical spawning migration. Ultimately, this research provides a validated biomarker panel for real-time broodstock health monitoring and strategic thermal management in hatcheries, offering an essential tool for the conservation of this 'conservation-dependent' species in the face of global climate change.

We hypothesized that heat stress induces a conserved, HSP90-centered chaperone interaction network across related cyprinid species, that selected candidate genes (*hsp90ba*, *Grp94*, *hsp90aa1*, and *Trap1*) would exhibit divergent, sex- and season-specific expressional plasticity in wild *R. frisii* exposed to contrasting natural thermal profiles during spawning migration.

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2. MATERIALS AND METHODS

2.1. Ethics Statement

All experimental procedures involving *R. frisii* were conducted in accordance with ethical guidelines for the care and use of aquatic animals. The experimental protocol was reviewed and approved by the Ethics Committee of the University of Guilan, Iran (Approval No. IR.GUILAN.REC.1404.160).

2.2. Study design

This study employed an integrated bioinformatics and experimental validation workflow to identify heat stress-associated biomarkers and gene regulatory networks related to heat shock proteins (HSPs) in *R. frisii*. Public transcriptomic datasets from closely related cyprinid fish were analyzed to identify candidate genes responsive to thermal stress (Figs. 1 and 2). The resulting candidate biomarkers were subsequently validated by quantitative real-time PCR (qRT-PCR) using liver tissues collected from wild *R. frisii* broodstock (Akbarzadeh *et al.* 2018).

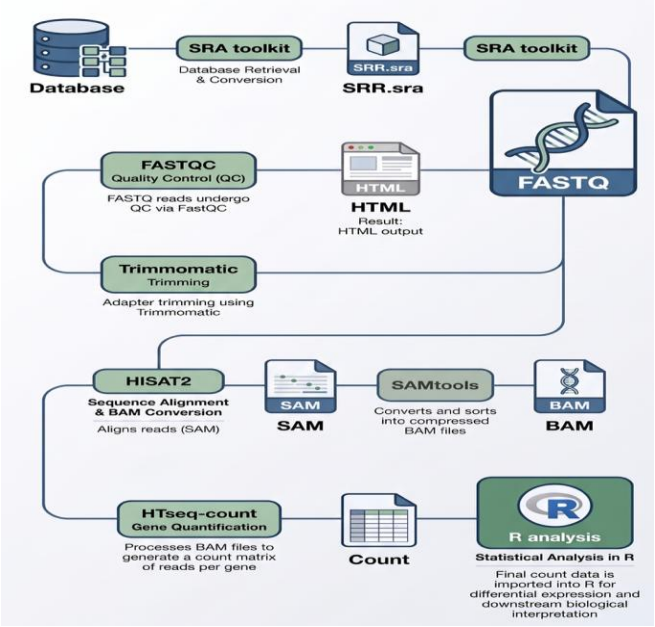

Fig 1. Comprehensive bioinformatics pipeline for RNA-Seq data processing and differential expression analysis.

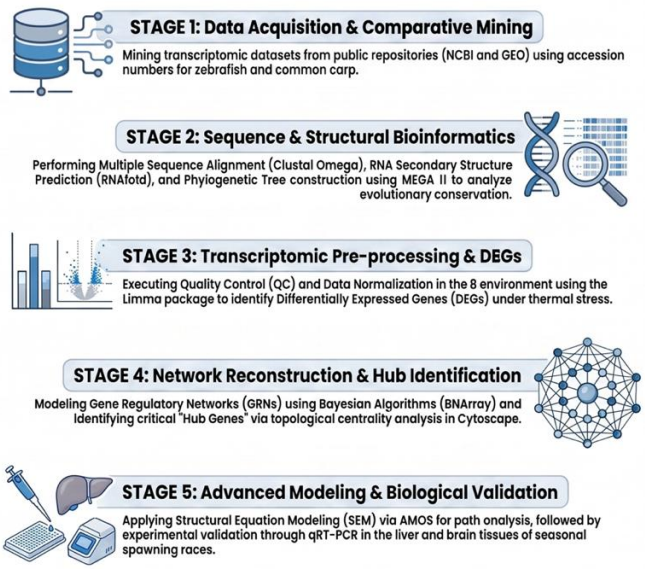

Fig. 2. Experimental framework for the molecular validation of identified hub genes in *R. frisii*

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2.3. Transcriptomic datasets

Two publicly available transcriptomic datasets were retrieved from the NCBI Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) databases. The first dataset (GSE220546) contained RNA-seq data from heat-stressed zebrafish (*Danio rerio*) embryos. In contrast, the third dataset (PRJNA1432439) consisted of RNA-seq data from common carp (*Cyprinus carpio*) exposed to acute heat stress. These species were selected, since *R. frisii* currently lacks publicly available transcriptomic datasets. In contrast, it belongs to the order Cypriniformes and shares conserved stress-response pathways (Table 1).

Table 1. Summary of high-throughput transcriptomic datasets used for cross-species data mining.

Feature	Dataset I (GSE220546)	Dataset II (PRJNA1432439)
Species	Zebrafish (*Danio rerio*)	Common carp (*Cyprinus carpio*)
Developmental stage	Embryo (1 day post fertilization)	Adult (4 months old)
Target tissue	Whole embryo	Intestine
Control temperature	27.0 °C	28.0 °C
Heat temperature	32.0 °C	38.5.0 °C
Replicates (n)	12 (6 Control / 6 Heat)	6 (3 Control / 3 Heat)
Sequencing platform	Illumina NovaSeq 6000	Illumina HiSeq 2500
Reference genome	GRCz11 (NCBI)	GCA_000951615.2 (NCBI)

2.4. Bioinformatics analysis

Raw transcriptomic data were processed using the R statistical environment. Quality assessment, normalization, and differential expression analyses were performed using standard RNA-seq workflows. Differentially expressed genes (DEGs) were identified according to adjusted *p*-value and fold-change thresholds. Genes belonging to the HSP family and their associated regulatory genes were extracted for downstream analyses.

2.5. Functional enrichment analysis

Functional annotation of significant DEGs was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses to identify biological processes and signaling pathways associated with heat stress.

2.6. Protein-Protein Interaction (PPI) network construction

To understand the functional interactions of the candidate genes, a Protein-Protein Interaction (PPI) network was reconstructed using the STRING database (v12.0). Associations were filtered using a high-confidence threshold (confidence score ≥ 0.7). The network was visualized in Cytoscape (v3.10.4). Highly connected candidate nodes (hubs) were identified and ranked using the cytoHubba plugin, incorporating topological algorithms including Maximal Clique Centrality (MCC), Degree, and Betweenness Centrality.

2.7. Experimental validation

R. frisii broodstock were collected during the spawning season. Water temperature was measured at the time of fish collection using both a mercury thermometer and a digital thermometer. The recorded water temperature was 19.3 °C during the Spring Strain sampling and 13.9 °C during the Autumn Strain sampling. Liver tissues were excised immediately after sampling, preserved in RNAlater, and stored at −80 °C until analysis. Total RNA was extracted using extraction kits. Total RNA was extracted from liver tissues using the TRIzol reagent (Thermo Fisher Scientific, USA). RNA concentration and purity were determined spectrophotometrically using a NanoDrop ND-1000, yielding an average concentration of 350 ± 45 ng μL^{-1} with 260/280 ratios between 1.85 and 2.02. RNA integrity was verified on a 1.2% agarose gel. To prevent genomic DNA contamination, 1ug of total RNA was treated with 1 µg of DNase I (RNase-free, Fermentas, USA) at 37 °C for 30 min, followed by enzyme inactivation at 65 °C for 10 min using 2.5 mM EDTA (Mohammady *et al*. 2021). To ensure precise orthology assignment in teleosts, reciprocal BLASTp searches were performed against the NCBI non-redundant database. To avoid cross-amplification of co-orthologs or duplicate paralogs, qRT-PCR primers for *hsp90ba*, *Grp94*, *hsp90aa1*, and *Trap1* were specifically designed targeting highly divergent 3'-untranslated regions (3'-UTR) or unique exon-exon junctions.

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2.8. Quantitative real-time PCR (qRT-PCR)

Expression levels of selected candidate genes were quantified using SYBR Green-based real-time PCR. *β-actin* was used as the internal reference gene, and relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Table 2. Primer sequences.

Primer	(5' → 3') sequence	Product size (bp)
B-Actin **F**	GCCACGGCTGGAGACACTCATC	180
B-Actin **R**	CCTGGCCTCCCTGTCTATCTTCC	
hsp90ba**F**	GCCACGGCTGGAGACACTCATC	240
hsp90ba**R**	CGAGCTCTGGTGATGGACGTG	
Grp94 **F**	CTGGGATCATGAGGCGACTGGGATA	220
Grp94 **R**	CTGCTCGAGCTACAGCATCTAGCTGTG	
hsp90aa1 **F**	CTGGGATCCTTGACTACCTTGAGCTGGA	190
hsp90aa1 **R**	GTCTGAGCTACAGCTCACTTTGCCTTG	
Trap1 **F**	GGCATAAGACACACCTGGAGA	210
Trap1 **R**	GCGCTAGTTACGGATCTCCAAGT	

The transcriptional stability of *β-actin* as an internal reference was validated across all seasonal and sex groups. Ct values of *β-actin* exhibited outstanding stability with no statistically significant variations among the experimental groups ($p > 0.05$; Mean Ct = 22.14 ± 0.35), confirming its suitability as a reference gene.

2.9. Statistical analysis

To evaluate the main effects of season (Spring vs. Autumn), sex (Male vs. Female), and their interactions on the relative mRNA expression of the candidate genes, a two-way analysis of variance (Two-Way ANOVA) was performed. Homogeneity of variances and normality were verified using Levene's and Shapiro-Wilk tests, respectively. Significant differences among groups were determined using Tukey's post hoc test. Statistical significance was defined as $p < 0.05$.

3. RESULTS

3.1. In Silico Transcriptomic Analysis

3.1.1. Functional enrichment analysis of candidate genes

The transcriptomic analyses were performed to characterize the molecular response to thermal stress and to identify conserved stress-responsive genes across cyprinid fish species. We analyzed two independent transcriptomic datasets, comprising heat-stressed zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*), to identify differentially expressed genes and functionally enriched biological processes. The resulting gene sets were subsequently examined with particular emphasis on the heat shock protein (HSP) family, followed by functional enrichment analysis and comparison of the major biological pathways activated under thermal stress. Finally, selected HSP-related hub genes identified from the comparative analysis were considered for subsequent validation in *R. frisii*

Table 3. Summary of datasets used for cross-species meta-analysis.

Feature	Dataset I (GSE220546)	Dataset II (PRJNA1432439)
Model species	Zebrafish (*Danio rerio*)	Common carp (*Cyprinus carpio*)
Total DEGs	1731	318
Up-regulated genes	1649	312
Down-regulated genes	81	6
Key hub genes identified	*diaph3, iqgap3, rock1, tfr1a* [159]	*serpinh1b, hsph1, dnajb4, atox1*
Primary functional focus	Cytoskeletal organization, cell signaling	Proteostasis, chaperone activity, autophagy

3.1.2. Visual profiling of cyprinid thermal response

The transcriptomic landscape of each dataset was presented through a four-grid, multi-panel figure (Figs. 1 and 2), ensuring data quality and illustrating expression shifts.

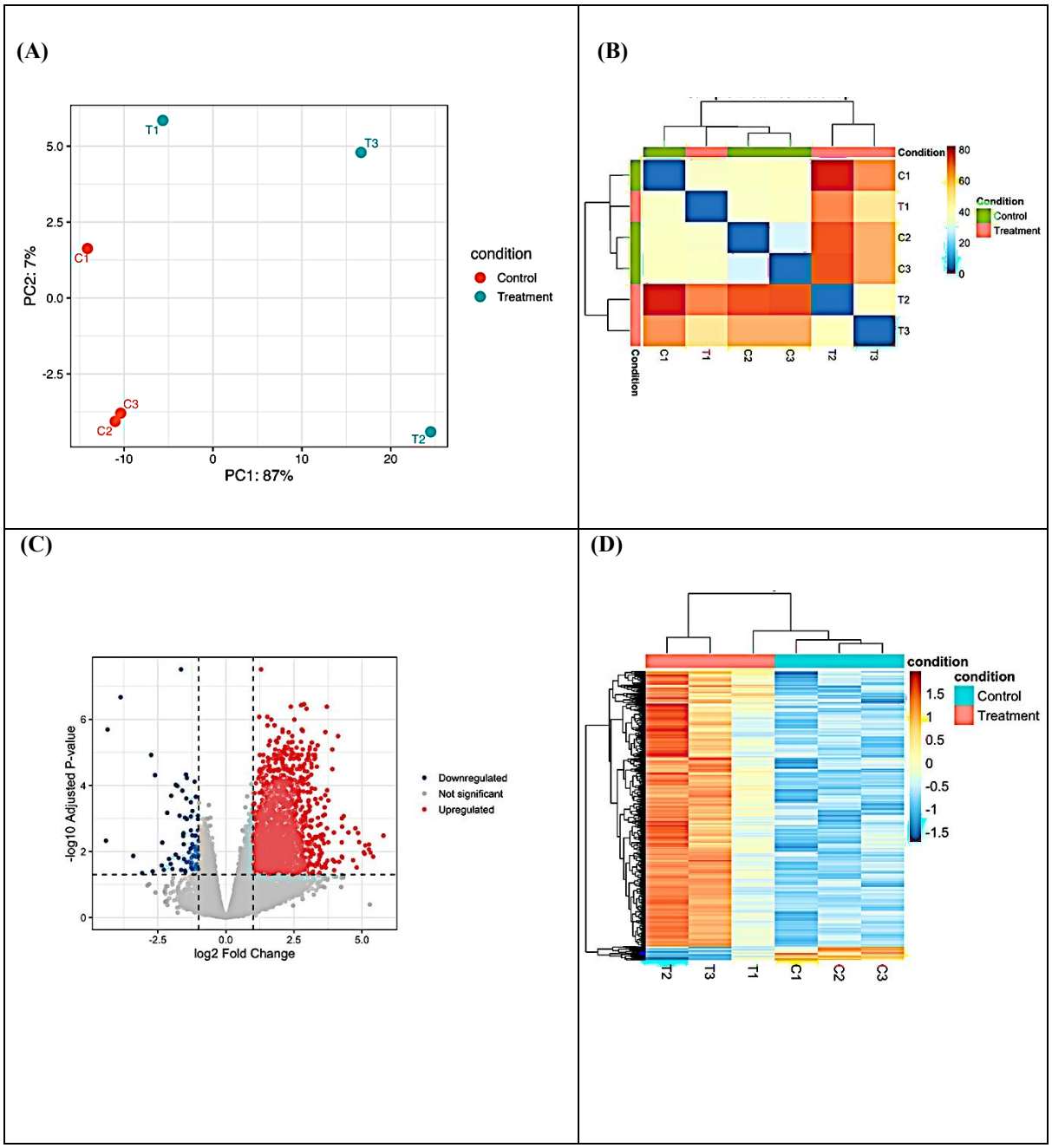

Fig. 3. (A) Principal Component Analysis (PCA) plot illustrating the distinct clustering of samples based on their treatment groups; (B) Sample distance heatmap showing the hierarchical relationship and correlation between biological replicates; (C) Volcano plot representing the distribution of differentially expressed genes (DEGs); red and blue/green dots indicate significantly up-regulated and down-regulated transcripts, respectively (|log2FC| > 1, Padj < 0.05); (D) Expression heatmap of target genes, where color intensity represents the Z-score of normalized expression levels.

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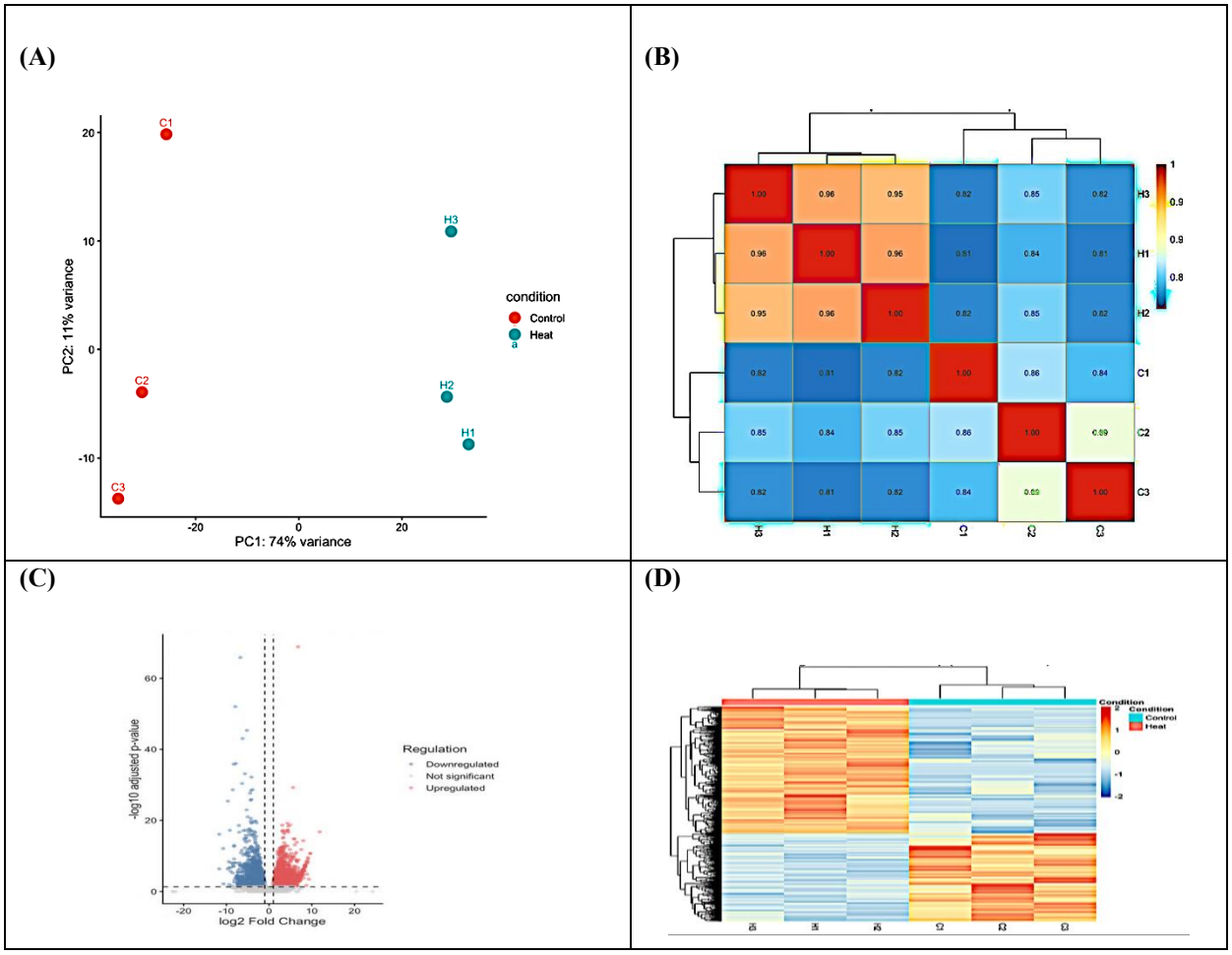

Fig. 4. (A) Principal Component Analysis (PCA) plot illustrating the distinct clustering of samples based on their treatment groups; (B) Sample distance heatmap showing the hierarchical relationship and correlation between biological replicates; (C) Volcano plot representing the distribution of differentially expressed genes (DEGs); red and blue/green dots indicate significantly up-regulated and down-regulated transcripts, respectively (|log2FC| > 1, Padj < 0.05); (D) Expression heatmap of target genes, where color intensity represents the Z-score of normalized expression levels.

3.1.3. Expression Profiling of the HSP Family across Datasets

Table 4. Differentially expressed HSP family genes identified through meta-analysis and their functional roles.

Dataset	Gene symbol	Regulation	Functional Role (Molecular Description)
GSE220546			Molecular chaperone; prevents protein aggregation and aids in refolding denatured proteins.
(Zebrafish - Heat)	*hsp90aa1.1 / .2*	Up	Cytosolic chaperone; stabilizes target proteins and coordinates signaling pathways.
	hspb9	Up	Small HSP; provides immediate protection against thermal proteotoxicity.
PRJNA1432439	*serpinh1b*	Up	Collagen-specific chaperone (HSP47); essential for proper folding of procollagen in the ER.
(Common carp - Heat)	*hsph1*	Up	Nucleotide Exchange Factor; acts as a co-chaperone to accelerate the HSP70 folding cycle.
	hspb6	Up	Small HSP; stabilizes the cytoskeleton and inhibits early-stage apoptosis.
	dnajb4 / dnajc24	Up	HSP40 co-chaperones; stimulate HSP70 ATPase activity for efficient protein rescue.
Selected hubs	*hsp90ba*	Up	Master regulator of cytosolic protein folding and stability in the liver.
Rutilus frisii	*Grp94*	Up	ER-resident chaperone; manages the unfolding protein response (UPR) in the liver.
	hsp90aa1	Up	Highly inducible cytosolic chaperone; buffers cells against thermal mutations.
	Trap1	Up	Mitochondrial HSP90; maintains mitochondrial integrity and prevents ROS-induced damage.

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3.1.4. Comparative functional enrichment (GO/KEGG)

To understand the biological mechanisms, a comparative functional analysis was performed. While life stages influenced the specific pathways, a conserved "proteostasis core" was evident in both datasets.

Table 5. Comparative functional enrichment (GO/KEGG) of thermal stress response in zebrafish and common carp datasets.

Category	Enriched Term	Zebrafish (GSE220546)	Common carp (PRJNA1432439)
Biological Process (BP)	Phosphorylation	105	12
	Cell adhesion	60	-
	Translation	-	11
	Multicellular organism development	-	14
	Intracellular signal transduction	50	-
	Autophagy	-	4
Molecular Function (MF)	Metal ion binding	340	-
	Nucleotide binding	170	-
	Protein binding	-	12
	Structural constituent of ribosome	-	10
	Kinase activity	110	-
Cellular Component (CC)	Membrane	505	8
	Cytoplasm	455	-
	Ribosome	-	9
	Cytoskeleton	100	-
	Endoplasmic reticulum	-	6
KEGG Pathway	Metabolic pathways	109	-
	Autophagy - animal	-	4
	MAPK signaling pathway	50	-
	TGF-beta signaling pathway	-	2.5

The comparative analysis, structured by dataset (Table 5), highlights a divergent scale of transcriptomic activation. The Zebrafish embryo dataset (GSE220546) exhibited a high-magnitude response, particularly in membrane (505 genes) and metal ion binding (340 genes), reflecting intense structural reorganization. In contrast, the adult common carp (PRJNA1432439) showed a more specialized and lower-count profile, focusing on protein quality control machinery, including ribosomal integrity and autophagy. This shift from 'structural development' in embryos to 'homeostatic recycling' in adults provides the biological rationale for using these datasets as complementary models to identify the core HSP hub genes.

3.1.5. Comparative Gene Ontology (GO) enrichment analysis

The functional significance of the differentially expressed genes (DEGs) was evaluated through Gene Ontology (GO) enrichment across three categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC).

Table 6. Comparative GO enrichment analysis between zebrafish (GSE220546) and common carp (PRJNA1432439) datasets.

GO category	Zebrafish (GSE220546)	Common carp (PRJNA1432439)	Comparative Insight
Biological process (BP)	Phosphorylation, cell adhesion, intracellular signal transduction, actin cytoskeleton organization.	Multicellular organism development, translation, autophagy, response to growth factor stimulus.	Zebrafish embryos focus on structural remodeling, while adult carp focus on proteostasis and cellular recycling.
Molecular function (MF)	Metal ion binding, protein kinase activity, transferase activity, nucleotide binding.	Protein binding, structural constituent of ribosome, actin binding.	Emphasis shifts from kinase signaling in Zebrafish to ribosomal integrity in Common Carp.
Cellular component (CC)	Cytoplasm, plasma membrane, cytoskeleton, membrane.	Ribosome, membrane, endoplasmic reticulum, lysosome.	Thermal stress primarily impacts the cytoskeletal framework in embryos and protein synthesis machinery in adults.

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The GO enrichment analysis revealed a distinct, life-stage-dependent strategy for managing thermal stress within the Cyprinidae family. In the Zebrafish embryo dataset (GSE220546), the biological processes were dominated by actin cytoskeleton organization and cell adhesion, reflecting a critical need to maintain morphological integrity during rapid developmental phases under heat. Conversely, the adult common carp dataset (PRJNA1432439) exhibited a shift toward autophagy and ribosomal structural components, indicating a prioritized investment in protein quality control and the degradation of damaged cellular debris.

3.2. Experimental validation in *Rutilus frisii*

R. frisii broodstock were collected from Anzali Wetland and a river in the southwest of the Caspian Sea basin, Guilan Province, Iran during the 2025–2026 spawning season. At the time of sampling, the recorded environmental water temperatures were 13.9 °C for the Autumn Strain and 19.3 °C for the Spring Strain. A total of 12 broodstock were included in the experimental validation, comprising six individuals from the Spring Strain (three males and three females) and six individuals from the Autumn Strain (three males and three females). Liver tissues were excised immediately after sampling, preserved in RNAlater solution, and stored at −80 °C until RNA extraction. Each biological sample was analyzed in three technical replicates during qRT-PCR analysis, and the mean Ct value was used for statistical analysis.

3.2.1. Functional enrichment analysis of candidate genes

To validate the biological relevance of the identified candidate genes in *R. frisii*, Gene Ontology (GO) enrichment analysis was performed using the differentially expressed genes identified from liver tissue. A total of 46 significantly enriched biological processes were detected. The most overrepresented categories were associated with response to external stimulus, immune system processes, and antigen processing, indicating that thermal stress activates multiple defense and adaptive pathways in the liver.

Table 7. Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in the liver of *R. frisii*

Category	GO term / Biological process	*p*-value	FDR
Biological process (BP)	Antigen processing and presentation	5.50E-09	5.40E-07
	Immune system process	5.20E-08	5.40E-07
	Response to external stimulus	4.80E-08	2.00E-06
	Response to wounding	3.30E-07	1.10E-05
	Response to chemical stimulus	2.20E-06	2.60E-05
	Monocarboxylic acid metabolic process	1.80E-06	3.20E-05
	Carboxylic acid metabolic process	7.10E-04	5.80E-03
	Organic acid metabolic process	7.40E-04	5.80E-03
	Biological quality regulation	7.10E-04	5.80E-03
	Multicellular organismal process	7.30E-04	5.80E-03
	Cellular ketone metabolic process	7.20E-04	6.20E-03
	Response to stimulus	7.50E-04	6.20E-03
	Response to stress	4.50E-03	3.40E-02
Molecular function (MF)	Organic acid transmembrane transporter activity	7.10E-07	2.60E-05
	Carboxylic acid transmembrane transporter activity	5.50E-07	2.60E-05
	Secondary active transmembrane transporter activity	2.60E-05	3.50E-04
	Active transmembrane transporter activity	3.50E-04	3.80E-03
	Substrate-specific transporter activity	3.80E-04	3.80E-03
	Ion transmembrane transporter activity	6.70E-04	5.80E-03
	Transporter activity	9.60E-04	6.50E-03
	Substrate-specific transmembrane transporter activity	9.40E-04	6.50E-03
	Transmembrane transporter activity	1.60E-03	9.40E-02
Cellular component (CC)	Cytoplasm	1.50E-02	1.30E-04
	Endoplasmic reticulum	2.40E-03	2.20E-03
	Extracellular region	6.30E-04	1.80E-03
	Integral component of plasma membrane	1.20E-04	2.40E-03
	Extracellular region part	1.70E-04	2.80E-03
	Organelle membrane	1.30E-03	1.70E-02
	Cytoplasmic part	2.80E-03	3.20E-02
	Plasma membrane part	4.20E-03	4.10E-02
	Plasma membrane	4.50E-03	4.60E-02

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Functional enrichment analysis (Table 7) revealed a sophisticated molecular response in the *R. frisii* liver, characterized by the activation of 46 significant biological processes. The most prominent response was centered on immune modulation and stimulus recognition, showing the highest statistical significance. Furthermore, a strong enrichment in transmembrane transport activities and metabolic reorganization (particularly organic and monocarboxylic acids) highlights the energetic and structural adjustments required for adaptation to external stressors. These results indicate that the liver serves as a critical hub for integrating environmental signals into a coordinated systemic defense, involving both molecular chaperones and immune machinery.

Among the significantly enriched biological processes, response to wounding represented the largest functional category (four genes), followed by the monocarboxylic acid metabolic process (three genes). In the Molecular Function ontology, the most significantly enriched terms were associated with organic acid transmembrane transporter activity, suggesting metabolic adjustments accompanying the cellular response to heat stress.

Table 8. Topological centrality scores of the identified hub genes within the thermal stress protein-protein interaction (PPI) network.

Clustering Coefficient	Betweenness	Closeness	EcCentricity	BottleNeck	EPC	Degree	MNC	DMNC	MCC
0.19608	60.06249	24.33333	0.33333	4	6.046	18	8	0.86462	81
0.13333	1.46638	18.48333	0.24	1	3.046	6	2	0.61446	3
0	0	14.46666	0.2	1	1.666	2	1	0	1
0.32632	66.26118	24.48333	0.24	2	6.149	20	9	1.46962	10201
0	94.36444	19.16666	0.24	2	2.642	8	1	0	4
0	0.84444	18.4	0.24	1	2.44	4	1	0	2
0	0	16.08333	0.24	1	1.686	2	1	0	1
0.31149	46.62164	24.91666	0.24	2	8.198	24	11	1.44924	20281
0	102.6344	19.66666	0.24	2	2.491	8	1	0	4
0.33666	11.64119	24.24	0.24	2	6.264	22	11	1.32341	6140
0.30434	14.06332	24.64	0.24	2	6.813	24	12	1.22934	4688
0.06143	3.11318	19.2	0.2	1	3.293	8	2	0.61446	4
0.33231	14.18403	26.33333	0.24	2	8.648	26	13	1.36949	21960
0.34683	14.00446	24.91666	0.24	1	6.986	24	12	1.40496	20424
0.22308	66.84146	29.91666	0.24	3	9.98	40	20	1.06846	19244
0	1.06143	16.44	0.2	1	1.913	4	1	0	2
0.14	22.93183	22.41666	0.24	2	4.184	16	6	0.84488	34
0	16.80829	20.4	0.24	1	2.631	6	1	0	3
0.34446	0.84444	21.4	0.24	1	4.426	10	4	1.03622	30
0.30669	42.11608	26.33333	0.24	2	8.606	26	12	1.4634	18121
0	0	16	0.24	1	1.838	2	1	0	1
0.26263	6.66032	22.83333	0.33333	1	4.961	12	6	0.84488	22
0.36364	0.80096	22.16666	0.24	1	4.622	12	6	1.14118	62
0.24614	46.96416	29.24	0.24	1	9.801	36	18	1.19001	19608
0.26606	90.69812	24.4	0.33333	1	6.439	22	10	1.26696	1604
0.2006	160.0662	33.66666	0.33333	1	11.264	42	26	1.04464	32694
0.24231	44.84136	26.41666	0.24	1	8.424	26	13	1.04639	2440
0.24108	98.08666	24	0.24	2	6.346	22	10	1.14624	4093
0.2136	121.8968	30.83333	0.33333	2	10.326	42	21	1.04003	11842
0.18438	260.3826	29	0.33333	4	9.088	34	14	1.16116	6634
0.19964	136.9266	28.83333	0.33333	2	9.206	34	16	1.00411	2894

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3.2.2. Network topology analysis

To identify the most influential regulatory genes, topological properties of the gene interaction network were evaluated using multiple centrality algorithms implemented in Cytoscape. Network parameters including Maximum Neighborhood Component (MNC), Maximum Clique Centrality (MCC), Degree, Closeness, Betweenness, Radiality, Stress, Edge Percolated Component (EPC), and related algorithms were compared for hub gene identification.

Network topology metrics such as neighborhood connectivity and MCC were used to estimate the local importance of each node, whereas branching characteristics reflected the overall network density and connectivity. Hub genes consistently identified by multiple algorithms were considered the most reliable candidates for subsequent experimental validation.

To identify the master regulators of the thermal response, the reconstructed PPI network was subjected to a multi-algorithm topological analysis (Table 8). The Maximal Clique Centrality (MCC) algorithm, known for its high accuracy in identifying essential nodes, was prioritized alongside Degree and Betweenness centralities. The top-ranked nodes exhibited exceptionally high MCC scores (up to 32,694) and connectivity (Degree up to 42), indicating their role as the 'Common Core' that orchestrates the cellular chaperone machinery.

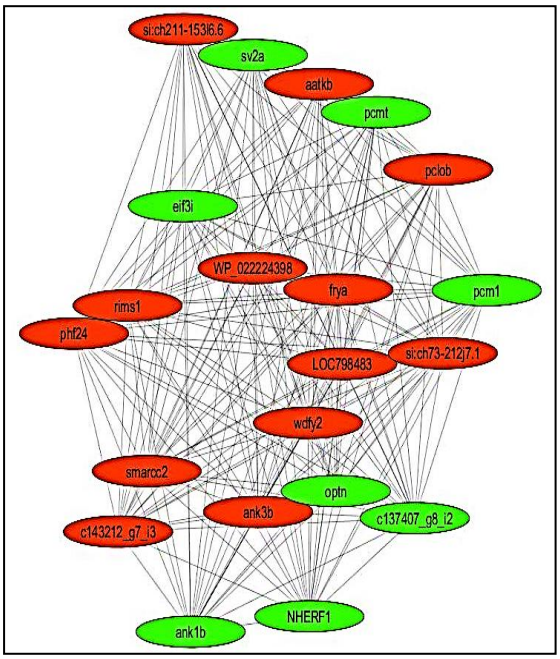

Fig. 5. Protein-Protein Interaction (PPI) network illustrating the chaperone interaction landscape in the liver of *R. frisii.* The nodes represent differentially expressed genes (DEGs), with green nodes indicating significant up-regulation and red nodes representing down-regulation. The central, highly connected nodes (hubs), such as *hsp90ba* and *Grp94,* orchestrate the cellular response, highlighting their roles as primary molecular sentinels in natural populations.

To decipher the molecular architecture of the thermal response in *R. frisii*, a Gene Regulatory Network (GRN) was reconstructed based on the validated hub genes (Fig. 3). The topological analysis revealed a highly interconnected network where molecular chaperones occupied the most central positions.

A striking pattern of predominant up-regulation (visualized in green) was observed among the hub genes, particularly within the HSF1-HSP axis, confirming the active recruitment of the proteostasis machinery. Conversely, specific metabolic and structural transcripts exhibited down-regulation (visualized in red), suggesting a strategic trade-off to prioritize cellular survival over energy-intensive processes during migration stress. The high connectivity (Degree) and centrality (MCC) of these genes, as summarized in Table 8, confirm their status as reliable biomarkers for assessing the physiological health of the Southern Caspian Sea populations.

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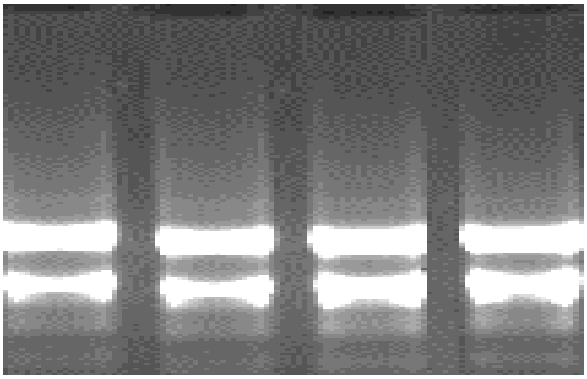

Fig. 6. Assessment of total RNA integrity extracted from the liver of *R. frisii*. Representative gel electrophoresis image showing intact 18S and 28S ribosomal RNA bands, confirming the high quality of the templates for downstream cDNA synthesis.

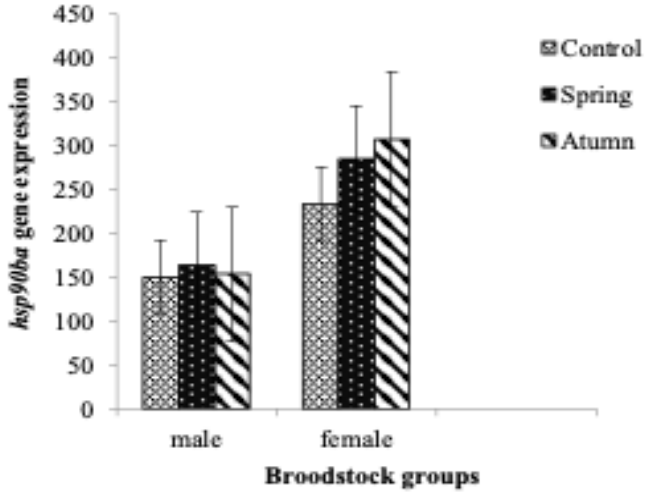

Fig. 7. Relative mRNA expression of *hsp90ba* in male and female *R. frisii*. Comparison across seasonal strains shows a robust up-regulation in the Autumn Strain (AS) compared to the Spring Strain (SS)

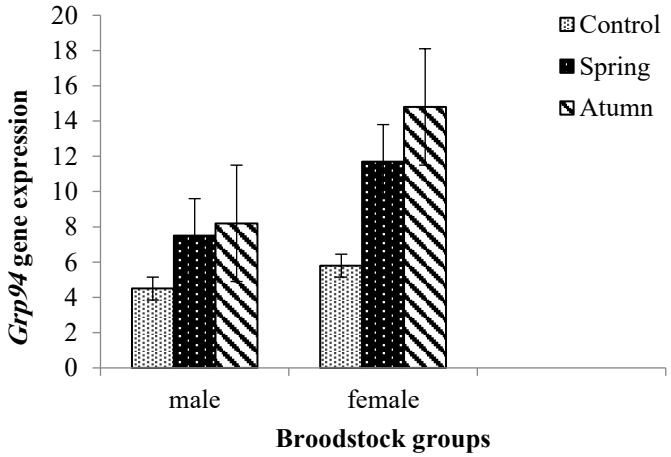

Fig. 8. Relative hepatic mRNA expression of *Grp94* in seasonal populations. The Endoplasmic Reticulum (ER)-resident chaperone exhibits significantly higher transcripts in the Autumn Strain, particularly among female spawners.

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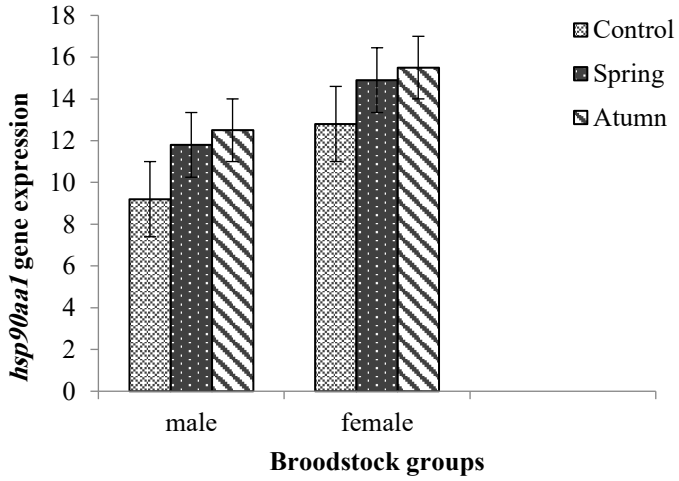

Fig. 9. Divergent expressional plasticity of the cytosolic chaperone *hsp90aa1*. Data represent fold-change differences between seasonal strains, highlighting the increased molecular defense in Autumn migrants.

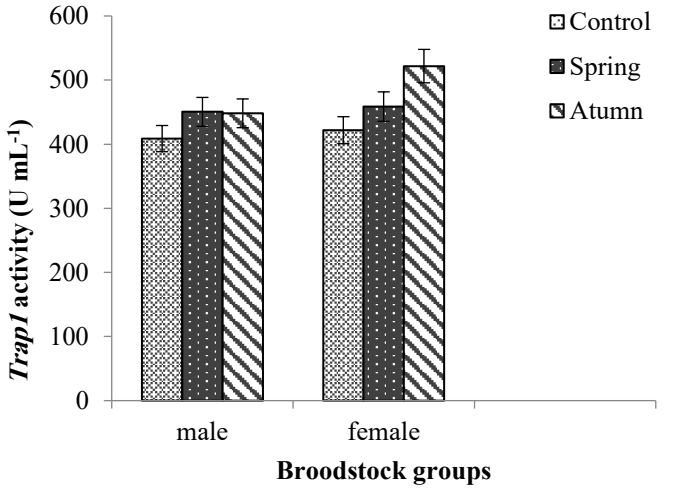

Fig. 10. Expression profile of the mitochondrial-localized *Trap1* gene. Significant seasonal and sex-specific variations are observed, with the highest expression recorded in the Autumn Strain females.

4. DISCUSSION

Global climate change and the increasing frequency of thermal fluctuations represent major challenges for aquatic ectotherms, particularly freshwater and migratory fish species that have limited capacity for behavioral thermoregulation. *R. frisii* is a commercially and ecologically important species inhabiting the Caspian Sea basin, where seasonal temperature variations and anthropogenic environmental pressures may affect its physiological performance and survival. In ectothermic organisms, temperature elevation directly influences cellular homeostasis by disrupting protein folding, increasing oxidative damage, and altering metabolic regulation. Therefore, understanding the molecular mechanisms underlying thermal adaptation is essential for predicting the resilience of fish populations under future climate scenarios. In the present study, transcriptomic-based analyses were applied to characterize heat-responsive molecular pathways and identify candidate genes associated with thermal stress tolerance in *R. frisii*. The results highlight the central role of stress-responsive networks, particularly heat shock protein-mediated mechanisms, in maintaining cellular stability under elevated temperatures (Chen *et al*. 2024).

Heat shock proteins (HSPs) represent one of the most conserved and evolutionarily ancient molecular defense systems responsible for maintaining cellular proteostasis under environmental stress conditions (Farasati *et al*. 2025b). In ectothermic organisms, including teleost fishes, temperature fluctuations impose a major physiological

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challenge because metabolic activity, protein stability, membrane fluidity, and enzymatic function are directly influenced by ambient temperature. Therefore, the ability to rapidly activate molecular chaperone systems represents a crucial adaptive mechanism for survival in variable aquatic environments (Volkoff & Rønnestad, 2020). The present study provides further evidence that the HSP family constitutes a highly conserved component of thermal stress adaptation in *R. frisii*, a cyprinid species naturally exposed to seasonal temperature variations in the Caspian Sea ecosystem.

The differential expression analysis performed in this study revealed extensive transcriptional modulation in response to heat stress, indicating that thermal exposure induces a coordinated molecular response rather than affecting isolated genes. Similar transcriptomic investigations in other fish species have shown that heat stress alters diverse biological processes, including protein folding, energy metabolism, oxidative balance, immune regulation, and apoptosis (Lyu *et al*. 2018). For example, RNA-seq analysis in Atlantic salmon demonstrated that thermal stress primarily affected molecular chaperones and stress-related pathways, with Hsp70 and Hsp90 members among the major responsive genes. These findings are consistent with the present results and indicate that despite differences in habitat and evolutionary history, fish species share conserved transcriptional strategies to cope with temperature-induced cellular challenges (Shi *et al*. 2019). Previous transcriptomic research on *R. frisii* has demonstrated that large-scale gene expression changes occur naturally during physiological transitions such as spawning migration (Farasati *et al*. 2025b). Thousands of differentially expressed transcripts in liver tissue of spawning females, highlighting the complexity of transcriptional regulation in this species. In agreement with these findings, the present study indicates that environmental stressors such as elevated temperature can also induce broad transcriptional reprogramming, suggesting that transcriptomic plasticity is a fundamental component of adaptation in *R. frisii*.

Functional enrichment analysis provides additional evidence that thermal stress affects multiple interconnected cellular pathways in *R. frisii*. Enriched biological processes associated with protein processing, stress response, regulation of metabolism, and cellular signaling suggest that heat exposure triggers a complex adaptive mechanism involving both protective and compensatory responses (Farasati *et al*. 2025a). Similar patterns have been reported in other aquatic species exposed to elevated temperatures, where activation of pathways associated with endoplasmic reticulum stress, oxidative stress response, autophagy, and energy metabolism contributed to maintaining cellular integrity (Li *et al*. 2025). Recent transcriptomic and multi-omics studies have also demonstrated that heat stress can modify metabolic pathways through coordinated regulation of HSPs, antioxidant systems, and signaling cascades such as PI3K/AKT/mTOR and MAPK pathways (Chen *et al*. 2023).

Analysis of the GSE220546 dataset revealed extensive gene expression remodeling following heat exposure, affecting pathways related to protein quality control, signal transduction, cytoskeletal organization, and metabolic regulation. The enrichment of MAPK signaling and protein phosphorylation pathways suggests that early responses to thermal stress are mediated through rapid signaling cascades that regulate downstream protective mechanisms, including HSP activation and cellular stress adaptation.

Alterations in cytoskeletal and protein-folding-related pathways further indicate that thermal stress affects fundamental aspects of cellular stability. Since elevated temperature can disrupt protein structure and membrane properties, thermal tolerance depends on maintaining overall cellular homeostasis rather than activation of individual stress genes (Metz *et al*. 2025). Although the analyzed dataset was generated from a model cyprinid species, the high conservation of stress-responsive pathways provides valuable comparative evidence for understanding thermal adaptation mechanisms in *R. frisii*. Moreover, transcriptomic investigations directly performed on *R. frisii* have demonstrated substantial gene expression plasticity during physiological transitions, supporting the importance of dynamic transcriptional regulation in coping with environmental challenges (Rasmus *et al.* 2023b).

The regulatory network analysis performed in this study provides further insight into the molecular interactions controlling heat-responsive genes. Unlike traditional single-gene approaches, network-based analyses allow identification of key regulatory components that coordinate multiple stress-associated pathways. The presence of hub genes related to molecular chaperoning and stress regulation suggests that these genes may serve as potential biomarkers for monitoring thermal stress responses in *R. frisii*. Similar approaches in fish transcriptomics have shown that regulatory networks involving transcription factors, stress-responsive genes, and signaling molecules provide a more comprehensive understanding of thermal adaptation mechanisms (Drown *et al.* 2022). Recent studies integrating transcriptome and regulatory analyses have identified central regulators such as heat shock

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transcription factors (HSFs) and endoplasmic reticulum stress-related genes as important controllers of thermal response pathways (Hook *et al*. 2025).

Analysis of the PRJNA1432439 dataset highlighted the importance of metabolic tissues, particularly liver and intestine, in regulating thermal tolerance through coordinated responses involved in protein folding, cellular stress management, and metabolic homeostasis. Elevated temperature disrupts protein stability and increases the accumulation of misfolded proteins; therefore, cells activate complementary mechanisms, including molecular chaperones, the unfolded protein response (UPR), and autophagy, to maintain proteome integrity.

The interaction between HSP-mediated protein refolding and autophagic degradation represents a key survival strategy during prolonged thermal stress. While HSP70 and HSP90 families assist in the recovery of damaged proteins, autophagy contributes to the removal of irreversible protein aggregates. In this context, the identification of stress-responsive genes such as HSP47 (*SERPINH1*) and *GRP94* highlights the involvement of endoplasmic reticulum-associated quality-control mechanisms in thermal adaptation. These findings suggest that heat tolerance in *R. frisii* is regulated through an integrated proteostasis network extending across cytoplasmic, mitochondrial, and endoplasmic reticulum compartments rather than through the activation of individual HSP genes alone (Akbarzadeh *et al*. 2018; Makvandi *et al*. 2022).

The experimental validation of candidate genes in *R. frisii* tissues provides essential evidence that transcriptomic patterns identified from public datasets reflect biologically meaningful responses in the target species. The expression analysis of selected HSP-related genes confirmed the involvement of key molecular chaperones in thermal stress adaptation, supporting the reliability of transcriptomic and network-based predictions. The agreement between computational analyses and tissue-level expression patterns indicates that these genes represent conserved components of the thermal stress response rather than dataset-specific alterations.

The observed regulation of HSP genes in *R. frisii* is consistent with previous studies demonstrating that HSP70, HSP90, and small HSPs play central roles in maintaining protein homeostasis, reducing oxidative damage, and protecting cellular functions under elevated temperature conditions. Moreover, recent studies on *R. frisii* have shown that gene expression profiles are strongly influenced by physiological status and environmental conditions, emphasizing the importance of molecular validation for understanding adaptation mechanisms in this species (Farasati *et al*. 2025a, b). Therefore, integrating transcriptomic analyses with tissue-based validation provides a stronger framework for identifying potential molecular biomarkers associated with thermal tolerance in *R. frisii* (Farasati *et al*. 2025a).

The co-activation and central network topology of *hsp90ba*, *Grp94*, *hsp90aa1*, and *Trap1* represent an organelle-specific proteostasis defense system. Under thermal stress, *hsp90aa1* acts as the primary cytosolic inducible chaperone, while *hsp90ba* maintains constitutive cytosolic folding. Concurrently, *Grp94* manages the unfolded protein response (UPR) in the endoplasmic reticulum (ER) essential for the high-rate vitellogenin synthesis in spawning females, while *Trap1* protects mitochondria from metabolic oxidative stress (ROS). This compartmentalized response ensures cell-wide proteome integrity during stressful spawning migration.

A significant finding of this study was the differential expression pattern of heat-responsive genes between seasonal strains of *R. frisii*. The stronger expression profile observed in the autumn strain suggests differences in molecular preparedness for environmental challenges. Seasonal variation in thermal history can influence physiological acclimatization and molecular responsiveness in ectothermic organisms. Fish populations exposed to different temperature regimes may develop distinct regulatory strategies, including altered expression thresholds of stress-response genes.

The distinct expression profiles observed between the spring and autumn strains may reflect, at least in part, the complex evolutionary history of Ponto-Caspian *Rutilus* lineages. Levin *et al.* (2025) documented patterns of genomic admixture and introgressive gene flow among several *Rutilus* lineages, including evidence of introgression involving *R. kutum*. Such evolutionary complexity provides a broader genomic context for interpreting phenotypic and transcriptional differences among closely related or geographically structured *Rutilus* populations. Nevertheless, the observed seasonal differences in the present study cannot be attributed to genetic divergence alone, because environmental temperature history, reproductive status, spawning stage, and migration history may also contribute to the observed expression patterns.

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The higher expression of candidate HSP genes in one seasonal group may indicate enhanced activation of cellular protection mechanisms; however, further physiological experiments are required to determine whether these differences represent genetic adaptation, environmental acclimatization, or interaction between both factors.

We explicitly acknowledge the limitations of using cross-species and cross-tissue comparisons. Genetic backgrounds, developmental stages (embryos vs. adults), and tissue-specific environments (intestine vs. liver) significantly influence transcriptomic signatures. Therefore, the conserved genes identified in this comparative framework represent conserved candidates of the core heat-response machinery rather than direct indicators of tissue-specific pathology in *R. frisii*.

The ecological significance of these findings is particularly important for *R. frisii*, a species naturally exposed to environmental fluctuations within the Caspian Sea ecosystem. Increasing water temperatures associated with climate change may influence reproductive success, growth performance, immune function, and population sustainability. Identification of heat-responsive biomarkers can provide valuable molecular tools for evaluating environmental stress conditions and improving conservation and aquaculture management strategies. Recent studies on thermally sensitive and commercially important fish species have emphasized that understanding the molecular mechanisms of thermal tolerance is necessary to predict species vulnerability under future warming scenarios. For example, investigations in *Tor putitora* demonstrated that elevated temperature exposure significantly modified expression patterns of multiple HSP genes across tissues, highlighting the importance of molecular biomarkers in assessing thermal adaptation capacity (Kaur *et al*. 2025).

Since sampling was conducted on wild spawning populations, interpreting expression differences between Spring and Autumn strains requires caution. These differences likely reflect a complex interaction of environmental temperature history, reproductive endocrine status, spawning stage, nutritional profiles, and migration history, rather than environmental temperature alone.

5. CONCLUSION

This study provides new insights into the molecular mechanisms underlying thermal stress response in *R. frisii* by integrating transcriptomic analyses, functional annotation, regulatory network analysis, and tissue-level validation. The results demonstrate that heat stress induces a coordinated molecular response involving stress signaling, protein quality control, and proteostasis pathways rather than activation of individual stress genes alone. The HSP90-centered chaperone network, including *hsp90aa1*, *hsp90ba*, *Grp94*, and *Trap1*, was identified as a key component of thermal adaptation and was further supported by expression validation in the *R. frisii* tissues. These findings highlight the potential of HSP90 family members as candidate heat-responsive HSP-related genes for assessing thermal resilience in *R. frisii*. Considering the increasing impact of climate change on aquatic ecosystems, the identified candidate genes and regulatory pathways provide valuable molecular resources for future studies on conservation, broodstock management, and development of climate-resilient aquaculture strategies for this commercially important species.

This study highlights the candidate heat-stress-responsive chaperone network in *R. frisii* by integrating comparative transcriptomics and field-based expression. The HSP90-related genes (*hsp90aa1*, *hsp90ba*, *Grp94*, and *Trap1*) were identified as candidate components associated with the thermal stress response, demonstrating sex- and season-specific expression variations. While these findings provide valuable genetic resources for future climate-resilient aquaculture and broodstock management, further controlled, standardized laboratory thermal challenge trials are mandatory to definitively evaluate their diagnostic biomarker performance in *R. frisii* (Metz *et al*. 2025).

From a conservation management perspective, the findings highlight the need to strengthen genetic conservation strategies for *R. frisii*, particularly for the vulnerable phytophilous spawning population (Rahbar *et al*. 2022). The differential expression of HSP90-related candidate genes under contrasting seasonal environmental conditions provides molecular evidence of physiological responses in naturally occurring populations and highlights their potential vulnerability to increasing environmental and thermal pressures. Given the ecological dependence of phytophilous spawners on shallow, vegetated spawning habitats, the degradation of these habitats may further increase the vulnerability of this population. Although the present field-based findings do not establish thermal adaptation or definitive heat-stress biomarker performance, they support the incorporation of molecular and genetic information into conservation and management programs (Akbarzadeh *et al*. 2018). Particular attention should therefore be given to maintaining the genetic diversity and reproductive habitats of vulnerable spawning

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populations, especially phytophilous spawning grounds, to reduce the risk of further population decline and loss of valuable genetic resources (Fao.org. 2026).

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