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Molecular and Physiological Insights Into Handling Stress Responses in Hybrid Grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*)

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ABSTRACT

Handling stress is a significant challenge in intensive aquaculture, often compromising fish welfare, immunity, and overall productivity. This study investigates the skin transcriptomic responses, blood plasma enzyme activities, and histological changes in the liver, spleen, and head kidney of hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) subjected to repeated handling stress over 10 consecutive days. RNA-sequencing of the skin identified 2521 differentially expressed genes (DEGs), showing significant enrichment in oxidative phosphorylation and cardiac muscle contraction pathways. Stress conditions upregulated genes such as *NADH ubiquinone oxidoreductase*, *ATP synthase*, *cytochrome b-c1 complex*, *cytochrome c oxidase*, *succinate dehydrogenase complex subunit B*, and *troponin T2d cardiac*, indicating enhanced mitochondrial activity and energy turnover. These molecular signatures correlated with physiological recovery in stress-resilient (handling-resilient [HR]) fish, as evidenced by elevated alanine transaminase levels and preserved hepatic morphology. In contrast, stress-sensitive (handling-sensitive [HS]) fish exhibited a pronounced reduction in hepatic cytoplasmic area ($48.75 \pm 0.47\ \mu m^2$) compared to the control ($62.47 \pm 0.20\ \mu m^2$) and HR ($67.82 \pm 0.24\ \mu m^2$) groups. Furthermore, HS fish showed severe splenic necrosis ($3.15 \pm 0.68\ \mu m^2$) compared to the control ($0.27 \pm 0.29\ \mu m^2$), reflecting impaired metabolic and antioxidant capacity. This integrated analysis elucidates the molecular and physiological impacts of handling stress and identifies key pathways linked to stress resilience. Implementing refined handling techniques is essential to mitigate stress-related health impacts and enhance welfare in hybrid grouper aquaculture.

1 | Introduction

The intensification of aquaculture has significantly boosted production efficiency but has simultaneously introduced complex challenges regarding the health and welfare of cultured species. Routine handling procedures, including grading, vaccination, transportation, and harvesting, are the primary stressors, particularly within high-density rearing systems [1, 2]. Numerous studies on commercially important species such as *Oncorhynchus mykiss*

[3], *Salmo salar* [4], *Lates calcarifer* [5], and *Arapaima gigas* [6] have demonstrated that handling induces a primary stress response characterized by elevated cortisol levels, which subsequently activate secondary metabolic and immune responses [7]. When handling is prolonged or frequent, these adaptive responses can become maladaptive, resulting in chronic physiological disruptions such as immunosuppression, metabolic imbalance, and increased disease susceptibility [8]. Handling stress is particularly relevant to the hybrid grouper, a crossbreed of the female tiger grouper and male giant grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*). This fish holds high economic value in Southeast Asia [9], yet it is frequently exposed to handling stress during live transportation and marketplace display in confined environments. These prolonged stressors can severely compromise its physiological resilience [10]. Consequently, a comprehensive understanding of the hybrid grouper's molecular and physiological responses is essential for optimizing welfare and productivity in commercial aquaculture settings [11, 12].

As the primary interface between the fish and its environment, the skin provides both physical protection and immunological defence [13]. Handling stress can compromise skin integrity, increasing susceptibility to infection and declining overall health [14, 15]. Recent transcriptomic studies in *Scophthalmus maximus*, *S. rhombus*, *Pelteobagrus fulvidraco*, and *Plectropomus leopardus* have revealed that the skin functions as a metabolic and immunological organ during stress [16, 17]. Transcriptomic profiling provides a high-resolution approach to dissect stress responses, revealing differential gene expression patterns associated with resilience or vulnerability [18, 19]. In other aquaculture species such as *O. mykiss*, crowding stress has been shown to activate proteolytic pathways while suppressing muscle growth regulators; similarly, in *Coregonus maraena*, repeated handling upregulated genes involved in immune modulation [10, 20, 21]. Understanding these molecular mechanisms in hybrid grouper could identify heritable traits for stress resilience, facilitating selective breeding and providing a source of minimally invasive biomarkers for stress assessment [22–24]. To complement transcriptomic insights, biochemical markers provide immediate evidence of stress-induced tissue damage and metabolic shifts. Enzymes such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) serve as indicators of hepatic function, tissue integrity, and anaerobic metabolism [25, 26]. Furthermore, plasma glucose (GLU) and cholesterol (CHOL) levels reflect the activation of the hypothalamic–pituitary–interrenal (HPI) axis and subsequent energy mobilization [12, 27, 28]. For instance, increased GLU levels observed in *Epinephelus moara* and *Acanthopagrus schlegelii* under stress confirm significant metabolic alterations linked to the HPI axis [28, 29]. Histopathological changes in organs such as the liver, head kidney, and spleen provide further evidence of stress-induced physiological alterations, including hepatocellular vacuolization, hematopoietic disruption, and splenic necrosis [30, 31].

Despite the growing body of literature on stress physiology and transcriptomics in aquaculture species, an integrated perspective on how repeated handling alters both the molecular and physiological landscapes of the hybrid grouper is currently lacking. Therefore, this study aims to characterize transcriptomic alterations in skin tissue alongside histopathological and biochemical responses in the liver, spleen, and head kidney of hybrid grouper subjected to repeated handling stress. By integrating molecular and physiological endpoints, this research seeks to uncover stress-responsive pathways and potential resilience markers, contributing to more sustainable and welfare-oriented aquaculture practices.

2 | Materials and Methods

2.1 | Ethical Statement

All animal handling procedures and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM, Certificate Number UPM/ACUC/AUP-R054/2022) prior to the commencement of the study. This study adheres to the strict minimum sample size; therefore, 30 hybrid groupers (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) were used to achieve reliable biological replication in compliance with approved animal ethics guidelines.

2.2 | Fish Management and Maintenance

Healthy hybrid groupers (mean weight: 82.37 ± 15.45 g; mean length: 16.90 ± 0.99 cm) were obtained from a commercial nursery in Sepang, Selangor, Malaysia. The fish were acclimatized for 4 weeks in a 500 L recirculating system equipped with physical and biological filtration at the Hatchery Unit, Institute of Bioscience, UPM, Serdang, Selangor, Malaysia. During acclimatization, fish were fed twice daily (0900 and 1700) to apparent satiation with commercial pellets (Dindings 920). Water quality was monitored daily: temperature, pH, and dissolved oxygen (DO) were measured using a Handheld Multi-Parameter Water Quality Meter Model WQC-30 (DKK-TAO Corporation, Tokyo, Japan), while salinity was verified with a Milwaukee MR100ATC hand refractometer (ATAGO, Japan). Total ammonia nitrogen was monitored using an API Test Kit (Mars Fishcare North America Inc., USA). Maintenance included biweekly filter cleaning and weekly 50% water exchanges to ensure optimal conditions.

2.3 | Experimental Design and Sampling

Following acclimatization, fish were individually tagged using colored cotton threads secured at the base of the second or third ray of the first dorsal fin for identification. The fish were then moved to two 250 L glass aquaria ($122 \times 46 \times 46$ cm^3) with aeration for a 48 h conditioning period. The experimental setup consisted of two groups ($n = 15$ fish per group): fish in the control group were maintained under standard conditions without handling; while fish in the treatment group were housed in a mesh cage fitted within the aquarium to facilitate standardized handling. The stress protocol, adapted from Martorell-Ribera et al. [20], was performed daily between 0900 and 1000 for 10 consecutive days. The procedure involved 1 minute of herding with a scoop net, followed by 5 minutes of air exposure (by lifting the internal cage), before returning the fish to the water. All fish were fed twice daily to satiation with commercial pellets (Dindings 920) after the daily stress induction. Physical and behavioral changes were observed and recorded throughout the experimental period. Based on the criteria established by Matusin et al. [23], fish in the treatment group were categorized into two phenotypes: handling-resilient (HR) – active fish exhibiting normal swimming and feeding

patterns; or handling-sensitive (HS)—fish exhibiting anorexia (loss of appetite), darkened body coloration, loss of equilibrium, solitary behavior, or gasping. The observations were recorded using a binary scoring system ("−" for absent, "+" for present). Total scores determined the final categorization of each fish.

2.4 | Samples Collection

At the end of the experimental period, fish ($n = 6$ per treatment group) were randomly selected for blood, skin, and organ (liver, spleen, and head kidney) sampling. Fish were anesthetised with tricaine methanesulfonate (MS-222) at 100 mg/L. Blood was collected via caudal puncture using a sterile syringe rinsed with 10% EDTA. Plasma was separated by centrifugation at 10,000 rpm for 10 min and stored at −80°C until further analysis. Following blood collection, the fish were then euthanised with a lethal dose of MS-222 at 200 mg/L. Skin tissues were excised from the dorsal region, rinsed with 1 × PBS, and immediately snap-frozen in liquid nitrogen for transcriptomic analysis. For histopathology, the liver, spleen, and head kidney were harvested and fixed in Bouin's solution for 20 h, followed by transfer to 70% ethanol for long-term preservation. The overall workflow is summarized in Figure 1.

2.5 | Transcriptomic Analysis

2.5.1 | RNA Extraction, Library Construction, and Sequencing

Total RNA was extracted from the skin samples of three experimental groups: HR (GHR1, GHR2, and GHR3), HS (GHS1, GHS2, and GHS3), and control (GC1, GC2, and GC3). RNA was extracted using TRIzol reagent (Invitrogen) and subsequently purified using the RNeasy Plus Kit (Qiagen), including an on-column DNase treatment to remove genomic DNA contamination. RNA integrity was verified via 1% agarose gel electrophoresis, and RNA purity was assessed using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA). Messenger RNA (mRNA) was captured using poly-T oligo-attached magnetic beads and then fragmented for cDNA synthesis. First-strand cDNA was synthesized using random hexamer primers, followed by second-strand synthesis. For directional (strand-specific) libraries, dUTP was incorporated during the second-strand synthesis and digested with USER enzyme prior to PCR amplification. Nondirectional libraries utilized dTTP and underwent standard end-repair, A-tailing, and adapter ligation. Libraries were quantified using a Qubit fluorometer and real-time PCR (RT-qPCR), while size distribution was validated using a Bioanalyzer. Sequencing was performed on the Illumina platform to generate paired-end reads, with base calling managed by the CASAVA pipeline.

2.5.2 | Sequence Quality Assessment, *De Novo* Assembly, and Functional DEGs Annotation

Raw FASTQ reads were firstly processed using FASTP to remove adapters, poly-N sequences, and low-quality reads. Quality metrics, including Q20, Q30, and GC-content, were calculated to ensure data integrity. High-quality clean reads were then aligned to the reference genome (downloaded from NCBI,https://www.ncbi.nlm.nih.gov) using HISAT2 v2.0.5. Transcripts were assembled using StringTie v1.3.3b in a reference-based approach. Gene expression levels were quantified using featureCounts v.1.5.0-p3 and normalized as FPKM (fragments per kilobase of transcript sequence per million mapped reads).

2.5.3 | Differential Expression and Functional Enrichment Analysis

Differential expression analysis was performed using the DESeq2 R package (version 1.20.0), which employs a negative binomial distribution model. *p*-Values were adjusted for multiple testing using the Benjamini–Hochberg approach to control the false discovery rate. Genes with an adjusted *p*-value (p_{adj}) < 0.05 were considered as significant differentially expressed genes (DEGs). Functional annotation of DEGs was subsequently performed through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses using the clusterProfiler R package. GO terms were categorized into three major categories: biological processes (BPs), cellular components (CCs), and molecular function (MF). Pathways and terms with a $p_{adj} < 0.05$ were considered significantly enriched.

2.6 | Validation via RT-qPCR

To validate the transcriptomic findings, five DEGs (three upregulated and two downregulated genes) were selected for quantitative

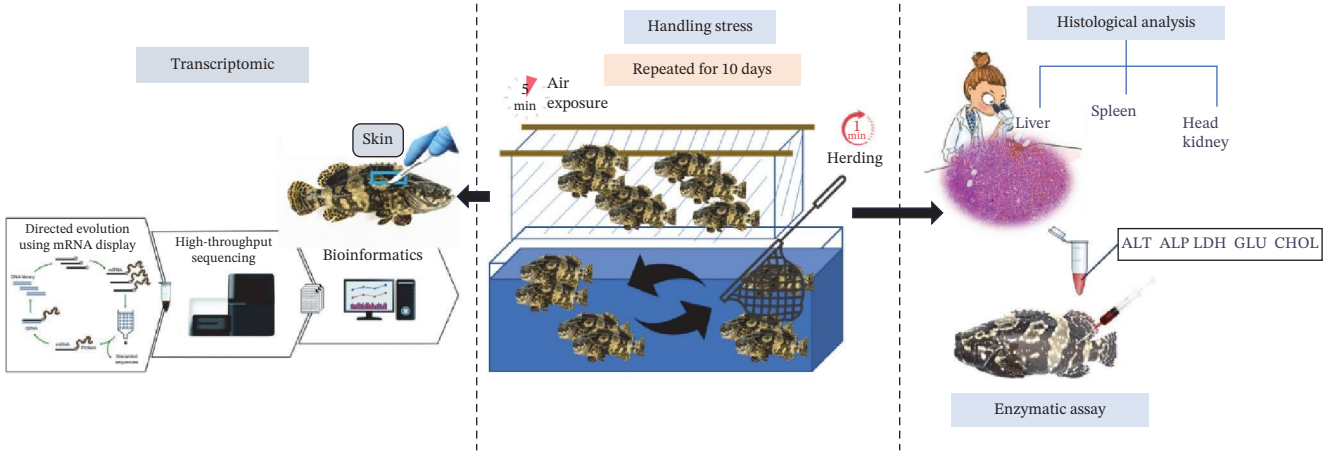


FIGURE 1 | Overview of the experimental design integrating skin transcriptomics, histopathology, and enzymatic assays to investigate handling stress responses in hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*).

RT-qPCR. RNA was isolated using the Monarch Total RNA Miniprep Kit (New England Biolabs) and reverse-transcribed into cDNA using SensiFAST cDNA Synthesis Kit (Bioline, UK) according to the manufacturer's protocol.

RT-qPCR was performed using 2x qPCRBIO SyGreen Mix Separate-ROX (PCR Biosystem, UK) on a Real-Time PCR Detection System X60B (Heal Force, China). Primers (Table 1) were designed using Primer3 and verified via NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and OligoAnalyzer (https://www.idtdna.com/calc/analyze) to ensure the absence of secondary structures or dimers. The PCR cycling conditions involved initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 20 s. Stability of housekeeping genes was evaluated using geNorm [32], NormFinder [33], and BestKeeper [34], identifying peroxisomal biogenesis factor 14, *pex14* as the most stable internal control [18]. Relative expression levels were calculated using the Pfaffl method [35], accounting for amplification efficiency. The correlation between RT-qPCR data and FPKM values was used to confirm the reliability of the RNA-Seq dataset.

2.7 | Biochemical and Enzymatic Assay

Blood plasma from the control, HR, and HS groups was analyzed for metabolic and stress indicators. The activities of ALT, ALP, LDH, along with levels of GLU and CHOL, were quantified using commercial enzymatic assay kits (BioREX MANNHEIM Diagnostics, Germany). All measurements were performed using a Biolis 24i Premium automatic biochemical analyser according to the manufacturer's protocols.

2.8 | Histological Analysis

Organ samples (liver, spleen, and head kidney) from three fish per group (control, HR, and HS) were processed at the Histopathology Laboratory, Faculty of Veterinary Medicine, UPM. Tissues were dehydrated, embedded in paraffin, and sectioned at 4 μm. Sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). For each sample, 10 random microscopic fields were examined at 200x magnification using a Nikon Eclipse Ci microscope camera (Nikon, Japan). Quantitative histomorphometry analysis was performed using ImageJ software (v1.54d, National Institute of Health, USA). Total tissue areas and specific regions of interest were calculated (μm^2) based on color threshold. Where present, necrotic areas or pathological lesions were manually outlined and measured to determine the extent of tissue damage.

2.9 | Statistical Analysis

Statistical analyses were performed using SPSS software version 29.0 (IBM Corporation, New York, USA). Data are presented as means ± standard deviations (SDs). Normality and homogeneity of variance were verified using the Shapiro–Wilk test and Levene's test, respectively. Differences between the control, HR, and HS groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post hoc test. The threshold for statistical significance was set at $p < 0.05$.

3 | Results

3.1 | Behavioral and Physical Observations

Water quality remained within the optimal range throughout the 10-day experimental period. Parameters were maintained as follows: temperature ranges from 24.4° to 27.8°C, salinity 22 to 25 ppt, pH 7.64 to 7.70, DO 4.85 to 6.80 mg/L, and ammonia 0.25 to 0.50 mg/L. There were no significant fluctuations in water quality between the control and treatment aquaria.

Throughout the experimental period, six distinct stress-related behavioral and physical changes were identified in the treatment group, which include loss of appetite, loss of equilibrium, gasping

TABLE 1 | The primer sequences of the selected genes were used for real-time reverse transcription quantitative PCR (RT-qPCR) validation.

Gene name	Primers	Sequence
NADH_ubiquinone oxidoreductase subunit C2, *ndufc2*	F	GGTTACACACCATGCCCTTG
	R	GGACACCTGATTTTAGCGGC
ATP synthase peripheral stalk subunit d, *atp5pd*	F	CTGAAGACTCGCAGTGATGC
	R	TGTGTGTCAGTAGGCTCAGG
Tubulin beta 5 transcript variant X1, *tubb5*	F	CGCATCTCTGAGCAGTTCAC
	R	ATTCACCCTCCTCTTCAGCG
Matrix metallopeptidase 2, *mmp2*	F	CATTCCAAAGCGTCTCGCAA
	R	CATGTCTCCCACAGAAGCGT
Glutamic pyruvate transaminase (alanine aminotransferase) 2, *gpt2*	F	ATCTTCATCCCACCACGAGC
	R	CTCTGCTGATCGGAGGTTCC
Peroxisomal biogenesis factor 14, *pex14*	F	CCAGTCAACCACACCAACAG
	R	TGGCTCACATCATCCTCCTC
Hypoxanthine phosphoribosyltransferase 1, *hprt1*	F	CCACAACCATACAGCAGCAG
	R	TAGTTTTGCGGGCACTTCAC

at the water surface, darkened body coloration, solitary behavior, and lethargy. No mortality was recorded during the study. Based on the cumulative frequency of these observations, fish were categorized into two distinct phenotypes (Table 2): HS—fish showing a high stress–response frequency with a total score of ≥ 4; HR—fish showing minimal stress indicators with a score of ≤ 2.

3.2 | *De Novo* Transcriptome Assembly and Annotation

To identify gene expression profiles associated with repeated handling stress, transcriptomes were sequenced from the skin tissues of the control (C), HR, and HS groups. A total of 842,226,356 raw reads were obtained across all groups (329,420,918 for control; 257,779,684 for HR; and 255,025,754 for HS). After the removal of adapter sequences and low-quality reads, 832,454,830 high-quality clean reads were retained for downstream analysis (Table 3). Quality control metrics confirmed the high reliability of the sequencing data: Q20 ranged from 97.16% to 97.82%; Q30 ranged from 92.94% to 93.99%; GC content was stable across samples (44.56%–46.39%); and the mean sequencing error rate was consistently low at 0.03%.

3.3 | Gene Expression Level and Sample Correlation Analysis

The distribution of gene expression levels based on FPKM values across the control, HR, and HS groups was visualized using a

TABLE 2 | Scoring of cumulative behavioural and physical stress indicators in hybrid grouper subjected to daily handling stress.

Fish ID	Loss of appetite	Equilibrium loss	Gasping	Darker body	Solitary behaviour	Lethargy	Total score	Phenotype
H1	−	−	−	−	−	−	0	HR
H2	+	+	+	+	+	+	6	HS
H3	−	−	−	−	−	−	0	HR
H4	+	+	+	−	+	+	5	HS
H5	+	+	+	+	+	+	6	HS
H6	+	+	+	+	+	+	6	HS
H7	+	−	−	+	+	+	4	HS
H8	−	−	−	−	−	−	0	HR
H9	+	−	−	+	+	−	3	—
H10	+	−	−	+	−	−	2	HR
H11	+	−	+	−	+	−	3	—
H12	+	−	−	−	+	−	2	HR
H13	+	−	−	−	−	−	1	HR
H14	+	−	+	+	+	+	5	HS
H15	+	−	−	−	+	−	2	HR

Note: Stress signs were scored as absent (−) or present (+). Individual fish (H1–H15) were categorized based on cumulative scores as handling-resilient (HR, score ≤ 2) or handling-sensitive (HS, score ≥ 4).

TABLE 3 | Overview of the sequenced data of hybrid grouper skin subjected to repeated handling stress.

Group	Sample	Raw reads	Clean reads	Clean bases (*G*)	Q20 (%)	Q30 (%)	GC (%)	Error (%)	Total mapped rate (%)
C	GC1	161 233 262	159 213 728	23.88	97.49	93.44	45.75	0.03	86.02
	GC2	85 192 928	84 415 356	12.66	97.66	93.64	46.31	0.03	88.53
	GC3	82 994 728	81 974 218	12.3	97.21	93.05	44.56	0.03	82.61
HR	GHR1	87 607 842	86 828 838	13.02	97.82	93.99	46.39	0.03	89.37
	GHR2	87 691 710	86 666 810	13.0	97.31	92.94	45.99	0.03	88.1
	GHR3	82 480 132	81 452 336	12.22	97.51	93.35	45.82	0.03	88.96
HS	GHS1	85 717 626	84 517 838	12.68	97.16	92.96	44.81	0.03	82.12
	GHS2	85 451 874	84 432 700	12.66	97.45	93.31	45.16	0.03	86.63
	GHS3	83 856 254	82 953 006	12.44	97.51	93.43	45.35	0.03	85.82

Abbreviations: C, control group; HR, handling-resilient; HS, handling-sensitive.

boxplot to compare gene expression patterns under repeated handling (Figure 2a). The reliability of the dataset was confirmed by high Pearson correlation coefficients (R^2) between replicates: 0.861–0.929 for control; 0.937–0.953 for HR; 0.909–0.935 for HS (Figure 2b). These values ($R^2 > 0.861$) indicated strong reproducibility within groups and high similarity in the overall skin transcriptomic profiles, supporting the validity of subsequent differential expression analyses. Principal component analysis (PCA) showed that while the HR and HS groups did not form entirely distinct clusters from the control group, the GHS replicates (GHS1–GHS3) showed a clear alignment along PC1 despite some dispersion (Figure 2c). Notwithstanding this variation, the internal correlation coefficients ($R^2 > 0.909$) remained within an acceptable range for high-throughput transcriptomic studies. Furthermore, a Venn diagram was used to visualize the distribution of genes across groups. A total of 10,892 genes were co-expressed across the C, HR, and HS groups, representing the core skin transcriptome of the hybrid grouper (Figure 2d).

3.4 | Identification of DEGs

To evaluate the molecular response to repeated handling, differential expression analysis was performed across the experimental groups. A total of 2521 significantly DEGs ($p < 0.05$) were identified across the skin tissue samples (Figure 3a). The comparison between HR and control groups identified 1207 significant DEGs, consisting of 496 upregulated and 711 downregulated genes (Figure 3). In contrast, the comparison between HS and control groups identified a higher number of transcriptional changes, with 1314 significant DEGs, comprising 561 upregulated and 753 downregulated genes (Figure 3). A hierarchical clustering heatmap was constructed to visualize the expression patterns of these DEGs across the three groups (Figure 4). The heatmap revealed distinct expression signatures, with the HS group showing pronounced deviations in gene expression compared to the control, indicating severe physiological response to the handling stressor.

3.5 | Functional Enrichment Analysis of DEGs

To further characterize the functional roles of the identified DEGs, GO enrichment analysis was performed across the three primary categories: BP, MF, and CC. Significant GO terms ($p_{adj} < 0.05$) for the HR vs. control and HS vs. control comparisons were summarized in Figure 5. In the HR vs. control comparison, the most significantly enriched category within CC was the organelle envelope, suggesting a cellular level adjustment to maintain homeostasis during stress. In contrast, the HS vs. control comparison revealed a distinct enrichment pattern, with the cytoskeleton emerging as the most significantly enriched category in CC. This shift potentially indicates a loss of structural integrity or significant cellular remodeling in the skin of HS fish.

To identify the metabolic and signaling networks altered by repeated handling, DEGs were mapped to the KEGG database (Figure 6). In the HR vs. control comparison, only two pathways were significantly enriched ($p_{adj} < 0.05$). Notably, oxidative phosphorylation was the most significantly enriched upregulated pathway, involving 35 upregulated DEGs. No significantly enriched pathways were identified among the downregulated DEGs for this group, suggesting that the resilience in hybrid grouper may be linked to the upregulation of mitochondrial energy production. In contrast, the HS vs. control comparison revealed 12 significantly

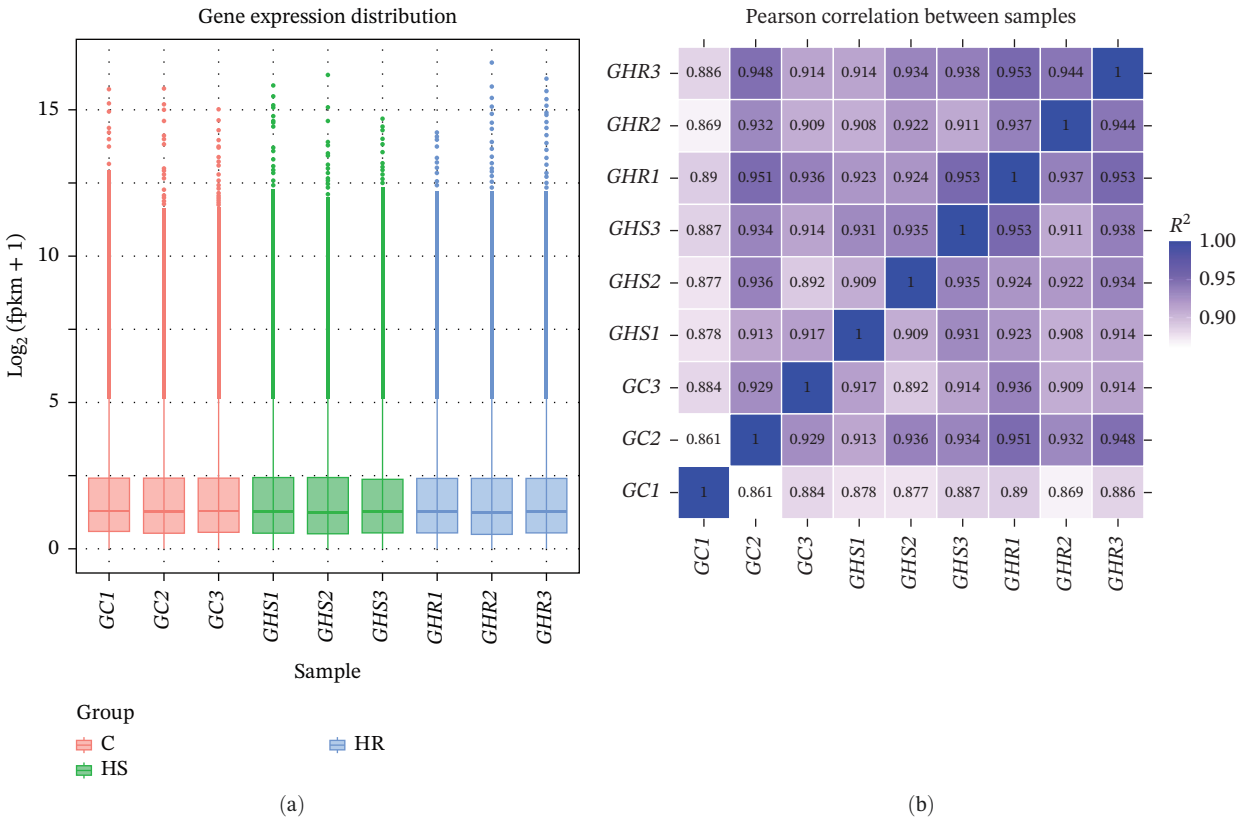
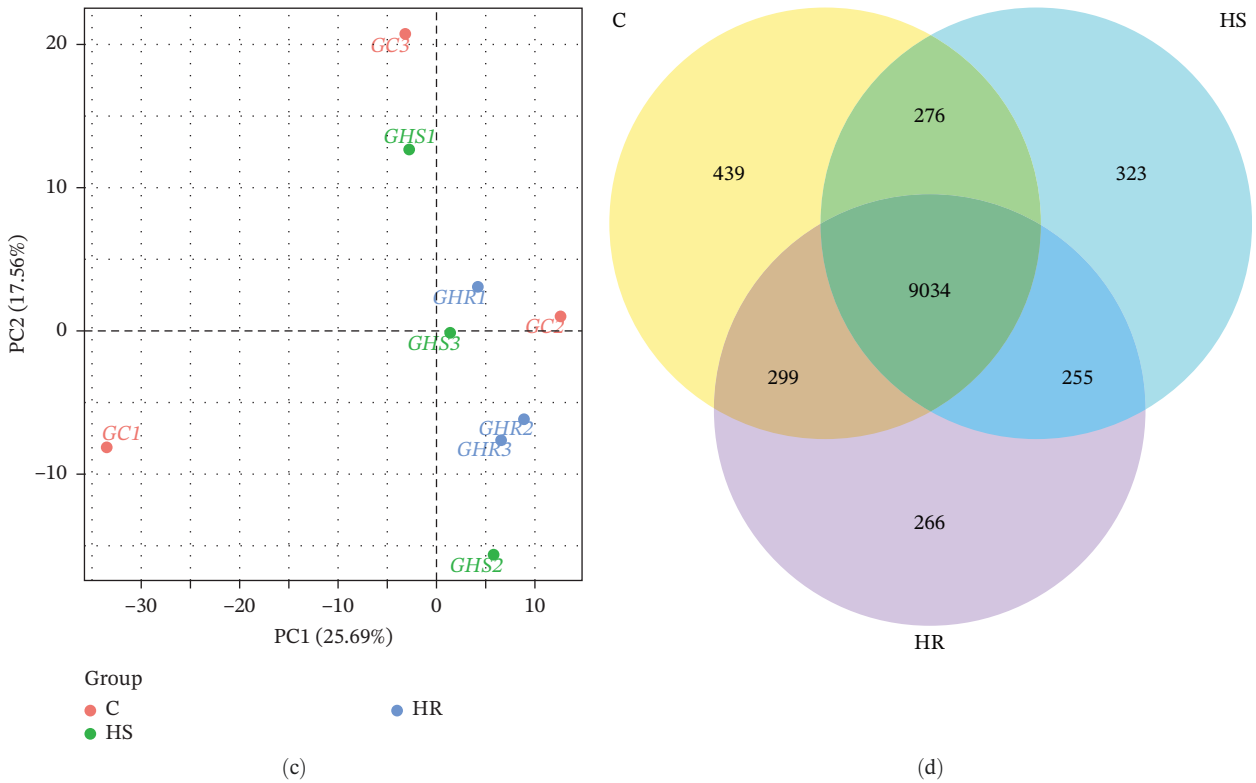
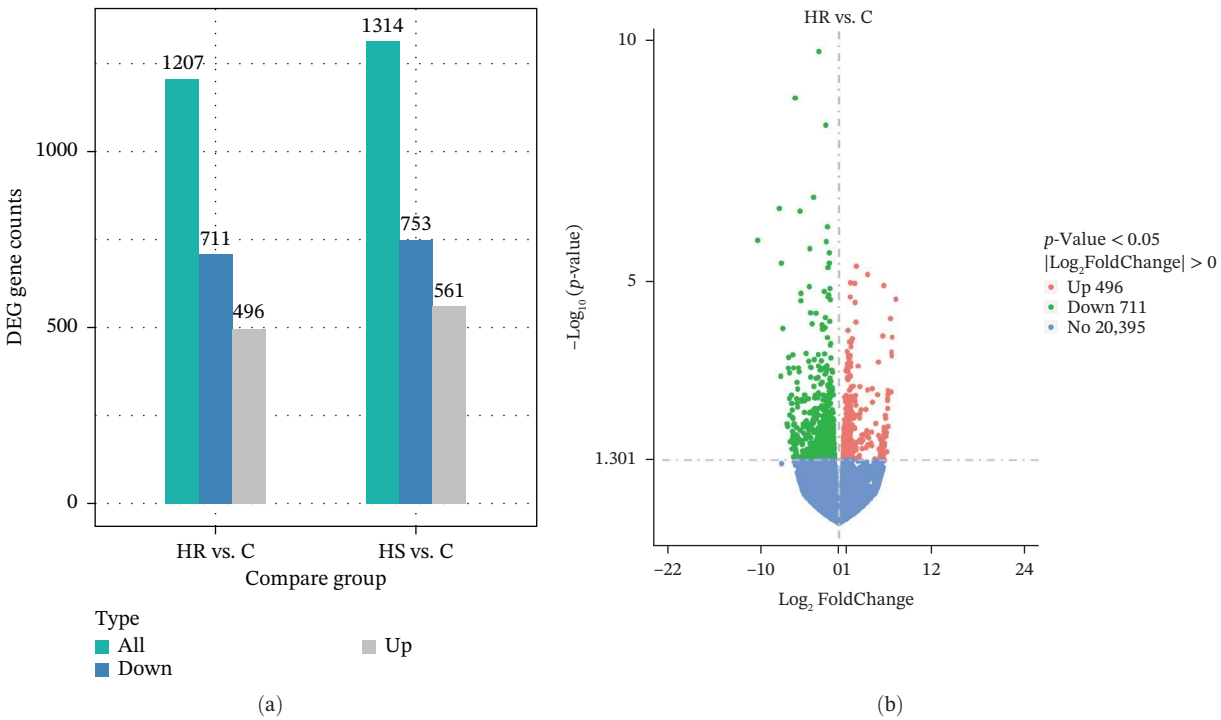


FIGURE 2 | (Continued)



(c) (d)

FIGURE 2 | Global gene expression and sample correlation analysis of the skin transcriptome in hybrid grouper subjected to repeated handling stress. (a) Boxplot showing the distribution of FPKM values across replicates; (b) Pearson correlation heatmap illustrating the transcriptomic similarity between samples. R^2 values represent the square of the Pearson correlation coefficient calculated from the FPKM values of all genes; (c) principal component analysis (PCA) showing the spatial distribution and clustering patterns of the control, handling-resilient, and handling-sensitive groups based on their expression profiles; (d) venn diagram showing the number of unique and co-expressed (overlapping) genes identified across the three experimental groups.



(a) (b)

FIGURE 3 | (Continued)

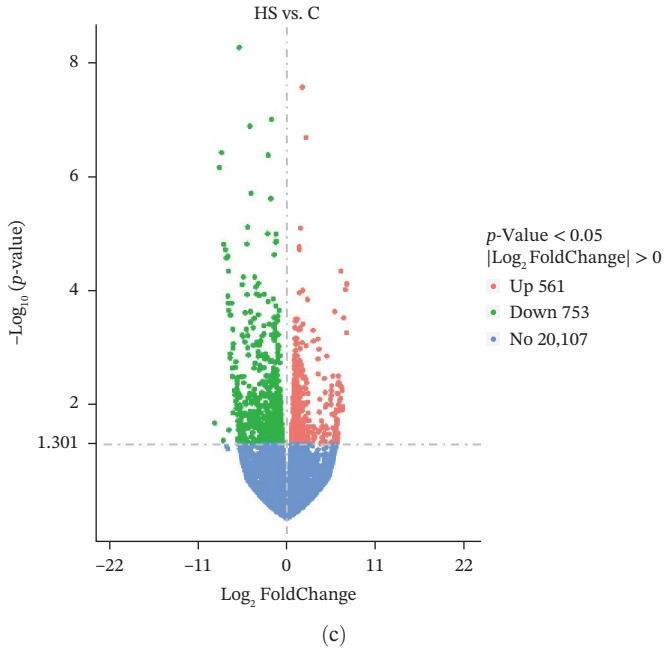


FIGURE 3 | Identification and quantification of differentially expressed genes (DEGs) in the skin tissue of hybrid grouper subjected to repeated handling stress. (a) Bar graph showing the total number of upregulated and downregulated DEGs in the HR (HR vs. control) and HS (HS vs. control) comparisons; (b,c) Volcano plots showing the distribution of fold changes and statistical significance for the HR vs. control (b) and HS vs. control (c) comparisons ($p < 0.05$). Each point represents an individual gene: red dots indicate significantly upregulated genes; green dots indicate significantly downregulated genes; blue dots represent genes with no significant differential expression.

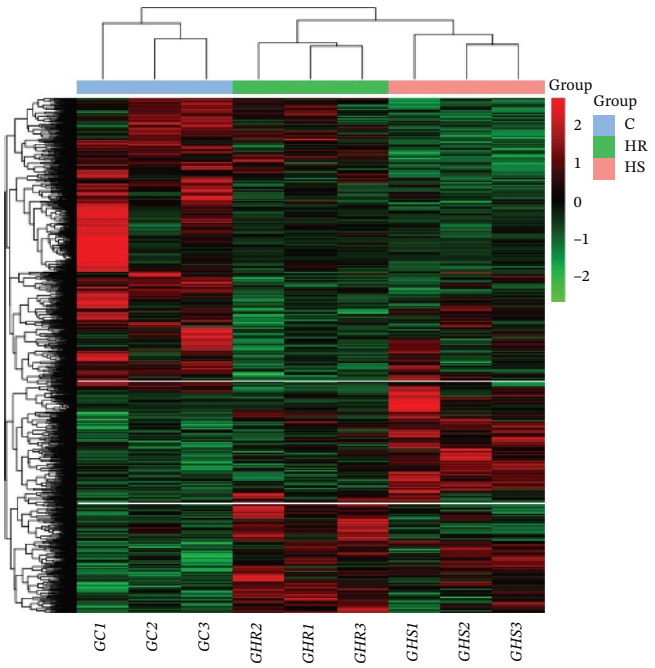


FIGURE 4 | Hierarchical clustering heatmap of differentially expressed genes (DEGs) in the skin of hybrid grouper. Heatmap illustrates the global expression profiles across nine samples, including control (GC1−3), handling-resilient (GHR1−3), and handling-sensitive (GHS1−3). Each row represents an individual DEG, while each column represents a biological replicate. The color scale indicates the relative expression levels, where red color denotes higher expression and green denotes lower expression.

enriched pathways ($p_{adj} < 0.05$). The carbon metabolism pathway was the most significantly enriched among the downregulated genes (19 downregulated DEGs), indicating a suppression of primary energy metabolism in HS fish. No significant enrichment was observed for upregulated DEGs in the HS group. Based on KEGG Orthology (KO) annotations, these enriched pathways were classified into two major functional networks: "metabolism" and "organismal systems" (Figure 7). These networks illustrated the complex interactions of cellular molecules and metabolic intermediates involved in the stress-response signatures of the hybrid grouper skin.

3.6 | Key Genes in Response to Handling Stress

Based on KEGG and GO enrichments, the candidate DEGs associated with stress response of the skin following 10-day repeated handling stress were presented in Figure 8. The stress response pathways of the significantly enriched genes in HR group were upregulated in oxidative phosphorylation such as NADH ubiquinone oxidoreductase subunits C2, A3, A5, A7, and B (*ndufc2*, *ndufa3*, *ndufa5*, *ndufa7*, and *ndufb6*), ATP synthase components (*atp5pf* and *atp5pd*), cytochrome b-c1 complex subunit Rieske and 8 (LOC125886998 and LOC125894313); cytochrome c oxidase subunit 5A, 5B, and 6A (LOC125887767, LOC125903063, and LOC125878961); succinate dehydrogenase complex subunit B (*sdhb*). Some genes were enriched in purine metabolism, including ATP synthase H + transporting mitochondrial F0 complex subunit G, *atp5l*; and aldolase A fructose-bisphosphate B, *aldoab*.

In contrast, the stress response pathways of the significantly enriched genes in HS group were downregulated in metabolism pathways such as pyruvate metabolism, arginine biosynthesis, valine, leucine, and isoleucine degradation, amino acid metabolism, carbohydrate metabolism, and oxidation–reduction that were mediated by genes, including myosin-7 (LOC125901781); aspartate aminotransferase cytoplasmic-like (LOC125885351); cytochrome b-c1 complex subunit (LOC125902759); troponin T

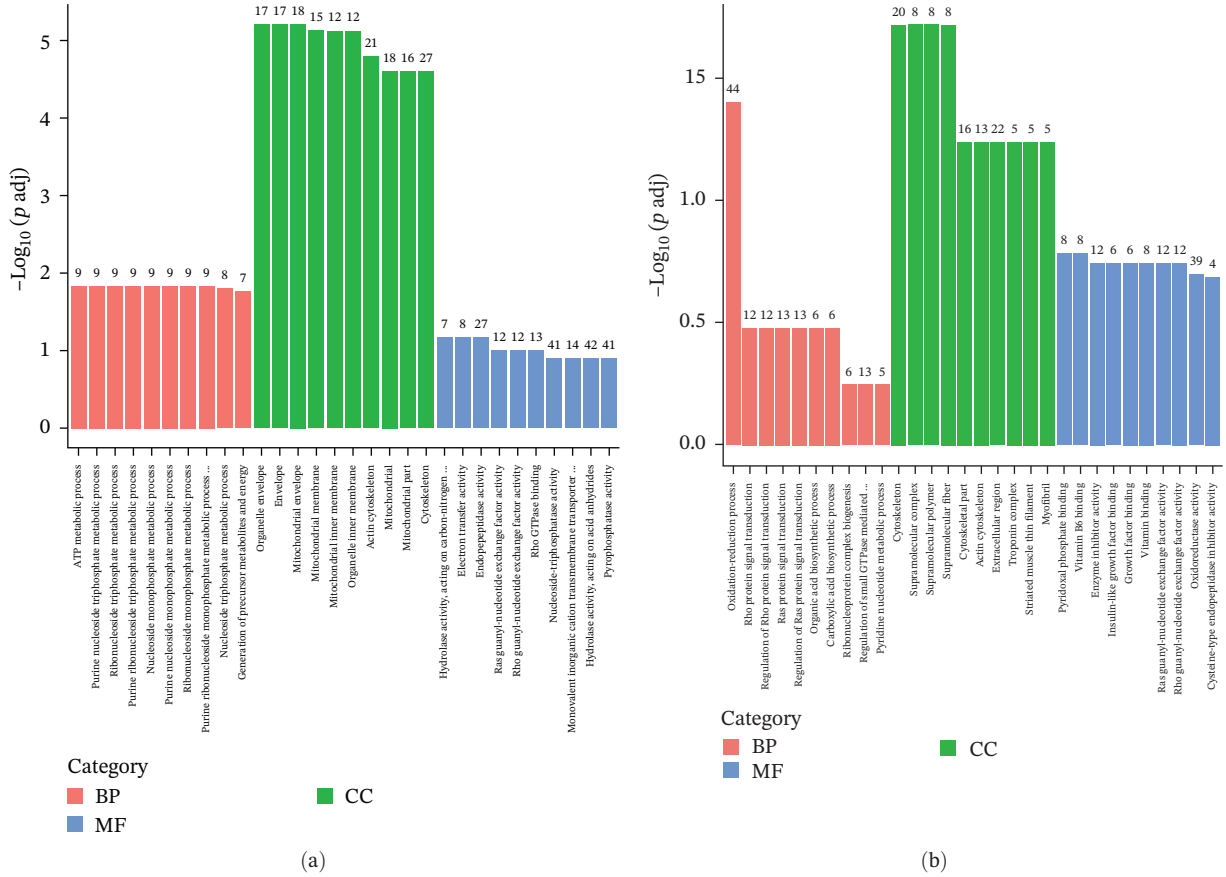


FIGURE 5 | Gene ontology (GO) functional enrichment analysis of DEGs in the skin of hybrid grouper subjected to repeated handling stress. DEGs were categorized into three primary functional domains: biological process (BP), cellular component (CC), and molecular function (MF). The *x*-axis represents the specific GO terms, while the *y*-axis indicates the total number of DEGs associated with each term. (a) Enriched GO terms in the handling-resilient vs. control comparison and (b) enriched GO terms in the handling-sensitive vs. control comparison.

cardiac muscle isoforms-like (LOC125897791); dihydrolipoamide dehydrogenase, *dldh;* ALT, *si_ch211–217a12.1*; aspartate aminotransferase cytoplasmic like (LOC125885351); 3-hydroxyisobutyrate dehydrogenase B, *hibadhb*; glutamic pyruvate transaminase 2, *gpt2*; glutamic–oxaloacetic transaminase 2B, *got2b*; guanosine monophosphate reductase, *gmpr*; sorbitol dehydrogenase, *sord*; LDH Ba, *ldhba*; isocitrate dehydrogenase NADP + 2, *idh2*; aldehyde dehydrogenase 4, 6 family member A1, *aldh4a1*, *aldh6a1*; malate dehydrogenase 2, *mdh2*; and apoptosis inducing factor mitochondrian associated 1, *aifm*.

Genes associated with cytoskeletal dynamics were upregulated in the HR group, including FYVE RhoGEF and PH domain-containing 5a, *fgd5a;* and Rho guanine nucleotide exchange factor, *arhgef10*. However, the HS group exhibited downregulation of guanine nucleotide-binding protein-like 2, *gnl2;* Ras homolog family member Q, *rhoq;* and insulin-like growth factor binding protein 2a, *igfbp2a*.

3.7 | Validation of DEGs via RT-qPCR

To validate the reliability of the RNA-Seq data, five DEGs associated with metabolism, apoptosis, and cellular processes were selected for RT-qPCR analysis. The selected genes included NADH: Ubiquinone Oxidoreductase Subunit C2 (*ndufc2*), ATP Synthase Peripheral Stalk Subunit D (*atp5pd*), Matrix Metallopeptidase 2 (*mmp2*), Tubulin Beta 5 Class I (*tubb5*), and Glutamic–Pyruvic Transaminase 2 (*gpt2*). The RT-qPCR results demonstrated that the expression trends of all five genes were highly consistent with the expression profiles obtained from the Illumina sequencing (Figure 9). Specifically, *ndufc2*, *atp5pd*, and *mmp2* exhibited significant upregulation, while *tubb5* and *gpt2* were downregulated.

3.8 | Plasma Biochemical and Enzymatic Profiles

The effect of 10-days repeated handling stress on the blood plasma biochemistry of hybrid grouper was illustrated in Figure 10a–e. Statistical analysis revealed that handling stress did not significantly alter the level of ALT, ALP, LDH, GLU, or CHOL compared to the control group ($p > 0.05$). Blood plasma LDH, GLU, and ALP levels did not differ in the control, HR, and HS groups. Nevertheless, ALT activity was elevated in the HR group relative to both the control and HS groups. Notably, CHOL levels appeared lower in the stressed group; however, this was accompanied by high variability between individual fish.

3.9 | Histopathological Examination

3.9.1 | Liver

Histological examination of liver specimens from hybrid grouper subjected to repeated handling stress revealed varying degrees of cellular alterations across the experimental groups (Figure 11). The

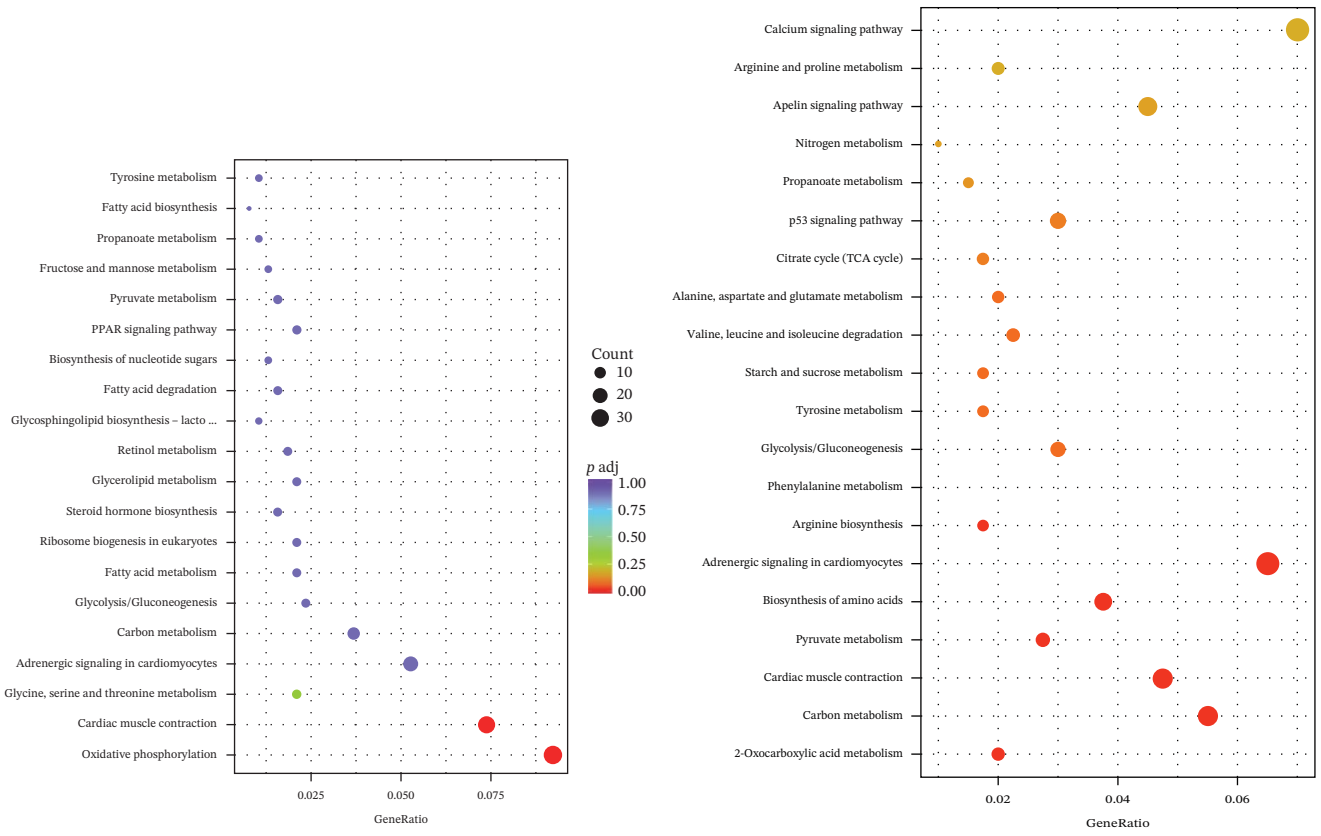


FIGURE 6 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment scatter plot of DEGs in the skin tissue of hybrid grouper. (a) Top 20 enriched KEGG pathways in the handling-resilient vs. control comparison and (b) Top 20 enriched KEGG pathways in the handling-sensitive vs. control comparison. The *x*-axis represents the ratio of the number of DEGs mapped to a specific pathway relative to the total number of genes annotated to that pathway. The *y*-axis lists the specific KEGG pathways. The size of each point is proportional to the number of DEGs associated with the pathway, while the color gradient (from red to purple) indicates the level of statistical significance based on the adjusted *p*-value ($p_{adj} < 0.05$).

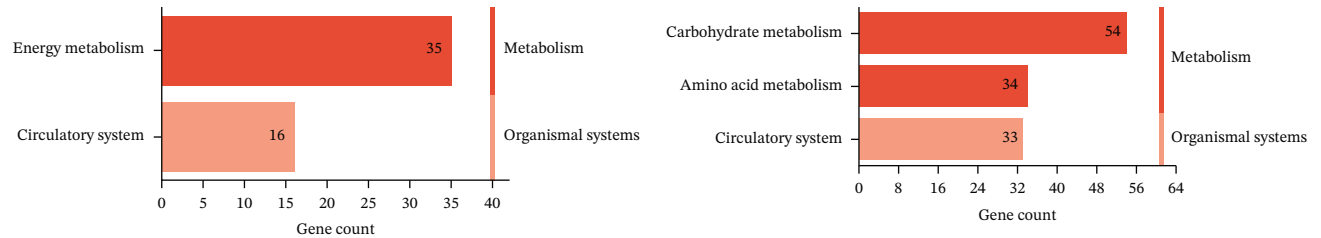


FIGURE 7 | Functional classification of significantly enriched KEGG Orthology (KO) terms for DEGs ($p_{adj} < 0.05$). Functional annotations for (a) handling-resilient vs. control and (b) handling-sensitive vs. control were grouped into two primary categories: metabolism and organismal systems. The *x*-axis represented the total number of DEGs annotated within each specific pathway, while the *y*-axis listed the KEGG pathways categorized under these functional networks.

HS group exhibited significantly higher levels of focal cytoplasmic degeneration ($48.75 \pm 0.47\,\mu m^2$) compared to the HR ($67.82 \pm 0.24\,\mu m^2$) and control ($62.47 \pm 0.20\,\mu m^2$) groups ($p < 0.05$, Table 4). These results indicated that repeated handling stress induces significant hepatic tissue damage in sensitive fish than in resilient fish.

3.9.2 | Spleen

Parenchymal tissue of the spleen showed moderate multifocal areas of necrosis in fish subjected to repeated handling stress (Figure 11). Splenic necrosis was significantly more extensive in the HS group ($3.15 \pm 0.68\,\mu m^2$) but absent in the HR group ($p < 0.05$, Table 4). However, minor necrotic areas were observed in a few fish within the control group ($0.27 \pm 0.29\,\mu m^2$), though these were negligible compared to the HS phenotype.

3.9.3 | Head Kidney

In contrast to the liver and spleen, the head kidney remained relatively unaffected by the 10-day stress protocol (Figure 11).

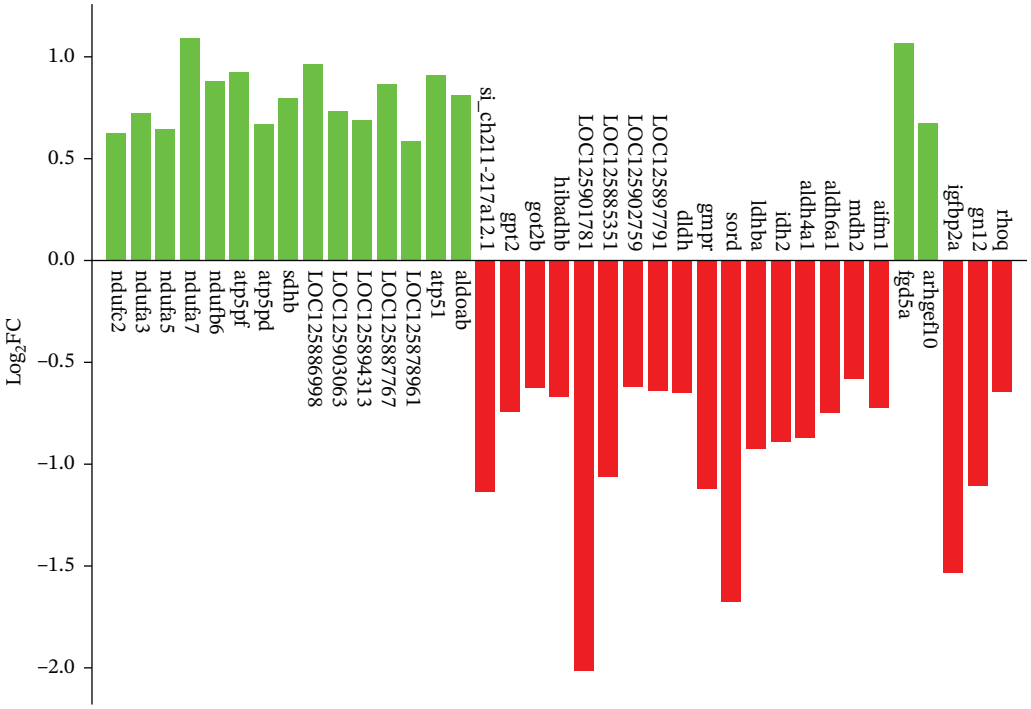


FIGURE 8 | Differentially expressed genes in the skin tissue of hybrid grouper subjected to 10-day repeated handling stress, showing upregulated (green) and downregulated (red) genes. The *x*-axis lists the key genes, while the *y*-axis indicates the log2 fold change expression values.

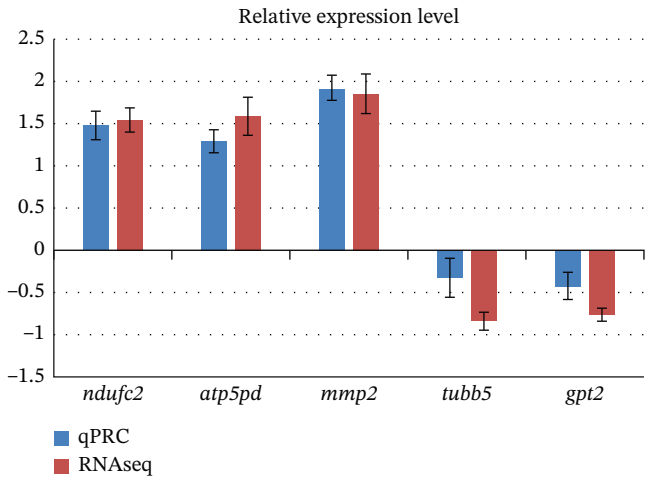


FIGURE 9 | Validation of RNA-seq data via RT-qPCR for selected differentially expressed genes (DEGs) in the skin of hybrid grouper. The *x*-axis lists the five DEGs involved in energy metabolism and cellular structural processes (*ndufc2*, *atp5pd*, *mmp2*, *tubb5*, and *gpt2*). The *y*-axis indicates the relative expression values. Red bars represent the RNA-seq data, while the blue bars represent the RT-qPCR results. The RT-qPCR was normalized to the reference gene peroxisomal biogenesis factor 14 (*pex14*), with relative expression levels calculated using the Pfaffl method. Data are presented as mean ± SD ($n = 3$).

The hematopoietic tissue maintained a normal architecture across all groups. Specifically, the area of melanomacrophage aggregates did not differ significantly between the control, HR, and HS groups ($p > 0.05$, Table 4), suggesting that the head kidney's structural and immune-filtering capacity was preserved despite repeated handling.

4 | Discussion

In intensive aquaculture, stressors such as sorting, harvesting, and transport are inevitable [36, 37]. These activities significantly impact fish health, often leading to immunosuppression and heightened disease vulnerability [31, 38]. In this study, a 10-day repeated handling protocol consisting of daily herding and 5-min air exposure was designed to simulate cumulative stressors encountered in aquaculture operations. While 5 min of air exposure may exceed standard netting durations, it reflects realistic stress scenarios such as prolonged cage lifting during grading, vaccination, harvesting, or transportation under suboptimal conditions. Findings from this study indicated that 10 days of repeated handling elicited a targeted physiological modulation rather than systemic failure. Interestingly, the absence of statistically significant differences in plasma enzyme activities (ALT, ALP, LDH, GLU, and CHOL) suggests the presence of robust physiological buffering and metabolic adaptation mechanisms that maintain homeostasis during stress exposure. Fish regulate internal biochemical conditions through coordinated endocrine and metabolic adjustments, allowing plasma enzyme activities to remain relatively stable despite environmental challenges. Such metabolic plasticity enables cultured species to mitigate cellular damage and preserve tissue integrity during sublethal stress conditions [39, 40]. This interpretation aligns with the transcriptomic data, where the modulation of metabolic regulation and oxidative stress pathways suggests adaptive physiological adjustments rather than severe, acute tissue disruption. Supporting this interpretation, Magnoni et al. [41] reported that *Oncorhynchus mykiss* exhibited no significant changes in oxidative stress biomarkers under hypoxia, indicating effective adaptation. Similarly, Sekar et al. [42] observed no significant variation in ALP activity in *Trachinotus mookalee* subjected to crowding stress, suggesting that certain enzymatic

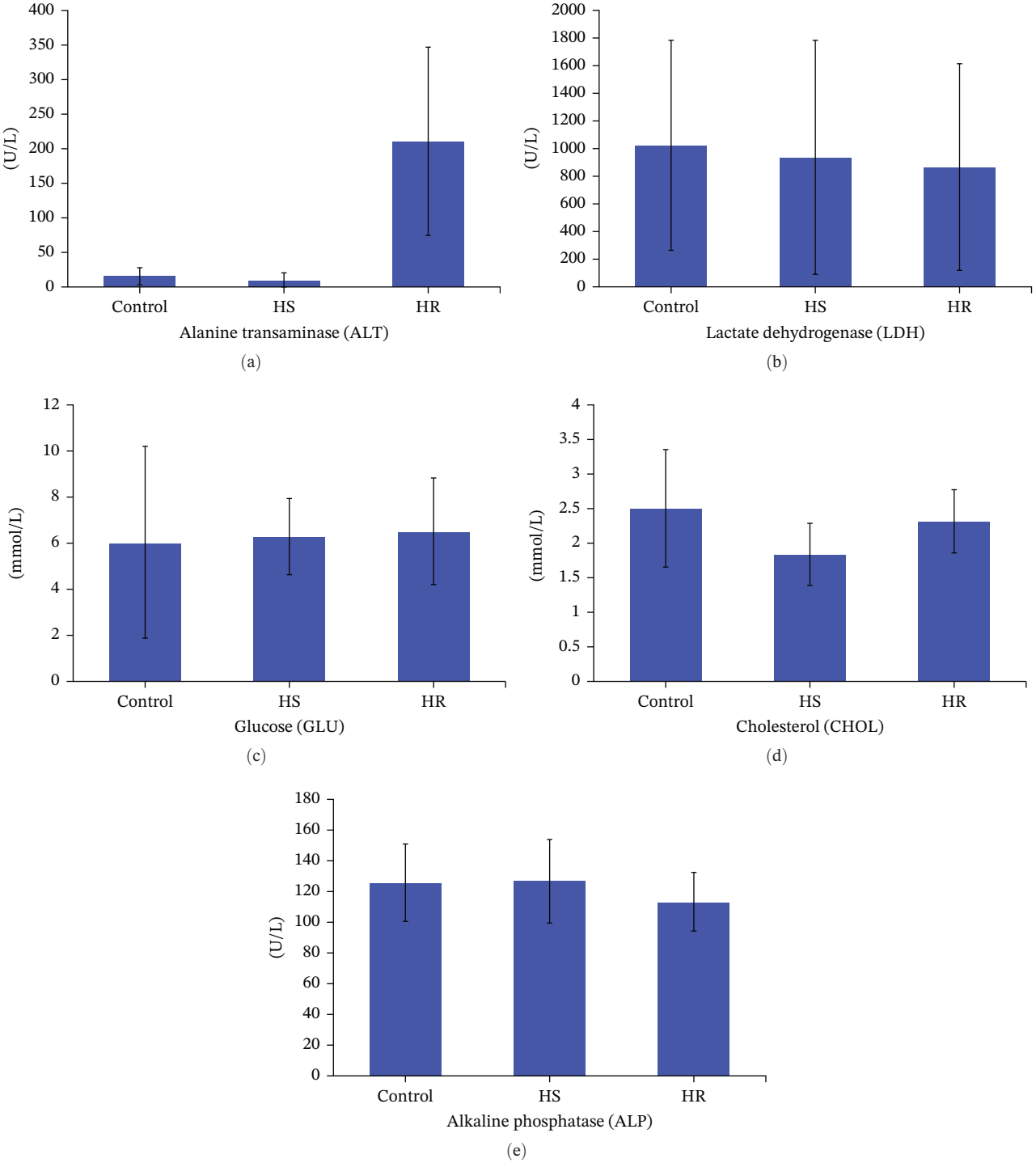


FIGURE 10 | Plasma biochemical and enzymatic profiles of hybrid grouper after 10 days of repeated handling stress. (a) Alanine aminotransferase (ALT); (b) lactate dehydrogenase (LDH); (c) glucose (GLU); (d) cholesterol (CHOL); (e) alkaline phosphatase (ALP). Blood plasma was analyzed from handling-resilient (HR), handling-sensitive (HS), and control groups. Data were expressed as mean ± SD ($n = 2–3$). Statistical analysis using one-way ANOVA followed by Tukey's HSD post hoc test revealed no significant differences among the experimental groups ($p > 0.05$).

biomarkers have limited responsiveness depending on the stress duration and the physiological tolerance of the species.

4.1 | Transcriptomic Responses to Repeated Handling Stress

This study provides a high-throughput analysis of the transcriptomic landscape in the skin of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) subjected to repeated handling stress. The skin, serving as both a physical barrier and an active immune interface, exhibited distinct transcriptional adaptations between HR and HS fish. The identification of 2521 DEGs underscores an active molecular remodeling of skin tissues aimed at mitigating stress impacts [16]. These transcriptional changes were corroborated by histological evidence. HR fish displayed minimal hepatic cytoplasmic degeneration and

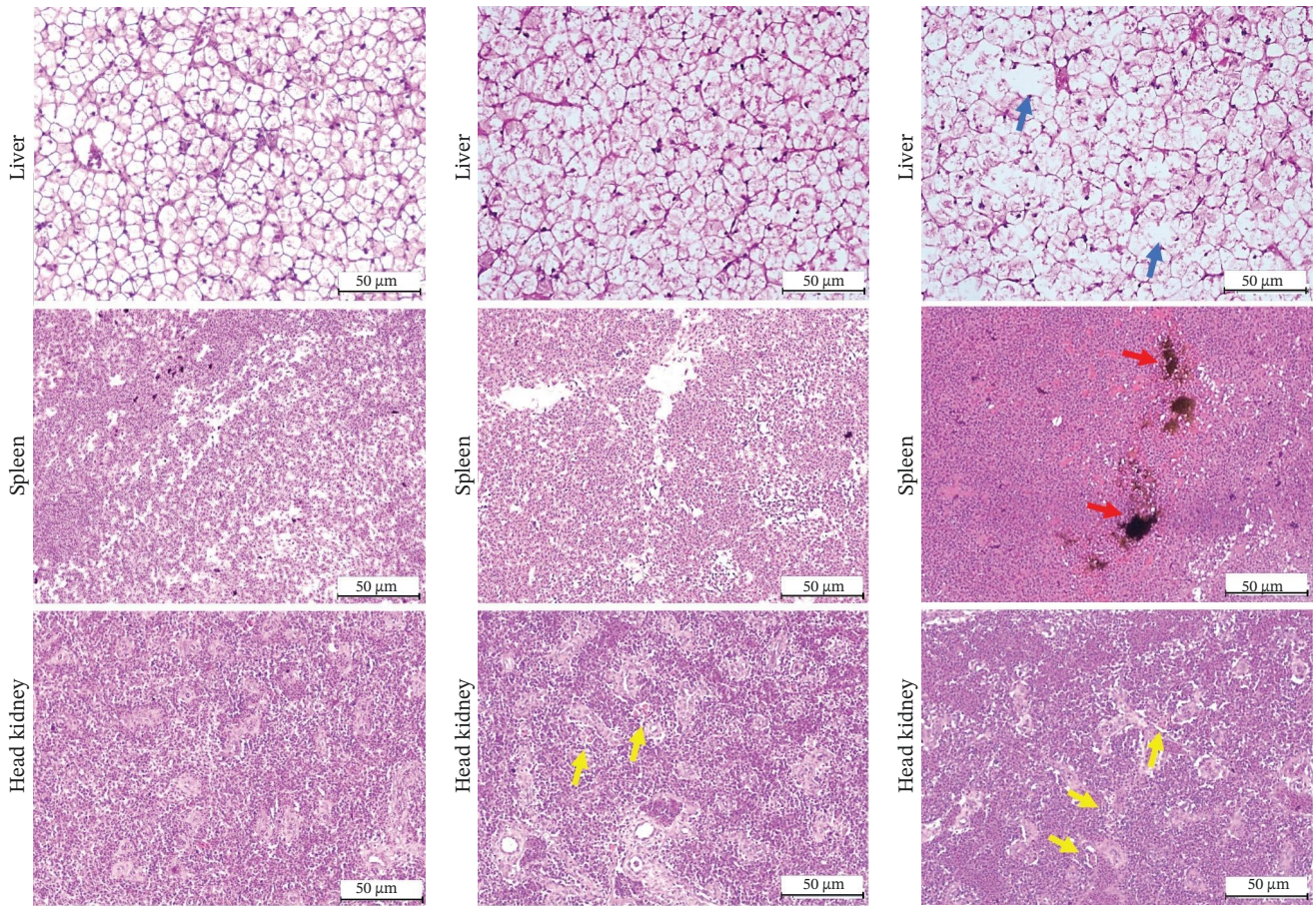


FIGURE 11 | Representative histological sections of liver, spleen, and head kidney from hybrid grouper following a 10-day repeated handling stress. Photomicrographs compare the control group (a), HR group (b), and HS group (c) Liver: blue arrows indicated focal areas of cytoplasmic hepatic degeneration. Spleen: red arrows indicated multifocal areas of necrosis within the splenic parenchyma. Head kidney: yellow arrows denoted melano-macrophage centers within the hematopoietic tissue. All sections were stained with hematoxylin and eosin (H&E) and captured at 200x magnification (scale bar = 50 μm).

TABLE 4 | Quantitative histomorphometric analysis of tissue alterations in the liver, spleen, and head kidney of hybrid grouper.

Phenotype	Liver cytoplasmic area	Spleen necrotic area	MMC total area in head kidney
HS	48.57 ± 0.47^{a}	3.15 ± 0.68^{a}	10.00 ± 4.56^{a}
HR	67.82 ± 0.24^{b}	0.00 ± 0.00^{b}	10.00 ± 4.18^{a}
Control	62.47 ± 0.20^{b}	0.27 ± 0.29^{b}	10.00 ± 6.12^{a}

Note: Data represented the mean area (μm^2) ± standard deviation of cytoplasmic regions in the liver, necrotic patches in the spleen, and melano-macrophage centers in the head kidney. Different superscript letters within the column indicated significant differences ($p < 0.05$) between the control, HR, and HS groups based on one-way ANOVA and Tukey's HSD test.

splenic necrosis, consistent with transcriptomic signatures of efficient metabolic regulation. In contrast, the pronounced tissue damage observed in HS fish correlated with the altered gene expression related to mitochondrial energy production, oxidative stress regulation, and immune signaling. These findings emphasized the skin's role as both a frontline defence and a reflective matrix for the fish's internal physiological state.

4.2 | Energy Metabolism and Adaptive Pathways in Resilient Fish

In the HR group, hybrid groupers exhibited a robust upregulation of oxidative phosphorylation pathways, characterized by the enhanced expression of genes including *ndufc2*, *ndufa3*, *ndufa5*, *ndufa7*, *ndufb6*, *atp5pf*, *atp5pd*, LOC125886998, LOC125894313, LOC125887767, LOC125903063, LOC125878961, and *sdhb*. This indicates elevated mitochondrial activity to meet the heightened energy demands induced by repeated handling stress. Such adaptations align with other findings in pearl gentian grouper and *Oncorhynchus mykiss*, where mitochondrial efficiency underpins stress tolerance [18, 43]. The upregulation of pathways related to cardiac muscle contraction such as *tnnt2d*, further emphasizes the physiological importance of efficient energy and oxygen delivery in maintaining homeostasis during stress [44–46].

The enrichment of purine metabolism, mediated by genes including *atp5l* and *aldoab*, highlights its critical role in stress adaptation. Purine nucleotides (ATP and GTP) are essential not only for energy production but also play pivotal roles in signal transduction and cellular integrity [47, 48]. This metabolic reprogramming likely supports the cellular repair processes that allow HR fish to sustain physiological stability during prolonged stress

[48]. Similar adaptive strategies have been documented in other teleosts; for instance, *Sebastes schlegelii* utilized purine catabolism to supplement energy production during prolonged starvation [49]; while *Pterygoplichthys pardalis* exhibited purine metabolism changes in response to handling stress [31]. Collectively, current findings suggest that enhanced energy metabolism supports the adaptive capacity of HR fish. This transcriptional shift observed indicates elevated mitochondrial capacity and increased ATP generation, which promote tolerance to repeated handling stress. This metabolic prioritization supports physiological maintenance and cellular repair, as evidenced by the preserved liver morphology and minimal splenic necrosis observed in the HR fish. The physiological relevance of these molecular signatures is further supported by enzymatic assays. The relatively higher ALT activity in the HR fish may represent an adaptive mobilization of hepatic amino acids for gluconeogenesis, a response consistent with metabolic adjustment rather than pathological damage. This aligns with observations in *O. mykiss*, where elevated ALT was linked to hepatic involvement in successful stress adaptation [43].

4.3 | Metabolic and Redox Dysregulation in Sensitive Fish

In contrast, HS fish exhibited a significant downregulation of pathways associated with metabolism and oxidation–reduction (redox), indicating impaired energy production, inefficient resource allocation, and antioxidant defence mechanisms under handling stress [28, 50]. Pathways including pyruvate metabolism, arginine biosynthesis, and valine, leucine, and isoleucine degradation were significantly downregulated, where pathway-associated genes such as *si_ch211–217a12.1*, *gpt2*, *got2b*, *hibadhb*, LOC125901781, LOC125885351, LOC125902759, LOC125897791, and *dldh* being suppressed. As pyruvate is the primary link between glycolysis and the tricarboxylic acid (TCA) cycle, its reduced availability likely restricts ATP generation, exacerbating energy deficits [51]. Similar patterns of compromised metabolism intensifying physiological stress responses have been reported in *Trachinotus ovatus* and *Cyclopterus lumpus* [50, 52].

The transcriptional repression of metabolic and redox-associated genes, including *gmpr*, *ldhba*, *idh2*, *aldh4a1*, *aldh6a1*, *mdh2*, *dldh*, *hibadhb*, and, *aifm1*, indicates a profound impairment of mitochondrial redox homeostasis and antioxidant defence [28]. Although reactive oxygen species (ROS) were not directly quantified, the transcriptional repression of these pathways suggests a reduced capacity to detoxify ROS, predisposing metabolically active tissues to oxidative injury and inflammatory signaling. Similar redox-driven metabolic collapse has been reported in *Penaeus vannamei* and *Mycropterus salmoides*, where mitochondrial dysfunction and ROS accumulation intensified tissue degeneration and inflammatory signaling [52–54]. Histopathological observations corroborated these findings. The HS fish showed significant hepatic cytoplasmic degeneration and splenic necrosis (Figure 11). Furthermore, the transcriptional repression of antioxidant and redox-associated genes in HS fish provides a mechanistic basis for the observed splenic necrosis. Under sustained stress, elevated ROS levels coupled with impaired cellular repair and immune signaling would likely predispose lymphoid tissues to degeneration. These pathological changes align with the observed transcriptomic suppression of metabolic pathways and mirror findings in other teleosts exposed to handling or hypoxic stress [7, 55]. Supporting this, enzymatic assays revealed lower CHOL levels, this suggests an impairment in energy mobilization and lipid metabolism under chronic stress. Furthermore, the shifts in CHOL levels highlight metabolic divergence during stress adaptation [56]. While GLU typically rises via cortisol-mediated mobilization [5], the absence of significant hyperglycemia in this study suggests a species-specific metabolic exhaustion or a regulatory transition toward hypoglycemia, contrasting with the acute responses observed in *Lates calcarifer* and *Salmo salar* [5, 30]. Lower CHOL levels in stressed fish further indicate alterations in lipid metabolism by triggering adrenergic stimulation of lipolysis and enhanced phospholipid hydrolysis, which may suppress β-oxidation as documented in *Scophthalmus maximus* under hypoxic conditions [56].

4.4 | Interplay Between Amino Acid and Carbohydrate Metabolism

The concurrent downregulation of amino acid and carbohydrate metabolism in HS fish underscores a synergistic energy deficit. Key genes involved in amino acid transamination and gluconeogenic precursor mobilization, specifically *si:ch211-217a12.1*, *gpt2*, and *got2b*, serve dual roles as metabolic bridges. They convert amino acids into essential intermediates, such as pyruvate and oxaloacetate, which feed directly into the TCA cycle. The reduction in amino acid and carbohydrate metabolisms suggests limited replenishment of critical metabolites, further restricting pyruvate production and subsequent ATP generation [50]. This cascading effect highlights the interconnected nature of metabolic networks, where disruptions in amino acid mobilization exacerbate deficits in carbohydrate processing. Similar inhibitory patterns have been reported in hybrid groupers under light stress [57] and in *T. ovatus* under hypoxia, where these pathways were suppressed during the stress phase but significantly upregulated during recovery [50].

In contrast, HR fish showed significant upregulation of oxidative phosphorylation and purine metabolism. This indicates sustained mitochondrial activity and efficient ATP production, which are vital for maintaining redox homeostasis under repeated handling stress [28, 49]. Likewise, previous studies demonstrated the centrality of oxidative stress modulation in adaptive responses to starved *Danio rerio* [58], hypoxic *Epinephelus coioides* [51], crowded *Acanthopagrus schlegelii* [28], and chronically stressed *Oreochromis niloticus* and *Sparus aurata* [59, 60]. Maintaining redox balance thus appears to be a critical determinant of stress resilience, enabling tolerant fish to meet the high energy costs of stress without compromising cellular health [59]. This was reflected in both their histological integrity and stable enzymatic profiles, indicating a dynamic interplay between molecular regulation and cellular health. This metabolic adaptability underscores their ability to meet the demands of prolonged stress, thereby mitigating its physiological impacts [52], which likely contributed to the absence of obvious histological damage and stable enzymatic profiles in these individuals. The stark contrast between the two groups emphasizes the importance of coordinated oxidative phosphorylation, amino acid, and carbohydrate metabolism in determining stress resilience.

4.5 | Cytoskeletal and Regenerative Responses

In the HR fish, the upregulation of genes associated with cytoskeletal dynamics, such as *fgd5a* and *arhgef10* in Ras and Rho guanyl-nucleotide exchange factor activity pathways, likely supports skin barrier integrity and structural resilience against mechanical handling [61]. In contrast, HS fish exhibited a downregulation of *gnl2*, *rhoq*, and *igfbp2a*. As growth factors are essential for tissue regeneration and angiogenesis, the suppression of *igfbp2a* suggests compromised cellular repair mechanisms in HS fish [62]. This impaired regenerative capacity, coupled with diminished immune signaling, underscores the vulnerability of HS fish to tissue damage, evidenced by the hepatic cytoplasmic degeneration and splenic necrosis observed in this study. These pathological changes are similar to the observations in *Astyanax altiparanae* [55] and *Salmo salar* [7] under similar handling stressors.

Notably, the stable morphology of the head kidney suggests that the primary HPI axis remained largely preserved. This indicates that the stress response in hybrid grouper manifests primarily through peripheral (skin and liver) metabolic and immune reprogramming rather than systemic endocrine disruption. In addition, the downregulation of genes related to cytoskeletal integrity in the HS fish, further explains the increased tissue fragility and diminished capacity for homeostatic recovery. In general, this study could serve as a baseline for elucidating the behavioral and physiological responses of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) to repeated handling stress by integrating transcriptomic, histopathological, and biochemical analyses. Nevertheless, several limitations may have affected the statistical significance of the results. In particular, the absence of statistical significance in enzymatic parameters likely reflects a limited dataset and interindividual variability, thereby constraining the detection of subtle yet biologically meaningful responses. Furthermore, the scarcity of comparative biochemical reference data for this hybrid species limits the robustness of specific interpretations. As such, the findings should be regarded as preliminary baseline data. Future investigations employing larger sample sizes and comprehensive reference datasets are essential to validate these observations and to strengthen inferences regarding metabolic regulation, stress resilience, and aquaculture optimization.

4.6 | Implications for Aquaculture Welfare

The implications of these findings for best management practices in aquaculture are significant. Optimizing handling techniques with the utilization of fish pumps during transfer and harvesting is highly recommended [63]. In parallel, the transition toward minimally invasive monitoring approaches offers substantial potential to enhance fish welfare while maintaining robust physiological assessments [64]. Rapid, minimally invasive screening techniques, such as surface-enhanced Raman spectroscopy (SERS) applied to epidermal mucus, provide a promising tool for discriminating tolerant and susceptible fish in response to stressors [24]. The industrial revolution, coupled with technological advancements, could be vital in handling stress through the incorporation of artificial intelligence (AI), the Internet of Things (IoT), and automation, particularly in the handling of fish during sorting, transfer, and harvesting [65]. For instance, AI can analyze data from monitoring systems to optimize water quality and fish behaviors, while IoT devices can provide real-time feedback on environmental conditions via underwater cameras and water sensors [66]. Automation can streamline sorting and transfer processes, minimizing human-induced stressors [67]. Technologies such as bio-loggers to monitor heart rate in farmed [68] and machine vision-based methods for size measurement, mass estimation, species and stock identification, gender identification, quality assessment, grading, behavior monitoring, and counting in fish farms are promising innovations [69].

5 | Conclusion

This study provides a comprehensive characterization of the molecular and physiological disruptions induced by repeated handling stress in hybrid grouper. HR fish maintained homeostatic stability through the upregulation of genes involved in energy metabolism, immune function, and cytoskeletal stability, while HS fish exhibited marked metabolic suppression, impaired redox balance, and reduced cellular repair capacity. Although plasma enzymatic biomarkers remained relatively stable, histopathological analysis confirmed significant internal damage in the sensitive phenotype, including hepatic cytoplasmic degeneration and splenic necrosis. This discrepancy suggests that while systemic biochemical parameters may appear compensated, peripheral tissues undergo profound degenerative changes during chronic stress. Consequently, these findings highlighted the high sensitivity of the skin transcriptome as a reflective matrix for internal physiological status. Ultimately, this research demonstrates the potential of skin transcriptome and epidermal mucus as a promising, minimally invasive biological matrix for welfare monitoring. Integrating these molecular insights with selective breeding, refined handling protocols, and Industry 4.0 technologies such as AI, automated sorting, and real-time sensor networks would offer a strategic pathway to enhance the resilience, welfare, and long-term sustainability of hybrid grouper aquaculture.

Author Contributions

Saleema Matusin: writing – original draft, data curation, formal analysis, investigation, methodology, visualization. **Annie Christianus**: writing – review and editing, funding acquisition, conceptualization, data curation, methodology, supervision. **Norazrin Ariffin**: investigation. **Annas Salleh**: writing – review and editing, formal analysis. **Chen Fei Low**: writing – review and editing, methodology. **Chou Min Chong**: conceptualization, methodology, data curation. **Ina Salwany Md Yasin and Muhammad Hafiz Abu Bakar**: visualization.

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Ethics Statement

All animal handling procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (Certificate Number UPM/ACUC/AUP-R054/2022) prior to the initiation of the experiments.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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