

Article

Metabolomic Signatures Uncover Domestication-Driven Hypoxia Tolerance in Common Carp (*Cyprinus carpio*)

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Abstract

Hypoxia represents a pervasive selective pressure in aquaculture systems, prompting aquatic organisms to develop distinct tolerance strategies through diverse physiological and biochemical responses. The common carp (*Cyprinus carpio*), a species characterized by extensive geographical distribution and a long history of artificial selection, exhibits considerable inter-varietal and inter-population variation in hypoxia tolerance. Nevertheless, the mechanistic basis underlying hypoxia tolerance in the context of domestication remains poorly understood. The present study employed mirror carp (*C. carpio* var. *specularis*), a highly domesticated strain with proven hypoxia tolerance, and Hebao red carp (*C. carpio* var. *wuyuanensis*), a locally distributed, minimally domesticated variety that is relatively sensitive to hypoxia, to investigate the metabolic responses and underlying mechanisms associated with hypoxia tolerance. Focusing on muscle and liver tissues, we performed separate statistical and bioinformatics analyses of the metabolic profiles derived from the two strains. The results revealed that hypoxia exposure induced only minor perturbations in energy metabolism in mirror carp, whereas Hebao red carp exhibited a profound metabolic depression in muscle, accompanied by signs of insufficient energy production in the liver. Notably, an enhanced nitric oxide (NO) pathway, implicated in vascular tone regulation and oxygen delivery, was observed in the liver of mirror carp but not in that of Hebao red carp. Collectively, these findings delineate distinct metabolic patterns between common carp strains subjected to differing degrees of domestication, which may account for their differential hypoxia tolerance. Moreover, this study offers a theoretical foundation for the selective breeding of hypoxia-resilient fish strains in aquaculture practices.



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Keywords: common carp; hypoxia tolerance; metabolomics; nitric oxide pathway

Key Contribution: Hypoxia-tolerant mirror carp show preserved muscle aerobic metabolism, enhanced hepatic gluconeogenesis, and activated NO-mediated oxygen delivery, whereas hypoxia-sensitive Hebao red carp exhibit metabolic depression and hepatic energy deficit.

1. Introduction

Aquatic hypoxia—dissolved oxygen (DO) dropping below physiological requirements—is a pervasive and growing selective pressure in aquaculture and natural waters [1]. Driven by oxygen's inherently low solubility and slow diffusion, it compromises growth, elevates disease risk, and causes massive mortalities in farmed fish [1,2]. For teleost fishes, hypoxia poses a severe challenge to aerobic metabolism, necessitating a complex array of behavioral, physiological, and biochemical adaptations to maintain energy homeostasis and survive oxygen deprivation [3]. Across species, a wide array of adaptive responses have been documented over decades of research, including metabolic suppression, maintenance of pH homeostasis, and alleviation of oxidative damage upon reoxygenation [4,5]. While the general physiological responses to oxygen deprivation have been well characterized, the majority of mechanistic studies have concentrated on acute physiological acclimation. However, the extent to which long-term artificial domestication has fundamentally reshaped the metabolic mechanisms underpinning hypoxia tolerance remains a critical and underexplored frontier in evolutionary physiology [6].

Domestication imposes strong targeted selection for desirable traits such as rapid growth and feed efficiency, often resulting in unintended genetic drift and altered stress responsiveness that may inadvertently compromise environmental resilience [6,7], including hypoxia tolerance, as energy allocation shifts toward somatic growth at the expense of stress coping mechanisms [7]. However recent evidence suggests that domestication can also drive the fixation of specific metabolic phenotypes that enhance survival under intensive farming conditions, where hypoxia is a recurrent stressor [8]. Understanding whether domestication has enhanced or eroded hypoxia tolerance, and identifying the underlying metabolic mechanisms, is therefore essential for developing sustainable breeding programs that balance production performance with environmental robustness [2].

The common carp (*Cyprinus carpio*) serves as an ideal model system to dissect the metabolic implications of domestication on hypoxia tolerance [8]. As one of the earliest and most widely cultured freshwater fish species, common carp has undergone centuries of artificial selection, resulting in numerous distinct strains with varying degrees of domestication intensity [8]. Among these, Mirror carp (*C. carpio* var. *specularis*) represents a highly domesticated lineage selected for decades for rapid growth and uniform morphology under high-density farming conditions, whereas Hebao red carp (*C. carpio* var. *wuyuanensis*) retains a more primitive genetic background and lower domestication intensity [8,9]. Previous transcriptomic analyses have revealed that these two strains exhibit divergent genetic backgrounds regarding hypoxia tolerance, with significant differences in the expression of hypoxia-inducible factors and stress-responsive genes [8,9]. However, gene expression changes do not always translate directly into functional metabolic outcomes, leaving the downstream metabolic consequences of these genetic differences largely uncharacterized [9]. This gap highlights the necessity of moving beyond transcriptomics to examine the actual physiological state through metabolomics, which captures the integrated output of regulatory networks and reflects real-time energy redistribution and redox balance [10–12].

Metabolomics provides a dynamic chemical snapshot of cellular physiology, offering direct insight into the functional endpoints of hypoxia adaptation that transcriptomics alone cannot reveal [11]. By profiling the metabolome, researchers can identify key metabolic shifts in energy metabolism, such as glycolytic flux, tricarboxylic acid (TCA) cycle activity, and lipid oxidation, as well as changes in antioxidant defenses and signaling molecules [4,10,12]. For instance, previous metabolomic studies in cyprinids have identified key metabolic shifts in energy metabolism and antioxidant defense during acute hypoxia and reoxygenation, including changes in glucose levels, lipid peroxidation, and antioxidant profiles such as Catalase and Glutathione-S-Transferase [4,10]. Furthermore, ¹H-NMR

metabolomics has distinguished the metabolic strategy of common carp under moderate hypoxia from the extreme anoxia tolerance of crucian carp, highlighting the reliance on metabolic suppression and specific adaptive mechanisms rather than ethanol fermentation [11,13]. Despite these advances, most studies have focused on acute physiological acclimation within single strains, failing to address how domestication history influences constitutive metabolic architectures or inducible responses across differently domesticated lines [10].

The liver and skeletal muscle serve as central hubs for metabolic responses to hypoxia, orchestrating energy production, storage, and glucose homeostasis via gluconeogenesis, glycogenolysis, and anaerobic glycolysis [9,14]. Yet, whether the superior hypoxia tolerance of Mirror carp over Hebao red carp reflects a constitutively distinct metabolic architecture under normoxia or an enhanced inducible capacity upon hypoxic challenge remains ambiguous [9,15]. Furthermore, the involvement of the nitric oxide (NO) pathway—a key regulator of vascular tone and oxygen delivery—in modulating strain-specific tolerance has not been fully characterized [16–18].

Emerging evidence in other teleosts underscores the importance of metabolic reprogramming and antioxidant defense in hypoxia acclimation [19,20], yet domestication-driven modifications to these processes remain largely unexplored. In common carp, prior biochemical work has focused primarily on temporal oxidative stress responses rather than on comparative metabolic configurations across differentially domesticated strains [4]. Elucidating these domestication-induced metabolic shifts is of practical significance for sustainable aquaculture, as hypoxia-induced mortality continues to constrain carp production [2]. Identifying reliable metabolic biomarkers associated with enhanced resilience could enable non-lethal screening and marker-assisted selection [7]. Accordingly, this study aims to decipher the metabolic underpinnings of differential hypoxia tolerance between Mirror and Hebao red carp, offering a theoretical basis for breeding hypoxia-resilient strains and advancing robust aquaculture practices under environmental change.

2. Materials and Methods

2.1. Fish Maintenance

Experimental fish were obtained from a single batch of Mirror carp and Hebao red carp hatched at the Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences. At 3 months of age, healthy individuals with intact body surfaces and a body weight of 197.5 ± 6.1 g were selected. A total of 30 individuals of each strain were randomly assigned to three replicate tanks (10 fish per tank) and acclimated for 7 days under controlled conditions: water temperature 25 °C, dissolved oxygen 6 mg/L, pH 7.0–8.0, ammonia nitrogen 0.3 mg/L, and nitrite nitrogen 0.01 mg/L. After acclimation, fish were subjected to subsequent experimental procedures.

2.2. Hypoxia Exposure

Experimental fish from each strain were randomly assigned to either normoxia (control) or hypoxia treatment groups. Feeding was suspended throughout the experimental period to eliminate dietary confounding effects. For the hypoxia group, fish were first introduced into the experimental tanks, and then aeration was terminated at the onset of the experiment, allowing the DO concentration to decline gradually and naturally. DO levels were recorded at 15 min intervals using an Orion Star A223 RDO optical dissolved oxygen meter (Thermo Fisher Scientific, Waltham, MA, USA). In the hypoxia treatment group, DO concentration was allowed to decrease progressively and subsequently maintained at 1.02 ± 0.06 mg/L for a duration of 24 h. In contrast, the normoxia group was maintained

at a constant DO level of 6.0 ± 0.1 mg/L throughout the experimental period by means of continuous aeration.

2.3. Tissue Sampling

Upon completion of the exposure period, fish were euthanized with an overdose of MS-222 (Sigma-Aldrich Company, Shanghai, China), with 10 individuals per group. Approximately 2 g of skeletal muscle and 2 g of liver tissue were excised from each individual across all treatment groups. Tissue samples were rinsed three times with ice-cold phosphate-buffered saline (PBS) (Shanghai Sangon Biotech Co., Ltd., Shanghai, China) to remove residual blood, immediately snap-frozen in liquid nitrogen, and stored at -80 °C until metabolite extraction.

2.4. LC-MS/MS Analysis

Untargeted metabolomic profiling was performed using an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole time-of-flight mass spectrometer (TripleTOF 6600, AB Sciex, Framingham, MA, USA). Chromatographic separation was achieved via hydrophilic interaction liquid chromatography (HILIC) on an ACQUITY UPLC BEH Amide column ($2.1 \text{ mm} \times 100 \text{ mm}$, $1.7 \mu\text{m}$; Waters, Milford, MA, USA) [21]. The mobile phase consisted of (A) 25 mM ammonium acetate and 25 mM ammonium hydroxide in water and (B) acetonitrile. The gradient elution program was as follows: 85% B held for 1 min, linearly decreased to 65% B over 11 min, reduced to 40% B in 0.1 min and held for 4 min, returned to 85% B in 0.1 min, followed by a 5 min re-equilibration period.

Data were acquired in both ESI positive and negative ionization modes. The ESI source parameters were configured as follows: ion source gas 1 (Gas1), 60 psi; ion source gas 2 (Gas2), 60 psi; curtain gas (CUR), 30 psi; source temperature, 600 °C; ion spray voltage floating (ISVF), ± 5500 V. For MS-only acquisition, the scan range was set to m/z 60–1000 Da with an accumulation time of 0.20 s per spectrum. For auto MS/MS acquisition, the scan range was m/z 25–1000 Da with an accumulation time of 0.05 s per product ion spectrum. Product ion spectra were acquired using information-dependent acquisition (IDA) in high-sensitivity mode. The collision energy (CE) was fixed at 35 V with a spread of ± 15 eV, and the declustering potential (DP) was set at ± 60 V.

2.5. Data Processing and Statistical Analysis

Raw MS data (.wiff.scan files) were converted to MzXML format using (version 3.0.8789) and subsequently processed with XCMS software (version 1.26.0) for peak detection, retention time correction, and chromatographic alignment. Metabolite identification was performed by matching accurate mass measurements (<25 ppm mass error) and MS/MS fragmentation patterns against an in-house reference standards database.

Following feature extraction, variables with nonzero measurement values in fewer than 50% of samples within at least one group were excluded. Multivariate statistical analyses were conducted using the MetaboAnalyst platform (www.metaboanalyst.ca, accessed on 25 August 2025). Data were subjected to Pareto scaling prior to principal component analysis (PCA) for unsupervised overview and orthogonal partial least-squares discriminant analysis (OPLS-DA) for supervised classification. Model robustness was evaluated by leave-one-out cross-validation (LOOCV) and response permutation testing ($n = 200$). Differential metabolites were identified based on the following criteria: variable importance in projection (VIP) values >1.0 derived from the OPLS-DA model, combined with a two-tailed Student's *t*-test *p*-value < 0.05 applied to the original data.

3. Results

3.1. Untargeted Metabolomic Profiling of Mirror Carp Muscle Under Hypoxia

Untargeted metabolomic analysis identified 65 metabolites exhibiting significantly altered abundance ($p < 0.05$) in the muscle of mirror carp following hypoxia exposure compared with the normoxia control (Supplementary Table S1; Figure 1A). Of these, 44 metabolites were significantly upregulated, including leucine, isoleucine, and arginine, whereas 21 metabolites were significantly downregulated, notably glutamate, betaine, carnitine, and taurine. Additionally, decreasing trends were observed for alanine and malic acid, although these changes did not reach statistical significance ($0.05 < p < 0.1$).

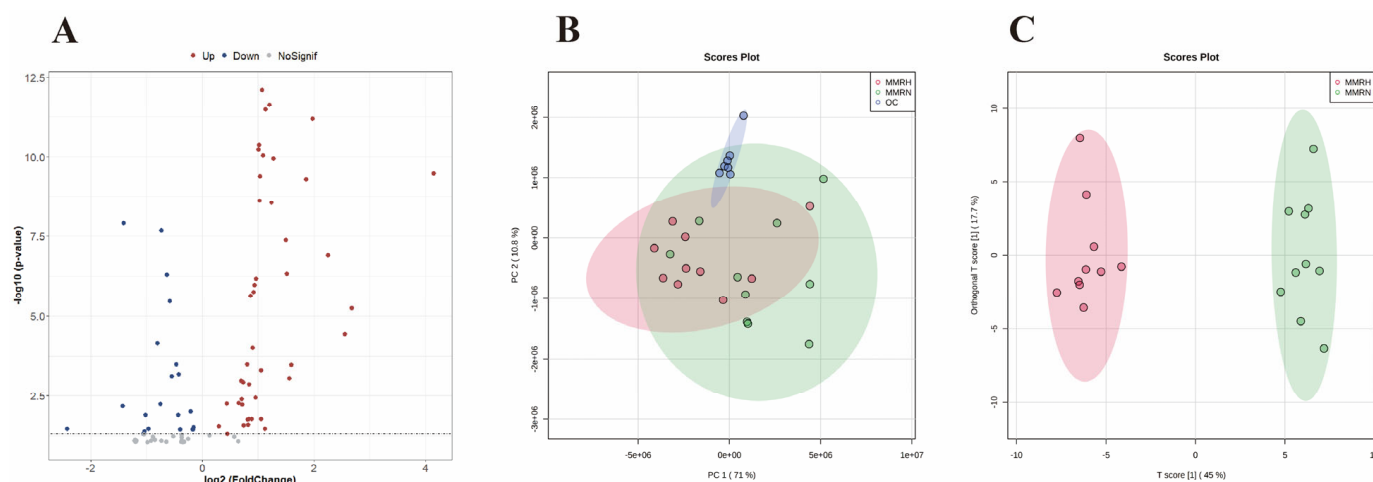


Figure 1. Statistical analysis of differential metabolites in muscle of mirror carp. (A) Volcano plot of $\log_2$ fold change and $-\log_{10}(p\text{-value})$ of differential metabolites. Red dots represent upregulated metabolites, while blue dots represent downregulated metabolites. Gray dots represent metabolites with p -value > 0.05 . (B) Principal component analysis (PCA) of differential metabolites. Different colors correspond to treatment groups and QC samples. (C) Orthogonal partial least squares discrimination analysis (OPLS-DA) of differential metabolites.

To characterize the global metabolic perturbation induced by hypoxia, multivariate statistical analyses were performed on the differential metabolite profiles. Unsupervised PCA revealed no outliers within the dataset (Figure 1B). A supervised OPLS-DA model was subsequently constructed, yielding robust parameters ($R^2Y = 0.957$, $Q^2 = 0.946$), which demonstrated a clear metabolic distinction between the hypoxia and normoxia groups (Figure 1C). Hierarchical clustering analysis of differential metabolites further confirmed accurate separation of the two experimental groups, indicating that the identified metabolic signatures provided a precise discrimination of hypoxia-induced alterations in mirror carp muscle (Figure 2). A correlation matrix was generated to evaluate the pairwise relationships among differential metabolites (Figure 3). Furthermore, KEGG pathway enrichment analysis revealed 37 significantly enriched metabolic pathways ($p < 0.05$), with valine, leucine, and isoleucine metabolism and arginine metabolism among the most prominently represented (Supplementary Table S1; Figure 4).

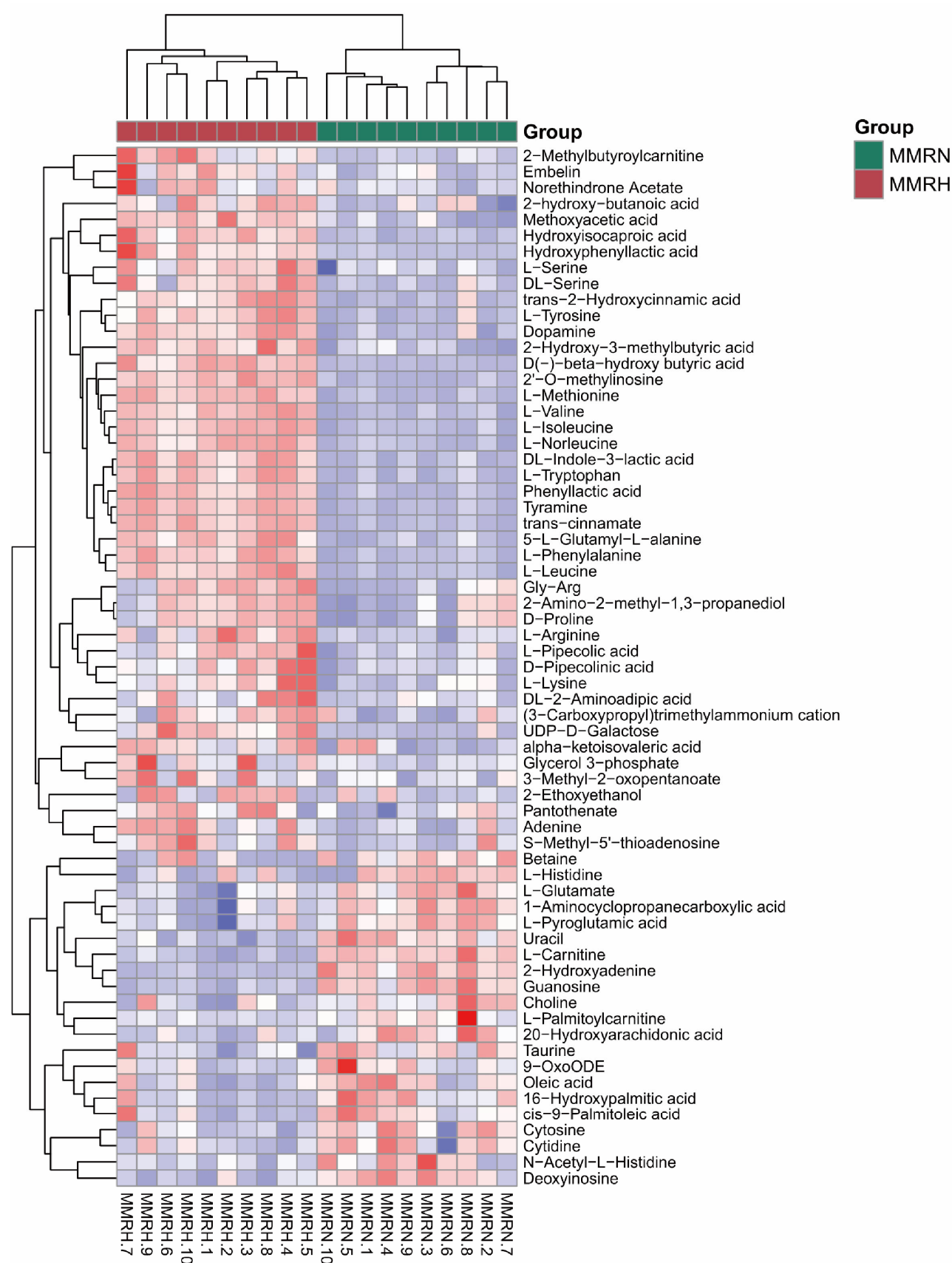


Figure 2. Hierarchical clustering plot of differential metabolites in muscle of mirror carp. Red color boxes represent upregulated metabolites, while blue color boxes represent downregulated metabolites. Darker colors indicate higher significance. MMRN, muscle samples of mirror carp under normoxia; MMRH, muscle samples of mirror carp under hypoxia.

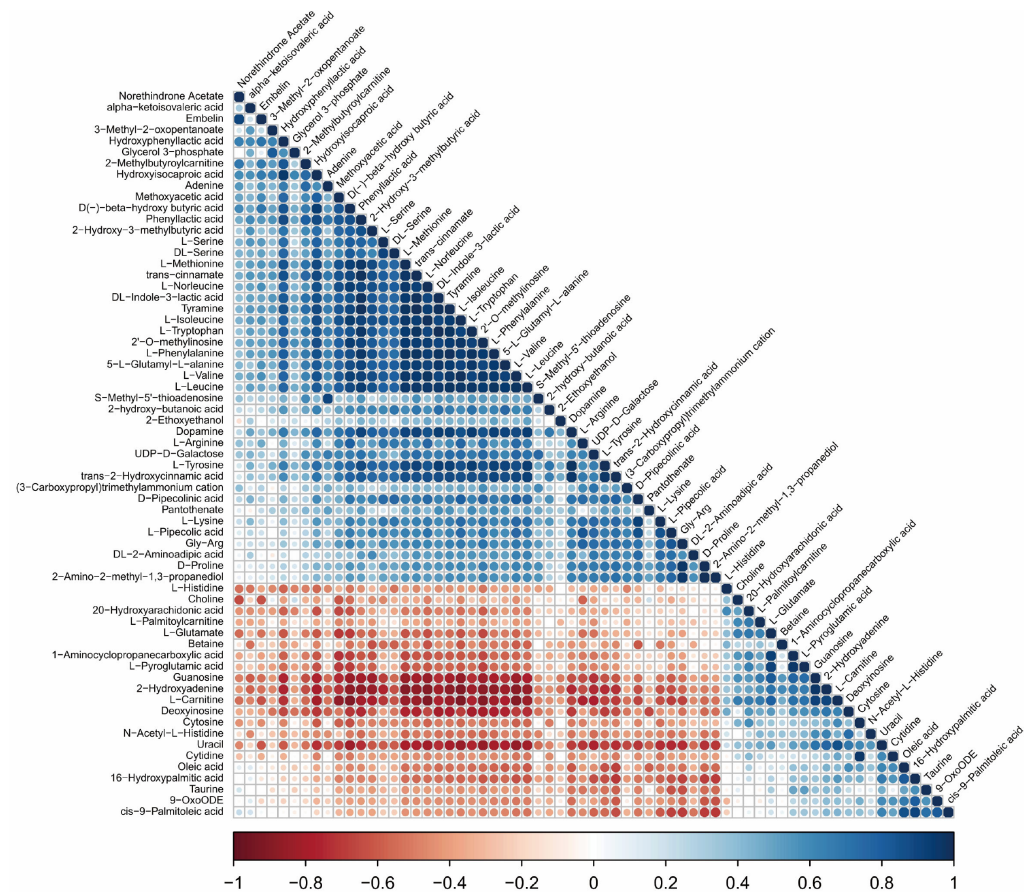


Figure 3. Correlation matrix of differential metabolites in muscle of mirror carp. Different colors and different sizes circles represent the magnitude of correlation. Deeper color represents higher degree of relevance between the metabolites, and larger circles indicate a stronger correlation.

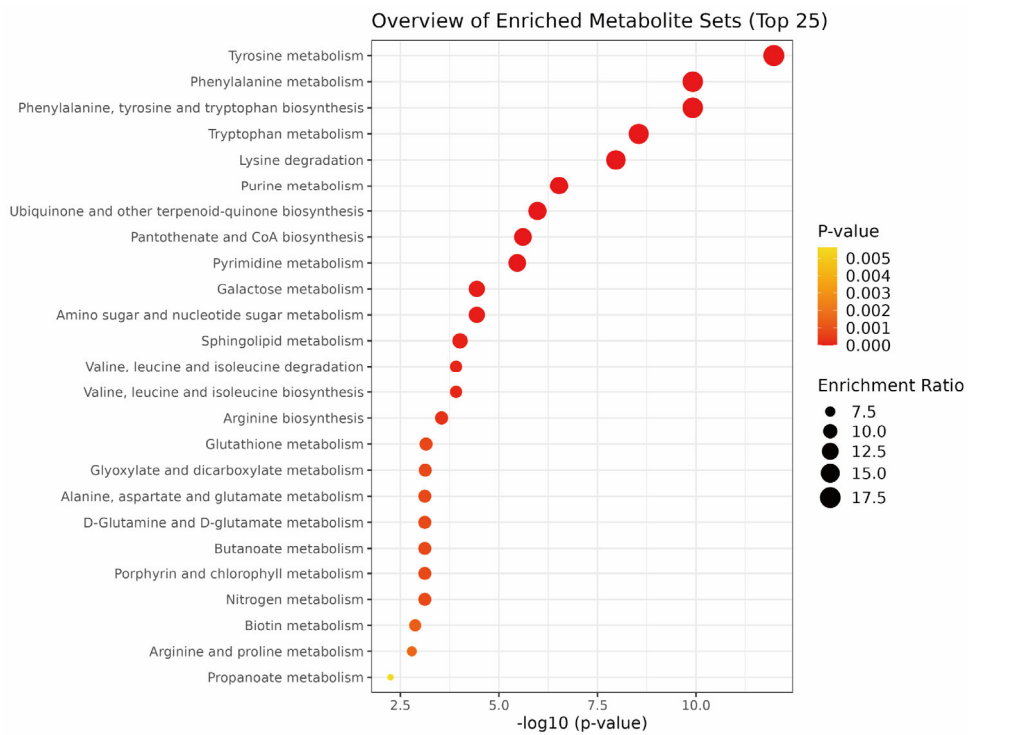


Figure 4. Top 25 significantly enriched KEGG metabolic pathways of differential metabolites in muscle of mirror carp.

3.2. Untargeted Metabolomic Profiling of Hebao Red Carp Muscle Under Hypoxia

In Hebao red carp muscle, untargeted metabolomics identified 66 significantly differential metabolites ($p < 0.05$) between the hypoxia and normoxia groups (Supplementary Table S2; Figure 5A), comprising 41 upregulated and 25 downregulated metabolites. Notably, the concentrations of malic acid, leucine, isoleucine, valine, betaine, alanine, and glyceraldehyde 3-phosphate were elevated under hypoxia stress, whereas carnitine and creatinine levels were significantly reduced. Marginal decreasing trends were observed for taurine, glutamate, and glutamine ($0.05 < p < 0.1$). Of particular interest, adenosine 5'-diphosphate (ADP) exhibited an increasing trend in the hypoxia group, suggesting enhanced energy turnover.

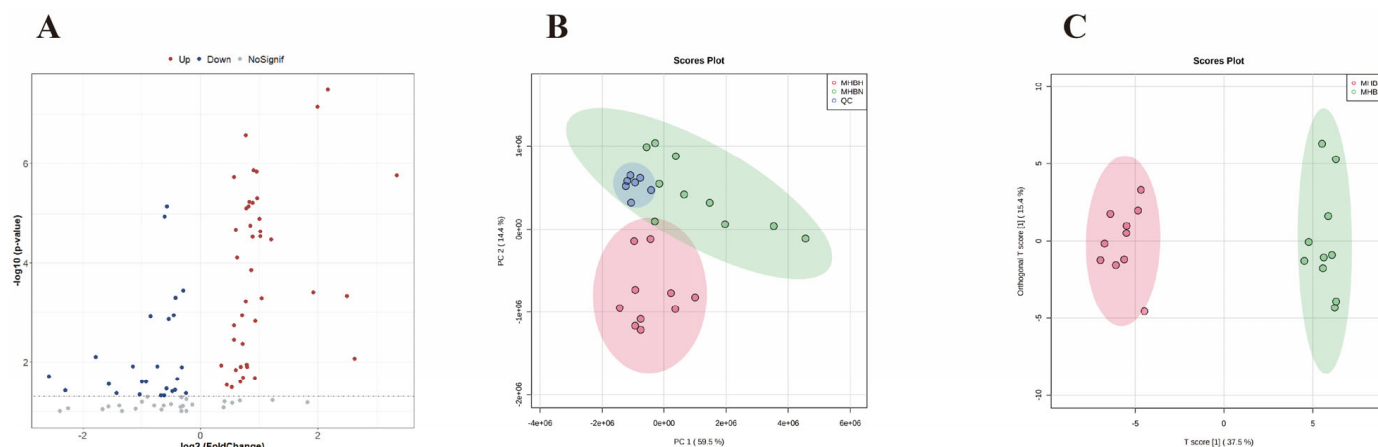


Figure 5. Statistical analysis of differential metabolites in muscle of Hebao red carp. (A) Volcano plot of log₂ fold change and $-\log_{10}(p\text{-value})$ of differential metabolites. Red dots represent up-regulated metabolites, while blue dots represent downregulated metabolites. Gray dots represent metabolites with $p\text{-value} > 0.05$. (B) Principal component analysis (PCA) of differential metabolites. Different colors correspond to treatment groups and QC samples. (C) Orthogonal partial least squares discrimination analysis (OPLS-DA) of differential metabolites.

Unsupervised PCA confirmed the absence of outliers within the metabolite profiles (Figure 5B). The OPLS-DA model ($R^2Y = 0.893$, $Q^2 = 0.859$) revealed a clear separation between hypoxia and normoxia groups, validating that the differential metabolic patterns reliably reflected hypoxia-induced metabolic reprogramming (Figure 5C). Hierarchical clustering analysis corroborated the precise classification of samples into two distinct groups (Figure 6), and a correlation matrix was constructed to illustrate inter-metabolite relationships (Figure 7). KEGG enrichment analysis identified 27 significantly associated pathways ($p < 0.05$), including beta-alanine metabolism and alanine, aspartate, and glutamate metabolism (Supplementary Table S2; Figure 8).

3.3. Untargeted Metabolomic Profiling of Mirror Carp Liver Under Hypoxia

A total of 100 metabolites were identified as significantly altered ($p < 0.05$) in the liver of mirror carp following hypoxia exposure (Supplementary Table S3; Figure 9A). Among these, 43 metabolites displayed elevated levels, including leucine, glutathione disulfide (GSSG), glutamine, dihydroxyacetone phosphate (DHAP), nicotinamide adenine dinucleotide (NAD^+), citrulline, and betaine. Conversely, 57 metabolites exhibited reduced levels, with particularly pronounced decreases in succinate, adenosine monophosphate (AMP), adenosine 3'-monophosphate, taurine, N^G , N^G -dimethylarginine (ADMA), and carnitine. Although not statistically significant ($0.05 < p < 0.1$), hypoxia exposure was associated with an increasing trend in lactate and a decreasing trend in arginine, suggesting shifts in anaerobic glycolysis and nitrogen metabolism.

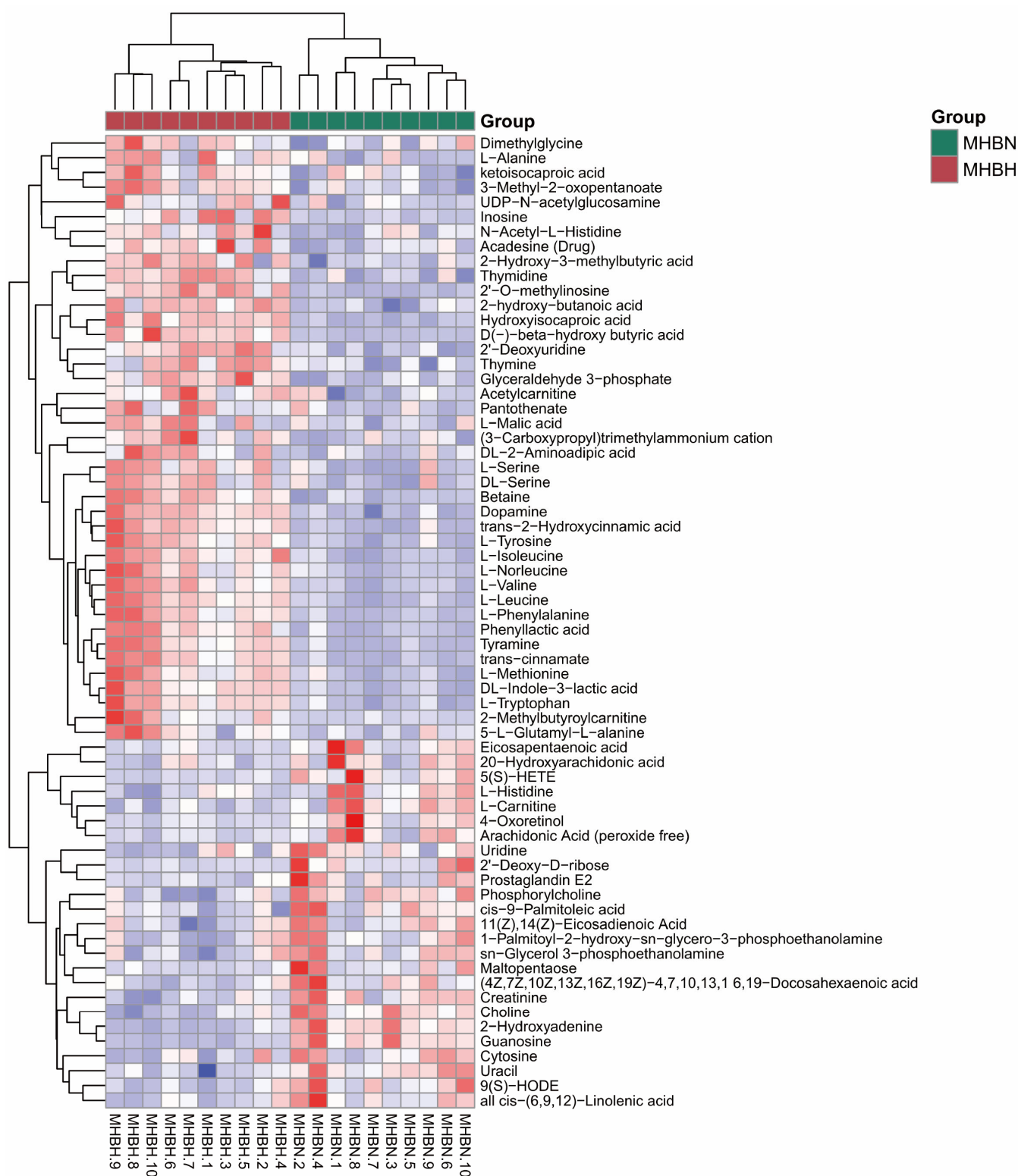


Figure 6. Hierarchical clustering plot of differential metabolites in muscle of Hebao red carp. Red color boxes represent upregulated metabolites, while blue color boxes represent downregulated metabolites. Darker colors indicate higher significance. MHBH, muscle samples of Hebao red carp under normoxia; MHBH, muscle samples of Hebao red carp under hypoxia.

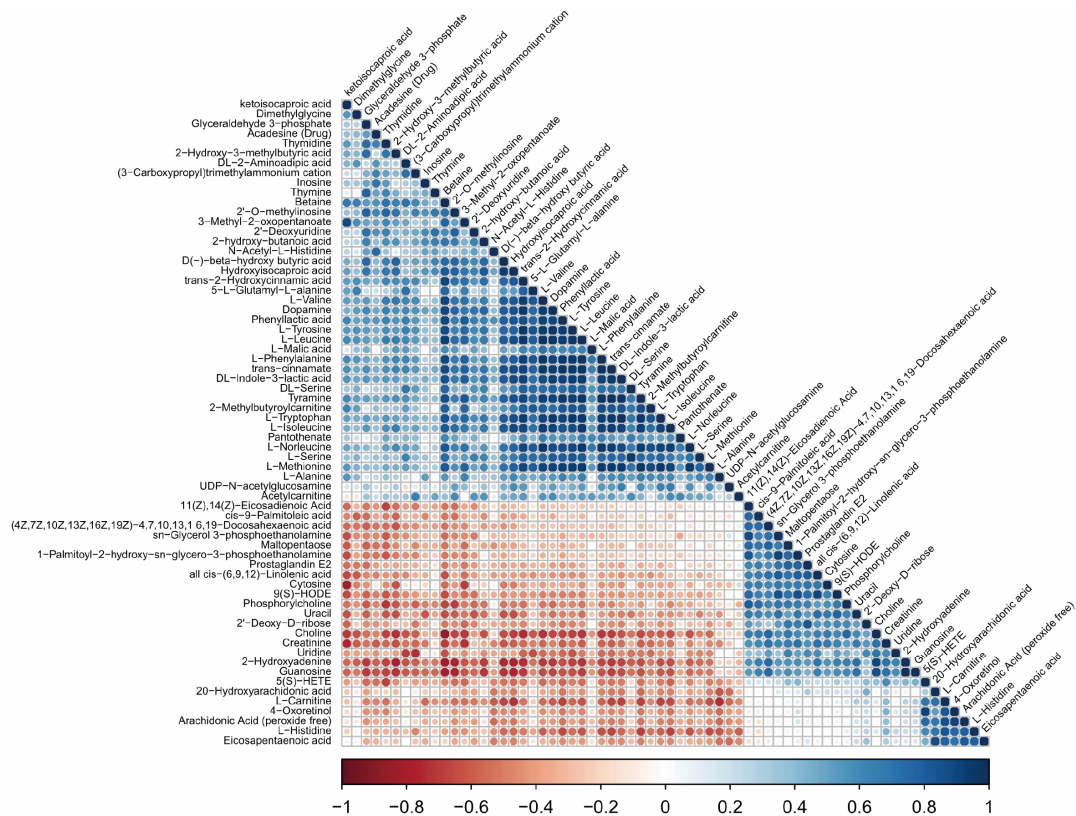


Figure 7. Correlation matrix of differential metabolites in muscle of Hebao red carp. Different colors and different sizes circles represent the magnitude of correlation. Deeper color represents higher degree of relevance between the metabolites, and larger circles indicate a stronger correlation.

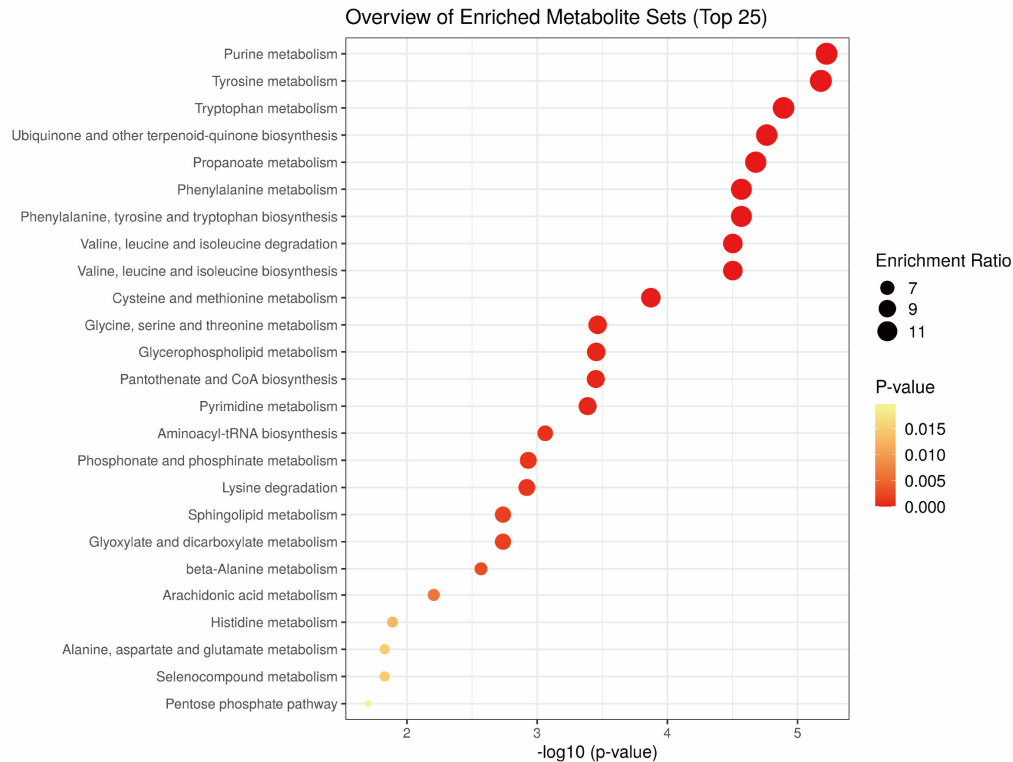


Figure 8. Top 25 significantly enriched KEGG metabolic pathways of differential metabolites in muscle of Hebao red carp.

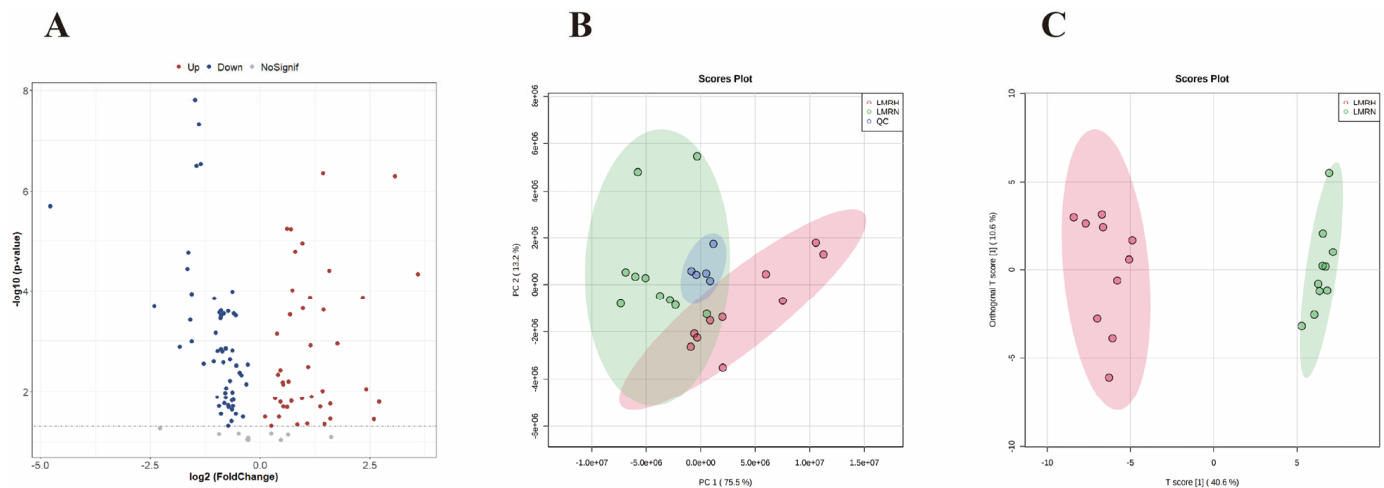


Figure 9. Statistical analysis of differential metabolites in liver of mirror carp. (A) Volcano plot of log₂ fold change and -log₁₀ (*p*-value) of differential metabolites. Red dots represent upregulated metabolites, while blue dots represent downregulated metabolites. Gray dots represent metabolites with *p*-value > 0.05. (B) Principal component analysis (PCA) of differential metabolites. Different colors correspond to treatment groups and QC samples. (C) Orthogonal partial least squares discrimination analysis (OPLS-DA) of differential metabolites.

PCA of the hepatic metabolite profiles confirmed that no outliers were present (Figure 9B). The OPLS-DA model ($R^2Y = 0.966$, $Q^2 = 0.958$) demonstrated pronounced separation along the first predictive component, indicating that the selected metabolites provided a highly accurate explanation of hypoxia-induced metabolic changes (Figure 9C). Hierarchical clustering analysis (Figure 10) and correlation matrix analysis (Figure 11) were performed to visualize the overall metabolite patterns and inter-metabolite associations. KEGG pathway enrichment analysis identified 38 significantly enriched metabolic pathways ($p < 0.05$), with particular enrichment in arginine and proline metabolism, the citrate cycle (TCA cycle), beta-alanine metabolism, and glutathione metabolism (Supplementary Table S3; Figure 12).

3.4. Untargeted Metabolomic Profiling of Hebao Red Carp Liver Under Hypoxia

In the liver of Hebao red carp, 91 metabolites displayed significantly altered abundance ($p < 0.05$) following hypoxia treatment (Supplementary Table S4; Figure 13A). Of these, 39 metabolites were significantly upregulated, including creatinine, ADP, betaine, nicotinamide adenine dinucleotide phosphate (NADP⁺), phosphoenolpyruvate, beta-alanine, leucine, and valine. In contrast, 52 metabolites were significantly downregulated, including lactate, maleic acid, succinate, taurine, aspartate, N^G, N^G-dimethyl-L-arginine (ADMA), α -glucose 1-phosphate, and α -glucose. Moreover, adenosine 3'-monophosphate exhibited a decreasing trend ($0.05 < p < 0.1$), while acetylneuraminic acid, GSSG, glycerol 3-phosphate, glutamate, phosphorylcholine, and DHAP displayed marginal increasing trends without reaching significance.

To assess whether the observed differential metabolite patterns could discriminate between experimental conditions, PCA was performed and confirmed the absence of outliers (Figure 13B). Subsequent OPLS-DA analysis yielded a well-fitted model ($R^2Y = 0.949$, $Q^2 = 0.936$) with clear separation along the first predictive component (Figure 13C), demonstrating robust metabolic differentiation between hypoxic and normoxic conditions. Hierarchical clustering results were presented as ordered heatmaps with associated dendrograms (Figure 14), and correlation coefficients between metabolites were displayed as a lower triangular matrix (Figure 15). KEGG pathway analysis uncovered 36 significantly enriched metabolic pathways ($p < 0.05$)

under hypoxia, encompassing the citrate cycle (TCA cycle), pyruvate metabolism, alanine, aspartate, and glutamate metabolism, nicotinate and nicotinamide metabolism, and glycolysis/gluconeogenesis (Supplementary Table S4; Figure 16).

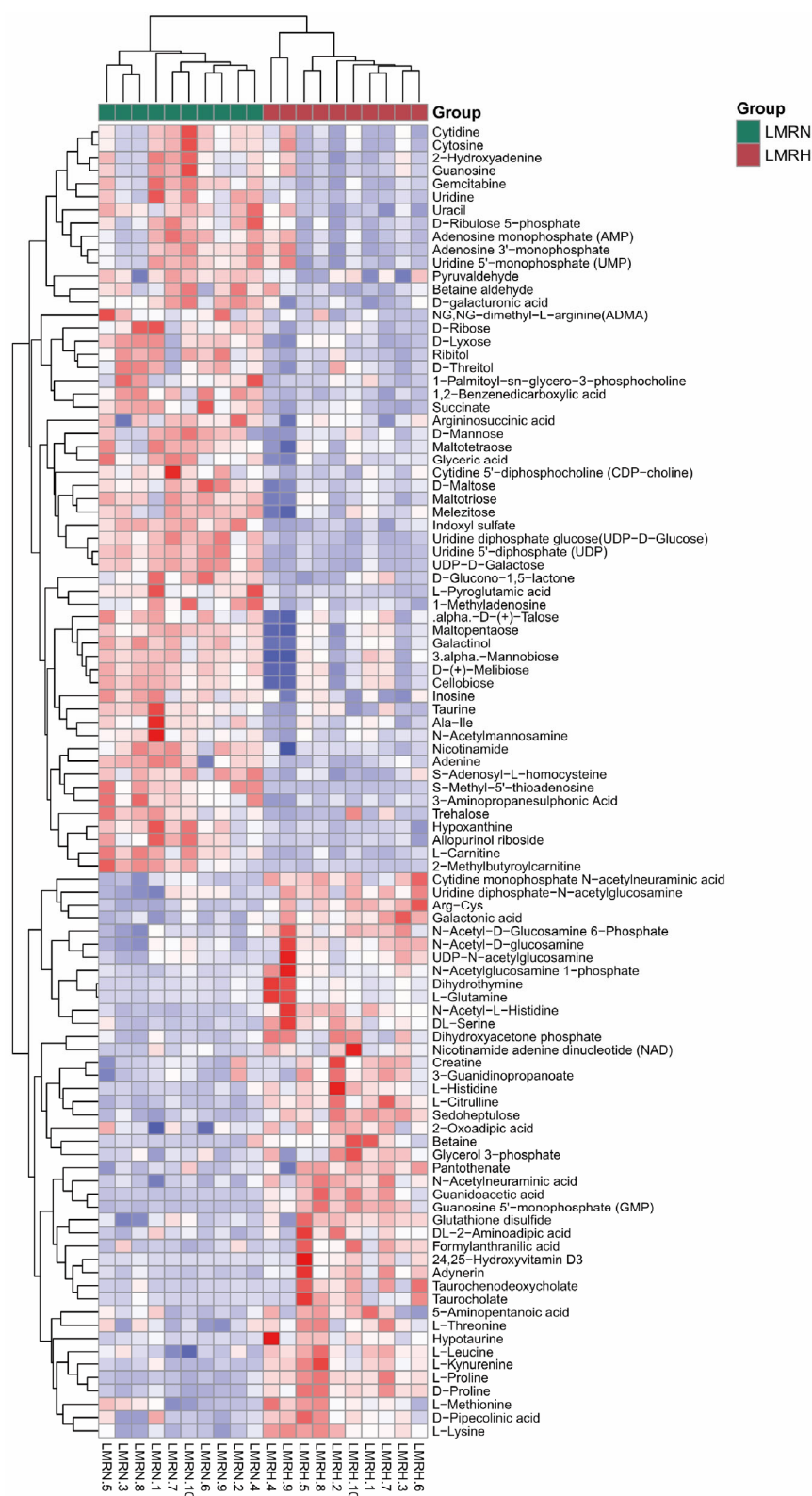


Figure 10. Hierarchical clustering plot of differential metabolites in liver of mirror carp. Red color boxes represent upregulated metabolites, while blue color boxes represent downregulated metabolites. Darker colors indicate higher significance. LMRN, liver samples of mirror carp under normoxia; LMRH, liver samples of mirror carp under hypoxia.

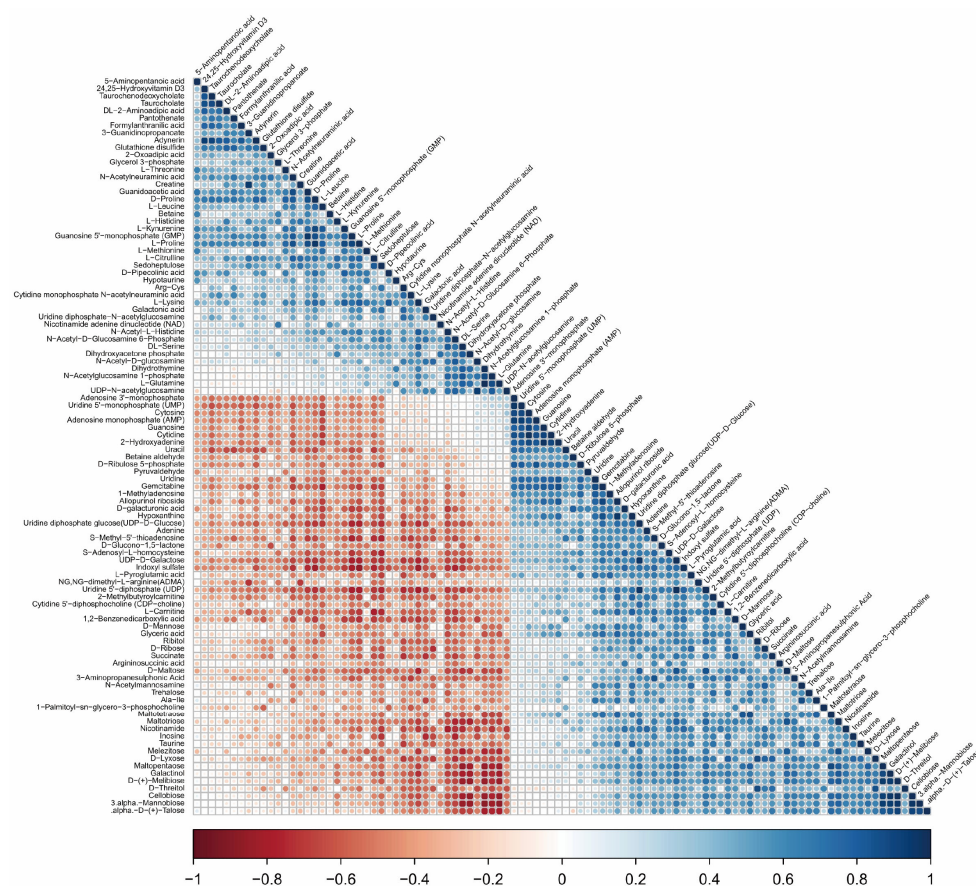


Figure 11. Correlation matrix of differential metabolites in liver of mirror carp. Different colors and different sizes circles represent the magnitude of correlation. Deeper color represents higher degree of relevance between the metabolites, and larger circles indicate a stronger correlation.

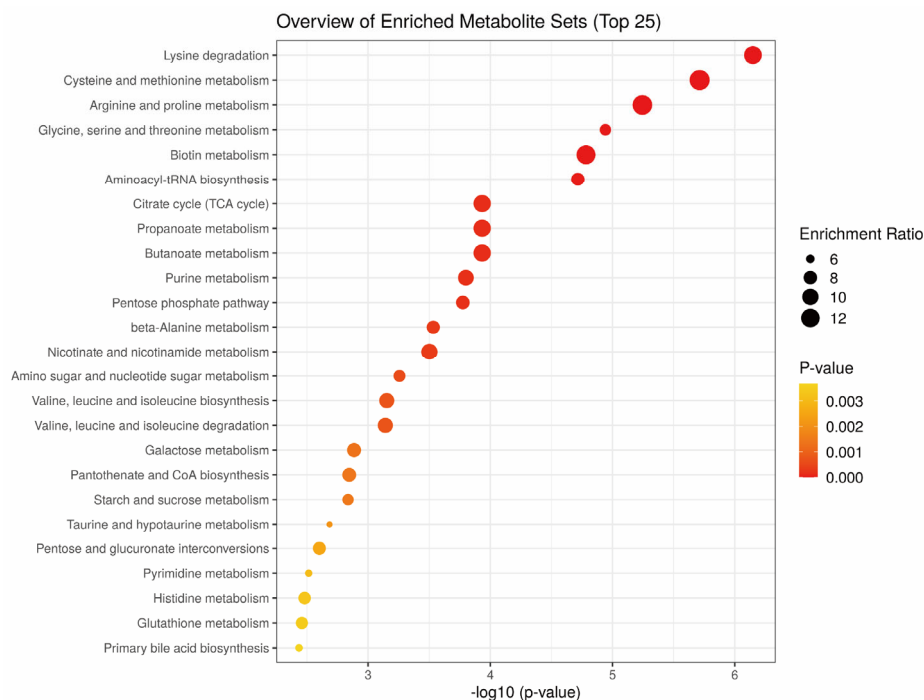


Figure 12. Top 25 significantly enriched KEGG metabolic pathways of differential metabolites in liver of mirror carp.

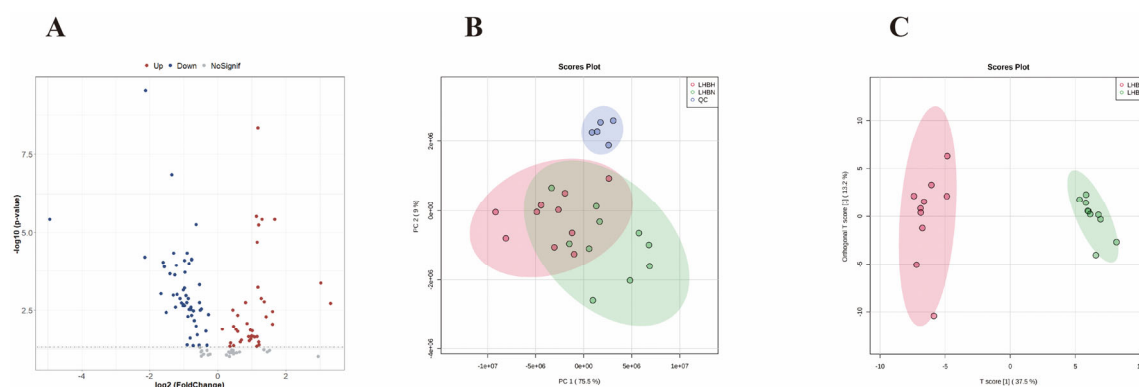


Figure 13. Statistical analysis of differential metabolites in liver of Hebao red carp. (A) Volcano plot of log2 fold change and $-\log_{10}(p\text{-value})$ of differential metabolites. Red dots represent upregulated metabolites, while blue dots represent downregulated metabolites. Gray dots represent metabolites with $p\text{-value} > 0.05$. (B) Principal component analysis (PCA) of differential metabolites. Different colors correspond to treatment groups and QC samples. (C) Orthogonal partial least squares discrimination analysis (OPLS-DA) of differential metabolites.

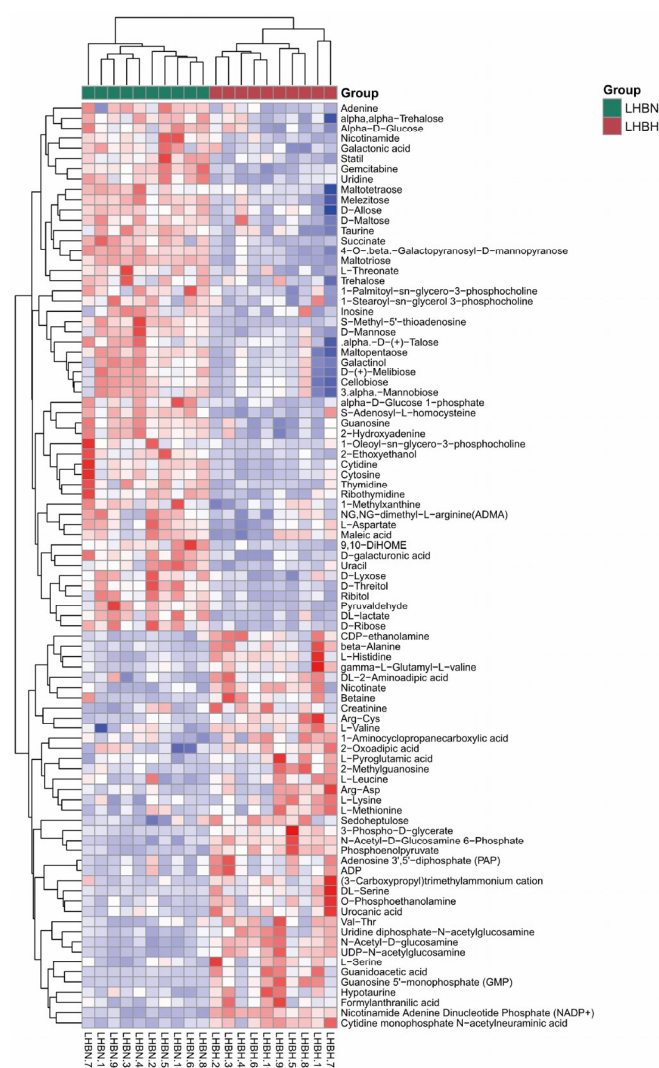


Figure 14. Hierarchical clustering plot of differential metabolites in liver of Hebao red carp. Red color boxes represent upregulated metabolites, while blue color boxes represent downregulated metabolites. Darker colors indicate higher significance. LHBN, liver samples of Hebao red carp under normoxia; LHBH, liver samples of Hebao red carp under hypoxia.

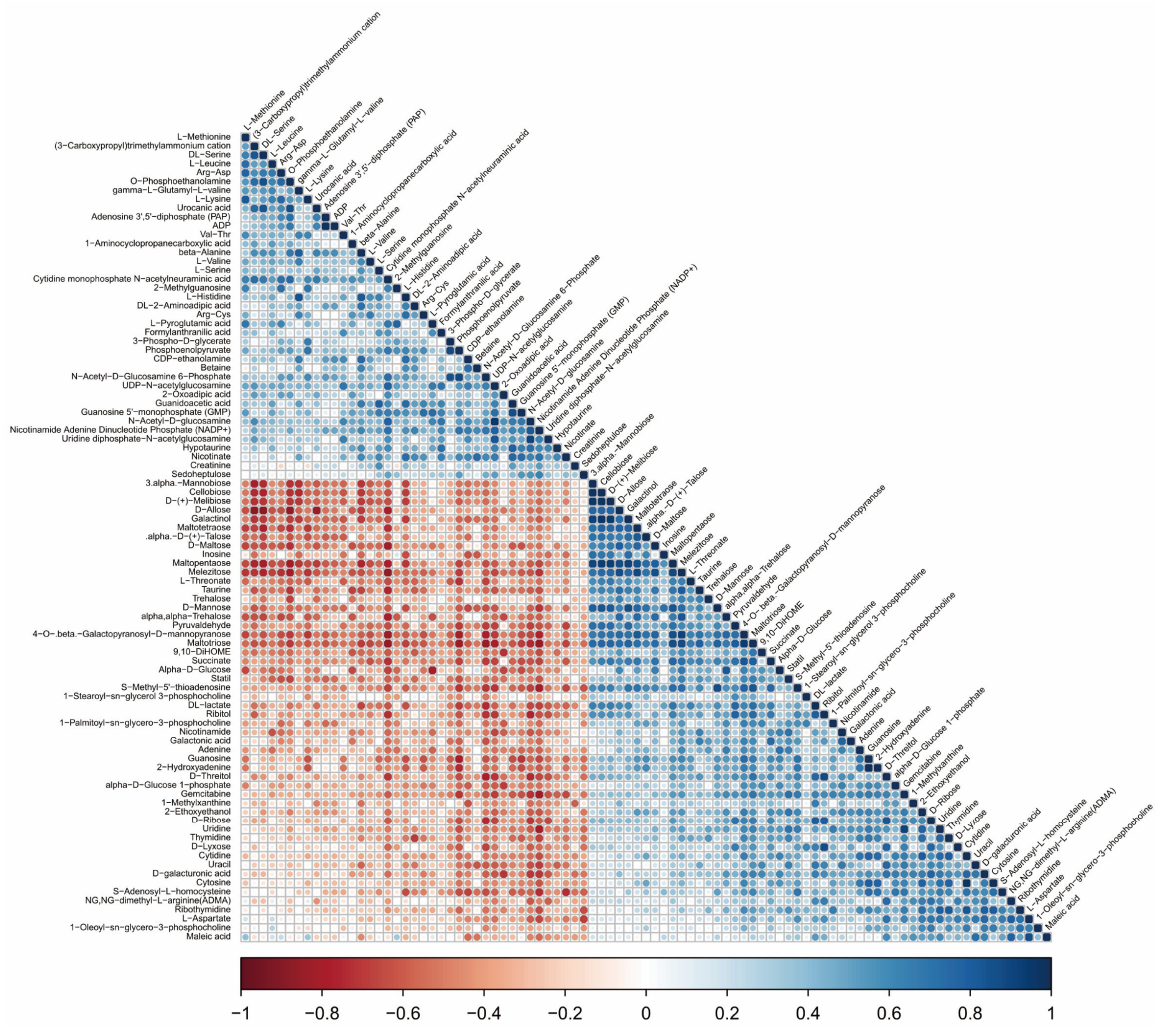


Figure 15. Correlation matrix of differential metabolites in liver of Hebao red carp. Different colors and different sizes circles represent the magnitude of correlation. Deeper color represents higher degree of relevance between the metabolites, and larger circles indicate a stronger correlation.

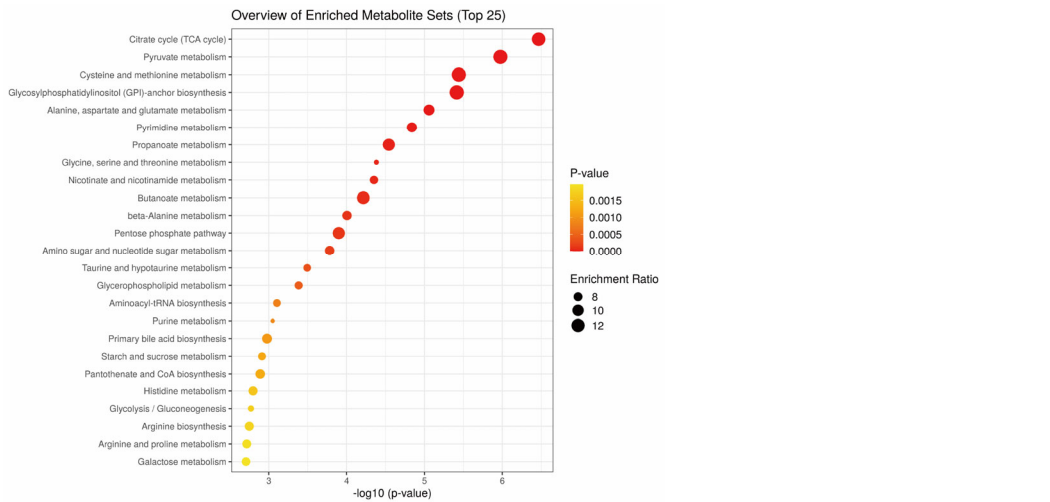


Figure 16. Top 25 significantly enriched KEGG metabolic pathways of differential metabolites in liver of Hebao red carp.

4. Discussion

4.1. General Energy Metabolism

ATP powers cellular reactions, with metabolism governed by the equilibrium between ATP production and consumption, dynamically shifting between aerobic and anaerobic pathways in response to oxygen availability [22,23]. Under normoxia, oxidative phosphorylation yields ~36 ATP per glucose molecule [24]; hypoxia induces a partial transition to anaerobic glycolysis and substrate-level phosphorylation, a conserved vertebrate adaptive strategy that varies in magnitude and tissue specificity among fish species [23,25,26].

Mirror carp muscle exhibited remarkable metabolic stability under hypoxia, with no significant changes in anaerobic metabolites (lactate, alanine) or TCA cycle intermediates, except a marginal maleic acid decrease. This resilience suggests sufficient physiological buffering—potentially via enhanced O₂ extraction or myoglobin stores—preserving aerobic metabolism under moderate hypoxic challenge [9,27]. In contrast, the liver displayed a distinct shift: succinate decreased while lactate and DHAP trended upward, indicating partial redirection toward anaerobic glycolysis. Hepatic lactate accumulation, rather than muscular, aligns with Cori cycle activity, consistent with previous common carp findings [9]. Concurrent NAD⁺ accumulation implies enhanced gluconeogenic flux, while reduced AMP and 3'-AMP suggest sustained adenylate energy charge sufficient to fuel gluconeogenesis, mirroring observations in hypoxia-tolerant grass carp [27]. Elevated hepatic glutamine likely results from increased transporter-mediated uptake under hypoxia, a response well documented in mammals and increasingly recognized in fish. Collectively, these metabolic adjustments suggest that the highly domesticated mirror carp effectively counteracted the hypoxic challenge, as evidenced by maintained aerobic capacity in muscle and enhanced gluconeogenesis in liver [28–30].

Hebao red carp—a less domesticated strain—exhibited a divergent strategy. Hepatic TCA intermediates (maleic acid, succinate) decreased significantly alongside NADP⁺ accumulation, confirming TCA and pentose phosphate pathway attenuation, while glycolytic intermediates (DHAP, phosphoenolpyruvate) rose—evidence of a pronounced switch from oxidative phosphorylation to anaerobic glycolysis [10]. Increased ADP and decreased glucose indicate energy insufficiency and impaired ATP production, consistent with hypoxia-intolerant phenotypes [31]. Intriguingly, hypoxia resulted in a significant decrease in hepatic lactate in Hebao red carp, a finding consistent with previous research demonstrating that both short-term and long-term hypoxia can induce decreases in lactate concentrations across most tissues except the brain [32,33]. Hepatic lactate declined, likely reflecting gluconeogenic reconversion; however, diminished glucose and glycogen suggest rapid extrahepatic consumption by high-demand organs [6]. Hebao red carp muscle showed elevated anaerobic metabolites (alanine, glyceraldehyde 3-phosphate) and reduced creatinine, implying suppressed metabolic rate. The absence of lactate accumulation supports metabolic rate depression (MRD)—a strategy enabling ATP demand-supply balance and preventing acidosis [23]. Intriguingly, TCA intermediates were maintained, with malic acid increasing significantly. Given that hypoxia typically upregulates PDK1, reducing TCA flux, we posit that glutamine anaplerosis replenishes TCA pools, as glutamine declined by >50%. This mechanism, established in hypoxic tumor cells and involving malate–aspartate shuttling, may sustain ATP production [34–38]. Definitive confirmation via ¹³C-glutamine tracing is warranted.

Overall, the two strains adopted opposing energy strategies: mirror carp exhibited metabolic resilience with preserved muscle aerobic capacity and hepatic gluconeogenic reinforcement; Hebao red carp relied on muscle MRD with hepatic energy shortfall. This divergence likely reflects differential domestication pressures, with intensive selection in

mirror carp favoring metabolic robustness, whereas Hebao red carp retains a primitive suppression-based response [9].

4.2. Antioxidative Metabolites

Hypoxia paradoxically induces oxidative stress through enhanced formation of reactive oxygen species (ROS), which plays a pivotal role in mediating tissue injury [39]. Recent studies on cyprinid fish have demonstrated that reoxygenation following hypoxia leads to particularly strong shifts in redox-related parameters and significant tissue damage, indicating that the cost of post-hypoxic recovery represents a substantial physiological burden [39]. Antioxidants represent a critical line of cellular defense, functioning to neutralize free radicals and mitigate oxidative damage. Among these, glutathione (GSH) constitutes one of the most essential intracellular antioxidative defense metabolites [40]. The glutathione peroxidase system is critically important in protecting cells from oxidative injury by reducing hydrogen peroxide (H_2O_2), with concomitant oxidation of GSH to glutathione disulfide (GSSG) [41,42]. Although the majority of generated GSSG is recycled intracellularly back to GSH by glutathione reductase, a fraction proportional to the GSSG concentration is excreted into bile [41]. Under conditions of oxidative stress, hepatic GSSG is preferentially excreted into bile, rendering biliary glutathione redox status a sensitive index of hepatic oxidant burden [41,43]. In the present study, elevated levels of GSSG were observed in the liver of both mirror carp and Hebao red carp, reflecting an active state of oxidative stress and serving as a reliable biomarker of oxidative damage. Notably, the presence of oxidative stress markers in both strains despite their divergent energy metabolism strategies suggests that ROS generation under hypoxia represents a universal challenge independent of metabolic coping strategy. This observation is consistent with findings in other cyprinid species, where intermittent hypoxia differentially affected metabolic parameters but induced oxidative stress markers across species regardless of their baseline antioxidant capacity [39]. Furthermore, studies in genetically improved farmed tilapia (GIFT) have demonstrated that antioxidant enzyme activities in the liver increased during acute hypoxia as a compensatory response, while malondialdehyde (MDA) content decreased, suggesting active hepatic antioxidant defense mobilization [44].

Taurine, a sulfonic amino acid with established antioxidative and membrane-stabilizing properties [45,46], is essential for the development and maintenance of skeletal muscle function [45,47]. Taurine enhances antioxidant defense capacity by inhibiting the lipid peroxidation cascade, thereby mitigating the consumption of GSH [48]. The present metabolomic profiles revealed significant decreases in taurine across both strains, indicating substantial consumption of this protective metabolite under hypoxia-induced oxidative stress. The consistent depletion of taurine across multiple tissues and both strains underscores its role as a first-line sacrificial antioxidant during acute hypoxic challenge and suggests that taurine supplementation may represent a viable nutritional strategy to bolster antioxidant capacity in aquaculture fish subjected to periodic oxygen fluctuations [49,50]. Furthermore, carnitine, which participates in redox signaling and modulates transcription factors including Nrf2, PPAR α , and NF- κ B while activating the vitagene network, was identified as significantly depleted in both muscle and liver of both strains. Recent metabolomic investigations have documented that the glycolysis pathway is activated while lipid metabolism-related pathways including peroxisome proliferator-activated receptor (PPAR) signaling are inhibited under hypoxia in freshwater fish, with associated reductions in fatty acid transport molecules such as carnitine palmitoyl transferase [25,51]. The reduction in carnitine observed in the present study likely reflects its dual role as both an antioxidant agent, particularly effective against lipid peroxidation, and as a substrate for fatty acid β -oxidation. Under hypoxic conditions, the suppression of lipid oxidation

pathways coupled with enhanced antioxidant demand creates a scenario of accelerated carnitine depletion, providing a mechanistic basis for this consistent observation across tissues and strains.

4.3. Regulatory Molecules Against Hypoxia

Hypoxia triggered a strain-specific decrease in hepatic arginine, the substrate for nitric oxide (NO) synthase, exclusively in mirror carp. NO modulates vascular tone and oxygen delivery via the HIF-mediated oxygen-sensing cascade [28]. NO synthase (NOS) converts arginine to NO and citrulline, a process endogenously inhibited by asymmetric dimethylarginine (ADMA) [52]. Elevated citrulline indicates increased NOS activity [52,53], while reduced ADMA reflects relieved suppression of NO generation [54]; these parallel shifts unequivocally demonstrate NO pathway activation. In mirror carp liver, concurrent citrulline increase and ADMA decrease provide metabolomic evidence for enhanced NO activity, conferring hemodynamic advantages via vasodilation and improved tissue oxygenation—partially explaining their superior hypoxia tolerance relative to Hebao red carp. The absence of this response in Hebao red carp suggests domestication may have reinforced hypoxia-sensing-vasoregulatory coupling, consistent with artificial selection shaping stress-response cascades in aquaculture species [55].

Branched-chain amino acids (BCAAs: valine, isoleucine, leucine) [56] increased significantly in both strains post-hypoxia. BCAAs regulate protein synthesis, growth, and metabolic homeostasis, comprising ~18% of proteinaceous residues. Their catabolic derivatives act as signaling molecules in protein formation and stress adaptation. Recent multi-omics in hybrid fish confirmed BCAA catabolism as a primary metabolic response to anaerobic conditions [12]. Hypoxia-induced BCAA accumulation likely reflects enhanced proteolysis to mobilize amino acid pools and direct signaling functions promoting stress-responsive gene expression. This pattern is evolutionarily conserved among cyprinids, as anoxia-exposed crucian carp exhibit widespread tissue amino acid accumulation [6].

Hypoxia elicited significant betaine changes across all groups, implicating osmotic disturbance [57,58]. Betaine, derived from choline via irreversible oxidation, functions as a compatible osmolyte stabilizing membranes and exerts antioxidant effects by increasing methionine bioavailability for GSH synthesis [59]. Elevated hepatic betaine suggests increased hepatocyte membrane permeability and compensatory betaine biosynthesis under acute hypoxic stress [60,61], consistent with osmotic responses in Manila clam and *Takifugu obscurus* gill under hypoxia [62]. Additionally, betaine exerts hepatoprotective effects against ischemia–reperfusion injury by interfering with prostanoid and TNF- α release from activated Kupffer cells, suggesting a dual role in osmotic protection and anti-inflammatory defense during hypoxic challenge [63].

4.4. Integrative Perspective: Domestication-Driven and Background-Associated Divergence in Hypoxia Coping Strategies

Mirror carp originated in Europe through selective breeding for reduced scales to facilitate processing, representing one of the oldest and most intensive domestication lineages in aquaculture [64]. In contrast, Hebao red carp is an indigenous Chinese strain recognized for its distinctive reddish skin and compressed body morphology, resulting from artificial selection for ornamental traits under a comparatively shorter and less intensive domestication regime [65]. Importantly, mirror carp and Hebao red carp are inherently distinct geographical strains with divergent genetic backgrounds, growth rates, body shapes, and environmental adaptation characteristics [65,66]. Given these substantial differences, the observed metabolic divergence cannot be exclusively attributed to domestication intensity. Rather, the dichotomy between proactive metabolic maintenance in mirror carp and reactive metabolic suppression in Hebao red carps likely reflects the interaction between

domestication-driven selection and intrinsic genetic predispositions shaped by distinct evolutionary histories. Hypoxia tolerance is increasingly recognized as a complex polygenic trait shaped by both natural selection and artificial domestication, with metabolic flexibility serving as a key determinant [67]. Studies in hypoxia-tolerant grass carp have demonstrated preferential utilization of fatty acid as alternative energy substrates and identified key metabolic biomarkers distinguishing tolerant from sensitive phenotypes [27]. Transcriptomic analyses in common carp have further elucidated hypoxia-responsive gene networks and adaptive metabolomics [9,68]. Future research integrating multi-omics approaches—including transcriptomics, metabolomics, and epigenomics—will be essential to dissect the relative contributions of domestication and genetic background to strain-specific hypoxia coping strategies, and to identify candidate biomarkers for marker-assisted selection in hypoxia-tolerant breeding programs.

5. Conclusions

This study employed untargeted metabolomics to characterize strain-specific metabolic reprogramming in mirror carp and Hebao red carp under hypoxia. Mirror carp displayed a resilient phenotype, preserving aerobic catabolism in muscle, enhancing hepatic gluconeogenesis with high adenylate energy charge, and selectively activating the NO/arginine/citrulline pathway to facilitate oxygen delivery. In contrast, Hebao red carp adopted a depression-based strategy, with muscle metabolic suppression and hepatic energy insufficiency (ADP accumulation, glucose depletion) approaching failure. Both strains exhibited oxidative stress (GSSG accumulation, taurine/carnitine depletion), indicating a universal challenge irrespective of coping strategy. The selective activation of the NO pathway exclusively in mirror carp provides a novel mechanistic basis for their enhanced hypoxia tolerance, suggesting that domestication has reinforced the coupling between hypoxia sensing and vasoregulatory responses. Although stable-isotope tracing, metabolic flux analysis, and multi-omics integration are warranted to fully elucidate the underlying mechanisms, our findings demonstrate fundamental divergences in metabolic network regulation between the two strains, which have been subjected to contrasting intensities of domestication selection. These results define the metabolomic basis of hypoxia tolerance in common carp and provide a theoretical framework for breeding low-oxygen-resilient aquaculture strains.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fishes11090533/s1>, Table S1. Differential metabolites (VIP > 1, $p < 0.1$) in muscle of mirror carp identified during hypoxia exposure. Table S2. Differential metabolites (VIP > 1, $p < 0.1$) in muscle of Hebao red carp identified during hypoxia exposure. Table S3. Differential metabolites (VIP > 1, $p < 0.1$) in liver of mirror carp identified during hypoxia exposure. Table S4. Differential metabolites (VIP > 1, $p < 0.1$) in liver of Hebao red carp identified during hypoxia exposure.

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