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# Comparative analysis of erythrocyte hemolysis, plasma parameters and metabolic features in red crucian carp (*Carassius auratus* red var) and triploid hybrid fish following *Aeromonas hydrophila* challenge

Ning-Xia Xiong [a], Sheng-Wei Luo [a,*], Lan-Fen Fan [b], Zhuang-Wen Mao [c], Kai-Kun Luo [a], Shao-Jun Liu [a,**], Chang Wu [a], Fang-Zhou Hu [a], Shi Wang [a], Ming Wen [a], Qing-Feng Liu [a]

[a] State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, PR China
[b] College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China
[c] Hunan Provincial Key Laboratory of Nutrition and Quality Control of Aquatic Animals, Department of Biological and Environmental Engineering, Changsha University, Changsha, 410022, PR China

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ABSTRACT

Aeromonas hydrophila can pose a great threat to survival of freshwater fish. In this study, *A. hydrophila* challenge could promote the erythrocyte hemolysis, increase free hemoglobin (FHB) level and generate malondialdehyde (MDA) production in plasma but decrease the levels of total antioxidant capacity (T-AOC), total superoxide dismutase (SOD), catalase (CAT), alkaline phosphatase (ALP) and lysozyme (LZM) of red crucian carp (RCC, 2 N = 100) and triploid hybrid fish (3 N fish, 3 N = 150) following *A. hydrophila* challenge. Elevated expression levels of heat shock protein 90 alpha (HSP90α), matrix metalloproteinase 9 (MMP-9), free fatty acid receptor 3 (FFAR3), paraoxonase 2 (PON2) and cytosolic phospholipase A2 (cPLA2) were observed in *A. hydrophila*-infected fish. In addition, *A. hydrophila* challenge could significantly increase expressions of cortisol, leucine, isoleucine, glutamate and polyunsaturated fatty acids (PUFAs) in RCC and 3 N, while glycolysis and tricarboxylic acid cycle appeared to be inactive. We identified differential fatty acid derivatives and their metabolic networks as crucial biomarkers from metabolic profiles of different ploidy cyprinid fish subjected to *A. hydrophila* infection. These results highlighted the comparative metabolic strategy of different ploidy cyprinid fish against bacterial infection.

## 1. Introduction

Environmental pollution may be considered to have an immunosuppressive effect on immune defense in fish, thus rendering fish more susceptible to pathogenic infection [1]. In addition, the releases of pollutants such as antibiotics and heavy metals may not only contaminate nature environment, but also promote the increasing numbers of resistant bacteria by significantly challenging the microbial population [2,3]. Apart from documented cases, microbial disease constitutes one of the largest aquaculture problems that can pose a great threat to survival of aquatic organisms, finally leading to significant economic losses [4,5]. Crucian carp (*Carassius auratus*) is one of the most important economic freshwater fish and abundant in lakes, rivers and reservoirs in China, which is popular with fish farmers [6]. Increasing evidences indicate that *A. hydrophila*, a gram-negative pathogen with various virulent factors, can sharply increase accumulative mortality of allogynogenetic crucian carp from 12 h to 24 h at the doses of $1 \times 10^8$ CFU $ml^{-1}$ [7,8]. Although immunoprophylaxis is an effective strategy in restricting the spread of pathogenic invasion [9], vaccine application cannot deal with all issues of infectious diseases and antibiotics bioaccumulation, which may easily trigger the emergence of antibiotic resistance bacteria (ARB) in water environment [10].

Triploidization is considered as a practical strategy for large-scale generation of sterile fish [11], but artificial induction of triploidy fish by pressure or temperature shock may decrease its disease resistance and weight gains [12,13]. Hybridization produces novel genotypes and phenotypes in hybrid offsprings [14,15], thus giving rise to species with novel capabilities and forming chimeric genes through the fusion of

* Corresponding author. College of Life Science, Hunan Normal University, Changsha, 410081, PR China.
** Corresponding author. College of Life Science, Hunan Normal University, Changsha, 410081, PR China.
*E-mail addresses:* swluo@hunnu.edu.cn (S.-W. Luo), lsj@hunnu.edu.cn (S.-J. Liu).

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**Table 1**
The primer sequences used in this study.

| Primer names' | Sequence direction (5' → 3') | Use |
|---|---|---|
| RT-18S-F | CCGACCCTCCCTCACG | qPCR |
| RT-18S-R | GCCTGCTGCCTTCCTTG | qPCR |
| RT-MMP9-F | GACATCCGCAACTATCAAACTT | qPCR |
| RT- MMP9-R | GGGGTAACATCACTCCAGACTT | qPCR |
| RT- FFAR3-F | ATGGCGTGGACAGTGGG | qPCR |
| RT- FFAR3-R | GCGAAACGGCAGGAAAA | qPCR |
| RT- PON2-F | GAGAACTTACCCAAAACTACCTT | qPCR |
| RT- PON2-R | CCTCAACAAATCGGAAAATC | qPCR |
| RT- HSP90α-F | AGCAGCCGATGATGGA | qPCR |
| RT- HSP90α-R | GGATTTGGCGATGGTTC | qPCR |
| RT- cPLA2-F | ATGGTGGGTTTGCTGGG | qPCR |
| RT- cPLA2-R | TGACTCCTGGACCGCTGA | qPCR |

pieces of various genomes [16]. Chimeric genes and nonsynonymous mutation in hybrid offsprings may generate structural changes at transcriptional level that can alter gene expressions, enzymatic activities and cellular signals upon stimulation [17,18]. In fish, recent reports suggest that hybrid fish can elicit a low susceptibility to pathogenic infection by comparing with those of their parental species [19,20]. Recently, bisexual fertile tetraploid cyprinid fish (4 N = 200) was generated via intergeneric hybridization of red crucian carp (RCC, *Carassius auratus* red var, 2 N = 100, ♀) and common carp (CC, *Cyprinus carpio* L, 2 N = 100, ♂) [21,22]. Then, sterile triploid cyprinid fish (3 N fish, 3 N = 150) was obtained by crossing of RCC (2 N = 100, ♀) and tetraploid cyprinid fish (4 N = 200, ♂) [23]. 3 N fish contained two sets of RCC chromosome groups and one set of CC chromosome group, which may provide a triploid hybrid animal model for studying the mechanisms of sterility, growth, and disease resistance.

In this study, the aims were to compare and characterize erythrocyte hemolysis, non-specific immunity, antioxidant status and metabolite profiles in plasma of RCC and 3 N, which may provide a new insight into immunometabolic status of different ploidy cyprinid fish.

## 2. Materials and methods

### 2.1. Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. We followed the laboratory animal guideline for the ethical review of the animal welfare of China (GB/T 35892–2018).

### 2.2. Animals

The different ploidy cyprinid fishes (RCC and 3 N) were obtained from an aquaculture base in Wang Cheng district (Changsha, China), which were acclimatized in 70 × 65 × 65 cm plastic aquarium (25 fishes/aquarium) with the diluted freshwater (pH 8.0, 23 ± 1 °C) for two weeks and fed with commercial diet twice daily till 24 h before challenge experiment, respectively. In addition, water quality was properly controlled to avoid pathogenic contamination during fish acclimation or immune challenge.

### 2.3. Ploidy analysis

Peripheral blood samples were withdrawn from caudal veins of RCC and 3 N fish, diluted and resuspended in pre-cooling ethanol, fixed overnight at 4 °C, washed with PBS and resuspended in PBS at a concentration of $1 \times 10^7$ cells/ml. Following staining of 4′,6-diamidino-2-phenylindole (DAPI), measurement of DNA content was analyzed by using a flow cytometer [24]. The experiment was performed in triplicate.

### 2.4. Immune challenge with A. hydrophila

Based on our previous studies, *A. hydrophila* strain was cultured at 28 °C for 24 h, centrifuged at 10000×*g* at 4 °C for 15 min and resuspended in 1 × PBS (pH 7.3) [25]. Then, RCCs and 3Ns (average length 23.2 ± 0.92 cm) were intraperitoneally injected with 100 μl suspension of $1 \times 10^8$ CFU $ml^{-1}$ *A. hydrophila* in PBS, which was served as infection group, while injection of 100 μl sterile PBS was used as the control group. Peripheral blood samples were withdrawn from caudal veins of RCC and 3 N fish at 8 h post-infection. The plasma was isolated by centrifugation at 2000×*g* for 10 min, immediately frozen in liquid nitrogen and preserved in −80 °C [26,27]. Each group contained six sets of plasma samples for biological replication. Each set of plasma was the single plasma isolated from peripheral blood of three different individuals.

### 2.5. Sample preparation and liquid chromatography-mass spectrometry (LC-MS) metabolomics

Plasma preparation for LC-MS metabolomics was performed as previously described [28,29]. In brief, plasma samples were precipitated in 80% methanol of all protein. Following protein removal, the filtrate samples were dried in vacuum. The above samples were analyzed by ultra performance liquid chromatography (UPLC) coupled to a premium quadrupole-time of flight mass spectrometer (QTOF MS) as described previously [30]. The separation was performed by using a reverse phase $C_{18}$ BEH column. The binary gradient elution system is composed of water (containing 0.1% formic acid, v/v) and acetonitrile (containing 0.1% formic acid, v/v). LC-MS metabolomics was operated in positive and negative ion mode (scan range 50–1000 *m/z*). QCs were injected at regular intervals throughout the analytical run.

### 2.6. LC-MS data processing and pathway analysis

Raw data were analyzed by progenesis QI software [31]. Then, the obtained LC-MS data were shown with *m/z*, peak retention time (RT) and peak intensity, while RT-m/z pairs were used to identify each ion. Resulting matrix was reduced by peak removals with more than 60% missing value (ion intensity = 0) in samples. Internal standard was used for QC analysis and the metabolites were annotated by using public metabolic database, which were subjected to partial least squares discriminate analysis (PLS-DA) [32–34]. The obtained differential metabolites was analyzed by hierarchical cluster analysis (HCA) and the results were represented as heat map. Threshold of Variable importance in projection (VIP) was set to 1 for ranking metabolites. HCA was performed on the log transformed normalized data and completed in R platform using distance matrix. The obtained differential metabolites were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

### 2.7. Erythrocyte hemolysis

Peripheral blood samples were withdrawn from caudal veins of RCC and 3 N after PBS treatment and *A. hydrophila* challenge. Then, blood samples were added to various concentration of sodium chloride solution (SCS). Following 2 h incubation at room temperature, erythrocyte hemolysis was observed. 0.9% SCS was used as the control, while 1.9% urea solution was used as complete hemolysis group [35]. The experiment was repeated in triplicate.

### 2.8. Free hemoglobin (FHB) content in plasma following A. hydrophila challenge

According to previous studies, FHB content in fish plasma was performed by using a free hemoglobin (FHB) detection kit (Nanjing Jiancheng Bioengineering institute, China) [36]. Briefly, fish plasma was

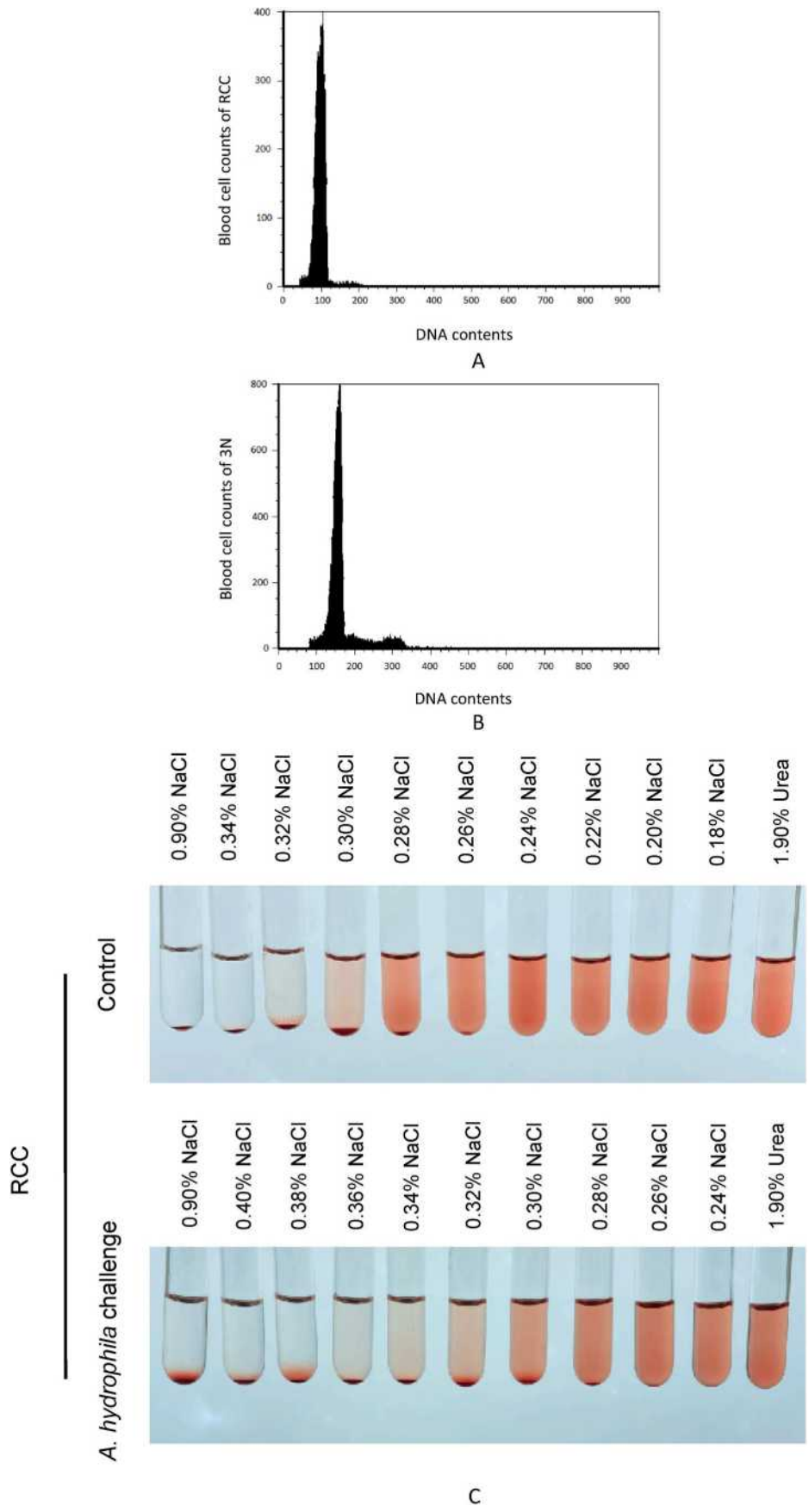



**Fig. 1.** Ploidy analysis, erythrocyte hemolysis and plasma FHB content in different ploidy cyprinid fish. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

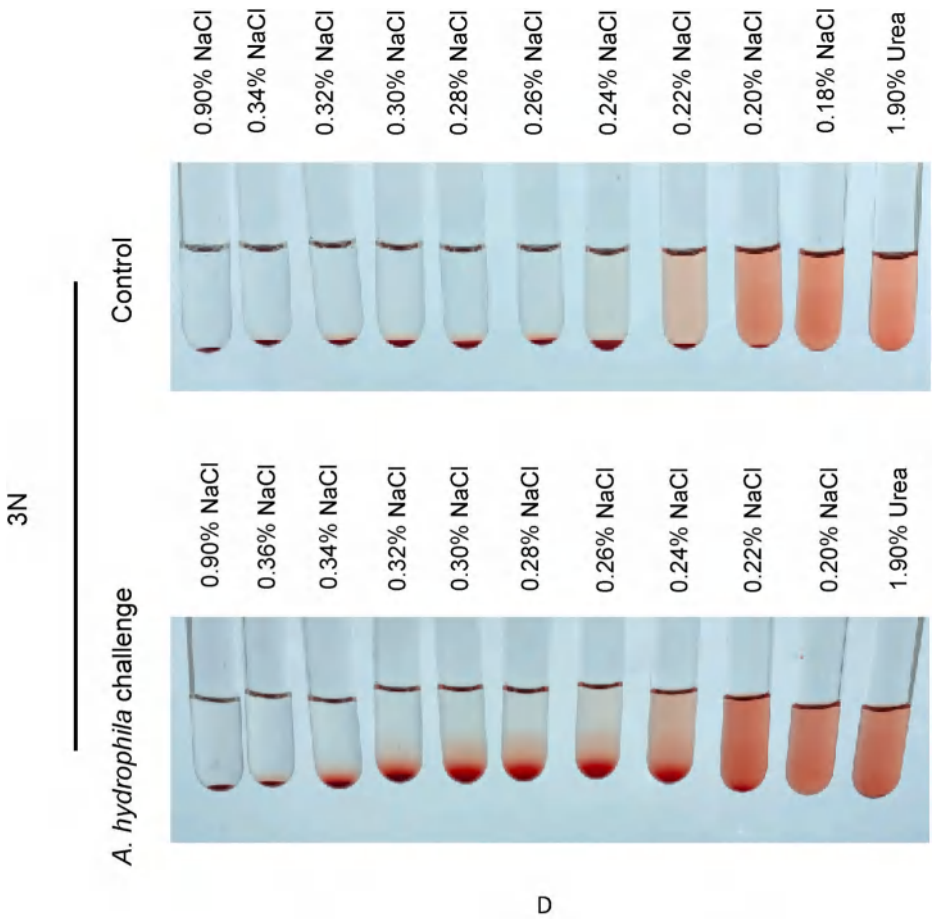



**Fig. 1.** (*continued*).

incubated with staining solution at 37 °C for 20 min and measured at 510 nm [37]. The results were given in microgram protein of FHB content per milliliter of plasma. The experiment was performed in triplicate.

### 2.9. Determination of plasma alkaline phosphatase (ALP) activity

Plasma ALP activity in fish was measured by using alkaline phosphatase (ALP) activity kit (Nanjing Jiancheng Bioengineering institute, China) [38]. The reaction compounds could be detected by the absorbance at 520 nm. The results were expressed as mU ALP activity per milliliter of plasma. The experiment was performed in triplicate.

### 2.10. Determination of plasma lysozyme (LZM) activity

Plasma LZM activity in fish was detected according to previous studies [39]. 0.02% (w/v) suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in 0.05 M phosphate buffer (pH 6.2) was used as substrate, while lyophilized hen egg white lysozyme was used as a standard. Plasma samples were added to the substrate at 25 °C. The results of enzymatic activity were expressed as U LZM activity per milliliter of plasma. The experiment was performed in triplicate.

### 2.11. Measurement of total superoxide dismutase (SOD) activity

According to the protocol of total SOD activity kit (Beyotime

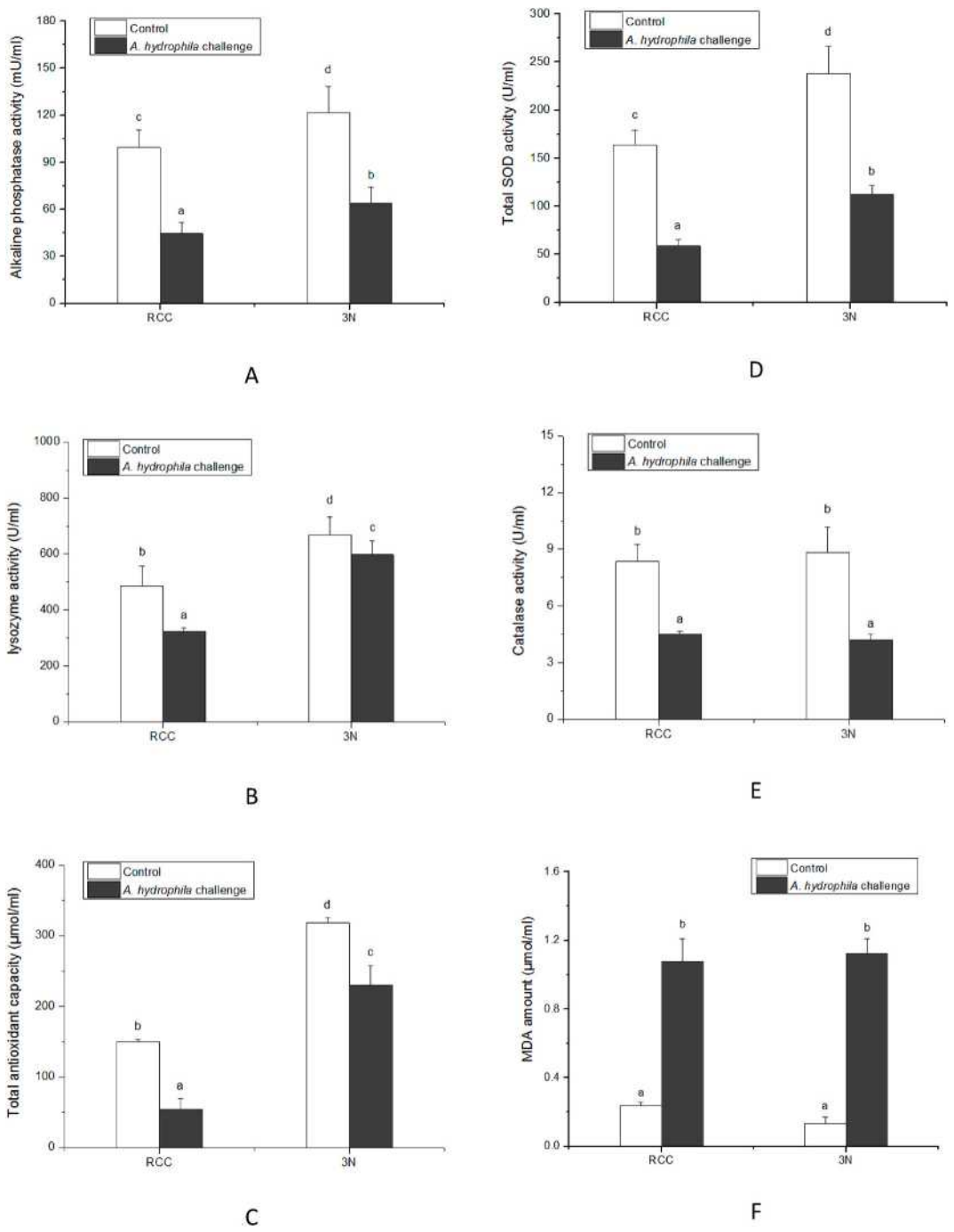



**Fig. 2.** Plasma parameters of RCC and 3 N following *A. hydrophila* challenge. Plasma ALP (A), LZM (B), T-AOC (C), total SOD (D), CAT (E) and MDA (F) were measured by a microplate reader. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

Biotechnology, Shanghai, China), the enzymatic activity in plasma was measured as the change in absorbance at 560 nm by using a Synergy 2 multi-detection microplate reader (Bio-Tek, USA) [40]. The results were given in units of SOD activity per milliliter of plasma, where 1 U of SOD is defined as the amount of enzyme producing 50% inhibition of SOD. The experiment was repeated in triplicate.

### 2.12. Measurement of catalase (CAT) activity

Determination of CAT activity was performed according to ammonium molybdate spectrophotometric method [41]. Based on protocol of catalase (CAT) activity kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the reaction compounds could be monitored by the absorbance at 405 nm. The results were given in units of CAT activity per milliliter of plasma, where 1 U of CAT is defined as the amount of enzyme decomposing 1 μmol $H_2O_2$ per second. The experiment was repeated in triplicate.

### 2.13. Measurement of total antioxidant capacity (T-AOC)

Total antioxidant capacity (T-AOC) can be expressed in term of the contribution to antioxidant compounds when taking into account varied rates of antioxidant reaction with 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [42]. Based on protocol of total antioxidant

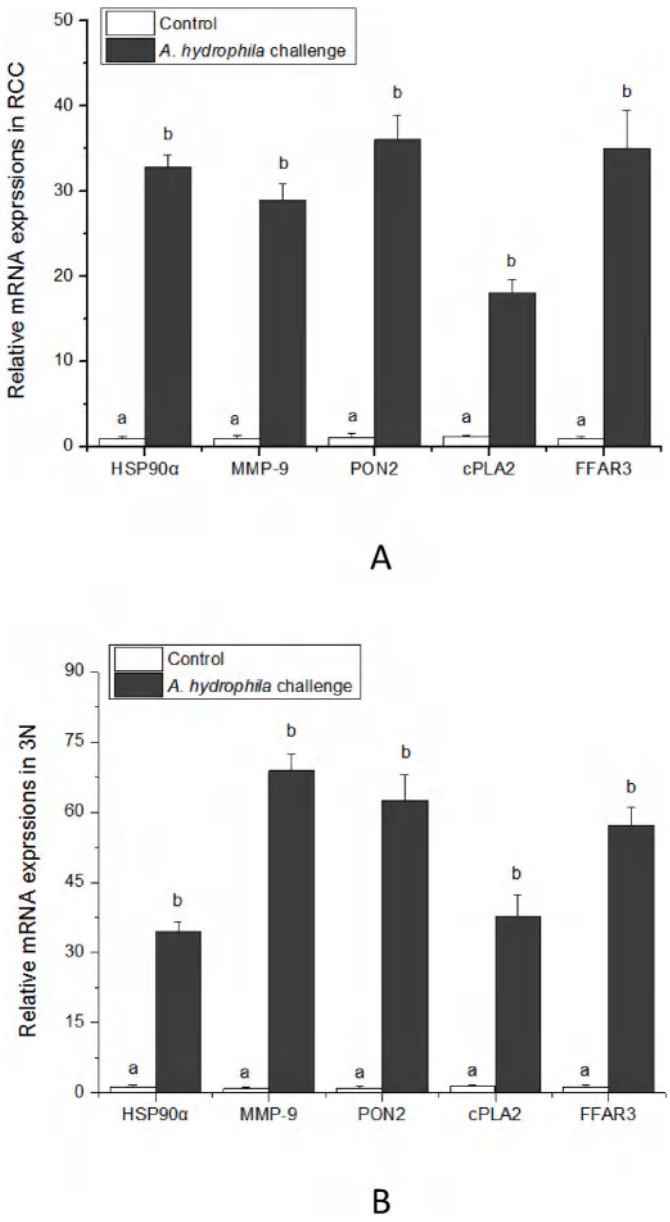



**Fig. 3.** Gene expressions in peripheral blood cells of RCC and 3 N following *A. hydrophila* challenge. Relative gene profiles of HSP90α, MMP-9, PON2, cPLA2 and FFAR3 in peripheral blood cells of RCC (A) and 3 N (B) were calculated by the $2^{-\triangle\triangle Ct}$ methods. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$).

capacity (T-AOC) assay kit with ABTS method (Beyotime Biotechnology, Shanghai, China), T-AOC level in plasma was measured [43]. Quenching of the cation can be measured at 734 nm. Trolox solution was used as a reference standard. The experiment was repeated in triplicate.

### 2.14. Measurement of malondialdehyde (MDA) production

Free MDA and lipid hydroperoxides can be determined by thiobarbituric acid (TBA) method [44]. According to protocol of lipid peroxidation MDA assay kit (Beyotime Biotechnology, Shanghai, China), plasma MDA amount was measured by using a Synergy 2 multi-detection microplate reader (Bio-Tek, USA) [45]. The concentration of MDA was expressed as micromole MDA per milliliter of plasma. The experiment was repeated in triplicate.

### 2.15. RNA isolation and cDNA synthesis

Total RNA isolation and cDNA synthesis were performed as previously described [46]. Total RNA was extracted from peripheral blood cells isolated from caudal veins of RCC and 3 N by using HiPure Total RNA Mini kit (Magen, China). After that, concentration and integrity of purified total RNA were determined by measurement of 260/280 nm absorbance and agarose gel electrophoresis, respectively. 1000 ng of purified total RNA was used for cDNA synthesis using Revert AidTM M-MuLV Reverse Transcriptase Kit (MBI Fermentas, USA).

### 2.16. Quantitative real-time PCR (qRT-PCR) assay

Expression profiles of heat shock protein 90 alpha (HSP90α), matrix metalloproteinase 9 (MMP-9), free fatty acid receptor 3 (FFAR3), paraoxonase 2 (PON2) and cytosolic phospholipase A2 (cPLA2) in RCC and 3 N were examined by using Applied Biosystems QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). qRT-PCR assay contained 1 cycle of 95 °C for 30s, 40 cycles of 95 °C for 15s, 60 °C for 35s, followed by 1 cycle of 95 °C for 30s, 60 °C for 60s. At the end of qRT-PCR amplified reactions, melting curve analysis was implemented to confirm credibility of each qRT-PCR analysis [47]. Besides, the expression of 18S rRNA (XR_003291850.1) was measured and used as internal control to normalize results of qRT-PCR analyses [48]. All primers were checked to be completely identical to sequences of target genes of both RCC and 3 N, and primer specificity was confirmed prior to qRT-PCR assay. Each sample was analyzed in triplicate. The primers were shown in Table 1 qRT-PCR results were measured with $2^{-\triangle\triangle Ct}$ methods [49].

### 2.17. Statistical analyses

The data analyses were measured by using SPSS 18 analysis program and represented as means ± standard deviation. All of the experimental data analyses were subjected to Student's *t*-test or one-way ANOVA (one-way analysis of variance). Further analysis of Duncan's multiple range test, only if the level of P-value $< 0.05$, the differences were considered statistically significant.

## 3. Results

### 3.1. Erythrocyte hemolysis and plasma FHB content in RCC and 3 N following A. hydrophila challenge

In Fig. 1A-B, the ploidy of RCC and 3 N was determined by flow cytometric analysis. In Fig. 1C, erythrocytes isolated from PBS-treated RCC began to lyse at the dose of 0.32% NaCl solution and occurrence of complete erythrocyte hemolysis was observed under 0.24% NaCl solution, whereas erythrocytes isolated from RCC following *A. hydrophila* challenge lysed at the dose of 0.36% NaCl solution and complete erythrocyte hemolysis was observed at 0.26% NaCl solution. In Fig. 1D, erythrocytes isolated from PBS-treated 3 N began to lyse at the dose of 0.22% NaCl solution and occurrence of complete erythrocyte hemolysis was observed following treatment with 0.18% NaCl solution, whereas erythrocytes isolated from *A. hydrophila*-infected 3 N may significantly lyse at the dose of 0.30% NaCl solution and complete erythrocyte hemolysis was observed under 0.20% NaCl solution. In Fig. 1E, plasma FHB level increased dramatically in RCC and 3 N following *A. hydrophila* challenge by comparing with those of the control.

### 3.2. Enzymatic activities and antioxidant status in plasma following A. hydrophila challenge

Enzymatic activities of plasma ALP and LZM in RCC and 3 N were investigated following *A. hydrophila* challenge. As shown in Fig. 2A-B, *A. hydrophila* challenge could significantly decrease the enzymatic

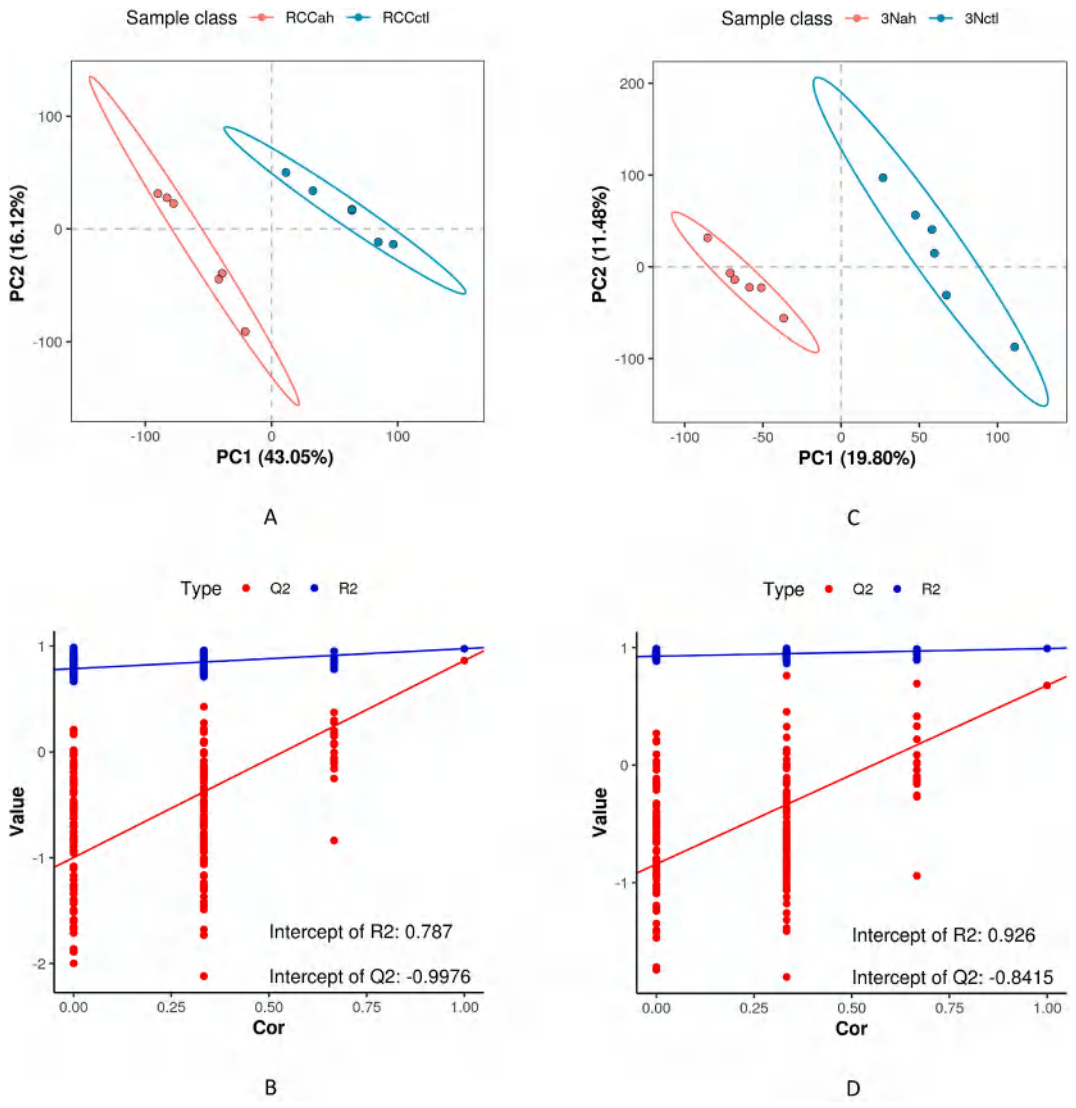



**Fig. 4.** PLS-DA analysis and permutation plot analysis of RCC (A–B) and 3 N (C–D) showing separation of the experimental groups.

activities of ALP and LZM, whereas the enzymatic activities were consistently higher in 3 N by comparing with those of RCC.

The antioxidant parameters in RCC and 3 N following *A. hydrophila* challenge were shown in Fig. 2C–F. T-AOC level, total SOD activity and CAT activity decreased significantly in RCC and 3 N following *A. hydrophila* challenge, while a sharp increase of MDA amount was detected.

### 3.3. Gene expressions determined by qRT-PCR assay

To investigate *A. hydrophila* challenge on the metabolic genes and stress indicator genes in RCC and 3 N, expression patterns of HSP90α, MMP-9, FFAR3, PON2 and cPLA2 were measured in peripheral blood. As shown in Fig. 3A-B, RCC and 3 N receiving *A. hydrophila* exhibited elevated levels of HSP90α, MMP-9, FFAR3, PON2 and cPLA2 by comparing with those of the control, respectively.

### 3.4. Metabolic profiling of different ploidy fish in response to *A. hydrophila* challenge

Based on PLS-DA analysis, clear separations of control samples and *A. hydrophila* challenge samples were observed in RCCah vs RCCctl and 3Nah vs 3Nctl (Fig. 4A–D). In Fig. 5A, a total of 2502 differential metabolites were detected in RCCah vs RCCctl, containing 517 increased metabolites and 1985 decreased metabolites. In contrast, a total of 1865 differential metabolites were detected in 3Nah vs 3Nctl, containing 1614 increased metabolites and 251 decreased metabolites. Then, the relative contents and relationship of top 60 differential metabolites (P-value $<$ 0.05, VIP $\geq$1) were analyzed by HCA-heatmap. As shown in Fig. 5B, relative levels of most metabolites decreased significantly in RCCah vs RCCctl, while a significant increase of stress-related hormones was observed, including estradiol-17-phenylprplonate, penbutolol, tetrahydrocortisone, taccalonolide A and cortisol. In contrast, relative contents of most metabolites increased dramatically in 3Nah vs 3Nctl, whereas levels of dibenz(a,h)acridine, pregnanolone and blestriarene B decreased significantly (Fig. 5C).

### 3.5. Differences in important metabolites and pathway analyses of different ploidy fish after *A. hydrophila* challenge

To further characterize crucial metabolic biomarkers in RCC and 3 N following *A. hydrophila* challenge, secondary metabolites in LC-MS-based metabolomics database were analyzed. In Fig. 6A, percentages of metabolic categories in RCCah vs RCCctl ranked lipids $>$ carbon sources $>$ nucleotides $>$ amino acids. In contrast, percentages of metabolic categories in 3Nah vs 3Nctl ranked lipids $>$ amino acids $>$ carbon

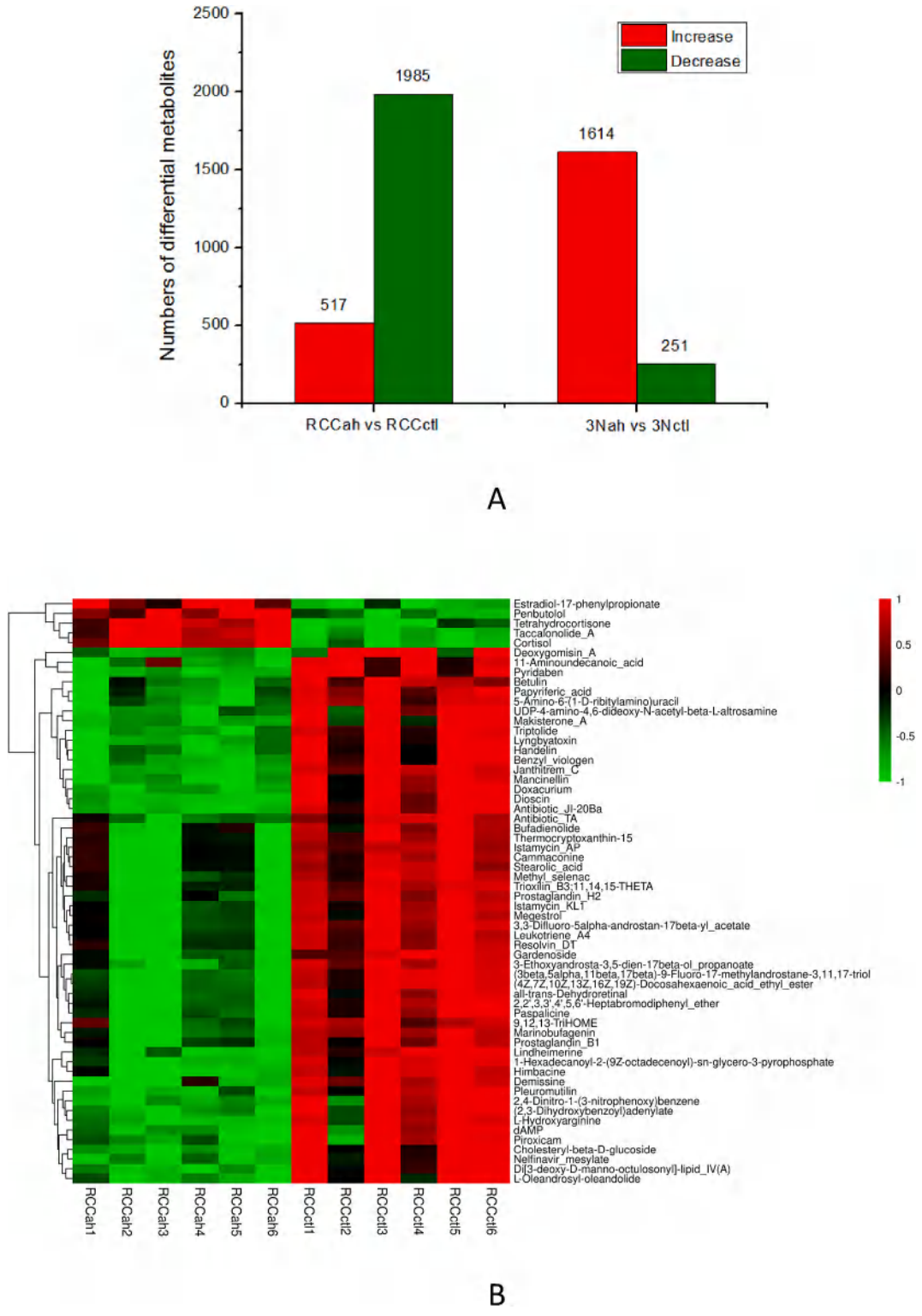



**Fig. 5.** Features of differential primary metabolites. (A) Numbers of differential metabolites in RCC and 3 N. (B-C) HCA-heatmap showing the relative contents and relationship of top 60 metabolites (P-value $< 0.05$, VIP $\geq 1$) in RCCah vs RCCctl and 3Nah vs 3Nctl.

sources > nucleotides (Fig. 6B). The numbers of increased and decreased metabolites in RCCah vs RCCctl and 3Nah vs 3Nctl were shown in Fig. 6C–F. Most numbers of metabolites were down-regulated in RCCah vs RCCctl, while most numbers of metabolites were up-regulated in 3Nah vs 3Nctl. These results suggested that different ploidy cyprinid fish may elicit a distinct metabolic response following *A. hydrophila* challenge.

To represent the relative contents of important metabolites (P-value $< 0.05$, VIP $\geq 1.5$) in RCCah vs RCCctl and 3Nah vs 3Nctl, HCA-heatmap of top 30 metabolites were investigated. As shown in Fig. 6G, the relative abundances of most stress-induced hormones, fatty acid derivatives and amino acids decreased dramatically in RCCah vs RCCctl, while maleic

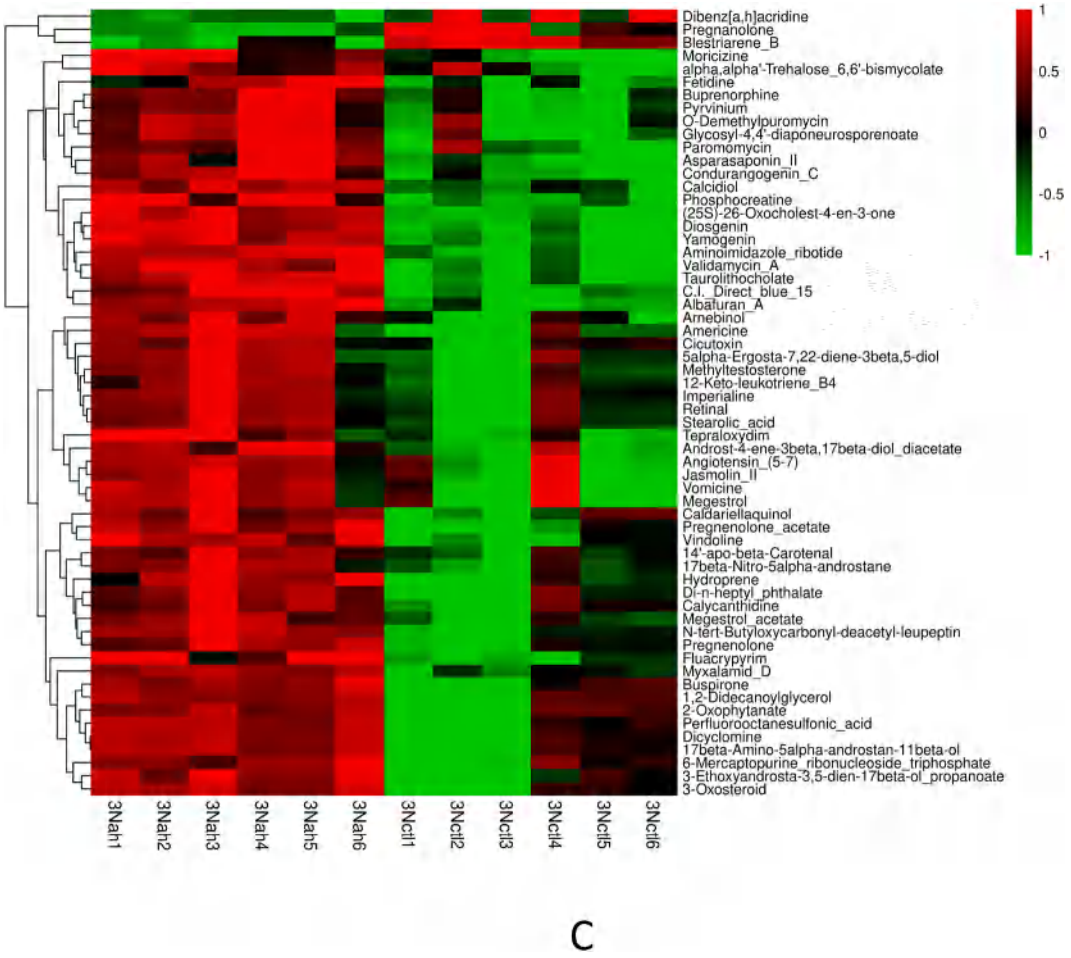



**Fig. 5.** (*continued*).

acid homopolymer and cortisol were up-regulated. In contrast, the relative contents of long chain poly unsaturated fatty acid (LC-PUFA), lysophosphatidylcholine (lysoPC), acylcarnitine and amino acids status increased significantly in 3Nah vs 3Nctl (Fig. 6H).

To compare and investigate the mechanisms linking *A. hydrophila*-induced metabolites of different ploidy cyprinid fish and their specific pathways, the obtained secondary metabolites were used for pathway enrichment by KEGG analysis. In Fig. 7A, differential metabolites in RCCah vs RCCctl belonged to five main categories, including "drug development", "environmental information processing", "human diseases", "metabolism" and "organismal systems", while the most assigned category was "metabolism". In addition, "Arachidonic acid metabolism" and "Biosynthesis of unsaturated fatty acids" were significantly enriched in RCCah vs RCCctl, while "Arachidonic acid metabolism" enriched the most metabolites (Fig. 7B). In Fig. 7C, differential metabolites in 3Nah vs 3Nctl belonged to seven main categories, including "cellular processes", "drug development", "environmental information processing", "human diseases", "metabolism" and "organismal systems", while the most assigned category was "metabolism". In Fig. 7D, "Choline metabolism in cancer" and "Aminoacyl-tRNA biosynthesis" were significantly enriched in 3Nah vs 3Nctl, while "Aminoacyl-tRNA biosynthesis" and "ABC transporters" enriched the most metabolites. The results suggested that active lipid metabolism may play a key role in metabolic processes of RCC and 3 N, while 3 N may have a broad class of metabolites following *A. hydrophila* challenge.

To further investigate the comprehensive analysis of significant metabolites profiles (P-value $< 0.05$, VIP $\geq 1$), metabolic pathway networks of RCCah vs RCCctl and 3Nah vs 3Nctl were shown in Fig. 8. The metabolic processes of glycolysis and tricarboxylic acid cycle appeared to be inactive in RCC and 3 N following *A. hydrophila* challenge, while relative levels of cortisol, leucine, isoleucine, glutamate and biosynthesis of fatty acid increased dramatically. *A. hydrophila*-infected RCCs exhibited a sharp increase of glucose, taurine, glutamine, maleic acid, LC-PUFAs, and lysophosphatidylethanolamine (LysoPE) along with decreased levels of detected long chain acylcarnitine. In contrast, *A. hydrophila*-infected 3Ns exhibited a significant increase of proline, lysine, tyrosine, acylcarnitine, glutaric acid derivatives, LC-PUFAs, LysoPC, lysophosphatidylserine (LysoPS) and long chain acylcarnitine.

## 4. Discussion

*A. hydrophila* is a serious pathogen that can cause disease outbreak in freshwater aquaculture [50]. Additionally, aerolysin is a significant virulent factor secreted by *A. hydrophila* that can bind to specific glycophosphatidylinositol (GPI)-anchored proteins on the surface of erythrocytes and cause a significant damage to fish erythrocytes by forming pores in cell membrane, then exhibiting a strong hemolytic activity and triggering deep wound infection [51,52]. In this study, erythrocytes of 3 N exhibited a stronger resistance against osmotic stress by comparing with those of RCC. *A. hydrophila* infection could accelerate erythrocyte hemolysis and increase plasma FHB level in RCC and 3 N, but erythrocytes of RCC exhibited a higher susceptibility to osmotic stress following *A. hydrophila* challenge by comparing with those of 3 N. These results suggested that erythrocytes of 3 N exhibited a higher resistance against *A. hydrophila*-induced hemolysis by comparing with those of RCC.

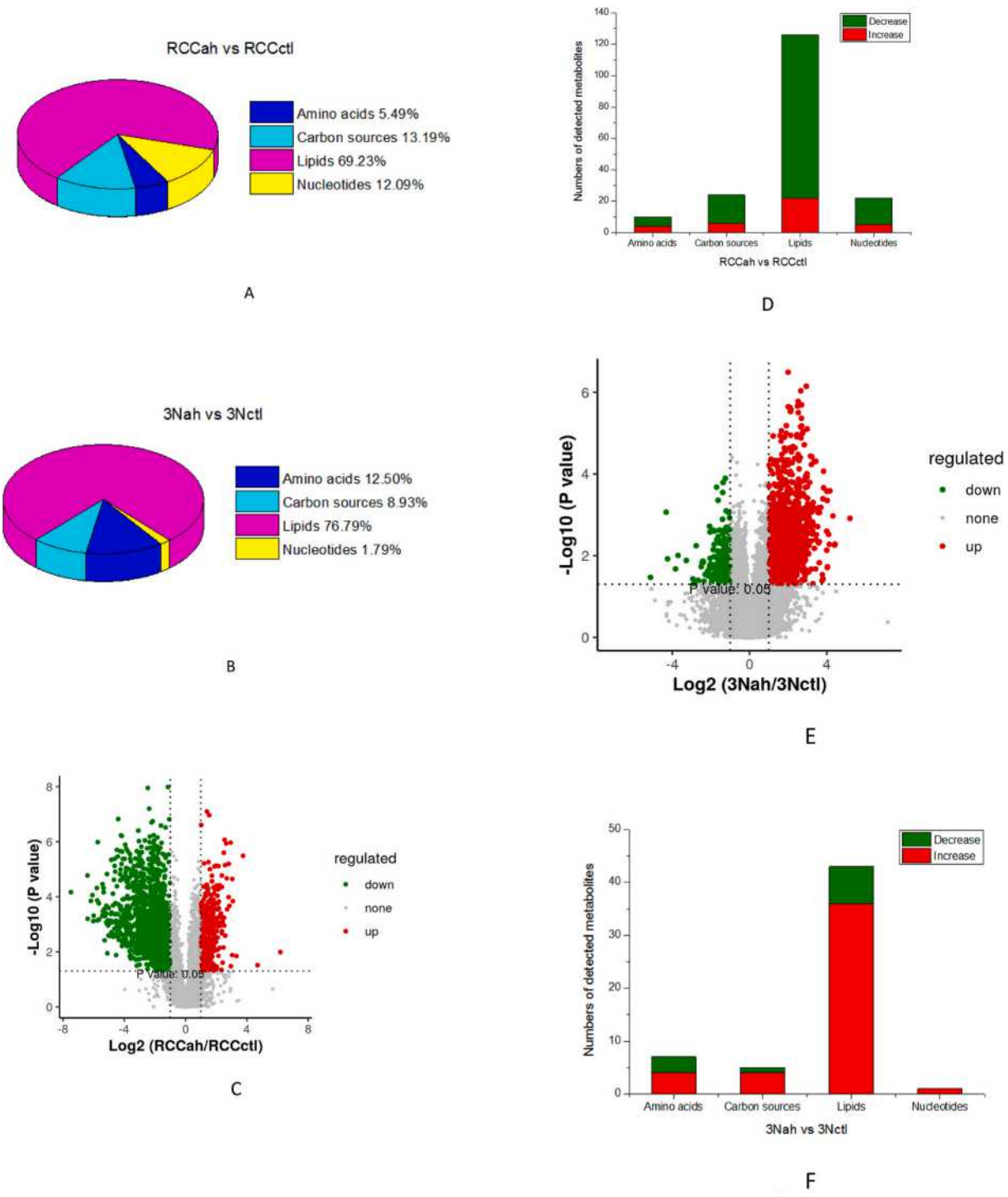
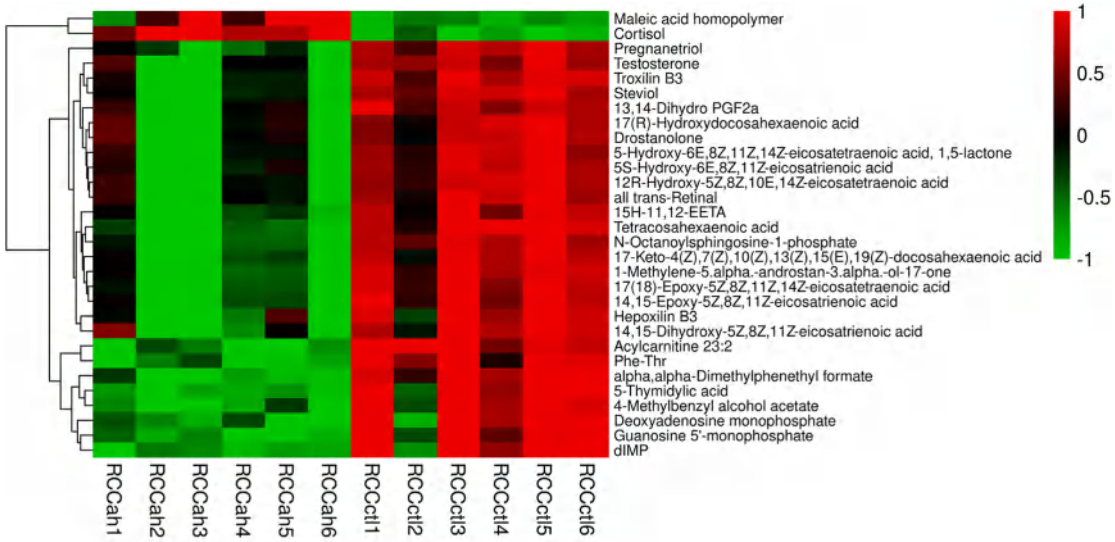



**Fig. 6.** Features of differential secondary metabolites. (A–B) Percentage of differential metabolites of RCCah vs RCCctl and 3Nah vs 3Nctl in four categories. (C–D) Numbers of decreased and increased metabolites in RCCah vs RCCctl. (E–F) Numbers of decreased and increased metabolites in 3Nah vs 3Nctl. (G–H) HCA-heap of top 30 metabolites (P-value $< 0.05$, VIP $\geq 1.5$) in RCCah vs RCCctl and 3Nah vs 3Nctl that showed major changes following *A. hydrophila* challenge.

Increasing evidences indicate that acute bacterial infection may impair host immunity, diminish phagocytic activity and dysregulate cellular signals, resulting in the delayed bacterial clearance [53]. Lymphoid organs, immune cells, humoral factors and cytokines are the major components of immune defense against foreign organisms or substances, including bacteria, viruses, parasites and toxins, which may render fish higher sensitive to stressors [54,55]. As lower vertebrates, fish may primarily rely on the innate immune response to pathogenic infection and their humoral response is predominantly characterized by LZM, complements, lectins, ALP and other antibacterial peptides [56]. LZM participates in bacteriolytic process, phagocytic activity as well as activation of complement cascades [57]. ALP is a macrophage lysosomal marker enzyme playing a vital role in fish immunity [58], which can lessen the toxicological effect of lipopolysaccharide (LPS) and prevent inflammation [59]. HSPs are the predominant stress-induced proteins in response to various kinds of environmental stress or inflammation [60,

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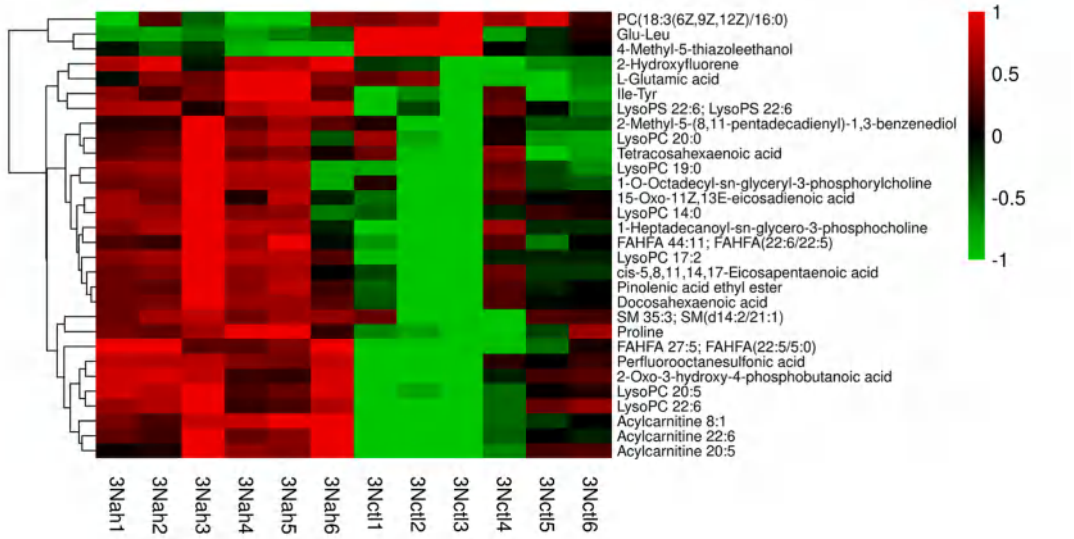


H

**Fig. 6.** (*continued*).

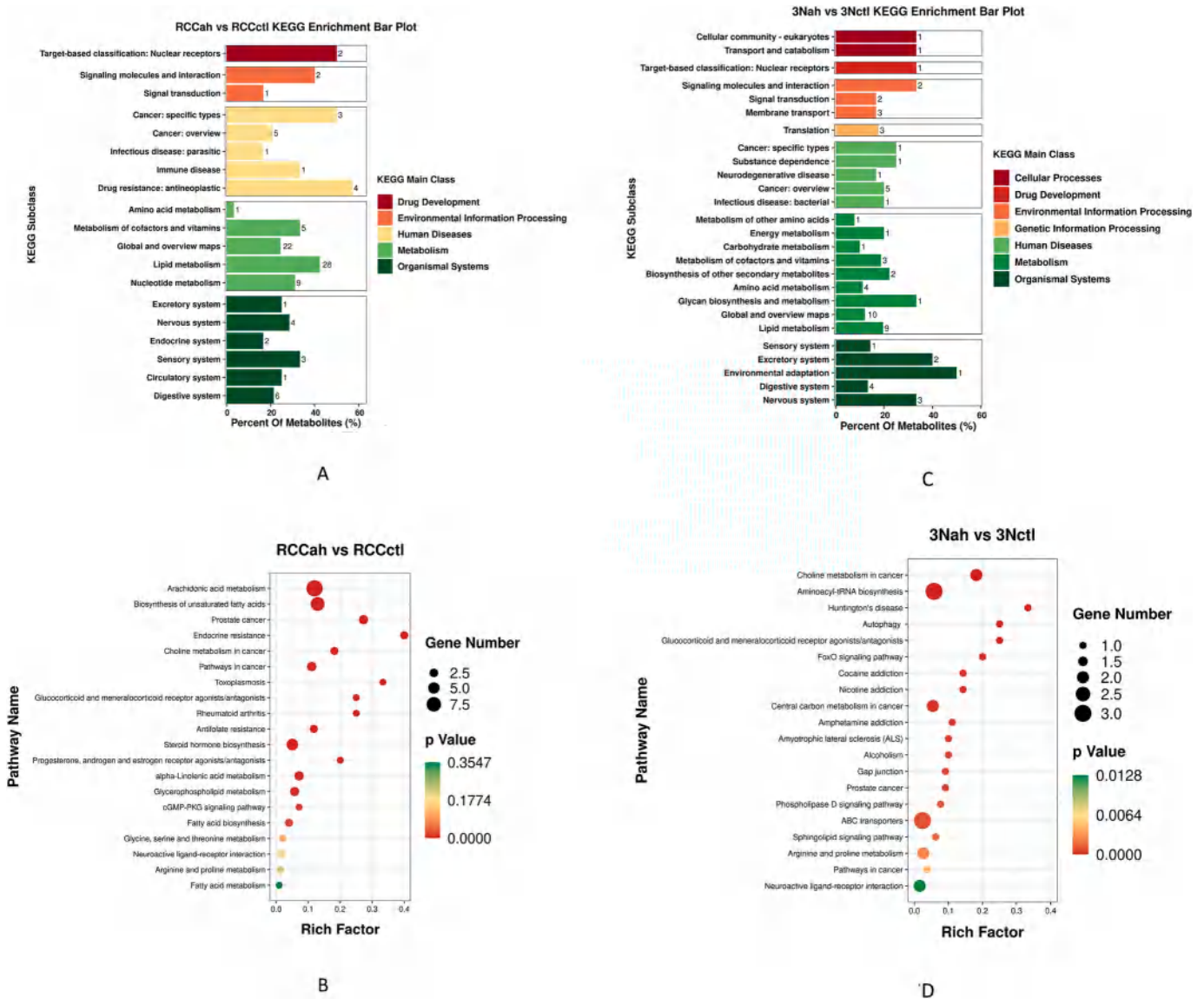


**Fig. 7.** KEGG analysis of plasma metabolites in RCCah vs RCCctl and 3Nah vs 3Nctl. KEGG annotation of detected metabolites in RCCah vs RCCctl (A) and 3Nah vs 3Nctl (C). Top 20 enriched metabolic pathways in RCCah vs RCCctl (B) and 3Nah vs 3Nctl (D).

61], which can exert immune activation and confer protection against infectious diseases [62]. MMP-9 can participate in degradation process of extracellular matrix in normal physiology, which can act as a protective molecule capable of defending against bacterial infection in mice or fish through macrophage migration during inflammatory response [63,64]. PON can mediate inflammatory response [65], while PON2 exhibits the highest hydrolytic activity toward acyl-homoserine lactones involved in immune defense against bacterial infection and oxidative stress [66,67]. In this study, decreased levels of plasma ALP and LZM activity along with elevated expressions of HSP90α, MMP-9 and PON2 were observed in RCC and 3 N following acute *A. hydrophila* infection, but the activities of ALP and LZM in 3 N were consistently higher than those of RCC, suggesting that plasma of 3 N showed a higher antibacterial activity during *A. hydrophila*-induced inflammatory response by comparing with that of RCC.

*A. hydrophila* infection can also induce oxidative stress in fish [68,69]. Although intracellular ROS level can bridge innate immunity with adaptive immune response [70,71], its excessive accumulation may elicit an adverse effect on the organisms, triggering lipid peroxidation, antioxidant imbalance and mitochondrial dysfunction [72,73]. The antioxidant enzymes and compounds can counteract cytokine-mediated toxicity [74], while severe oxidative stress can damage antioxidant defense in fish [75], leading to turbulence of fatty acid β-oxidation and immune response [76]. In this study, down-regulations of T-AOC level, total SOD and CAT activity along with elevated levels of MDA amount were observed in RCC and 3 N following *A. hydrophila* challenge. These results implied that acute *A. hydrophila* infection may lead to collapse of host defense in fish undergoing severe inflammatory response.

Vertebrate blood is playing a central role in bridging endocrine system to immune regulation, in which various forms of immune cells can produce hormones and participate in immune signals under the control of central nervous system [77]. Moreover, fish blood parameters, including cortisol, glycemina, hematocrit and antioxidant enzymatic activities, can act as potential biomarkers of environmental stressors and chemical toxins [78]. In general, exposure to stressors may also cause teleostean non-specific response in hormone levels, metabolite contents, hydromineral balance and hematological changes, which may enable fish to maintain homeostasis and counteract stress-evoked adverse effects [79]. However, the comparative mechanisms linking *A. hydrophila* infection to metabolic response in different ploidy fish are still unclear.

Amino acids not only serve as preferential energetic substrates together with fatty acids [80,81], but also participate in immune

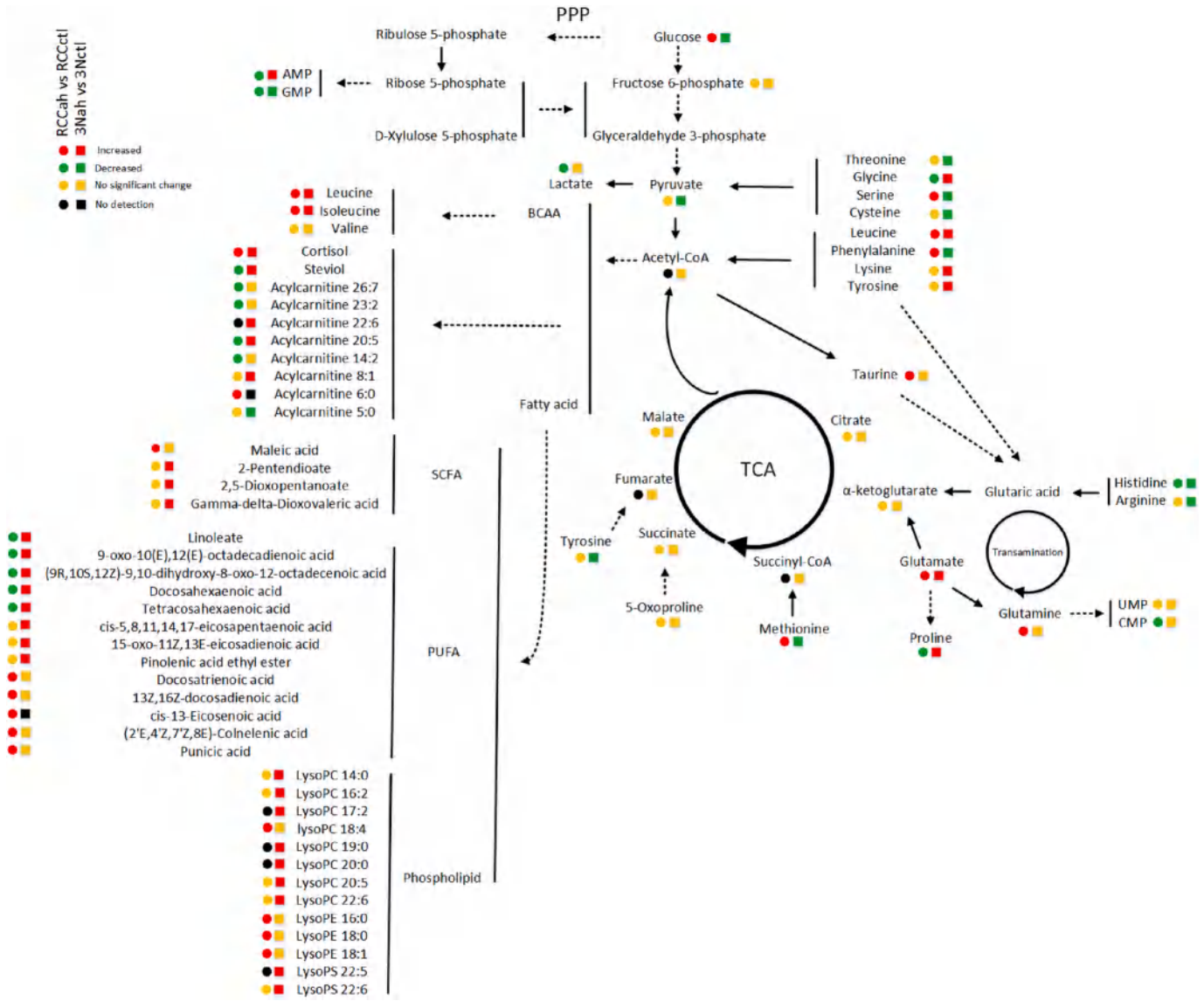


**Fig. 8.** The metabolic pathway network of different ploidy cyprinid fish following *A. hydrophila* challenge.

regulation [82]. Glutamate, an energy substrate in leucocyte metabolism, can modulate immune system by acting on metabotropic receptors in central and peripheral nervous system [83]. In addition, glutamate can act as an immediate precursor for glutathione synthesis, playing an important role in the removal of oxidants and regulation of the immune response in fish [84,85]. Branch-chain amino acids (BCAAs) can regulate antibody production and alter the balance between inflammatory and anti-inflammatory cytokines by lymphocytes [86,87], while BCAAs restriction may lead to complete absence of protein synthesis or proliferation of lymphocytes, resulting in immune impairment [88,89]. Previous studies demonstrate that leucine can enhance innate immunity and eliminate invading bacteria by modulating teleostean metabolic products [90], while administration of isoleucine can enhance immune function and antioxidant capacity in fish [91].

Fatty acids are commonly presented in organisms as three main classes of esters: triglycerides, phospholipids and cholesterol esters, serving as key metabolites for energy supply and structural components of cell membranes [92]. Free fatty acids are important energy sources for most tissues, which are transported by binding to plasma albumin [93]. Increasing findings suggest that free fatty acids can elicit a direct influence on immune cell activation and fatty acid-driven inflammatory mediator production [94]. Short chain fatty acid (SCFA) can regulate immune cell activation and modulate innate immune response along with synergistic up-regulation of cytokines via FFAR3 activation [95–97]. Although PUFA cannot be denovo-synthesized by vertebrates and must be obtained in the diets, the most biologically active essential fatty acids (EFA), including arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), can be synthesized in fish via sequential desaturation and elongation of linoleic acid (18:2n-6) and linolenic acid (18:3n-3) [98]. Currently, emerging evidences demonstrate that PUFAs can possess the ability to regulate immune response, promote macrophage function as well as exert a significant antimicrobial activity against a variety of invading pathogens [99]. Evidences are emerging that cPLA2 serving as disease marker can modulate normal physiology and disease pathogenesis in many tissues by catalyzing the hydrolysis of phospholipids to arachidonic acid and lysophospholipids (LPLs) [100]. LPLs such as LysoPC, LysoPE and LysoPS can possess a direct antibacterial activity, regulate macrophage activation, promote phagocytic clearance of apoptotic cells as well as mediate inflammatory resolution [101–104]. Additionally, plasma acylcarnitine can promote the transport of long chain fatty acids and facilitate mitochondrial fatty acid β-oxidation, whose expression levels can serve as a marker of disease status [105, 106]. Plasma cortisol is considered as stress-related hormonal indicator

[107], which can restore homeostasis upon *in vitro* stimulation [108], elicit multiple effects on energy metabolism [109,110] as well as modulate the up-regulated expression of HSPs [111]. In this study, *A. hydrophila* challenge can dramatically increase cPLA2 expression and PUFA contents in different ploidy cyprinid fish, but 3 N may have a broad class of fatty acid derivatives. In addition, the difference in fatty acid derivatives and plasma acylcarnitine in RCC and 3 N may reflect the differential regulation of mitochondrial fatty acid β-oxidation in response to *A. hydrophila* challenge, which can serve as metabolic indicators of different ploidy cyprinid fish following *A. hydrophila* infection.

In summary, we compared and characterized erythrocyte hemolysis, plasma parameters, gene expressions and metabolic features of different ploidy cyprinid fish. Our findings revealed that erythrocytes of 3 N showed a higher resistance against *A. hydrophila*-induced hemolysis by comparing with those of RCC. Acute *A. hydrophila* infection could decrease non-specific immunity and antioxidant status along with elevated level of inflammatory regulators in different ploidy fish. In addition, we further identified fatty acid derivatives and their metabolic networks as crucial biomarkers in RCC and 3 N after *A. hydrophila* challenge. Thus, the information presented in this study could provide a novel insight into differences in metabolic strategy of different ploidy fish to cope with bacterial infection.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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