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Histological and transcriptomic responses of two immune organs, the spleen and head kidney, in Nile tilapia (*Oreochromis niloticus*) to long-term hypersaline stress

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Abstract

Hyperosmotic stress can adversely affect fish immunity, but little is known about the histological and transcriptomic responses of immune organs in fish in a hyperosmotic environment. This study evaluated the effects of long-term hypersaline conditions (16‰) on the growth, histology and transcriptomics of the two main immune organs, the spleen and head kidney, in Nile tilapia *Oreochromis niloticus* relative to those reared in freshwater for eight weeks. No differences in weight gain and specific growth rate were found between fish reared under these two salinities. Hyperosmotic stress induced a congestive or enlarged spleen. Platelet- and coagulation-related gene expression was significantly decreased in tilapia at 16‰. The red cell distribution width and value of the mean corpuscular hemoglobin were significantly greater in fish at 16‰ salinity than in control fish in freshwater. A large volume of melano-macrophages in the spleen and pigment deposition in both the spleen and head kidney were observed in the histological sections in fish at 16‰ salinity. Transmission electron microscopic results showed abnormal macrophages with deposition granules in the spleen and head kidney and more neutrophils in the head kidney of fish at 16‰ than in control fish. In total, 772 and 502 genes were annotated for significantly different expression in the spleen and head kidney, respectively, and corresponded to five and one significantly changed immune system pathways, respectively. The complement pathway in the spleen was significantly down-regulated at 16‰. This study indicates that long-term exposure of Nile tilapia to a hyperosmotic environment can induce splenomegaly, reduce coagulation function, enhance phagocytic activity and down-regulate the complement pathway in the spleen. The spleen is a more sensitive organ for immune responses to chronic ambient salinity stress than the head kidney in Nile tilapia.

38 **Key words:** Nile tilapia *Oreochromis niloticus*, immune organ, hematology,

39 phagocytosis, transcriptomics, spleen, head kidney

1. Introduction

Immune ability in most vertebrates comprises both innate and acquired immunity. In teleost fish, the head kidney and spleen are the two largest lymphoid and immunocompetent organs [1, 2]. The main cells in the head kidney are macrophages, which aggregate into melano-macrophage centers (MMCs), and lymphoid cells, which are found at all developmental stages and mostly exist as B cells [3]. The spleen contains a system of splenic ellipsoids, MMCs and lymphoid tissue with a positive function in phagocytosis and the capture of antigens [4]. The head kidney and spleen have a hematopoietic function equivalent to erythropoiesis in the bone marrow until adulthood [3, 5]. Physiological and structural changes usually occur in these two organs in teleost fish under environmental stress. Exposure to cadmium chloride (20.93 mg/L) for 120 h can cause significant changes in MMCs and free macrophages in the spleen and kidney of *Oreochromis mossambicus* [6]. A high temperature together with pre-exposure to 5 μg/L cadmium increases the lipid peroxidation levels in the spleen of zebrafish [7]. The relative spleen weight and spleen lysozyme activity are decreased, and total immunoglobulin expression is increased, in the Eurasian perch *Perca fluviatilis* after acute stress exposure for 72 h [8]. Exposure to endosulfan reduces the relative spleen weight and spleen cell viability but increases macrophage activity in the spleen of *Oreochromis mossambicus* [9]. However, no research has used a combined approach of histology and transcriptomics to investigate the immune response of the spleen and head kidney to ambient stress in teleost fish.

As one of the important environmental factors, salinity directly affects the physiological status and immune function of aquatic organisms. The complexity of estuarine water and regional variation make water salinity unpredictable and bound to induce a complex adaptation process of aquatic organisms [10]. In *Tilapia*

mossambicus, acute hyperosmotic stress can increase phagocytosis, respiratory burst activity and humoral immune reactions in the spleen and head kidney [11]. Similarly, salinity stress can enhance immune responses in the kidney of the striped catfish *Pangasianodon hypophthalmus* [12]. The blood parameters of teleost fish are also important indicators to detect abnormalities related to environmental stress and disease [13]. Ambient salinity also influences hematology in many fish species such as the great sturgeon *Huso huso* [14], cobia *Rachycentron canadum* [15] and *Notopterus notopterus* [16] and shortnose sturgeon *Acipenser brevirostrum* [17]. Meanwhile, chronic salinity stress can increase susceptibility to microbial infection in striped catfish [18] and streptococcus infection in Nile tilapia *Oreochromis niloticus* [19]. However, little is known about the fundamental immune response of immune organs to long-term salinity stress in teleost fish.

Nile tilapia is one of the most important commercial freshwater fish in aquaculture worldwide due to its fast growth rate, relatively low production cost and high tolerance to adverse conditions. The tolerance of a wide range of salinity makes it an important species in brackish water aquaculture [20], and it has become a model species to study salinity adaptation in aquatic organisms. However, Nile tilapia in brackish water have shown lower immunity and higher disease susceptibility than in freshwater and result in disease outbreak and metabolic disorder [19, 21, 22]. Previous research has mostly focused on the aspects of growth, antioxidant status, osmoregulation and feed utilization of Nile tilapia during salinity adaptation, and few studies have concerned autogenous immune responses in immune organs that are fundamental to overcome the poor performance of Nile tilapia in brackish cultivation. Therefore, as a good model for salinity adaptation, the understanding of the immune response of the spleen and head kidney in Nile tilapia to long-term salinity stress can

provide a theoretical reference and a practical guideline for a variety of euryhaline teleost fish in scientific research and aquaculture operations.

In the present study, the growth performance, hematology, histology and transcriptomics were analyzed to evaluate the comprehensive response of the spleen and head kidney in Nile tilapia between freshwater and hyperosmotic environments. The results of this study will provide an in-depth understanding of the immune status of immune organs in a euryhaline fish under long-term salinity stress.

2. Materials and methods

2.1. Experimental fish and conditions

The sex-reversed all-male Nile tilapia juveniles were obtained from a private hatchery in Shenzhen, Guangdong, China. After one-week acclimation in tanks (300 L) at the Biological Station of East China Normal University, the tilapia were randomly assigned to six glass tanks (200 L) at a density of 18 fish per tank. Three tanks were filled with freshwater, and the salinity in the other three tanks were gradually increased to 16‰ at a daily rate of 4‰ by adding sea salt. After the salinity reached the target value, the experiment was initiated with 18 fish (6.41 ± 0.09 g) in each tank (200 L) for 49 days. During the trial, tilapia were fed to satiation with a commercial diet (32% protein and 4% lipid, TONGWEI CO., LTD, Sichuan, China) twice daily (0800 and 1500 h). One hour after feeding, the uneaten diet was removed by siphon and the daily water exchange rate was 30% of the tank volume. The incoming fresh water and brackish water were aerated thoroughly before entering the water recirculation system. The photoperiod was maintained at 12 h light and 12 h dark, and water-quality parameters were monitored. During the whole trial, the dissolved oxygen concentration was 7.7-8.9 mg/L, the pH averaged 8.06 ± 0.23, ammonia-N

was <0.05 and water temperature averaged 27 ± 2 °C.

2.2. Sample collection

At the end of the trial, all fish were anesthetized in 30 ppm MS-222 and then were counted and weighed for survival, weight gain and the specific growth rate. The blood of three fish was individually sampled from each tank randomly by caudal sinus puncture with a 1-mL plastic syringe and then was transferred to 1.5-mL tubes coated with lithium heparin as the anticoagulant for hematological determination. Five spleens from each tank were weighed, corresponding to the body weight of each fish. The spleen and head kidney were quickly dissected and then were frozen in liquid nitrogen for transcriptomic analysis. The spleen and head kidney were cut into small cubes and were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for histology assay. The animal ethics protocol was approved by the East China Normal University Experimental Animal Ethics Committee (No. F20140101). The weight gain and relative spleen weight were calculated as follows:

Weight gain (WG, %) = [final weight (g) - initial weight (g)] /initial weight (g) × 100

Specific growth rate (SGR, % day^{-1}) = [ln(final weight) - ln(initial weight)] /days × 100

Relative spleen weight (RSW, %) = (wet spleen weight) /(wet body weight) × 100

2.3. Hematological assay

The white blood cell count (WBC, 10^9/L), red blood cell count (RBC, 10^{12}/L), hemoglobin (HGB, g/L), hematocrit (HCT, %), mean corpuscular volume (MCV, fl), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/L), red cell distribution width (RDW, %), platelet count

(PLT, 10^9/L), mean platelet volume (MPV, fl), platelet distribution width (PDW, fl) and plateletcrit (PCT, %) were investigated using an automated hematology analyzer (BC-2800vet, Shenzhen, Mindray Bio-Medical Electroics, China).

2.4. Paraffin sections of the spleen and head kidney

The excised spleen and head kidney samples were fixed in paraformaldehyde (4%) for 24 h. The fixed spleen and head kidney were then dehydrated in ascending concentrations of alcohol and cleaned in xylol, followed by vacuum-embedding in paraffin. The embedded spleen and head kidney were sectioned with a rotary microtome at 5 μm. The tissue slices of the spleen and head kidney were stained with hematoxylin and eosin (HE). The stained sections were analyzed using the BX51 system (OLYMPUS, Tokyo, Japan), and digital images were taken using Image-Pro plus 6.0.

2.5. Transmission electron microscopic observation

For transmission electron microscopy (TEM) analysis, the spleen and head kidney were fixed in glutaraldehyde (2.5%) for 3 h and then were washed three times with phosphate buffer (pH 7.4). Tissues were post-fixed in 1% osmic acid (0.1 M phosphate buffer, pH 7.4) at 20 °C for 2 h and then were washed with the same buffer and method. Dehydration was conducted using an ascending series of ethanol solutions (50%, 70%, 80%, 90%, 95% and 100%) and acetone (100%) before transfer to a 1:1 mixture of acetone and 812 embedding medium (90529-77-4, SPI, West Chester, PA, USA) for 3 h. Penetration occurred overnight in a 2:1 mixture of acetone and 812 embedding medium and for 6 h in the 812 embedding medium. The specimens were transferred into gelatin capsules containing the embedding medium overnight in an oven at 37 °C. The capsule embedding was completed in the oven for

48 h at 60 °C. Ultrathin sections were cut at 60-80 nm using a Leica ultramicrotome (EM UC7, Leica Microsystems, Germany) and a diamond knife on an Ultra 45 °C (Daitome AG, Nidau, Switzerland). Sections were stained with both uranyl acetate and lead citrate for 15 min to observe the ultrastructure using a transmission electron microscope (HT7700, Hitachi, Tokyo, Japan).

2.6. RNA extraction, transcriptome library preparation and Illumina sequencing

Total RNA was extracted from the spleen and head kidney with three replicates using TRIzol ® Reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (Takara, Japan). The quality and quantity of total RNA were assessed using a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, DE, USA).

The RNA-seq transcriptome library was prepared following the TruSeq^{TM} RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA from the spleen and head kidney, respectively. Messenger RNA was isolated according to the poly A selection method using Oligo (dT) beads and then was fragmented using fragmentation buffer. Double-stranded cDNA of the two tissues was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). T4 DNA ligase buffer was used to end-repair the double-stranded cDNA. A single (A) was added using Klenow buffer. Adaptor-modified fragments were selected by gel-purification, and PCR amplification was performed for 15 cycles. After being quantified by TBS380, the paired-end RNA-seq sequencing library was sequenced using Illumina HiSeq 4000. The SRA number for data uploaded into NCBI is SRP132530 for head kidney and SRP132531 for spleen.

2.7. Differential expression analysis and functional enrichment

187 The high-quality trimmed sequences were used for further mapping to the tilapia
188 genome (GenBank accession No. 8126) with Hisat 2. To identify differential
189 expression genes between the two different treatments in two tissues, the expression
190 level of each transcript was calculated according to the fragments per kilobase of exon
191 per million mapped reads (FRKM) method. RSEM
192 (http://deweylab.biostat.wisc,edu/rsem/) was used to quantify gene abundance.
193 Differential expression analysis was conducted using R statistical package software
194 EdgeR (Empirical analysis of Digital Gene Expression in R,
195 http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html). KEGG was
196 performed for functional-enrichment analysis in the metabolic pathways at
197 Bonferroni-corrected $P \leq 0.05$ compared with the whole-transcriptome background.
198 KEGG pathway analysis was carried out using KOBAS
199 (http://kobas.cbi.pku.edu.cn/home.do).

200 *2.8. Statistical analysis*

201 Statistical analysis was carried out using SPSS statistics 20 (IBM, Armonk, NY,
202 USA). All data are presented as the means ± standard error (SE). The results were
203 subjected to check for normality and homogeneity of variance by Levene's equal
204 variance test. Independent sample t test was performed to examine significant
205 differences between two treatments. *P*-value less than 0.05 was considered
206 statistically significant and marked as "*"; those treatments less than 0.01 were
207 marked as "**".

208 **3. Results**

209 *3.1. Growth performance*

210 Tilapia in freshwater showed slightly higher WG and SGR than those in brackish

water. The RSW of tilapia in brackish water was significantly higher than that in freshwater ($P < 0.05$). Tilapia under the two ambient salinities both showed 100% survival (Table 1).

3.2. Hematological parameters

Significantly higher MCV ($P < 0.01$) and MCH ($P < 0.05$) were detected for tilapia in brackish water than for those in freshwater (Figure 1). Tilapia in freshwater showed significantly higher MPV ($P <0.01$) and PDW ($P <0.05$) than those in brackish water (Figure 2). No significant difference was found in other hematological parameters between the two ambient salinities.

3.3. Paraffin sections of the spleen and head kidney

Erythrocytes were increased in the spleen section with a significant reddish color in brackish water than in freshwater (Figure 3-A, C). The spleen of tilapia in brackish water showed significantly more macrophages and a larger volume of melano-macrophage centers (Figure 3-B, D). The deposition of pigments such as melanin in both the spleen and head kidney of tilapia was more conspicuous in brackish water than in freshwater (Figure 3-B, D and Figure 4-B, D).

3.4. Transmission electron microscopic observations

Ambient salinity exhibited no significant influence on the lymphocyte ultrastructure in both the spleen and head kidney (Figure 5-B, D and Figure 6-B, E). In brackish water, macrophages showed a chaotic ultrastructure and more secondary lysosomes and debris (Figure 5-A, C and Figure 6-A, D). Neutrophils were significantly increased in the head kidney of tilapia in brackish water compared with tilapia in freshwater.

3.5. Transcriptomic analysis in the spleen

A total of 320.52 million reads were obtained, including 61.16, 55.49 and 51.02 million reads from control spleens, respectively, and 50.50, 56.32 and 46.03 million reads from spleens of tilapia under salinity stress, respectively. After the filtering, a total of 314.15 million reads (98.01% of total reads) were used for downstream transcriptome assembly, which contained 60.03, 54.11 and 50.02 million reads from control spleens, respectively, and 49.52, 55.25 and 45.22 million reads from spleen of tilapia under salinity of16, respectively. In total, 27,088 genes were annotated, and the expression levels of 772 genes were significantly different in the spleen between brackish water and freshwater ($P < 0.05$). Among these 772 genes, 398 genes were up-regulated, and 374 genes were down-regulated in brackish water compared with that in freshwater.

Twenty pathways were significantly changed ($P < 0.05$) in the spleen using the Kyoto encyclopedia of genes and genome database (KEGG) (Table 2). Five pathways were categorized into the immune system, containing complement and coagulation cascades, antigen processing and presentation, natural killer cell-mediated cytotoxicity, intestinal immune network for IgA production and hematopoietic cell lineage involving 25 significantly changed annotated genes in these five immune pathways (Figure 7). Among those pathways, the complement and coagulation cascade pathway showed an overall down-regulation by chronic salinity stress in the spleen (Figure 8).

3.6. Transcriptomics of the head kidney

A total of 318.79 million reads were obtained, including 53.23, 51.27 and 55.83 million reads from control head kidneys, respectively, and 53.71, 55.91 and 48.84 million reads from head kidney of tilapia under salinity stress, respectively. After the filtering, a total of 312.41 million reads (98.00% of total reads) were used for

downstream transcriptome assembly, which contained 52.23, 50.36 and 54.78 million reads from control head kidneys, respectively, and 52.58, 54.58 and 47.88 million reads from head kidney of tilapia under salinity stress, respectively. In total, 27,088 genes were annotated, and the expression levels of 502 genes were significantly different in the head kidney between fish in brackish water and freshwater ($P < 0.05$). Among these 502 genes, 266 genes were up-regulated and 236 genes were down-regulated in tilapia in brackish water compared with those in freshwater.

Thirteen significantly changed pathways ($P < 0.05$) were obtained, including one immune system (antigen processing and presentation) involving two significantly changed annotated genes (Table 3) (Figure 7).

4. Discussion

The relative spleen weight describes the relative size of the spleen to the body size and is an indicator of immune activation [23]. In our study, Nile tilapia in brackish water showed significantly higher RSW than in freshwater. Meanwhile, more red blood cells were detected in the spleen of fish in brackish water, indicating that long-term salinity stress can induce splenomegaly, which reflects active erythrocyte production in the spleen. Similarly, the infection of Nile tilapia by *Vibrio vulnificus* in a low-salinity environment could induce splenomegaly along with congestion and infiltration of epithelioid cells [24]. At a high water temperature (33 °C), splenomegaly is more frequently seen in gilthead seabream *Sparus aurata* than at a low temperature (25 °C) [25]. Environmental alterations can lead to an increase in the spleen volume, permitting the organism to maintain its organic functions in a balance although numerous chronic diseases may also occur [26]. Therefore, in our study, splenomegaly is an apparent phenotype of tilapia to adapt long-term hyperosmotic

stress. Blood is a patho-physiological indicator of body health, and the hematological parameters give an indication of any abnormality under environmental stress [27, 28]. In our study, MCV and MCH were higher in the blood of fish in brackish water than in freshwater. Spleen is a vital organ for hematopoiesis and immunity, and red cell numbers increased significantly in spleen of tilapia under hyperosmotic stress by HE staining in our study. Therefore, this change in hematology may be associated with the adjustment of hematopoiesis and immune function in the spleen during chronic salinity stress [29].

Coagulation cascades were significantly changed by ambient salinity in the transcriptomics analysis of the spleen given the important role of the spleen in hemostasis [30]. The gene expression levels of coagulation factor 1Xb, coagulation factors II and V, serpin peptidase inhibitor clade C/D, fibrinogen alpha/beta/gamma chain, protein Z/C, plasminogen and plasminogen activator were significantly down-regulated in the spleen of Nile tilapia in brackish water compared with those in freshwater. The decreased expression of coagulation-related genes in the spleen of brackish water means the attenuation of blood clotting function or dissolution of blood clots [31]. Platelets play a major role in the regulation of hemostasis, and their activation results in platelet aggregation at the injury site [32]. Furthermore, our study shows that the low coagulation function in the spleen reduced the contents of PLT, MPV, PDW and PCT. The overall down-regulation of platelet indicators suggests that blood coagulation is significantly impacted by chronic salinity stress in Nile tilapia. Similarly, chronic hyperosmotic stress can also deplete the number of thrombocytes in striped catfish [33]. In our study, although the growth performance and survival of Nile tilapia were not significantly influenced by ambient salinity stress, the immune organs and hematological parameters both showed significant changes at 16‰.

Cytologically, the spleen is a lymphatic gland and is very rich in melano-macrophages to aid phagocytosis in the immune response. Numerous melano-macrophages can form clusters and become melano-macrophage centers [34]. In our study, the spleen of Nile tilapia in brackish water assembled a large volume of melano-macrophage centers with more deposition of pigments, including lipofuscin, melanin and hemosiderin. In addition to salinity, starvation can also induce the deposition of melano-macrophages in the organs of different fish such as dogfish *Carassius auratus*, rainbow trout *Oncorhynchus mykiss* and olive flounder *Paralichthys olivaceus* [35-37]. The function of melano-macrophages in teleosts are similar to that in human macrophages to metabolize toxic and waste substances and perform immune functions in hematopoietic tissues [38]. The increase in macrophages and pigments corresponds to the change in the pathological and physiological conditions in fish [36, 39]. The change in the number and volume of melano-macrophage centers in the spleen of fish is related to environmental stress and tissue catabolism [40]. The development of any adventitious melano-macrophage centers is also related to chronic inflammatory lesions [41]. IL-1β and cox-2 are two key mediators of the inflammatory response [42, 43]. In the present study, when Nile tilapia were stressed at 16‰, the mRNA expression of interleukin-1 beta (IL-1β) and cyclo-oxygenase 2 (COX-2) in the spleen were significantly higher than those in freshwater. This indicates that high salinity can trigger the immune response and inflammatory reaction in the spleen of Nile tilapia. Similarly, the histologic section also showed obvious melanin deposition in the head kidney.

Macrophages can be enlarged by active phagocytosis of heterogeneous materials, such as cell debris, pigments, and neutral mucopolysaccharide [37, 44]. TEM analysis of the spleen and head kidney showed that 16‰ salinity induced larger and fuzzy

ultrastructures of macrophage that include large and irregular lysosomes and deposition granules as a result of active phagocytosis. Similarly, a study on brown trout *Salmo trutta* transferred from freshwater to seawater shows an increase in the phagocytic activity of head kidney leukocytes [45]. Neutrophils play a crucial role in the generation of immune responses in fish due to the activation of a non-specific immunity mechanism [46]. In our study, the number of neutrophils in the head kidney of Nile tilapia in brackish water was significantly increased compared with that in freshwater. At the infection sites, endothelial cells capture bypassing neutrophils through the endothelial cell lining where the neutrophils are activated for subsequent interaction with microbes [47]. The increase in neutrophils in the head kidney in brackish water indicates the enhanced ability against pathogen infection. Our results suggest that long-term hypersaline stress can induce some damage and inflammation in the spleen and head kidney, although a significant impact on growth performance of Nile tilapia was not detected.

Transcriptome analysis in the spleen showed that chronic salinity stress induced five significantly changed pathways of the immune response. The complement and coagulation system shows several interconnections due to the common role in innate defense against external threats [30]. In our study, the mRNA expression levels of complement 3, 5, 8 and 9 were all significantly down-regulated in brackish water compared with those in freshwater. A high level of complement activation is usually produced during blood clotting [48]. Complement activity can also modulate the aggregative properties of platelets [49]. The decrease in complement, coagulation and platelets indicates a decline in the immunity of Nile tilapia in brackish water. In another study, acute exposure of tilapia to saline water resulted in the increased expression of complement C3 protein at 1 h and 24 h in serum, but it was gradually

359 decreased at 4 h and 8 h [50]. Similarly, after acclimation at low salinity for 100 days,
360 the alternative complement activity in the gilthead seabream was significantly lower
361 than the control group in sea water [51]. The effect on the humoral innate immune
362 parameters by ambient salinity differed over acclimation time. In addition to the
363 complement pathway, antigen processing and presentation pathway, as well as natural
364 killer cell-mediated cytotoxicity pathway, the intestinal immune network for IgA
365 production and hematopoietic cell lineage were also significantly changed. Compared
366 with the spleen, the immune response in the head kidney of gene expression was less
367 conspicuous. Only one immune pathway (i.e., antigen processing and presentation)
368 was significantly changed by salinity stress. Cathepsin L is a typical cysteine
369 proteinase found in lysosomes and is considered to play an important role in
370 cathepsins B and H in the degradation of both endogenous and exogenous proteins
371 taken up by lysosomes [52, 53]. The cathepsin L pathway in the spleen and head
372 kidney was significantly up-regulated in brackish water compared with that in
373 freshwater, possibly due to the enhancement of phagocytosis in these two tissues.
374 Briefly, all immunity related parameters of tilapia form the aspects of gene expression,
375 histology and hematology have been altered under long-term hyperosmotic stress. In
376 this study, growth performance and survival of tilapia under stress of salinity 16 were
377 not significant influenced by salinity stress, reflecting the importance of immune
378 response in maintaining normal growth and survival for dealing with environmental
379 stress for teleost.

380 **Conclusions**

381 Long-term hypersaline stress can induce splenomegaly, reduce coagulation function,
382 enhance phagocytic activity and down-regulate the complement pathway in the
383 immune organs of Nile tilapia, although no significant influence on growth

384 performance and survival was detected. Long-term or intensive stress by ambient
385 salinity can induce a more serious negative impact on immunity and may ultimately
386 influence growth performance and survival. Therefore, the primary measure to cope
387 with various ambient stress is to enhance the immune system function possibly by
388 proper environmental management.

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Figure legends

Figure 1

Effects of two ambient salinities (0 and 16‰) on hemocyte hematology in Nile tilapia *Oreochromis niloticus*. The values represent means ± SEM. The single asterisk (*) indicates a significant difference ($P < 0.05$), and "**" indicates an extremely significant difference ($P < 0.01$). Zero represents the salinity of 0‰ and 16 represent the salinity of 16‰. The values of WBC, RBC, HGB, HCT, MCV, MCH, MCHC and RDW are the means of 9 replicates. WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

Figure 2

Effects of two ambient salinities (0 and 16‰) on platelet hematology in Nile tilapia *Oreochromis niloticus*. The values represent means ± SEM. The single asterisk (*) indicates a significant difference ($P < 0.05$), and "**" represent an extremely significant difference ($P < 0.01$). Zero represents a salinity of 0‰ and 16 represents the salinity of 16‰. Values of PLT, MPV and PDW are the means of 9 replicates. PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit.

Figure 3

Transverse section of the spleen in Nile tilapia *Oreochromis niloticus* at 0‰ (A and B) and 16‰ (C and D) with 200- and 50-μm scale bars. a, melano-macrophage centers. b, pigments of melanins.

Figure 4

549 Transverse section of the head kidney in Nile tilapia *Oreochromis niloticus* at 0‰ (A
550 and B) and 16‰ (C and D) with 200- and 50-μm scale bars. a, pigments of melanins.

551 **Figure 5**

552 Ultrastructural organization in the spleen of Nile tilapia *Oreochromis niloticus* at 0‰
553 (A and B) and 16‰ (C and D) with transmission electron microscopy
554 magnification × 3.0 k (A and C) and × 1.0 k (B and D). a, melano-macrophage; b,
555 lymphocyte; c, erythrocyte.

556 **Figure 6**

557 Ultrastructural organization in the head kidney of Nile tilapia *Oreochromis niloticus*
558 under 0‰ (A, B and C) and 16‰ (D, E and F) with transmission electron
559 microscopy magnification × 3.0 k (A, B, D and E) and × 1.0 k (C and F). a,
560 melano-macrophage; b, lymphocyte; c, neutrophils.

561 **Figure 7**

562 Heat map of significantly changed genes involved in significantly changed immune
563 system pathways in the spleen and head kidney under chronic salinity stress.

564 **Figure 8**

565 Pathway of complement and coagulation cascades. The green frames represent the
566 down-regulated genes. The frames in both red and green indicated that these genes
567 had more than one unigene; some of them were up-regulated, while others were
568 down-regulated during ambient salinity stress in the spleen of Nile tilapia.

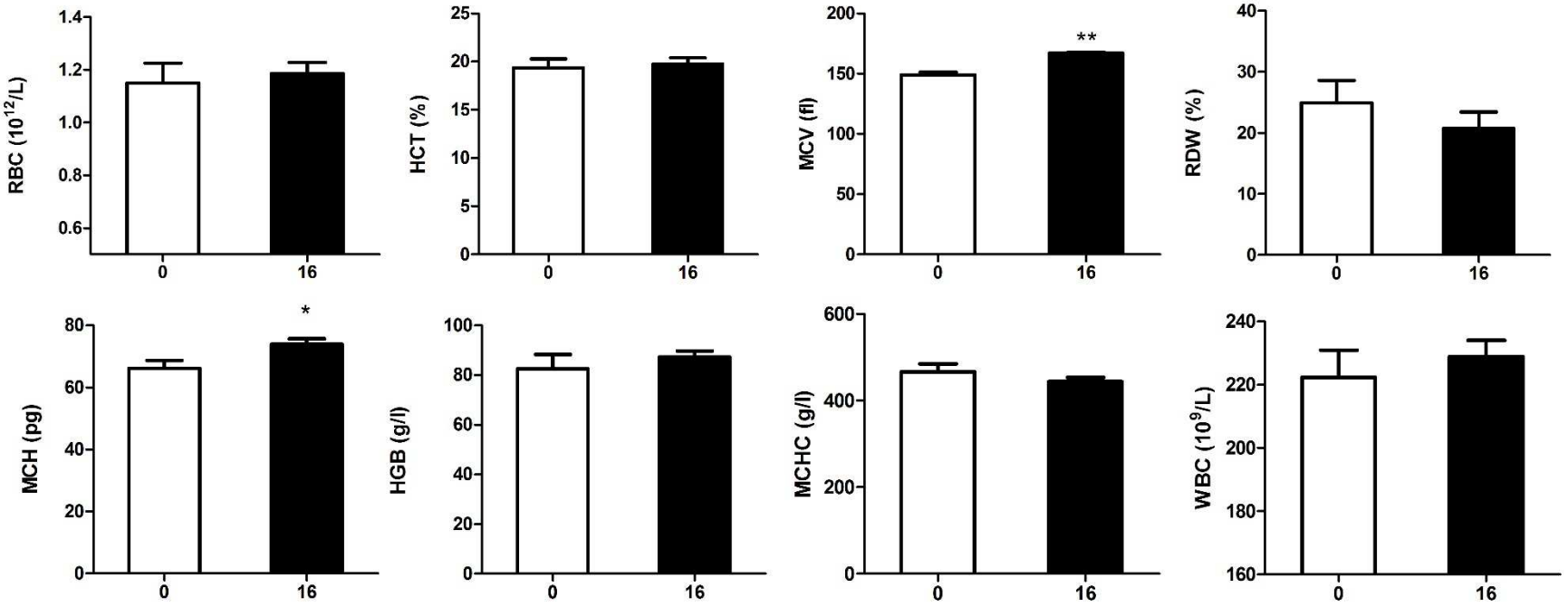
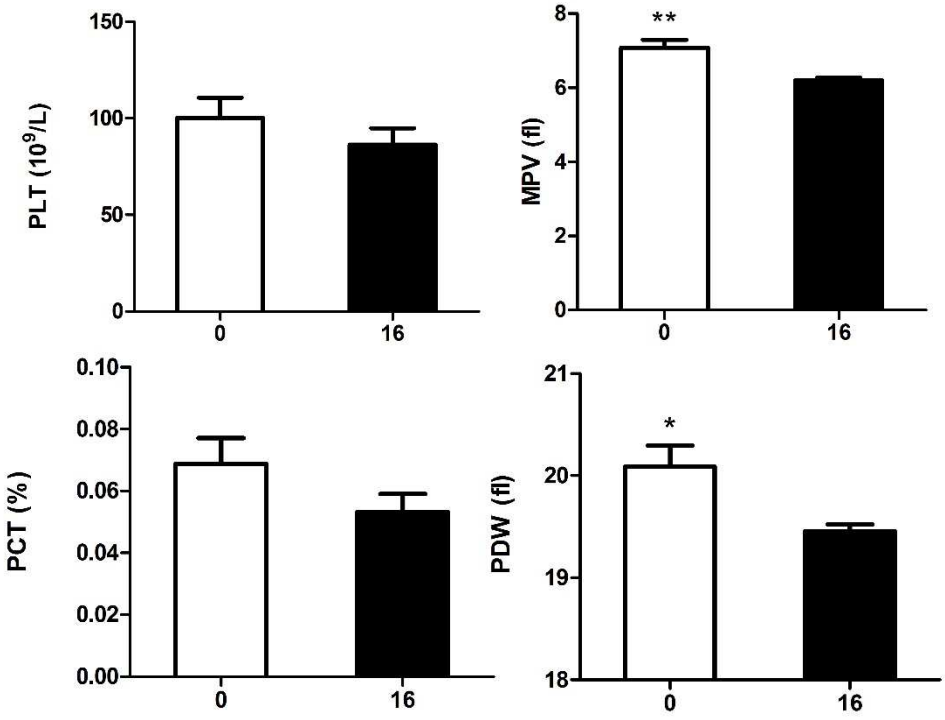


Figure 1



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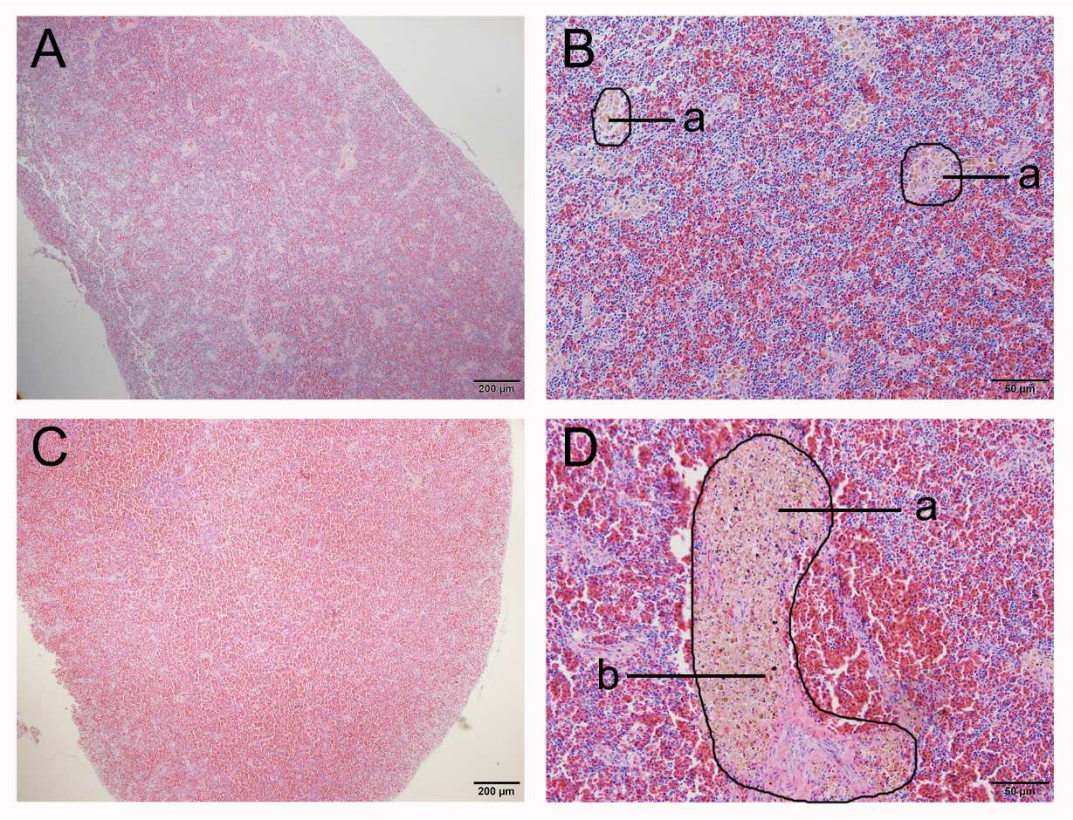

Figure 3

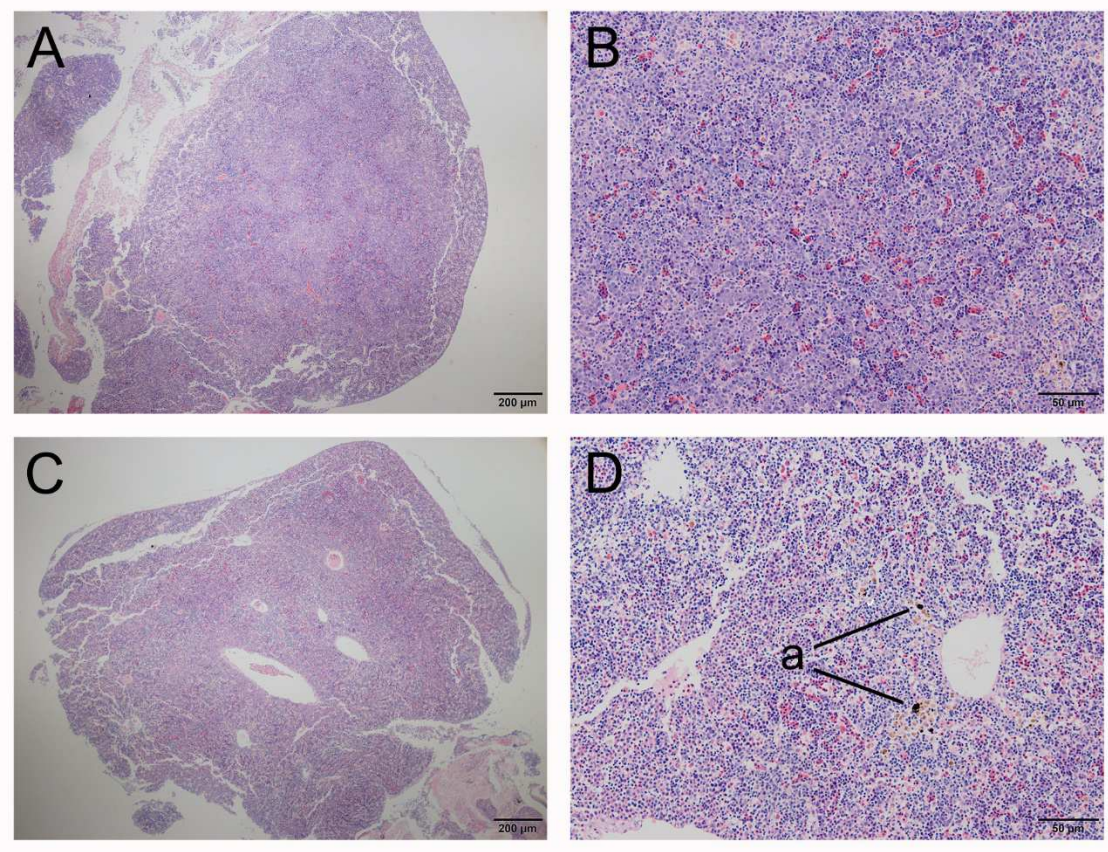

Figure 4

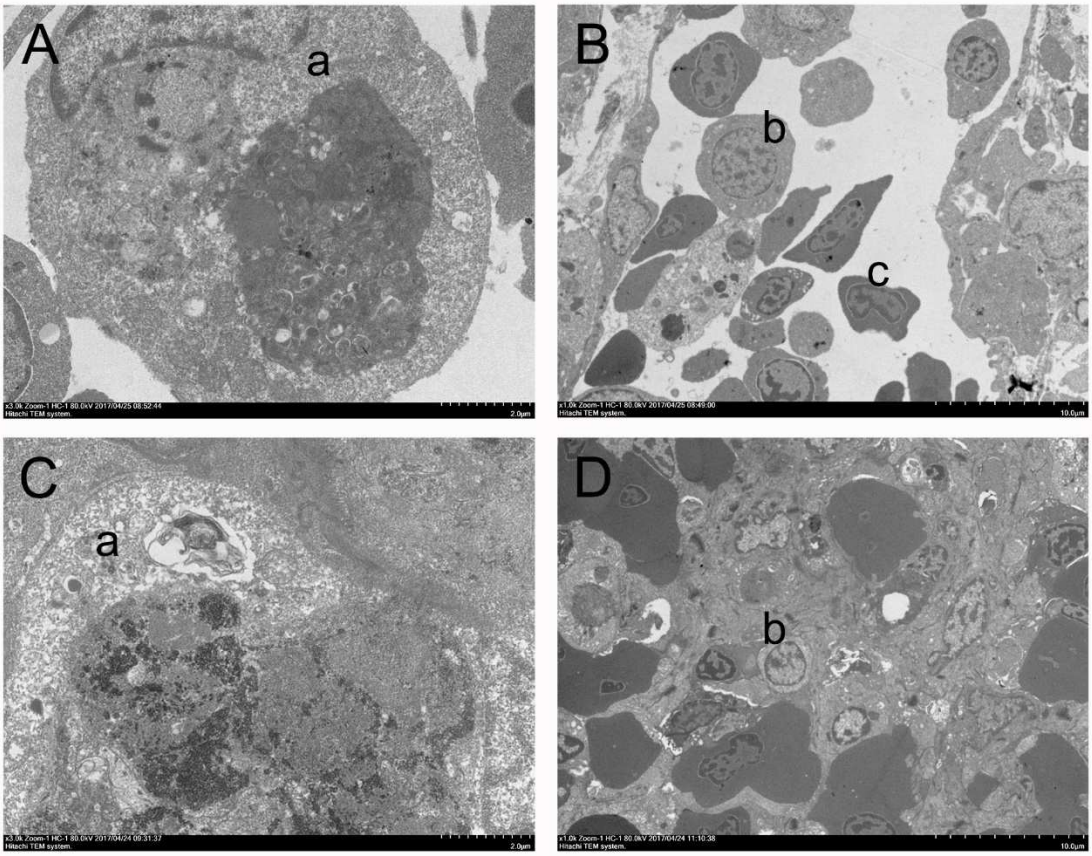

Figure 5

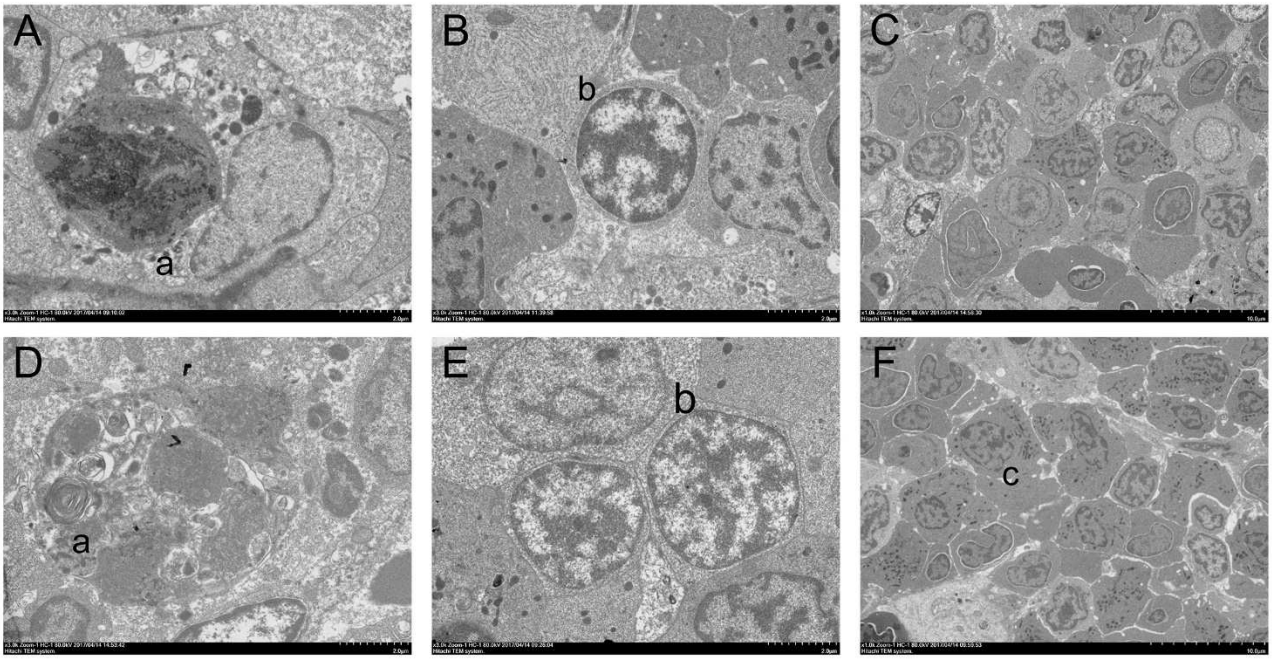

Figure 6

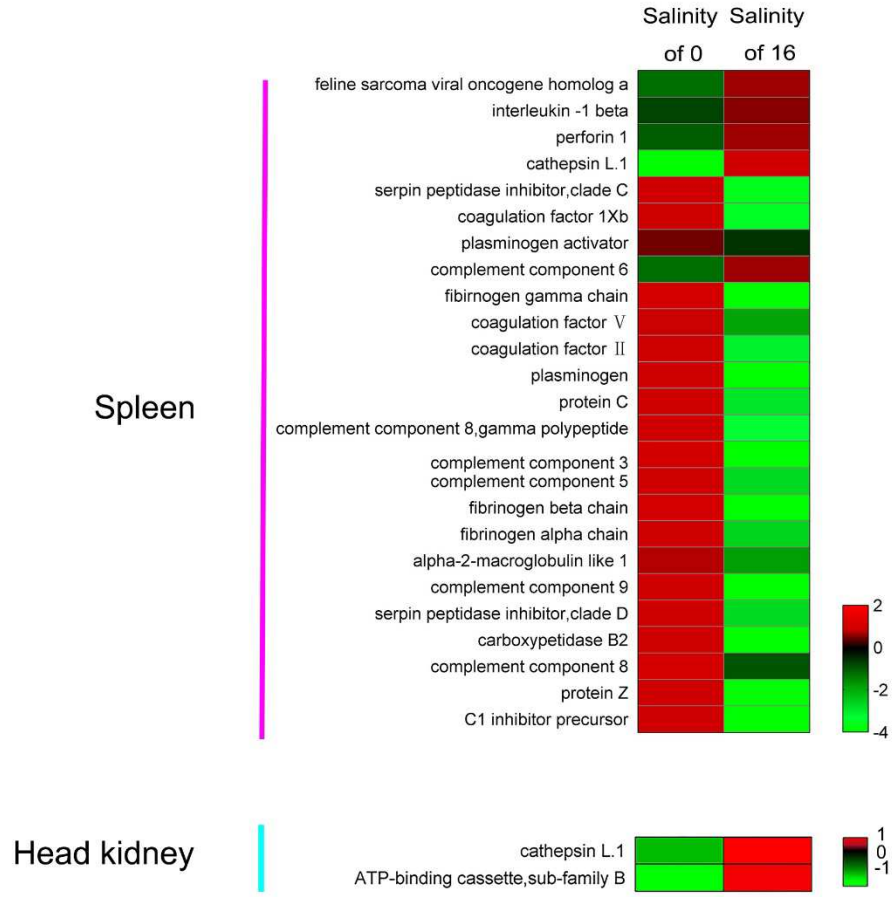

Figure 7

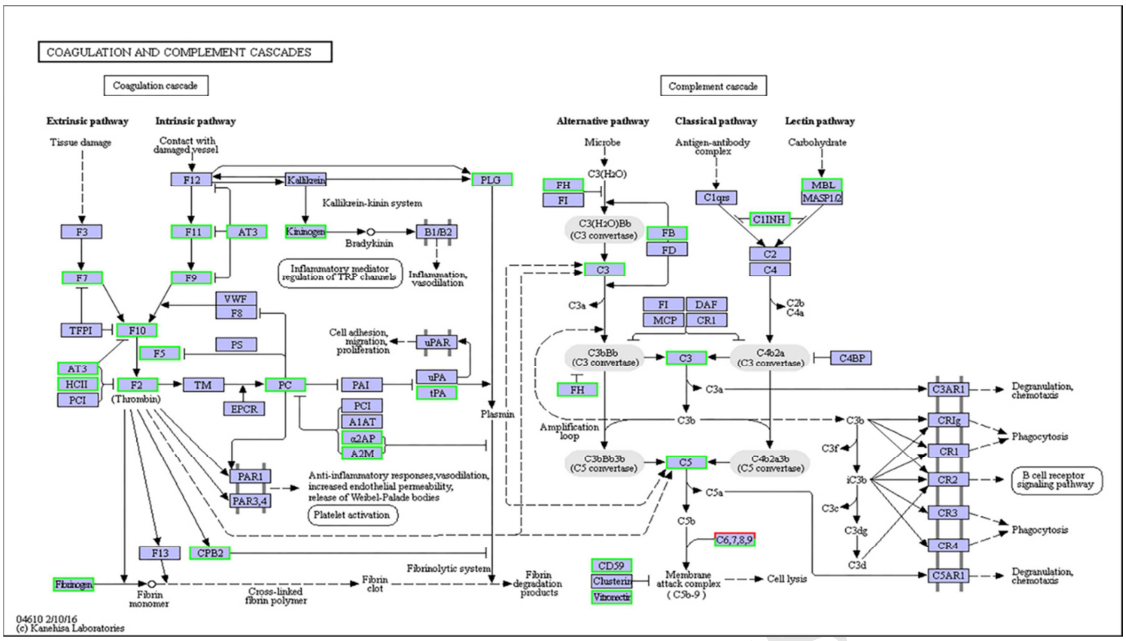

Figure 8

Table 1
Growth performance, survival and relative spleen weight of Nile tilapia in two environmental salinities for 49 days

Parameters	Salinity	
	0	16
Initial weight	6.41 ± 0.09	6.41 ± 0.09
Final weight	31.05 ± 0.41	29.04 ± 0.63
Weight gain (%)	383.21 ± 6.89	353.69 ± 10.26
Specific growth rate (%)	3.28 ± 0.03	3.15 ± 0.05
Relative spleen weight (%)	0.24 ± 0.02	0.31 ± 0.02*
Survival (%)	100	100

"*" indicates significant difference ($P < 0.05$).
The values of the weight gain and specific growth rate are the means of 3 replicates.
The values of the relative spleen weight are the means of 15 replicates.

Table 2
Significantly changed pathways ($P < 0.05$) in the spleen by transcriptomics analysis of Nile tilapia under chronic salinity stress

Name of pathway	Category	Input number	Background number	P-value
Complement and coagulation cascades	Immune system	36	105	0.001
Antigen processing and presentation		24	163	0.001
Natural killer cell-mediated cytotoxicity		15	185	0.003
Intestinal immune network for IgA production		9	85	0.004
Hematopoietic cell lineage		7	76	0.022
Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism	4	12	0.002
Tyrosine metabolism		6	40	0.004
Phenylalanine metabolism		4	20	0.008
Cysteine and methionine metabolism		6	58	0.025
Tryptophan metabolism		5	54	0.049
Methane metabolism	Energy metabolism	5	40	0.017
Carbon fixation in photosynthetic organisms		4	33	0.035
Phagosome	Transport and catabolism	31	285	0.001
Cell adhesion molecules (CAMs)	Signaling molecules and interaction	28	324	0.001
Glycolysis/Gluconeogenesis	Carbohydrate metabolism	8	87	0.015
Fat digestion and absorption	Digestive system	6	58	0.021
Naphthalene degradation	Xenobiotics biodegradation and metabolism	2	5	0.021
Isoquinoline alkaloid biosynthesis	Biosynthesis of other secondary metabolites	3	19	0.037
Drug metabolism-cytochrome P450	Xenobiotics biodegradation and metabolism	5	50	0.038
Degradation of aromatic compounds	Global and overview maps	2	8	0.043

Table 3
Significantly changed pathways ($P < 0.05$) in the head kidney by transcriptomics analysis of Nile tilapia under chronic salinity stress

Name of pathways	Category	Input number	Background number	P-value
Antigen processing and presentation	Immune system	11	163	0.002
Glycolysis/Gluconeogenesis	Carbohydrate metabolism	9	87	0.001
Pyruvate metabolism		4	53	0.035
Pentose phosphate pathway		3	34	0.046
Cardiac muscle contraction	Circulatory system	17	118	0.001
Adrenergic signaling in cardiomyocytes		18	247	0.001
Steroid hormone biosynthesis	Lipid metabolism	8	56	0.001
Cell adhesion molecules (CAMs)	Signaling molecules and interaction	17	324	0.001
Nicotinate and nicotinamide metabolism	Metabolism of cofactors and vitamins	4	38	0.013
Protein digestion and absorption	Digestive system	9	175	0.019
Ovarian steroidogenesis	Endocrine system	5	73	0.027
Biosynthesis of amino acids	Global and overview maps	6	100	0.028
Phagosome	Transport and catabolism	12	285	0.029

1. Long-term hyperosmotic stress can induce splenomegaly and reduce coagulation function in Nile tilapia *Oreochromis niloticus*.
2. Long-term hyperosmotic stress can enhance the deposition of pigment and the ability of phagocytic both in the spleen and the head kidney.
3. Complement pathway showed significant down-regulation in the spleen under long-term hyperosmotic stress.
4. The spleen is more sensitive organ for immune responses to chronic hyperosmotic stress than the head kidney.