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RESEARCH

# Chronic heat stress attenuates innate immunity and increases susceptibility to *Aeromonas hydrophila* in Siberian sturgeon (*Acipenser baerii*)

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## Abstract

Global warming elevates water temperatures, subjecting sturgeons, a cool-water fish species, to prolonged heat stress, particularly during summer months. This study investigated the effects of chronic heat stress on innate immune function and disease resistance in Siberian sturgeon (*Acipenser baerii*). Fish were acclimated stepwise from 21 to 26 °C and maintained at 26 °C for 21 days, followed by an *Aeromonas hydrophila* (abbreviated *A. hydrophila*) challenge. Fish maintained at 21 °C were used as the control group. Compared to the 21 °C controls,sturgeons exposed to 26 °C exhibited significantly reduced bacteridal activity, alternative complement hemolytic activity ($ACH_{50}$), and myeloperoxidase (MPO) activity during heat exposure; in addition, following *A.hydeophila* challenge, the 26 °C group also showed significantly lower levels of ACH50, MPO, reactive oxygen species (ROS) production, and phagocyytic activity in peripheral blood leukocytes (PBLs). Serum immunoglobulin (IgM) and lysozyme levels fluctuated temporally but decreased markedly after bacterial infection in the 26 °C group. In the post-*A. hydrophila* challenge, the 26 °C group exhibited a significantly lower survival rate (25.93%) than the 21 °C group (62.96%). Heat stressed sturgeons also displayed strong upregulation of pro-inflammatory cytokines including interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), and interleukin-8 (*IL-8*) in the spleen and head kidney after infection, while the expression of major histocompatibility complex class II β (*MHCIIβ*) was upregulated in the head kidney but downregulated in the liver. These results showed that chronic heat stress attenuates innate immunity in *Acipenser baerii* and exacerbates inflammatory dysregulation upon infection, ultimately weakening disease resistance. A water temperature of 25–26 °C is commonly adopted in sturgeon aquaculture based on satisfactory short-time survival and growth performance. Nevertheless, our study suggests that under the experimental conditions, maintaining water temperatures below 26 °C may be advantageous for Siberian sturgeon. At 26 °C, innate immunity was significantly affected compared to the 21 °C control.

**Keywords** *Acipenser baerii* · Chronic heat stress · Innate immune indices · Leukocyte phagocytosis · Respiratory burst activity · Immune gene expression

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## Introduction

Sturgeons, representing the ancient group of chondrostean fishes, are among the oldest extant primitive fish species, often referred to as “living fossils” (Onofri et al. 2024). Owing to their premium flesh and lucrative caviar, they have become a significant aquaculture species in China, which now accounts for over 85% of world sturgeon production and is the leading global exporter of caviar (Chen et al. 2025).

As typical cool-water fish species, sturgeons thrive between 18 and 24 °C, with water temperatures exceeding 28 °C markedly reducing their feed intake and growth (Mims et al. 2002). In recent years, intensified summer heat waves under global warming have increased water temperatures, exposing sturgeons to chronic thermal stress. Such conditions frequently trigger disease outbreaks and mass mortality, imposing substantial economic losses.

As poikilotherms, sturgeons depend on physiological and behavioral mechanisms to adapt to fluctuating environmental temperatures. Research on fish has documented that both chronic and acute heat stress compromises immune homeostasis, suppresses innate defenses, and increases susceptibility to pathogens (Nardocci et al. 2014). Notably, aquatic animals, especially ancient species like sturgeons, rely critically on their innate immune system, in which key components, such as lysozyme, antimicrobial peptides, and complement exhibit distinct regulatory mechanisms (Whyte et al. 2007).

However, current research on the immunological impacts of heat stress in sturgeons remains limited and highly species-dependent. For instance, chronic heat stress at 22–30 °C in Dabry’s sturgeon (*Acipenser dabryanus*) activates hepatic antioxidant systems, increases apoptosis, and downregulates lysozyme activity (Liu et al. 2025). In contrast, Siberian sturgeon (*Acipenser baerii*) under 20–28 °C heat stress exhibit splenic histopathological changes and upregulated immune factor expression (Tan et al. 2024), while Russian sturgeon at 30 °C show contradictory responses, with reduced complement activity but elevated lysozyme (Lyz) levels (Aversa-Marnai et al. 2025). These species-specific discrepancies suggest that immune responses to thermal stress cannot be generalized across sturgeons, underscoring the need for targeted investigations on individual commercially important species. Moreover, it is generally accepted among farmers that sturgeons grow faster at 25–26 °C. However, it remains unknown whether this optimal temperature for growth might inadvertently compromise immune competence, potentially increasing disease susceptibility. Therefore, this study investigated *Acipenser baerii*, a major farmed species in China, to elucidate the mechanisms of innate immune responses to chronic heat stress (21–26 °C) and subsequent bacterial challenge with *A. hydrophila* at cellular and molecular levels. The findings will provide theoretical and technical guidance for disease prevention during high-temperature periods, enhance understanding of environmental adaptive immunity in chondrostean fish, and support efforts to improve sturgeon resilience and aquaculture productivity.

## Materials and methods

### Experimental fish and acclimation

Healthy juvenile Siberian sturgeons (average weight, 280 ± 31 g) were obtained from the Shidu Sturgeon Breeding Base in Beijing and acclimatized in a recirculating aquaculture system at 21 ± 1 °C for two weeks prior to the experiment. During all experiment stages, fish were fed the commercial sturgeon pellets (2 mm) twice daily. The chemical composition of the basal diet fed to sturgeon was formulated to meet the following specifications (guaranteed analysis): crude protein ≥ 41%, crude lipid ≥ 6%, crude ash ≤ 16%, crude fiber ≤ 4%, and moisture ≤ 12%. Additionally, the diet contained ≥ 0.8% total phosphorus and ≥ 2.0% lysine. Water quality parameters (ammonia, nitrite, pH, and dissolved oxygen (DO)) were monitored and maintained within optimal ranges for sturgeons (Table 1). All animal handling and manipulation procedures were conducted in accordance with the guidelines for the Care and Use of Experimental Animals (GB/T 35892–2018) established by the national quality management department of China, and approved by the institutional ethical review committee of Beijing Academy of Agriculture and Forestry Sciences.

## Experimental design and temperature regimes

The schematic figure of the experimental design was shown in Fig. 1. The experiment consisted of two temperature treatments. Each temperature group was reared in a separate water circulation system, housed in six independent experimental tanks, each stocked with 16 fish. In the 21 °C group, the fish were maintained at 21 °C throughout the experimental period. In the 26 °C group, the temperature was increased by 1 °C, from 21 °C per day until reaching 26 °C and then maintained at 26 °C for 21 days. In detail, the acclimation to 26 °C was achieved using a controlled, stepwise warming protocol. Submersible aquarium heaters were placed in the main sump tank of the recirculating system and set to increase the water temperature by 1 °C per day, starting from the baseline of 21 °C. The actual temperature in each tank was cross-checked daily with an independent calibrated thermometer. On days 1, 7, 14, and 21 of the temperature treatment, six random fish were sampled for serum immune parameters analysis. On day 21, the *A. hydrophila* challenge was performed immediately following sample collection.

## Bacterial challenge and sampling scheme

Sturgeon bacterial pathogen strain *A. hydrophila* NX830 (obtained from National Aquatic Pathogen Collection Center, China) was cultured in TSA medium and resuspended in PBS to a density of $1 \times 10^7$ CFU/mL.

Within each temperature group, fish were divided into challenge and control subgroups. The bacterial challenged fish were intraperitoneally injected with 200 μL of *A. hydrophila* suspension (at a concentration corresponding to 1/2 of the 96-h median lethal dose (LD50), which was derived from preliminary test), and the control fish received an equal volume of PBS. In detail, in the challenged subgroup, three tanks (9 fish per tank) were used for recording cumulative mortality over 7 days post-infection; and the three additional tanks (6 fish per tank) were designated for tissue sampling at 24-h post-*A. hydrophila* challenge. In the control subgroup, three tanks (6 fish per tank) served as PBS-infected controls and were sampled at 24-h post-infection (hpi).

**Table 1** Water quality parameters across temperature treatments (mean ± SD)

| Temperature treatment | DO (mg/L) (min–max) | $NO_2^-$-N (mg/L) (min–max) | $NH_3$-N (mg/L) (min–max) | Measurement frequency |
|---|---|---|---|---|
| 21 °C | 7.85 ± 0.52 (5.8–7.9) | 0.031 ± 0.018 (0.008–0.058) | 0.077 ± 0.042 (0.025–0.128) | Every 2 days |
| 26 °C | 7.42 ± 0.58 (6.2–8.5) | 0.035 ± 0.012 (0.006–0.068) | 0.085 ± 0.032 (0.018–0.155) | Every 2 days |

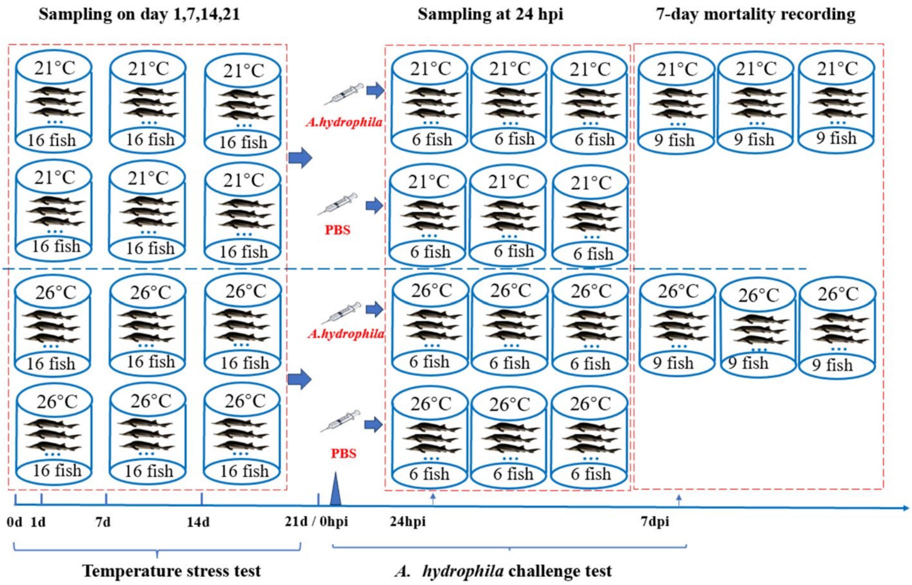

**Fig. 1** Schematic figure of the experimental design

## Sample collection

On day 1, 7, 14, and 21 of temperature stress and 24 hpi, blood samples were collected from the caudal vein of six sturgeons in each temperature group for innate immune-related parameters analysis. For serum preparation, blood samples were left at room temperature for 2 h, then refrigerated at 4 °C for 12 h, and then centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant (serum) was carefully collected and stored at − 80 °C.

On day 21 (0 hpi) and 24 hpi, blood samples were collected from six random sturgeons per temperature group for the detection of PBLs ROS content and phagocytic activity. Whole blood was mixed with an equal volume of heparin sodium anticoagulant and RPMI-1640 medium (Solarbio, China), gently layered over 4 mL of 65% Percoll solution, and centrifuged at 840 × g for 30 min at 4 °C. The leukocyte-rich interphase was collected, resuspended in PBS, and centrifuged again at 840 × g for 5 min at 4 °C. After three washing steps, the purified leukocytes were collected for further assays. Each experimental group included three biological replicates, with each replicate composed of pooled PBLs from two individual fish to meet the cell quantity required for the experiment.

In addition, the spleen, head kidney, and liver tissues were harvested at 0 hpi and 24 hpi, quick-frozen in liquid nitrogen, and stored at − 80 °C for subsequent RNA extraction and quantitative real-time polymerase chain reaction (qPCR) analysis.

## Innate immune indices

### Serum bactericidal activity

Serum bactericidal activity was quantified via a plate-count assay. *A. hydrophila* was cultured overnight at 28 °C, and the resulting colonies were suspended in PBS. The bacterial suspension was adjusted to an optical density (OD) of 1.0 using a microplate reader and subsequently diluted 1:1000.

Next, 20 μL of serum was mixed with 100 μL of the diluted bacterial suspension and incubated at 28 °C for 1 h. After incubation, 50 μL of the mixture was plated in duplicate onto Luria–Bertani (LB) agar plates and incubated at 28 °C for 20 h. Colonies were then counted, with 0.85% saline serving as the negative control. The bactericidal activity was calculated using the following formula:

$$Bactericidal\ rate\ (\%) = (1 - [colony\ count\ in\ test\ group]/[colony\ count\ in\ control\ group]) \times 100$$

### Determination of serum lysozyme content

Lysozyme content was measured using a lysozyme assay kit (A050-1–1, Nanjing Jiancheng Bioengineering Institute, China) with a blank control method (three tubes: blank, standard, test). Briefly, 0.2 mL of distilled water, lysozyme standard solution, and sample were added to 2 mL of wall-lysing microbe suspension, respectively. The mixtures were incubated in a 37 °C water bath for 15 min, then immediately placed in an ice-water bath below (<0 °C) 3 min. Transmittance was measured at 530 nm with distilled water as the blank, and the transmittance of each tube after incubation was recorded as T15 (the transmittance value after 15 min of incubation at 37 °C). Lysozyme content was calculated as:

Lysozyme content = (transmittance of test tube $UT_{15}$ − transmittance of blank tube $OT_{15}$)/(transmittance of standard tube $ST_{15}$ − transmittance of blank tube $OT_{15}$) × standard tube concentration (10 μg/mL, i.e., 200 U/mL) × sample dilution factor.

### Serum complement alternative pathway hemolytic activity (ACH50)

$ACH_{50}$ was conducted according to (Yano 1992). Rabbit erythrocytes were used as target cells, and EGTA-$Mg^{2+}$–gelatin veronal buffer (GVB) served as a $Ca^{2+}$ chelator to inhibit the classical complement pathway. After the incubation of fish serum and EGTA-$Mg^{2+}$–GVB, the OD of the supernatant was then measured at 414 nm, with the tube (containing no serum) serving as the blank control.

Hemolytic activity ($Y$) was determined as the ratio of the sample's OD value to that of the 100% hemolysis control. A graph was plotted with $\log[Y/(1-Y)]$ on the x-axis and the log of the corresponding serum volume ($x$, mL) on the $y$-axis. From this graph, the serum volume ($K$) required to achieve 50% hemolysis was determined. $ACH_{50}$ activity was calculated as:

$$ACH_{50} = (1/K) \times serum\ dilution\ factor\ (units/mL)$$

All measurements were performed in triplicate, and the results were averaged.

### Measurement of MPO, superoxide dismutase (SOD), and IgM levels

Serum MPO, SOD, and IgM levels were measured using commercial detection kits (H508-1–1, A001-1–1, H109-1–1; Nanjing Jiancheng Bioengineering Institute, China) following the manufacturers' protocols.

### Determination of intracellular ROS levels in PBLs

Intracellular ROS levels were assessed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorescent probe, following the method of Luo et al. (2015) with minor modifications. Isolated PBLs were adjusted to a concentration of $1 \times 10^6$ to $1 \times 10^7$ cells/mL. A 500 μL aliquot of the cell suspension was incubated with 5 μL DCFH-DA stock solution (1 mM, final working concentration 10 μM, D6883, Sigma-Aldrich, Germany) at room temperature in the dark for 30 min. ROS-associated fluorescence was then measured by flow cytometry. Data analysis was performed using FlowJo 10.8.1. The mean fluorescence intensity (MFI) of each sample was recorded as an indicator of intracellular ROS content. The ROS response to bacterial challenge was calculated as the absolute change from pre-challenge baseline: $\Delta ROS = MFI_{24hpi} - MFI_{0hpi}$. Data are presented as the mean of three biological replicates per group.

### Phagocytic activity of PBLs

Phagocytic activity was determined following the method of Wang Xiaowen et al. (2017). *A. hydrophila* was cultured on LB medium for 16 h, washed with PBS, and centrifuged at 10,000 × g for 5 min before resuspension in RPMI Medium 1640 (31,800, Solarbio, China). The bacterial suspension was inactivated by incubation at 65 °C for 1 h, then labeled with fluorescein isothiocyanate (FITC) in a DMSO solution (final FITC concentration, 100 μg/mL) at 30 °C for 1 h. After labeling, the bacteria were washed five times with PBS and resuspended in RPMI medium 1640 to a final concentration of $1 \times 10^8$/mL.

Next, 100 μL of the FITC-labeled *A. hydrophila* suspension was mixed with 1 mL of leukocyte suspension ($1 \times 10^6$/mL) and incubated at 28 °C for 1 h. Leukocytes were then centrifuged at 500 × g for 10 min, washed three times with PBS, and incubated with 1 mL of 0.125% trypan blue for 5 min to quench extracellular fluorescence, followed by 2–3 additional PBS washes. Cells were resuspended in 0.9% normal saline for flow cytometry analysis (Partec, Germany). Leukocytes from Siberian sturgeon not exposed to FITC-labeled bacteria served as negative controls. Data were analyzed by Flowjo 10.8.1: leukocytes were gated on forward scatter (FSC) vs. side scatter (SSC) plots, and FITC-positive cells (phagocytosed bacteria) were quantified using the FL1 channel. More than 10,000 leukocytes were analyzed per sample, and phagocytic activity was expressed as the percentage of PBLs phagocytosing A. hydrophila. Each experimental group contained three biological replicates, with results presented as the mean value.

### Expression levels of immune related genes

Total RNA was extracted from samples collected 24 hpi using RNAiso Plus (9108, Takara, Japan) and reverse-transcribed into cDNA (RR092A, Takara, Japan) for qPCR analysis. Relative mRNA levels of *IgM*, *IL-1β*, *IL-6*, *IL-8*, and *MHCIIβ* in Siberian sturgeon were quantified using SYBR Green-based qPCR assays (RR820A, Takara, Japan). Specific primers for all investigated genes were listed in Table 2. Each experimental group included three biological replicates, with each replicate composed of pooled tissues from two individual fish to reduce biological variability. For each sample, three technical replicates were performed to ensure measurement accuracy. The *18S* ribosomal RNA, a housekeeping gene, served as the internal control to normalize the template amounts (Wang et al. 2021), and relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

## Statistical analysis

All experimental data are presented as mean values accompanied by standard deviations (mean ± SD). A two-way analysis of variance (ANOVA) was conducted in GraphPad v6.01 to examine the effects of temperature (21 °C vs 26 °C) and time (day 1, 7, 14, and 21) on immunological parameters, as well as the combined effects of temperature and bacterial challenge on PBL ROS contents, phagocytosis, and immune-related gene expression in various tissues. Post hoc multiple comparisons were performed as follows: pairwise comparisons between two temperature groups were conducted at each time point using Bonferroni's correction; pairwise comparisons among the four time points (1, 7, 14, 21 days) during temperature stress were performed using Tukey's HSD test. A probability threshold of $P < 0.05$ was established as the criterion for statistical significance throughout all analyses.

# Results

## Effect of chronic heat stress on the survival rate of juvenile Siberian sturgeon

After the *A. hydrophila* challenge, the average 7-day survival rate was 62.96% in the 21 °C group and 25.93% in the 26 °C group ($P = 0.0032$) (Fig. 2).

## Serum immune parameters of juvenile Siberian sturgeon under chronic heat stress

During chronic heat stress, two-way ANOVA (Table 3) showed no significant main effects of temperature and time on serum bactericidal rate, but a significant temperature × time interaction was observed ($F$ (3, 30) = 3.069, $P = 0.0429$, $\eta^2 p = 0.2348$, Table 4). Post hoc analysis indicated that the serum bactericidal activity of sturgeon in the 26 °C group was significantly lower than that in the 21 °C group on days 7 and 14 of heat stress ($P < 0.05$, Fig. 3A). After *A. hydrophila* challenge, the bactericidal rate was extremely significantly affected by challenge status, and both temperature groups showed a significant decrease in serum bactericidal rate at 24 hpi compared with 0 hpi ($P < 0.05$). No significant main effect of temperature or temperature × challenge interaction was found, and there was no significant difference in serum bactericidal rate between the 26 °C and 21 °C groups observed at 24 hpi ($P > 0.05$, Fig. 4A).

For serum lysozyme activity during chronic heat stress, two-way ANOVA revealed no significant main effects of temperature and time, and no temperature × time interaction ($F$ (3, 30) = 1.655, $P = 0.198$, $\eta^2 p = 0.1420$). The mean lysozyme activity in the

**Table 2** List of primers used for qRT-PCR

| Primers | GenBank accession | Primer sequence 5′–3′ | Tm (°C) | Annealing temperature (°C) | Product size(bp) |
|---|---|---|---|---|---|
| 18SF | AY904463.1 | TGCCCTATCAACTTTCGATGG | 60.2 | 59.6 | 121 |
| 18SR |  | CTGCCTTCCTTGGATGTGGT | 59.6 |  |  |
| qAbIL-1βF | MZ467298.1 | TGATGGAAGGTCACGGGGT | 60.3 |  | 164 |
| qAbIL-1βR |  | CAGCATCATGGCTGACAGCTC | 61.4 |  |  |
| qAbIL-6F | XM_033993799.3 | CACTGTCAGCGTTGCTGGTTC | 61.4 |  | 117 |
| qAbIL-6R |  | GCCACTCGGCTAAAGATTCCC | 62.7 |  |  |
| qMHCIIβF | JQ288773.1 | CGATGCCCGAATCTGAAAG | 58.8 |  | 123 |
| qMHCIIβR |  | GCACCAAAATGCGACCTGTA | 59.4 |  |  |
| qAbIL-8F | MK140599.1 | CATCCATCCCAGGCAGATC | 57.9 |  | 129 |
| qAbIL-8R |  | TTGACCCAGCGGGCAGTT | 61.8 |  |  |
| qIgMF | KC734557.1 | AGCAAGTTGACCGTGCCTACC | 61.7 |  | 118 |
| qIgMR |  | CAGTGGAATCTGCGTTTCTGG | 60.6 |  |  |

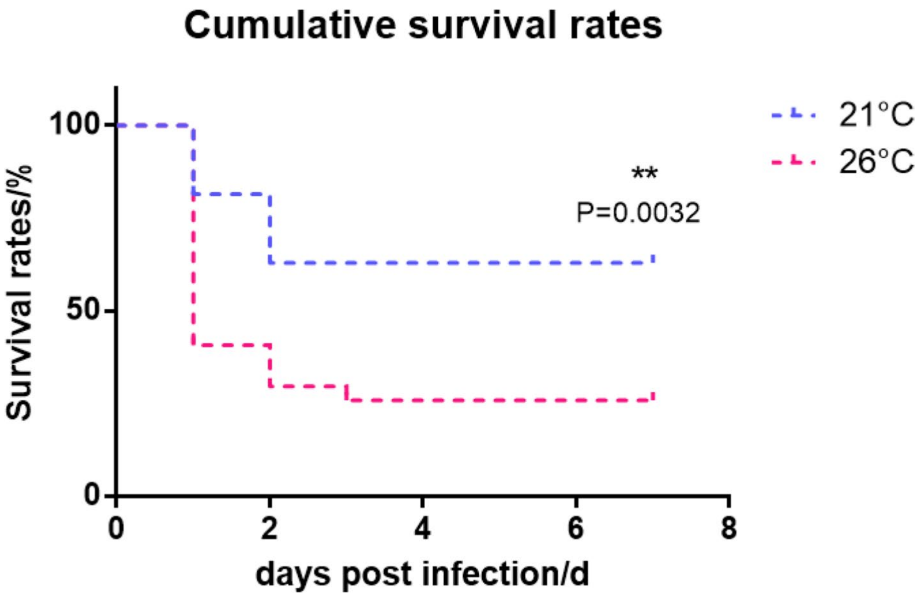

**Fig. 2** The survival rates of sturgeons in different groups after *A. hydrophila* infection. The experiment consisted of two treatment groups with three replicates each, and each tank (replicate) was stocked with nine fish. The survival curve was analyzed as time-to-event using the Kaplan–Meier method with log-rank test (GraphPad v6.01)

26 °C group was slightly higher than that in the 21 °C group at all time points except day 7, with no statistical difference ($P > 0.05$, Fig. 3B). After *A. hydrophila* challenge, a significant temperature × challenge interaction was detected ($F$ (1, 10) = 6.308, $P = 0.0308$, $\eta^2p = 0.387$), while the main effects of temperature and challenge status were not significant. Post hoc comparison showed that the serum lysozyme activity in the 26 °C group was significantly lower than that in the 21 °C group at 24 hpi ($P < 0.05$, Fig. 4B).

During chronic heat stress, $ACH_{50}$ was significantly affected by the main effect of time and temperature × time interaction, while the temperature effect was not significant. Post hoc analysis found that serum $ACH_{50}$ in the 26 °C group was significantly higher than that in the 21 °C group on day 7 of heat stress ($P < 0.05$), but declined to a level lower than the 21 °C group on day 14 (Fig. 3C). After *A. hydrophila* challenge, two-way ANOVA showed extremely significant main effects of temperature and challenge status on $ACH_{50}$, with a marginal temperature × challenge interaction. $ACH_{50}$ decreased significantly in both temperature groups after the challenge ($P < 0.05$), and the 26 °C group had a significantly lower $ACH_{50}$ level than the 21 °C group at 24 hpi ($P < 0.05$, Fig. 4C).

For serum MPO activity during chronic heat stress, the main effect of time was highly significant, the temperature effect was marginal, and no temperature × time interaction was observed ($F$ (3, 30) = 0.016, $P = 0.997$, $\eta^2p = 0.0016$). MPO activity in the 26 °C group was slightly lower than that in the 21 °C group during the entire chronic heat stress period with no statistical significance ($P > 0.05$, Fig. 3D). After *A. hydrophila* infection, significant main effects of temperature and challenge status were found, with no significant temperature × challenge interaction. Serum MPO activity decreased significantly in both temperature groups after the challenge ($P < 0.05$), and the 26 °C group showed significantly lower MPO activity than the 21 °C group both before and after the challenge ($P < 0.05$, Fig. 4D).

**Table 3** Results of two-way ANOVA examining the effects of temperature, time, and their interaction on immunological parameters during chronic heat stress

| Immunological parameter | Temperature $F$(df), $P$, $\eta^2p$ | Time $F$(df), $P$, $\eta^2p$ | Temperature × Time $F$(df), $P$, $\eta^2p$ |
|---|---|---|---|
| $ACH_{50}$ | $F$ (1, 10) = 2.471, $P$ = 0.147, 0.0175 | $F$ (3, 30) = 64.39, $P$ < 0.0001, 0.8656 | $F$ (3, 30) = 4.346, $P$ = 0.012, 0.3029 |
| SOD | $F$ (1, 10) = 6.909, $P$ = 0.025, 0.2360 | $F$ (3, 30) = 58.44, $P$ < 0.0001, 0.8539 | $F$ (3, 30) = 3.160, $P$ = 0.039, 0.2402 |
| MPO | $F$ (1, 10) = 4.951, $P$ = 0.050, 0.0784 | $F$ (3, 30) = 17.23, $P$ < 0.0001, 0.6327 | $F$ (3, 30) = 0.016, $P$ = 0.997, 0.0016 |
| Lyz | $F$ (1, 10) = 0.133, $P$ = 0.723, 0.0048 | $F$ (3, 30) = 2.228, $P$ = 0.105, 0.1822 | $F$ (3, 30) = 1.655, $P$ = 0.198, 0.1420 |
| IgM | $F$ (1, 10) = 3.359, $P$ = 0.097, 0.0937 | $F$ (3, 30) = 2.313, $P$ = 0.096, 0.1879 | $F$ (3, 30) = 3.732, $P$ = 0.022, 0.2718 |
| Serum bactericidal rate | $F$ (1, 10) = 10.32, $P$ = 0.3981, 0.2734 | $F$ (3, 30) = 0.4453, $P$ = 0.7224, 0.0426 | $F$ (3, 30) = 3.069, $P$ = 0.0429, 0.2348 |

**Table 4** Results of two-way ANOVA examining the effects of temperature, challenge, and their interaction on immunological parameters after *A. hydrophila* infection

| Immunological parameter | Temperature $F$(df), $P$, $\eta^2$p | Challenge $F$(df), $P$, $\eta^2$p | Temperature × challenge $F$(df), $P$, $\eta^2$p |
|---|---|---|---|
| ACH50 | $F$ (1, 10) = 10.89, $P$ = 0.0080, 0.764 | $F$ (1, 10) = 508.7, $P$ < 0.0001, 0.981 | $F$ (1, 10) = 4.677, $P$ = 0.0559,0.319 |
| SOD | $F$ (1, 10) = 0.491, $P$ = 0.4993, 0.049 | $F$ (1, 10) = 137.4, $P$ < 0.0001, 0.932 | $F$ (1, 10) = 0.068, $P$ = 0.8001, 0.007 |
| MPO | $F$ (1, 10) = 6.649, $P$ = 0.0275, 0.306 | $F$ (1, 10) = 63.79, $P$ < 0.0001, 0.864 | $F$ (1, 10) = 0.179, $P$ = 0.6815, 0.018 |
| Lyz | $F$ (1, 10) = 0.000, $P$ = 0.9883, 0.000 | $F$ (1, 10) = 2.479, $P$ = 0.1464, 0.199 | $F$ (1, 10) = 6.308, $P$ = 0.0308, 0.387 |
| IgM | $F$ (1, 10) = 21.80, $P$ = 0.0009, 0.693 | $F$ (1, 10) = 2.006, $P$ = 0.1870, 0.167 | $F$ (1, 10) = 8.616, $P$ = 0.0149, 0.463 |
| serum Bactericidal rate | $F$ (1, 10) = 4.9, $P$ = 0.0512, 0.537 | $F$ (1, 10) = 182.4, $P$ < 0.0001, 0.9506 | $F$ (1, 10) = 2.778, $P$ = 0.1265, 0.2174 |

Significant main effects of temperature and time, as well as a significant temperature × time interaction ($F$ (3, 30) = 3.160, $P = 0.039$, $\eta^2p = 0.2402$) for serum SOD activity were detected during chronic heat stress. SOD activity in the 26 °C group was lower than that in the 21 °C group on days 1, 7, and 14, and recovered to the level of the 21 °C group on day 21 (Fig. 3E). Following *A. hydrophila* challenge, the main effect of challenge status was extremely significant, and both temperature groups exhibited a significant decrease in SOD activity ($P < 0.05$). No significant temperature effect or temperature × challenge interaction was detected, and no significant difference in SOD activity between the two groups was observed post-challenge ($P > 0.05$, Fig. 4E).

During chronic heat stress, IgM content showed a significant temperature × time interaction ($F$ (3, 30) = 3.732, $P = 0.022$, $\eta^2p = 0.2718$), while the main effects of temperature and time were not significant. Post hoc analysis revealed that serum IgM levels

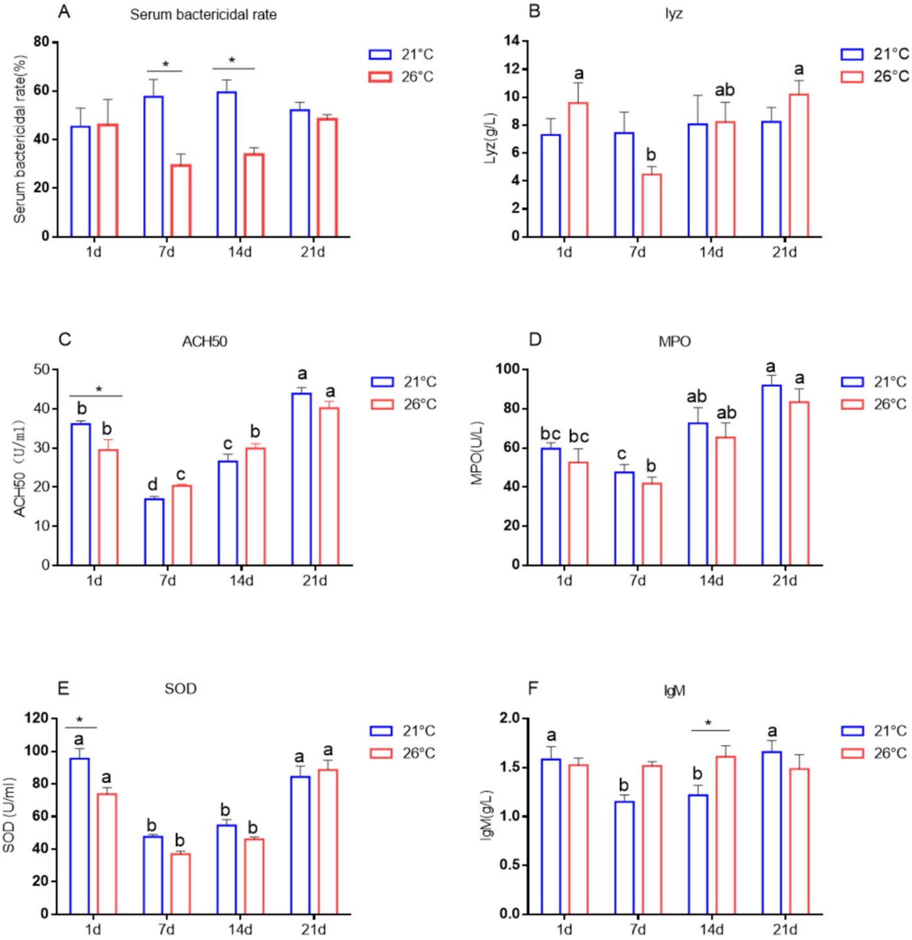

**Fig. 3** The serum immune parameters of sturgeons ($n = 6$, biological replicates, individual fish) in different temperature groups at different time points of heat stress period. A. Serum bactericidal rate; B. Lyz activity; C. ACH50; D. MPO activity; E. SOD activity; F. IgM content.Note: different lowercase letters indicate significant differences in means at different time points within the same temperature group, and asterisks (*) indicate a significant difference between the two temperature groups at the same time point

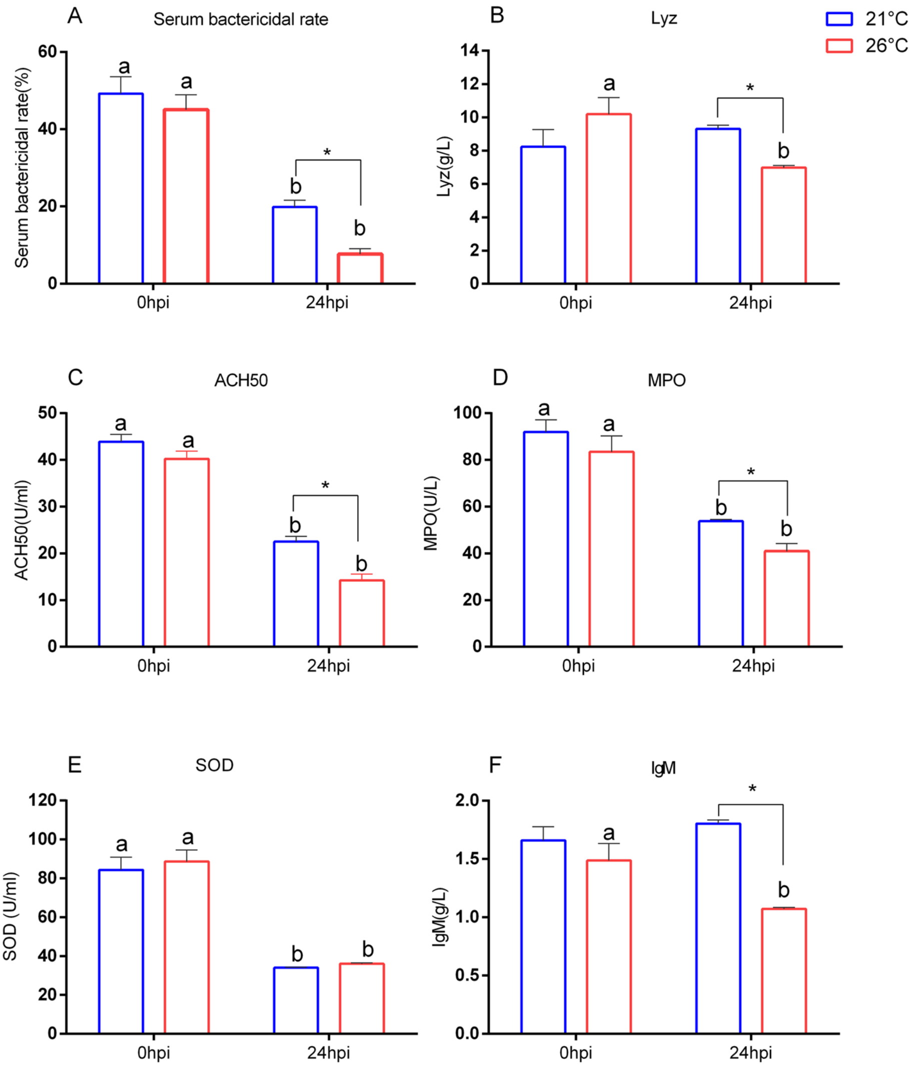

**Fig. 4** The serum immune parameters of sturgeons ($n = 6$, biological replicates, individual fish) in different temperature groups before and after *A. hydrophila* infection.A. Serum bactericidal rate; B. Lyz activity; C. ACH50; D. MPO activity; E. SOD activity; F. IgM content. Note: different lowercase letters indicate significant differences in means at different time points within the same temperature group, and asterisks (*) indicate a significant difference between the two temperature groups at the same time point

in the 26 °C group were significantly higher than those in the 21 °C group on d 7 and 14 of heat stress ($P < 0.05$), and slightly lower in the 26 °C group on day 21 with no statistical difference (*$P > 0.05$, Fig. 3F). After *A. hydrophila* challenge, two-way ANOVA showed a significant main effect of temperature and a significant temperature × challenge interaction ($F$ (1, 10) = 8.616, $P = 0.0149$, $\eta^2 p = 0.463$), while the challenge effect was not significant. Serum IgM level in the 26 °C group decreased compared with the pre-challenge level, while no significant change was observed in the 21 °C group;

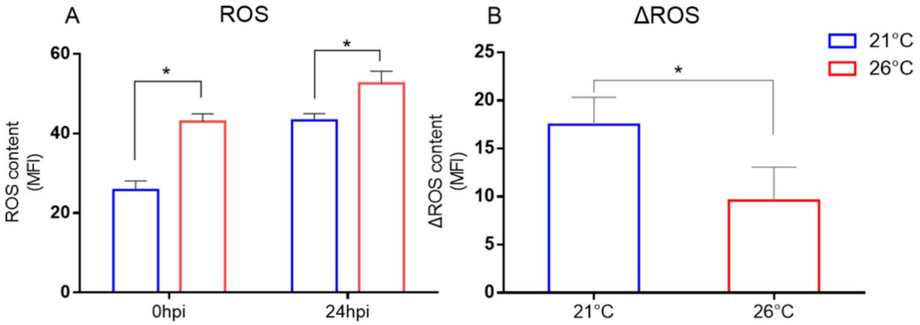

**Fig. 5** The total ROS levels of sturgeon PBLs before and after *A. hydrophila* infection in two temperature groups (A) and the respiratory burst response of PBLs by *A. hydrophila* infection (B). A two-way ANOVA was conducted in GraphPad v6.01 to examine the effects of temperature (two level, 21 °C vs 26 °C) and challenge on ROS content and an independent *t*-test was used for statistical analyses of ΔROS between the two groups ($n = 3$, biological replicates, pooled cell samples). Note: different lowercase letters indicate significant differences in means at different time points within the same temperature group, and asterisks (*) indicate a significant difference between the two temperature groups at the same time point

additionally, IgM content in the 26 °C group was significantly lower than that in the 21 °C group at 24 hpi ($P < 0.05$, Fig. 4F).

## Effects of chronic heat stress on ROS and phagocytic activity of juvenile Siberian sturgeon PBLs upon *A. hydrophila* challenge

Subsequent to the *A. hydrophila* challenge, PBLs in both temperature groups displayed an increasing trend in total ROS levels. The 26 °C group demonstrated significantly higher ROS levels than the 21 °C group ($P < 0.05$) before and after bacterial challenge. However, the net ROS production (ΔROS), representing the respiratory burst response upon *A. hydrophila* challenge in the 26 °C group, was significantly lower than that in the 21 °C group (Fig. 5).

Two-way ANOVA revealed a significant main effect of temperature on the phagocytic activity of PBLs ($F$ (1, 10) = 16.98, $P = 0.0021$), with significantly higher phagocytic rates observed at 21 °C compared to 26 °C (Fig. 6B). The main effect of bacterial challenge was not statistically significant ($F$ (1, 10) = 0.4584, $P = 0.5137$), and there was no significant interaction between temperature and challenge ($F$ (1, 10) = 0.6401, $P = 0.4423$). Before bacterial challenge, no significant difference in the phagocytic rate of PBLs was observed between the 21 °C and 26 °C groups. Following infection, the phagocytic rate in the 21 °C group increased over the subsequent 24 h, whereas the rate in the 26 °C group decreased. At 24 hpi, the phagocytic rate in the 26 °C group was significantly lower than that in the 21 °C group ($P < 0.05$).

## Effects of chronic heat stress on the expression of immune-related genes in *A. hydrophila* infected Siberian sturgeon

qPCR results revealed temperature-dependent and organ-specific differences in gene expression (Fig. 7). Both *IL-6* and *IL-8* mRNA levels were significantly upregulated in the spleen of sturgeon from both the 21 °C and 26 °C groups following *A. hydrophila* infection compared to the PBS-injected controls, with a greater induction observed in

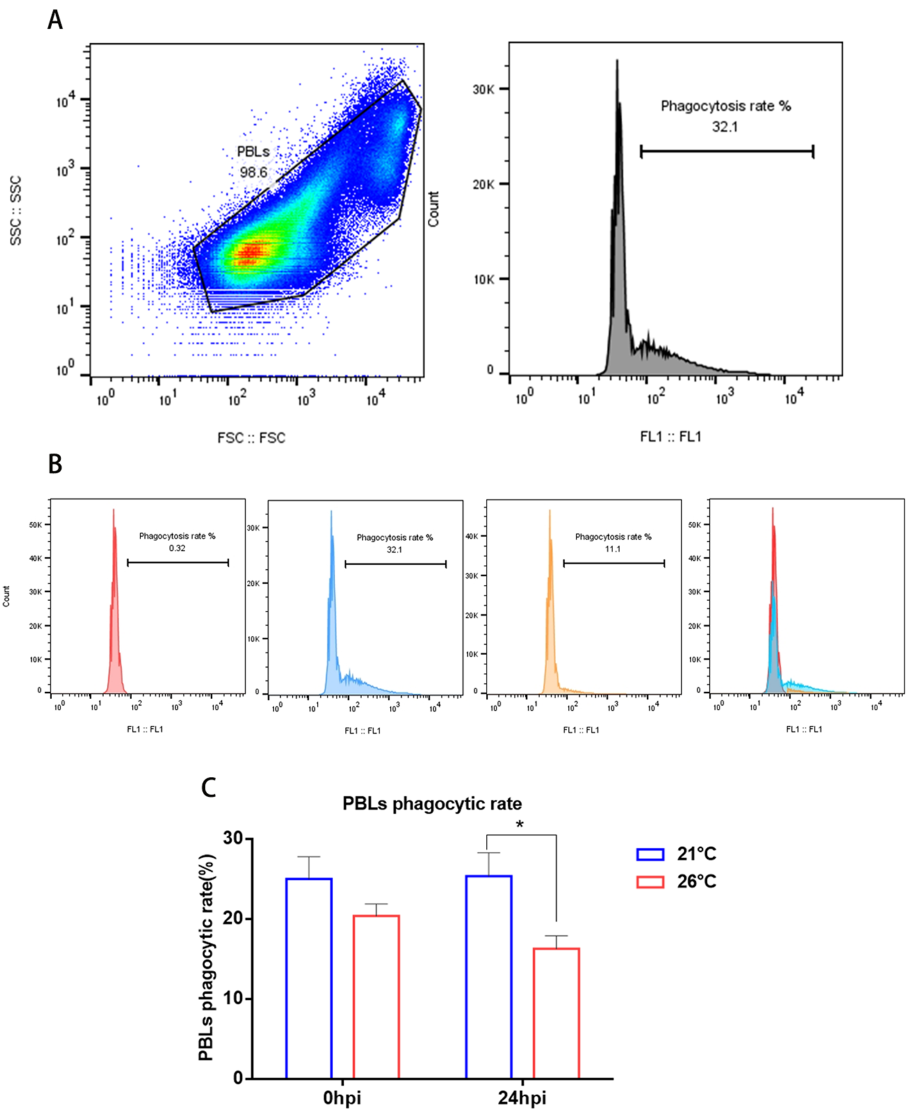

**Fig. 6** Flow cytometric analysis of phagocytosis in PBLs of sturgeons before and after *Aeromonas hydrophila* infection in two temperature groups ($n=3$, biological replicates, pooled cell samples). **A** PBLs were gated based on a two-dimensional lattice figure of FSC–SSC. Histogram overlay showing the fluorescence intensity (FL1) of the gated PBLs. **B** Merged histogram showing phagocytosis results of random chosen samples from each group following *A. hydrophila* infection (Flowjo 10.8.1). Note: different lowercase letters indicate significant differences in means at different time points within the same temperature group, and asterisks (*) indicate a significant difference between the two temperature groups at the same time point

the 26 °C group ($P < 0.05$). In contrast, the expression levels of *IL-1β*, and *IgM* were significantly upregulated only in the 26 °C group ($P < 0.05$).

In the head kidney, the 26 °C group exhibited a pronounced upregulation of *IL-1β*, *IL-6*, *IL-8*, *MHCIIβ* mRNA, compared to both the PBS-injected group and the 21 °C challenged

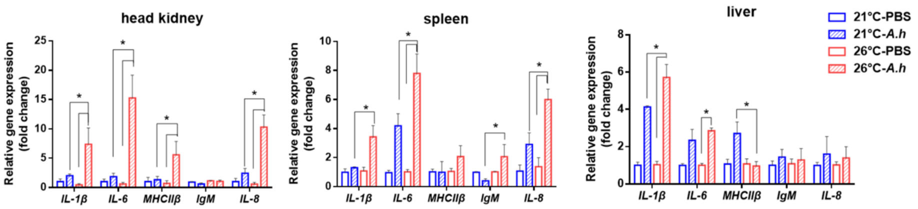

**Fig. 7** Expression levels of immune genes in the spleen, head kidney, and liver tissues of Siberian sturgeon infected with *A. hydrophila* at 21 °C or 26 °C group. To reduce individual variability, six randomly sampled fish were pooled in pairs to create three biological replicates for real-time qPCR analysis ($n=3$, biological replicates, pooled tissue samples), with each replicate assayed in three technical replicates. Note: In Fig. 7, asterisks (*) indicate a significant difference in immune factor expression levels between any two groups

group ($P<0.05$). However, *IgM* gene expression showed no significant differences across any of the groups.

In the liver, *A. hydrophila* infection upregulated *IL-1β* and *IL-6* mRNA levels in both the 21 °C and 26 °C groups ($P<0.05$). However, only the 21 °C group showed significant upregulation of the *MHCIIβ* post-*A. hydrophila* challenge. No significant changes were observed in *IgM* or *IL8* expression across any of the groups.

## Discussion

As poikilothermic organisms, fish are highly susceptible to water temperature perturbations, which can disrupt their physiological processes and suppress immune function when temperatures exceed their optimal range (Pörtner and Peck 2010; Seppala and Jokela 2011; Wang et al. 2018). This study assessed the effects of chronic heat stress on the immune and antibacterial function of *Acipenser baerii*, with one limitation being that the experiment was conducted during winter, constraining the achievable temperature range and meaning the 5 °C differential may not fully replicate summer thermal conditions.

### Integrated impact of chronic heat stress on disease resistance

Our study demonstrated that chronic exposure to 26 °C significantly compromises the disease resistance of *Acipenser baerii*, as evidenced by the markedly reduced survival rate (25.93%) following *A. hydrophila* challenge compared to the 21 °C control group (62.96%). This finding aligns with previous reports in Russian sturgeon (*Acipenser gueldenstaedtii*) showing increased susceptibility to *A. hydrophila* at 30 °C (Aversa-Marnai et al. 2025), and similar results have been documented in Nile tilapia (*Oreochromis niloticus*) with increased mortality at higher temperatures (Chen et al. 2011). The reduced disease resistance likely results from the combined effects of decreased innate immune parameters, attenuated leukocyte functions, and disruption of immune organ structural integrity, notably the spleen and head kidney (Tan et al. 2024).

### Dynamic changes in serum immune parameters

Previous research on *Oreochromis niloticus* has demonstrated that individuals reared at elevated temperatures (29 °C and 31 °C) exhibit significantly lower serum bactericidal activity compared to those maintained at 25 °C (Chen et al. 2011). Consistent with these findings, our study observed a similar impact of high-temperature stress on serum bactericidal activity in sturgeon. This suggests that elevated temperatures may perturb the composition of crucial serum components, such as complement proteins and antimicrobial peptides, thereby compromising pathogen defense mechanisms. Lysozyme, a key hydrolytic enzyme targeting the peptidoglycan layer of Gram-positive bacterial cell walls (Hikima et al. 2002), and IgM, the major, a major antibody in fish and a vital indicator of immune responsiveness (Sunyer 2013), both exhibited a distinct trend: initial elevation under chronic heat stress (acute phase immune activation) followed by a decline (immune functional attenuation), with a significant reduction in the 26 °C group post *A. hydrophila* infection. This pattern is consistent with findings in Siberian hybrid sturgeon exposed to elevated transport temperatures (Jiang et al. 2025). Reduced lysozyme activity and suppressed immunoglobulin secretion have also been reported in thermally stressed hybrid sturgeon (*A. baerii* ♀×*A. schrenkii* ♂) and rainbow trout (*Oncorhynchus mykiss*), respectively (Köllner and Kotterba 2002; Liu et al. 2025).

The complement system is a critical component of innate immunity, contributing to pathogen clearance through opsonization and direct lysis via classical or alternative pathways. $ACH_{50}$ reflects the functional capacity of the alternative complement system. An early decline in $ACH_{50}$ activity on day 1 in the 26 °C group and a further drop post bacterial infection aligns with findings in *Acipenser gueldenstaedtii* under combined chronic thermal stress and *A. hydrophila* infection (Aversa-Marnai et al. 2025). MPO, a heme-containing peroxidase primarily localized in neutrophils that mediates microbial elimination (Davies and Hawkins 2020), was slightly reduced in the 26 °C group during heat stress and significantly lowered post-infection, suggesting chronic heat stress affects neutrophil function—consistent with decreased serum bactericidal activity.

Serum SOD activity was significantly reduced by heat stress at 26 °C on day 1, remained lower than the 21 °C group for 14 days, and recovered to normal levels by day 21. No significant difference in serum SOD activity was observed between the two groups post *A. hydrophila* infection, but intracellular ROS levels in PBLs were elevated in the 26 °C group (Fig. 5), validating oxidative stress despite restored serum antioxidant enzyme activity. This suggests other antioxidant components may be compromised or ROS production exceeds the antioxidant system's neutralization capacity, consistent with previous reports of chronic heat stress- induced ROS elevation in fish (Iftikar and Hickey 2013; Yang et al. 2024). Notably, Siberian sturgeon exhibits a robust antioxidant capacity under short-term gradual temperature increase (Wang et al. 2023), which may explain the recovery of SOD activity after prolonged heat stress. It is important to note that this study focused on serum SOD activity and PBL ROS production, and we did not comprehensively assess the status of all components of the antioxidant system. Future research could explore the involvement of other enzymatic and non-enzymatic antioxidants to elucidate the complete picture of antioxidant system dynamics under chronic heat stress.

## Attenuation of PBLs immune functions

PBLs encompassing lymphocytes, monocytes, granulocytes, and macrophages, are central components of the innate immune system. Granulocytes and macrophages, acting as primary nonspecific immune cells, mediate bactericidal effects through phagocytosis and respiratory burst (Pilsczek et al. 2010). Phagocytic activity of PBLs is a key indicator of fish immune immunity (Ellis 2001), and our results showed significantly lower net ROS and phagocytic capacity in the 26 °C group—consistent with findings in hybrid sturgeon (*Huso dauricus* ♀×*Acipenser schrenckii* ♂) at 28 °C compared to 23 °C and 18 °C (Wang et al.

2015). Similarly, research on *Oreochromis niloticus* indicated that respiratory burst activity in PBLs was higher at 25 °C compared to higher temperature groups (29 °C and 31 °C) (Wang et al. 2020). Collectively, elevated temperatures may alter immune cell membrane fluidity and compromise cellular energy supply, subsequently weakening immune cell function and compromising the functional capacity of PBLs in Siberian sturgeon at 26 °C.

## Tissue-specific change of immune gene expression

Excessively high temperatures are known to induce liver congestion and steatosis in fish, triggering inflammatory responses and dysregulation of lipid metabolism (Yang et al. 2025). Our investigation revealed that chronic high-temperature stress significantly upregulated the transcription of inflammatory factors (*IL-1β*, *IL-6*), chemokine *IL-8*, and antigen-presenting molecule *MHCIIβ* in both the spleen and head kidney of *A. hydrophila*-infected sturgeon. These responses were more pronounced in the head kidney. While *IL-1β* and *IL-6* mRNA were also significantly elevated in the liver. However, the liver demonstrated relatively minor changes in immune gene expression compared to other immune organs, suggesting tissue-specific sensitivity to thermal stress. Notably, the spleen showed an exclusive upregulation of *IgM* gene expression in the high-temperature group, indicating that elevated temperatures may impair the initiation phase of adaptive immunity.

These findings align with previous research in spotted seabass (*Lateolabrax maculatus*) which demonstrated that prolonged exposure to high water temperatures induces liver inflammation (Yang et al. 2024). Similarly, thermal stress at 28 °C significantly elevated immune-related factors (*TGF-β*,*IL-1β*, and *IL-8*) in the spleen of Siberian sturgeon (Tan et al. 2024), and studies on *Oreochromis niloticus* under thermal stress also reported upregulation of *IL-1β* (Esam et al. 2022). Collectively, these observations underscore the capacity of elevated temperatures to amplify inflammatory responses to pathogenic infection in fish. We propose that the upregulated pro-inflammatory cytokine expression under chronic heat stress may represent a compensatory protective immune activation in response to bacterial invasion, while the concurrent attenuation of innate immune effector functions (e.g., leukocyte phagocytosis, complement activity) renders this activation maladaptive, ultimately leading to inflammatory dysregulation and increased tissue damage risk (Tan et al. 2024).

In conclusion, although a temperature of 26 °C may support acceptable growth rates in practical aquaculture over short durations, our findings demonstrate that chronic heat stress—specifically, a stepwise increase from 21 to 26 °C maintained for 21 days—significantly compromised the disease resistance of Siberian sturgeon. This reduction is likely a consequence of attenuated innate immune function, dysregulated inflammatory cytokine homeostasis, and impaired PBLs function. These results underscore the importance of incorporating immune competence metrics into thermal management strategies in aquaculture, moving beyond sole reliance on growth parameters. To mitigate disease outbreaks during summer heatwaves, proactive measures such as feed supplementation with immunostimulants and the provision of shading or cooling for fish ponds are recommended. Furthermore, future research should explore screening for heat-resistant sturgeon varieties or developing targeted immunomodulators to enhance thermal tolerance based on the observed thermal stress-induced alterations in immune factors.

**Author contribution** Xiaowen Wang, Hua Zhu: conceived and designed the experiments. Xiaowen Wang: performed the experiments, Xiaowen Wang and Wei Wang analyzed the data and interpreted the results.

Huijuan Li, Rong Zhang and Lili Liu: contributed reagents/materials/analysis tools. Xiaowen Wang and Hua Zhu: wrote and revised the paper.

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**Data availability** The dataset that was utilized in this study is available upon reasonable request from the corresponding author.

## Declarations

**Competing interest** The authors declare no competing interests.

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