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Pseudomonas plecoglossicida hijacks host glycolysis to induce hypoglycemic shock in large yellow croakers (*Larimichthys crocea*)

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ABSTRACT

Bacterial sepsis frequently progresses to hypoglycemia, a metabolic condition strongly associated with increased mortality. In this study, large yellow croakers were injected with phosphate-buffered saline (PBS) to establish a control group (CG) or exposed to an equivalent dose of *Pseudomonas plecoglossicida*, after which infected fish were categorized into moribund group (MG) or survival group (SG). Results showed that hepatic glycogen was rapidly depleted across all infected croakers, but severe hypoglycemia emerged only in the MG group, with blood glucose reduced to 0.80 mmol/L. Infection produced substantial oxidative and redox disruption, including accumulation of lipid peroxides, depletion of reduced glutathione (GSH), and a decline in the NAD⁺/NADH ratio, although these fluctuations were markedly attenuated in the SG group compared to the MG group. MG group exhibited widespread metabolic deterioration, characterized by reduced intermediates across glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation, decreased glycerides and fatty acids, clear mitochondrial swelling with cristae loss, and strong suppression of G6pc transcription. SG group displayed a distinct metabolic reconfiguration involving altered abundance of 26 lipid species (18 increased and eight decreased) and 13 carbohydrates (nine increased and four decreased). The most pronounced fluctuations were observed in the glycogen-shunt products, maltose elevated

47.78-fold relative to the CG and 7 995-fold relative to the MG, and D-(+)-trehalose elevated by 964.08-fold and 1 106.38-fold, respectively. Functional analyses identified *pptse6* (ACRRS2_13720), a type VI secretion system (T6SS) effector, as a key virulence gene in *P. plecoglossicida*, whose absence reduced bacterial virulence by 808-fold. Another competitive T6SS effector, TreS, contributed to maltose and D-(+)-trehalose synthesis, with its deletion lowering production of these metabolites by 7.75-fold and 6.75-fold, respectively. Overall, these findings demonstrated that *P. plecoglossicida* causes profound disruption of central energy metabolism in large yellow croakers and induces hypoglycemia, with activation of the glycogen shunt closely associated with survival.

Keywords: Hypoglycemia; Sepsis; *Pseudomonas plecoglossicida*; Large yellow croaker; Glycogen shunt; Tse6

INTRODUCTION

As a critical physiological indicator, blood glucose requires stringent clinical surveillance during sepsis, especially given infection-associated hypoglycemia, which correlates strongly with increased mortality (Mitsuyama et al., 2022). Fish exhibit carbohydrate metabolism patterns that differ markedly from those of mammals, with carnivorous species showing especially limited capacity for dietary carbohydrate utilization

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(Polakof et al., 2012). Due to these feeding-associated metabolic constraints, carnivorous fish display significantly weaker glycemic regulation than omnivorous fish and mammals, making them more susceptible to fluctuations in circulating glucose (Li et al., 2022). The large yellow croaker (*Larimichthys crocea*), a representative carnivore, requires approximately 24 hours to restore normoglycemia after exposure to a 30 mg glucose administered per 100 g body weight in juvenile fish, a dose equivalent to only 10%–20% of the minimum glucose supplementation used in humans (Gu & Xu, 2011; Li et al., 2017). This markedly inefficient glycemic control indicates that infection-induced hypoglycemia is likely to be fatal in this species, positioning large yellow croakers as an optimal vertebrate model for investigating the mechanisms underlying infection-associated hypoglycemic pathogenesis.

Visceral white spot disease (VWSD), a major bacterial threat to large yellow croaker aquaculture, is caused by *Pseudomonas plecoglossicida*, an aerobic, Gram-negative bacillus with exceptional environmental resilience (Xu et al., 2024). This pathogen can survive under nutrient-limited conditions and exhibits striking metabolic versatility in nutrient-rich environments, including rapid conversion of glucose into 2-keto-gluconic acid, a precursor for erythorbic acid synthesis (Dharni et al., 2014; Hou et al., 2020; Mao et al., 2024). Although capable of efficient metabolic activity *ex vivo*, *P. plecoglossicida* generates severe pathology in vertebrates, as evidenced by clinical manifestations, such as pneumonia in a 5-year-old boy (Lin et al., 2024). In aquaculture settings, this pathogen has led to devastating economic losses (He et al., 2025a; Yan et al., 2022), with carnivorous fish experiencing disproportionately high mortality during outbreaks (He et al., 2025b; Mao et al., 2024). Detection rates in infected large yellow croakers can approach 90%, with mortality reaching 50% in severe outbreaks (Duan et al., 2024; Xu et al., 2015).

VWSD progression is frequently accompanied by hypoglycemia-related behavioral symptoms, including lethargic swimming, impaired equilibrium, and rolling movements (Sun et al., 2020). Hypoxemia, commonly observed in infected fish, appears to arise as a consequential symptom of hypoglycemia-induced metabolic collapse (Rebollada et al., 2025; Sun et al., 2023). Histopathological lesions, especially extensive granulovacuolar degeneration in the liver, spleen, and kidney, indicate profound impairment of energy metabolism (Sun et al., 2020). Transcriptomic analyses have further revealed significant hyperactivation of glycolysis and the citrate cycle post-infection, whereas suppression of these pathways is associated with enhanced host resistance to *P. plecoglossicida* (He et al., 2023; Zhang et al., 2021). Collectively, current evidence strongly implicates hypoglycemia as a central feature of VWSD pathogenesis.

This study applied controlled infection in large yellow croakers, a natural host for *P. plecoglossicida*, to establish control, moribund, and survival groups, and integrated mass spectrometry imaging with multi-omics analyses to define the pathological consequences of infection-associated hypoglycemia in vertebrates. This study also investigated differential engagement of carbohydrate metabolism as a potential anti-infection strategy and delineated immune response networks shaped by carbohydrate flux partitioning. Overall, the findings of this study provide a mechanistic framework that advances current understanding of infection-induced hypoglycemia and inform the development of strategies to mitigate its impact.

MATERIALS AND METHODS

Artificial infection assay

Large yellow croakers without external lesions were obtained from a commercial fishery in Ningde, China, with an average length and weight of 15.05 ± 0.52 cm and 52.1 ± 4.20 g, respectively. Fish were acclimated for 14 days in a recirculating system equipped with two oxygen pumps per unit and maintained at 18°C. On day 14, 10 individuals were randomly selected for gill filament microscopy and spleen polymerase chain reaction (PCR) to confirm pathogen-free status. The PCR assay utilized species-specific primers targeting *P. plecoglossicida* (Izumi et al., 2007).

Pseudomonas plecoglossicida was cultured in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 18°C and 220 r/min. Bacterial cells were washed, resuspended in phosphate-buffered saline (PBS), and diluted to the required concentrations for experimental infection.

Sixty fish were randomly selected and evenly divided into three groups. Two groups received intraperitoneal injection of 10^3 or 10^4 colony-forming units (cfu) of bacteria, and one group received an equivalent volume of PBS. Each group was maintained in a separate recirculating system for continuous monitoring. Mortality was recorded every 24 h, and dead individuals were removed immediately. No feed was provided during the trial. The assay was conducted in three independent runs.

Following exposure to the half-lethal dose (LD_{50}), fish exhibiting floating behavior or immobility but with persistent gill movement were classified as the moribund group; fish that remained alive at 7 days post-infection were classified as the survival group; and fish injected with PBS were classified as the control group. Visceral tissues and blood samples were collected from all groups for subsequent analyses, and each sample was tested in triplicate.

The infection assay using Δ *ptpse6* mutants followed the same procedure as those used for the wild-type strain.

All animal protocols were approved by the Animal Ethics Committee of Jimei University (Approval No. JMU202403021).

Blood oxygen saturation detection

Blood oxygen saturation (SaO_2) was measured following Sun et al. (2023). In brief, equal volumes of toluene and large yellow croaker blood were mixed and subjected to Sephadex G-75 purification to isolate hemoglobin. Isolated hemoglobin was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and its molar absorption coefficient (ϵ) was determined using a Hemoglobin Assay Kit (Nanjing Jiancheng, China). Hemoglobin derivatives were generated under oxygen or nitrogen, and corresponding ϵ values were measured.

Normoxic conditions were established by supplying each recirculating aquaculture system with two oxygen pumps, after which blood was collected from the fish as the normoxic control. Hypoxic conditions were generated by removing oxygen pumps and covering the water surface with a plastic film to restrict gas exchange. Fish were monitored hourly, and blood was collected once clear signs of asphyxiation appeared; these samples served as the hypoxic control. Three biological samples were collected for each control group.

Blood from the moribund and survival groups was collected, with three samples per group. A portion of each sample was used to quantify total hemoglobin concentration (cHb) using a

Hemoglobin Assay Kit (Nanjing Jiancheng, China). The remaining blood was used to measure oxyhemoglobin (O₂Hb) concentration via absorbance measurements. Blood oxygen saturation (SaO₂) was calculated using the formula: SaO₂=cO₂Hb/cHb.

Red blood cell counting

Blood samples were collected from three large yellow croakers as three independent replicates. According to the Erythrocyte Dilution Assay Kit (Nanjing Jiancheng, China), each 10 µL sample was diluted with 2 mL of diluent and examined under an optical microscope using a standard blood cell counting plate.

Histological investigation

Following Sun et al. (2024b), visceral tissues from large yellow croakers were fixed in 4% immunohistochemical fixative (Labcead, China), embedded in paraffin, sectioned into thin slices, and stained with hematoxylin-eosin (H&E) (Servicebio, China), Prussian blue (DAB enhanced) (Servicebio, China), VG staining solution (Servicebio, China), AB-PAS dye (Servicebio, China), or HBFP dye (Servicebio, China). Stained sections were observed and imaged under an optical microscope, and 10 random fields were selected for quantification, with vacuolated cells expressed as a percentage of total cells. Liver samples were immersed in fixative (Servicebio, China), embedded in resin, polymerized, sectioned into ultrathin slices, and examined by transmission electron microscopy (TEM).

Hepatic biochemical analysis

Glycogen levels were quantified using a Liver and Muscle Glycogen Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocols. In brief, liver tissue was homogenized in 3-fold KOH, heated at 100°C for 20 min, and centrifuged at 10 000 ×g for 10 min at 4°C. The resulting supernatant was collected and diluted with chromogenic solution. After boiling and cooling, absorbance was measured at 620 nm.

Lipid peroxide (LPO) levels were measured using an LPO Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocols. Liver tissue was homogenized in 10-fold PBS at 4°C, then centrifuged at 845 ×g for 10 min at 4°C. The resulting supernatant (200 µL) was successively incubated with buffer 1 and buffer 2 at 45°C for 60 min, followed by centrifugation at 1 500 ×g for 10 min at 4°C. The mixture was diluted with absolute ethanol, with 200 µL of the diluent then measured at 586 nm.

Reduced glutathione (GSH) was quantified using a GSH and Glutathione Disulfide (GSSG) Assay Kit (Beyotime Biotechnology, China) following the manufacturer's protocols. Liver tissue was homogenized in 10-fold PBS at 4°C and centrifuged at 845 ×g for 10 min at 4°C, with the resulting supernatant used to measure total GSH and GSSG levels. GSH was calculated as total GSH – 2×GSSG.

Mitochondrial complex V activity was measured with a Complex V ELISA Kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. Liver tissue was homogenized in 10-fold PBS at 4°C and centrifuged at 845 ×g for 10 min at 4°C. Subsequently, 10 µL of supernatant was mixed with 40 µL of sample diluent buffer and 100 µL of horseradish peroxidase (HRP)-conjugate reagent. After incubation at 37°C for 60 min, the samples were successively incubated with chromogen solutions A and B at 37°C for 15 min in the dark, and absorbance was measured at 450 nm

after adding 50 µL of stop solution.

NAD⁺ and NADH levels were quantified using an NAD⁺/NADH WST-8 Kit (Beyotime Biotechnology, China) according to the manufacturer's protocols. Briefly, livers were homogenized in 20-fold NAD⁺/NADH extraction buffer, and total NAD and NADH were measured sequentially. NAD⁺ was calculated as NAD_{total} – NADH.

Multi-omics analysis

Metabolite extraction was performed following Wu et al. (2022). In brief, liver tissue (25±1 mg) was mixed with beads and 200 µL of H₂O, vortexed for 30 s, homogenized at 35 Hz for 4 min, and sonicated for 5 min in a 4°C water bath, with this cycle repeated three times. The homogenate was mixed with 480 µL of extraction solution (MTBE:MeOH, 5:1 (v/v)) containing deuterated internal standards, vortexed for 30 s, sonicated for 10 min in a 4°C water bath, and incubated at –40°C for 1 h. Samples were then centrifuged at 900 ×g for 15 min at 4°C. Supernatants were collected and evaporated to dryness in a vacuum concentrator. The dried extract was reconstituted in 200 µL of DCM:MeOH (1:1, v/v), vortexed for 30 s, sonicated for 10 min at 50 Hz and 4°C, and centrifuged at 13 800 ×g for 15 min at 4°C. A 75 µL aliquot of supernatant was transferred into glass vials for analysis. Quality control (QC) samples were prepared by mixing equal aliquots from all samples.

For lipid analysis, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an ultra-high-performance liquid chromatography (UHPLC) system (Vanquish; Thermo Fisher Scientific, USA) equipped with a Phenomenex Kinetex C18 column (2.1 mm×100 mm, 2.6 µm; Phenomenex, USA) coupled to an Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo Fisher Scientific, USA). Mobile phase A consisted of H₂O/ACN (6:4, v/v) with 10 mmol/L HCOONH₄, and mobile phase B consisted of IPA/ACN (9:1, v/v) with 10 mmol/L HCOONH₄. Injection volume was 2 µL.

The Orbitrap Exploris 120 mass spectrometer was used to acquire MS/MS spectra under Xcalibur control (Thermo Fisher Scientific, USA). Full-scan spectra were monitored continuously. ESI source settings were as follows: sheath gas 30 Arb, Aux gas 10 Arb, capillary temperature 320°C, full MS resolution as 60 000, MS/MS resolution 15 000, stepped normalized collision energy (SNCE) 15/30/45, and spray voltage 3.8 kV (positive) or –3.4 kV (negative).

Raw data files were converted to mzXML format using 'msconvert' in ProteoWizard (Smith et al., 2006). Peak detection, extraction, alignment, and integration were carried out using the CentWave algorithm in XCMS, with minfrac set to 0.5 and annotation cutoff set to 0.3. Lipid identification was performed by spectral matching against the LipidBlast library using R and XCMS.

Mass spectrometry imaging-based MALDI-MSI analysis

Liver tissue from large yellow croakers was embedded in 2% carboxymethyl cellulose (CMC; Aladdin, China) using a dry ice-isopentane cooling environment. After solidification, embedded tissue was cryosectioned and mounted onto slides, which were dried in a vacuum dryer for 30 min. Tissue locations were marked, and optical images were scanned.

Dried sections on ITO-coated glass slides were coated using an HTX TM sprayer (Bruker, Germany). For positive-ion lipid detection, slides were sprayed with 10 mg/mL CHCA (α-cyano-4-hydroxycinnamic acid) dissolved in ACN-H₂O (7:3,

v/v). For negative-ion lipid and carbohydrate detection, slides were coated with 5 mg/mL NEDC (N-(1-naphthyl) ethylenediamine dihydrochloride) dissolved in methanol-water (6:4, v/v). Sprayer conditions were 75°C, 0.1 mL/min flow rate, and 10 psi for positive-ion mode, and 90°C, 0.08 mL/min flow rate, and 10 psi for negative-ion mode. Each slide received four matrix passes with 10 s drying between passes.

MALDI timsTOF MSI experiments were performed on a prototype Bruker timsTOF flex MS system (Bruker, Germany) equipped with a 10 kHz SmartBeam 3D laser. Laser power was set to 65% and held constant throughout acquisition. Mass spectra were acquired in positive or negative mode across an *m/z* range of 50–1 200 Da. Spatial resolution was set to 50 μ m, and each pixel spectrum was generated from 400 laser shots. Spectra were normalized using the root-mean-square method, and signal intensity in each image was displayed as normalized intensity. Raw data were processed in SCI^{LS}™ Lab 2024 and converted into pixel points on the imaging map.

Microbiology incubation

The growth curve assay was performed following Wang et al. (2023). Briefly, *P. plecoglossicida* was inoculated into TSB and cultured for 16 h at 28°C and 220 r/min. The bacterial suspension was washed three times with PBS and adjusted to 10⁸ cfu/mL. A 10 μ L aliquot was mixed with 190 μ L of minimal salt medium (MSM; Ararat, China) supplemented with 2% glucose (BBI, China), maltose (BBI, China), or trehalose (BBI, China). The mixture was transferred to a 96-well microplate and incubated in a microplate reader (SYNERGY H1; Biotek, USA) at 28°C for 48 h, with OD₆₀₀ measured hourly. Four independent biological replicates were prepared for each condition.

For the plate incubation assay, 10 μ L of bacterial suspension (10⁸ cfu/mL) was transferred to a MSM agar plate supplemented with 2% glucose (BBI, China), maltose (BBI, China), or D-(+)-trehalose (BBI, China). All plates were incubated in a thermostatic incubator at 28°C for 96 h.

For the tolerance assay, *P. plecoglossicida* was cultured for 24 h in MSM containing 2% glucose, supplemented with 100 μ mol/L, 200 μ mol/L, 500 μ mol/L, or 1 mmol/L maltose, or 100 μ mol/L, 200 μ mol/L, 500 μ mol/L, or 1 mmol/L D-(+)-trehalose.

Genome sequencing, assembly, annotation, and analysis

Genome sequencing followed the method of Koren et al. (2017). Genomic DNA from *P. plecoglossicida* was extracted using the Easyseq DNA Purification Kit (TransGen Biotech, China). DNA quality was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA), and the samples with 260/280 ratios above 1.8 were selected for further analysis.

High-quality DNA was sheared to an average fragment size of 10 kb using Megaruptor 3 (Diagenode, USA). Fragmented DNA was amplified using a PacBio Ultra-Low DNA Input Kit (Pacific Biosciences, USA) following the manufacturer's recommendations. Amplified DNA was converted into a PacBio library using the SMRTbell Express Template Prep Kit v3.0 (Pacific Biosciences, USA). T-overhang SMRTbell adapters were ligated, and the library was purified with two rounds of AMPure PB bead cleanup (Pacific Biosciences, USA). Library concentration was measured with Qubit, and fragment size distribution was confirmed using a Femto Pulse system. The library was sequenced on a single SMRT Cell 8

M using the PacBio Sequel IIe platform with a 30 h movie time. Circular consensus sequencing was performed in real time with SMRT Link v.10.1 (Pacific Biosciences, USA) using default parameters.

Raw reads were filtered with Seqkit v.2.0.0 and assembled using Unicycler, followed by correction with Pilonjin. Coding sequences (CDS) were predicted with Glimmer (<http://ccb.jhu.edu/software/glimmer/index.shtml>) and Prodigal. tRNAs were predicted using tRNAscan-SE v.2.0 (<http://trna.ucsc.edu/software/>), and rRNAs were predicted using Barrnap (<https://github.com/tseemann/barrnap>). Gene Ontology (GO) annotations were conducted using Blast2GO.

Seamless gene knockout

Seamless gene knockout in *P. plecoglossicida* followed the method of Sun et al. (2024b). Using genomic DNA from strain NZBD9 as the template, 400 bp upstream and downstream fragments of *tse6* or *treS* were amplified using the primers *tse6*_{F1/R1}, *tse6*_{F2/R2}, *treS*_{F1/R1}, and *treS*_{F2/R2} (Supplementary Table S1). Plasmid pK18mobSacB was linearized with HindIII and BamHI (Takara Biotech, Japan). The amplified upstream and downstream fragments were ligated into the linearized plasmid using an OK Clon DNA Ligation Kit III (Accurate Biology, China). Recombinant constructs pK18mobSacB-*tse6* and pK18mobSacB-*treS* were introduced sequentially into *Escherichia coli* DH5 α and *P. plecoglossicida* competent cells. Positive clones were successively screened using kanamycin and 10% sucrose. Modified genotypes were verified by PCR and DNA sequencing using primers *tse6*_{F3/R3} and *treS*_{F3/R3} (Supplementary Table S1). The resulting knockout strains were designated Δ *tse6* and Δ *treS*.

Maltose and D-(+)-trehalose quantitation

Wild-type *P. plecoglossicida* and the Δ *treS* mutant were cultured in MSM supplemented with 2% glucose at 28°C and 220 r/min for 16 h. The bacterial suspension was centrifuged at 5 000 $\times g$ for 10 min at 28°C, and the supernatant was concentrated using 3K protein ultrafiltration tubes (Millipore, USA). Maltose and D-(+)-trehalose concentrations were determined using a commercial detection kit (Aidisheng, China).

Biolog identification

Pseudomonas plecoglossicida was streaked on TSA and incubated at 28°C for 16 h. A single colony was inoculated into TSB and cultured at 28°C and 220 r/min for 16 h. Subsequently, 100 μ L of the bacterial suspension was transferred to each well of the Biolog GEN III identification plate, which was loaded into the GEN III MicroStation automated microbial identification system (Biolog ID, USA) and incubated at 28°C for 24 h. Data were analyzed using MicroStation software.

Gene expression analysis

RNA was extracted from infected liver tissue using an Eastep® Super Total RNA Extraction Kit (Promega, China). RNA templates were reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Biotech, China). Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) was performed using QuantStudio 6 Flex (Life Technologies, USA) as described in previous research (Yang et al., 2025). Primers are listed in Supplementary Table S1. Expression levels were normalized to 16S rDNA, and relative expression was calculated using the 2^{- $\Delta\Delta$ Ct} method.

Statistical analyses

Data were expressed as mean±standard deviation (SD) from at least three independent experiments. Omics data were filtered with the chi-square test, and significance was defined as $P<0.05$ and $\log_2$ fold change ≥ 1 . Other datasets were analyzed in SPSS v24.0 software (USA) using one-way analysis of variance (ANOVA) with Dunnett's test. Statistical significance was set at $P<0.05$.

RESULTS

Pseudomonas plecoglossicida induces hypoglycemia in large yellow croakers

The injection dose required to yield both fatal and nonfatal outcomes was established through gradient dose

experiments, which identified a median lethal dose (LD_{50}) of $4.44\times 10^3\pm 2.79\times 10^3$ cfu/fish (Figure 1A). This dose was used to generate three groups: control group (PBS) (CG), moribund group (MG), and survival group (SG). The CG and SG groups retained normal hepatic coloration and physiological oxygen saturation (SaO_2), whereas the MG group exhibited pronounced hepatic pallor and significantly reduced SaO_2 (Figure 1B; Supplementary Figure S1). Erythrocyte counts, myocardial ischemia, visceral vascular rupture, and iron distribution showed no significant intergroup differences (Supplementary Figures S2–S4), indicating that hypoxemia is directly associated with mortality in large yellow croakers, rather than secondary to anemia.

Extensive vacuolar degeneration was observed in visceral organs of infected fish, most prominently in the liver

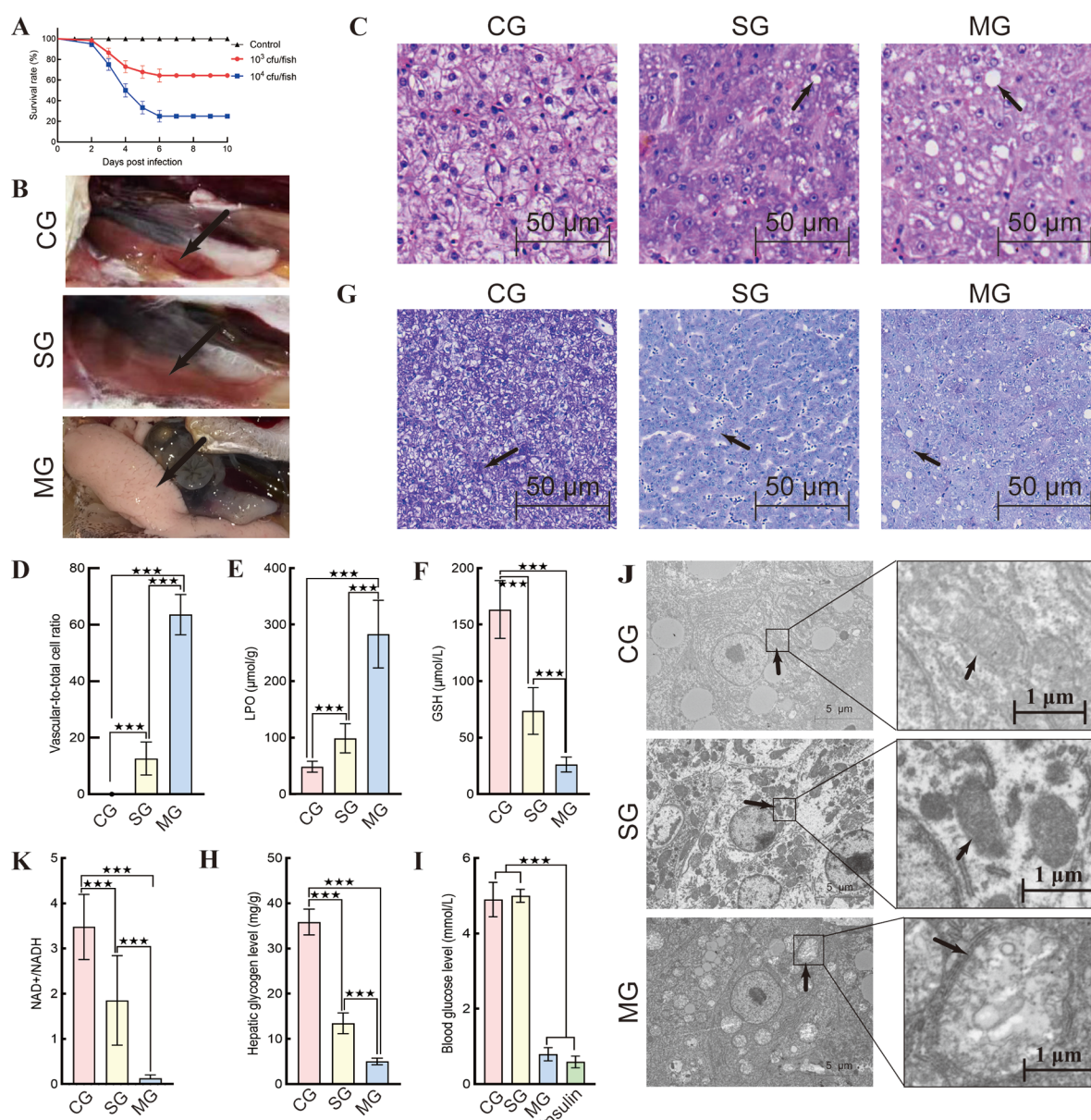


Figure 1 *Pseudomonas plecoglossicida* induced hypoglycemia in large yellow croakers

A: Mortality rates of croakers challenged with varying concentrations of *P. plecoglossicida*. B: Macroscopic liver morphology across experimental groups. Arrows indicate livers. C: Histopathological features of H&E-stained hepatic tissues. Arrows indicate vacuolated cells. D: Proportion of vacuolated cells relative to total hepatocytes. E: Hepatic LPO levels. F: Hepatic GSH levels. G, H: Hepatic glycogen levels. Arrows indicate glycogen. I: Blood glucose levels across experimental groups. J: Ultrastructural changes in hepatocytes. Arrows indicate mitochondria. K: NAD⁺/NADH ratio across experimental groups. D, E, F, H, I, K: $n=3$. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

(Figure 1C; Supplementary Figure S5). Hepatocytes from the CG group showed no vacuolation, while the SG group displayed moderate pathology ($12.66 \pm 5.84\%$ vacuolated cells) and the MG group showed severe pathology ($63.54 \pm 7.13\%$ vacuolated cells) (Figure 1C, D). This gradient indicates progressive energy metabolism impairment that aligned with survival outcomes.

LPO accumulation followed a consistent pattern, with the lowest levels in the CG group, intermediate levels in the SG group, and the highest levels in the MG group, which reached 5.8-fold relative to the CG group and 2.87-fold relative to the SG group (Figure 1E). GSH levels showed an inverse correlation, with the highest content in the CG group, measuring 2.21-fold that of the SG group and 6.21-fold that of the MG group (Figure 1F). Hepatic glycogen concentration was 35.87 mg/g tissue in the CG group, 2.66-fold higher than in the SG group and 7.10-fold higher than in the MG group (Figure 1G, H; Supplementary Figure S6). These results demonstrated substantial depletion of hepatic energy reserves and pronounced oxidative stress during infection. Under physiological conditions, hepatic glycogen degradation maintains blood glucose homeostasis (Sharafifard et al., 2025). Here, blood glucose levels were 4.93 mmol/L and 5.02 mmol/L in the CG and SG groups, respectively, but declined precipitously to 0.80 mmol/L in the MG group, matching levels in insulin-overdosed fish (Figure 1I), indicating a fatal hypoglycemic event.

Mitochondrial ultrastructure differed markedly across groups. The CG and SG groups maintained intact cristae, whereas the MG group displayed swollen mitochondria with fragmented cristae, consistent with severe organelle injury (Figure 1J). The NAD^+/NADH ratio in the CG group (3.48) was 1.88-fold higher than in the SG group and 26.77-fold higher than in the MG group, demonstrating profound mitochondrial dysfunction and energy deficiency in moribund fish (Figure 1K).

Collectively, these physiological and pathological observations indicate that infection-induced hypoglycemia contributes critically to mortality in large yellow croakers and is closely associated with mitochondrial impairment.

Excessive hepatic glucose consumption triggers hypoglycemia

Mass spectrometry imaging revealed a pronounced depletion of hepatic glucose in the MG group compared to the CG and SG groups (Figure 2A). In contrast, the SG group accumulated substantially higher levels of D-(+)-trehalose and maltose than both the CG and MG groups (Figure 2B), indicating that survival outcomes may depend on glucose diversion into alternative metabolic pathways.

To systematically map carbohydrate remodeling, high-throughput profiling of 65 carbohydrates, filtered for ≥ 2 -fold variation, was conducted, with results revealing distinct remodeling patterns. Notably, compared to the CG group, the SG group showed four decreased and nine increased carbohydrates, while the MG group showed 18 decreased and nine increased carbohydrates (Figure 2C, D). Comparison of MG and SG profiles identified 17 decreased and five increased carbohydrates (Figure 2E). Several metabolic shifts, including elevated pyruvate and lactate, reflected enhanced anaerobic respiration but did not associate directly with survival.

However, certain metabolites were strongly linked to

adverse outcomes, including elevated oxoadipic acid and 2-isopropylmalic acid in the MG group, indicative of excessive amino acid mobilization; activation of mevalonic acid (MVA) consistent with cholesterol or terpenoid utilization; and depletion of glucuronic acid, gluconic acid, and D-(+)-galacturonic acid, reflecting compromised antioxidant capacity (Figure 2F). The most striking and significant alteration was observed in glucose metabolism. Hepatic glucose in the MG group dropped to 0.19 ± 0.05 nmol/g, accompanied by substantial reductions in key glycolytic intermediates, including D-glucose-6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (FDP), and phosphoenolpyruvic acid (PEP), indicating severe glucose depletion and glycolytic inhibition (Figure 2G). In contrast, the SG group maintained glucose at 1.66 ± 0.48 nmol/g, statistically comparable to the CG group (1.25 ± 0.53 nmol/g), with only a modest reduction in G6P, consistent with relative glycolytic stability (Figure 2C). Although the MG group exhibited elevated adenosine triphosphate (ATP) and guanosine triphosphate (GTP) levels (ATP/GTP ratio 3.66), both the CG and SG groups contained only 29.38%/22.94% of MG ATP and 48.47%/43.89% of MG GTP, with ATP/GTP ratios of 2.22 and 1.91, respectively (Figure 2C–E). Notably, glucose-derived disaccharides (maltose and trehalose) diverged sharply among groups, with maltose/trehalose at 5.02/0.01 nmol/g (CG), 239.85/8.32 nmol/g (SG), and 0.03/0.01 nmol/g (MG) (Figure 2C–E). These results demonstrate that the differential diversion of glucose through distinct metabolic pathways is associated with survival and mortality in infected fish.

Mobilization of hepatic glycerolipids (GLs) and fatty acids (FAs)

Mass spectrometry imaging indicated substantial depletion of hepatic FAs in the MG group, whereas glyceride changes were comparatively subtle (Figure 3A, B). To clarify this discrepancy, comprehensive hepatic lipidomic analysis was conducted. Compared to the CG group, the MG group exhibited 53 increased and 168 decreased lipid species, whereas the SG group exhibited 19 increased and 32 decreased lipid species (Figure 3C, D). Among these, 28 lipids showed no significant differences between the SG and MG groups and were thus excluded from further analysis (Figure 3E). After filtering, the SG group displayed alterations in only three GLs (0.56% of total) and three FAs (4.35%), whereas the MG group showed severe reductions in 24 GLs (4.44%) and 21 FAs (30.43%), along with increases in two GLs (Figure 3F, G). These findings suggest extensive mobilization of FAs and GLs in the MG group, while the SG group maintained near-stable lipid homeostasis. The modest 4.44% reduction in GLs in the MG group may explain the limited glycerolipid changes observed in mass spectrometry imaging.

Hepatic enzyme-metabolite conjugation

Enzymes serve as pivotal catalysts governing energy metabolic flux. A total of 72 energy-related enzymes were identified across glycolysis (EMP), the TCA cycle, oxidative phosphorylation (OXPHOS), glycogenolysis, gluconeogenesis, glyceride degradation, and β -oxidation (Figure 4A). After filtering for significance ($P < 0.05$, fold change ≥ 1.2), clear expression differences emerged. Compared to the CG group, the SG group showed increased Hk1, Gapdh, Pc1, and Echs1 expression, corresponding to EMP, gluconeogenesis, and β -oxidation (Figure 4B). These increases indicated metabolic

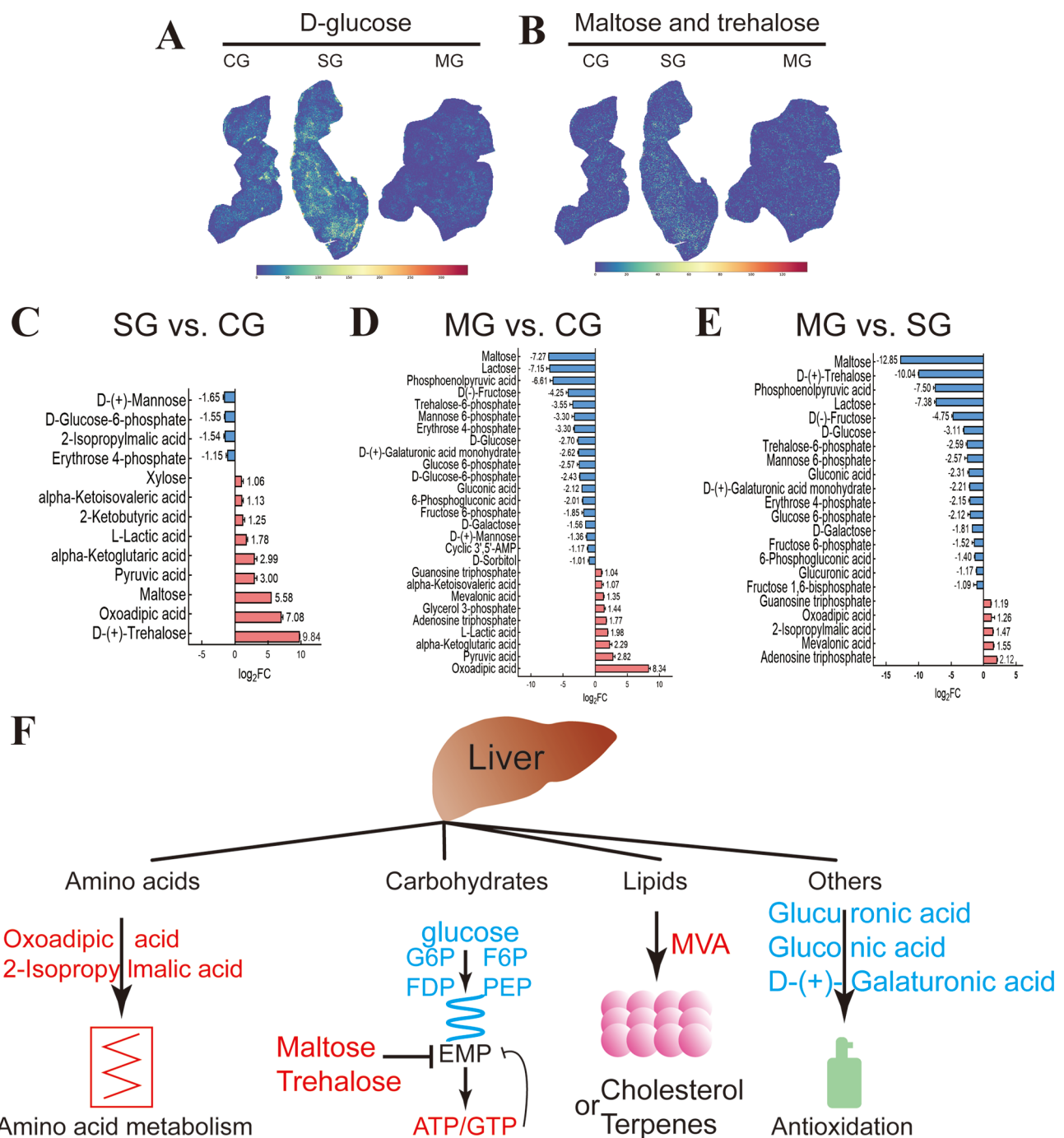


Figure 2 Quantitative analysis of hepatic carbohydrates in *P. plecoglossicida*-infected large yellow croakers

A, B: Mass spectrometry images of livers. A: Glucose; B: Maltose and trehalose. C–E: Differential carbohydrate abundances across groups. C: SG vs. CG; D: MG vs. CG; E: MG vs. SG. F: Schematic of biological processes enriched by different carbohydrates. G6P: Glucose-6-phosphate; F6P: Fructose 6-phosphate; FDP: 1,6-fructose diphosphate; PEP: Phosphoenolpyruvic acid; EMP: Glycolysis; MVA: Mevalonic acid. Red font indicates an increase in carbohydrate content, blue font indicates a decrease.

plasticity that supported immune activity by dynamically reprogramming energy pathways for host protection during infection. In contrast, the MG group showed increased *Cs* and *Echs1* expression but decreased *Cox2*, *Mdh*, *Pfk1*, *Acsm*, and *G6pc* expression (Figure 4C), consistent with impaired glycolysis, forced FA catabolism, and mitochondrial impairment. The most pronounced divergence appeared in the MG group compared to the SG group, with the MG group displaying broad reductions in *Pgm1*, *G6pc*, *Gapdh*, *Aco2*, *Mdh1*, *Atp1*, *Atp7*, *Gk3*, *Acsm*, *Acad5*, *Acat*, *Pc1*, and *Pck2* expression (Figure 4D). These collective shifts indicate a

systemic failure of energy production in the MG group, characterized by multi-pathway disruption and profound metabolic collapse.

Glycogen shunt may contribute to defense against *P. plecoglossicida*

The glycogen shunt in the SG group generated substantial amounts of maltose and D-(+)-trehalose (Figure 2D), prompting an evaluation of their effects on *P. plecoglossicida*. *In vitro* assays showed that neither maltose nor D-(+)-trehalose supported bacterial growth as sole carbon sources

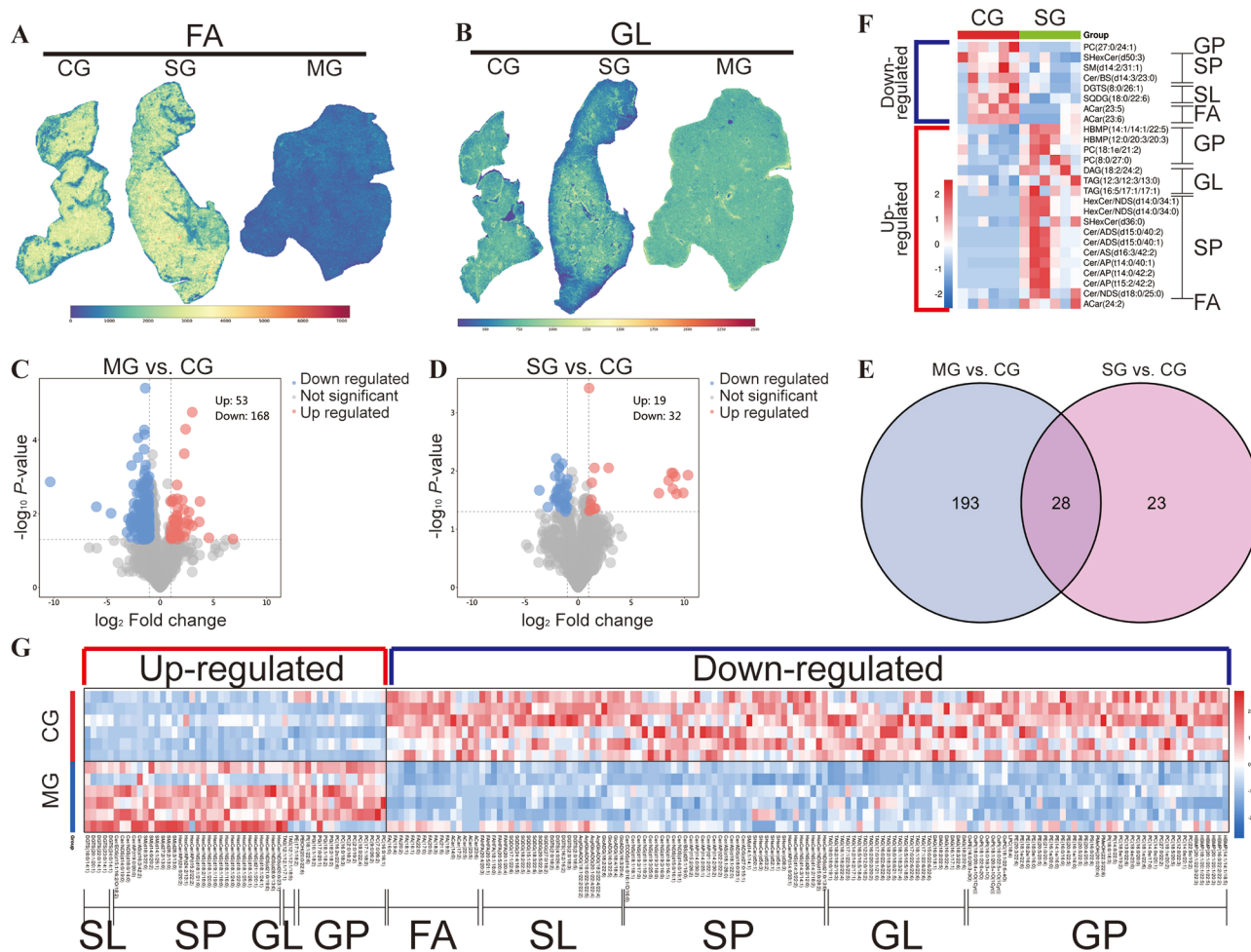


Figure 3 Quantitative analysis of hepatic lipids in *P. plecoglossicida*-infected large yellow croakers

A, B: Mass spectrometry images of livers. A: FAs; B: GLs. C, D: Volcano plot of varied lipids. C: MG vs. CG; D: SG vs. CG. E: Venn diagram of intersection between groups. F, G: Heatmap of lipids. F: SG vs. CG; G: MG vs. CG. SL: Saccharolipids; SP: Sphingolipids; GL: Glycerolipids; GP: Glycerophospholipids; FA: Fatty acids.

(Figure 5A, B; Supplementary Table S2) and neither disaccharide inhibited bacterial proliferation (Supplementary Figure S7). These findings suggest that maltose and D-(+)-trehalose likely support host protection rather than exerting direct antimicrobial activity.

Because vertebrates lack the capacity to synthesize maltose and D-(+)-trehalose (Argüelles, 2014; Attfield & Bell, 2003), whole-genome sequencing of *P. plecoglossicida* was performed using third-generation sequencing technology. The assembled genome was 5.53 Mb in size with a GC content of 62.66%. In total, 4 985 genes were annotated (Figure 5C), representing 239 more genes than reported previously from second-generation sequencing (Huang et al., 2018). Given that the type VI secretion system (T6SS) has been recognized as a key virulence determinant in *P. plecoglossicida* (Tao et al., 2020), targeted analysis of the T6SS pathogenicity island was conducted. Genomic screening revealed a PAAR-domain effector homologous to *tse6*, a *P. aeruginosa* toxin that degrades NAD⁺ and NADP⁺ in target cells (Whitney et al., 2015) (Figure 5D). To avoid confusion with the *P. aeruginosa* gene, the locus in *P. plecoglossicida* was designated *pptse6* (ACRRS2_13720). A strain lacking *pptse6* was successfully generated and verified (Supplementary Figure S8). The LD₅₀ of Δ *pptse6* was $3.58 \times 10^6 \pm 6.62 \times 10^5$ cfu/fish, representing an 808-fold attenuation compared to the wild-type strain

(Figure 1A, 5E). Downstream of *pptse6*, the *dcrB* gene was identified, encoding a DUF4946 domain-containing adaptor protein (Supplementary Figure S9), followed by *vgrG*, forming a *pptse6-dcrB-vgrG* arrangement analogous to that in *P. aeruginosa* (Quentin et al., 2018). The *treS* gene was located directly downstream of *vgrG*. Deletion of maltose in culture supernatants from 664.29 ± 262.54 μ g/mL to 85.71 ± 12.37 μ g/mL and D-(+)-trehalose from $1\ 160.20 \pm 200.99$ μ g/mL to 171.98 ± 53.70 μ g/mL (Figure 5F), demonstrating efficient disaccharide synthesis by TreS. Transcriptional patterns indicated reduced *pptse6* and elevated *treS* in the SG liver compared to the MG liver (Figure 5G). Molecular docking predicted competitive binding between TreS and DcrB at LYS600 of VgrG (Figure 5H, I). These results suggest that the glycogen shunt may be functionally associated with the selective assembly of ppTse6 and TreS during *P. plecoglossicida* infection.

DISCUSSION

Clinical data show that severe hypoglycemia (blood glucose <2.2 mmol/L) significantly elevates mortality in septic patients (Mitsuyama et al., 2022), consistent with the onset of coma and irreversible neurological injury when circulating glucose falls below this threshold (Liu & Gao, 2024). Carnivorous fish maintain substantially lower baseline glucose levels than

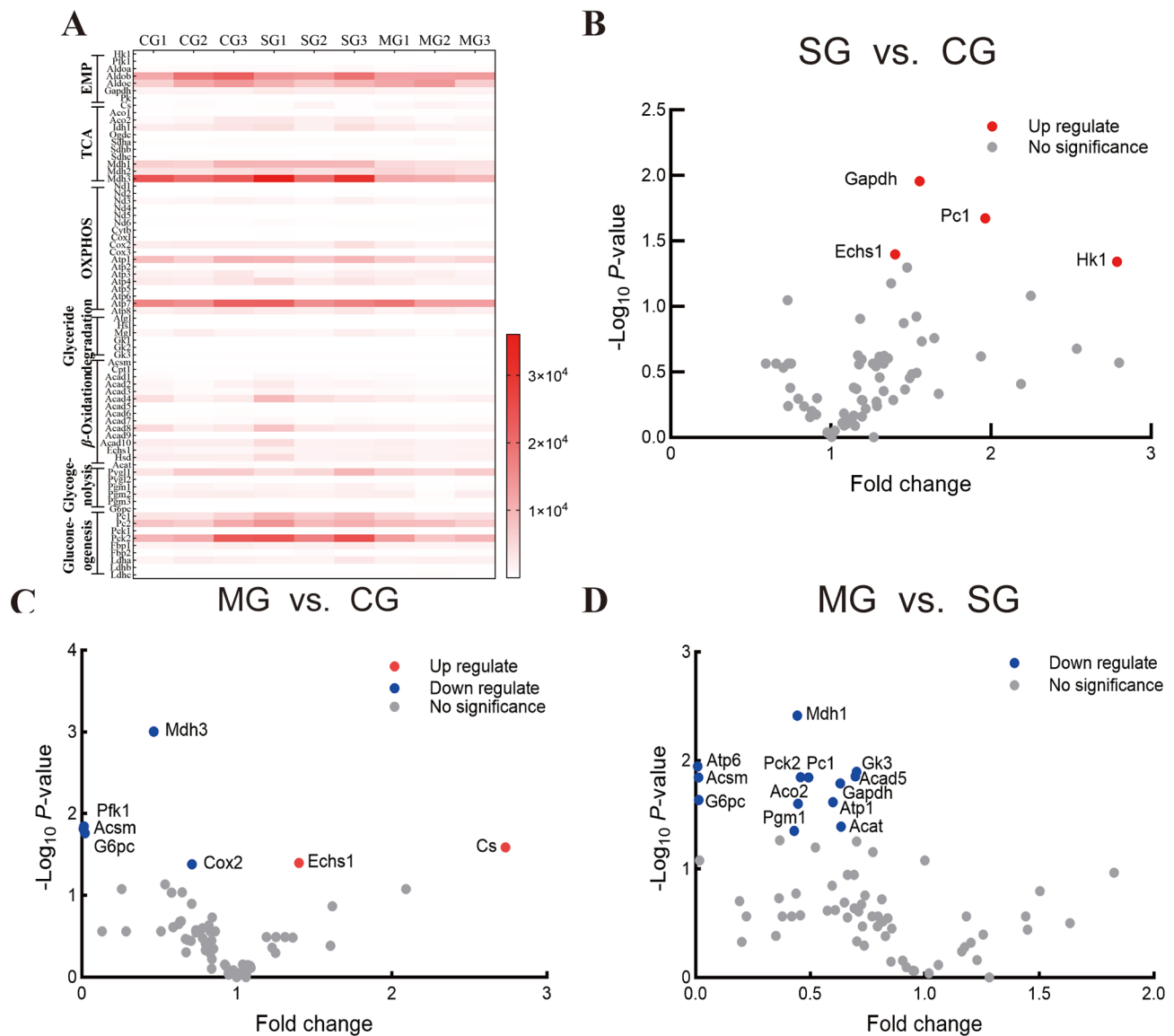


Figure 4 Protein expression pattern in the liver of large yellow croakers

A: Expression pattern of proteins related to energy metabolism. B–D: Volcano plot of differentially expressed proteins related to energy metabolism. B: SG vs. CG; C: MG vs. CG; D: MG vs. SG.

mammals, averaging approximately 3.5 mmol/L (Polakof et al., 2012). Hypoglycemia has been documented in species such as rainbow trout under hypoxic or pharmacological stress (Polakof et al., 2012). Their slow glucose turnover prolongs restoration of homeostasis, heightening vulnerability to infection-driven metabolic stress (Bever et al., 1981; Garin et al., 1987). In this study, *P. plecoglossicida*-infected large yellow croakers developed critically low glucose concentrations of 0.8 mmol/L (Figure 1I), a level far below the mammalian danger threshold. Sluggish glucose turnover also implies that infected large yellow croakers endure hypoglycemic periods far exceeding mammalian tolerance, likely exacerbating depletion of energy intermediates. This extended and severe hypoglycemic state highlights the large yellow croaker as a suitable vertebrate model for resolving the mechanisms underlying infection-induced hypoglycemia.

In response to infection, host metabolic networks undergo rapid reprogramming to sustain the energetic demands of immune activation (Krejčová et al., 2025). During early *P. plecoglossicida* infection, large yellow croakers exhibited significant transcriptional up-regulation of genes involved in

glycolysis, the TCA cycle, and oxidative phosphorylation, consistent with elevated ATP demand (Zhang et al., 2021). However, *P. plecoglossicida* diverts host glucose to fuel 2-ketogluconate biosynthesis (Sun et al., 2024a), exacerbating the metabolic burden in infected fish. As carbohydrate availability declined, extensive mobilization of FAs and GLs was observed (Figure 3F, G), indicating a compensatory shift toward lipid catabolism. In parallel, glucose was redirected to support a Warburg-like metabolic profile, as evidenced by intracellular L-lactate accumulation (Figure 2C, D), suggesting preferential glycolytic flux despite reduced energetic efficiency. Similar dual engagement of glycolysis and aerobic glycolysis has been described in white spot syndrome virus (WSSV)-infected shrimp (Su et al., 2014), underscoring the broader relevance of this strategy among pathogens. Although the precise role of the Warburg effect in *P. plecoglossicida* remains unclear, it appears insufficient to account for host mortality. Instead, systemic energetic failure likely results from mitochondrial collapse, linked to reduced expression of Aco2, Mdh1, Atp1, and Atp7, and disrupted glycemic regulation mediated by G6pc (Figure 1J, 4D). Proteomic analysis

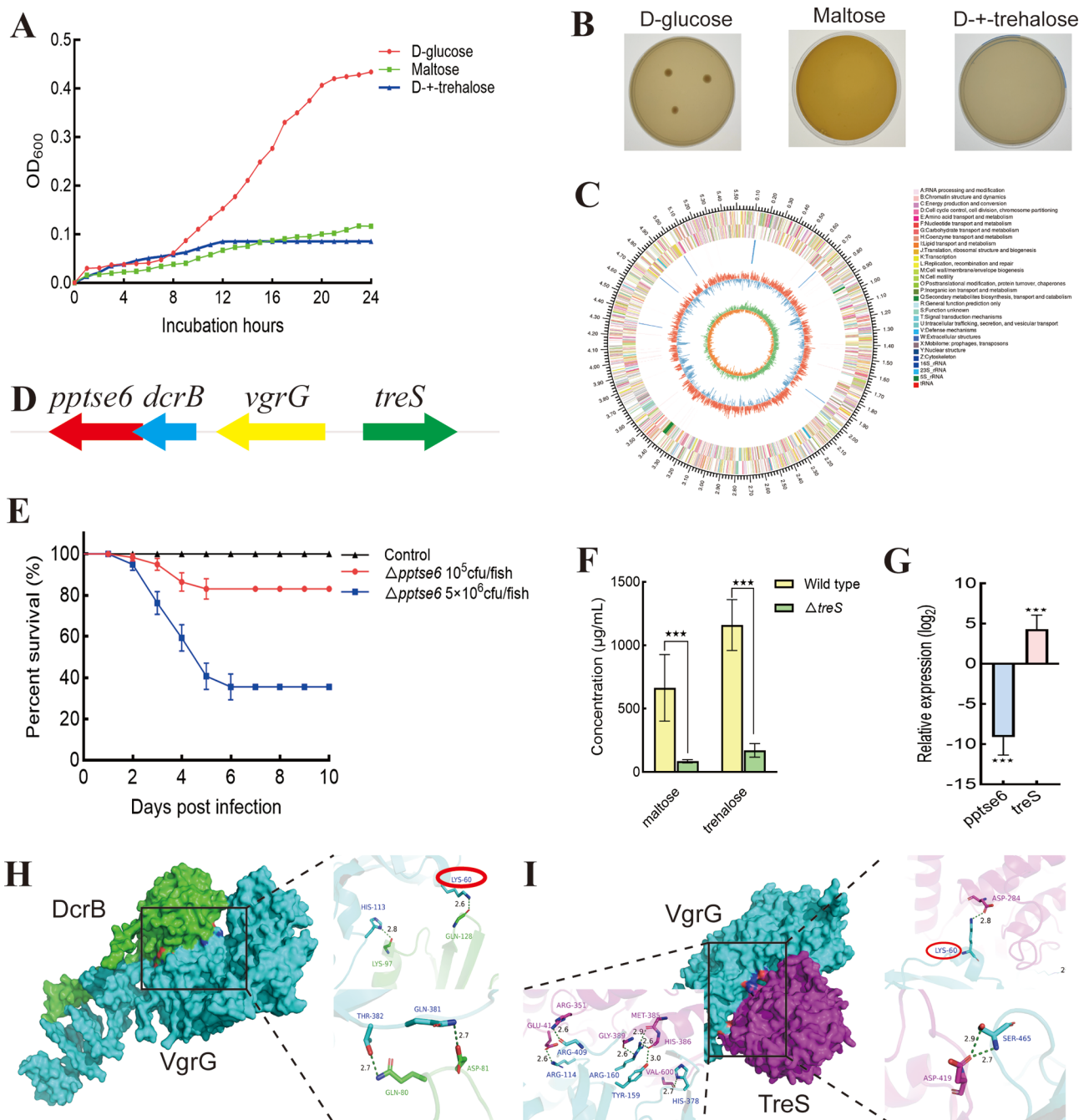


Figure 5 *Pseudomonas plecoglossicida* synthesizes but cannot utilize maltose or D-(+)-trehalose

A, B: *Pseudomonas plecoglossicida* cannot utilize maltose or D-(+)-trehalose. A: Growth curve; B: Incubation plate. C: Genomic mapping of *P. plecoglossicida*. D: Schematic of VgrG location. E: Mortality of large yellow croakers challenged with different doses of $\Delta pptse6$. F: Concentrations of D-(+)-trehalose and maltose in *P. plecoglossicida* culture supernatant. G: Expression levels of *pptse6* and *treS* in SG vs. MG. H&I: Molecular docking of VgrG and effectors. H: VgrG and DcrB; I: VgrG and TreS. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

revealed broader suppression of metabolic enzymes in the MG group, including *Gk3* and *Aco2*, which participate in multiple energy-producing pathways. Their expression remained relatively stable or slightly elevated in SG compared to CG but showed mild reductions in MG compared to CG, producing a clear divergence between MG and SG despite minimal differences against the CG baseline. This inverse regulation suggests divergent metabolic adaptation trajectories that may underlie differential survival outcomes. Coordinated expression patterns of these metabolic effectors may serve as prognostic indicators of host resilience, warranting targeted validation.

Glycogenolysis appeared unavoidable during infection (Figure 1H), supporting immune activity while simultaneously amplifying inflammatory signaling (Ma et al., 2020; Thwe et al., 2019). Although this pathway primarily generates ATP, it concurrently lowers the NAD⁺/NADH ratio, creating a feedback constraint that inhibits subsequent glucose utilization and promotes inflammatory output (Tang et al., 2024). Our prior work detected severe inflammatory activation in moribund fish (Tang et al., 2020), consistent with the marked glycolytic suppression observed under *P. plecoglossicida*-driven metabolic interference (Figure 1I, 7). Remarkably, a subset of croakers countered this disruption by engaging a glycogen-

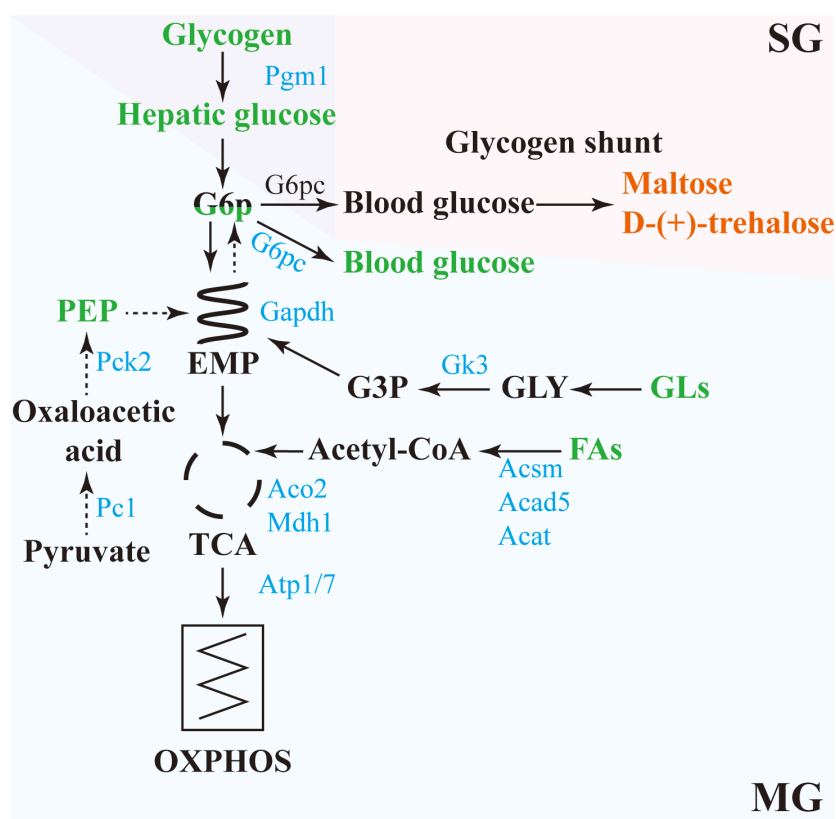
Although vertebrates lack the enzymatic machinery to synthesize maltose and D-(+)-trehalose (Argüelles, 2014; Attfield & Bell, 2003), these disaccharides can be metabolized via intestinal maltase and trehalase (Tjo et al., 2025). During controlled infection, large yellow croakers were isolated from natural environmental sources of these sugars, such as plants and insects, implicating endogenous microbial biosynthesis. The liver, harboring the highest *P. plecoglossicida* load (10^7 cfu) (Tang et al., 2020), likely served as a production site. Upon accessing hepatic glucose, *P. plecoglossicida* secreted TreS via T6SS to synthesize both maltose and D-(+)-trehalose (Figure 5F). Expression of TreS and the T6SS effector ppTse6 appeared conditionally segregated, suggesting that mutually exclusive virulence programs may modulate host outcomes. Gut microbes also represent a potential source of disaccharide production. Previous research has reported that *P. plecoglossicida* disrupts intestinal flora stability, with survival positively correlated with *Actinobacteria* dominance (>30%) (Li et al., 2020). *Thermophilic actinomycetes* convert

This study demonstrated that *P. plecoglossicida* exploited host energy metabolism—particularly glucose utilization—to induce profound hypoglycemia in large yellow croakers. Conversely, engagement of a glycogen shunt that produced maltose and D-(+)-trehalose appeared to stabilize glycemic control and enhance host resistance. A critical virulence factor, the T6SS effector ppTse6, was identified in *P. plecoglossicida*. Notably, its deletion resulted in an 808-fold reduction in its pathogenicity. These findings not only advance strategies for preventing VWSD but also deepen our understanding of infection-induced hypoglycemia in vertebrates.

The *Pseudomonas plecoglossicida* NZBD9 genome was uploaded to the GenBank SRA database under BioProjectID PRJNA1272058 and Science Data Bank under accession number 31253.11.sciencedb.i00139.00301.

Supplementary data to this article can be found online.

The authors declare that they have no competing interests.



Blue font indicates down-regulated proteins; green font indicates decreased carbohydrates and lipids; red font indicates increased carbohydrates. PEP: phosphoenolpyruvic acid; EMP: glycolysis; TCA: tricarboxylic acid cycle; OXPHOS: oxidative phosphorylation. Red font indicates an increase in substance content, green font indicates a decrease, and blue font indicates protein expression down-regulation.

AUTHORS' CONTRIBUTIONS

Y.J.S. and Q.P.Y. conceived and initiated the project. Y.J.S., X.Z.P., and H.J.T. carried out most experiments. Y.J.S., J.G.H., and Q.P.Y. wrote and revised the manuscript with input from all other authors. All authors read and approved the final version of the manuscript.

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