

Research article

gys1 regulates maternal glycogen reserve essential for embryonic development in zebrafish

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ARTICLE INFO

Keywords:

gys1
Glycogen synthesis
Embryonic development
Untargeted metabolomics

ABSTRACT

The reserve of glycogen is essential for embryonic development. In oviparous fish, egg is an isolated system after egg laying with all the required energy deposits by their mothers. However, the key regulated factor mediates the storage of maternal glycogen reserve which support for embryogenesis in the offspring is largely unknown. Glycogen synthase (GYS) is a central enzyme for glycogen synthesis. In our previous study, we generated a *gys1* knockout zebrafish line, showed an embryonic developmental defect in F₃ generation. In this study, firstly we determined that the *gys1* was maternal origin by backcrossing the F₂ mutant with wildtype lines. PAS staining and glycogen content measurement showed that glycogen reserve was reduced both in ovaries and embryos in the mutant group compared to wildtypes. Free glucose measurement analysis showed a 50 % of reduction in *gys1* mutant embryos compared to wildtype embryos at 24 hpf; showed an approximal 50 % of reduction in *gys1* mutant adults compared to wildtypes. Micro-injection of 2-NBDG in embryos and comparison of fluorescent signal demonstrated that glucose uptake ability was decreased in the mutant embryos, indicating an impaired glucose metabolism. Untargeted metabolomics analysis then was employed and revealed that key modified metabolites enriched into vitamin B pathway, carbohydrate and unsaturated fatty acid pathways. These results demonstrated that *gys1* played a role on glycogen metabolism, involved into the maternal glycogen reserve which essentially contribute to embryonic development.

1. Introduction

Glycogen serves as the main carbohydrate and energy reserve across animal phyla [1]. Glycogen is a branched polymer of glucose stores mainly in liver and skeletal, with a little amount store in brain and heart. The breakdown of liver glycogen is one the main source to generate glucose during fasting periods to maintain blood glucose level [2,3]. Muscle glycogen is directly utilized through glycolysis to maintain muscle activities [4]. In the heart and brain, glycogen is an energy substrate that can generate anaerobic energy during short-term oxygen deficiency contributing to survival [5].

During embryogenesis, glycogen is one of the main sources of ATP and intermediate substrates required for the progression of

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<https://doi.org/10.1016/j.heliyon.2024.e31149>

Received 10 August 2023; Received in revised form 10 May 2024; Accepted 10 May 2024

Available online 14 May 2024

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embryonic development. In viviparous animals, the glucose is stored as glycogen in placenta to support the high energy demand of development and growth of the fetus, especially before implantation [6–9]. Reduced placental glycogen content is frequently associated with fetal development and growth retardation [10]. Distinct from vivipara, oviparous animals like insect and fish, their eggs become closed and isolated from the environment after egg laying with all energy conversion taking place during embryogenesis [11]. During fish and insect oogenesis, their mother must deposit lipids, protein, carbohydrates which are required for all the posterior embryonic development [11]. The mutation of branching and debranching enzyme *GlyS* and *GlyP* in *Drosophila* caused semi-lethality during larval period [12]. The knockdown of glycogen synthase kinase (GSK-3) in Red flour Beetle (*Tribolium castaneum*) caused impaired embryogenesis [11]. Zebrafish (*Danio rerio*), which is a lecithotrophic organism, the development of the eggs rely on the nutrition stores in yolk until pharynges stage around 5 dpf (day post fertilization) [13]. The required glycogen mainly stored in the yolk which is maternally provided to meet the high energetic demand during fertilization, gastrulation, and organogenesis [14,15]. However, the key regulated factor mediates synthesis and storage of maternal glycogen reserve which support for embryogenesis in the offspring is largely unknown.

Glycogen metabolism is governed by evolutionarily conserved glycogen synthase and phosphorylase by the concerted action of branching enzyme and de-branching enzymes [2,16]. Mammals possess two genes to encode glycogen synthase, *Gys1* which is with its broad expression in the variety tissues of skeletal muscle, cardiac muscle, adipose tissue, brain, gonad and most other cells which are capable of synthesize glycogen [17,18], and *Gys2*, which restricts its expression in the liver [19,20]. These two paralog genes are both located in the genome of zebrafish. In the previous study, we established two mutant lines *gys1* and *gys2* via CRISPR/Cas9 system in zebrafish (*Danio rerio*). We found that the mutation of *gys2* is compatible with the development and growth in zebrafish in both F₂ and F₃ generation, while the mutation of *gys1* has no effect on the development and growth of F₂ fishes, but the F₃ mutant embryos delayed its development and displayed abnormalities with its mortality rate reached to 95 % by 48 hpf (hour post fertilization) [21]. Hence, we hypothesized that *gys1* probably function as an essential regulatory factor to deposit the glycogen during the oogenesis which is essential for embryonic development in the next generation. To address this, we firstly confirmed the maternal effect of *gys1* by backcrossing and we therefore aimed to investigate the cause for the observed mortality in F₃ embryos from *gys1*-deficient zebrafish. We found that glycogen reserve was decreased, glucose metabolism was impaired in both F₂ adult and F₃ embryo mutants, glucose uptake was decreased in embryo mutants. Untargeted metabolomics analysis of 24 hpf embryos revealed that modified metabolites enriched into vitamin B, carbohydrate and unsaturated fatty acid pathways involves into the glycogen anabolism network which contributes to the embryonic development. Our study suggested that *gys1* mediated glycogen reserve during the oogenesis, and the glycogen metabolism is essential for the embryonic development in the next generation.

2. Materials and methods

2.1. Zebrafish maintenance and mutant genotyping

This mutant line was produced by our group and reported previously [21] according to the CRISPR/Cas9 protocol in zebrafish as reported [22]. The target site located in the fourth exon of *gys1* DNA sequence, which caused a truncated protein with 267 amino acids by Cas9 protein. Specific oligonucleotide primers (Fw:5'- TCCTTATGACAAGAAGACTGTGTTCC-3' and Rv:5'- CTGATTGCACGGTCTCC-3') were designed to amplify a 489 bp fragment of the *gys1* region containing the target site of the mutation using RT-PCR to characterize the genotypes of fishes. Briefly, F₁ heterozygous *gys1*^{+/-} were incrossed and maintained in a mixed genotype population to get F₂ offsprings with all the genotypes for the following experiment and rule out density effect. Genomic DNA of F₂ fish tails of was extracted and amplified with the designed primers and confirmed the sequence by the first-generation sequencing. F₃ wildtype and mutant embryos were obtained from the incrossed of genotyped F₂ wildtype and mutant adult fish respectively. All the zebrafish embryos and mutants were maintained in a photoperiod of 14:10 (light/dark) in aquatic habitat system (AquaNeering, US) at a temperature of 28.5 °C ± 1 °C.

2.2. Cell apoptosis

F₃ *gys1* wild type and mutant embryos were anesthetized with tricaine (sigma-aldrich) at 24 hpf were fixed by 4 % paraformaldehyde (Sangon Biotech, Shanghai) overnight at 4 °C. Then embryos were washed dehydrated with gradient methanol. Cell apoptosis of embryos were determined followed by the construction of Click-iT™ Plus TUNEL Assay Kit (Invitrogen, US). Embryos were stained with 10 µg/ml DAPI (Invitrogen, US) solution for 10 min. The stained embryos were then washed with PBST and visualized by laser confocal microscopy (ZEISS, LSM710 N) at excitation of 350 nm.

2.3. Periodic acid-schiff (PAS) staining

After genotyped, the fish were anesthetized with tricaine (sigma-aldrich) and sacrificed, the ovaries of F₂ *gys1* WT and MT fish were dissected and fixed in Bouin's solution (Sangon Biotech, Shanghai) overnight at 4 °C. *gys1* WT and MT F₃ embryos at 48 hpf were collected and fixed with 4 % paraformaldehyde overnight. Then all the samples were dehydrated with gradient ethanol and embedded with paraffin at 55 °C after washing. The embedded blocks were sectioned and stained with Glycogen Periodic Acid Schiff (PAS/Hematoxylin) Stain Kit (Solarbio, Beijing) followed by the instruction. Photographs were taken under microscope (ZEISS, AxioScope 5). PAS positive area was pick and average staining intensity was analyzed by Image J image analysis software [23].

2.4. Glycogen measurements

24 hpf *gys1* WT and MT embryos, livers and ovaries from 180 dpf *gys1* WT and MT adult zebrafish were extracted with the extraction buffer provided in the glycogen content assay kit (Solarbio, BC0345). The glycogen contents were determined followed by the manual instruction of the kit.

2.5. Glucose measurements

120 dpf F₂ *gys1* WT and MT adult zebrafish were fasted for 48 h and then anesthetized with tricain (sigma-aldrich). 1 µl of blood was taken from the fishes from the tail and diluted 100 times with the provided 0.05 M sodium phosphate (pH = 7.4) buffer in the kit. Serum was obtained by centrifugation at 2350 g for 10 min at 4 °C. F₃ *gys1* WT and MT embryos at 24 hpf were collected and homogenized in 300 µl assay buffer, placed on ice for 1 h until the measurement. Free glucose was measured followed by the manual instruction of the Amplex™ Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, US).

2.6. RT-qPCR

Total RNA of liver from 120 dpf normally feeding WT and MT adults and 24 hpf embryos with different genotypes were extracted with Easstep® Super Total RNA Extraction Kit (Promega, US). cDNAs were synthesized using *Evo M-MLV* Reverse Transcription Kit II (Takara, Japan). Quantitative RT-PCR was performed using FastStart Essential DNA Green Master (Roche, Switzerland). Thermal cycling and fluorescence monitoring were performed in LightCycler® 480 II qPCR machine (Roche, Switzerland). The designed primers were shown in [Supplementary Table 1](#).

2.7. Glucose uptake

12 nL fluorescent glucose analog 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG, 0.1 mg/ml) was microinjected into the yolk of F₃ WT and MT embryos at 24 hpf (MCE, US) and incubated at 28.5 °C. After 1.5 h incubation, injected embryos were anesthetized with tricaine as describe by Jensen et al. [24] and subjected to Laser Confocal Microscopy (ZEISS, LSM710 N) to visualize the glucose uptake of the embryos at excitation of 488 nm.

2.8. Untargeted metabolomics analysis

180 embryos (about 80 mg) at 24 hpf were collected from WT and MT groups respectively (6 replicates for each group), transferred into 2-mL centrifuge tubes and chilled on ice to anesthetize and suppress enzymatic activity. Moisture was then removed, and samples were frozen in liquid nitrogen, and stored at −80 °C.

Metabolome extraction was performed as follows: samples were mixed with cold methanol/acetonitrile/water solution (2:2:1, v/v), degassing was achieved by sonication for 30 min to remove the oxygen in the solvent, the supernatant was vacuum dried after centrifuged to avoid oxidation of metabolites. The extraction was separated by ultra-high performance liquid chromatography system (UHPLC, 1290 Infinity, Agilent). The mass spectrometer analyses were performed on a Mass spectrometer (Q Exactive, Thermo) in both positive and negative ionization modes by electrospray ionization. Raw data were converted into mzXML files using ProteoWizard, and XCMS software was used for peak alignment and peak area extraction. The extracted data were evaluated for data pre-processing and experimental data quality evaluation. Data analysis is carried out, including statistical analysis of metabolite identification results, analysis of differences between groups and bioinformatics analysis of differential metabolites. For KEGG pathway enrichment, the differently expressed metabolites were blasted against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://geneontology.org/>) to retrieve their COs and were subsequently mapped to pathways in KEGG. The corresponding KEGG pathways were extracted. The analysis was performed by R (v 3.6.3).

2.9. Data analysis

GraphPad Prism (version 6.01) was used for the statistical analysis and graphs making. Datas are represented as the mean ± SEM. Sample sizes and statistical methods are specified in figure legend. Statistical significance was determined using a student's *t*-test. Survival curves was visualized by Kaplan-Meier plots, and statistical analysis was determined by Log-rank (Mantel-Cox) test. The statistical significance of KEGG pathway enrichment was determined by Fisher's Exact Test, the whole metabolites of each pathway were taken as background dataset.

3. Results

3.1. *gys1* is a maternal factor for zebrafish embryonic development

Nutrient factors for embryonic development in zebrafish are mainly maternally provided [13], we firstly validated that if the effect of *gys1* on embryonic development was maternal or not. We performed back cross as showed in [Fig. 1a](#), embryos produced by the F₂ *gys1* male mutant crossed with F₂ *gys1* female wild type siblings, underwent normal development and differentiation without any

morphological defects. While the embryos produced by the F₂ female *gys1* mutant crossed with F₂ male wild type siblings, showed delayed of development with its pericardial cavity enlarged by 24 hpf (Fig. 1a). After hatching, the embryos displayed phenotypes including brain necrosis, small eyes, curved body axis and reduced pigmentation (Fig. 1a). The first time point of a severe lethality appeared was at around 16–24 hpf, mortality rate reached to 95 % by 48hpf (Fig. 1b), which showed consistence with our previous results and confirmed a maternal effect of *gys1* gene [25].

3.2. Loss of maternal *gys1* promotes cell apoptosis in embryos

Since the mortality mainly occurred around 24 hpf, we then performed TUNEL staining on F₃ *gys1* WT and MT embryos to assess cell apoptosis at this time point. The TUNEL staining of the whole embryos showed that the apoptosis mainly emerged in the nervous system, tailbud and yolk sac (Fig. 2a). The positive TUNEL staining showed 2.5-fold of increase with cell apoptosis in *gys1* mutant embryos compared to wild type siblings with statistical significance (Fig. 2b). Previous study showed that number of apoptotic cells

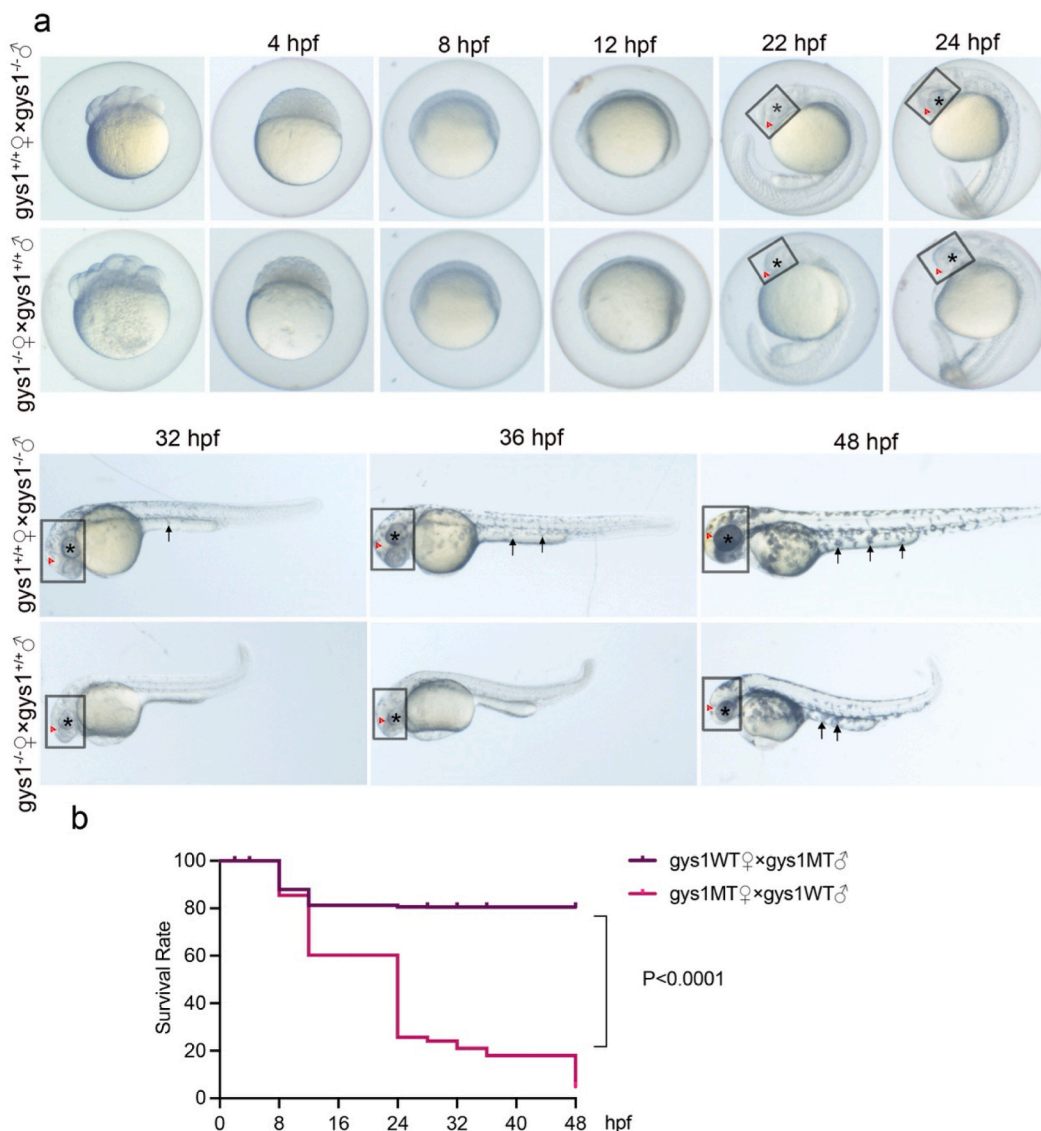


Fig. 1. Maternal *gys1* mutant embryos exhibit morphological defect and high mortality rate. (a) Bright-field images showing delayed embryonic development of maternal *gys1* mutant embryos from *gys1*^{-/-} ♀ cross with *gys1*^{+/+} ♂, in contrast to time-matched parental *gys1* mutant embryos from *gys1*^{-/-} ♂ cross with *gys1*^{+/+} ♀ from 2 hpf to 48 hpf. Small head phenotype was indicated in rectangles, brain necrosis was indicated with red arrows, small eye phenotype was indicated with asterisks, reduced pigmentation phenotype was indicated with black arrows. (b) Survival Rate of embryos from *gys1*^{-/-} ♀ cross with *gys1*^{+/+} ♂ (n = 1175) and *gys1*^{-/-} ♂ cross with *gys1*^{+/+} ♀ (n = 885) from 0 hpf to 48 hpf, survival rate was plotted by Kaplan-Meier method, Log-rank (Mantel-Cox) test showed p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

maintain at a low level in the nervous system and tailbud at 24 hpf, which indicated that the loss of *gys1* gene caused cell apoptosis in the embryos [26].

3.3. Loss of *gys1* reduced the glycogen reserve both in F2 ovaries and F3 embryos

Due to the board expression pattern of *gys1* in zebrafish, the global knockout of *gys1* should eliminate glycogen synthesis in various tissues as the mice model lacking GSY1 [27]. But if the maternal deficiency of *gys1* could affect the glycogen storage in the embryos of the next generation is uncertain. To address this, then we directly compared the glycogen contents in both F₂ ovaries and F₃ embryos. PAS staining showed that ovarian glycogen reserve in *gys1* mutant was 10 % significantly decreased compared to WT groups (Fig. 3a). Meanwhile, glycogen accumulation in F₃ whole embryo showed dramatical reduction (67 %) in mutant group compared to WT group at 48 hpf (Fig. 3b). In addition, the staining also showed that *gys1* WT ovaries contained full growth (FG) stage follicles and fewer primary growth (PG) and pre-vitellogenic (PV) stage follicles, while in the *gys1* MT ovaries contained no FG follicles and more PG and PV stage follicles (Fig. 3a). For further confirmation, we determined the glycogen contents from tissue extraction, found that both the glycogen levels in liver (64 %) and ovary (63 %) were reduced in mutants compared to wildtypes (Fig. 3c and d). Glycogen contents from embryo extraction at 24 hpf also showed an approximal 57 % reduction in mutants compared to wildtypes (Fig. 3e). These results, which is identical to the GYS1 mice knockout model with impaired glycogen synthesis in various tissues [27], indicated that the high mortality rate in the F₃ mutant embryos probably due to the reduction of glycogen reserve and fail to energetically support the embryonic development (Fig. 1)

3.4. Maternal loss of *gys1* impaired glucose metabolism in adults and embryos

Mice with deletion of *Gys1* showed impaired of glucose metabolism [28]. Hence, we sought to address that if the reduced glycogen content would affect glucose metabolism in zebrafish. Firstly, we compared the free glucose concentration in the plasma of F₂ adults and F₃ embryos at 24 hpf. Glucose measurement showed a 53 % reduction of plasma free glucose in F₂ mutants (Fig. 4a) and a 36 % reduction of free glucose in F₃ embryos at 24 hpf (Fig. 4c) compared to wild type siblings, which suggested impaired of glucose metabolism due to the decrease of glycogen reserve in *gys1* mutant. They also displayed dysregulated mRNA expression levels of genes involved in gluconeogenesis, glycolysis and glycogenolysis. Genes involved into gluconeogenesis including *g6pca.1*, *pck1* and *pck2* are dramatically increased in the mutants of both embryos and adults (Fig. 4b and d). *pklr* and *pygl* involved into glycolysis also up regulated in the mutant adults, but with no changes in embryos (Fig. 4b and d). No changes were observed in both *ugp2b* and *gsk3ba* in the mutant adults (Fig. 4b), but *ugp2b* was slightly increased in embryos (Fig. 4 d). The changes of mRNA levels of these genes confirmed an impaired glucose metabolism phenotype in the mutant due to the loss of *gys1*.

3.5. Maternal loss of *gys1* attenuated the glucose uptake in mutant embryos

With the indication of impaired of glucose metabolism due to the loss *gys1*, we tried to assess the ability of glucose uptake from yolk in F₃ *gys1* MT embryos. With the indicate of 2-NBDG [29], which is a fluorescent glucose analog, enable us to visualize dynamic embryonic glucose uptake *in vivo*. We injected same amount of 2-NBDG analog into the yolk of *gys1* WT and MT embryos at 24 hpf. After 1.5 h post microinjection, we observed fluorescent signal diffused into the trunk from yolk in the WT embryos, whereas the diffused fluorescent signal was relatively weak in the mutant group (Fig. 5a). We then compared the fluorescent intensity found that the signal is 80 % reduced with significant statistical difference between wildtype and mutant group (Fig. 5b), demonstrated that the glucose uptake ability is impaired in the *gys1* mutant embryos. In agreement with mice, the glucose uptake ability is also impaired in muscle due to the knockout of *GYS1* [28].

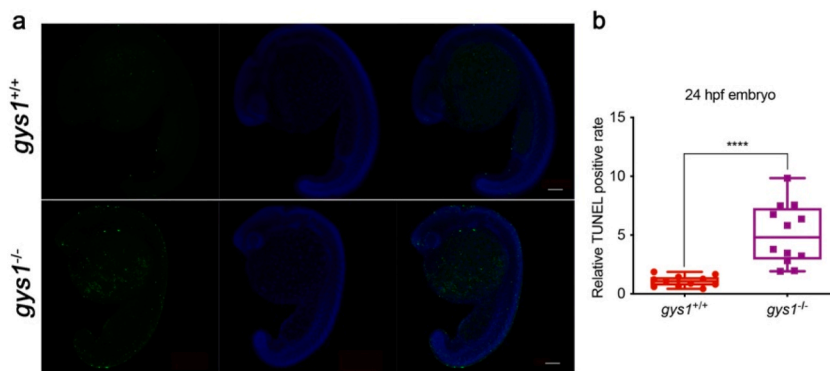


Fig. 2. Loss of maternal *gys1* promotes cell apoptosis in embryos. (a) TUNEL (green) and DAPI (blue) staining of F₃ *gys1* WT and *gys1* MT embryos at 24 hpf. (b) Unpaired *t*-test statistical comparison of TUNEL positive rate in *gys1* WT and MT embryos at 24 hpf ($n = 12$, $***P < 0.001$), Scale bar = 100 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

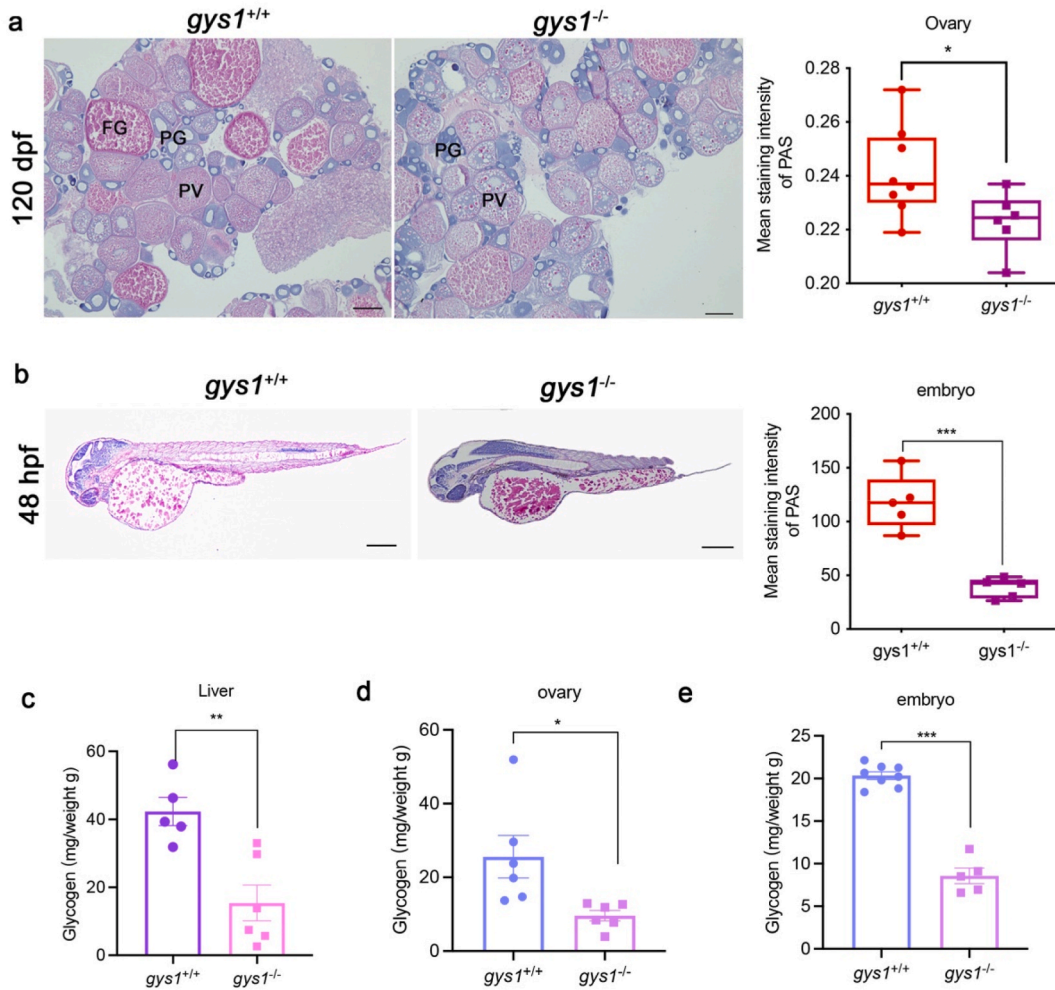


Fig. 3. Maternal loss of *gys1* caused reduced of ovarian (F₂) and embryonic (F₃) glycogen reserve. (a) Representative photographs of PAS glycogen staining in ovaries of F₂ *gys1* WT and *gys1* MT (left) in adults; Unpaired *t*-test statistical analysis of average PAS staining intensity in F₂ *gys1* WT and *gys1* MT ovarian glycogen staining (right, *gys1*WT n = 8, *gys1*MT n = 7, Scale bar = 200 μm **P* < 0.05). PG: primary growth stage; PV: pre-vitellogenic stage; FG: full growth stage. (b) Representative photos of PAS glycogen staining in F₃ *gys1* WT and *gys1* MT embryos at 48 hpf (left). Unpaired *t*-test statistical analysis of average staining intensity analysis of glycogen accumulation in embryos (right, *gys1*WT n = 5, *gys1*MT n = 5, Scale bar = 200 μm, ****p* < 0.001). (c–e) Glycogen content (mg) per weight (g) analysis of liver (*gys1*WT n = 5, *gys1*MT n = 6, ***p* < 0.01), ovary (*gys1*WT n = 6, *gys1*MT n = 6, **p* < 0.05) in 180 dpf adults and 24 hpf embryo (*gys1*WT n = 8, *gys1*MT n = 8, ****p* < 0.001). Unpaired *t*-test was used as the statistical analysis.

3.6. Metabolomic profile changed in *gys1* mutant embryos

Metabolic profiles remodeling the early mouse embryo development [30]. Due to the reduced glycogen reserve, impaired glucose metabolism and reduced glucose uptake, we further seek to analyze the altered metabolites between *gys1* WT and MT group to identify changed metabolites and metabolic signaling pathways which are involved into the glycogen metabolism essential for the embryonic development mediated by *gys1* signaling. We performed mass spectrometry analysis to the extraction from *gys1*WT and MT line embryos at 24 hpf due to the high mortality rate of the mutant embryos afterwards. The quality of the data has been summarized in [Supplementary Table 2](#). We detected total 1640 metabolites in the two groups by UHPLC-Q-TOF MS, with 66 metabolites showed significantly different contents, in which 37 metabolites were upregulated while 29 metabolites were downregulated in the mutant group compared to wild-type. As previous study [31], these metabolites mainly enriched into metabolic pathways including vitamin B metabolism, carbohydrate metabolism, lipid metabolism, amino acid metabolism, others are enriched into endocrine system, cell growth and death, nucleotide metabolism. KEGG pathway enrichment showed that 9 pathways are significantly modified ([Fig. 6](#)), biosynthesis of unsaturated fatty acids, beta-alanine metabolism, vitamin B6 metabolism pathways showed most significance among the 9 enriched modified KEGG pathways ([Fig. 6](#)).

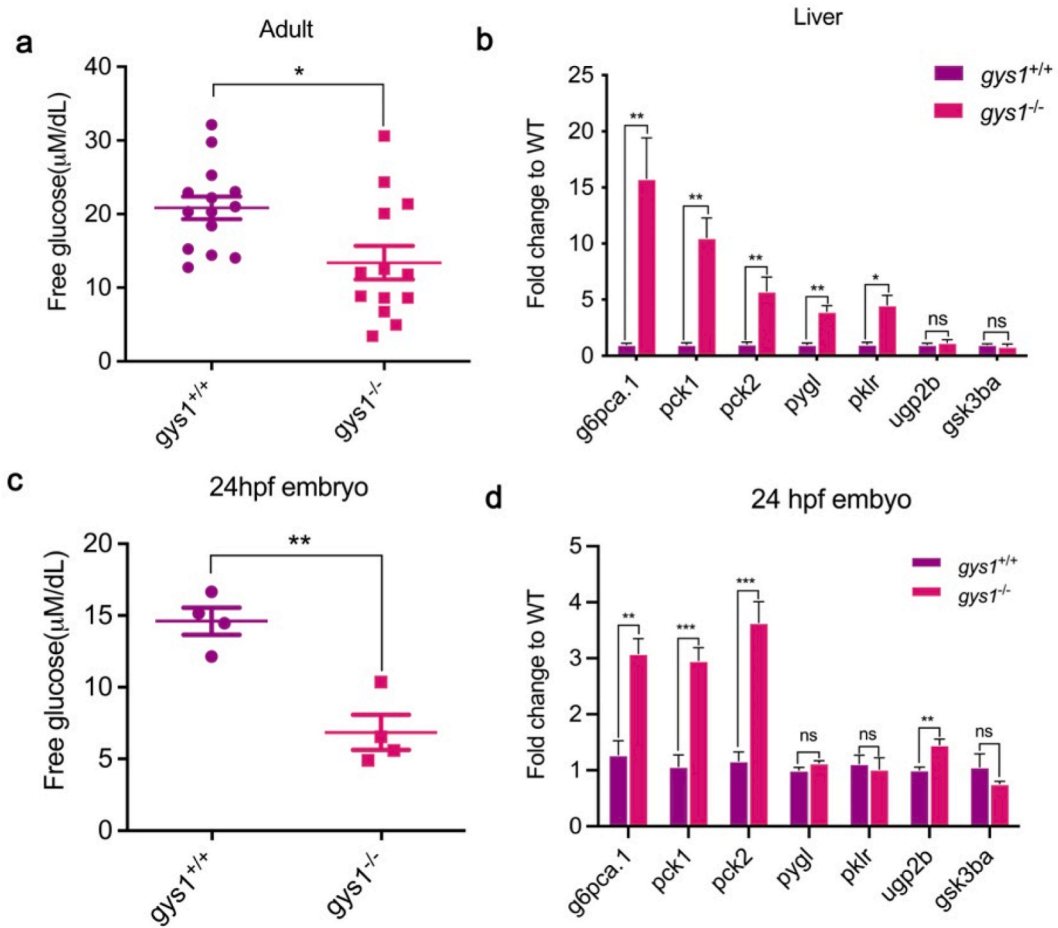


Fig. 4. Loss of *gys1* impaired the glucose metabolism in zebrafish. (a and c) Plasma free glucose levels in 120 dpf F₂ adults (*gys1* WT n = 14, *gys1* MT zebrafish n = 14) and F₃ embryos at 24 hpf (*gys1*WT n = 4, *gys1*MT n = 4). (b and d) Loss of *gys1* impaired the mRNA levels of glucose metabolism related genes in 120 dpf adult liver (n = 4) and embryos (n = 4), respectively. Unpaired *t*-test was used as the statistical analysis. Gluconeogenesis genes: *g6pca.1* glucose-6-phosphatase catalytic subunit 1a; *pck1* phosphoenolpyruvate carboxykinase 1; *pck2* phosphoenolpyruvate carboxykinase 2 (mitochondrial), Glycolysis genes: *pkfr* pyruvate kinase L/R; *pygl* phosphorylase, glycogen, liver; Glycogenolysis and glycogen synthesis genes: *ugp2b* uridine diphosphate glucose pyrophosphorylase; *gsk3ba* glycogen synthase kinase 3 beta. (**p* < 0.05 ***p* < 0.01 ****p* < 0.001 *****p* < 0.0001 ns: no significant difference).

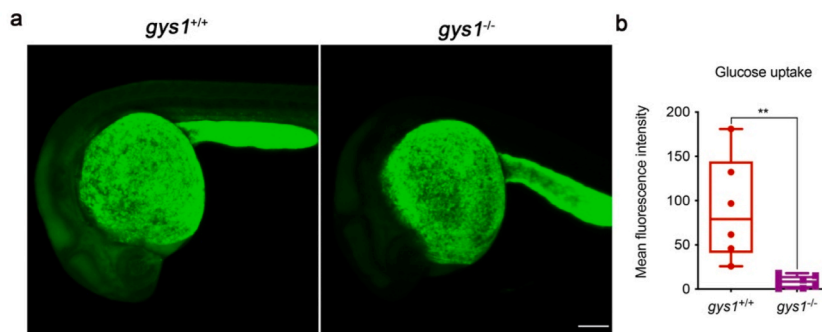


Fig. 5. The glucose uptake ability is reduced in F₃ *gys1* mutant embryos. (a) Representative images showing the glucose uptake after microinjection of 2-NBDG in embryos. (b) Average fluorescent intensity analysis of glucose uptake (*gys1*WT n = 6, *gys1*MT n = 7, ***p* < 0.01). Unpaired *t*-test was used as the statistical analysis.

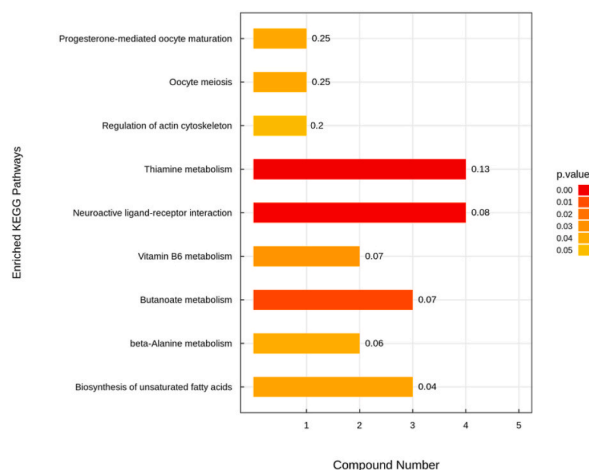


Fig. 6. Enriched KEGG pathway analysis revealed by Untargeted metabolomics. KEGG pathway analyzed with MetaboAnalyst 5.0 for the different expressed metabolic profiles between *gys1* WT and MT at 24 hpf. p value was determined by Fisher's Exact Test and indicated on the right. Compound number represents to the number of differentially expressed metabolites contained in each KEGG metabolic pathway.

Table 1
Significantly different metabolites in the positive ion mode.

Name	VIP	FC	p-value	m/z	rt(s)
Glu-His	2.015	1.439	0.000003	267.11	348.27
Thiamine	11.599	1.511	0.000056	265.11	333.56
Cytidine 5'-diphosphocholine	2.201	3.277	0.000067	489.10	428.98
5-(2-hydroxyethyl)-4-methylthiazole	4.122	1.476	0.000075	144.05	333.59
Arg-Val	1.208	0.519	0.000082	274.18	364.94
Bendiocarb	3.102	2.049	0.000120	167.09	129.56
Lys-Val	1.174	0.600	0.000129	246.17	383.71
Fluconazole	1.416	0.624	0.000262	307.10	340.11
Phenylacetaldehyde	1.243	0.710	0.000277	103.05	245.76
Tectoridin	1.157	0.392	0.000712	463.13	331.41
2-amino-1-phenylethanol	4.134	0.733	0.000740	120.08	245.80
DL-phenylalanine	3.222	0.745	0.000764	166.08	244.86
Pro-phe	1.172	2.339	0.001839	263.14	180.36
L-saccharopine	4.264	1.611	0.001896	277.14	436.42
Digoxigenin bisdigoxoside	1.245	0.370	0.004003	673.36	399.36
Cinoxacin	1.117	2.192	0.004144	263.09	202.22
DL-arginine	6.879	0.704	0.005691	175.11	493.54
Arg-Thr	1.007	0.602	0.006068	276.16	392.18
Xanthorhamnin	1.359	1.258	0.006955	809.18	243.60
Diacetyl	1.000	1.908	0.012424	87.04	224.00
Acetylcholine	2.635	1.958	0.013127	146.11	224.00
Guanine	1.265	0.715	0.014179	152.05	252.65
DL-tyrosine	2.165	0.713	0.014337	182.08	289.49
Flavone base + 3o, c-hex-feruloylhex	1.506	0.743	0.016302	771.22	245.16
Phe-pro	1.089	0.216	0.017219	263.13	225.47
5-methylbenzotriazole	2.259	1.553	0.019835	134.06	108.81
Pseudojervine	1.478	0.368	0.019837	588.35	378.71
3-hydroxybutyric acid	1.295	1.156	0.019905	87.04	359.78
N-acetyl-D-glucosamine	2.491	0.711	0.020127	204.09	250.24
4-Aminobutyric acid (GABA)	1.292	1.153	0.021526	104.07	359.77
Pyridoxine	6.365	1.639	0.021813	170.08	109.50
Eszopiclone n-oxide	1.316	1.388	0.027497	809.20	235.95
2,6-di-tert-butyl-4-hydroxymethylphenol	3.071	0.121	0.029190	219.17	28.40
Juglone	2.491	0.456	0.031933	175.05	234.54
D-sorbitol	1.380	0.676	0.033600	165.05	289.43
DL-octopamine	1.304	0.745	0.035500	136.07	289.46
(-)-norepinephrine	4.212	1.632	0.035871	152.07	109.05
Pyridoxal phosphate	1.529	1.460	0.035916	150.05	51.29
Formononetin	2.845	1.385	0.046163	559.13	210.59

4. Discussion

In the present study, we verified that *gys1* functioned as a mediator on glycogen metabolism, which is required for embryonic development in zebrafish. The loss of *gys1* decreased glycogen reserve in both F₂ ovaries and F₃ embryos, and glucose uptake, caused impaired glycogen metabolism and metabolic profile changes in vitamin B metabolism, carbohydrate metabolism and unsaturated fatty acid metabolism pathway.

The first time point of a severe lethality appeared around at 16–24 hpf in *gys1* mutant group (Fig. 1b). In mammals, glucose uptake by embryos is low from fertilization until 8-cell age and before compaction, too much glucose is toxic, around the morula stage glucose uptake starts to increase and is dramatically higher by the blastocysts stage [32,33]. In zebrafish, metabolic profiling changes study showed that the levels of glucose and glucose-6-phosphate, galactose, fructose and myo-inositol, amino acids and ribose are elevated at 24 hpf compared to early stage, indicating a sign of increase in energy demand [31]. This explained occurrence of a high lethality at around 24 hpf in *gys1* mutant, a time point that energetic demand is highly demanded while the energetic support in the mutant is insufficient due to low reserve of glycogen. Consequently, we confirmed that carbohydrate metabolism is impaired in the *gys1* mutant group as glucose levels in adults and embryos are decreased, and the ability of glucose uptake is reduced. Untargeted metabolomics analysis confirmed that carbohydrate metabolites including Udp-*n*-acetylglucosamine, *n*-acetyl-D-glucosamine, ribitol, D-sorbitol are all down regulated and secondary glucose metabolites D-psicose and aloenin were significantly affected (Tables 1 and 2), suggests metabolic fuel of carbohydrate metabolism are downregulated due to the decreased glycogen reserve in the *gys1* mutants. However, previous metabolomics glucose is elevated at 24 hpf compared to the very early stage to support the embryonic development [31], which provide further evidence of insufficient energetic deposit fail to meet the high energetic demand for embryogenesis at around 24 hpf eventually lead to the lethality. Consistently, *Gys1*-KO mice display reduced glucose turnover and muscle glucose uptake, insulin resistance [28]. *GYS1*-deficiency in human is extremely rare. A Japanese girl with a deficiency of *GYS1* gene who had been suffering from recurrent attacks of exertional syncope accompanied by muscle weakness and pica since age 5 year, and died of cardiac arrest at age 12, which pointed that *GYS1* plays an essential role in development [34]. Taken together, these data physiologically supported that the high lethality in *gys1* mutant is due to low maternal glycogen reserve and impaired glycogen metabolism in between generations, further pointed the view that glycogen reserve mediated by *gys1* essential for embryonic development which could be evolutionary conserved.

Then we sought to identify the modified metabolites that caused high lethality and abnormality in embryos, trying to illustrate the key metabolic pathways safeguard embryogenesis in zebrafish. The most significant enriched pathway is the biosynthesis of unsaturated fatty acids (Fig. 6), related metabolites including gamma-linolenic acid and linolenic acid are significantly modified with 2-fold of increase in the mutant group compared to wildtype group (Table 2). Unsaturated fatty acid is essential for the embryonic development not only in bovine and cows but also in chicks and fish [31,35,36]. Linoleic acid (LA) is the predominant n-6 polyunsaturated fatty acid, the treatment of LA retarded the bovine oocyte maturation and resulted in a significant lower percentage of cleaved embryos and blastocyst yield in a dose depend manner [37], which showed good alignment with our mutant that enhanced of LA impaired the embryonic development. In accordance with our result, the elimination of glycogen synthesis would result in an improvement of lipid accumulation in human and mice [38,39]. Glycogen storage disease patient which was accompanied with over accumulation of glycogen showed increased free fatty acids and abnormal fatty acid profiles in physiological levels, PPAR signaling pathway, fatty acid biosynthesis, lipid synthesis were modified in transcriptome levels [40]. In terms of our mutant, the failure of depositing sufficient glycogen due to the loss of *gys1*, impaired glucose metabolism, eventually caused unsaturated fatty acid metabolism disorder, accumulated excess LA, which probably lead the death of the mutant embryos.

Another interesting pathway is vitamin B6 pathway, in which both metabolites pyridoxine and pyridoxal phosphate were up regulated. Correlatively, metabolites involved in thiamine pathway (which is also called vitamin B1) include thiamine, pyridoxal phosphate (PLP), 5-(2-hydroxyethyl)-4-methyl-thiazole, and DL-tyrosine were all up-regulated with a 1.5-fold change, except DL-tyrosine down regulated with 0.7-fold-change (Tables 1 and 2). Although KEGG analysis showed failed to show statistical significance of both pathways, with vitamin B6 pathway were on the edge of statistical significance ($p = 0.07$). Both enrichment of these two pathways and the upregulated metabolites pointed to the view that vitamin B metabolism is enhanced in the mutant line. Vitamin B is a coenzyme of glycogen phosphorylase and requires for the glycogen degradation in rats and human [41,42]. The mutant enhanced vitamin B pathway is probably a fitness to intensify the glycogen phosphorylation and degradation to meet the high energetic demand during embryogenesis due to the decreased glycogen reserve.

In summary, our results demonstrated that *gys1* functions as a branching enzyme contribute to glycogen deposit during the oogenesis in the adult which is maternally essential for embryonic development in offspring. And inherited disorder of glycogen metabolism caused by deficiency of *gys1* enzyme led to aberrant glycogen storage and utilization, consequently followed by disorders of carbohydrate, vitamin B, unsaturated fatty acid metabolic pathways. To our knowledge, this is the first work demonstrated *gys1* mediates the glycogen metabolism and reserve inter between two generations, in fish. We clarified that maternal nutrition environment, which is essential for embryonic development, would help to improve the egg quality and embryonic development in breeding production and conservation. The limitation of present work is that we didn't networks among carbohydrate, vitamin B, and unsaturated fatty acid metabolism which is essential for embryonic development, further work needs to contribute efforts to illustrate and integrate the networks.

Ethical declarations

All animal procedures of this research were conducted in compliance with ethical standards and were approved by the Animal

Table 2
Significantly different metabolites in the negative ion mode.

Name	VIP	FC	p-value	m/z	rt(s)
4-[2-[(2-ethyl-2,3-dihydroxybutanoyl)oxymethyl]anilino]-4-oxobutanoic acid	3.067	2.621	0.000058	352.14	336.77
Cinobufagin	1.318	4.959	0.000109	487.25	321.94
Progesterone	4.107	0.480	0.000652	313.24	141.12
Citramalic acid	1.171	1.792	0.000745	207.05	333.32
Neomangiferin	1.419	1.970	0.000784	583.12	359.36
Rutarensin	1.390	1.740	0.001954	657.16	360.62
His-Pro	1.926	1.833	0.002120	251.10	317.90
2,6-di- <i>tert</i> -butylphenol	2.139	1.859	0.002497	205.16	29.14
.gamma.-linolenic acid	6.469	2.125	0.004538	277.22	98.78
1-(9z,12z-octadecadienoyl)-2-hydroxy- <i>sn</i> -glycero-3-phosphoethanolamine	13.487	3.101	0.006684	476.28	161.92
Mevalonic acid	3.564	1.607	0.006896	147.07	340.73
Ginkgotoxin	3.043	2.084	0.008587	150.06	105.93
D-arabitol	1.131	1.594	0.011965	101.02	72.48
Linolenic acid	15.082	1.974	0.013786	277.22	47.09
3,3',4,5'-Tetrahydroxy-trans-stilbene	1.250	2.224	0.016702	487.13	157.88
Ile-Asp	1.431	0.535	0.017624	245.11	359.84
1,3-cyclobutanedicarboxylic acid, 2,4-diphenyl-, 1-(1-naphthalenyl) ester, (1.beta.,2r,3.alpha.,4r)-	1.050	1.676	0.017848	421.17	215.64
D-psicose	1.019	0.506	0.018908	179.04	110.62
Orotate	1.489	1.773	0.019943	155.01	222.15
Gly-His	2.011	0.689	0.022277	211.08	364.76
Aloenin	1.239	1.601	0.025198	819.22	392.24
Ribitol	10.878	0.484	0.025372	151.06	236.08
(2-acetyloxy-3,6-diphenylcyclohexyl) acetate	1.209	0.368	0.031782	351.18	37.11
N-Acetyl-L-alanine	1.505	1.612	0.034078	130.05	333.98
Palmitic acid	25.946	0.483	0.039361	255.23	48.00
Udp- <i>n</i> -acetylglucosamine	9.749	0.546	0.041088	606.07	421.37
Propiolic acid	1.795	0.149	0.048396	69.00	26.71

Research and Ethics Committee of Shanghai Ocean University under protocol SHOU-DW-2-2020-027.

Data availability statement

Data will be available upon request.

CRediT authorship contribution statement

Jie Chen: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Xiao Ji:** Writing – original draft, Visualization, Validation, Methodology, Data curation. **Jing Gao:** Visualization, Methodology, Data curation. **Jiao Huang:** Investigation, Data curation. **Jianfeng Ren:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We acknowledge for the funding provided by Shanghai Municipal Government to Shanghai Ocean University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e31149>.

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