frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 10 October 2023
DOI 10.3389/fmars.2023.1243767

OPEN ACCESS

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RECEIVED 21 June 2023
ACCEPTED 27 September 2023
PUBLISHED 10 October 2023

CITATION
Zhao Z, Zhao Q, Wang H, Wei L, Wang S, Li S, Yuan D and Wang Z (2023) Integrated transcriptomic and metabolomic analyses identify key factors in the vitellogenesis of juvenile Sichuan bream (*Sinibrama taeniatus*). *Front. Mar. Sci.* 10:1243767. doi: 10.3389/fmars.2023.1243767

Integrated transcriptomic and metabolomic analyses identify key factors in the vitellogenesis of juvenile Sichuan bream (*Sinibrama taeniatus*)

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Vitellogenesis is the most important stage of ovarian maturation in fish, involving the synthesis and transport of essential yolk substances and their complex mechanisms and coordination process. The liver is the main tissue involved in the vitellogenesis of oviparous animals, but studies of vitellogenesis in fish rarely include the liver and ovary as a whole. The aim of this study was to explore the molecular mechanism and associated regulatory factors behind vitellogenesis in Sichuan bream (*Sinibrama taeniatus*). The different stages of oogenesis were first identified by successive histological observations. Then, ovary and liver tissues that developed to 115 days (stage II, previtellogenesis stage), 165 days (stage III, vitellogenesis stage) and 185 days (stage IV, late-vitellogenesis stage) were collected for transcriptomic and metabolomic analyses, and serum testosterone (T), 17β-estradiol (E_2), vitellogenin (Vtg), triiodothyronine (T_3), and thyroxine (T_4) levels were measured at the corresponding stages. We found that energy redistribution during vitellogenesis is mainly regulated through glycolysis, fatty acid biosynthesis and the citrate cycle pathway. In the liver, energy metabolism was promoted by activating glucolipid metabolic pathways to provide sufficient ATP, but at the same time, the ovary tends to retain nutrients rather than decompose them to produce energy. In addition, we have identified several key factors involved in the metabolism of neutral lipids, polar lipids, amino acids and vitamins, which are involved in the assembly and transport of important yolk nutrients. The initiation of vitellogenesis was found to be associated with a surge in serum E_2 levels, but the sustained increase in Vtg levels in the late stage may be due more to upregulation of the estrogen receptor. These results provide valuable information about the regulation of ovarian development in cultured fish.

KEYWORDS

Sinibrama taeniatus, vitellogenesis, vitellogenin, transcriptomics, metabolomics Sinibrama taeniatus, metabolomics

1 Introduction

The process of vitellogenesis in teleosts is generally characterized by the synthesis and secretion of vitellogenin (Vtg) by the liver under estrogen stimulation, its transportation to the ovaries through blood circulation, uptake by growing oocytes, and subsequent processing into yolk proteins (YPs) (Kime, 1993; Lubzens et al., 2017). As the primary precursors of YPs, Vtg is the main source of polar lipids and amino acids nutrition in oocytes (Finn and Fyhn, 2010). Additionally, Vtg functions as a specialized carrier of lipid soluble vitamins, inorganic ions and hormones (Babin et al., 2007; Lubzens et al., 2017). The process of vitellogenesis in fish represents a crucial and intricate stage of oogenesis, involving morphological and structural alterations in oocytes (Tyler and Sumpter, 1996; Le Menn et al., 2007), as well as the coordination and allocation of nutrient and energy reserves within the organism (Perez et al., 2007). These factors collectively determine the nutritional status of eggs and subsequently impact offspring survival (Hiramatsu et al., 2015).

The causes of vitellogenesis are intricate and dynamic (Hiramatsu et al., 2013), necessitating a comprehensive understanding of the underlying mechanisms and coordinated processes to enhance species reproduction management and conservation. Previous studies have elucidated the prevailing vitellogenesis pattern, contingent upon the regulation of the hypothalamic-pituitary-gonadal (HPG) axis (Nagahama and Yamashita, 2008; Grier et al., 2009; Levavi-Sivan et al., 2010). The hypothalamus mediates the transmission of crucial signals from the central nervous system through the release of gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH), ultimately inducing estradiol (E_2) secretion in the ovaries (Zohar et al., 2010). E_2 forms dimers with the estrogen receptors, guiding Vtg synthesis in the liver and subsequent release into the circulation. Then, Vtg is incorporated into the developing oocyte to support its growth (McBride et al., 2015).Factors that control vitellogenesis in fish may affect the HPG axis at different levels, leading to the initiation or delay of development (Good and Davidson, 2016). The previous studies have generally concluded that vitellogenesis is directly regulated by E_2 *in vivo*, as evidenced by the progressive increases in plasma FSH levels and follicular *fshr* mRNA expression, both of which enhance pituitary-to-ovarian signaling, thereby further promoting E_2 synthesis (Luckenbach et al., 2008; Guzman et al., 2014). In the endocrine regulation of the ovary, StAR, Cyp1a1, Hsd3 and Cyp19a1 proteins appear to be common targets of gonadotropin-mediated upregulation of expression during the vitellogenesis stage (Ings and van der Kraak, 2006; Kagawa et al., 2003; Nakamura et al., 2003; Montserrat et al., 2004; Kazeto et al., 2006; Zhang et al., 2013). In addition to sex hormones, there is increasing evidence of a cross-talk between the reproductive and thyroid systems in teleosts due to the fact that thyroid hormones often exhibit seasonal rhythms associated with reproduction (Duarte-Guterman et al., 2014; De Vincentis et al., 2018). The administration of triiodothyronine (T_3) in goldfish significantly enhanced the expression of estrogen receptor alpha (ERα) in the liver, thereby promoting an increase in Vtg levels through its interaction with E_2 (Nelson and Habibi, 2016). The field of nutrigenomics believes that ensuring adequate body nutrition and lipid reserves can affect vitellogenesis via nerve center regulation of gene expression (Taranger et al., 2010; Sullivan and Yilmaz, 2018). The addition of 8% fish oil to the diet of spotted scat (*Scatophagus argus*) compared to 8% soybean oil can effectively promote the accumulation of yolk in the ovaries by regulating the expression of genes related to steroid hormones, glucose and lipid metabolic pathways, with an increased proportion of n-3 long chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), in the ovaries (Wang et al., 2022). Meanwhile, dietary supplementation with fish oil increased the serum vitellogenin levels of female spotted scat and upregulated the expression of the reproduction-related genes *vtga*, *vtgb*, and *zp4*, as well as *fabp2* and *mfsd2ab*, which are related to lipid absorption and transport in the liver (Wang et al., 2021).

China is the world's largest aquaculture producer, and traditional carp species occupy an important share of the inland aquaculture industry (Tacon and Metian, 2015; Edwards et al., 2019; Newton et al., 2021). The long time required for ovarian maturation of carp species, especially before vitellogenesis (Sehgal and Toor, 1995; Song and Wessel, 2005), has significantly limited the development of the aquaculture industry. Therefore, investigating the molecular mechanisms that initiate vitellogenesis and seeking regulation is an unavoidable issue in aquaculture production. In oviparous vertebrates, the liver plays a crucial role in gonadal development and is primarily responsible for synthesizing and providing essential nutrients during vitellogenesis to support oocyte growth (Levi et al., 2009; Xu et al., 2016; Gloux et al., 2019). However, studies on fish vitellogenesis often separate the ovaries from the liver (Osachoff et al., 2016; Xu et al., 2016; Bertolini et al., 2020; Meng et al., 2022), and only a few reports on crustacean aquatic animals have combined them to investigate the mechanisms underlying vitellogenesis (Feng et al., 2022; Li et al., 2022). Sichuan bream (*Sinibrama taeniatus*) is a small economic Cyprinidae fish endemic to the upper reaches of the Yangtze River (Luo et al., 2014), usually breeding once or twice a year under natural conditions, with the youngest female sexually mature individual in the field over one year old (Li et al., 2015). At present, by optimizing breeding conditions in the laboratory, we can make adults spawn once every 2 weeks, and the sexual maturation time of juveniles has been greatly reduced compared to those in the wild at the same time points. Based on its easy-to-regulate characteristics, Sichuan bream can serve as a good model for exploring key factors in the ovarian development of Cyprinidae fish. The purpose of this study was to explore the biological changes related to the ovarian maturation process, especially the vitellogenesis stage, in juvenile Sichuan bream by regularly measuring their growth performance, ovarian development status, serum steroid hormones, thyroid hormones and Vtg levels and the combination of transcriptomic and metabolomic analysis of ovary and liver. The results of this study will help us gain insight into the biological changes that occur during ovarian development and vitellogenesis to help regulate the captive breeding of economic fish.

2 Materials and methods

2.1 Ethics statement

All experimental protocols were approved by Southwest University, and the study was carried out under the protocols of the Institutional Animal Care and Use Committee of Southwest University.

2.2 Experimental fish and sample collection

The experimental fish (a total of 350 individuals) were artificially bred *Sinibrama taeniatus*, with the parents being 3 females and 3 males from one population collected from natural rivers of the Upper Yangtze River. Fish were maintained in indoor tanks (50 × 40 × 30 cm) with a water circulating system at 26°C under a 14 L:10 D lighting cycle and fed twice daily using commercial feed.

The experiment lasted from November 2021 to May 2022, and the sampling times were as follows: samples were collected every 2 days from 1-30 days after hatching, every 5 days from 30-60 days, and every 10 days after 60 days. Ten fish were dissected after anesthesia with tricaine methanesulfonate (MS-222) each time. Individual measurements of body length and body weight were taken. Ovaries were removed and weighed to determine the gonadosomatic index (GSI = [gonad weight/body weight] × 100) and then fixed in Bouin's solution for further histological analysis to determine the timing of each stage of oogenesis.

At 115 (stage II), 165 (stage III), and 185 days (stage IV) after hatching, another 6 female individuals were sampled. Blood, ovary and liver samples of each individual were collected as follows: (a) blood samples were centrifuged (4°C, 4000 r/min, 10 min) to collect serum and stored at -80°C for the determination of serum sex hormones and vitellogenin levels; (b) parts of the ovary were fixed in Bouin's solution for further histological analysis; (c) remaining ovary and liver samples were each divided into 3 portions and stored at -80°C for RNA isolation, transcriptome analysis and metabolome analysis.

2.3 Histological analysis

Fixed ovary tissues were dehydrated with serial dilutions of ethanol, embedded in paraffin, sectioned serially at 5-8 μm in thickness (Leica RM 2235), and then double stained with hematoxylin and eosin for histological analysis to distinguish the different stages of ovarian development (Lowerre-Barbieri et al., 2011; Yin et al., 2019). All micrographs were taken using a light microscope (Nikon Eclipse 80i), and oocyte diameters were recorded.

2.4 Serum biochemical analyses

Fish ELISA Kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China) were used to determine serum concentrations of 17β-estradiol (E_2), testosterone (T), vitellogenin (Vtg), triiodothyronine (T_3), and thyroxine (T_4) according to the manufacturer's instructions. Measure the absorbance (OD) value using a microplate reader (Rayto RT-6100, USA) at a wavelength of 450nm to calculate the concentration. Serum samples from six fish in each group were tested.

2.5 Transcriptomic analysis

Total RNA from stage II, stage III, and stage IV ovaries and liver tissues were extracted using Trizol reagent (TaKaRa Bio, Japan) according to the manufacturer's protocol, and the quality of total RNA was assessed using an Agilent 2100 Bioanalyzer system (Agilent Technologies, USA). For more detailed instructions, refer to our previous work (Yuan et al., 2020). The qualified ovary and liver samples (total RNA concentration ≥ 10 μg; RNA integrity number ≥ 8) were used for sequencing library construction with a paired-end sample preparation kit (Illumina Inc., USA), and the library was sequenced on an Illumina HiSeq 2000 sequencer.

Before proceeding to the subsequent analysis step, the raw sequencing data were filtered to remove the noise reads that contained adapters, ploy-N reads, and low-quality reads by fastp (Chen et al., 2018). The resulting clean reads were subsequently mapped onto a *Sinibrama taeniatus* genome using the aligner HISAT2 (Kim et al., 2019). Gene expression quantification was calculated using RNA-Seq by Expectation-Maximization (RSEM), and they were converted to Fragments Per Kilobase of exon model per Million mapped fragments (FPKM), thereby generating normalized gene/transcript expression levels (Li and Dewey, 2011). The DESeq2 R package (Love et al., 2014) was used to analyze differential expression between the two groups with adjusted *P* values. The threshold for significant differential expression was established at adjusted *P* value < 0.05 and absolute $\log_2$FC ≥ 1. The clusterProfiler R package (Wu TZ. et al., 2021) was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of differentially expressed genes (DEGs), and results were visualized using ggplot2 package.

2.6 Experimental validation by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with TB Green ® Premix Ex TaqTM II (Takara Bio) on a QuantStudio 1 (Thermo Fisher, USA). Ten DEGs were selected in each of the ovary and liver samples. According to the genome and transcriptome data, the primers of the target gene were designed (see Supplementary Table A.1). β-actin was used as the internal reference gene, and the $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of genes.

2.7 Metabolomics analysis

Ovary and liver tissues from stage II, stage III and stage IV were used for metabolomic analysis. A 25 mg sample was weighed into an EP tube, and 500 μL extract solution (methanol: acetonitrile: water = 2:2:1, with isotopically labeled internal standard mixture) was added. Then, the samples were homogenized at 35 Hz for 4 min and sonicated for 5 min in an ice-water bath. The homogenization and sonication cycle were repeated 3 times. Then, the samples were incubated for 1 h at -40°C and centrifuged at 12000 rpm (RCF = 13800 (×g), R = 8.6 cm) for 15 min at 4°C. The resulting supernatant was transferred to a fresh glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

LC-MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to an Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo) (Wang et al., 2016). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonium hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The autosampler temperature was 4°C, and the injection volume was 2 μL. The Orbitrap Exploris 120 mass spectrometer was used for its ability to acquire MS/MS spectra in information-dependent acquisition (IDA) mode by the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as follows: sheath gas flow rate, 50 Arb; Aux gas flow rate, 15 Arb; capillary temperature, 320°C; full MS resolution, 60000; MS/MS resolution, 15000; collision energy, 10/30/60 in NCE mode; spray voltage, 3.8 kV (positive) or -3.4 kV (negative).

The raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program that was developed using R and based on XCMS for peak detection, extraction, alignment, and integration (Smith et al., 2006). Then, an in-house MS2 database (BiotreeDB) was applied for metabolite annotation. The cutoff for annotation was set at 0.3. The compounds identified in positive mode (POS) and negative mode (NEG) were consolidated prior to subsequent analysis.

The resulting data were fed into the SIMCA16 software package (Umetrics, Umea, Sweden) for principal component analysis (PCA) and orthogonal projections to latent structures–discriminate analysis (OPLS–DA). In the OPLS-DA model, R^2Y is used to represent the interpretation rate, and Q^2 is used to represent the prediction rate of the model. Theoretically, the closer R^2Y and Q^2 are to 1, the better the model is. The variable importance in the projection (VIP) was sorted according to the overall contribution of each variable to the OPLS-DA model, and those metabolites with a VIP ≥ 1 and P value < 0.05 were considered significant differences. The differentially expressed metabolites (DMs) were annotated with KEGG pathway analysis using metaboanalyst software (www.metaboanalyst.ca).

2.8 Statistics

All data are presented as the mean value ± standard deviation. One-way ANOVA was used for comparisons between groups, followed by Duncan's multiple range test, and $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS 23 software (SPSS, Chicago, IL, USA).

3 Results

3.1 Stage of oogenesis

Several key stages of oogenesis in Sichuan bream were identified by histological observation. Within 40 days after hatching, there were only primordial germ cells in the gonads, which cannot be used to distinguish between males and females, and the germ cells subsequently pass through the following four developmental stages that eventually lead to egg maturation. Stage I: 40-60 days, the ovary is filled with clusters of oogonia undergoing vigorous mitosis (Figure 1A). Stage II: 60-150 days, oocytes enter the primary growth stage. The nucleus lacks a large central nucleolus, and multiple small nucleoli begin to appear on the inside of the nuclear membrane margins (Figure 1B). In addition, oocyte size continues to increase, and protoplasts continue to accumulate at this stage (Figure 1C). Stage III: 150-180 days, the appearance of yolk vesicles (or cortical alveoli) marks the entry of the oocyte into the secondary growth stage and the initiation of vitellogenesis (Figure 1D). Subsequently, there is a rapid accumulation of yolk material within the cell accompanied by an increase in the number of yolk vesicles (Figure 1E). Stage IV: After 180 days, entering the stage of late-vitellogenesis. Yolk granules dominate the ooplasm of the largest oocytes (Figure 1F). Following yolk accumulation, artificial induction can be employed to facilitate ovary maturation.

3.2 Growth performance

Body length, body weight, GSI and oocyte diameter were recorded during oogenesis, and all values showed a gradual increase throughout the developmental stages. Notably, the increase in body length and body weight slowed or even "paused" upon entering stage III (Figure 2A). In contrast, ovarian development was significantly promoted in stage III compared to the long stage II, as reflected by the increase in GSI and oocyte diameter (Figure 2B), indicating that the material and energy metabolism in the body is undergoing dramatic changes at this critical stage.

3.3 Serum biochemistry

The levels of serum testosterone (T), 17β-estradiol (E2), vitellogenin (Vtg), triiodothyronine (T3), and thyroxine (T4) were measured during stages II, III and IV of oogenesis. Serum T levels were

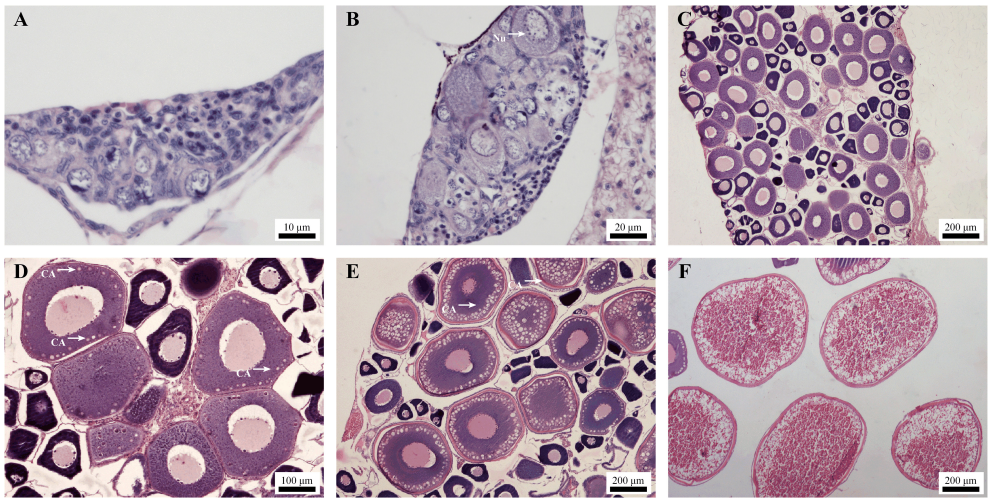

FIGURE 1
Micrographs of ovaries at different developmental stages. **(A)** On day 45, ovary in stage I; **(B)** On day 60, ovary in early-primary growth stage. Nu, Nucleolus; **(C)** On day 100, ovary in mid- primary growth stage; **(D)** On day 150, ovary in early-vitellogenesis stage. CA, cortical alveoli; **(E)** On day 165, ovary in mid-vitellogenesis stage; **(F)** On day 185, ovary in late-vitellogenesis stage.

maintained at low levels at different stages, and there was no significant difference between the stages ($P > 0.05$) (Figure 3A). Compared to stage II, serum E2 levels increased significantly in stages III and IV ($P < 0.05$), but there was no significant difference between stages III and IV ($P > 0.05$) (Figure 3B). Serum Vtg levels continued to increase in stages II, III and IV, with significant differences between all groups ($P < 0.05$) (Figure 3C). In addition, the two serum thyroid hormones levels exhibited distinct patterns. Serum T3 levels demonstrated a high degree of similarity across the three developmental stages ($P > 0.05$) (Figure 3D), while T4 levels followed the same trend as Vtgs and continued to rise during vitellogenesis, with significant differences between groups ($P < 0.05$) (Figure 3E).

3.4 Transcriptomic analysis

The RNA-Seq analysis was conducted on ovarian and liver samples collected from three distinct developmental stages. After preprocessing and filtering the low-quality sequences, 144.06 M, 149.55 M and 152.75 M total clean reads were obtained for ovaries at stages II, III and IV, respectively, while 137.29 M, 130.23 M and 127.74 M total clean reads were obtained for liver samples at the corresponding stages. The Q20, Q30 and total mapping values of all samples were analyzed to show the high quality of sequencing and alignment (Supplementary Table A.2).

The results of differential expression analysis showed that the comparison of stage II and stage IV resulted in the highest number of DEGs in the ovary, with 1150 DEGs upregulated and 1054 DEGs downregulated (Figure 4A). Conversely, the lowest number of DEGs was obtained in the comparison of stage III and stage IV ovaries. Notably, 609 (581 + 29) DEGs produced in stage II versus stage III and in stage II versus stage IV are identical, maybe implying that the ovary undergoes similar regulation in the two stages after entering vitellogenesis compared to the previtellogenesis stage (Figure 4B). The number of DEGs in the liver did not exhibit significant variation across different stages, with the highest number of DEGs observed in the comparisons between stages III and IV (Figure 4C). Additionally, the quantitative relationship between the comparison groups in the liver exhibited less variability compared to that in the ovaries (Figure 4D).

In this paper, we focused on the differences between pre- and post-vitellogenesis, so we performed enrichment analysis for the

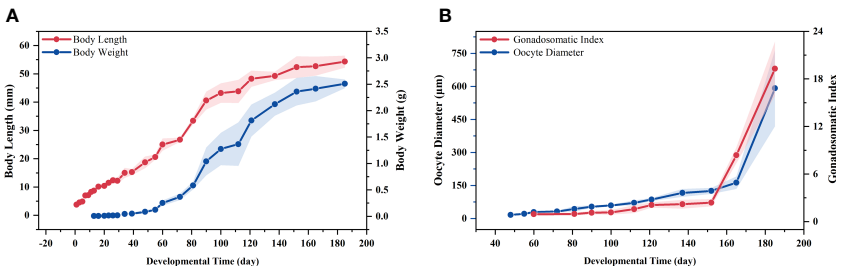

FIGURE 2
Changes in biometric parameters during oogenesis. **(A)** Body length and body weight; **(B)** Gonadosomatic index (GSI) and oocyte diameter.

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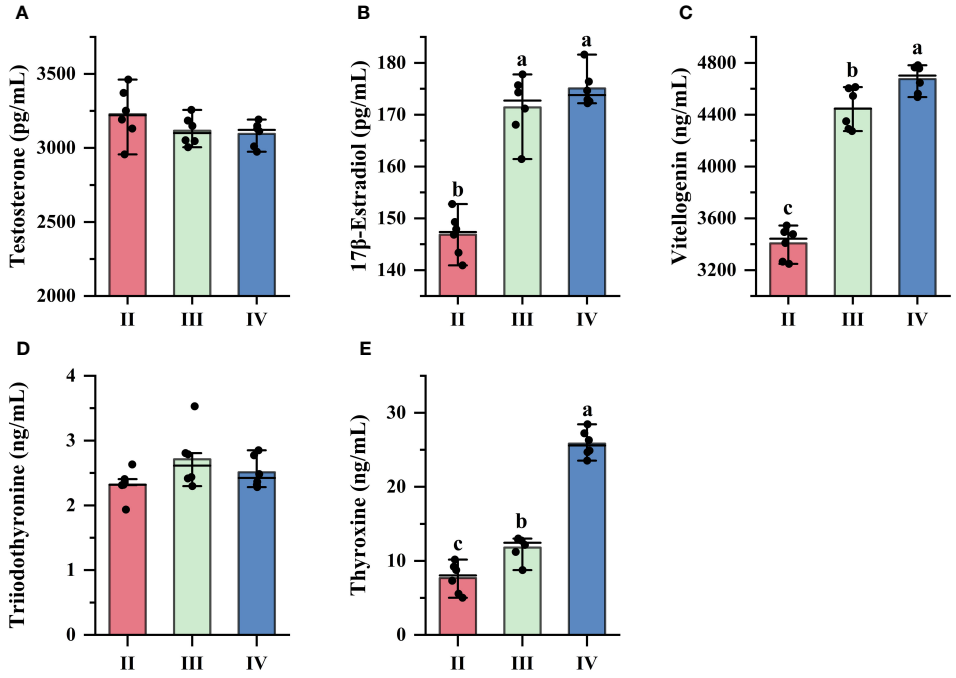

FIGURE 3
Changes in serum biochemical levels. **(A)** Testosterone; **(B)** 17β-estradiol; **(C)** Vitellogenin; **(D)** Triiodothyronine; **(E)** Thyroxine. The x-axis indicates different developmental stages of the ovary. [a,b,c] Mean values with unlike letters were significantly different ($P < 0.05$).

DEGs generated in the comparison of stage II with stage III and stage II with stage IV to explore their functional categories. The top 15 GO terms showed that DEGs in the ovary (Figure 4E) were mainly associated with "binding", including "nucleic acid binding" and "organic cyclic compound binding" after the start of vitellogenesis. In the comparison between stage II and stage III, DEGs also affected the "response to steroid hormone" and the "response to lipid". DEGs in the liver (Figure 4F) were mainly related to "catalytic activity" and "oxidoreductase activity" during vitellogenesis, and DEGs in stage IV particularly affected "amino acid activation" and "amino acid metabolic processes".

In the comparison of stage II with stages III and IV, DEGs in the ovary were significantly enriched in 24 and 34 pathways by KEGG analysis (Figure 5A), while DEGs in the liver were enriched in 89 and 80 pathways (Figure 5B), respectively. During vitellogenesis, ovarian development is controlled by a complex regulatory network, in which the functions of DEGs are mainly related to environmental information processing, and several signal transduction pathways, such as the "PI3K-Akt signaling pathway", the "MAPK signaling pathway" and the "Rap1 signaling pathway", are significantly enriched. In addition, three hormone-related pathways were enriched in stage IV, including "ovarian steroidogenesis", "thyroid hormone signaling pathway" and "cortisol synthesis and secretion". In the liver, DEGs were mainly enriched in pathways related to energy metabolism, such as "glycolysis/gluconeogenesis", "fatty acid biosynthesis" and "citric acid cycle (TCA cycle)". In addition, pathways related to lipid metabolism, amino acid metabolism and vitamin metabolism, such as "Glycerophospholipid metabolism", "Alanine, aspartate and glutamate metabolism" and "Retinol metabolism", were also significantly enriched, which indicates involvement of the synthesis or assembly processes of yolk nutrients in the liver.

To explore the key factors regulating vitellogenesis, we focused on DEGs in the ovary and/or liver involved in cellular morphological changes, the endocrine system, carbohydrate metabolism, lipid metabolism, amino acid metabolism, cofactors and vitamin metabolic pathways. Heat maps were constructed using Z score (Auman et al., 2007) normalized values to reflect the expression patterns of DEGs at different stages (Figures 5C, D).

3.5 Validation of RNA-seq data by qRT-PCR

The RNA-Seq data was validated by selecting 10 DEGs from the ovary (*rbp1*, *pla2g3*, *got2*, *slc2a1*, *pkm*, *pfkp*, *pfkfb3*, *hsd17b1*, *cyp1a1*, *pnpla4*) and liver (*fbp1, agpat1, apob, vtg1, esr1, fbp2, adpgk, hsp90b, gpt2, lipin1*) for qRT-PCR analysis, respectively. The results showed highly similar trends between the RNA-Seq and qRT-PCR results (Figures 6A, B), indicating the reliability of the RNA-Seq results.

3.6 Metabolomic analysis

To facilitate comparison between groups, metabolites obtained from positive and negative mode identifications were rearranged and combined for subsequent analysis. The normalized data obtained were analyzed by PCA and OPLS-DA using multivariate analysis. The PCA score plots show the trends of the data from the ovaries and liver at different stages (Figures 7A, B), which means that the ovaries and liver have distinct metabolic profiles at different

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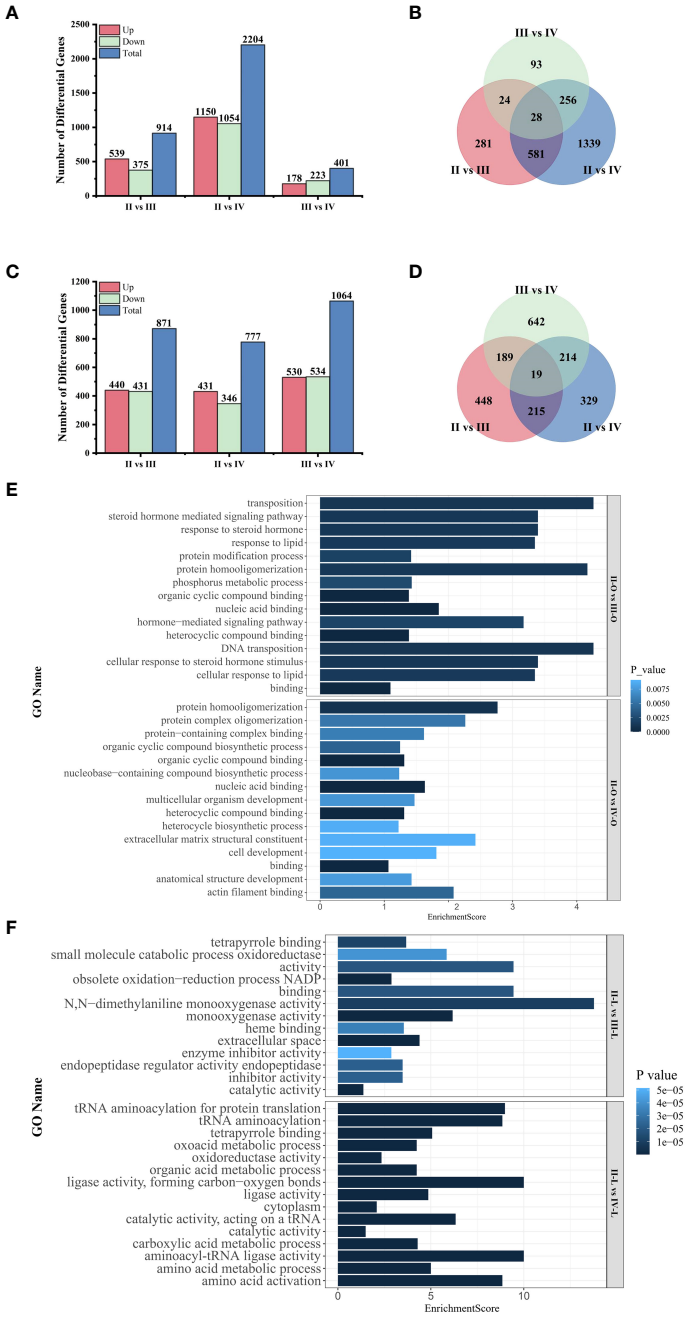

FIGURE 4
Transcriptome analysis among the comparison groups. **(A)** Number of DEGs in the ovary; **(B)** Venn diagram of DEGs in the ovary; **(C)** Number of DEGs in the liver; **(D)** Venn diagram of DEGs in the liver; **(E)** GO enrichment analysis of DEGs in the ovary; **(F)** GO enrichment analysis of DEGs in the liver.

stages. The OPLS-DA score plots showed significant separation between all comparison groups (Supplementary Figure A.1). The values of the classification parameters R^2Y and Q^2 were both stable with adequate goodness-of-fit and predictive ability, which indicates that subsequent DMs could be analyzed. DMs between groups were screened with VIP ≥ 1 and P value < 0.05 as criteria and successfully annotated by metabolite identification level 2 rather than level 1. The number of DMs in the ovary and liver when comparing stages is shown in Figures 7A, B.

Pathway analysis of DMs was performed using MetaboAnalyst software to analyze their impact on the ovary and liver during vitellogenesis. Multiple DMs involved in carbohydrate metabolism (L-Lactic acid, Glucose 6-phosphate, Glucose 1-phosphate, Palmitic acid, Isocitric acid, Citric acid, Succinic acid, Acetyl-CoA, Fumaric acid), lipid metabolism (Citicoline, Acetylcholin, O-Phosphoethanolamine, CDP-Ethanolamine, Glyceryl-phosphorylethanolamine, Glycerophosphocholine), amino acid metabolism (Glycine, L-Glutamic acid, L-Histidine, D-Aspartic

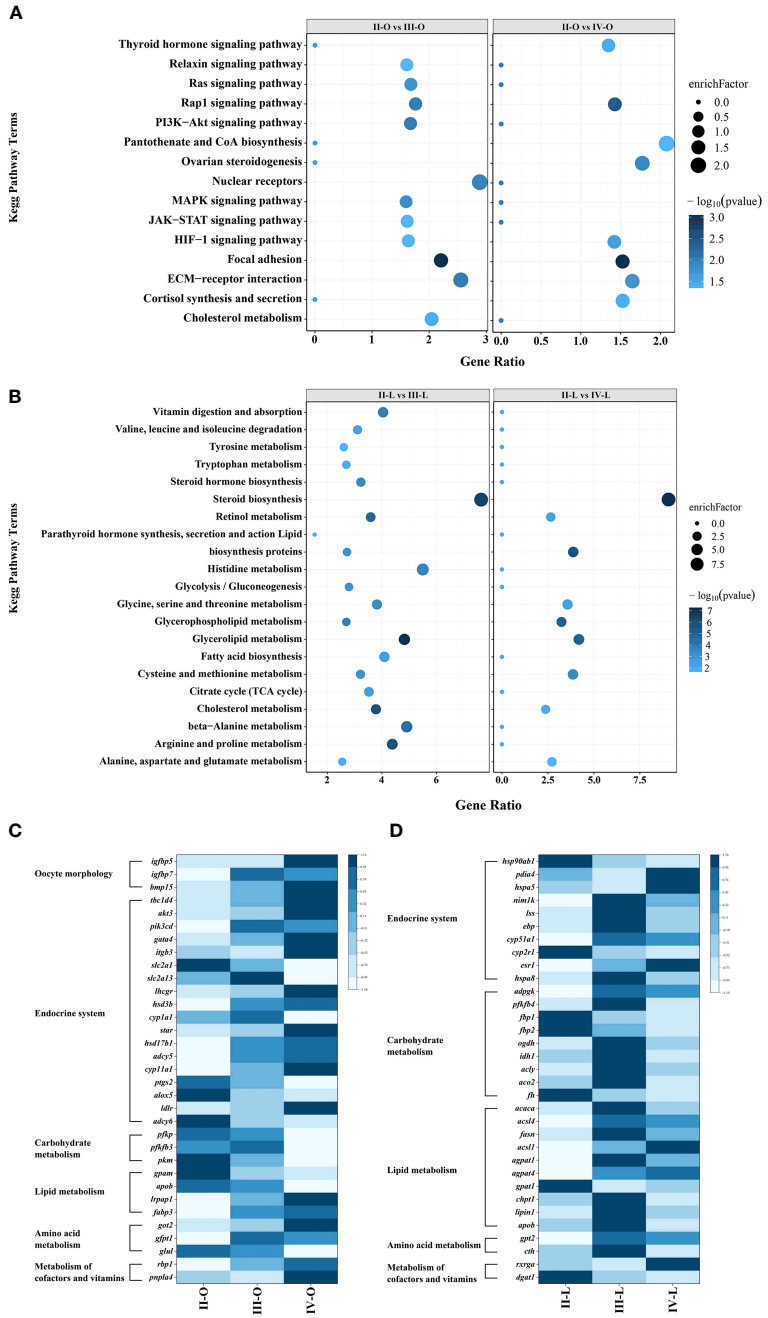

FIGURE 5
KEGG enrichment analysis and heat map of the DEGs. **(A)** Enriched KEGG pathways in the ovary; **(B)** Enriched KEGG pathways in the liver; **(C)** Heat map of DEGs involved in key pathways in the ovary; **(D)** Heat map of DEGs involved in key pathways in the liver.

acid, L-Prolin, L-Threonin, L-Tryptophan, S-Adenosyl-L-homocysteine, N-Acetyl-L-methionine, L-Methionine, S-Adenosylhomocysteine, L-Phenylalanine), metabolism of cofactors and vitamins (9-cis-Retinoic) and steroid hormone biosynthesis (Taurin, Cholesterol sulfate, Cholesterol) were screened by correlating transcriptome KEGG enrichment results, and heatmaps were constructed to visualize their expression patterns at different stages (Figures 7C, D).

4 Discussion

In most teleosts, ovarian development is a dynamic process with both stages and continuity (Patino and Sullivan, 2002). In this paper, on the basis of existing studies (Lowerre-Barbieri et al., 2011; Yin et al., 2019), the different developmental stages of the Sichuan bream ovary were determined by the histological characterization of oocyte renewal. Female juvenile Sichuan bream can complete yolk

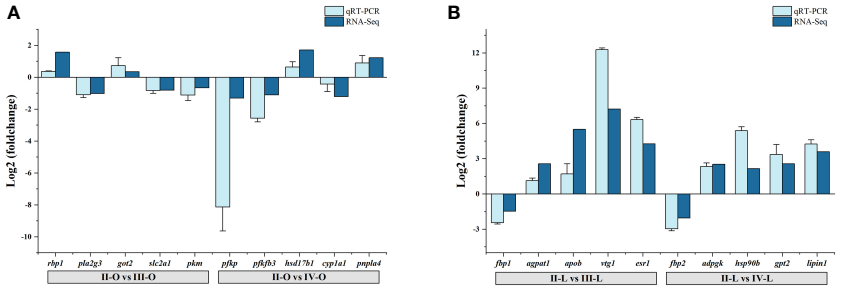

FIGURE 6
qRT-PCR validation of DEGs generated from the RNA-Seq results. **(A)** DEGs in the ovary; **(B)** DEGs in the liver.

accumulation and reach sexual maturity in 6 months under laboratory culture conditions, which is much shorter than the sexual maturation time of individuals in the field (Li et al., 2015). Perhaps due to the complexity of oocyte vitellogenesis, it requires a longer preparation time (stage II), which occupies approximately half of the length of the ovary maturation time. Therefore, this study investigated the influence of the liver and ovary on vitellogenesis during oocyte maturation mainly by combining transcriptomics and metabolomics.

4.1 Thyroid hormones regulate ovarian development

The thyroid hormones (THs), primarily thyroxine (T_4) and triiodothyronine (T_3), play pivotal roles in regulating physiological processes such as growth, morphogenesis, and reproduction in vertebrates (Stacey et al., 1984; Brown et al., 2014). The correlation between circulating THs and reproductive cycles in teleosts has been demonstrated by several studies. The peak plasma THs levels were observed during gametogenesis in certain species, such as rainbow trout (Osborn et al., 1978) or brook trout (White and Henderson, 1977), while climbing perch (Chakraborti and Bhattacharya, 1984) and sea lamprey (Sower et al., 1985) exhibited peak THs levels during spawning. Researchers gradually recognized the potential crosstalk mechanism between the thyroid and reproductive axes and attempted to modulate reproductive physiological processes in fish with THs, which achieved significant outcomes (Lam and Loy, 1985; Soyano et al., 1993). We found that key DEGs involved in "thyroid hormone synthesis" in the liver (*hsp90b*, *pdia4*, *hspa5*, *nim1k*) and "thyroid hormone signaling pathway" in the ovary (*itgb3*, *pik3cd*, *akt3*, *tbc1d4*, *gata4*) were both significantly upregulated, which would promote the synthesis and function of THs (Yi et al., 2020). The serum T_3 level in Sichuan bream was found to be much lower than that of T_4, which is consistent with the observed trend in other carp species such as grass carp (Ma et al., 2021) and Chinese rare minnow (Li et al., 2014). In addition, we noted that serum T_4 levels, but not T_3 levels, rose consistently during vitellogenesis, paralleling changes in serum Vtg levels. T_4 is the primary form of thyroid hormone synthesized in organisms and it is converted into the more biologically active T_3 through the iodothyronine deiodinase (Dio2) (St Germain et al., 2009; Habibi et al., 2012).

Previous studies have demonstrated that T_4 exerts a stimulatory effect on metabolism and the bioactivity of sex hormones, while also facilitating the uptake of vitellogenin by tilapia oocytes (Lam, 2007). Therefore, it can be inferred that T_4 could be necessary for fish reproduction. Undeniably, T_3 has also been shown to be involved in the synthesis of vitellogenin in fish liver. The administration of thyroid hormones with distinct biological activities to Poecilia reticulata (Lam and Loy, 1985) and goldfish (Nelson and Habibi, 2016) can promote vitellogenesis through synergistic effects on the HPG axis. In our study, although serum T_3 levels exhibited an increase during vitellogenesis, the observed change was not statistically significant. This lack of significance may be attributed to the potent biological activity of T_3 (Deal and Volkoff, 2020), which mitigates any pronounced surge in demand for it. Future research should incorporate hormone treatment experiments to investigate further the underlying physiological mechanisms through which thyroid hormones regulate vitellogenesis.The growth of oocytes occurs throughout ovarian development, particularly during vitellogenesis, when their diameter significantly increases to accommodate the accumulation of material in cells due to nutrient intake (Tyler and Sumpter, 1996; Lubzens et al., 2017). It is widely recognized that THs are involved in promoting oocyte growth by stimulating the expression of insulin-like growth factors (IGFs) (Eivers et al., 2004; Molla et al., 2019). We detected increased expression of IGF-binding proteins (IGFBPs), including igfbp5 and igfbp7, in stages III and IV of the ovary. These proteins are known to interact with IGFs and play a role in IGF signaling *in vivo* (Artico et al., 2021; Waters et al., 2022). In addition to the TH-IGF pathway, bone morphogenetic factor 15 (bmp15) is thought to be a key factor in the regulation of primary oocyte growth and granulosa cell development in mammals and teleosts (Juengel et al., 2004; Moore and Shimasaki, 2005). Transcriptomic data showed that bmp15 is significantly upregulated in ovarian stage IV, which coincides with the stage of rapid oocyte growth. Interestingly, while the ovaries gained additional growth, the rapid growth of the body seemed to be inhibited, including the factors of body length and body weight. This is not uncommon in fish. Some scholars believe that the early stage of fish sexual maturation is accompanied by the acceleration of

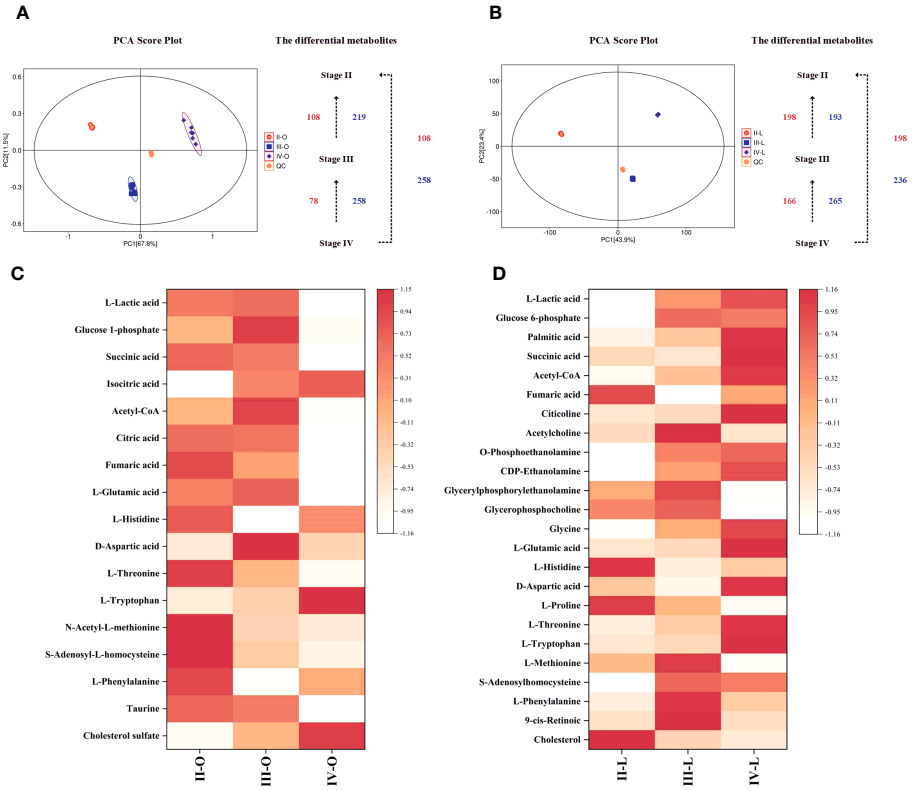

FIGURE 7
Metabolomic analysis among the comparison groups. **(A)** PCA score plot and DMs in the ovary; **(B)** PCA score plot and DMs in the liver; **(C)** Heat map of Screened DMs in the ovary; **(D)** Heat map of Screened DMs in the liver.

somatic cell growth, and in the later stage, somatic cell growth decreases because resources are directed to gonadal development (Norberg et al., 2001). For most teleosts, drastic physiological changes often occur in this critical growing stage, regulating energy metabolism through the liver and reallocating accumulated reserves for vitellogenesis (Bertolini et al., 2020).

4.2 Energy metabolism in the liver and ovary

After entering the vitellogenesis stage, liver DEGs are significantly enriched in pathways related to ATP production, which may be indispensable for the initiation of vitellogenesis. L-lactic acid and glucose 6-phosphate were found to be upregulated by metabolomic assays in both stage III and stage IV in the liver, indicating that the liver is undergoing vigorous glucose metabolism (Boles, 1998; Yao et al., 2019). Combined with the results of transcriptomic analysis, we found that the upregulation of DEGs catalyzes several key unidirectional links in the glycolytic pathway, including ADP-dependent glucokinase (*adpgk*) (Grudnik et al., 2018) and 6-phosphofructo-2-kinase (*pfkfb4*) (Yi et al., 2019). Meanwhile, fructose-1,6-bisphosphatases (*fbp1* and *fbp2*) were downregulated in the unidirectional link of gluconeogenesis, implying that the liver supplies energy to the organism by promoting glycolysis and inhibiting the gluconeogenesis pathway during vitellogenesis. In addition, fatty acids are important substrates for energy metabolism, and we found that palmitic acid, an important intermediate in fatty acid biosynthesis (Zhang et al., 2023), was significantly upregulated in stage IV in the liver. Combined with the results of transcriptome analysis, the process of fatty acid biosynthesis in the liver was comprehensively and profoundly promoted during vitellogenesis, as reflected by the significant upregulation of acetyl-CoA carboxylase (*acaca*), long-chain fatty acid-CoA ligase (*acsl4*, *acsl1*), and fatty acid synthase (*fasn*), which catalyze almost all of the key steps in fatty acid biosynthesis (Mu et al., 2021). Carbohydrates and fatty acids are eventually metabolized to acetyl coenzyme A, which then enters the citrate cycle (TCA cycle) to produce ATP for life activities (Zheng et al., 2021). Our study showed that during vitellogenesis, several DMs (succinic acid, acetyl-CoA) and DEGs (*ogdh*, *idh1*, *acly*, *aco2*) are upregulated in the liver, contributing to the release of energy via TCA cycle pathway. Fish ovarian development is an energy-intensive process (Song et al., 2018), and our study shows that energy metabolism in the liver was promoted by upregulating glucolipid metabolic pathways to provide sufficient ATP for vitellogenesis.

The ovary undergoes completely different regulation from the liver in pathways related to energy metabolism. We detected by transcriptome analysis that 6-phosphofructo-2-kinase (*pfkfb3*), ATP-dependent 6-phosphofructokinase (*pfkp*) and pyruvate kinase (*pkm*) were significantly downregulated in stage IV and

that they inhibited ovarian glucose catabolism. The bidirectional glucose transporter protein GLUTs, located on the cell membrane, are encoded by the SLC gene and responsible for glucose transport between the cell and the blood (Augustin, 2010; Levy et al., 2010). Herein, *slc2a1* and *slc2a13* were downregulated in stage IV ovaries, which inhibited glucose transport and supported our hypothesis to some extent. DMs involved in the TCA cycle, such as succinate, acetyl coenzyme A, citrate and fumarate, were also found to be downregulated during vitellogenesis, suggesting that oocytes tend to retain nutrients rather than decompose them to produce energy at this stage.

4.3 Molecular cargo biosynthetic processes

It has been suggested that the start of puberty in fish is associated with the level or rate of accumulation of lipid stores (Taranger et al., 2010). Several DEGs and DMs involved in lipid metabolism are upregulated in the liver during vitellogenesis, activating the "glycerophospholipid metabolism" and "glycerol ester metabolism" pathways, which contribute to the synthesis and transport of key lipid components. As an essential component of oocyte nutrition, neutral lipids are stored in the cells in the form of oil droplets in some fish (Patino and Sullivan, 2002). This lipid component has been shown to consist mainly of triglycerides (TAG), which are assembled in the liver with apolipoproteins (Apob) to form very low-density lipoproteins (VLDL) and are released into the bloodstream as the main lipid component secreted by the liver (Shelness and Ledford, 2005; Hiramatsu et al., 2013). A recent study reported a conserved pathway for TAG synthesis via "glycerol-3-phosphate" (Li et al., 2018). We detected significant upregulation of acylglycerophosphate acyltransferase (*agpat1*), Phosphatidate phosphatase (*lpin1*) and *apob* expression in the liver during vitellogenesis, which together facilitate the synthesis and assembly of neutral lipids in the liver.

Circulating VLDL enters the oocyte mainly by the following two pathways: 1) receptor (Vldlr)-mediated endocytosis; and 2) processing of VLDL to low-density lipoprotein by ovarian lipoprotein lipase (LPL), with the released free fatty acids (FFAs) that enter the oocyte to regenerate neutral oil droplets (Hiramatsu et al., 2013). Differential expression of *vldr* and *lpl* was not found in this study, but *lrpap1* expression was found to be upregulated in both stage III and stage IV. Past studies have speculated that Lrpap1 might function as a chaperone protein to promote the correct folding of LPLs and the formation of intramolecular disulfide bonds (Van Leuven et al., 1998), and its upregulation may be related to the formation of lipid droplets by VLDL in the ovarian absorption circulation. In addition, expression of the fatty acid binding protein (*fabp3*) was upregulated in ovarian stage IV, which has been widely shown to contributed to the intracytoplasmic transport of free fatty acids after crossing the oocyte plasma membrane (Liu et al., 2007; Thirumaran and Wright, 2014). These DEGs contribute to neutral lipid synthesis and transport and support the concept of a non-endocytotic pathway of oocyte lipidation in Sichuan bream.

In addition to neutral lipids, polar lipids are synthesized in the liver and stored in the lipovitellin structural domain of Vtg, which becomes the main lipid component carried and transported by Vtg (Lubzens et al., 2017). The main component of polar lipids is phosphatidylcholine, which is barely hydrolyzed during the maturation stage of the oocyte and serves as an essential yolk nutrient store for the development of the offspring (Yilmaz et al., 2016). Past studies have reviewed the general pattern of synthesis of phosphatidylcholine using fatty acids and acetyl-CoA as substrates (Jackowski et al., 2000). We found that DEGs, including *agpat4*, *gpat1*, and *chpt1*, are upregulated in stage III and/or stage IV, which helps the liver synthesize essential lipid components during vitellogenesis to supply Vtg formation.

We found that many DMs and DEGs were enriched in amino acid synthesis and metabolism-related pathways in both stage III and stage IV. The levels of glutamic acid, glycine, histidine, aspartic acid, proline, threonine, tryptophan, methionine, cysteine, and phenylalanine were found to be significantly elevated in the liver during vitellogenesis by metabolomic assays, suggesting that the liver's requirement for amino acids is associated with increased protein synthesis. Past studies have identified Vtg proteins from English Sole (*Pleuronectes vetulus*) by native PAGE (Roubal et al., 1997), with the highest glutamate content (12.6%) and lowest histidine abundance (2.5%). In addition, we compared the amino acid composition of Vtg in various fish (Tao et al., 1993; Fazielawanie et al., 2013; Maltais et al., 2014) and found that alanine or glutamic acid was generally higher in fish, and there were lower levels of glycine, histidine, aspartic acid, proline, threonine, methionine and phenylalanine. The increase in glutamate in organisms is critical, and high levels of glutamate may lead to the production of other amino acids, such as aspartate and alanine, via glutamate-oxaloacetate transaminase (Got) and glutamate pyruvate transaminase (Gpt), and these amino acids may eventually serve as substrates for the synthesis of Vtg or participate in other biological processes (Korsgaard, 1990). We detected significant upregulation of *got2* and *gpt2* in stage IV ovaries and livers, respectively, suggesting a conversion of glutamate to other amino acids; meanwhile, aspartate was an upregulated DM in stage IV ovaries, in agreement with our transcriptome results. In addition, cysteines were also found to be associated with the Vtg structure; its two small structural domains (β'-c and Ct) together contain 14 highly conserved cysteine residues (Lubzens et al., 2017).

In recent years, Vtg has received some attention as an important carrier of lipolytic vitamins such as vitamin A and vitamin E. In particular, vitamin A is transported into the egg in the form of retinol to regulate gene transcription in developing embryos (Balmer and Blomhoff, 2002) or determine egg quality (Izquierdo et al., 2001; Palace and Werner, 2006). We found that the DM 9-cis-retinoic was upregulated in stages III and IV of the liver; this metabolite is activation form of retinol (Kane, 2012). Its specific precursor 9-cis-retinal is an important isomer of retinol and coexists in the circulation (Saeed et al., 2017). Information on the transport and uptake of the vitamin by developing oocytes in fish is scarce, and we speculate that 9-cis-retinoic may be the predominant form present and transported in Sichuan bream. Additionally, 9-cis-retinoic is a high-affinity ligand for retinoid X receptors (RXRs). We found that *rxrga* is upregulated in liver stage IV, which mediates the activity of 9-cis-retinoic acid and agrees with our hypothesis.

Retinol-binding protein 1 (Rbp1) is an intracellular regulator of vitamin A metabolism and retinoid transport (Levi et al., 2008). We found significant upregulation of *rbp1* in both stages III and IV in the ovary, which facilitates vitamin A uptake and storage by oocytes. Moreover, significant upregulation of *pnpla4* was detected in ovarian stage IV, which has been reported to regulate access to retinol from retinol-ester storage depots in vertebrates (Holmes, 2012).

4.4 Vitellogenesis: response to estrogen

It has been well confirmed that the progression of vitellogenesis is usually accompanied by a rapid increase in circulating E_2 to induce Vtg production in the liver (Lubzens et al., 2010). DEGs involved in "ovarian steroidogenesis" were significantly enriched in the comparison between stage II and stage IV of the ovary, where *star*, *cyp11a1*, *hsd3b* and *hsd17b1* were significantly upregulated and mediated the process of transporting cholesterol from the cytoplasm to the mitochondrial inner membrane and converting it to E_2 through a series of enzymatic reactions (Nyuji et al., 2020). This mechanism is supported by the serum E_2 levels at different stages, which increased significantly ($P < 0.05$) after the onset of vitellogenesis compared to stage II. We found that while serum E_2 levels increased significantly, T remained at a lower level throughout the vitellogenesis process. Although we detected the upregulation of taurine in stages III and IV of the ovaries, this metabolite has been shown to be closely associated with elevated T levels in fish and mammals (Yang et al., 2013; Garcia-Reyero et al., 2014), suggesting that T acts as a substrate and is actively involved in E_2 synthesis rather than being released directly into the bloodstream. In this study, while serum Vtg levels were found to be significantly elevated in both stages III and IV ($P < 0.05$), E_2 levels did not show significant differences between stages III and IV, suggesting that other key factors may be involved in the persistent elevation of Vtg levels.

Traditionally, it is widely accepted that E_2 exerts its effects through binding to the intracellular nuclear estrogen receptors (ERs). E_2 forms dimers with ERs and subsequently bind to the estrogen response elements in the promoter region of the Vtg gene, leading to transcription and translation through genomic effects (Gruber et al., 2004). To date, three estrogen receptor subtypes have been described for fish, including estrogen receptor 1 (*esr1*), estrogen receptor 2b (*esr2b*) and estrogen receptor 2a (*esr2a*) (Wang et al., 2021). Vtg synthesis in teleosts is generally thought to be mediated primarily by *esr1* (Cerda et al., 2008). However, it has been reported that knockdown of ERα, ERβ1, and ERβ2 in fish all led to a significant decrease in Vtg gene expression levels (Nelson and Habibi, 2010; Griffin et al., 2013). In addition, unique but also overlapping roles in the estrogen-dependent actions of these two receptor subtypes were found in mice lacking ERα, Erβ, or both (Matthews and Gustafsson, 2003). Knockdown of ERs in goldfish liver cells (Nelson and Habibi, 2010) and zebrafish embryos (Griffin et al., 2013) revealed that stimulation with E_2 increased the expression of ERβs in particular, which activated ERα and ultimately contributed to vitellogenesis. The present study found that *esr1*, but not *esr2*, was upregulated in stages III and IV of the liver, consistent with changes in Vtg levels, confirming the important role of *esr1* in Vtg production. However, further investigation is needed to understand the role of ERβs.

In recent years, the role of the membrane-bound G protein-coupled estrogen receptor (GPER) in fish vitellogenesis has been well define (Wu XJ. et al., 2021). Activation of GPER by E_2 leads to rapid activation of G proteins and cell membrane-associated secondary signaling such as generation of cAMP and EGFR-signaling, which may stimulate *vtg* transcription through rapid nongenomic effect (Filardo and Thomas, 2005; Prossnitz and Barton, 2011). Previous study have demonstrated that knockdown of the zebrafish *gper* gene leads to delayed ovarian development and a significant decrease in serum Vtg level (Wu XJ. et al., 2021). Furthermore, treatment with GPER agonist (G-1) or antagonist (G-15) significantly activated or inhibited the expression of zebrafish Vtg genes in the liver, suggesting that GPER plays a crucial role in regulating vitellogenesis (Chen et al., 2019). We found significant upregulation of *gper* expression in both stage III and stage IV of the liver (results not shown), which is similar to the expression profile of *esr1* and parallel to changes in serum Vtg levels. Therefore, it is reasonable to believe that GPER, as an essential estrogen membrane receptor, contributes significantly to vitellogenesis in *Sinibrama taeniatus*.

5 Conclusion

This study identified the time required for ovarian maturation in juvenile Sichuan bream (*Sinibrama taeniatus*) under laboratory culture conditions and explored the complex process of vitellogenesis by combining growth performance, serum biochemistry, transcriptomic and metabolomic analyses. The dynamic changes in gene expression and metabolites revealed that energy reallocation during vitellogenesis is mainly regulated by carbohydrate and lipid metabolic pathways, and several key factors involved in the assembly and transport of important yolk nutrients have been detected. In addition, we found that the initiation of vitellogenesis was associated with a surge in serum E_2 levels, but the sustained increase in Vtg levels at the late stage appears to be more attributable to the upregulation of estrogen receptors, which should be further confirmed in future studies. Overall, this study seems to be the first report to investigate the complex metabolic processes that occur in the liver and the ovary during vitellogenesis by transcriptomics and metabolomics in fish, providing valuable information about the regulation of ovarian development in fish.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA949537.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Southwest University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZW and ZZ contributed to conception and design of the study. ZZ, QZ and LW collected the samples. HW performed the bioinformatics analysis. SW and SL performed the molecular experiments. ZZ wrote the manuscript. QZ and DY reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Chongqing Technology Innovation and Application Development Special Key Project (Grant No. 2023TIAD-KPX0067) and the Ministry of Agriculture of China (Grant No. 4141900024).

Acknowledgments

Thanks to all the authors for contributing to this article. We are also grateful to all the laboratory members for their technical advice and helpful discussions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1243767/full#supplementary-material

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