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The molecular mechanism of ovary development in *Thamnaconus septentrionalis* induced by rising temperature via transcriptomics and metabolomics analysis

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The greenfin horse-faced filefish, *Thamnaconus septentrionalis*, which is widely distributed in the Indo-West Pacific Ocean, is an important commercial fish species with characteristic blue-green fins, rough skin, and a spine-like first dorsal fin. Recently, *T. septentrionalis* has captured the attention of conservation and development due to its sharply declining population, and its great economic value as an important marine aquaculture fish species. To protect the resources of *T. septentrionalis* and develop its fishery industry, artificial breeding is essential and has been carried out gradually in local farms in China. A previous study has shown that temperature can effectively influence the gonadal development in *T. septentrionalis* in its overwintering state. However, the molecular mechanisms underlying the effects of temperature on the gonadal development in *T. septentrionalis* are still unrevealed and worth further study. In the present study, we systematically investigated the effects of temperature on ovary development in *T. septentrionalis* through transcriptomics and metabolomics analysis. The results showed that temperature rises significantly regulated the metabolic status of *T. septentrionalis* and promoted ovary development and maturation process through the regulation of the pathways of ECM-receptor interaction, regulation of actin cytoskeleton, oocyte meiosis, steroid hormone biosynthesis, lipid metabolism, ABC transporters, protein digestion and absorption, biosynthesis of amino acids pathways, etc. The histological, scanning electron microscope, and transmission electron microscope analyses showed that the oocytes in the ovaries of *T. septentrionalis* experiencing the temperature rises developed from phase II to III with increased gonadal somatic index and 17 β -estradiol level. The present study for the first time elucidated the

potential molecular regulatory mechanism of ovary development in *T. septentrionalis* induced by temperature and will provide valuable information for the artificial breeding of *T. septentrionalis* and conservation of *T. septentrionalis* fishery resources in the future.

KEYWORDS

Thamnaconus septentrionalis, ovary development, molecular mechanism, transcriptomics, metabolomics

1 Introduction

The greenfin horse-faced filefish *Thamnaconus septentrionalis* is a temperate water fish of Tetraodontiformes distributed widely in the Indo-West Pacific Ocean (Bian et al., 2020). Due to its appealing taste, rich nutritional benefits, and high economic value, *T. septentrionalis* has become an important commercially cultured marine fish species in Japan, Korea, and China (Bian et al., 2020). The natural resources of *T. septentrionalis* have rapidly decreased over the past decades, mainly as a result of serious overfishing and habitat loss. To develop the fishery industry of *T. septentrionalis* and meet market demands, artificial breeding is essential and has increased rapidly in recent years. With the rapid expansion of the *T. septentrionalis* aquaculture industry and market demands, the seedling production of the species was substantially enhanced.

The natural breeding period of *T. septentrionalis* is fairly late, in May–June along Shandong coastal waters, and parent fishes in the waters are affected by low temperature in winter and have a longer gonad development time (Mao et al., 2024). Therefore, the fry coming from parent fish cultivated under normal temperatures cannot be cultivated to commercial fish size in the same year. Reproductive regulation of parent *T. septentrionalis* through temperature rises could prolong the culture and growth period and likely achieve commercial fish sizes to meet the market demands. In teleosts, gonad development is influenced by many environmental factors (water temperature, photoperiod, food availability, etc.), and water temperature could significantly influence gonadal development and maturation (Urbatzka et al., 2011; Flores et al., 2020). During the artificial breeding period, the parent fish must undergo a three-stage process of temperature dropping (November), temperature rising for overwintering (December to January), and temperature rising for ripening (February) to facilitate successful spawning. In a previous study, we found temperature rises for overwintering could break ovary maintenance in stage II and start the development (Mao et al., 2024). However, the molecular mechanism underlying the induction of gonadal development in *T. septentrionalis* through temperature rises has been scarcely studied.

In addition, the aquaculture industry and sustainability have been increasingly impacted by climate change such as global warming (McKenzie et al., 2021; Yadav et al., 2024). The

temperature rise induced by global warming affects the reproductive biology and population structure of commercially important tropical fish species (Brulé et al., 2022; Lema et al., 2024). The climate-induced temperature changes influenced the growth performance, fecundity, and population size of the marine fish *Gadus morhua* and *Zoarcetes viviparus* (Pörtner et al., 2001). Wanzenböck et al. (2022) found that ocean warming has an effect on population polymorphism and range shifts in stickleback by altering its fecundity and offspring development. Recent studies have also been conducted to explore the temperature-dependent effects on ovary development in teleosts. A high-temperature treatment intensified and promoted the emergence of sex-differentiated females in *Leporinus friderici* (Souza et al., 2024), induced Nile tilapia female sex reversal (Zhao et al., 2020), and increased the breeding probability in female coral reef damselfish *Acanthochromis polyacanthus* (Spinks et al., 2021). Besides the promoting effects on ovarian development (Souza et al., 2024; Zhao et al., 2020), a high-temperature treatment could also negatively affect the ovarian development in female pupfish by impairing ovarian steroidogenesis (Housh et al., 2024), and delayed ovarian development in the lesser sand eel with oocyte maturation over 2 months (Wright et al., 2017).

Considering the variability in responses of teleost reproductive systems to thermal challenges and the effects of climate change on aquaculture and fisheries, it is crucial to explore the effects of temperature rises on other fish species. Our preliminary study found that green horse-faced filefish is a thermo-sensitive species vulnerable to gonadal development disturbance due to rising temperature, and is suitable for scientific research on temperature-induced reproductive development in fish. Thus, we applied transcriptomics and metabolomics techniques to systematically elucidate the molecular mechanisms of ovary development in *T. septentrionalis* induced by temperature rises in the present study. Histological and ultrastructural analyses of *T. septentrionalis* ovaries were conducted in this study. The concentration of two key steroid hormones (17 β -estradiol and testosterone) was quantified in *T. septentrionalis* plasma. The major findings in the present study will reveal the critical biological pathways and the genes involved in ovary development in *T. septentrionalis*, which will provide theoretical scientific evidence for the artificial breeding of *T. septentrionalis*, enrich the

scientific knowledge of the molecular mechanism underlying teleost ovary development, and potentially provide theoretical scientific evidence for the conservation efforts in fish stocks or fisheries management under global warming.

2 Materials and methods

2.1 Experiment animals, design and sampling

Three-year-old female parent greenfin horse-faced filefish *T. septentrionalis* without a reproductive history were collected from a local aquatic hatchery located in Yantai, China. Our preliminary investigation found that the ovaries of female fish will be maintained at stage II in the process of *T. septentrionalis* breeding without appropriate temperature stimulation (Supplementary Figure 1), which provided an important basis and evidence for conceiving the experiment design and determining the sampling time points in this study. Furthermore, the experiment was carried out under the same culture condition as recorded in the preliminary investigation to exclude the potential influence of the other factors (e.g. diet, temperature, salinity, and photoperiod) on ovarian development of the *T. septentrionalis* used in this study. During the overwintering period controlled by water temperature rises, *T. septentrionalis* ovaries ($n = 6$ for each group) were sampled before the water rising (the LO group), while those sampled after the water rising were designated as the HO group. Detailed information on the experimental design and sampling in this study are presented in Supplementary Figure 2. Prior to sampling, fish were not fed for 24 hours (h) and anesthetized with 0.05% MS-222. The body's full length, body weight, and gonad weight were measured and listed in Table 1. The gonadal somatic index (GSI) of each fish was measured as described in Flores et al. (2015). Using heparinized syringes, the blood samples were collected from the caudal vein of 12 fish ($n = 6$ for the LO group, $n = 6$ for the HO group) and centrifuged for 15 min at 800 g to separate plasma for hormone extraction and quantification of 17 β -estradiol (E2) and testosterone (T) by enzyme-linked immunosorbent assay (ELISA) following the method as described in Sánchez-Hernández et al. (2013). Then, the tissues of the ovaries were collected for further molecular and cellular analysis. For each ovary sample, approximately 1 cm³ of ovary tissue was preserved in Bouin's solution for histopathological analysis and 1 cm³ of ovary tissue was preserved in 2.5% glutaraldehyde solution for the scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses. Further ovary tissues of *T. septentrionalis* ($n = 3$ /group for transcriptomic analysis; $n = 6$ /group for metabolomics analysis)

were collected and preserved at -80°C after quick freezing in liquid nitrogen until being further processed.

2.2 Histological analysis of *T. septentrionalis* ovaries

The ovary tissues of *T. septentrionalis* were dissected and further sampled for histological examination. Briefly, the ovary tissues were excised and fixed in Bouin's solution for less than 24 h, and the fixative solution was replaced several times with 70% ethanol in which samples were eventually stored. All tissue samples were then processed using paraffin histological techniques and stained with hematoxylin and eosin (H and E) as described in previous studies (Johnson et al., 2009; Sharma et al., 2023). The prepared slides were carefully examined and documented with an Olympus BX53 microscope equipped with an Olympus DP73 digital camera (200 $\times$, 400 $\times$).

2.3 SEM and TEM analysis of *T. septentrionalis* ovary

The gonads were removed and individually immersed in sodium cacodylate buffer (0.1 M, pH 7.4) supplemented with 2.5% glutaraldehyde. After 24 h at 4°C, tissue samples were washed three times for 5 min with the sodium cacodylate buffer and post-fixed in the same buffer containing 1% osmium tetroxide (OsO₄) for 1.5 h. For the SEM analysis, the tissue samples were dehydrated in a graded ethanol series (30%–100%), critical point dried using CO₂, mounted on metallic stubs using carbon stickers, sputter-coated with gold, and examined using a HITACHI SU8100 SEM. For the TEM analysis, the tissue samples were dehydrated in an ascending acetone series and embedded in Epon 812. Ultra-thin sections were cut in a microtome and contrasted with uranyl acetate and lead citrate, followed by examination using a HITACHI HT7800 electron microscope with a HITACHI TEM system.

2.4 RNA extraction, library construction, transcriptome sequencing, and analysis

RNA extraction, library preparation, sequencing, and bioinformatics analyses were performed at Beijing Novogene Technology Co., LTD (Beijing, China). The sequencing library was prepared using the NEBNext mRNA Sample Prep Reagent Set for Illumina (NEB, USA). Qubit 2.0 (Life Technologies,

TABLE 1 The physiological indexes of the sampled *Thamnaconus septentrionalis* in this study.

Group	Total length (cm)	Body weight (g)	Ovary weight (g)	GSI (%)
LO	30.73 $\pm$ 2.54	526.57 $\pm$ 93.56	9.71 $\pm$ 1.92	1.84 $\pm$ 0.05 ^b
HO	32.53 $\pm$ 1.04	557.12 $\pm$ 73.61	13.22 $\pm$ 1.11	2.33 $\pm$ 0.28 ^a

The different superscript letters indicated the significance of the GSI between the two groups ($P < 0.05$).

Carlsbad, CA, USA) was used for preliminary library quantification, and qRT-PCR was conducted for accurate quantification of the effective concentration of the library (>2 nM) to ensure the quality of the library. The libraries were subjected to paired-end HiSeq sequencing and generated 6G of data for each sample. Novaseq 6000 was used with the PE150 sequencing strategy. Trimmomatic software (v. 0.39) was used to remove reads with sequencing adapters (Bolger et al., 2014), reads with N (uncertain base) content $>10\%$, and reads with low-quality base ($Q \leq 20$) content $> 50\%$. FASTP (version 0.19.7) served as the low-quality reads removal software with the parameter FASTP-Q 20-U 50-N 15-L 150. The clean reads were aligned to a reference genome using HISAT2 software (Pertea et al., 2016), based on the bowtie2 method (Langmead, 2010), and genes were annotated.

The annotated genes were counted using RNASeq by Expectation Maximization v1.3.0 (Li and Dewey, 2011), and FPKM values were determined to obtain transcript levels. Differentially expressed genes (DEGs) among the samples were identified using the DESeq2 R package and based on $FDR < 0.05$ and $|\log_2(\text{fold change})| > 1$ (Love et al., 2014). The DEGs were clustered in heatmaps and subjected to Gene Ontology (GO) enrichment for functional analysis (<http://www.geneontology.org/>) based on molecular function, biological process, and cellular component terms using GoSeq (v. 1.22) (Young et al., 2010). For KEGG pathway enrichment analysis (Kanehisa et al., 2004), a hypergeometric test was applied to identify the pathways significantly enriched in DEGs based on $FDR < 0.05$. R software and a self-written script were used for pathway enrichment analysis using Benjamini–Hochberg-corrected FDR values.

2.5 Untargeted metabolomics analysis of *T. septentrionalis* ovaries

2.5.1 Sample collection and preparation

The fish ovary tissues were quickly frozen in liquid nitrogen immediately after dissection. Then, the tissue was cut on dry ice (~ 80 mg) and placed in an Eppendorf tube (2 mL). The tissue samples, with 200 μL of H_2O and five ceramic beads, were homogenized using the homogenizer, and 800 μL methanol/acetonitrile (1:1, v/v) was added to the homogenized solution for metabolite extraction. The mixture was centrifuged for 20 min (14,000 g, 4°C), and the supernatant was dried in a vacuum centrifuge. For LC-MS analysis, the samples were re-dissolved in 100 μL acetonitrile/water (1:1, v/v) solvent and centrifuged at 14,000 g at 4°C for 15 min, and then the supernatant was injected.

2.5.2 LC-MS/MS analysis

Analysis was performed using a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) at Shanghai Applied Protein Technology Co., Ltd. For HILIC separation, samples were analyzed using a 2.1 mm $\times$ 100 mm ACQUITY UPLC BEH Amide 1.7 μm column (Waters, Ireland). In both ESI positive and negative modes, the mobile phase contained A = 25 mM ammonium acetate and 25 mM

ammonium hydroxide in water and B = acetonitrile. The gradient was 95% B for 0.5 min and was linearly reduced to 65% for 6.5 min, and then was reduced to 40% for 1 min, and then increased to 95% for 0.1 min, with a 3 min re-equilibration period employed. The ESI source conditions were set as follows: Ion Source Gas1 (Gas1): 60, Ion Source Gas2 (Gas2): 60, curtain gas (CUR): 30, source temperature: 600°C , IonSpray Voltage Floating (ISVF) ± 5500 V. In the MS-only acquisition, the instrument was set to acquire over the m/z range of 60–1000 Da, and the accumulation time for the TOF MS scan was set at 0.20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25–1000 Da, and the accumulation time for the product ion scan was set at 0.05 s/spectra. The product ion scan was acquired using information-dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was fixed at 35 V with ± 15 eV; declustering potential (DP), 60 V (+) and -60 V (–); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.

2.5.3 Data processing and statistical analysis

The raw MS data were converted to MzXML files using ProteoWizard MSConvert before being imported into freely available XCMS software. For peak picking, the following parameters were used: centWave $m/z = 10$ ppm, peakwidth = c (10, 60), and prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, and minfrac = 0.5 were used. CAMERA (Collection of Algorithms of MEtabolite pRoFile Annotation) was used for annotation of isotopes and adducts. In the extracted ion features, only the variables with more than 50% of the non-zero measurement values in at least one group were kept. Compound identification of metabolites was performed by comparing the accuracy m/z value (< 10 ppm) and MS/MS spectra with an in-house database established with available authentication standards.

After sum-normalization, the processed data were analyzed in an R package, where it was subjected to multivariate data analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Seven-fold cross-validation and response permutation testing were used to evaluate the robustness of the model. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. Student's t-test was applied to determine the significance of differences between two groups of independent samples. The metabolites were considered to be significantly different between the LO and HO groups when $VIP > 1$ and $P\text{-value} < 0.05$. Pearson's correlation analysis was performed to determine the correlation between two variables.

2.6 Validation of representative DEGs with qRT-PCR assays

To verify the reliability of the sequencing data, the transcriptional profiles of 12 DEGs involved in critical fish gonadal development processes with significant alterations were further analyzed by qRT-

PCR assays. Total RNAs were isolated from the same subset of fish ovary samples using Trizol reagent (TaKaRa, Japan). The extracted RNAs were treated with RNase-free DNase I (TaKaRa, Japan) to remove contaminating genomic DNAs. The transcripts of the DEGs were analyzed and validated by qRT-PCR assays using the PrimeScriptTM RT reagent Kit (TaKaRa, Japan) as described in Li et al. (2019). The qRT-PCR assays were carried out in triplicate. The transcripts of the target genes were normalized by the geometric mean of three endogenous reference genes, including β -actin, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and elongation factor 1-alpha (*ef1a*), according to the MIQE guidelines (Bustin et al., 2009). The qRT-PCR primers used in this study were designed in the online software qPrimerDB 2.0 (Li et al., 2025) and are listed in Supplementary Table 1. The gene expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method as described in Livak and Schmittgen (2001). Data were shown as the mean $\pm$ standard deviation (SD) and analyzed via the one-way analysis of variance (ANOVA), followed by Duncan's multiple range test using the SPSS statistics software (version 20.0, SPSS, USA). Differences were considered significant when $P < 0.05$.

3 Results

3.1 The physiological index of the sampled female *T. septentrionalis*

No significant difference was found in body length, full length, body weight, and gonad weight between the LO and HO groups, while the GSI was significantly increased in the HO group compared to the LO group (Table 1). Compared to the LO group, the concentrations of E2 (1.86-fold, $P < 0.05$) and T (1.54-fold, $P < 0.05$) were significantly increased in the plasma of *T. septentrionalis* in the HO group (Figure 1).

3.2 Histological, SEM, and TEM analyses of *T. septentrionalis* ovary

In the previous histological staining analysis, the oocyte development stages of *T. septentrionalis* were described in detail and classified into six stages (Zhang, 2011). In the present study, the histological observation showed that the oocytes in the LO ovaries were predominantly in phase II with a minority of oocytes in phase I, indicating the LO ovaries were in stage II. Then, after the temperature rise, the oocytes in the HO ovaries started to develop with increased cell volume and the partial oocytes transitioned to phase III, indicating the development of the HO ovaries to stage III (Figure 2). The SEM analysis showed that the overall structure of the sampled ovaries was tree-branch-like and nearly spherical oocytes could be clearly observed (Figures 3A, B). Similar to the histological results, the oocytes in the LO ovaries were mainly in phase II, while those in HO ovaries developed in a stepwise fashion to phase III. In addition, the ultrastructure of the nucleolus, nuclear membrane, and radiation zone could be clearly observed in the sampled ovaries in the TEM analysis (Figures 3C, D). Compared to the oocytes in the LO ovaries, the nuclei in the HO ovaries were enlarged with more condensed nucleoli, and big vacuoles appeared in the HO ovaries.

3.3 Transcriptome responses of *T. septentrionalis* ovary to rising temperature

3.3.1 Quality assessment of sequencing data

A total of 271,432,936 raw sequencing reads were obtained by the transcriptomics analysis of ovary tissues from the LO and HO groups. After removing low-quality and junction sequence reads, a total of 262,230,538 clean reads were obtained. The results of transcriptomics

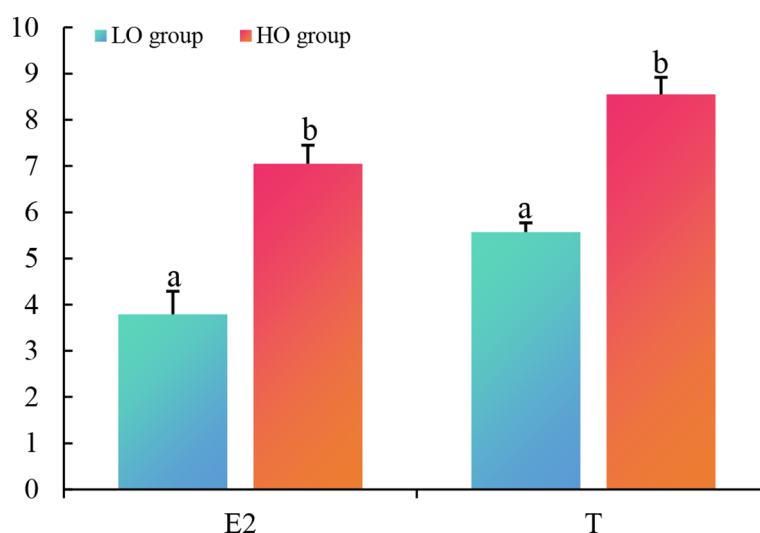


FIGURE 1

The concentrations of plasma 17 β -estradiol (E2) and testosterone (T) in the LO and HO groups. Statistics significance ($P < 0.05$) was indicated by the different letters on bars. LO, *T. septentrionalis* ovaries treated with low temperature; HO, *T. septentrionalis* ovaries treated with high temperature.

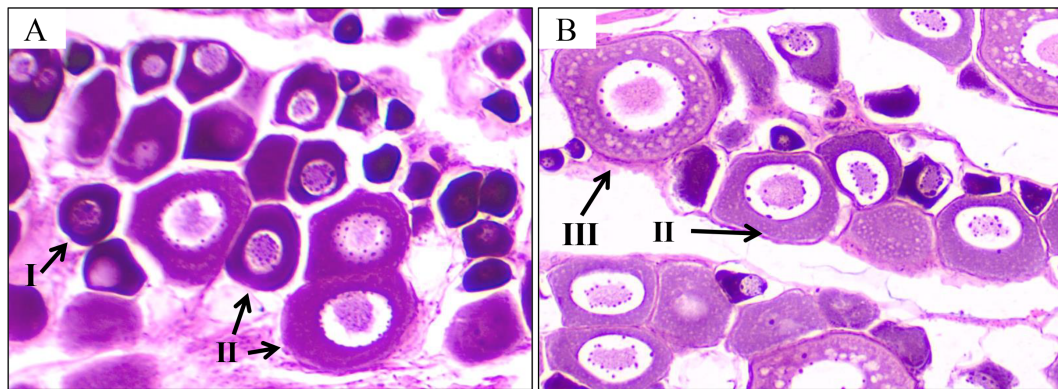


FIGURE 2

Histological observation of *T. septentrionalis* ovaries in the LO (A) and HO (B) groups. I, oocyte in phase I; II, oocyte in phase II; III, oocyte in phase III; scale bar: 100 μ m.

quality analysis showed that the average GC content in the LO and HO groups was 53.15% and 53.18%, respectively; the average Q30 values were 91.52% and 91.36%, respectively; and the average Q20 values were 96.66% and 96.56%, respectively. The clean reads were

mapped to the *T. septentrionalis* genome; the average mapping rates of the LO and HO groups were 81.16% and 81.03%, respectively, indicating that the quality of transcriptome sequencing was good and reliable (Supplementary Table 2).

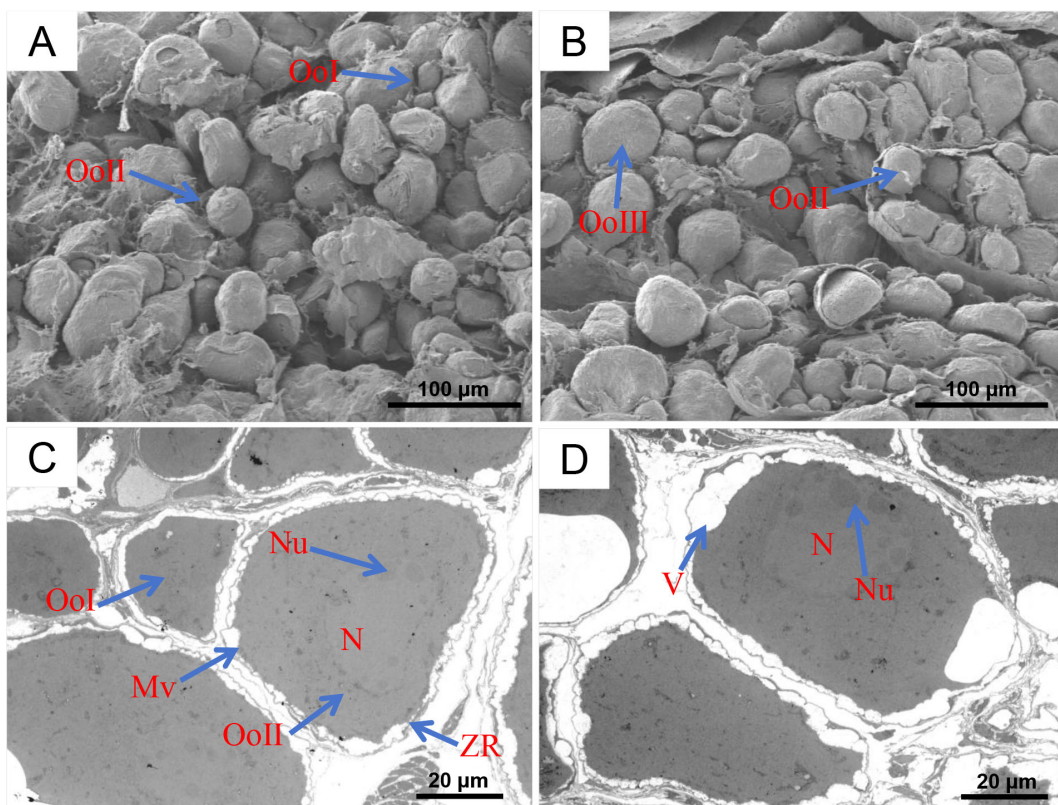


FIGURE 3

SEM and TEM analysis of *T. septentrionalis* ovaries in the LO and HO groups. SEM photograph of *T. septentrionalis* ovaries in the LO (A) and HO (B) groups. TEM analysis of the *T. septentrionalis* ovaries in the LO (C) and HO (D) groups. OoI, oocyte in phase I; OoII, oocyte in phase II; OoIII, oocyte in phase III; Nu, nucleolus; CA, cortical alveoli; V, vacuole; Oo, oocyte; Mv, microvilli; ZR, zona radiata. Scale bar: 2 μ m. SEM, scanning electron microscope; TEM, transmission electron microscope.

3.3.2 Identification and enrichment analysis of DEGs

In the comparative analysis of the transcriptomic sequencing of the LO and HO groups under the screening conditions of $FDR < 0.05$ and $|\log_2 FC| \geq 1$, a total of 146 DEGs were identified, including 97 upregulated and 49 downregulated genes (Figure 4A). In order to deeply understand the biological processes in which the DEGs were involved, GO enrichment analysis was conducted. The results showed that the DEGs were mainly divided into three functional categories: biological process, molecular function, and biological adhesion. The biological processes mainly include proteolysis, cell adhesion, and DNA metabolic processes. The cellular components mainly comprise the extracellular matrix, parts of the extracellular region, and the extracellular region. The molecular functions are mostly related to the metalloproteinase activity, metallopeptidase activity, and structural constituents of the extracellular matrix (Figure 4B). KEGG pathway enrichment analysis suggested that a total of 146 DEGs were divided into six categories, which included metabolism, organic system, human diseases, environmental information processing, cellular processes, and genetic information processing, of which six KEGG pathways, including ECM-receptor interaction, focal adhesion, vascular smooth muscle contraction, regulation of actin cytoskeleton, oocyte meiosis, and steroid hormone biosynthesis, were significantly enriched (Figure 5).

3.4 qRT-PCR assays of critical DEGs in *T. septentrionalis* ovaries responding to rising temperature

In order to verify the reliability and accuracy of the RNA-Seq results, 12 DEGs in *T. septentrionalis* ovaries enriched in the ECM-receptor interaction (*collagen $\alpha 1$* , *femp2*, and *integrin $\alpha 1$*), focal adhesion (*collagen $\alpha 2$* and *vinculin*), regulation of actin cytoskeleton (*protein phosphatase 1* and *myosin*), steroid hormone

biosynthesis (*hsd3b* and *hsd17b1*), oocyte meiosis (*adenylate cyclase 8*, *cyclin-E2*, and *calmodulin-1*) pathways were subjected to qRT-PCR analysis. The transcripts of *collagen $\alpha 1$* (2.16-fold), *FEMP2* (3.58-fold), *integrin $\alpha 1$* (2.78-fold), *collagen $\alpha 2$* (5.62-fold), *vinculin* (13.68-fold), *protein phosphatase 1* (15.21-fold), *myosin* (9.16-fold), *hsd3b* (6.21-fold), *hsd17b1* (3.62-fold), and *calmodulin-1* (2.69-fold) were significantly increased in *T. septentrionalis* ovaries responding to rising temperature (Supplementary Figure 3). The transcripts of *adenylate cyclase 8* (0.15-fold) and *cyclin-E2* (0.28-fold) were significantly decreased in *T. septentrionalis* ovaries after temperature rises (Supplementary Figure 3). The qRT-PCR results showed that the DEGs exhibited a similar expression trend as that of RNA-seq results (Supplementary Figure 3), reflecting that the transcriptomic results were accurate and reliable in the present study.

3.5 Metabolomic responses of *T. septentrionalis* ovaries to rising temperature

3.5.1 Identification and multivariate statistical analysis of metabolites

The effects of rising temperature on ovary metabolism in *T. septentrionalis* were studied using non-targeted metabolomics analysis using the LC-MS platform. The ionization source of LC-MS was ESI, including two ion modes: positive ion mode (POS) and negative ion mode (NEG). The peak retention time (RT) and peak area of the total ion chromatogram of all the quality control (QC) samples overlapped well, indicating that the analysis system was stable and reliable (Supplementary Figure 4). In the positive and the negative ion modes, a total of 558 and 543 effective peaks were identified, respectively. The effective peaks were compared to the databases of the metabolomes (MS1 and MS2), which identified 558 and 543 metabolites in the positive and negative ion modes, respectively. The 1,101 metabolites were classified into 15

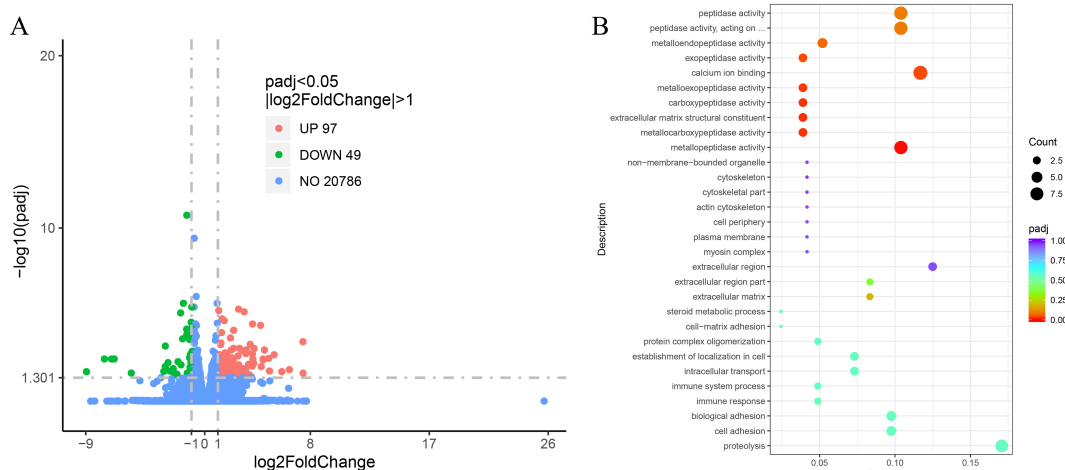


FIGURE 4

(A) The volcano map of the DEGs between the LO and HO groups. Red dots: significantly upregulated DEGs, green dots: significantly downregulated DEGs. (B) GO enrichment analysis of the DEGs between the LO and HO groups. DEGs, differentially expressed genes; GO, Gene Ontology.

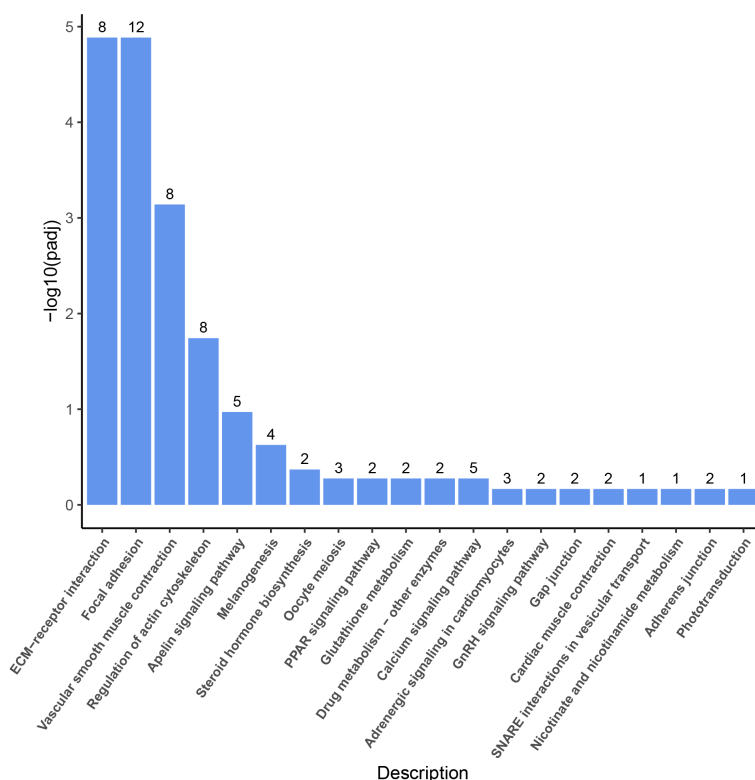


FIGURE 5

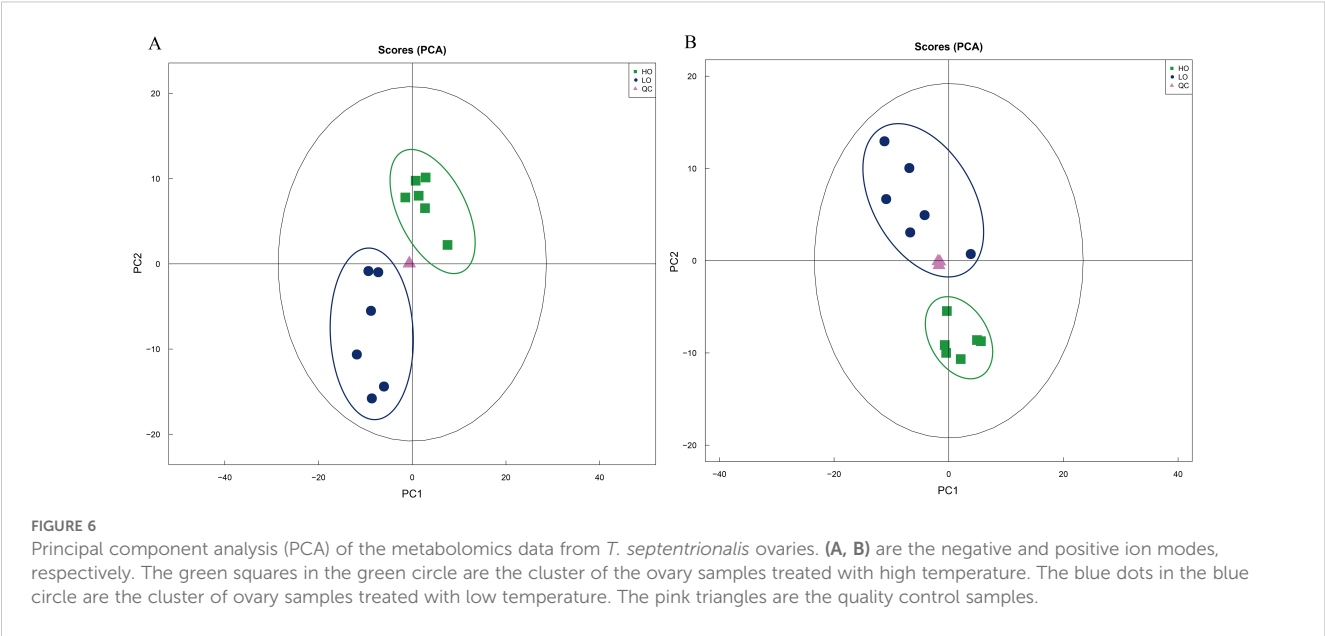
KEGG pathway enrichment analysis of the DEGs between the LO and HO groups. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes. Each column indicates for one pathway with the number of DEGs in it.

superclasses including lipids and lipid-like molecules; organic acids and derivatives; undefined, organoheterocyclic compounds; organic oxygen compounds; benzenoids; nucleosides, nucleotides, and analogs; organic nitrogen compounds; phenylpropanoids and polyketides; alkaloids and derivatives; homogeneous non-metal compounds; lignans, neolignans, and related compounds; organoheterocyclic compounds; organometallic compounds; and organosulfur compounds (Supplementary Figure 5).

The PCA results showed that the LO and HO groups were significantly differentiated in the negative and positive ion modes (Figure 6), respectively. The cross-validation of the OPLS-DA model showed that R^2_X , R^2_Y , and Q^2 were 0.544, 0.994, and 0.77 in the positive ion mode respectively, and those in the negative ion mode were 0.468, 0.979, and 0.798, respectively. Q^2 was greater than 0.5, indicating the model had good prediction ability. Then, the OPLS-DA model was established with a permutation test, and the order of the categorical variable Y was randomly changed to obtain the R^2 and Q^2 of the random model. All the Q^2 points were lower than the original black Q^2 points from left to right, which indicated the model's reliability with no overfitting and further ensured that the metabolomics data and instrument analysis system was reliable and stable in the present study.

3.5.2 Analysis of SDMs and their metabolic pathways

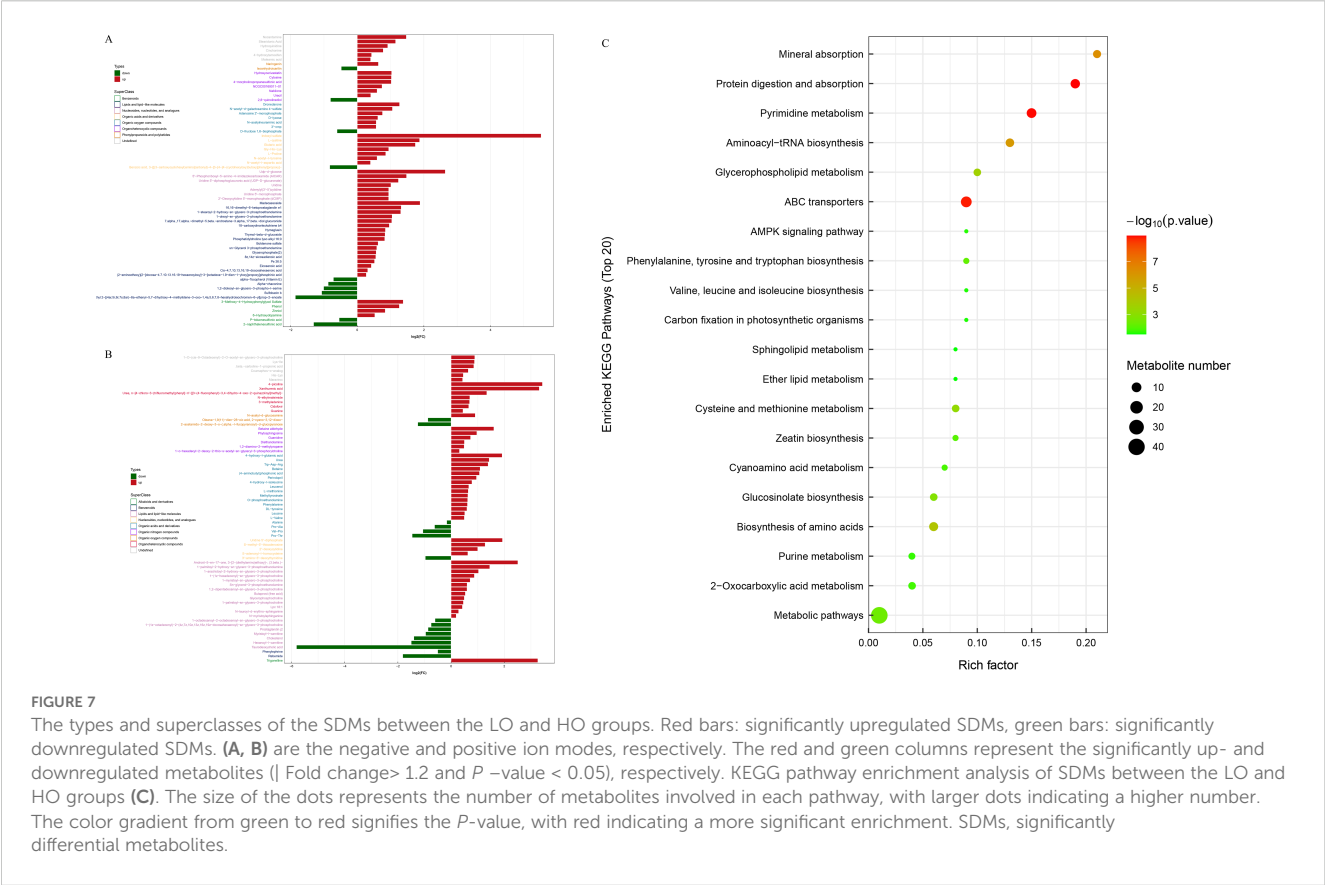
According to the screening conditions ($VIP > 1$ and $P < 0.05$), the significantly different metabolites (SDMs) between the two groups were obtained, and their fold changes (FCs) were calculated. The results showed that 65 SDMs were identified in the HO vs. LO groups, among which 54 were upregulated, and 11 were downregulated under the negative ion mode (Figure 7A). There was a total of 69 SDMs in the HO vs. LO groups under the positive ion mode, among which 53 were upregulated, and 16 were downregulated (Figure 7B). Based on the screening conditions of $VIP > 1$ and $P < 0.05$, a total of 25 KEGG pathways were significantly enriched by the SDMs between the LO and HO groups, and the top 10 significantly enriched pathways were protein digestion and absorption, pyrimidine metabolism, ABC transporters, mineral absorption, aminoacyl-tRNA biosynthesis, biosynthesis of amino acids, glycerophospholipid metabolism, cysteine and methionine metabolism, glucosinolate biosynthesis, and metabolic pathways (Figure 7C). In addition, three significantly enriched pathways, glycerophospholipid metabolism, ether lipid metabolism, and sphingolipid metabolism, belonged to the Pathway Hierarchy 2 "Lipid metabolism" category.



4 Discussion

The greenfin horse-faced filefish *T. septentrionalis* is a valuable commercial fish species widely distributed in the Indo-West Pacific Ocean and has become an important marine aquaculture fish species in China. In recent years, there has been a sharp decline in the population resources and overfishing of *T. septentrionalis*.

Studies have focused on the reproductive development and artificial propagation of *T. septentrionalis* under industrial aquaculture conditions. However, the molecular regulatory mechanism underlying the reproductive development in *T. septentrionalis* has been scarcely studied until now. In the present study, we systematically studied the molecular regulatory mechanism of temperature-induced ovary development in *T. septentrionalis*



under industrial aquaculture conditions. The key genes, metabolites, and the biological pathways contributing to the temperature-induced development of *T. septentrionalis* were revealed. In addition, the present study found that rising temperature promotes ovarian maturation in female *T. septentrionalis* primarily by enhancing the synthesis of E2 and metabolic regulation during the overwintering period.

The transcriptomics results showed that the pathways of “ECM-receptor interaction”, “Focal adhesion”, and “Regulation of actin cytoskeleton” were overtly modulated in *T. septentrionalis* ovaries after temperature rises. ECM is an important structure essential for normal tissue homeostasis (Bonnans et al., 2014) and participates in regulating cellular adhesion, proliferation, and differentiation (Liu et al., 2015). The ECM-receptor interaction pathway plays a crucial regulatory role in ovarian development and oocyte proliferation, differentiation, and maturation (Irving-Rodgers and Rodgers, 2005; Cassel, 2019; Curry and Osteen, 2003; Dong et al., 2021). ECM must be involved in ovarian development as ECM provides structural support for follicles and regulates follicle proliferation, survival, and differentiation, and steroid formation to promote follicle development and maturation (Cha et al., 1991). Wang et al. (2024) showed that the genes (*collagen* and *Integrin*) involved in the ECM-receptor interaction pathway were significantly upregulated in *Procambarus clarkii* ovaries, indicating their important regulatory function in promoting ovarian development and oocyte maturation. Yue et al. (2004) demonstrated that integrins could initiate the reorganization of cytoskeletal protein in mouse oocytes and were likely involved in transmembrane signal transduction and egg activation during fertilization. Pallotta et al. (2022) suggested that the FRAS1-related extracellular matrix protein (FEMP), an important ECM protein, is increased in female embryonic gonads of the leopard gecko *Eublepharis macularius* and participate in the gonad differentiation and embryonic development in *E. macularius*. Gautier et al. (2008) suggested that FREM proteins participate in the development of various tissues (e.g. pharyngeal arches, hypochord, musculature, fin) in zebrafish *Danio rerio*. In the present study, the transcripts of three genes (*collagen α1*, *Integrin α1*, and *femp2*) involved in the ECM-receptor interaction pathway were significantly increased in *T. septentrionalis* ovaries after temperature rises, which suggested the critical function of the ECM-receptor interaction pathway in the ovarian development in *T. septentrionalis* in response to rising temperature.

In addition, focal adhesion and the regulation of actin cytoskeleton pathways were also significantly influenced in *T. septentrionalis* ovary after temperature rises. Focal adhesion regulates cell morphology, migration, and proliferation during ovarian development (Wang et al., 2024). The actin cytoskeleton plays an important role in regulating meiotic maturation and fertilization (Santella et al., 2014). In the present study, four genes (*Protein phosphatase 1*, *Myosin*, *Vinculin*, and *collagen α2*) involved in these two pathways were significantly upregulated in *T. septentrionalis* ovaries after temperature rises. Mishra and Joy (2006) suggested that protein phosphatase (PP) may be involved

in the PKC (protein kinase C) modulation of steroidogenesis, oocyte maturation, and ovulation in animals. Vinculin is a cytoskeletal protein that localizes to cell-ECM and cell-cell adhesion sites, with crucial roles in the maintenance and regulation of cell adhesion and migration (Paz et al., 2007), and is involved in the PKC modulation of the cytoplasmic compartment during the development of oocytes (Johnson and Capco, 1997). Myosins were found to play a role in regulating the migration of zebrafish primordial germ cells (Blaser et al., 2006). Combining the results of this study and previous findings, the present study suggests that focal adhesion and regulation of actin cytoskeleton pathways were significantly involved in the temperature-induced ovarian development in *T. septentrionalis*.

Furthermore, the steroid hormone biosynthesis pathway was significantly influenced in *T. septentrionalis* after temperature rises. Two key steroidogenesis-related genes (*hsd3b* and *hsd17b1*) were significantly increased in *T. septentrionalis* ovaries after temperature rises in this study. Hasegawa et al. (2022) found rapid upregulation of *hsd3b* expression during oocyte maturation in Amur sturgeons *Acipenser schrenckii*. Recent studies also established that *hsd17b1* participates in regulating gonad differentiation and ovarian development by regulating the synthesis of sex steroid hormones (e.g. E2 and testosterone) in teleosts including Japanese sardine *Sardinops melanostictus* (Nyuji et al., 2024), olive flounder *Paralichthys olivaceus* (Zou et al., 2020), and Siberian sturgeon *Acipenser baerii* (Lasalle et al., 2024). Cai et al. (2023) also reported enhanced transcripts of *hsd3b* and *hsd17b1*, suggesting an important role in ovarian development in common carp *Cyprinus carpio* by catalyzing the synthesis of E2. E2 stimulates hepatic cells to synthesize and secrete vitellogenin to promote oocyte development in female fish (Hiramatsu et al., 2015; Hara et al., 2016; Maldonado-García et al., 2018). It is also known that E2 increases from the first stage of ovarian development and peaks during puberty in coho salmon *Oncorhynchus kisutch* and yellow tail flounder *Limanda ferruginea* (Campbell et al., 2006; Manning et al., 2008). In the present study, the transcripts of *hsd3b* and *hsd17b1* were significantly increased in *T. septentrionalis* ovaries after temperature rises, which contributed to enhancing the synthesis of E2. Then, the significantly increased E2 level promoted the development of *T. septentrionalis* ovaries with oocytes developed from stage II to stage III as indicated by the histological analysis and the significantly increased GSI. The combined results suggested that rising temperature could regulate sex-related steroid hormone synthesis such as E2 by influencing the steroid hormone biosynthesis pathway to promote the development and maturation of *T. septentrionalis* ovaries (Figure 8). Moreover, the findings of the stimulation effects of rising temperature on *T. septentrionalis* ovary development were consistent with recent studies, which showed that a temperature rise promoted ovarian development in *Leporinus friderici* (Souza et al., 2024), *Oreochromis niloticus* (Zhao et al., 2020), and *Acanthochromis polyacanthus* (Spinks et al., 2020).

In addition, the “Oocyte meiosis” pathway was significantly altered with three genes (*Adenylate cyclase type 8*, *Cyclin-E2*, and

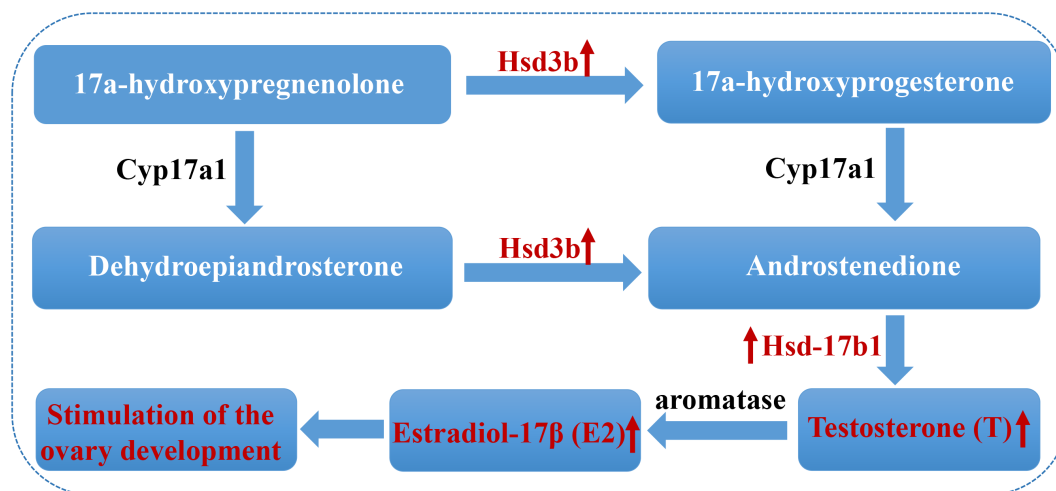


FIGURE 8

The potential mechanism of promoting ovarian development in *Thamnaconus septentrionalis* by increasing the E2 level via regulation of the key steroidogenic genes. The two significantly upregulated genes (Hsd3b and Hsd-17b1) and the increased levels of hormones (E2, T) were labeled in red and indicated by red arrows. Hsd3b, 3β-hydroxysteroid dehydrogenase; hsd17b1, 17β-hydroxysteroid dehydrogenase 1; E2, Estradiol-17β; T, Testosterone.

Calmodulin-1) differentially expressed in *T. septentrionalis* ovary after temperature rises in this study. Distinct expression patterns of *Adenylate cyclases* (ACs) and *Cyclins* were observed during oocyte growth and maturation of ricefield eels *Monopterus albus* (Meng et al., 2022), reflecting the roles of ACs and *Cyclins* in regulating fish oocyte meiosis and ovarian development. Tang et al. (2019) reported the significant downregulation of AC8 in Nile tilapia *Oreochromis niloticus* and suggested its involvement in regulating ovary development in Nile tilapia under photoperiod conditions. In the present study, the transcripts of AC type 8 and G2/S1-specific *Cyclin-E2* decreased significantly in *T. septentrionalis* ovaries after temperature rises, which was consistent with previous studies (Meng et al., 2022; Tang et al., 2019), further suggesting that ACs and *Cyclin-E2* play regulatory roles in fish ovary development. Moreover, the transcripts of *calmodulin 1* (CALM1) involved in the oocyte meiosis pathway were significantly increased in *T. septentrionalis* ovaries after temperature rises in this study. Previous studies showed that CALM1 is a prototypical calcium sensor and promotes the secretion of steroid hormones (Levors et al., 1991; Chin and Means, 2000). Moreover, CALM1 has been reported to regulate androgen production in chicken follicular membrane cells (Xu et al., 2016). Thus, the increased transcripts of CALM1 suggest its critical role in regulating temperature-induced ovarian development in *T. septentrionalis*.

In female animals, reproduction and metabolism are tightly connected and reciprocally regulated, with energy metabolism tuned to meet reproductive needs during the reproductive cycle (Fontana and Torre, 2016). In fish, reproduction requires energy investment to metabolically support ovary maturation and the production of massive eggs in females. Gonad development was found to be accompanied by metabolic and energy-demanding processes, and metabolic changes were closely related to the

reproductive cycle to sustain this energy-demanding process specific to the stage of gonadal development or period of growth in female goldfish (Ladisa et al., 2022). In addition, Ladisa et al. (2022) found enhanced lipid metabolism, particularly glycerophospholipid and sphingolipid metabolism, and fatty acids biosynthesis during the reproductive cycle of the mature female goldfish *Carassius auratus*. In the present study, we also found that lipid metabolism was significantly regulated by the rising temperature, with three pathways, “Glycerophospholipid metabolism”, “Ether lipid metabolism”, and “Sphingolipid metabolism”, significantly enhanced after the temperature rise. The lipid content of ovaries accounts for a large proportion of the fish’s weight, ranging from 12.2% to 25.5% of the wet weight in wild *Tilapia nilotica* (James Henderson and Tocher, 1987). Furthermore, elevated lipid synthesis during the peak of vitellogenesis and lipid mobilization contributed to supporting maximal gonadal maturation (Ladisa et al., 2022). Thus, we suggest that the higher temperature had promoting effects on the maturation of *T. septentrionalis* ovary partially by enhancing fish lipid metabolism.

In addition, three metabolic pathways involved in protein and amino acids including “Protein digestion and absorption”, “Biosynthesis of amino acids pathways”, and “Cysteine and methionine metabolism” were also enhanced by the rising temperature in *T. septentrionalis* ovaries. Zhu et al. (2020) revealed the metabolic changes of fish bodies in harmony with ovarian development and indicated that there were increased active amino acids, lipid metabolism, and energy dynamics in the fish body during ovary development from stage II to IV in female Chinese sturgeon *Acipenser sinensis*. Urich et al. (2020) showed that the development of oocytes likely resulted from the differences in energy metabolism, protein synthesis, phospholipid production, and elevated levels of amino acids in the gonads of largemouth

bass *Micropterus salmoides*. Thus, the results in this study revealed active metabolism of lipids, proteins, and amino acids in response to the rising temperature, which likely played an important role in promoting the development of *T. septentrionalis* ovaries.

It was also noteworthy that the ABC transporter pathway was found to be significantly enriched in the *T. septentrionalis* ovaries in response to rising temperature by the metabolomics analysis. The role of the ABC transporter pathway in fish reproductive processes has increasingly attracted research interest in recent years. Huang et al. (2023) found that ABC transporters were involved in regulating gonad development and oocyte maturation in zebrafish using comparative transcriptome analysis. Shu et al. (2023) showed that the ABC transporter pathway played a crucial role in follicular development and oocyte maturation in the ovaries of *Ctenopharyngodon idellus*. Sun et al. (2023) found that the ABC transporter pathway was significantly involved in gonad differentiation and development in golden pompano *Trachinotus blochii*. Previous studies have established that ABC transporters transport ligands across cellular lipid membranes, which is critical for many aspects of cell physiology, including the uptake of nutrients and energy generation (Linton, 2007; Wilkens, 2015). Thus, in the present study, the higher-temperature-induced ovarian development in *T. septentrionalis* during the overwintering period was closely related to the active processes of nutrition uptake and energy metabolism, which needs to be further studied.

5 Conclusion

In the present study, the potential underlying molecular mechanism of higher-temperature-induced ovary development in *T. septentrionalis* during the overwintering period was systematically investigated at the cellular and molecular levels. The transcriptomic and metabolomic analyses revealed the key genes, metabolites, and critical regulatory pathways significantly associated with ovary development in *T. septentrionalis*. The histological and ultrastructural analyses showed the rising temperature contributed to the development of oocytes in *T. septentrionalis*, which was further supported by the significantly increased plasma E2 concentration. In terms of female gonadal development, rising water temperature was suitable for the development of *T. septentrionalis* ovaries. The major findings in the present study will provide theoretical scientific evidence for the artificial breeding of *T. septentrionalis*, enrich the scientific knowledge of the molecular mechanism underlying teleost ovary development, and potentially provide theoretical scientific evidence the conservation efforts in wild fish stocks or fisheries management under global warming.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was approved by Experimental animal Ethics Committee of Yantai University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Conceptualization, Formal Analysis, Funding acquisition, Writing – original draft. WX: Data curation, Formal Analysis, Writing – review & editing. XZ: Data curation, Formal Analysis, Methodology, Writing – review & editing. JW: Writing – review & editing. XC: Data curation, Writing – review & editing. XY: Formal Analysis, Writing – review & editing. JZ: Funding acquisition, Writing – review & editing. YW: Funding acquisition, Writing – review & editing. LL: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1556002/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Histological observation of *T. septentrionalis* ovaries. (A) ovary of 6-month-old *T. septentrionalis*; (B) ovary of 12-month-old *T. septentrionalis*; (C) ovary of 18-month-old *T. septentrionalis*; (D) ovary of 24-month-old *T. septentrionalis*; (E) ovary of 3-year-old *T. septentrionalis* before rising temperature for overwintering; (F) ovary of 3-year-old *T. septentrionalis* after the overwintering period. Oo(II): oocyte in phase II; Oo(III): oocyte in phase III; Scale bar: 25 μ m.

SUPPLEMENTARY FIGURE 2

Detailed information of the experimental design and sampling in this study.

SUPPLEMENTARY FIGURE 3

Comparison of qRT-PCR and RNA-Seq results for gene expression of 12 DEGs in *T. septentrionalis* ovaries responding to temperature treatment. The qRT-PCR and the RNA-Seq results are represented by the red and blue curves, respectively. DEGs, differentially expressed genes.

SUPPLEMENTARY FIGURE 4

Total Ion Chromatography (TIC) of QC samples. The x and y axis represents the time and the sum of ionic strength respectively at each time point. (A) TIC of QC samples in POS mode; (B) TIC of QC sample in NEG. QC, quality control.

SUPPLEMENTARY FIGURE 5

The classification of the 1,101 metabolites detected in *T. septentrionalis* ovaries.

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