Transcriptome analysis of the *Larimichthys polyactis* under heat and cold stress

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Abstract

The small yellow croaker (*Larimichthys polyactis*) is an important marine economic fish that is widely distributed in the East Sea, Yellow Sea and Bohai of China. However, the wild populations of small yellow croaker are severely depleted, and there is currently a developing large-scale artificial propagation of this fish for aquaculture. However, the current variety of small yellow croaker that is cultivated is not capable to coping with large fluctuations in temperature. Therefore, it is important to understand the molecular mechanisms that are activated in response to temperature stress in the small yellow croaker. Here, we conducted transcriptomic analysis of the liver of small yellow croaker under heat and cold stress. A total of 270,844,888, 265,727,006 and 259,666,218 clean reads were generated from heat temperature group, low temperature group and control group, respectively, and comparing expression of genes in these transcriptomes,10,878 unigenes that were differential expressed were identified. Sixteen of the differentially expressed unigenes were validated by qRT-PCR. Pathway enrichment analysis identified that the ER pathway, immune signaling pathway and metabolic response pathway were affected by temperature stress. The results of this study provide a comprehensive overview of temperature stress-induced transcriptional patterns in liver tissues of the small yellow croaker. In addition, these results can guide future molecular studies of heat and cold stress response in this species for improving the stock used for aquaculture.

Keywords: *Larimichthys polyactis*; Heat stress; Cold stress; Liver transcriptomic; Illumina sequencing

1. Introduction

Water temperature is considered to be the 'abiotic master factor' for fish (Brett J R 1971), impacting biological processes in aquatic animals, including development, growth, reproduction, metabolism, behavior, and geographic distribution (Donaldson M R et al. 2008; Somero G N 2010). Although fish can adapt to variations in water temperature through physiological plasticity or micro-evolution, severe diseases or death occurs when they are exposed to temperatures exceeding their thermal tolerance capability (Whitehead A et al. 2011; Céline Bellard et al. 2012). Due to the intensification of global warming, extremely low winter temperatures and high summer temperatures have led to mass mortality in farmed fish and damaging the economic profit of aquaculture farms. This has negatively affected the economic development of several countries where the economy is dependent on aquaculture, including China, Israel, and South America (Ibarz A et al. 2010). Therefore, investigating the mechanisms underlying temperature adaptation and tolerance in fish species that are used for aquaculture is very important.

The small yellow croaker (*Larimichthys polyactis*) belongs is a warm-temperate near-bottom migratory fish. It is widely distributed in the East Sea, Yellow Sea and Bohai of China (Li Z et al. 2011). The small yellow croaker is nutritious and its meat is of high quality, making it an important marine economic fish. However, due to overfishing and deterioration of the environment in its native habitat, the wild resources of the small yellow croaker are severely depleted (Chen WM and Cheng QQ 2013). Artificial breeding techniques of the small yellow croaker have been developed recently, laying the foundation for large-scale artificial propagation of this species (Liu F et al. 2019). The wild population of small yellow croaker migrates based on water temperature, remaining in areas that are suitable for their survival during the different seasons (Johnson J A and Kelsch S W 1998). However, fish in

aquaculture cannot move beyond the cultured space and area, therefore they cannot migrate to higher or lower temperature to survive changes in temperature. The current method to combat this problem is through adjusting water temperature using thermostat, but this method increases production costs. Therefore, identifying economic methods to increase survival of small yellow croaker under changing temperatures is important for sustainable aquaculture.

Transcriptomics approaches are robust and reliable for identifying genetic pathways that are important for response to various conditions, facilitating the exploration of global gene expression changes caused by abiotic stress. Multiple studies have identified gene expression responses elicited by temperature stress in various fish species, such as rainbow trout (*Oncorhynchus mykiss*) (Li Y et al. 2017), zebrafish (*Danio rerio*) (Long Y et al. 2013), grass carp (*Ctenopharyngodon idellus*) (Yang Y et al. 2016), yellow drum (*Nibea albiflora*) (Dongdong X et al. 2018), channel catfish (*Ictalurus punctatus*) (Ju Z et al. 2002), gilthead sea bream (*Sparus aurata*) (Mininni A N et al. 2014), and large yellow croaker (*Larimichthy crocea*) (Qian B and Xue L 2016).

However, transcriptomic profile of *L. polyactis* under heat and cold temperatures stress remains to be elucidated. In the present study, RNA-Seq analysis was conducted to evaluate the effects of temperature stress on *L. polyactis*. The liver is involved in lipid, glucose and protein metabolism of fish (Tan P et al. 2017), but also regulates stress response. Therefore, we focused on the liver tissues for transcriptome analysis. Investigating the molecular mechanisms underlying temperature stress response in *L. polyactis* will contribute to understanding its adaptation to changes in temperature and promote the development of temperature-tolerant breeds for this species.

2. Materials and methods

87 **2.1 Experimental design and sampling**

88 One hundred and eighty small yellow croakers (55.0 ± 0.6 g) were obtained from the Marine
89 Fishery Institute of Zhejiang Province (Xishan Island, Zhoushan, China). The fish were randomly
90 divided into three groups with three tanks per group: heat temperature group (HTS), low temperature
91 group (LTS) and control group (CT). Fish were cultured in aerated water tanks (0.5 m^3) with 20 fish in
92 each tank, and the tank had a flow through sea water supply maintained at 20 °C. After 14 days of
93 acclimation, fish were placed under heat and cold stress by altering the temperature of the water in the
94 tank at a constant rate of 2 °C per 1 h. In the heat temperature group, temperature was increased from
95 20 °C to 32 °C, and the cold temperature group was decreased from 20 °C to 6 °C. Liver tissues were
96 sampled from five fish per group at 6 h, and samples were immediately flash-frozen in liquid nitrogen,
97 and then stored at −80 °C for further analysis. All the procedures performed on animals were approved
98 by the Guidelines for the Care and Use of Laboratory Animals in China.

99 **2.2 RNA isolation, library preparation and sequencing**

100 Total RNA of liver tissues was extracted using Trizol reagent (Invitrogen, CA, USA) in
101 accordance with the manufacturer's procedure. The total RNA quantity and purity were determined by a
102 Bioanalyzer 2100 and RNA 1000 Nano LabChip Kit (Agilent, CA, USA) and samples with an RNA
103 integrity number (RIN) above 7.0. Poly(A) RNA was purified from total RNA (5μg) using poly-T
104 oligo-attached magnetic beads with the two rounds of purification. The mRNA was then fragmented
105 into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were
106 reverse-transcribed to create the final cDNA library for the mRNA-Seq sample preparation kit
107 (Illumina, San Diego, USA), with an average of 300 bp (±50 bp) insert size for the paired end libraries.
108 The paired-end sequencing was performed on Illumina Hiseq4000 (LC Sceiences, USA) following the

vendor's recommended protocol and the raw data files were deposited to NCBI's Sequence Read Archive (SRA). The accession numbers are SRR10082705 (CT1), SRR10082704 (CT2), SRR10082698 (CT3), SRR10082697 (CT4), SRR10082696 (CT5), SRR10082703 (LTS1), SRR10082702 (LTS2), SRR10082701 (LTS3), SRR10082700 (LTS4), SRR10082699 (LTS5), SRR10082695 (HTS1), SRR10082694 (HTS2), SRR10082693 (HTS3), SRR10082692 (HTS4), SRR10082691 (HTS5).

2.3 *De novo* assembly, unigene annotation and functional classification

Cutadapt (Martin M 2011) and in house perl scripts were used to remove the reads that contained adaptor contamination, low quality bases and undetermined bases. Sequence quality of the clean reads were verified by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) including the Q20, Q30 and GC-content. All downstream analyses were based on clean reads, which were of high quality. *De novo* assembly of the transcriptome was performed with Trinity 2.4.0 (Grabherr et al. 2011). Trinity groups transcripts into clusters based on shared sequence content.

All assembled unigenes were aligned against the non-redundant (Nr) protein (http://www.ncbi.nlm.nih.gov/), Gene ontology (GO) (http://www.geneontology.org), SwissProt (http://www.expasy.ch/sprot/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and eggNOG (http://eggnogdb.embl.de/) databases using DIAMOND (Buchfink B et al. 2014) with a threshold of E-value<0.00001. The assembled transcriptome was used as the reference for the differential expression analysis.

2.4 Differential expression analysis

The expression levels of a unigene was calculated using the Transcripts per million (TPM) method (Günter P. Wagner et al. 2012) and unigenes that were significantly up-regulated or down-regulated

were identified for three comparisons: HTS–LTS, HTS–CT, and LTS–CT. The differentially expressed unigenes were selected with |log2 (fold change) | >1, with statistical significance ($P< 0.05$) using R package edgeR (Robinson M D et al. 2010) and the False Discovery Rate (FDR) set to < 0.001. According to these criteria, smaller FDR and larger ratio indicates a larger difference of the expression between the compared groups. Finally, the different expression unigenes (DEUs) that were identified were used to analyze differences in Gene ontology (GO) functional categories and KEGG pathways. GO function analysis (including GO functional classification annotation and GO functional enrichment analysis) for DEUs were performed by mapping all DEUs to each term of the GO database (http://www.geneontology.org/) and calculating the gene numbers each GO term had. All of raw data about transcriptome were submitted to the NCBI GEO database.

2.5 Validation using quantitative real-time PCR

To examine the reliability of the RNA-Seq results, sixteen DEUs were selected based on their potential functional important for validation using quantitative real-time RT-PCR (qRT-PCR). The housekeeping gene *β-actin* was used as the reference gene. Suitable primers (Table 1) were designed using Primer Express 6.0 and synthesized by GENEWIZ (Suzhou, China) Co., Ltd. PCRs for each sample were performed as triplicates with the SYBR Green dye (TaKaRa, Dalian, China) and StepOnePlus™ Real-Time PCR System according to the manufacturer's protocol. RNA samples that were used for the RNA-seq experiment were selected for this experiment. The qRT-PCR conditions were as follows: 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 34 s and dissociation curve analysis was carried out to determine the target specificity. The relative expression ratio of the target genes versus *β-actin* was calculated using $2^{-\triangle\triangle CT}$ method (Schmittgen T D and Livak K J 2008). Relative mRNA expression levels were statistically analyzed using SPSS 21 software.

3. Results

3.1 Transcriptome assembly and annotation

To identify genes that are differentially expressed under temperature stress, transcriptomes were generated from liver samples of animals placed in the heat temperature group (HTS), low temperature group (LTS) and control group. HTS was exposed to 32 °C and LTS was exposed to 6 °C, while the control group was maintained at 20 °C. In total, 276,893,528, 271,842,088, and 265,003,108 raw reads were obtained from HTS, LTS and CT, respectively. A total of 270,844,888, 265,727,006 and 259,666,218 clean reads were generated by filtering the raw reads from HTS, LTS and CT, respectively. We predicted 86,584 transcripts from the clean reads and N50 length was 1,773 bp (Table 2). The length distribution of the transcriptome libraries is shown in Fig. 1.

The value of Pearson's correlation coefficients (R) for sample expression was 0.6-0.97 for CT1, CT2, CT3, CT4 and CT5, 0.69-0.977 for HTS1 HTS2 HTS3 HTS4 and HTS5, and 0.741-1 for LTS1 LTS2 LTS3 LTS4 and LTS5 (Fig. 2). This highlighted that there was strong correlation of gene expression patterns in the replicate liver transcriptomes, demonstrating that collection methods were accurate. Unigenes were functionally annotated using NCBI_nr, GO, KEGG, Pfam, Swiss-Prot and eggNOG (Table 3).

3.2 Differential expression analysis

We constructed a heat map to identify patterns of unigene expression across the three treatment groups (Fig.3). Of these, 1,843 unigenes were expressed only in HTS-CT, 774 unigenes were expressed only in LTS-CT, and 1,248 unigenes were expressed only in LTS-HTS, and expression of 664 unigenes were shared across all groups. Compared to CT, 4,033 unigenes were upregulated and 3530 were

175 downregulated in HTS, 1,997 unigenes were upregulated and 2,528 were downregulated in LTS, and
176 4,053 unigenes were upregulated and 2,774 were downregulated in both HTS and LTS (Fig.4).

177 **3.3 GO enrichment and KEGG pathway analysis**

178 To evaluate the biological and functional implications of the differentially expressed unigenes
179 (DEUs) under heat and cold stress, GO and pathway enrichment analysis were performed using the GO
180 and KEGG databases. There were 29 significant GO categories that were enriched in HTS, LTS and CT
181 groups ($p < 0.01$) belonging to three categories: biological process (10 subclasses), cellular component
182 (6 subclasses) and molecular function (13 subclasses) (Fig.5). KEGG analysis identifies the associated
183 biological signaling pathways of unigenes (Fig. 6), and we found that 13 pathways were significant
184 enriched under HTS, LTS and CT groups ($p < 0.01$). “Protein processing in endoplasmic reticulum
185 (ER)” was the most enriched pathway, with 256 DEUs in this pathway. Other representative pathways
186 including “JAK-STAT signaling pathway”, “NOD-like receptor signaling pathway”, “Retinol
187 metabolism”, “Spliceosome”, and “Cysteine and methionine metabolism” were also identified.

188 **3.4 Validation of DEUs by using qRT-PCR**

189 Quantitative real-time PCR was performed on 16 DEUs to validate the expression patterns of the
190 DEUs that were identified by RNA-Seq. The qRT-PCR results were significantly correlated with the
191 RNA-Seq results ($p < 0.01$) (Table 4), and all 16 genes showed the identical up-regulated and
192 down-regulated patterns in both qRT-PCR and RNA-Seq experiments (Fig.7).

193

194 **4. Discussion**

195 **4.1 The effect of temperature on the small yellow croaker**

196 Water temperature plays an important role in the survival of small yellow croaker. Studying

197 temperature tolerance is important to generate strains that are more resistant to changes in temperature.
198 Under laboratory conditions, temperature tolerances of fish are usually measured either through
199 methods where the temperature is dynamic (i.e., critical thermal methodology, CTM) or static (i.e.,
200 incipient lethal temperature, ILT) (Beitinger T L and Bennett W A 2000). Temperature tolerance has
201 been studied extensively in several fish species. For example, the CTM temperature of redhorse
202 suckers (*Moxostoma erythrurum*), sheepshead Minnow (*Cyprinodon variegatus*) and eastern
203 mosqyitofish (*Gambusia holbrooki*; Poeciliidae) is 35.4°C (Reash R J et al. 2000), 45.1°C (Beitinger B
204 T L 1997) and 40.1°C (Meffe G K et al. 1995), respectively. Previous reports have suggested that the
205 mortality rate of small yellow croaker increases at temperatures higher than 32°C, thus we used 32°C
206 for the HTS condition in our study. For the LTS, 6°C was selected based on the ILT temperatures of
207 *Larimichthys crocea* (Mu F S et al. 2017).

208 **4.2 Difference in gene expression patterns**

209 In the present study, the liver transcriptome was assembled and gene expression differences in the
210 HTS, LTS, and CT groups were compared without a reference genome, as the small yellow croaker
211 genome has not been assembled. We predicted 86,584 transcripts and functionally annotated
212 approximately 37,702 unigenes, which was similar to other fish species, such as Atlantic Salmon
213 (*Salmo salar*) (Micallef G et al. 2012) and Oujiang color common carp (*Cyprinus carpio* var. color)
214 (Du J et al. 2019). N50 can be selected as a criterion to examination the quality of gene splice. Since
215 we did not have a genome of small yellow croaker, we estimated the N50 length using genomes of
216 large yellow croaker, which is closely related species with it. The estimated N50 length was 1,773 bp in
217 the small yellow croaker, which was similar to the N50 length of 1,943 bp in the large yellow croaker
218 (Shijun X et al. 2015). The N50 value indicated that our assembled transcriptome was suitable for

219 analysis of DEUs between three experiment groups. We next identified DEUs across the different
220 treatment conditions, and found that heat treatment led to more up-regulated genes, and cold treatment
221 led to more down-regulation of genes. DEUs included the Heat shock proteins (HSPs) and the Relaxin
222 Family Peptide Receptors 3 (RXFP3).

223 **4.2.1 Heat Shock Proteins family proteins**

224 HSPs, also known as stress proteins and extrinsic chaperones, are expressed in all organisms when
225 exposed to stress (R.J. Roberts et al. 2010). In aquatic animals, they play a fundamental role in immune
226 response (Dong C W et al. 2006) and inhibition of apoptosis (Sandilands J et al. 2010). In the heat
227 stress group, 24 HSPs genes were significantly up-regulated ($p< 0.01$), and *Hsp30* had the highest
228 expression level change (10.83-fold), followed by *Hsp90aa1* (10.11-fold), *Hsp70* (9.91-fold) and
229 *Hsp27* (hspb1, 6.65-fold). These genes have been extensively investigated in other fish species. When
230 rainbow trout blood was heat shocked *in vitro*, both *Hsp70* and *Hsp30* mRNA levels increased
231 significantly (Currie S et al. 2000). *Hsp90aa1* expression is associated with stress-induced
232 cytoprotection (Di-An F et al. 2016). *Hsp90aa1* regulates the folding of proteins under stress, as well
233 as protein trafficking, transcriptional regulation, and epigenetic regulation of gene expression
234 (Csermely P et al. 1998; Pearl L H and Prodromou C 2006). *Hsp27* has antiapoptotic effects through its
235 function in the mitochondria during thermotolerance (Samali A et al.2001).

236 Multiple HSPs were differentially expressed in the small yellow croaker exposed to stress. *Hsp70*
237 and *Hsp90* promotes the degradation of damaged proteins and *Hsp90a* regulates immune signaling
238 pathway to prevent the damage. Moreover, HSPs also regulate energy metabolism, lipid, and
239 carbohydrate levels to response to changing of temperatures. Therefore, our transcriptomic analysis
240 suggests that increases expression of HSPs protects the small yellow croaker from heat stress.

241 **4.2.2 Relaxin Family Peptide Receptors 3**

242 Temperature stress affects the physiology of fish at multiple levels, and can affect the central
243 nervous system and brain activity. Under temperature stress, fish display behaviors indicative of stress
244 and avoidance, such as erratic swimming, abnormal posture, and aggregative behavior (Quigley J T and
245 Hinch S G 2006). Spatial and temporal ambient temperature variations directly influence cellular
246 biochemistry and thus the physiology of fish, which are ectoderms (Burg V D and E. H 2005).
247 Neurotransmitters regulate activity in fish (Ortiz M and Lutz P L 1995) and brain monoamine
248 neurotransmitters induce agonistic behavior and stress reactions (Winberg S and Nilsson G E 1993).
249 Therefore, the cranial nerve response of fish is important in its response to changes in temperature.
250 In the DEUs analysis, we found that *RXFP3* expression changed significantly under temperature
251 stress ($p< 0.01$), which was up-regulated in HT vs LT (6.24-fold), down-regulated in LT vs CT
252 (6.51-fold) and up-regulated in HT vs CT (1.63-fold). *RXFP3* regulates a wide range of behaviors,
253 including feeding, stress responses, arousal, and cognitive processes (Bathgate R A D et al. 2013).
254 *RXFP3* has primarily been studied in mammals (Ganella D E et al. 2013; Kania A et al. 2014), and very
255 few studies have investigated its function marine fish (Fiengo M et al. 2013). For this reason, it is
256 unclear whether *RXFP3* regulates neuroendocrine response to temperature. Our findings suggest that
257 *RXFP3* plays an important role in temperature response. The mechanisms underlying this need further
258 investigation.

259 **4.3 Pathways that are differentially expressed under temperature stress**

260 A number of enriched pathways were found in the different treatment conditions. These included
261 protein processing in endoplasmic reticulum pathway, immune signaling pathway and metabolic
262 response pathway, and details are discussed below.

263 **4.3.1 Protein processing in endoplasmic reticulum pathway**

264 Endoplasmic reticulum stress (ERS) is a vital mechanism of cellular self-defense, but strong and
265 long-lasting ERS leads to irreversible cell damage (Qing D and Zhen Z 2009). ER is a subcellular
266 organelle where proteins are folded with the help of lumenal chaperones. Accumulation of misfolded
267 proteins in the ER causes ER stress and activates a signaling pathway called the unfolded protein
268 response (UPR). Studies of UPR targets engaged in endoplasmic reticulum-associated protein
269 degradation (ERAD) reveal an intimate coordination between efficient ERAD requires an intact UPR,
270 and inducing UPR increases ERAD capacity. Conversely, loss of ERAD leads to constitutive UPR
271 induction (Wodicka L 2000). It is well established that the number of correctly folded protein
272 significantly increases under heat stress. There are three ER transmembrane proteins in the small
273 yellow croaker: endoplasmic reticulum kinase (PERK), active transcription factor 6 (ATF6) and IRE1,
274 which promote cell survival by reducing the level of misfolded proteins (Lin J H et al. 2007). These
275 expression levels of these genes were up-regulated in animals that were under heat stress. Due to the
276 continued ERS, the ER function is severely impaired and the organelle elicits apoptotic signals, which
277 can be identified by the up-regulation of C/EBP homologous protein (CHOP). During ERAD, both the
278 Heat Shock Proteins family proteins (*Hsp40*, *Hsp70*, *Hsp90*) are up-regulated. HSPs, such as *Hsp40*
279 and *Hsp70*, can interact with misfolded proteins, preventing them from forming aggregates (Fink A L
280 1999). In the goby (*Gillichthys mirabilis*), *Hsp70* and *Hsp90* were strongly up-regulated during heat
281 shock and recovery from stress (Buckley and B. A 2006). In the study of Maraena whitefish
282 (*Coregonus maraena*), unfolded protein response (the major ER stress pathway) was one of two
283 characteristic pathways that were induced during acute and gradual heat stress (Alexander R et al.
284 2018). Our results are consistent with the above studies, showing that *Hsp40*, *Hsp70* and *Hsp90* were

285 up-regulated under heat stress.

286 In lower temperature stress experiment, *PERK* was the only ER transmembrane protein kinases
287 that were down-regulated, and the expression levels of *CHOP* was similar to the control. Protein
288 Disulfide Isomerase (PDI) recognizes unfolded and partially folded proteins. Due to expression levels
289 were down-regulated of PDIs family associated proteins, the function of PNGase and DUB were
290 undermined causing the damage of ERAD function. However, up-regulation of *Hsp40* could
291 compensate for the degradation level of ERAD ensuring the recovery and maintenance of liver function.
292 In conclusion, ER function may be important for the temperature stress response of the small yellow
293 croaker.

294 **4.3.2 Immune signaling pathway**

295 Studies of immune pathways often focus on disease (Maekawa S et al. 2017). However, stress
296 response and immune dysfunction are increasingly being linked through similar molecular pathways
297 (Philip A M and Vijayan M M 2015). In general, temperature affects the immune system in teleosts
298 (Bowden T J et al. 2007; Rebl A et al. 2013). Here, we also found changes in the expression of genes
299 belonging to the Jak-STAT and NOD-like receptor signaling pathways under temperature stress.

300 The JAK-STAT signaling pathway is one of the ubiquitous signaling pathways in metazoans, and
301 is involved in proliferation, differentiation, survival, apoptosis of cells, and mediates immune disorders
302 (Song Z et al. 2012). JAK-STAT is negatively regulated by Protein Tyrosine Phosphatase (PTP),
303 Suppressor of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS). TC-PTP can
304 phosphorylate JAK1, JAK3, STAT1, STAT3, and STAT5 (Pouliot P et al. 2009), and the Kinase activity
305 of JAKs and activation of STATs are inhibited by SOCS (Morales J K et al. 2010), and the transcription
306 of STATs is inhibited by PIAS (Ivashkiv L B and Hu X 2004). Under heat stress, the expression levels

of TC-PTP and PIAS were up-regulated, which likely promotes the expression levels of SOCS and activate anti-apoptotic programs. Under cold stress, STATs were down-regulated, which likely reduced the expression levels of SOCS.

In the NOD-like receptor signaling pathway, many genes were up-regulated after temperature experiment, including *NOD2* and signal mediators (*TRIP6, Nemo, NFKB* and *JNK*). The expression levels of *RIP2, CARD8, PSTPIP1* and *HSP90* family were up-regulated under heat stress and down-regulated in lower temperature stress. These expression changes are likely to indirectly influence the immune system. Thermal stress experiment in the Indian major carp catla (*Catla catla*) also found the activation of the NOD signaling pathway during thermal stress (Madhubanti Basu et al. 2015). Therefore, these immune signaling pathways are likely to play a role in temperature stress response in the small yellow croaker.

4.3.3 Metabolic response pathway

The metabolism of fish is dependent on the environmental temperature, and changes metabolic rate is one of the most rapid cellular responses to the change of temperature (Somero G N 2010a). Lipid peroxidation and antioxidant defense are affected by temperature (Bagnyukova T V et al. 2007), altering energy metabolism, lipid, and carbohydrate levels through heat-shock proteins (Buckley and B. A 2006a; Vergauwen L et al. 2010).

In present study, many biological processes were significantly altered when the small yellow croaker were exposed to temperature stress. Metabolic response pathways, such as “Glutathione metabolism”, “Carbon metabolism”, “Cysteine and methionine metabolism”, “Arginine and proline metabolism”, “Retinol metabolism”, “Oxidative phosphorylation”, “Lipid and steroid metabolism”, “Fructose and mannose metabolism”, “Amino acid metabolism” and “Carbohydrate metabolism” were

329 enriched in DEUs of animals exposed to temperature stress. This indicates that the regulation of
330 metabolic processes plays a key role of temperature response of the small yellow croaker. In particular,
331 metabolism of amino acids, carbohydrate and lipid were significantly influenced by the temperature
332 treatment, such as glycine, arginine, fructose, glycolate and glucose (Rawles S D et al. 2012). Under
333 heat stress, ATP-generating enzymes and *glucose-6-phosphatase* were up-regulated, suggesting that
334 heat stress led to a rapid production of ATP. This may be due in part to the requirement for ATP for the
335 function of molecular chaperones (Fink A L 1999a). Under cold stress, genes involved in lipid
336 metabolism were down-regulated, repressing fatty acid synthase and ceramide kinase expression levels.
337 Exposure to cold modifies lipid metabolism by lowering total saturated fatty acids in juvenile red
338 drum (Craig S R et al. 1995). Combined together, metabolic response pathways may play an
339 important role in the small yellow croaker's response to temperature stress.
340

341 **5. Conclusions**

342 In conclusion, our transcriptome analysis demonstrated that temperature stress significantly
343 altered gene expression in the small yellow croaker. A large number of DEUs were identified between
344 animals that were exposed to control, heat and low temperatures. In addition, *RXFP3* was identified as
345 a candidate gene that mediates temperature response. DEUs were enriched in the ER pathway, immune
346 signaling pathway and metabolic response pathway. By identifying candidate genes and cellular
347 pathways involved in temperature stress response, we provide important insights for future strategies to
348 generate small yellow croaker breeds that are tolerant of temperature stress for aquaculture purposes.
349
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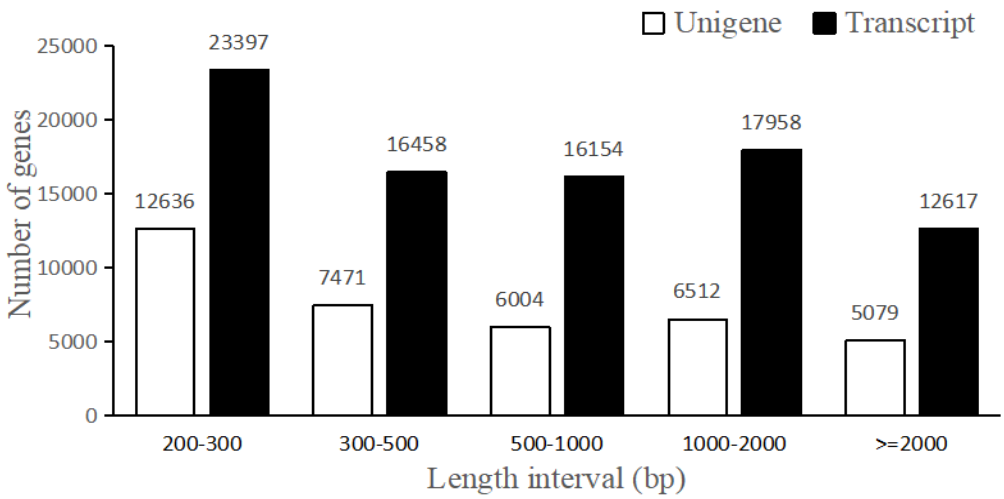
1 **Figures**



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3 **Fig. 1： Length distributions of the transcripts and unigenes**

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Fig. 2： Pearson correlation between samples

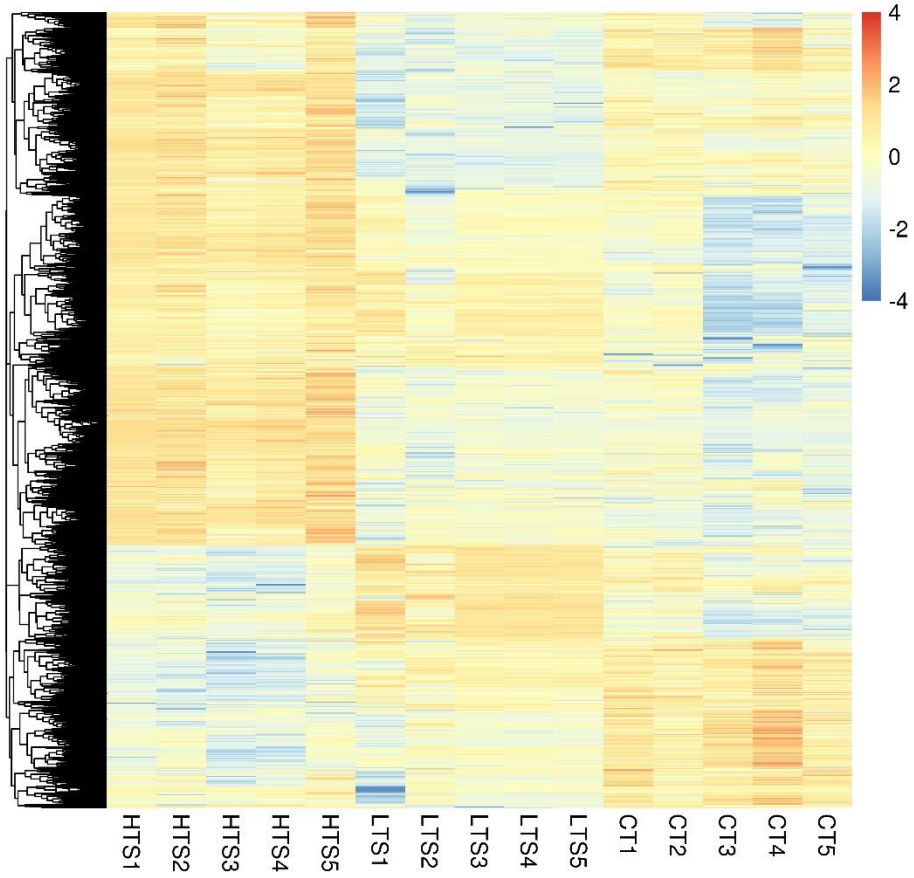


Fig. 3： Heat map of the differentially expressed unigenes

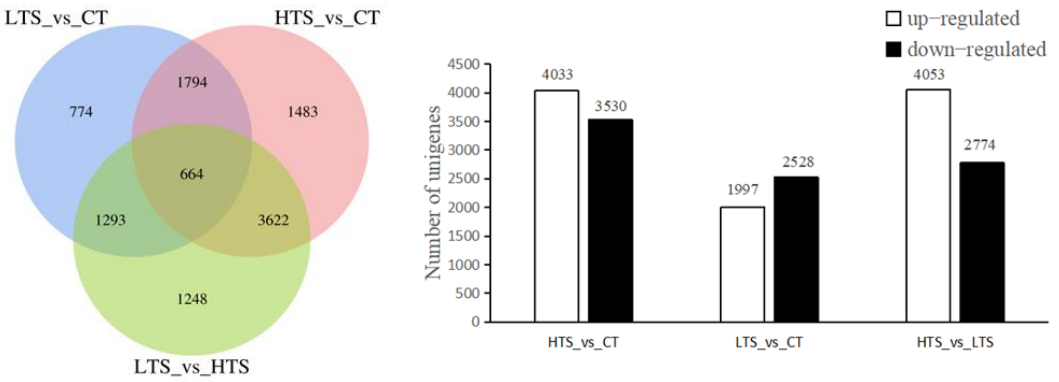


Fig. 4： Venn diagrams of differential expressed unigenes and differentially expressed unigenes in different groups

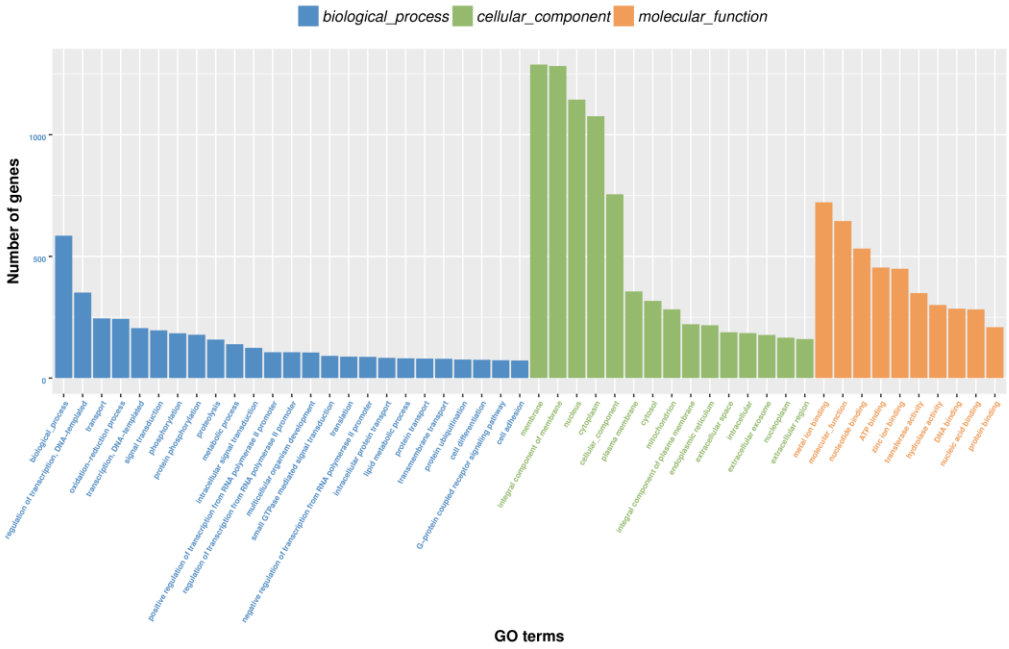
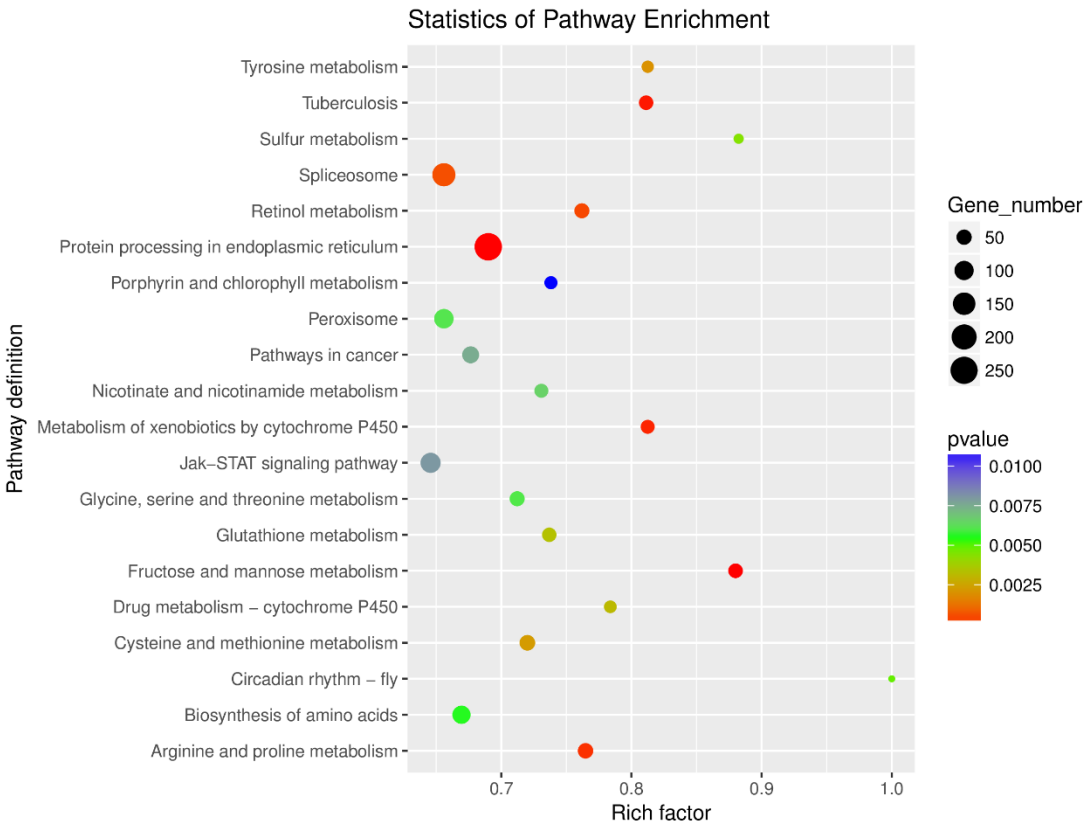


Fig. 5： Gene ontology (GO) classifications of DEUs in HTS-LTS-CT



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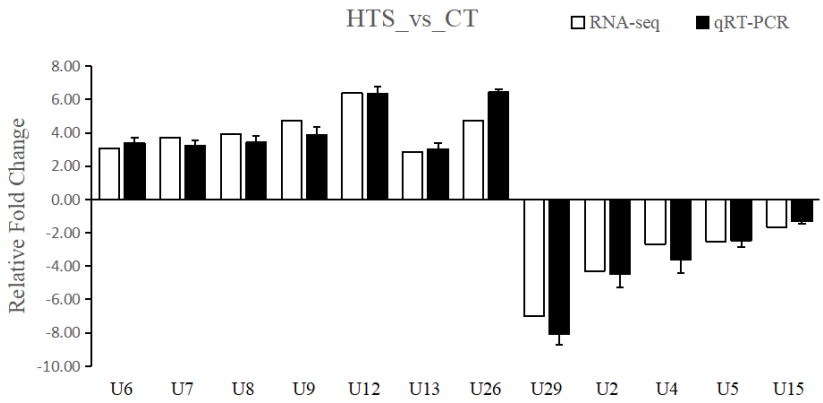
81 **Fig. 6： Scatterplot of enriched KEGG pathways for DEUs in HTS-LTS-CT**



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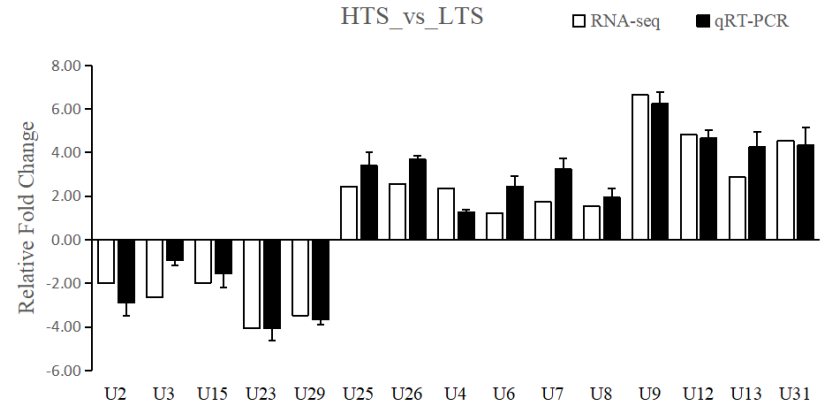

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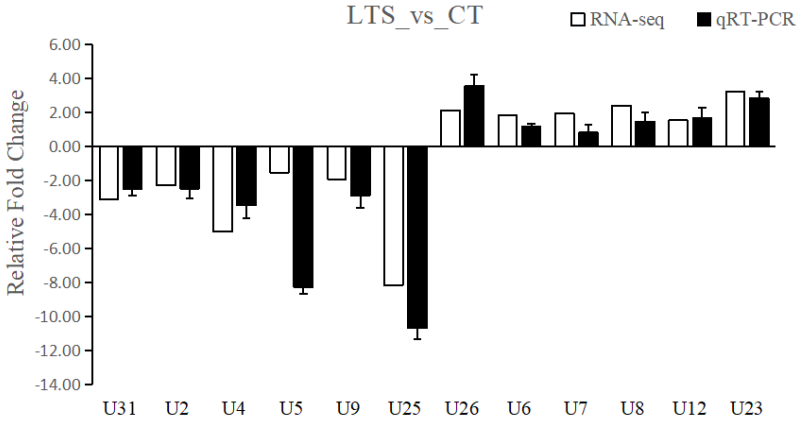

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85 **Fig. 7：Comparison of the expressions of RNA-Seq and qRT-PCR results**

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1 **Tables**

2 **Table 1： Primers used for qRT-PCR verification of differently expressed genes**

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Length (bp)
U2	TGACAGCAGACGAAGAGT	TCCACAGAGGCATCAGAG	147
U3	TTCACCTTCTTCTGCTTCC	GTTCACTCTGTATTCGTTCTG	435
U4	AGGAGGAGAATGCTGTCA	TGAACAACCACGGATGAG	161
U5	AGTGTTCGTGGCATTCAT	TCTTCTGGTCCTCTGTGT	448
U6	ACTCTAGTGTGCCTGTGT	CTGCTTCTCCTCATCTGAC	362
U7	GACAGCCGCACTAAGATT	TGAAGAAGGAGGAAGAAGAC	430
U8	CTGGAGTGCTGGAGAGTA	GTGCCGTAGTAAGTGTAGG	366
U9	CAGAACAGCCGCATCTTC	ACACCGTCCTTGGTCTTC	311
U12	CTATCTTGTCACCAGCATT	TCACTCAGTCCTTCCTCT	106
U13	GTCTTCTCTTCGCACTCTT	GCCTCATCGTCTGTTCTC	124
U15	GACGCAATGACAGCAATG	TCCTCTCACCACCATAGC	181
U23	ATGACTACAGCAGTGATGAC	GGAGAACAAGAAGATGAAGATG	143
U25	CTGGCTGCTGGACTAATC	AGAGGAGTGAAGGCTGAG	170
U26	TCCTCCTCCTCCTCTTCA	GTCACCTCCACTTCCATAC	110
U29	GCCATTGACGAACATAAGG	CTCCAACTCATCCAGATAGG	354
U31	AACACTGAGCACAACTATGA	AGAGGTGAACTGAGGACTT	302
β-actin	GACCTGACAGACTACCTCATG	AGTTGAAGGTGGTCTCGTGGA	292

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Table 2： Overview of assembly results

Index	All number	GC (%)	Min. Length (bp)	Median Length (bp)	Max. Length (bp)	N50	Total Assembled Bases
Transcripts	86,584	48.05	201	574	13,564	1773	87,499,743
Unigenes	37,702	47.17	201	445	13,564	1777	35,075,401

24 **Table 3： Summary statistics of transcriptome annotation**

Database	Number	Ratio (%)
All	37,702	100.00
GO	14,976	39.72
KEGG	9,587	25.43
Pfam	13,179	34.96
eggNOG	16,580	43.98
NR	18,272	48.46

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38 **Table 4: The correlation coefficient of the results among RNA-Seq and real-time PCR**

Group	Pearson correlation coefficient	Sig.
HTS - CT	0.988	0.000
HTS - LTS	0.960	0.000
LTS - CT	0.870	0.000

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