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RESEARCH ARTICLE

Comparative analysis of transcriptomics of *Fasciola hepatica* at different developmental stages

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ABSTRACT

This study explored the transcriptome differences in *Fasciola hepatica* at different developmental stages and identified functional genes related to growth and development during juvenile stages. DNBSEQ eukaryotic strand-specific transcriptome resequencing technology was used to sequence the transcriptomes of *Fasciola hepatica* eggs, juveniles, and adults. Additionally, the genes that were highly expressed during the juvenile stage were validated using qRT-PCR. The Q20 values of all three phases of sequencing were above 98%, and the Q30 values were above 94%. The differentially expressed genes (DEGs) in pairwise comparisons were analyzed by GO functional classification and the KEGG pathway database. Many immune-, growth-, and development-related pathways were found, which might be related to cell proliferation, development, and host immune evasion by *Fasciola hepatica*. In addition, five DEGs with high expression levels during the juvenile stage were identified: Cathepsin B, Glutathione S-transferase mu, heat shock protein 67B2, Kunitz-CH, and Legumain. Validation analyses revealed that these genes play key roles in maintaining normal growth, development, and immunological processes in liver *Fasciola hepatica*. RNA-seq was used to analyze the biological characteristics of the DEGs at different developmental stages concerning GO functional classification and KEGG metabolic pathways. Five DEGs with high expression during the juvenile stage were identified. These genes are related to the growth, development, and immune function of *Fasciola hepatica*, which provides a theoretical basis for subsequent research on the proteomics of *Fasciola hepatica* and the screening of candidate genes for early diagnosis.

Keywords: *Fasciola hepatica*; different developmental stages; transcriptomics; differential genes; growth and development.

INTRODUCTION

Hepatic fascioliasis is a zoonotic parasitic disease endemic to millions of people worldwide (Pedroza-Gómez *et al*., 2021; Serrat *et al*., 2023; Zumaquero-Ríos *et al*., 2013). During the growth and development of *Fasciola hepatica*, limnaeids are required as intermediate hosts and mammals as terminal hosts. The eggs of *F. hepatica* fall into the water along with the feces of the terminal host, where they develop into a miracidium. When the miracidium meets the intermediate host, the limnaeid, it rapidly enters the body of the host and undergoes three developmental stages: sporocyst, rediae, and cercaria, which take approximately four to seven weeks (Becerro-Recio *et al*., 2023; Rathinasamy *et al*., 2018). The cercariae then escape from the snail and attach to the aquatic weeds, shedding their tails and forming metacercariae. Mammals are infected by accidentally ingesting the metacercariae by feeding on aquatic weeds (Corrales *et al*., 2021; Wang & Mitchell, 2022). After the metacercariae are ingested by the organism, they undergo an asymptomatic incubation period and enter the acute and chronic clinical phases (Young *et al*., 2010).

Early detection of *F. hepatica* infection is key to preventing and treating *F. hepatica* disease, reducing treatment costs while achieving optimal treatment outcomes. Currently, there are many limitations in the prevention, treatment, and diagnosis of hepatic fascioliasis. Prevention and treatment of hepatic fascioliasis are limited to the eradication of snails in the pasture and deworming with medication; however, in recent years, some *F. hepatica* have begun to develop resistance to anti-Fasciola drugs (Cwiklinski & Dalton, 2018; Davey *et al*., 2022; Stuen & Ersdal, 2022). Currently, methods for diagnosing hepatic fascioliasis include etiological, molecular biology, and immunological diagnosis. Pathogenetic diagnostic methods can only be used in the advanced stages of infection with hepatic fascioliasis (Mokhtarian *et al*., 2018). Molecular biology diagnostic methods are complex, time-consuming, and not suitable for rapid clinical diagnosis (Calvani *et al*., 2017; Mezo *et al*., 2022). Currently, immunological diagnostic methods are in the laboratory research

phase (Alvarez Rojas *et al*., 2014; Gordon *et al*., 2012). Therefore, there is an urgent need to establish a sensitive and specific early diagnostic method to provide technical support for the early diagnosis and prevention of hepatic fascioliasis in domestic animals.

With the rapid development of high-throughput sequencing technology, transcriptomic sequencing has been widely used to study differential gene expression in organisms at different developmental stages. Currently, transcriptomic studies of trematode parasites have also received increasing attention; however, the genomic dataset of *F. hepatica* is relatively small compared with that of other trematodes, especially for early stage organisms (before adult worms), which limits the early diagnosis of hepatic fascioliasis. In 2020, Rosa *et al.* enriched iron acquisition, immune regulation, and other parasitic functions in 256 *Paragonimus* (Rosa *et al*., 2020). In 2017, Zhang *et al*. performed a gene expression analysis of buffalo livers infected with *Fasciola gigantica* at different developmental stages and screened 496, 880, and 441 differential genes at 3, 42, and 70 dpi, respectively (Zhang *et al*., 2017a). In 2020, Limpanont *et al*. discussed the transcriptome analyses of adult *Schistosoma* males to control and prevent schistosomiasis in the Mekong River (Limpanont *et al*., 2020). In 2023, Wu *et al*. used multi-omics techniques to analyze the transcriptomic, proteomic, and metabolomic changes in the livers of mice infected with *Clonorchis sinensis* (Wu *et al*., 2023). In 2015, Cwiklinski *et al*. assessed the genome-wide polymorphisms and transcriptional profiles of *F. hepatica* to identify key features (Cwiklinski *et al*., 2015). In 2017, McNulty *et al*. provided a reference genome sequence for *F. hepatica* isolated from sheep, confirming the presence of *Neorickettsia* in adult organs and tissues (McNulty *et al*., 2017). Recent advances in trematode-related genomics and bioinformatics have led to a better understanding of the relation between the biology of trematode parasites at the biological and molecular levels and have provided the basis for the search for drug targets, vaccine development, and early diagnosis.

In this study, the DNBSEQ eukaryotic strand-specific transcriptome sequencing technology was used to sequence the transcriptomes of *F. hepatica* at different developmental stages. The aim was to analyze the differences in metabolic levels and regulatory mechanisms involved in different developmental stages of *F. hepatica* in the terminal host. In addition, we screened for differentially expressed genes in the early stages of *F. hepatica* (juveniles) and verified them using qRT-PCR.

MATERIALS AND METHODS

Samples were collected from *F. hepatica* at three different developmental stages

Adults and eggs were collected from the liver and gallbladder of naturally infected cows in Wushenqi Banner, Inner Mongolia. Juveniles were collected from the livers of a cow that died acutely from an early infection with *F. hepatica* in Wushen Banner, Inner Mongolia. The collected eggs, juveniles, and adults were washed 2–3 times with normal saline. They were placed in normal saline for 2 hours, and after removing the intestinal contents, they were placed in a cryopreservation tube, and they were quick-frozen in liquid nitrogen for 30 min, and then stored in a -80! freezer for later use. Before transcriptome sequencing, worms at three different stages of development were identified by the molecular biology technique of *F. hepatica* and confirmed as *F. hepatica*.

RNA extraction and cDNA library construction

Egg, juvenile, and adult samples were ground into a powder under the protection of liquid nitrogen and then transferred to 1.5 mL of TRIzol reagent to fully lyse the cells. RNA was extracted using the MGIEasy Total RNA Extraction Kit (BGI, Beijing, China). A cDNA library was constructed using the DNBSEQ high-throughput sequencing (Thompson *et al*., 2020) reagent set process (BGI, Beijing, China). Machine sequencing was performed by combined probe-anchored polymerization.

Sequencing data analysis

In this study, SOAPnuke, a BGI filtering software, was used to filter out low-quality, linker-contaminated, and unknown bases with excessive N content from raw reads, and clean reads were obtained. Clean reads were aligned to the reference genome sequence using HISAT. After the new transcript was obtained, its protein-coding potential was predicted using CPC, and the new transcript predicted to have protein-coding potential was added to the reference gene sequence to obtain complete reference sequence information, which was analyzed based on this reference sequence.

Annotation analysis of differentially expressed genes

Pairwise comparisons of adults, juveniles, and eggs of *F. hepatica* revealed that the differentially expressed genes were annotated separately by GO function and then classified and enriched according to the three GO databases of cell composition, molecular function, and biological processes. At the same time, KEGG enrichment analysis was performed on differentially expressed genes to enrich the pathways with considerable differences, and the biological regulatory pathways of significant differential changes in *F. hepatica* at different developmental stages were found. According to the results of differential gene detection, hierarchical clustering analysis was carried out using the heatmap function in R software for the union of differential genes. Functional pathways with high confidence and more detailed and comprehensive functional clustering were obtained.

qRT-PCR validation

In this study, five differentially expressed genes with high expression levels in the juvenile stage of *F. hepatica* were screened and verified using qRT-PCR. According to the RNA extraction solution instructions (Wuhan servicebio technology CO., LTD., China), the total RNA of *F. hepatica* was extracted from the three developmental stages, and the RNA with excessive concentration was diluted in an appropriate proportion to achieve a final concentration of 200 ng/μL. A 20 μl reaction system was prepared, mixed and centrifuged, and reverse transcription was completed on a common PCR instrument, reverse transcribing RNA into single-stranded cDNA. The PCR system was prepared and amplified using real-time PCR. The PCR amplification system was as follows: pre-denaturation: 95°C for 30 s; 40 cycles: 15 s at 95°C, 30 s at 60°C. Melting curve: 65°C to 95°C. Fluorescence signals were acquired once per 0.5°C temperature rise to ensure that a single product was amplified in each reaction.

RESULTS

Sequencing data status and quality assessment

The base number of a single sample filtered at each development stage was not less than 6 Gb, the original reads of each sample through splicing reached more than 43 million, the clean reads obtained from the original reads after filtering reached more than 42 million, and the proportion of filtered reads exceeded 90%. The Q20 values of the three sequencing stages were above 98%, and the Q30 values were all above 94%. The results indicated that the data reliability of this study was high (Table 1).

Table 1. Primers and internal control primers for qRT-PCR detection

Primer name	Primer sequences (5'–3')	Fragment length (bp)	Annealing temperature (°C)
F. hepatica -ACTIN-F	CTGTGGTTCCTGTTGGGCTT	142	60
F. hepatica -ACTIN-R	TCCACCACCGCAACCATT	142	60
D915_004890-F	TCAGGGAGTCATGGTCGGTAT	222	60
D915_004890-R	GCTTTCGGGTTCAGTGGTG	222	60
D915_008429-F	TCAGTGGCAACTCGGGATA	109	60
D915_008429-R	GAGTTTTTGTGCCAGTTCCATTTC	109	60
D915_010279-F	CTTGGGCGAAGGCGTATGA	127	60
D915_010279-R	CGGAAATCAACGGCATAGCA	127	60
D915_011152-F	AACACCCAGTTGTCAAAGGAAA	247	60
D915_011152-R	CCAACCGAGAATACGAACCATA	247	60
D915_000805-F	CCGTGCTCTTAGCCACTCTGAT	122	60
D915_000805-R	CCAAGCCCAACTTCGTATTCC	122	60

Table 2. Statistics on the quality of filtered reads

Sample	Total Raw Reads (M)	Total Clean Reads (M)	Total Clean Bases (Gb)	Clean Reads Q20 (%)	Clean Reads Q30 (%)	Clean Reads Ratio (%)
Adult 1	43.69	42.22	6.33	98.4	94.64	96.64
Adult 2	47.19	42.78	6.42	98.32	94.33	90.66
Adult 3	45.44	42.29	6.34	98.38	94.56	93.08
Eggs 1	43.69	42.4	6.36	98.38	94.59	97.05
Eggs 2	45.44	43.18	6.48	98.31	94.38	95.03
Eggs 3	45.44	42.82	6.42	98.47	94.83	94.24
Juveniles 1	45.44	42.53	6.38	98.35	94.52	93.6
Juveniles 2	43.69	42.13	6.32	98.38	94.56	96.44
Juveniles 3	45.44	43.5	6.52	98.41	94.74	95.73

Correlation analysis of differentially expressed genes of *F. hepatica* at different developmental stages

Screening results of differentially expressed genes of F. hepatica at different developmental stages

The DEseq2 software was used to analyze differential gene expression in *F. hepatica* adults, juveniles, and eggs. The DEseq2 method was based on the negative binomial distribution principle, and the differentially expressed genes were detected according to the method described by Love *et al*. (Love *et al*., 2014) (Figure 1).

Differential gene expression results of F. hepatica at different developmental stages

In this study, the differentially expressed genes in the transcriptome of *F. hepatica* at three stages (F1), juvenile (F2), and egg (F3) were statistically analyzed. The results showed that 11,236 differentially expressed genes were expressed during the three developmental stages. A total of 4,040 genes were significantly differentially expressed between adults and juveniles. Among them, 1,757 genes were upregulated in the juvenile stage, and 2,284 genes were upregulated in the adult stage. A total of 8,079 genes were

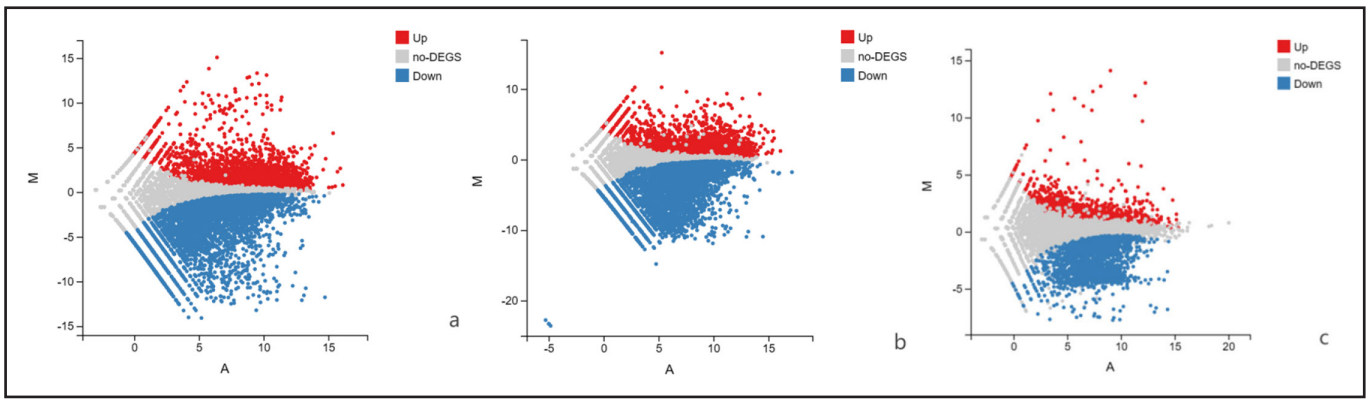

Figure 1. Differential MA distribution among groups. a: Juveniles vs eggs; b: Adults vs eggs; c: Adults vs juveniles. Q value (Adjusted P-value) <= 0.05 was used as the criterion to screen differentially expressed genes (DEGs), with the X-axis representing the A value (the average expression level after $\log_2$ transformation) and the Y-axis representing the M value (the difference fold after $\log_2$ transformation). Red represents upregulated DEGs, blue represents downregulated DEGs, and gray represents non-DEGs.

significantly differentially expressed between the adults and eggs. Among them, 3,726 genes were upregulated at the egg stage and 4,353 genes were upregulated at the adult stage. A total of 7,957 genes were significantly differentially expressed between juveniles and eggs. Among them, 3,617 genes were upregulated at the egg stage and 4,340 genes were upregulated at the juvenile stage. The results showed that the number of genes upregulated in the egg stage was higher than in the other two stages (Figure 2).

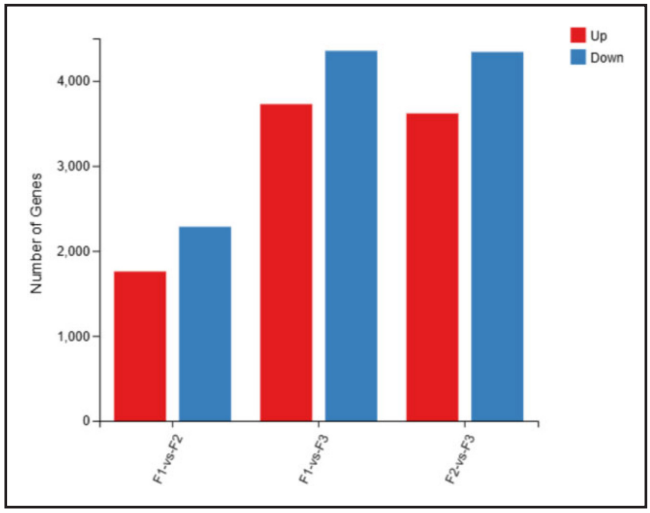

Figure 2. Statistical analysis of the number of differentially expressed genes in the transcriptome of *F. hepatica* compared in pairs at three stages: adult (F1), juvenile (F2), and egg (F3).

GO analysis of differentially expressed genes of *F. hepatica* at different developmental stages

To analyze the differences in the gene expression levels of *F. hepatica* at different developmental stages and predict the key biological processes, cellular components, and molecular functions of *F. hepatica* at different developmental stages, the differentially expressed genes obtained after pairwise comparisons were subjected to GO analysis. Enrichment analysis was performed using the phyper function in the R software. The enrichment bubble chart shows the enrichment degree of the GO Term from three dimensions, and the top 20 GO Terms with the smallest Q values were used by default (Figure 3).

Adult and juvenile stages were mainly enriched in cilium organization, plasma membrane-bound cell projections, and intrinsic components of the membrane. The adult and egg stages were mainly enriched in non-membrane-bound organelles, cytoskeleton, structural constituents of ribosomes, and cilia. The juvenile and egg stages were mainly enriched in the structural constituents of ribosomes and nucleoside-triphosphatase activity.

KEGG analysis of differentially expressed genes of *F. hepatica* at different developmental stages

Enrichment analysis was performed using the phyper function in R software according to the KEGG Pathway annotation classification. The enrichment bubble chart displays the enrichment of the KEGG Pathway in three dimensions, and the top 20 GO Terms with the smallest Q values were plotted by default (Figure 4).

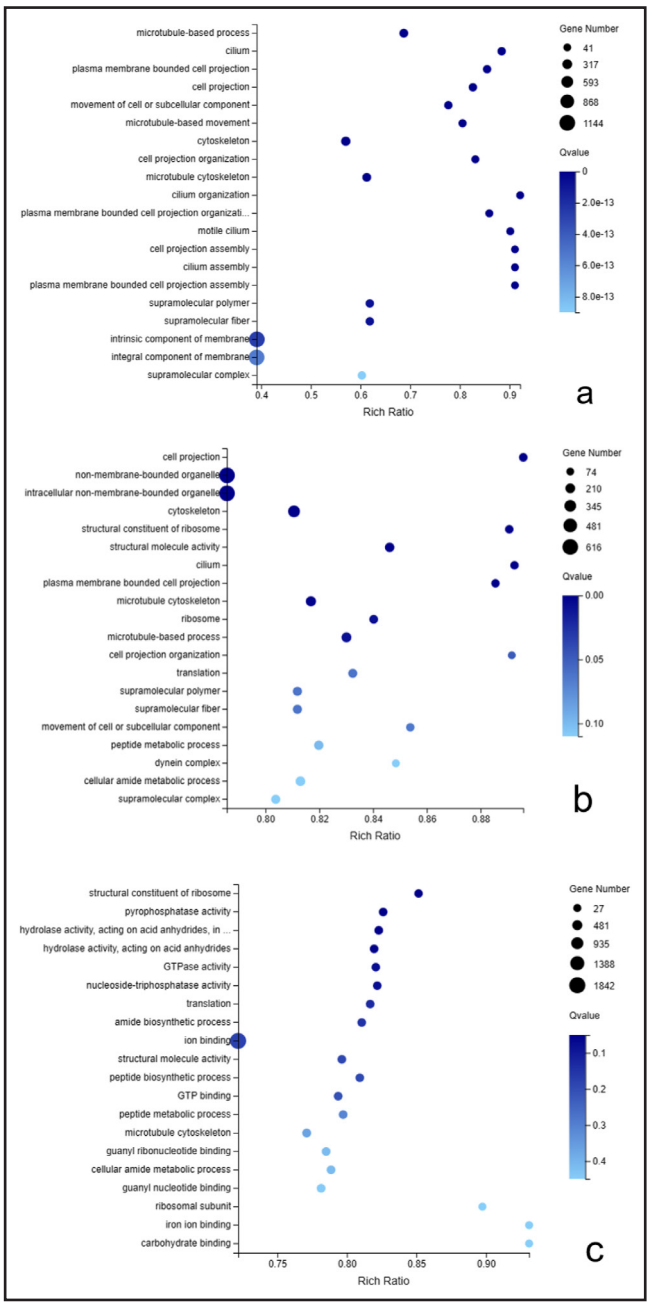

Figure 3. GO enrichment bubble map. A. adult versus juvenile; B. adult versus eggs; C. juvenile versus eggs. The X-axis is the Rich Ratio (the ratio of the number of genes annotated to an entry in the selected gene set to the total number of genes annotated to the entry of this species; the calculation formula is rich ratio = term candidate gene number/term gene number). The Y-axis represents the GO Term, the size of the bubble represents the number of differential genes annotated to a GO term, the color represents the enrichment Q value, and the darker the color, the smaller the Q value.

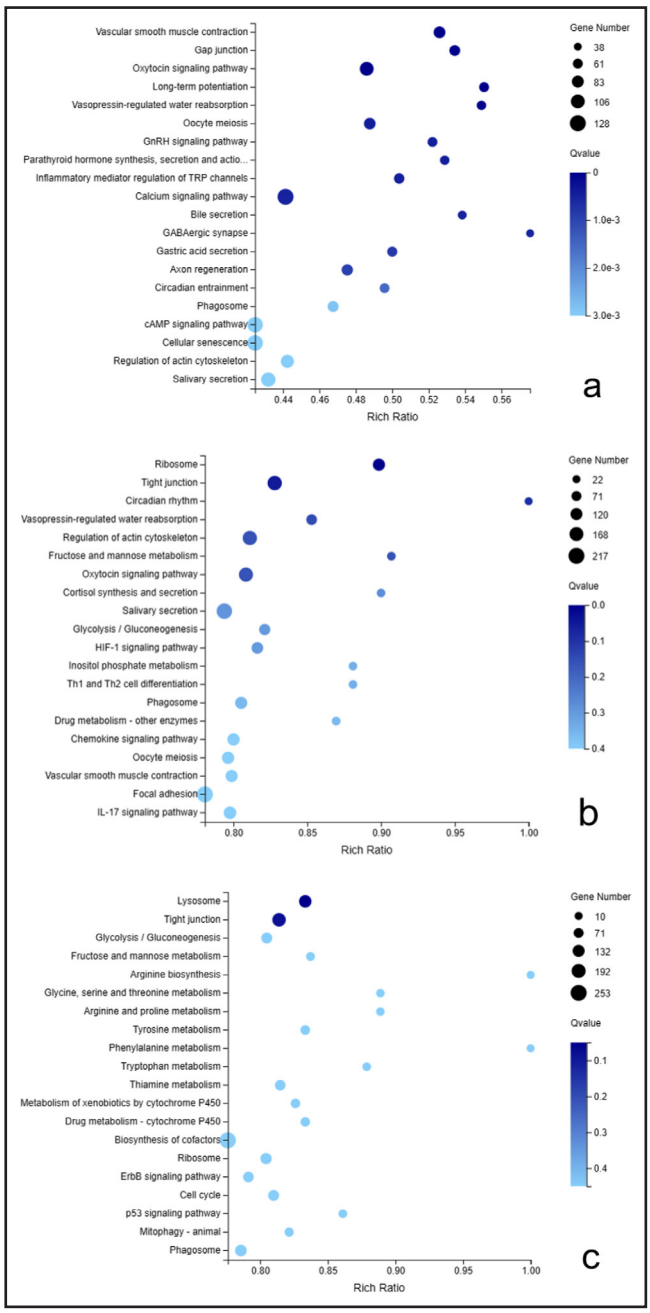

Figure 4. KEGG Pathway enrichment bubble map. D. adult versus juvenile; E. adult versus eggs; F. juvenile versus eggs. The X-axis is the Rich Ratio (the ratio of the number of genes annotated to an entry in the selected gene set to the total number of genes annotated to the entry of this species; the calculation formula is rich ratio = term candidate gene number/term gene number). The Y-axis represents the KEGG Pathway, the size of the bubble represents the number of differential genes annotated to a certain KEGG pathway, the color represents the enrichment Q value, and the darker the color, the smaller the Q value.

Adult and juvenile stages were mainly enriched in the gap junction and oxytocin signaling pathways. Ribosomes, regulation of the actin cytoskeleton, glycolysis/gluconeogenesis, and IL-17 signaling pathway were enriched in the adult and egg stages. Additionally, lysosomes, glycolysis/gluconeogenesis, and ribosomes were enriched in the juvenile and egg stages.

Analysis of genes related to the growth and development of *F. hepatica* in the juvenile stage

Five important genes related to growth and development in the juvenile stage were analyzed using qPCR. The results showed that different levels of differential gene expression occurred during the juvenile stage. The expression levels of Cathepsin B, Glutathione S-transferase (GST) mu, heat shock protein 67B2, Kunitz-CH, and Legumain were relatively high during the juvenile stage, and relatively low during the adult and egg stages (Table 3).

Table 3. Important genes related to the growth and development in the juvenile stage

Gene name	Gene enrichment expression folds			Protein description
	Adult	Juvenile	Egg	
D915_011152	0.57	3.12	0.78	Cathepsin B
D915_008966	1.00	15.12	1.68	GST mu
D915_010279	1.00	340.65	2.73	Heat shock protein 67B2
D915_000805	0.97	3.69	1.53	Kunitz-CH
D915_008429	0.77	1.41	0.87	Legumain

DISCUSSION

Due to the differences in the gene expression of *F. hepatica* at different developmental stages, transcriptome sequencing analysis was performed on *F. hepatica* at three different developmental stages in the terminal host to explore significant differences in gene expression, metabolic pathways, and functional clustering during the growth and development of *F. hepatica* at different developmental stages. Considering the important role of sample consistency in the accuracy of transcriptome data, the samples collected in this study were from the same species and three biological replicates were set for each developmental stage to ensure the accuracy of transcriptome sequencing data.

GO functional enrichment analysis showed that ribosomes were significantly enriched in the three developmental stages of *F. hepatica*, indicating that they were involved in the growth and development of *F. hepatica* throughout its life cycle, including regulation of cell growth, proliferation, differentiation, apoptosis, and DNA repair (Hayashi *et al*., 2014; Wang *et al*., 2015; Zhang *et al*., 2019). The developmental stages of adults, juveniles, and eggs are significantly enriched in cilium organization, which may be involved in the cell cycle and body development of *F. hepatica* (Hickey *et al*., 2023). In addition, compared with that in juveniles and eggs, adults were significantly enriched in plasma membrane-bound cell projections and the cytoskeleton, which are related to the growth and development of worms. Compared with that in eggs, juveniles showed significant nucleoside triphosphatase activity, which may be related to the intake of nucleosides as nutrients during growth and development (Elzoheiry *et al*., 2018; Skelly *et al*., 2022).

Functional enrichment of the KEGG pathway showed that compared with that in eggs, adults were significantly enriched in the IL-17 signaling pathway and regulation of the actin cytoskeleton. IL-17 is a proinflammatory cytokine involved in innate and adaptive immune responses that has been implicated in a variety of diseases (Fu *et al*., 2016; Haçariz *et al*., 2015). Studies have shown that

the IL-17 signaling pathway plays a key role in controlling the local parasite burden (Rodríguez *et al*., 2015; Sinton *et al*., 2023). Therefore, we hypothesized that the significant enrichment of IL-17 in the adult stage might protect the parasite from host immunity. Actin is located in the membrane of *F. hepatica* and in the muscles around the abdominal sucker (Tansatit *et al*., 2006), and is mainly involved in the regulation of the actin cytoskeleton, tight junctions, adhesion, platelet activation, and other related pathways. Adults were significantly enriched in actin cytoskeletal regulation, which may be due to the adhesion of *F. hepatica* to the host intestine, liver, and bile ducts (Boukli *et al*., 2011). Juveniles and adults were significantly enriched in glycolysis/gluconeogenesis compared with that in eggs, indicating that juveniles transitioned from aerobic to anaerobic carbohydrate metabolism during the development of *F. hepatica* from juveniles to adults (Robb *et al*., 2022). Adults were significantly enriched in the oxytocin signaling pathway, which may play an important role in many processes of *F. hepatica* development, such as cell growth and differentiation (Abdulai-Saiku & Vyas, 2021; Chatterjee *et al*., 2016).

qRT-PCR validation analysis showed that Cathepsin B, GST mu, heat shock protein 67B2, Kunitz-CH, and Legumain were highly expressed during the juvenile stage. The high expression of Cathepsin B during the juvenile stage may be related to the rapid growth and development of juveniles in the host (Peterkova *et al*., 2023; Robb *et al*., 2022). GST is an antigenic protein secreted by parasites present in almost all animals. As an immune evasion molecule in helminths, GST is transiently expressed on the surface of worms and plays an important role in the detoxification and removal of harmful molecules, which is conducive to the continuous survival of worms in the host (Cervi *et al*., 1999; López Corrales *et al*., 2021; Piedrafita *et al*., 2007; Xu *et al*., 2020). The GST mu isoenzyme in *F. hepatica* participates in the detoxification of various substrates by chemically binding to glutathione (Fernández *et al*., 2015; Zhang *et al*., 2017b). GST mu is highly expressed in the juvenile stage, and may be involved in oxidative stress metabolism and cellular detoxification in the juvenile stage, ensuring the survival of juveniles in their hosts (Fernández *et al*., 2000; Miles *et al*., 2022; Valdes-Fernandez *et al*., 2023). The effect of heat shock protein 67B2 on the growth and development of *F. hepatica* is currently unknown; however, it is highly expressed in the juvenile stage, may be involved in the body's immune system, and plays a potential role in severe environmental stress conditions (Park *et al*., 2020; Son *et al*., 2023). The Kunitz gene is typically located in the secretory protein (ES) or secretory tissue of *F. hepatica* and acts at the host-parasite interface (Cwiklinski *et al*., 2021). Kunitz-CH is expressed at a higher level in the juvenile stage, which may be related to its active secretion during part of the life cycle of *F. hepatica* (Cwiklinski *et al*., 2023), and is involved in the immune escape mechanism of the juvenile stage (Falcón *et al*., 2014). Legumain belongs to the peptidase C13 family, a protein that is an important component of the excretory secretion product of *F. hepatica* and plays an important role in the biology and pathogenesis of *F. hepatica*. The expression level of legumain in the juvenile stage is higher than that in the other two stages, and is expected to become a new vaccine or drug target (Cwiklinski & Dalton, 2022; Robinson *et al*., 2009) Through the verification and analysis of five differentially expressed genes in the juvenile stage, we found that these genes were mostly related to the growth, development, and immune function of *F. hepatica*, which lays a foundation for the early diagnosis of *F. hepatica*.

CONCLUSION

In this study, RNA-seq technology was used to screen a total of 11,236 differentially expressed genes from three developmental stages of *F. hepatica*. The analysis revealed the differences in the metabolic levels of *F. hepatica* at different developmental stages in the terminal host. Important functional clustering among worms at different developmental stages was also explored. Five differentially expressed genes with high expression during the juvenile stage were identified. It has been verified that these genes are mostly related to the growth, development, and immune function of *F. hepatica*, which lays the foundation for theoretical research, drug target discovery, early immunological diagnosis, and prevention of *F. hepatica*.

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Ethics Approval

Animal procedures were performed in accordance with the National Standard Guidelines for Ethical Review of Animal Welfare (GB/T 35892-2018) and were approved by the Animal Care and Use Committee of Inner Mongolia Agricultural University. All efforts were made to take the least painful approach to animal handling and to avoid or reduce stress, pain, and injury to the animals used in the experiment.

Conflict of Interest

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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