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## Original Article

# Mechanism of the Passage of *Angiostrongylus cantonensis* Across the Final Host Blood-Brain Barrier Using the Next-Generation Sequencing

\*Yue Guo<sup>1,2</sup>, Hai Yan Dong<sup>1,2</sup>, Hong Chang Zhou<sup>1,2</sup>, Zhong Shan Zhang<sup>1,3</sup>, Yu Zhao<sup>1</sup>, Yu Jie Zhang<sup>1</sup>

1. School of Medicine, Huzhou University, Huzhou Cent Hosp, Zhejiang, China
2. Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, Huzhou University, Huzhou, Zhejiang, China
3. School of Life Sciences, Huzhou University, Zhejiang, China

Received 20 Feb 2021  
Accepted 09 Apr 2021

**Keywords:**  
*Angiostrongylus cantonensis*;  
Transcriptional sequencing;  
The fourth stage larvae;  
Adult stage

**\*Correspondence Email:**  
[guoyue66@126.com](mailto:guoyue66@126.com)

### Abstract

**Background:** Multicellular parasites *Angiostrongylus cantonensis* larvae develop in the final host rat brain at the fourth stage (L4) and migrate to the lungs by the adult stage. The potential mechanism of its blood-brain barrier (BBB) passage remains unclear.

**Methods:** By using Illumina Hiseq/Miseq sequencing, we obtained the transcriptomes of 3 groups of adult males and 3 groups of female of *A. cantonensis* to generate similarly expressed genes (SEGs) between 2 genders at the adult stage. Next 2 groups of L4 expressed genes were used to compared with SEGs to create differentially expressed genes (DEGs) between 2 life stages to unlock potential mechanism of BBB passage.

**Results:** In total, we obtained 381 581 802 clean reads and 56 990 699 010 clean bases. Of these, 331 803 unigenes and 482 056 transcripts were successfully annotated. A total of 3 166 DEGs between L4 and adults SEGs were detected. Annotation of these DEGs showed 167 were down-regulated and 181 were up-regulated. Pathway analysis exhibited that calcium signaling pathway, the ECM-receptor interaction, focal adhesion, and cysteine and methionine metabolism were highly associated with DEGs. The function of these pathways might be related to BBB traversal, as well as neuro-regulation, interactions between parasite and host, environmental adaptation.

**Conclusion:** This study expanded the regulatory characteristics of the two important life stages of *A. cantonensis*. This information may provide a better appreciation of the biological features of the stages of the parasitic *A. cantonensis*.



## Introduction

The zoonotic nematode, *Angiostrongylus cantonensis*, is a lungworm that affects rats and is the causal agent of human angiostrongyliasis. Asia and the Pacific Islands are the main endemic regions of angiostrongyliasis. This disease has spread to many other non-endemic countries, including France, Germany, the Caribbean region (including Jamaica), Brazil, and South Africa etc. (1, 2). At present, *A. cantonensis* has become a threat to global health as at least 3000 cases of angiostrongyliasis have been reported in 31 countries (1, 2).

This parasite usually thrives in the lungs of the final host (rat) at the adult stage. Although humans are nonpermissive hosts, a successful infection may lead to eosinophilic meningitis and eosinophilic meningoencephalitis, also known as angiostrongyliasis (2). The third stage of *A. cantonensis* (L3) is infectious to the final host as the parasite completes its development in the intermediate host (snail). Inside the body of the rat (final host), *A. cantonensis* first migrates into the brain and then moves into the lungs. At the fourth and fifth stages (L4 and L5), *A. cantonensis* is in the brain of the final host and can cause eosinophilic meningitis and eosinophilic meningoencephalitis. While at the adult stage, *A. cantonensis* resides in the lung of the final host. The mechanism of the nematode migration remains unclear, especially across the blood-brain barrier (BBB).

In recent years, the rapid development of next-generation sequencing (NGS) has enabled extensive genomic understanding of parasite. NGS provides researchers with opportunities to identify differentially expressed genes across different tissues, cells, stages, genders, and species. In the field of parasitic research, NGS allows researchers to better understand the molecular and biochemical processes involved in the development, reproduction, drug-resistance and parasite-host interactions of several parasites including mul-

ticellular animal parasites like *Schistosoma mansoni* (3, 4), *S. japonicum* (5), *Haemonchus contortus* (6), *Ascaris suum* (7) and *Clonorchis sinensis* (8) etc., plus parasitic protozoa including *Leishmania donovani* (9) and malaria parasite (10, 11) etc.

In this study, we used Illumina Hiseq/Miseq sequencing to better understand the mechanism of *A. cantonensis* passage across the BBB and the transcriptomic differences between the 2 life stages by comparison the transcriptome of L4 with the similarities of the transcriptome of adult males and females.

## Materials and Methods

### Ethics statement

All animal work was conducted in strict compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals (as approved by the State Council of the People's Republic of China).

Apple snails *Pomacea canaliculate* and Sprague-Dawley rats (SD) were employed as the middle and final hosts of *A. cantonensis*.

### Parasites

L4 were harvested from the *A. cantonensis*-positive rat brain; here, 2 rats were used in the experiment, and each one provided 200 L4 nematodes. Adult stage *A. cantonensis* were collected from 3 *A. cantonensis*-positive SD rats. Each rat provided one pair of males and females, but 2 genders was separated into 2 different groups in the following steps. In total, 2 groups of L4, 3 groups of male adults and 3 groups of female adult worms were harvested. All collected worms were washed three times with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), then soaked in an RNA hold reagent (Transgene Biotech, Beijing, China), and stored in a liquid nitrogen container.

### RNA isolation and sequencing

Total RNA from samples was isolated according to the manufacturer's instructions, and its quality was tested using a Nanodrop 2000 machine (Thermo Scientific CA) to determine concentration and OD values. The integrity of total RNA was confirmed visually by agarose gel electrophoresis. Polyadenylated mRNA was purified from the total RNA using Oligo-dT beads, which can specifically bind to the poly A tail of mRNA. Next, mRNAs were randomly digested into 1.5-2kb kb fragments using the Ambion RNA fragmentation kit. Fragmented mRNAs were used as templates to synthesize single-stranded cDNA using random primers, following the synthesis of double-stranded cDNA. Then, template DNA fragments were end-repaired using End Repair Mix, and adenosine was added to the 3' ends to ligate the adapter. After the enrichment of the cDNA library by 15 cycles of PCR, PCR products were purified by 2% agarose gel electrophoresis. Target DNA bands were extracted using a Qiagen gel extraction kit. After quantification, bridge PCR was conducted to generate different clusters in cBot. Then, Illumina Hiseq/Miseq sequencing was performed.

### Reads quality control

The file produced by Hiseq/Miseq sequencing was in the form of FASTQ, which included read information as well as sequencing. Raw data of Illumina sequence was assessed for quality based on % ATGC content and average base quality (12, 13). Raw data would then be filtered to obtain clean data by using SeqPrep (<https://github.com/jstjohn/SeqPrep>) and Sickle (<https://github.com/najoshi/sickle>), when the following perform was conducted, including removing the adapter sequences, low quality reads, high N-rate sequences, and other contaminated data, which might seriously affect the quality of subsequent assembly (14).

### De novo assembly and annotation

*De novo* assembling of L4 clean data was obtained by using the Trinity software (<http://trinityrnaseq.sourceforge.net/>, version number: trinityrnaseq-r2013-02-25), when contig and singleton information was produced. After completion of ORF (open reading frame) prediction to the transcripts by Trinity, *De Novo* Assembling was conducted by Blast X (Version 2.2.25), querying databases including NCBI non-redundant protein sequences (Nr), Swissprot, Gene Ontology (GO), and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The E-value cutoff was  $1.0 \times 10^{-5}$ .

After the annotation, the gene expression levels of all samples were estimated by RSEM (<http://deweylab.biostat.wisc.edu/rsem/>). Software edgeR was used to analyze gene expression similarity or differences between groups (<http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>). First, SEGs between male and female adult *A. cantonensis* were obtained ( $FDR \geq 0.005$  and  $\log_2|FC| < 1$ ). Then, the transcriptome of L4 and SEGs were compared to produce DEGs among the L4 stage and adult stage ( $FDR < 0.005$  and  $\log_2|FC| \geq 1$ ). In addition, GO annotation, KEGG annotation, enriched GO, and enriched KEGG annotations of DEGs were conducted.

## Results

### Raw and clean sequencing, unigene, and transcript data

L4 group produced 98 352 654 raw reads and 14 752 898 100 raw bases. The male adult group provided 135 194 092 raw reads and 20 414 307 892 raw bases. The female adult group generated 149 010 616 raw reads and 22 500 603 016 raw bases. The L4, female adult, and male adult groups of *A. cantonensis* produced a total of 97 671 628 clean reads and 14 466 272 625 clean bases, 135 014 748 clean reads and 20 200 781 653 clean bases, and 148

895 426 clean reads and 22 323 644 732 clean bases, respectively. Assembly results revealed 331 803 unigenes and 482 056 transcripts were successfully generated. Annotation of differ-

entially expressed unigenes between L4 and SEGs exhibited 167 unigenes were down-regulated and 181 unigenes were up-regulated, and top 10 of 2 items are showed in Table 1.

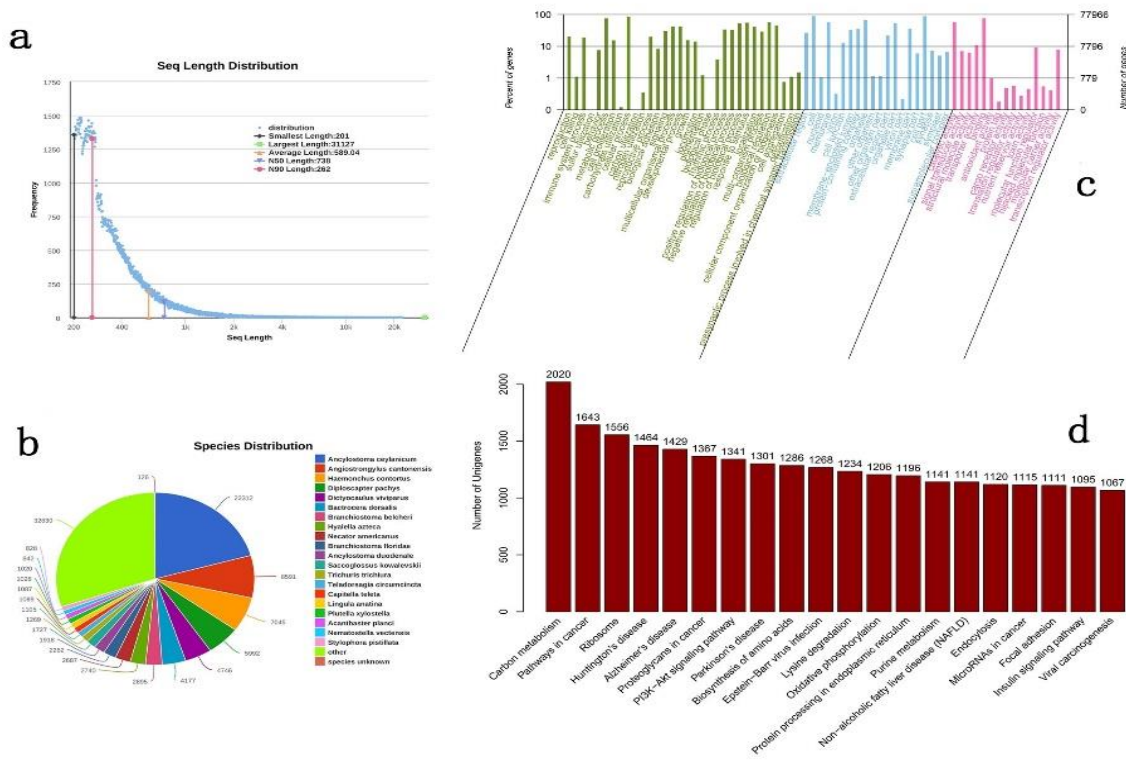
**Table 1:** Top 10 up-regulated and 10 down-regulated unigenes between the 4<sup>th</sup> stage and adult stage of *Angiostrongylus cantonensis*

Name	log <sub>2</sub> FC(L4/SameA FAM)	Pval	fdr	regu- late	signifi- cant	mean_ L4	mean_SameAF AM
TRINI- TY_DN11419_c0_g2	9.354651	8.40E- 44	2.66E- 40	up	yes	286.919 5	0.33841
TRINI- TY_DN6098_c4_g1	8.324563	2.72E- 13	5.07E- 11	up	yes	78.0761 3	0.143855
TRINI- TY_DN51540_c0_g1	7.883322	1.81E- 08	1.30E- 06	up	yes	121.230 1	0.413869
TRINI- TY_DN44440_c0_g1	6.46189	5.14E- 14	1.08E- 11	up	yes	75.0448 3	0.752465
TRINI- TY_DN13578_c0_g1	5.99984	1.23E- 20	1.18E- 17	up	yes	84.2291	1.217789
TRINI- TY_DN11501_c0_g1	5.902901	1.86E- 12	2.67E- 10	up	yes	19.2766 3	0.223838
TRINI- TY_DN289_c0_g3	5.071188	9.48E- 20	6.01E- 17	up	yes	290.059 5	8.530917
TRINI- TY_DN37627_c0_g1	5.057034	2.51E- 15	7.22E- 13	up	yes	29.9750 5	0.803415
TRINI- TY_DN64161_c1_g1	4.903323	1.14E- 07	6.57E- 06	up	yes	7.70673	0.160869
TRINI- TY_DN4227_c0_g3	4.724724	3.01E- 09	2.65E- 07	up	yes	7.56612 5	0.189929
TRINI- TY_DN6322_c19_g1	-1.06081	0.0047 92	0.0458 4	down	yes	34.6852	72.46527
TRINI- TY_DN12792_c1_g1	-1.06994	0.0049 73	0.0468 51	down	yes	18.8987	39.78487
TRINI- TY_DN11775_c1_g2	-1.08027	0.0027 96	0.0319 92	down	yes	23.8321 5	50.50293
TRINI- TY_DN32866_c0_g1	-1.09877	0.0037 66	0.0389 66	down	yes	47.8841	102.6685
TRINI- TY_DN431_c0_g1	-1.13066	0.0022 72	0.0283 2	down	yes	11.7379 5	25.82027
TRINI- TY_DN43921_c0_g2	-1.17851	0.0025 93	0.0307 5	down	yes	22.9445 5	52.05986
TRINI- TY_DN22816_c0_g1	-1.19753	0.0013 92	0.0193 35	down	yes	14.0594	32.37419
TRINI- TY_DN11227_c0_g1	-1.22181	0.0019 37	0.0251 3	down	yes	13.6105 3	31.87829
TRINI- TY_DN24730_c0_g2	-1.27217	0.0008 17	0.0126 8	down	yes	12.1162	29.4052
TRINI- TY_DN18003_c2_g1	-1.28597	0.0019 84	0.0254 27	down	yes	8.67839 5	21.30571

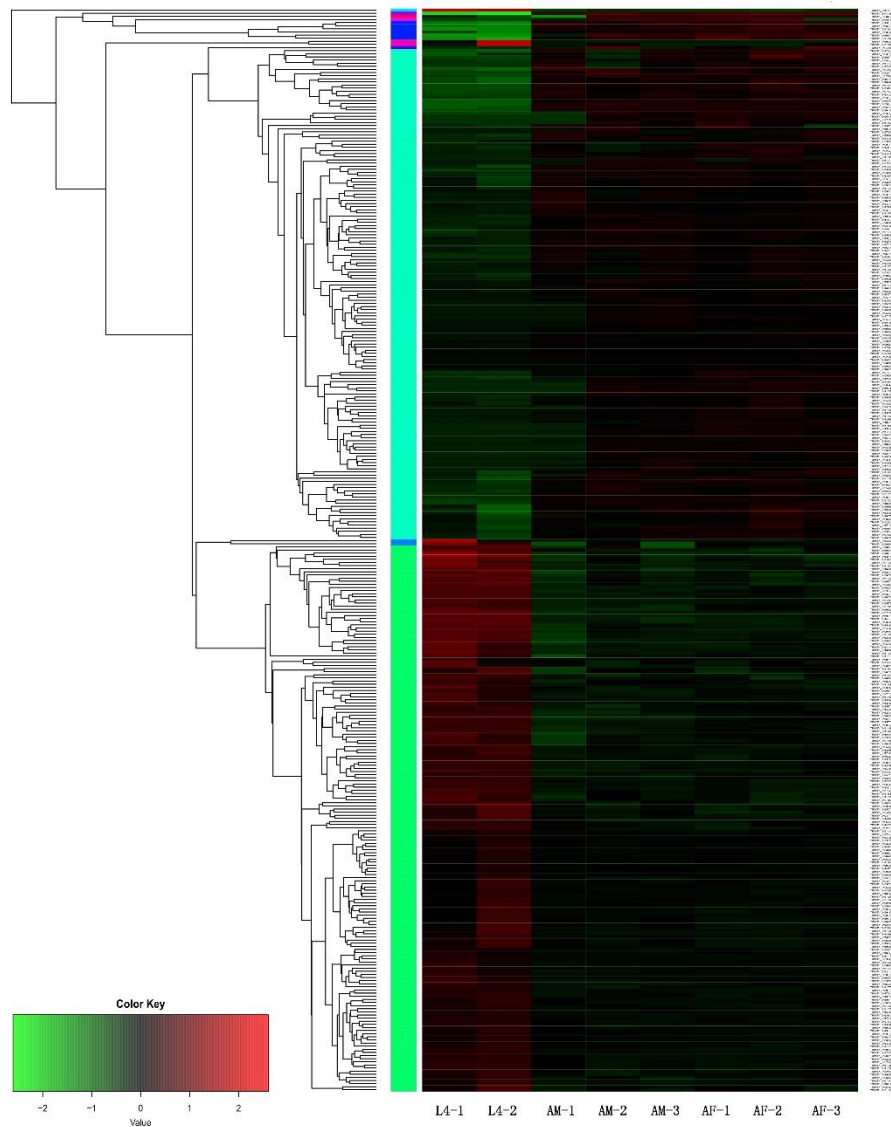
**GO and KEGG annotation of all unigenes**

GO annotation included three term types, biological processes, cellular components, and molecular functions, as shown in Fig. 1c. The top 10 most related terms in biological processes were: cellular process (67235 unigenes, 0.862363081%), metabolic process (59751 unigenes, 0.766372521%), biological regulation (44426 unigenes, 0.569812482%), response to stimulus (43327 unigenes concerned, 0.555716594%), regulation of biological process (41591 unigenes, 0.533450478%), cellular component organization or biogenesis (34930 unigenes, 0.448015802%), developmental process (32936 unigenes, 0.422440551%), multicellular organismal process (32859 unigenes, 0.421452941%), localization (320320.410845753), and positive regulation of biological processes (26529 0.340263705)

(Fig. 2). The top five related terms in cellular components were cell (70361 unigene related, 0.902457481%), cell part (70297 unigene related, 0.901636611%), organelle (52372 unigene related, 0.671728702%), membrane (44298 unigenes related, 0.568170741%), and organelle part (41972 unigene related, 0.538337224%). In addition, the top 5 highly related terms in molecular function were composed by the following: binding (60017 unigenes related, 0.769784265%), catalytic activity (45315 unigene related, 0.581214889%), transporter activity (8395 unigene related, 0.10767514%), molecular function regulator (7232 unigenes related, 0.092758382%), and transcription regulator activity (6103 unigenes related, 0.078277711%). The top 20 related pathways provided by unigenes KEGG are shown in Fig. 1d.



**Fig. 1:** Annotation of unigenes in different databases. (a). Unigene length distribution (b). KOG annotation of unigenes. (c). GO annotation results for unigenes. (d). The top 20 related KEGG pathways for unigenes



**Fig. 2:** Heatmap for differentially expressed genes between the 4-th stage larvae and adult stages of *A. cantonensis*

**GO annotation and KEGG annotation of DEGs between L4 and SEGs of adult worms**

The following 10 terms were highly expressed in L4 compared with SEGs of adult groups, including cell (63 unigenes), cell part (63 unigenes), binding (59 unigenes), cellular process (58 unigenes), organelle (55 unigenes), multicellular organismal process (52 unigenes), developmental process (51 unigenes), biological regulation (50 unigenes), response to a

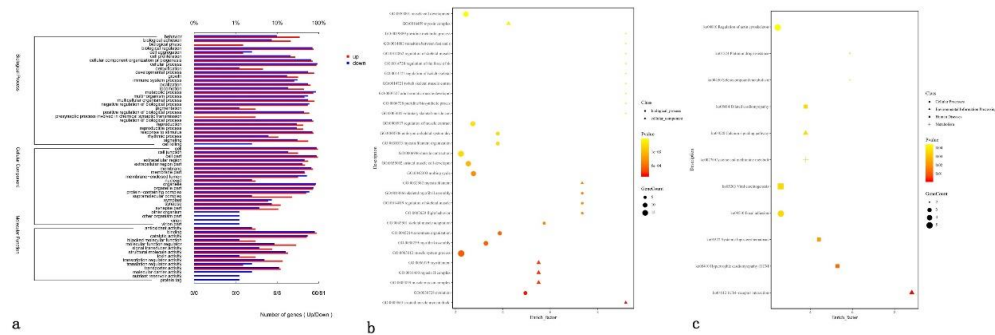
stimulus (50 unigenes), and metabolic process (49 unigenes), as shown in Fig. 3a.

Furthermore, the results indicated that the following 11 GO items were significantly enriched, including three FROM cellular component classes and eight biological processes. The former was composed of GO:0036379 (myofilament), GO:0016460 (myosin II complex), GO:0005859 (muscle myosin complex), and GO:0005863 (striated muscle myosin thick filament). The latter included: GO:0030239 (myofibril assembly),

GO:0045214 (sarcomere organization), GO:0007629 (flight behavior), GO:0030728 (ovulation), GO:0003012 (muscle system process), GO:0043501 (skeletal muscle adaptation), and GO:0014819 (regulation of skeletal muscle contraction), as shown in Fig. 3b.

The following KEGG pathways were significantly enriched: ko04512 (ECM–receptor interaction), ko05410 (hypertrophic cardio-

myopathy), ko05322 (systemic lupus erythematosus), ko04510 (focal adhesion), ko05203 (viral carcinogenesis), ko00270 (cysteine and methionine metabolism), ko04020 (calcium signaling pathway), ko05414 (dilated cardiomyopathy), ko00450 (seleno compound metabolism), ko01524 (platinum drug resistance), and ko04810 (regulation of actin cytoskeleton), as shown in Fig. 3c.



**Fig. 3:** Annotation of differentially expressed genes between L4 and SEGs of adult *A. cantonensis*. (a). Overview of GO annotation of DEGs. (b). Enriched GO annotation of DEGs (c). Enriched KEGG annotation of DEGs

## Discussion

Brain parasitism is a rare phenomenon, suggesting that the parasite needs to transverse across the BBB and invade into the brain of the final host, and only a few parasites maintain this ability, such as *A. cantonensis*, *Toxoplasma gondii*, *Trypanosoma Brucei*, *S. spp.*, and *Taenia solium*. Among all these parasites, *A. cantonensis* is special. *T. gondii* (14) and *T. brucei* (16) are protozoan parasites, their BBB passage might employ a different way from the multicellular parasite *A. cantonensis*. Although the multicellular parasite *Schistosoma spp.* (17, 18) and *T. solium* (19) migrate into the final host brain infrequently, this phenomenon is ectopic parasitism. Brain parasitism of *A. cantonensis* is inevitability, unlike *T. gondii* and *T. brucei*.

In this study, 2 groups of L4 larvae (brain stage) and 3 groups of both male and female adults were used to unlock potential mechanism of BBB passage *A. cantonensis* by the next-generation sequencing. The results

showed that many pathways were differentially expressed between the L4 and adult stages, including the calcium signaling pathway, ECM–receptor interactions, focal adhesion, and cysteine and methionine metabolism etc. as showing in Fig. 3b and 3c, enriched GO annotation of DEGs and enriched KEGG annotation of DEGs.

The calcium signaling and its pathway is universal across eukaryotic cells, protozoa, and multi-cell worms. This pathway regulates many cellular processes of the protozoa, including growth, differentiation, programmed cell death, exocytosis, endocytosis, phagocytosis, and recycling (20–22). Calcium in the free-living worm *Caenorhabditis elegans* is multifunctional and is linked to its development, fertility, proliferation, behavior, and lifespan (22, 23). In multicell parasites, calcium is fundamental for muscular contractility, parasite neuromusculature, host-parasite interaction, parasite development, and movement (24–26). The calcium signaling pathway can also be utilized as a target to kill the parasite. Praziquantel, a

schistosomiasis drug, disrupts the  $\text{Ca}^{2+}$  homeostasis in adult *Schistosoma* (27).

Furthermore, ECM–receptor interaction and focal adhesion pathway were also differentially expressed in the 2 life stages of *A. cantonensis*. The ECM-receptor interaction pathway and focal adhesion can alter the reproduction of *C. elegans* (28). The ECM-receptor interaction can act as an immune-related pathway for parasites to adapt to the host body (29) or play a role in schistosome-host interactions (30). Focal adhesion has been considered an essential step in cell migration (31). Interestingly, the focal adhesion kinase (FAK) of the host might be related to parasite translocation. FAK dysregulation can facilitate the transmigration of *Toxoplasma gondii* (32). FAK-deficient cells are significantly more susceptible to *T. cruzi* invasion (33).

The physiological state of L4 is different from that of the adult. The former is in the larval stage with the reproductive system is not well developed, whereas the latter has a mature reproductive system. There might be differences in the physiological regulation of the two by the nervous system. The calcium signaling pathway might play an essential role in the neuro-regulation, including muscle tissue activity, reproductive system activity, and even metabolism.

Besides, although both L4 and adults live inside the body of the final host, the former live in the brain and the latter in the lung. The composition of immune cells in the brain is also different from that of the rest of the body. The different immune cell compositions in the brain and lung, might lead to different interactions, inducing differential gene expression in the parasite.

Furthermore, the brain and lungs also differ in the concentration of ions.  $\text{K}^+$  and  $\text{Na}^+$  maintain higher concentrations in the brain than in the lung, whereas the  $\text{Ca}^{2+}$  concentration is higher in the lung. This suggested that the living environments in the brain and lungs are different. Therefore, the calcium signaling

pathway may be differentially expressed in the two life stages to adjust the absorption and discharge of ions.

In addition, both adult males and adult females are mature worms and need to produce sperm and eggs. L4 lives inside the brain without mature sexual organs. This difference might also be detected in the transcriptome at 2 stages, and ECM–receptor interaction might be involved.

Given the complexity of brain parasitism caused by multicellular worm *A. cantonensis*, many pathways and genes were related with this phenomenon. Meanwhile, each pathway may be multi-functional.

## Conclusion

Our study has elaborated on existing findings, however, further work exploring the complexity of the *A. cantonensis* transcriptome at different life stages is warranted.

## Acknowledgements

This research was supported by the Natural Science Funds of Zhejiang Province (LQ15C040003), grants from the Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, Huzhou University (Grant No. HUZUL201902& HUZUL20190208), as well as research funds of Huzhou University (2019XJKJ41&2019XJKJ43).

## Conflict of interest

The authors declare that there is no conflict of interest.

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