Main Pathways and Ion Channels Differentially Expressed in the Transcriptome of Male and Female Adult *Angiostrongylus cantonensis* Using a Deep Sequencing Approach

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**Abstract**

**Background:** The adult stage is an important period in the life cycle of *Angiostrongylus cantonensis*, as it is at this stage that male and female worms produce thousands of fertilized eggs daily.

**Methods:** To explore the transcriptional details of adult male and female *A. cantonensis*, three groups of male and female adult worms were collected, and their transcriptome profiles were analyzed using an Illumina next-generation sequencing platform. A total of 283,910,174 clean reads were obtained, and 137,626 unigenes and 237,059 transcripts were then generated. Unigenes were successfully annotated by querying the Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), NCBI non-redundant protein sequences (NR), PfAM, STRING, and SWISS-PROT databases. Then, differentially expressed genes (DEGs) between the 2 genders were identified. The GO and KEGG databases were used for DEG annotation, and a number of DEG annotations were enriched.

**Results:** The results obtained from querying DEGs using the GO and KEGG databases revealed that male and female adult worms exhibited differences in metabolism and production. Protein phosphorylation, ion transport, and calcium transport were all significantly enriched according to GO annotation. A number of other pathways were also enriched according to KEGG enrichment annotation, including the pentose phosphate pathway, nitrogen metabolism, oocyte meiosis pathway, neuroactive ligand-receptor interaction, calcium signaling pathway, transforming growth factor β (TGF-β) signaling pathway etc.

**Conclusion:** We hypothesized that the nervous system of the worm plays a key role in the physiological regulation of adult *A. cantonensis*, and based on this, the function of the calcium-signaling pathway should be investigated.
Introduction

Angiostrongyliasis is a disease caused by the rat lungworm *Angiostrongylus cantonensis* that was initially described by Chen (1935) in rats from China (1-3). The first case of human angiostrongyliasis was recorded in Taiwan in 1944. Since then, the disease has been documented worldwide with approximately 3,000 cases reported (4, 5). Adult *A. cantonensis* parasites reside within the lungs of rats. Male and female lungworms exist together in the pulmonary arteries of rats, and females produce thousands of fertilized eggs daily that are released within rat feces.

Both male and female *A. cantonensis* are filiform and tapered at the anterior end. The adult male nematodes are 15.5-23.0 mm in length and 0.25-0.35 mm wide. The esophagus of these worms is 0.29-0.35 mm long, and the intestine is wide, but thin-walled. The excretory pore is located in the region of the esophageal-intestinal junction. Females are larger than male adults, and they commonly measure 18.5-33.0 mm in length and 0.28-0.5 mm in width. Both males and females share similar head, esophageal, and intestinal morphologies; however, some morphological differences are obvious. Adult females exhibit characteristic structures such as an entangled uterus and intestine. However, the causes of these morphological and structural differences are unclear, particularly at the transcriptional level.

In recent years, next-generation sequencing (NGS) platforms provided by Solexa (Illumina) have enabled researchers to define the parasite transcriptome and genome (6, 7). NGS is a powerful tool that can be used to reveal transcription differences in different genes, cells, tissues, life stages, and genders during the process of parasite development and immigration. To determine the transcriptome differences between the two sexes of *A. cantonensis*, we employed an Illumina-based NGS strategy to sequence a single worm obtained from a rat host.

Methods

Experimental animals and sample collection

All Sprague Dawley (SD) rats were raised with free access to water and food at the Laboratory Animal Center, Medicine School, Huzhou University in 2019. Animal procedures were conducted in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (as approved by the State Council of the People’s Republic of China). All efforts were made to minimize discomfort and suffering in the animals during handling and manipulation.

SD rats (7-week-old, 120 g) were infected with 200 3rd stage *A. cantonensis* larvae. The rats were euthanized at 45 days post-infection to collect adult worms located in the rat pulmonary arteries, and each pair of male and female adult worms used in our experimental studies were obtained from the same rat host. The worms were washed three times with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and stored in RNA hold reagent (Transgene Biotech, Beijing, China) to extract RNA.

RNA extraction, cDNA library preparation, and sequencing

Trizol Reagent (Invitrogen, catalog number: 15596026) was used to suspend the worms, and RNA extraction was conducted according to the manufacturer’s protocols. Briefly, individual worms were suspended in Trizol (1 mL, 4 °C, and 10 min). A homogenizer was then used to facilitate lysis. The worm suspension was centrifuged at 12,000 × g for 10 min at 4 °C to collect the clear supernatant. The collected supernatant was mixed with chloroform and then centrifuged at 12,000 × g for 10 min at 4 °C. Next, the upper aqueous phase was
collected, and RNA was precipitated using isopropanol. The obtained RNA pellet was washed with 75% ethanol and centrifuged at 7,500 \( \times \) g for 5 min at 4 °C. RNA from each sample was quantified using a NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher).

RNA integrity was determined by agarose gel electrophoresis, and the RIN value was determined using an Agilent 2100. cDNA libraries were constructed when the total amount of RNA was not less than 5 g or 200 ng/L at an OD 260/280 of between 1.8 and 2.2.

Oligo (dT) was used to purify RNA from crude RNA for downstream library construction, sequencing, and analysis of transcriptome information. Fragmentation buffer was added to randomly fragment the complete RNA sequence into approximately 200 bp fragments. cDNA was synthesized by reverse transcriptase using random primers and mRNA as a template, and stable double-stranded DNA fragments were then constructed. The related adapter was added to the end of the dsDNA for sequencing using an Illumina Hiseq/Miseq.

Bioinformatics analysis

Clean data creation

The raw image data obtained from Illumina sequencing were converted into sequence reads by base calling, and the read results were stored in the FASTQ file format. Clean data were created by screening the raw reads using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) software programs. Clean reads were assembled into long sequences with Trinity (http://trinityrnaseq.sourceforge.net/, version: trinityrnaseq-r2013-02-25).

De novo transcriptome annotation

Prior to the process of annotation, the open reading frame (ORF) of the clean reads was predicted using Trinity. The obtained sequence information was annotated using BlastX (Version 2.2.25) by querying the NR, String, Swiss-Prot, and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. The E value was < 1.0×10-5. The NCBI_NR database, which is composed of Swiss-Prot, PIR (Protein Information Resource), PRF (Protein Research Foundation), and PDB (Protein Data Bank), was also used to estimate the similarities between the transcript sequence of this species and that of another species. GO (Gene Ontology, http://www.geneontology.org) annotation was performed to obtain gene information regarding BP (Biological Process), MF (Molecular Function), and CC (Cellular Component) using blast2go (http://www.blast2go.com/). Additionally, COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG/) and KEGG (http://www.genome.jp/kegg/) analyses were also employed during the annotation process.

Differentially expressed gene information

After the annotation of all concerned transcriptome samples, the gene expression levels were estimated using RSEM (http://deweylab.biostat.wisc.edu/rsem/). Differentially expressed genes (DEGs) were identified using edgeR (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html). GO annotation, KEGG annotation, enriched GO, and enriched KEGG annotations of DEGs were conducted.

Results

The 6 groups provided 284,204,708 reads and 42,914,910,908 bases, where 135,194,092 reads and 20,414,307,892 bases were acquired from the male group and 149,010,616 reads...
and 22,500,603,016 bases were acquired from the female group.

After filtering, all 6 groups of males and females produced 283,910,174 clean reads and 42,524,426,385 total clean bases. Of these groups, 3 male groups provided 135,014,748 clean reads and 20,200,781,653 total clean bases, and each male worm yielded 45,004,916 clean reads and 6,733,593,884 clean bases on average. Each female worm yielded 49,631,809 clean reads and 7,441,214,911 clean bases (Table 1).

Successful assembly of the clean data generated 137,626 unigenes and 237,059 transcripts. The length distribution of the unigenes is shown in Fig. 1, and 55.54% of the unigenes (76437) possessed a length of \(-1\)400 bp, while 19.10% of these genes (26288) exhibited a length of 401-600 bp.

### Table 1: Annotation characteristics of unigenes and transcripts

| Type                   | Unigene   | Transcripts |
|------------------------|-----------|-------------|
| Total sequence (N):    | 137,626   | 237,059     |
| Total sequence base    | 85,474,278| 229,035,411 |
| Percent GC             | 43.83     | 43.12       |
| Largest                | 15,869    | 15,869      |
| Smallest               | 201       | 201         |
| Average                | 621.06    | 966.15      |
| N50                    | 825       | 1,750       |
| N90                    | 268       | 367         |

The obtained unigenes and transcripts were annotated by querying the GO, KEGG, NR, PFAM, STRING, and SWISS-PROT databases, and the results included 58,524 unigenes and 124,109 transcripts that were successfully annotated in NR, 33,045 unigenes and 68,740 transcripts from GO, 29,242 unigenes and 62,699 transcripts from KEGG, 20,769 unigenes and 54,538 transcripts from PFAM, 41,314 unigenes and 88,741 transcripts from STRING, and 33,480 unigenes and 69,661 transcripts from SWISS-PROT.

The unigene GO annotation results are provided in Figure 1b, and the top 10 most related terms were cell (31,387, cellular component), cell part (31,359, cellular component), cellular process (29,703, biological process), organelle (29,055, cellular component), metabolic process (26,028, biological process), binding (25,985, molecular function), organelle part (23,569, cellular component), biological regulation (22,618, biological process), and response to stimulus (21,640, biological process).

Figure 1c illustrates the KOG annotation of unigenes, including [A] RNA processing and modification (3,097 unigenes related), [B] chromatin structure and dynamics (2,580 unigenes related), and [J] translation, ribosomal structure, and biogenesis (1,881 unigenes related) as the top 3 functional categories.

The KEGG annotation revealing the top 20 related pathways is presented in Figure 1d, and these include ribosome, carbon metabolism, protein processing within the endoplasmic reticulum, and others. The entire KEGG annotation result set is shown in Fig. 1e.
Gene expression levels were calculated using RSEM software (http://deweylab.biostat.wisc.edu/rsem/), and the heat map illustrates the gene expression of the 6 samples at the organismal level, as shown in Figure 2. Using the edgeR software (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html), DEGs were generated from the analysis of gene read counts produced by RSEM. Selection criteria were set as FDR < 0.05 and log₂|FC| ≥ 1. Unigenes that satisfied these criteria were considered as DEGs. A heatmap of these DEGs is shown in Fig. 2c.
Fig. 2: Overview of adult female and male *Angiostrongylus cantonensis*. (a). The morphology of female and male adult *A. cantonensis* (b). Principal component analysis plots highlight the transcriptomic signatures of male and female adults (c). Volcano plot of DEGs in female and male adults (d). Heat map for DEGs illustrating the hierarchical clustering of the DEGs between the two sexes of *A. cantonensis*.

Figure 3a shows the GO annotation of unigenes, and these include the three categories: biological process, cellular component, and molecular function. The top 4 highly related items in the biological processes were cellular process (431 upregulated genes and 766 downregulated genes in females), metabolic process (391 upregulated genes and 682 downregulated genes in females), biological regulation (363 upregulated genes and 669 downregulated genes in females), and regulation of biological processes (341 upregulated genes and 644 downregulated genes in females). In the cellular component category, the top ranking items included cell part (454 upregulated genes and 800 downregulated genes in females), cell (454 upregulated genes and 800 downregulated genes in females), organelle (422 upregulated genes and 750 downregulated genes in females), organelle part (353 upregulated genes and 624 downregulated genes in females), membrane (324 upregulated genes and 590 downregulated genes in females), membrane part (243 upregulated genes and 439 downregulated genes in females), and membrane-enclosed lumen (225 upregulated genes and 399 downregulated genes in females). Additionally, in the category of molecular function, GO annotation identified the top 4 terms as binding (395 upregulated genes...
and 706 downregulated genes in females), catalytic activity (283 upregulated genes and 501 downregulated genes in females), molecular function regulator (78 upregulated genes and 135 downregulated genes in females), and transporter activity (72 upregulated genes and 126 downregulated genes in females).

GO enrichment annotation revealed that several categories were significantly enriched in the female group, including protein phosphorylation (GO:0006468), regulation of protein phosphorylation (GO:0001932), positive regulation of protein phosphorylation (GO:0001934), ion transport (GO:0006811), cation transport (GO:0006812), and calcium transport (GO:0006816).

Further analyses revealed that among them, 41 pathways were remarkably enriched. The pathways belonged to 6 classes that included organismal systems (14 pathways included), metabolism (including 5 pathways), human diseases (5 pathways), genetic information processing (1 pathway), environmental information processing (12 pathways), and cellular processes (4 pathways) that included the pentose phosphate pathway (ko00030), glutathione metabolism (ko00480), nitrogen metabolism (ko00910), lysine degradation (ko00310), vascular smooth muscle contraction (ko04270), the phosphatidylinositol signaling system (ko04070), the oxytocin signaling pathway (ko04921), the oocyte meiosis pathway (ko04114), glutamatergic synapse (ko04724), dopaminergic synapse (ko04728), serotonergic synapse (ko04726), neuroactive ligand-receptor interaction (ko04080), calcium signaling pathway (ko04020), the TGF-β signaling pathway (ko04350), the Notch signaling pathway (ko04330), the MAPK signaling pathway (ko04011), the AMPK signaling pathway (ko04152), and the longevity regulating pathway (ko04212) (Fig. 4).

Fig. 3: Annotation of differentially expressed genes between male and female *A. cantonensis* and qPCR analysis of related genes. (a). Overview of GO annotation of DEGs. (b). Enriched KEGG annotation of DEGs.
Discussion

By using a high-throughput sequencing system, this study provided a comprehensive transcriptome-wide survey of male and female adult *A. cantonensis*. Transcriptome differences between the 2 genders are primarily related to metabolism, production, and other factors. A number of pathways may regulate the metabolism and production of the adult worm, and these include neural-related pathways, the calcium signaling pathway, the TGF-β signaling pathway, the Notch signaling pathway, and the MAPK cascade.

GO annotation of DEGs revealed that genes involved in metabolic and biosynthetic processes and the regulation of biological processes were more active in female adults. Enriched GO annotation of DEGs also identified that protein phosphorylation (GO:0006468), regulation of protein phosphorylation (GO: 0001932), and positive regulation of protein phosphorylation (GO: 0001934) were upregulated in females. KEGG analysis indicated that the pentose phosphate pathway (ko00030), glutathione metabolism (ko00480), nitrogen metabolism (ko00910), lysine degradation (ko00310), the oxytocin signaling pathway (ko04921), and the oocyte meiosis pathway (ko04114) were also highly expressed. The former four pathways may be related to metabolism, and the latter pathways may be related to the production of eggs.

These results suggest that female adult worms require abundant nutrition to produce thousands of eggs per worm pair each day. This transcriptomic finding was consistent with those for *C. elegans*, *S. mansoni*, and *S. japonicum* (8-10).

In free-living female worms (*C. elegans*), DEGs between males and females were enriched in functions that are characteristic of maternal developmental processes such as reproduction, embryo development, gene regulation, and metabolism (8-10). The human parasites *S. japonicum* and *S. mansoni* always ex-
hibit higher expression of genes involved in transcriptional regulation, mRNA splicing, DNA repair, germ cell proliferation, and cell cycle regulation. These genes influence ribosome function, chromosome processes, oocyte maturation, cell cycle regulation, transcription, translation, DNA repair, primary metabolic process, and folic acid binding and are elevated in comparison to those in male worms (8, 9, 11, 12). This phenomenon indicates that female adult worms require abundant material for egg production.

For the male transcripts, muscle formation and movement regulation were enriched in *S. japonicum*, *S. mansoni*, *S. mekongi*, and *Dirofilaria immitis* a filarial nematode (9, 13-18). Here *A. c*. exhibited similar characteristics according to GO enriched annotation of DEGs. These characteristics included enrichment for skeletal system development (GO:0001501) and microtubule cytoskeleton organization (GO:0000226). Additionally, KEGG enriched annotation of DEGs revealed enrichment for vascular smooth muscle contraction (ko04270), and the phosphatidylinositol signaling system (ko04070), indicating that the adult male *A. c.* may express genes related to muscle activity.

We also observed that neurotransmitters and neural-related pathways were enriched for DEGs between adult males and females, and these included glutamatergic synapses (ko04724), dopaminergic synapses (ko04728), serotonergic synapses (ko04726), and neuroactive ligand-receptor interactions (ko04080). Although previous studies have shown that neuropeptidergic signaling is essential for worm locomotion, feeding, host-finding, regeneration, and reproduction (19, 20), other research highlighted a role for neuropeptides in planarian germline development (8) and identified the susceptibility of developing *C. elegans* to anoxia (21) and the pairing of male and female worms (22). Knowledge regarding the mechanism and function of neurotransmitters and neural-related pathways in worm physiology is lacking. We theorized that *A. c.* DEGs enriched in neural-related pathways may reflect different physiological activities regulated by these pathways, including sperm production, egg production, and pairing.

In both vertebrate and worm cells, Ca²⁺ acts as a ubiquitous second messenger that plays an essential role in physiological processes such as neuronal secretion, muscle contraction, cell proliferation or differentiation, and aging of organisms (23, 24). Ca²⁺ also provides a molecular basis for *Caenorhabditis elegans* to sense mechanical stress and facilitates temperature signaling that modulates thermotaxis and cold tolerance (25) (26). Dysfunction or dysregulation of Ca²⁺ signaling leads to a number of neurologic disorders. The calcium hypothesis of neurodegeneration states that dysregulation of Ca²⁺ signaling greatly affects the development of neurodegenerative processes. Previous studies have shown that TGF-β and Notch signaling pathways can modify Ca²⁺ channels in the *Caenorhabditis elegans* strain CB55 that carries a truncating mutation in the unc-2 gene. This worm serves as an ortholog for human neurologic disorders (27).

In this study, the calcium-signaling pathway (ko04020) was enriched in DEGs between male and female *A. c.*, suggesting that it may be related to neuronal activity. Additionally, the TGF-β signaling pathway (ko04350), Notch signaling pathway (ko04330), MAPK cascade (GO:000165), MAPK signaling pathway (ko04011), and AMPK signaling pathway (ko04152) were all enriched according to either GO enrichment annotation or KEGG enrichment annotation of DEGs between male and female *A. c.*

In *C. elegans* and *Drosophila melanogaster*, TGF-β-related proteins balance germ cell proliferation and differentiation (28, 29). The Notch pathway in *Schistosoma Mansoni* is involved in parasite oogenesis and embryogenesis (30). As an evolutionarily conserved energy sensor, AMPK is involved in the regulation of energy metabolism in many organisms, including pigs (*Sus scrofa*), mice (*Mus musculus*), flies (*Drosophila*).
In conclusion, males and females differ in body size and structure to allow for their specific physiological needs. Male adults must produce sperm, while female adults must produce eggs and lay zygotes. These activities are all regulated by neurons through different pathways, and the Ca\(^{2+}\) - related pathway might play a key role directly or indirectly by either coupling to or combing with other pathways. For instance, the Ca \((^{2+}\)\)-dependent activator protein specific for secretion can regulate neurotransmission at the neuromuscular junction in *C. elegans* (33, 34).

**Conclusion**

In this study, we provided a detailed overview of the transcriptome of adult male and female of *A. cantonensis*. Although this study aimed to reveal the transcriptome difference between the 2 genders and a number of DEGs were obtained through this analysis, further studies are required to provide more details.

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**Conflict of interest**

The authors have no competing interests with the publication of this work.

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