Developmental Proteomics Reveals the Dynamic Expression Profile of Global Proteins of *Haemaphysalis Longicornis* (Parthenogenesis)

Wenge Liu

Lanzhou Veterinary Research Institute

Jin Luo

Lanzhou Veterinary Research Institute

Qiaoyun Ren

Lanzhou Veterinary Research Institute

Qilin Wang

Lanzhou Veterinary Research Institute

Jing Li

Animal Disease Prevention and Control Center of Qinghai Province

Peiwen Diao

Lanzhou Veterinary Research Institute

Runlai Cao

Lanzhou Veterinary Research Institute

Gaofeng Zhang

Lanzhou Veterinary Research Institute

Wenwei Luo

Lanzhou Veterinary Research Institute

Peiwen Liu

Lanzhou Veterinary Research Institute

Wen Li

Hami animal husbandry workstation, Xinjiang Uygur Autonomous Region

Guiquan Guan

Lanzhou Veterinary Research Institute

Jianxun Luo

Lanzhou Veterinary Research Institute

Hong Yin

Lanzhou Veterinary Research Institute

Hui Wang

Institute for Biomedical Engineering

Li Xiang

Wensu animal workstation, Xinjiang Uygur Autonomous Region

Guangyuan Liu (✉ liuguangyuan2002@sina.com)

Lanzhou Veterinary Research Institute

Research

Keywords: Haemaphysalis longicornis, proteomic, development, iTRAQ, differential protein

Posted Date: January 4th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1213265/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background: Ticks are important parasites that cause more diseases than most other animal parasites. *Haemaphysalis longicornis* is used as an experimental animal model for the study of three-host ticks due to its special life cycle and easy maintenance in the laboratory and in its reproduction. The life cycle of *H. longicornis* goes through a tightly regulated life cycle to adapt to the changing host and environment, and these stages of transition are also accompanied by proteome changes in the body.

Methods: In this study, the aim was to use the isobaric tags for relative and absolute quantification (iTRAQ) technique to systematically describe and analyze the dynamic expression of protein and the molecular basis of the proteome of *H. longicornis* in seven differential developmental stages (eggs, unfed larvae, fed larvae, unfed nymphs, fed nymphs unfed adults, and fed adults).

Results: A total of 2,059 proteins were identified, and their expression profiles were classified at different developmental stages. In addition, it was found that tissue and organ development-related proteins and metabolism-related proteins showed that they were involved in different physiological processes throughout the life cycle through the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the differentially expressed proteins (DEPs). More importantly, we found that the upregulated proteins of fed adult ticks were mainly related to yolk absorption, degradation, and ovarian development-related proteins. The abundance of the cuticle proteins in the unfed stages were significantly higher compared with those of the fed ticks in the previous stages.

Conclusions: In short, the protein spectrum changes identified in this study provide a reference proteome for future studies of tick functional proteins and provide candidate targets for elucidating tick development and developing new tick control strategies.

Introduction

Ticks are globally-distributed, blood-sucking ectoparasites that are related to tick-borne diseases. The impact of tick-borne diseases is staggering, especially in developing countries, where tick-borne diseases affect about 80% of the world's cattle, with economic losses estimated to be between US \$13.9 billion and US \$18.7 billion [1, 2].

Haemaphysalis longicornis is an important species of ticks, with a four stage life cycle: egg, larva, nymph, and adult [3]. In particular, larva, nymph, and adult need to be parasitic on the host, sucking blood to obtain nutrition and energy reserves, and then undergo molting as they proceed to the next stage or lay eggs. In the life cycle of *H. longicornis*, which spans for about four months, the body shape of *H. longicornis* will change greatly as well as many organs and tissues, which are all accompanied by the rise and fall of gene expression in *H. longicornis* [4]. The differential expression of these proteins in specific periods provides the molecular basis and condition guarantee for the maintenance and body changes of *H. longicornis* in a specific period.

In previous studies, two-dimensional electrophoresis (2-DE) construction, time-of-flight mass spectrometry (MALDI-TOF-MS), and two-dimensional differential gel electrophoresis (2D-DIGE) combined with MALDI-TOF-MS, have been used in tick studies. In addition, a lot of proteins in the tick midgut, salivary gland, and ovary and a variety of tick-related proteins have been successfully identified [5–7].

The isobaric tags for relative and absolute quantification (iTRAQ) technology is an advanced and accurate protein sequencing technology, which can determine the protein components in a sample more accurately and comprehensively as compared to traditional methods. Precise mass spectrometry detection method is an important prerequisite for comparative proteomics analysis, and as a quantitative proteomics method, iTRAQ can generate information about the abundance of hundreds of proteins at a time. In addition, it allows repeated multiplexing of parallel biology or techniques (eight iTRAQ markers) in a liquid chromatography with tandem mass spectrometry (LC-MS)/MS experiment, thus overcoming the difference between measurements in a single MS-based shot bullet spectrum analysis [8–10].

At present, iTRAQ has been successfully applied to the study of differentially expressed proteins (DEPs) in the salivary glands of *H. longicornis*. Researchers found that the expression of some proteins related to energy production was continuously down-regulated during salivary gland degeneration, while some proteins related to DNA or protein degradation were up-regulated through this method. In addition, the expression of some proteins related to apoptosis or autophagy also changed [11, 12].

However, there is still no study that confirms and analyzes the overall protein expression pattern of *H. longicornis* at the whole developmental stages. Therefore, the purpose of this study was to use eight-fold iTRAQ markers and LC-MS/MS to further understand the functional differences between different stages of the life cycle of *H. longicornis*, more importantly, to identify and quantify the protein content in these stages, and to find out the key proteins in the development of *H. longicornis*. This study not only provides a comprehensive and reliable data platform for tick prevention and control but also provides more potential targets for the development of tick vaccine.

Experimental Procedures

Rabbits and parasites

The clean New Zealand white rabbit was obtained from the Experimental Animal Center of the Lanzhou Veterinary Research Institute. The *H. longicornis* isolate used was the monoclonal strain of clean *H. longicornis*, which was cultivated and preserved by the Lanzhou Veterinary Research Institute [13].

The collection of *H. longicornis* at different developmental stages

Referring to the method in the previously described study [13], ticks in different starvation stages (unfed larvae, unfed nymphs, and unfed adults) were bitten on clean New Zealand white rabbits until they were engorged. Afterwards, the ticks were collected into breathable glass tubes and placed in an incubator with a humidity of 80% and a temperature of 27 °C, waiting for them to enter the next life stage-molting or spawning.

In short, seven different developmental stages of *H. longicornis* (parthenogenesis) were obtained and washed in bromogeramine, alcohol, and deionized water successively. After that, the samples were placed in an Eppendorf tube in triplicate and preserved at −80°C.

Protein extraction

Protein extraction was performed according to Wang's research and made some adjustments [11]. As follow, about 2 g from each sample was put into the 1.5-mL centrifuge tube, and a 5-mm magnetic bead and an appropriate amount of Lysis Buffer 3 were added, respectively. Then, ethylenediaminetetraacetic acid (EDTA) was added, the sample with a final concentration of 1 mM was vortexed, and the tube was allowed to rest for 5 min. Afterwards, dithiothreitol (DTT) was added, for a final concentration of 10 mM, and was shaken with a tissue grinder for 2 min (power, 50 Hz; time, 120s). The supernatant was centrifuged at 25,000 × g at 4 °C for 20 min, and the final concentration was 10 mM DTT. The tube was then placed in a 56-°C water bath for 1 h. Then, iodoacetamide (IAM) was added to a final concentration of 55 mM at room temperature and was allowed to stand for 45 min. Acetone, at a volume four times that of the IAM, was added, and the solution was allowed to stand at −20 °C for 2 h. The steps were repeated three times until the supernatant was colorless. Then, the solution was centrifuged at 25,000 × g and 4 °C for 20 min, and the supernatant was discarded. Afterwards, a 5-mm magnetic bead was added as well as a proper amount of Lysis Buffer 3 to allow the solution to precipitate. Then, the solution was shook for 2 min with a tissue grinder (power, 50 Hz; time, 120 s), centrifuged at 25,000 × g 4 °C for 20 min, and the supernatant was collected for quantitative analysis.

Proteolysis and peptide labeling

Protein solution (100 µg) was taken from each of the seven samples, then 2.5 µg of the trypsin enzyme was added at a protein to enzyme ratio of 40: 1, and the solution was hydrolyzed at 37 °C for 4 h. Then, trypsin was added according to the above ratio, continuing the enzymatic hydrolysis at 37 °C for 8 h, and then desalinating the hydrolyzed peptides using a Strata X column and vacuum dried. According to the number of samples, a certain amount of iTRAQ labeling reagent was taken out. After the reagent was restored to room temperature, 50 µL of isopropanol was added to each tube. After vortex shock, the peptide sample was dissolved with 0.5 M tetraethylammonium bromide (TEAB) and added to the corresponding iTRAQ labeling reagent. Different sample peptides were labeled with different iTRAQ tags, and the solution was allowed to stand at room temperature for 2 h.

LC-MS/MS analysis

The sample was separated by a Shimadzu LC-20AB liquid phase system with a 5-µm, 4.6 x 250-mm Gemini C18 column. The dried peptide sample was redissolved and injected with 2 mL of mobile phase A (5% ACN pH 9.8) and eluted at a flow rate gradient of 1 mL/min: 5% mobile phase B (95% acetonitrile [can], pH 9.8) for 10 min, 5–35% mobile phase B for 40 min, 35–95% mobile phase B for 1 min, mobile phase B for 3 min, and 5% mobile phase B for 10 min. The elution peak was monitored at a 214-nm wavelength, and one component was collected every minute. Combined with the chromatographic elution peak map, 20 components were obtained, and then freeze-dried.

The dried peptide sample was re-dissolved with mobile phase A (2% ACN, 0.1% FA), centrifuged at 20,000 × g for 10 min, and the supernatant was injected. The separation was carried out by the nanoliter liquid chromatograph of Shimadzu Company LC-20AD. The sample was first enriched and desalted in a trap column, and then connected in series with a self-assembled C18 column (75-µm inner diameter, 3.6-µm column size, 15-cm column length). The separation was carried out at a flow rate of 300 nL/min through the following effective gradient: 0–8 min, 5% mobile phase B (98% ACN, 0.1% formic acid [FA]); 8–43 min, mobile phase B increased linearly from 8–35%; and 43–48 min, mobile phase B increased from 35–60%. In 48–50 min, the mobile phase B rose from 60–80%; in 50–55 min, the mobile phase B rose to 80%, the mobile phase B, 55-65 minutes, and 5%, the mobile phase B. The end of the nanoliter liquid phase separation was directly connected to the mass spectrometer.

The peptides separated by liquid phase were ionized by a nanoESI source, and then entered into a tandem mass spectrometer Q-Exactive HF (Thermo Fisher Scientific, San Jose, CA) for data-dependent acquisition (DDA) mode detection. The main parameters were as follows: the ion source voltage was set to 1.6 kV; the scanning range of the first-stage mass spectrometry was 350~1,600 m/z; the resolution was set to 60,000 m; the initial m-stroke z of the second-stage mass spectrometry was fixed to 100; and the resolution was 15,000. The screening conditions for the parent ions of the secondary fragmentation were as follows: the charge was 2+ to 6+, and the parent ions with peak intensity of more than 10,000 were in the top 30. The ion fragmentation mode was higher-energy C-trap dissociation (HCD), and fragment ions were detected in Orbitrap. The dynamic exclusion time was set to 30 s, and the automatic gain control (AGC) was set to first-level 3E6 and second-level 1E5.

Database search and bioinformatics analysis

The original MS data was converted into a general format (.mgf) file with Proteome Discoverer 1.4, and then the data file was used to query the tick-related database. ProteinPilot protein software 4.5 (AB SCIEX) was used for further identification and quantification of proteins. In order to filter the results, we used an error detection rate of less than 0.01 for the identification, and for the quantification, a confidence level of 95% or an unused confidence score greater than 1.3a was used. For the DEPs, values | log²-fold change | > 1 were regarded as up-regulated or down-regulated proteins, respectively.

Functional classification of the DEPs was carried out according to GO annotation and <http://www.geneontology.org> analysis. The DEPs were divided into three categories, namely, molecular function, biological processes, and cellular components. The KEGG (<http://www.kegg.jp/kegg/>) was used to predict the molecular functions, biological processes, and important DEP pathways.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Six proteins (*CRK*, *flotillin*, *Mo-25*, *dystrophin*, *septin-1*, and *septin-2*) were randomly selected from the protein library built by iTRAQ, and these genes were compared at the transcriptional level by RT-qPCR. The first cDNA strand was synthesized using the total RNA from the samples from seven stages (egg, unfed larva, fed larva, unfed nymph, unfed adult, and unfed adult) using the PrimeScript Reverse Transcriptase kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. The RT-qPCR was carried out in accordance with the methods described earlier and relative transcript levels were calculated using the 2^{ΔΔCt} method [13]. The information on the primers of the selected gene and the reference gene beta-actin is shown in Table 1.

Table 1
Primer of the six genes for quantitative reverse transcription polymerase chain reaction

Gene	Primer name	Primer sequence (5'-3')
<i>Flotillin</i>	<i>Flotillin F</i>	CCATCAAGGACATCAGCGAT
<i>Mo-25</i>	<i>Flotillin R</i>	TATGCAGCCTTCTTGAGCTC
<i>Dystrophin</i>	<i>Mo-25 F</i>	GCACACGTTACCAACAGTA
<i>CRK</i>	<i>Mo-25 R</i>	ACAAGATCGCAATCCTCCTG
	<i>Dystrophin F</i>	AACGTGCACGTGTCCATGC
	<i>Dystrophin R</i>	GAGGTTCTGCAGGAGGTTGA
	<i>CRK F</i>	AGAACGAAGTCGGCGTCTTT
<i>Septin-1</i>	<i>CRK R</i>	ATGTCGGGGAACATCTGGT
<i>Septin-2</i>	<i>Septin-1 F</i>	TGTTATCAGCATGGTCCTGA
	<i>Septin-1 R</i>	AAGCAGCTCAAGGAGTCCGT
	<i>Septin-2 F</i>	AACAGCTTGTCAGCAAATC
	<i>Septin-2 R</i>	GACGTTGCCCTCTTGCAATT
<i>β-actin</i>	<i>β-actin F</i>	CGTTCCTGGGTATGGAATCG
	<i>β-actin R</i>	TCCACGTCGCACTTCATGAT

Statistical Analysis

All statistical data were processed with GraphPad Prism software version 7 (GraphPad, CA, USA), and the data were analyzed by Student's t-test. $P < 0.05$ was considered statistically significant.

Results And Discussion

The development of *H. longicornis* is accompanied by many changes in the host, such as sucking blood, molting, and spawning. In the whole process, the occurrence, development, and degeneration of tissues and organs are also accompanied by changes in the protein profile. The lack of detailed and comprehensive protein dynamic spectrum during the development of *H. longicornis* limits the research of development of *H. longicornis*, the screening of drug resistance genes, and the development of anti-tick drugs and other related studies. The iTRAQ quantitative proteomics is a powerful and novel tool for characterizing protein changes among different samples, which has been well verified in a variety of organisms, and helps people to analyze the protein profiles of many organisms under different conditions and different developmental stages [14–16]. Therefore, in this experiment, in order to clearly understand the differences in the physiological functions of *H. longicornis* at different developmental stages, especially, in order to identify, quantify, and compare the protein expression profiles of *H. longicornis* at different developmental stages, we used iTRAQ quantitative proteomics technology to analyze the related functions of *H. longicornis* proteins at different developmental stages.

Overview of main data and protein identification

In this study, iTRAQ was used to identify the proteomic at different stages of the life cycle of *H. longicornis*; that is, egg, unfed larvae, fed larvae, unfed nymph, fed nymph, unfed adult, and fed adult. In the three repeated experiments, a total of 2,056 proteins were identified from 4,405 peptides, which were matched with 2,608,862 spectra at a false discovery rate of 1% (Table 2). As shown in Figure 1A, the number of proteins identified in the three repeated experiments was 1,325; 1,333; and 1,504, respectively, while the 812 proteins were identified to be shared in the three repeats. Most of the proteins were identified by one peptide; specifically, 939.7 ± 45.28 (45.71%) proteins were identified based on one peptide. More than 218.7 ± 8.74 (10.64%) proteins were identified based on two peptides, 87 ± 4.35 (4.25%) proteins were identified based on three peptides, and about 810.5 ± 24.35 (39.45%) proteins were identified by more than three peptides (Figure 1B).

Table 2 Spectra, peptide, and protein identified by the isobaric tags for relative and absolute quantification (iTRAQ)						
	Total-spectra	Spectra	Unique-spectra	Peptide	Unique-peptide	Protein-identified
Repeat 1	839,950	7,932	7,109	2,769	2,663	1,325
Repeat 2	829,948	8,019	7,163	2,781	2,673	1,333
Repeat 3	938,964	8,951	7,961	2,939	2,844	1,504
Total	2,608,862	24,902	22,233	4,405	4,295	2,056

The sequence coverage of a specifically identified protein is estimated as the percentage of matching amino acids between the identified peptides with more than 95% confidence divided by the total number of amino acids in the protein sequence. The sequence coverage of 473.7 ± 38.35 (37.47%) proteins was less than 0–10%, while the sequence coverage of 393 ± 15.57 (23.15%) proteins was 10–20%. Moreover, 825 ± 15.57 (40.15%) proteins were determined to have a sequence coverage of more than 40%. The MS data has been deposited in iProX (Integrated Proteome Resources, <http://www.iprox.org/>) under the main accession number OMIX707 (Figure 1C).

Protein quantification

The *H. longicornis* tick has a complicated life cycle, and the molecular basis of its growth and development is still poorly understood. In order to study the protein profile during its growth process, we have obtained samples from seven differential development stages (egg, unfed larvae, fed larvae, unfed nymph, fed nymph, unfed adult, and fed adult). A proteomic analysis was performed, and the proteins with $|\log^2\text{-fold change}| > 1$ and $P < 0.05$ were considered DEPs. A pairwise comparison of the proteins of each successive stage was conducted, and it was found that compared with those of eggs, the unfed larvae had 124 up-regulated proteins and 83 down-regulated proteins; compared with those of hungry young ticks, there were 94 up-regulated proteins and 79 down-regulated proteins in the fed larvae; compared with the those of fed larvae, the unfed nymph had 88 up-regulated proteins and 89 down-regulated proteins; compared with those of unfed nymph, there were 86 up-regulated proteins in the ticks and 101 down-regulated proteins in the fed nymph; compared with those of fed nymph, there were 99 up-regulated proteins and 88 down-regulated proteins in the unfed adult; and compared with those of unfed adult, there were 70 up-regulated proteins and 87 down-regulated proteins in the fed adult. Figure 2 shows the number of DEP at different developmental stages.

RT-qPCR analysis

The transcriptional levels of six genes (*CRK*, *flotillin*, *Mo-25*, *dystrophin*, *septin-1*, and *septin-2*) were examined at seven stages. Through the analysis of the results, we found that the change of their transcriptional levels was not consistent with the trend of protein levels in iTRAQ at different developmental stages (Table 3 and Fig. 3).

Table 3
The protein expression ratio of the six genes

Protein	ID	Mean_Ratio	Mean_Ratio	Mean_Ratio	Mean_Ratio	Mean_Ratio	Mean_Ratio
		HLUL-VS-HLEE	HLFL-VS-HLUL	HLUN-VS-HLFL	HLFN-VS-HLUN	HLUA-VS-HLFN	HLFA-VS-HLUA
Septin-2	Cluster-28249.0	1.62	1.55	0.65	1.6	0.57	2.1
Flotillin	tr B7QHS0 B7QHS0_IXOSC	2.43721658	0.46803437	1.37488235	0.73256603	1.04877345	0.97843442
dystrophin	tr B7P346 B7P346_IXOSC	0.71111262	1.25660552	0.85444462	0.88173327	1.00682484	3.54506418
Septin-1	tr B7PN20 B7PN20_IXOSC	0.69083567	2.00483335	0.46407305	2.19979251	0.4197441	2.42521246
protein Mo25	tr B7PG66 B7PG66_IXOSC	1.25887208	1.27575866	0.68020405	1.10685157	0.90366207	0.95552281
CRK	tr B7Q2R0 B7Q2R0_IXOSC	0.88433373	1.47140064	0.57627263	1.80638402	0.7186571	1.56348104
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

The reason for this result is mainly attributed to the fact that RNA level is only a moderate proxy for protein abundance and does not fully represent protein expression abundance. These results highlight that it is necessary to analyze the differentiation mechanism components of *H. longicornis* at the protein level, which are involved in basic biological processes such as signal transduction, substance transport, catalytic activity, metabolism, and so on.

Expression profile of the identified proteins

Chitin-binding proteins

In this experiment, three chitin binding proteins (Cluster-30738.173199, Cluster-30738.190566, and Cluster-30738.187492) were identified. Among them, the two peritrophic membrane chitin-binding proteins shared the same expression trend; that is, they were decreased in the process of egg hatching into unfed larva, and then increased significantly with blood sucking. On the other hand, in the process of entering the next stage after blood sucking, it showed a significant downward trend, and then reached a peak in the fed adult (Table 4).

Table 4
Chitin-binding proteins

Protein	ID	Mean_Ratio	Mean_Ratio	Mean_Ratio	Mean_Ratio	Mean_Ratio	Mean_Ratio
		HLUL-VS-HLEE	HLFL-VS-HLUL	HLUN-VS-HLFL	HLFN-VS-HLUN	HLUA-VS-HLFN	HLFA-VS-HLUA
Peritrophic membrane chitin-binding protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.190566	0.58	2.69	0.52	2.07	0.3	3.4
Chitin-binding peritrophin-A, putative [<i>Ixodes scapularis</i>]	Cluster-30738.187492	1.13	0.43	1.2	0.8	0.66	1.62
Peritrophic membrane chitin-binding protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.77125	1.19	1.71	0.65	2.85	0.23	2.41
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

The peritrophic membrane (PM) is an important organ of blood-sucking arthropods, which provides protection for the microvilli of digestive tract epithelial cells and as a sturdy barrier to protect the intestinal tract from physical damage caused by the structure of food intake and the invasion of parasites and other pathogens [17]. Moreover, many studies have used histochemical and biochemical techniques to show the presence of chitin on the perineal membrane [18, 19]. A previous study found that the PM of *H. longicornis* was significantly different

between the larvae and the adult stages, and the presence of PM chitin-binding proteins was observed [20]. Similarity, in this study, we identified two kinds of PM chitin-binding proteins, both of which showed the same upward trend in the process of blood intake, which is consistent with previous studies, and can also explain their protective role in the process of blood uptake.

Digestion-related proteins

The digestion of blood provides energy and nutrients for maintaining the growth and metabolism of ticks, which is a complex process, requiring the cooperation of a variety of proteins to process and deal with the hemoglobin ingested, and then into their own nutrients [21]. In this study, a variety of proteins related to digestion were found, which would help to use dynamic strategies to explain and clarify the blood digestion process during the development of *H. longicornis*.

The differences in the expression of proteins related to digestion were analyzed in the different stages in the experiment. Interestingly, we found four trypsin proteins (Cluster-30738.179855, Cluster-30738.158249, Cluster-30738.86970, and Cluster-30738.127284). They increased significantly from unfed nymph to fed nymph as well as in unfed adult to fed adult. In addition, we also found three carboxypeptidases proteins (Cluster-30738.164810, Cluster-30738.108012, and Cluster-30738.136271). The expression of Cluster-30738.164810 showed low abundance in both eggs and larva stages, and then increased rapidly from unfed nymph and lasted until fed adult. In addition, the expression abundance of two proteins (Cluster-30738.108012 and Cluster-30738.136271) increased from eggs to the larva stage. After that, they showed upward trends from unfed stages to corresponding fed stages. Leucine aminopeptidase (Cluster-30738.169581) increased from eggs to the unfed larva stage, then began to decrease gradually and decreased to the lowest level until unfed adults (Table 5).

Table 5
Digestion-related proteins

Protein	ID	Mean_Ratio HLUL-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
Trypsin	Cluster-30738.179855	1.2	1.51	0.9	1.03	1.02	1.14
Trypsin-3	Cluster-30738.158249	1.21	0.84	0.62	1.69	0.78	1.54
trypsin-1	Cluster-30738.86970	0.51	0	0.43	4.41	0.48	2.03
Trypsin2	Cluster-30738.127284	0.77	0	0.63	1.37	0.52	2.58
Carboxypeptidas	Cluster-30738.164810	0.38	0.1	3.95	0.45	2.2	2.44
Serine carboxypeptidase	Cluster-30738.108012	1.14	1.76	0.75	1.22	0.93	1.15
Serine carboxypeptidase 2	Cluster-30738.136271	1.19	1.61	0.81	1.26	1.14	0.66
Leucine aminopeptidase	Cluster-30738.169581	1.49	1.28	1.25	0.96	0.94	0.66
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

Vitellogenin proteins

During the development of ticks, vitellogenin (Vg) is synthesized as a high-molecular weight precursor in body fat, gut, and ovary. After that, the Vg is released into the hemolymph and absorbed and accumulated by oocytes through receptor-mediated endocytosis. At this time, it is named Vt, which is an important source of nutrients for embryonic development [22, 23]. In this study, six vitellogenin proteins were identified: Vg1, Vg2, Vg3, Vg4, Vg5, and Vg6 (Cluster-30738.183992, Cluster-30738.173105, Cluster-30738.197239, Cluster-30738.175424, Cluster-30738.173278, and Cluster-30738.195117). Among them, Vg2, Vg3, and Vg6 showed the same expression pattern. The expression abundance of these Vg proteins increased significantly from egg hatching to unfed larva but began to decrease during the development of unfed larva to fed larva and increased again during molting into unfed nymph. Then, after sucking blood to the fed nymph stage, the content decreased again. While molting into the unfed adult stage, their content increased again, and then declined again after the last bloodsucking into the engorged adult stage. However, the expression abundance of Vg1 increased significantly from unfed nymph to fed nymph and from

unfed adults to fed adults. The results of the Vg4 and Vg5 showed that the expression abundance increased significantly from unfed larva to fed larva and from unfed adults to fed adults (Table 6).

Table 6
Vitellogenin (Vg)-related proteins

Protein	ID	Mean_Ratio HLUL-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
Vitellogenin-1	Cluster-30738.173246	0.52	0.44	0.66	1.46	0.66	1.99
Vitellogenin-2	Cluster-30738.151517	1.57	0.73	2.93	0.39	2.32	0.44
Vitellogenin-3	Cluster-30738.197239	1.25	0.87	1.53	0.67	1.3	0.98
Vitellogenin-4		/	1.57	1.06	0.92	1.3	1.14
Vitellogenin-5		/	1.95	1.66	0.73	0.96	2.01
Vitellogenin-6	Cluster-30738.195117	1.43	0.97	1.7	0.66	1.47	0.51
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

As early as 2010, scientists successfully annotated Vg1, Vg2, and Vg3 in *H. longicornis* and identified the protein size of these three Vgs. Also, they observed a rapid increase in Vg2 and Vg3 transcription levels in the body fat on the second day in feeding, a significant increase in Vg1 transcription in the midgut on the fourth day, and an increase in the mRNA expression of Vg2 in the ovary from the fourth day in feeding. To explore their role in the development of *H. longicornis*, through RNAi technology, it was found that the knockdown of Vgs could significantly affect the full blood weight of ticks in field teaching, and Vgs are necessary for egg weight and oviposition [23].

In 2018, scientists explored the ovariogenesis of *Boophilus microplus* and identified seven Vt peptides, which are the corresponding products of five different Vgs (Vg1, Vg2, Vg3, Vg4, and Vg5). They were observed to increase during the feeding phase, and most of which increased rapidly at the end of blood feeding [24].

In this study, it was found that the six Vgs showed different expression patterns in the different developmental stages of *H. longicornis*, suggesting that they may play different roles in different tissues and physiological processes, which needs to be further explored in the future.

Cuticle proteins

The cuticle of ticks is an important defense tissue, which can resist bad weather and other physical injuries, *H. longicornis* need undergo two molts in its lifetime, during which a lot of cuticle-related proteins undergo change [25]. In the blood-sucking process of ticks, the cuticle protein begins to increase, while in the molting process, the old epidermal protein will be absorbed, and the content will decrease; at the same time, it will gradually synthesize new cuticle proteins until the end of molting. In Liu's paper, the cuticle protein CPR1 is involved in the molting process of *H. longicornis* and is regulated by miRNA [13].

Thirteen cuticle proteins were found in this study. From the comparison of seven different developmental stages, the expression of these cuticle proteins showed two expression patterns: the first pattern, Cluster-30738.125201, Cluster-30738.137608, Cluster-30738.103058, Cluster-30738.134573, Cluster-167128.2, and Cluster-30738.125201 began to increase significantly in the process of hatching from eggs to larva. After being engorged, it showed a downward trend, and then showed an upward trend in the process of developing to the next stage of unfed. After that, this wavy mode of expression continued until fed adult. Meanwhile, the other class of cuticle had seven members, namely, Cluster-699.0, Cluster-30738.145384, Cluster-30738.187998, Cluster-30738.172572, Cluster-30738.167454, Cluster-30738.145385, and Cluster-30738.165925, which showed a downward trend in the process of egg development to unfed larva. Then, in the subsequent development process, it showed a significant upward trend from unfed stage to the corresponding fed stage and a significant downward trend in the process from the fed stage to the next unfed stage (Table 7) .

Table 7
Cuticle-related proteins

Protein	ID	Mean_Ratio HLUL-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.125201	4.29	0.55	2.44	0.45	3.2	0.37
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.137608	1.22	1.06	1.29	0.88	1.51	0.96
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.103058	2.16	0.64	2.65	0.34	2.05	0.57
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.134573	3.23	0.88	2.01	0.49	1.97	0.45
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-699.0	0.81	1.32	0.85	2.33	0.43	6.79
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.145384	0.59	9.2	0.21	4.2	0.16	2.11
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.187998	0.63	1.55	1.28	1.44	0.68	9.31
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.172572	0.76	1.58	0.58	1.86	0.67	10
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-167128.2	1.16	0.82	1.37	0.71	2.16	0.49
Cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.167454	0.83	4.28	0.41	2.88	0.29	2.3
cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.145385	0.5	7.85	0.22	5.51	0.17	1.61
cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.165925	1.34	2.82	0.43	1.88	0.47	1.11
cuticle protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.125201	4.29	0.55	2.44	0.45	1.14	0.37
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

In this study, cuticle-associated proteins showed different expression patterns—one part showed an upward trend in the satiety stage, and the other showed a downward trend. This opposite expression trends implies that there may be great differences in the structure and function of the cuticle proteins, which need to be further analyzed in terms of their protein structure, family classification, and related functional studies.

Membrane proteins

Biological process is a circular network, and membrane protein is an important hub in the network, which plays an important physiological role in organisms, such as cell proliferation and differentiation, energy conversion, signal transduction, and material transport. In addition, most drugs also achieve a therapeutic effect by interacting with membrane proteins [26].

A total of 12 membrane proteins were found in this study, which were divided into three patterns by the expression patterns in different developmental stages in the *H. longicornis*: the expression of the first class, Cluster-30738.172187, was relatively steady at different developmental stages, and there was no obvious stage specificity. The second class, Cluster-30738.179201, Cluster-30738.171068, Cluster-30738.174970, Cluster-30738.209558, Cluster-30738.172113, and Cluster-30738.171857, showed a significant upward trend from egg development to unfed larva, and then decreased significantly from unfed stage to the corresponding fed stage, while its expression abundance increased significantly from the fed stage to the next unfed stage. The third class, Cluster-30738.179418, Cluster-30738.180943, Cluster-30738.180943, Cluster-30738.77125, and Cluster-30738.172989, had a common pattern, and its expression abundance increased significantly from the unfed stage to the corresponding fed stage and reached a peak at the fed adult (Table 8).

Table 8
Membrane proteins

Protein	ID	Mean_Ratio HLEE-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
Peritrophic membrane chitin-binding protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.190566	0.58	2.69	0.52	2.07	0.3	3.4
Integral membrane protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.172187	0.85	0.97	1.03	1.04	1.15	0.99
PREDICTED: mitochondrial import inner membrane translocase subunit TIM14-like isoform X2 [<i>Octopus bimaculoides</i>]	Cluster-30738.179418	0.79	1.4	0.74	1.38	0.72	1.57
TPA_inf: membrane protein [<i>Amblyomma variegatum</i>]	Cluster-30738.179201	1.54	0.93	1.29	0.92	1.29	2.22
Basement membrane-specific heparan sulfate proteoglycan core protein, partial [<i>Stegodyphus mimosarum</i>]	Cluster-30738.171068	1.31	1.43	1.15	0.84	1.49	0.93
antigen B membrane protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.180943	0.74	4.41	0.42	3.39	0.26	2.09
membrane protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.174970	1.64	0.78	1.69	0.68	1.38	0.71
antigen B membrane protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.180943	0.74	4.41	0.42	3.39	0.26	2.09
PREDICTED: plasma membrane calcium-transporting ATPase 2-like, partial [<i>Cyprinodon variegatus</i>]	Cluster-30738.209558	1.64	0.52	1.75	0.85	1.39	0.71
membrane-bound O-acyltransferase domain-containing protein 2 [<i>Parasteatoda tepidariorum</i>]	Cluster-30738.172113	1.3	1.03	1.05	0.73	1.21	1.49
Vesicle-associated membrane protein 1, partial [<i>Stegodyphus mimosarum</i>]	Cluster-30738.171857	3.17	0.53	2.11	0.45	3.66	0.1
peritrophic membrane chitin binding protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.77125	1.19	1.71	0.65	2.85	0.23	2.41
adipocyte plasma membrane-associated protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.172989	1.15	1.78	0.99	1.08	0.68	1.16
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

Salivary proteins

Salivary gland is an important osmoregulation organ of ticks. Whether for a long time away from the host or during the feeding period of the host, the salivary glands are essential for maintaining the growth, development, and metabolism of ticks [27]. Furthermore, salivary glands

and saliva play key roles in the transmission of pathogenic microorganisms to the host [28]. By using the psiblast tool, scientists built the TickSialoFam (TSF) database, a publishable database that can help annotate tick sialo transcriptomes[29].

Under the stimulation of blood sucking, the salivary glands will develop and enlarge rapidly, and this process will also be accompanied by changes in a large number of salivary gland-related proteins. A total of five salivary gland-associated proteins were identified in this experiment, and they were classified into two classes according to their expression patterns in seven different developmental stages. The expression abundance of the first class, Cluster-30738.172529, Cluster-30738.173721, and Cluster-30738.175111, increased rapidly in the process of blood sucking, and the expression of these proteins would continue to increase with the process of development in *H. longicornis*. The expression abundance of the second class, Cluster-30738.164072, increased rapidly in the early stage of development (eggs hatched into unfed larva) and increased significantly during the development from unfed nymphs to fed adult (Table 9).

Table 9
Salivary proteins

Protein	ID	Mean_Ratio HLUL-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
Secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.172529	0.91	1.53	0.8	1.4	0.72	6.98
Salivary protein Sal4 [<i>Rhipicephalus</i>]	Cluster-30738.164072annulatus	5.75	0.9	1.04	0.91	1.94	0.18
salivary sulfotransferase, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173721	0.72	1.78	0.49	2.8	0.27	3.85
TPA_inf: salivary gland protein 223 [<i>Amblyomma variegatum</i>]	Cluster-30738.175111	0.84	1.43	0.86	2.98	0.35	3.87
TPA_inf: secreted salivary protein 1024 [<i>Amblyomma variegatum</i>]	Cluster-30738.171953	1.02	2.05	1.12	0.94	2.15	0.56
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

Secreted proteins

Secreted proteins (SP) present in parasites contribute directly or indirectly to the survival of parasites. In addition, parasites need to adapt to different hosts as well as to physiological changes during development, and SP proteins play an important role [30] in these processes.

In this experiment, a total of 37 secreted proteins were identified, and many proteins also showed a strong regularity and specific up-regulated expression at different developmental stages. These secreted proteins were mainly divided into three classes by collating the data. The first class, including a total of 12 proteins (Cluster-30738.87074, Cluster-30738.200760, Cluster-30738.71323, Cluster-30738.173742, Cluster-30738.63453, Cluster-175252.0, Cluster-30738.196486, Cluster-30738.177356, Cluster-30738.174134, Cluster-30738.182142, Cluster-30738.149846, and Cluster-30738.173637), showed a significant growth trend from the eggs stage hatched into unfed larva. After that, its expression abundance also showed a significant growth trend in the process from the fed stage to the next unfed stage. The second class, which consisted of 11 proteins (Cluster-30738.40780, Cluster-30738.172492, Cluster-30738.173758, Cluster-30738.236193, Cluster-30738.4675, Cluster-30738.168968, Cluster-30738.173651, Cluster-30738.173029, Cluster-30738.170909, Cluster-30738.173549, and Cluster-30738.172529), seemed to have some relationship with the process of satiety, and the expression of these proteins was significantly up-regulated in the starvation stage and the corresponding satiety stage and reached the peak at the stage of engorged adult. In the third class, including a total of 15 proteins, the regularity of these proteins seemed to be closer to the specificity of each developmental stage of *H. longicornis*, mainly including Cluster-30738.81824, Cluster-30738.201894, Cluster-30738.171064, Cluster-30738.236147, Cluster-30738.92518, Cluster-30738.58441, Cluster-30738.179348, Cluster-30738.171953, Cluster-30738.170773, Cluster-30738.176166, Cluster-30738.4778, Cluster-30738.170966, Cluster-30738.169678, and Cluster-30738.160134 (Table 10).

Table 10
Secreted proteins

Protein	ID	Mean_Ratio HLUL-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.87074	1.25	1.19	1.73	0.49	2.47	0.51
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.81824	0.79	0.29	9.8	0.23	7.77	0.1
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.200760	3.11	0.93	2.39	0.69	1.95	0.44
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.201894	2.31	0.91	1.12	1.19	0.7	1.47
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.40780	0.68	1.45	0.54	1.65	0.57	2.11
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.171064	1.02	1.44	0.98	1.18	1.08	0.86
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.236147	0.65	0.57	5.42	0.53	4.73	0.55
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.71323	5.5	0.6	2.14	0.52	2.08	0.45
secreted cysteine rich protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173742	3.36	0.41	2.49	0.19	10	0.1
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.172492	0.67	2	0.41	1.15	0.96	1.44
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173758	0.88	1.32	0.74	3.13	0.38	1.92
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.63453	2.44	0.55	1.98	0.88	1.09	0.64
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-175252.0	2.41	0.73	1.02	0.85	1.03	0.94
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.92518	0.64	2.01	1.66	0.68	1.75	0.88
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.236193	0.35	2.85	0.26	2.99	0.78	2.05
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.4675	0.86	1.81	0.83	2.93	0.33	4.13
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.168968	1.12	1.2	0.51	2.52	0.5	3.2
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173651	0.8	2.19	0.77	1.6	0.7	1.25
TPA_exp: hypothetical secreted protein 1652 [<i>Amblyomma variegatum</i>]	Cluster-30738.196486	1.29	1.2	1.96	0.56	1.89	0.57
putative secreted protein [<i>Ixodes scapularis</i>]	Cluster-30738.58441	1.23	1.66	0.77	1.03	1.11	0.81

*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.

Protein	ID	Mean_Ratio HLUL-VS- HLEE	Mean_Ratio HLFL-VS- HLUL	Mean_Ratio HLUN-VS- HLFL	Mean_Ratio HLFN-VS- HLUN	Mean_Ratio HLUA-VS- HLFN	Mean_Ratio HLFA-VS- HLUA
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173029	0.79	1.06	0.8	1.6	0.93	3.04
putative secreted salivary gland peptide [<i>Ixodes scapularis</i>]	Cluster-30738.177356	1.72	0.94	2.88	0.46	2.59	0.22
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.170909	1.01	1.79	0.59	2.2	0.37	1.29
TPA_inf: putative secreted glycine-rich protein [<i>Amblyomma variegatum</i>]	Cluster-30738.174134	1.47	1.33	1.27	0.87	1.65	0.68
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.179348	0.43	0.57	0.94	2	0.92	1.7
TPA_inf: secreted salivary protein 1024 [<i>Amblyomma variegatum</i>]	Cluster-30738.171953	1.02	2.05	1.12	0.94	2.15	0.56
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.170773	0.7	4.1	0.31	1.46	0.57	2.07
glycine proline-rich secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.176166	1.4	0.84	0.86	0.83	1.71	1.4
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173549	0.69	4.01	0.39	4.32	0.16	6.96
secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.4778	1.02	1.88	1.15	0.76	1.5	0.62
Secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.170966	0.6	1.19	0.66	0.86	1.12	0.86
TPA_inf: hypothetical secreted protein 790 [<i>Amblyomma variegatum</i>]	Cluster-30738.169678	0.96	1.3	1.08	0.84	1.94	0.65
Secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.182142	1.26	1.32	1.62	0.72	1.35	0.59
Secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.149846	1.54	0.87	1.37	0.89	1.13	0.86
secreted salivary gland peptide, putative [<i>Ixodes scapularis</i>]	Cluster-30738.172529	0.91	1.53	0.8	1.4	0.72	6.98
TPA_inf: hypothetical secreted protein 790 [<i>Amblyomma variegatum</i>]	Cluster-30738.160134	0.59	0.52	2.42	0.74	2.54	0.31
glycine proline-rich secreted protein, putative [<i>Ixodes scapularis</i>]	Cluster-30738.173637	1.64	1.28	1.73	0.63	1.73	0.27
*HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.							

GO analysis of the DEPs

Functional classification of the DEPs was carried out through GO analysis. We have identified 41, 45, 41, 44, 44, and 45 GO terms respectively in the HLUL vs. HLEE, HLFL vs. HLUL, HLUN vs. HLFL, HLFN vs. HLUN, HLUA vs. HLFN, and HLFA vs. HLUA groups (HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult). Among these GO terms, there were 19 biological process GO items, 14 cell component GO items, and eight molecular function GO items in UL vs. EE. The GO terms in the

FL vs. UL included 23 biological process terms, 14 cell component terms, and eight molecular function terms. The GO terms in the UN vs. FL included 19 biological process terms, 14 cell component terms, and eight molecular function terms. In FN vs. UN, there were 20 biological process terms, 14 cell component terms, and eight molecular function terms. For the GO terms in the UA vs. FN, there were 22 biological process terms, 14 cell component terms, and eight molecular function terms. The GO terms in FA vs. UA included 23 biological process terms, 14 cell component terms, and eight molecular function terms. In order to further explore the functions and properties of the up-regulated and down-regulated proteins in the different developmental stages of *H. longicornis*, we performed clustering and abundance analyses of these GO terms. The figure shows up to 20 rich GO terms in each group and up to three main GO cluster graphs (Figures 4 and 5).

Compared with eggs and unfed larvae ticks, the results of the GO analysis showed the following: for the molecular function: catalytic activity, binding, transport activity, structural molecular activity, and molecular function regulation; for the cell composition: cells, cell components, organs, membrane components, and organ components; and for the biological process: cellular process, metabolic process, regulation of biological functions, stimulus response, and recognition of cell composition/biological inheritance.

Similarly, we also found in the results that compared with the starvation phase, the GO analysis results in the fed stage were as follows: in the cell composition: cell membrane, ribosomes, RNA-induced silencing complex (RSIC), RNAi effector complex; in the molecular function: synthesis, enzyme activity, inhibitor enzyme activity, peptidase activity, synthetase activity, and epidermal composition; and in the biological processes: organic substance biosynthetic, organic substance catabolic, cofactor metabolic, cellular biosynthetic, and carbohydrate derivative metabolic.

In the unfed phase, compared with the previous fed phase, the enrichment results of the GO entries were as follows: in the cell composition: DNA packaging complex, protein-DNA complex, nucleosome, and chromatin; in the molecular function: lipid transporter activity, transporter activity, protein heterodimerization activity, structural consistent of cuticle, and protein kinase activity; and in the biological process: microtubule-based process, homeostatic process, protein-DNA complex assembly, and cellular component organization.

KEGG analysis of the DEPs

In order to further determine the biological pathways in which these differential proteins are involved in the development of *H. longicornis*, the HLUL vs. HLEE, HLFL vs. HLUL, HLUN vs. HLFL, HLFN vs. HLUN, HLUA vs. HLFN, and HLFA vs. HLUA groups (HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult) were analyzed and identified 2,112; 2,124; 4323; 3,371; 2,846; and 1,998 channels, respectively (Figures 6 and 7).

Among these pathways, it was found that compared with the unfed stage, the signal pathways enriched by the up-regulated proteins in the fed stage mainly included the digestive system, immune system, endocrine system, environmental adaptation, and infectious diseases (viral, and bacterial), signal transduction, cellular community-eukaryotes, cell growth and death, and transport and catabolism.

In the unfed stage, compared with the previous fed stage, the main enriched signal pathways included the excretory system, nervous system, aging, development, cardiovascular diseases, folding, sorting and degradation, replication and repair, and cell motility.

Conclusions

This is the first in-depth overview of the protein spectrum of *H. longicornis* (parthenogenesis), which could be of great significance for revealing the molecular architecture of ticks with complex life cycle. Our data provides strong molecular support for the use of *H. longicornis* as a powerful model for studying tick development and reveal a group of proteins. These proteomes have expanded to play a key role in biological regulation such as digestion, molting, ovarian development and immunomodulation. Overall, this is a report on the overall proteomics of *H. longicornis*, which will help us to understand the complex process of tick development, and the membrane-associated proteins and secretory proteins described in this paper will also help to find new target proteins and provide a theoretical basis and candidates for improving tick control strategies.

Abbreviations

iTRAQ
Isobaric tags for relative and absolute quantification
DEPs
Differentially expressed proteins
KEGG
Kyoto encyclopedia of genes and genomes
2-DE

two-dimensional electrophoresis
LC-MS)/MS
liquid chromatography with tandem mass spectrometry
MALDI-TOF-MS
time-of-flight mass spectrometry
EDTA
ethylenediaminetetraacetic acid

Declarations

Supplemental data

No supplemental data.

Acknowledgements

This work was supported by the funding from the National Key R&D Program of China (2019YFC1200502–2019YFC1200500–2017YFD0501200), The National parasitic Resource Center Project (NPRC-2019-194-30), the National Science Foundation of China (31572511), the Central Public-interest Scientific Institution Basal Research Fund (Y2019YJ07-04 and Y2018PT76), the Agriculture Research System of MOF and MARA (CARS-37), ASTIP (CAAS-ASTIP-2016-LVRI) and the Jiangsu Co-innovation Center Program for the Prevention and Control of Important Animal Infectious Disease and Zoonoses supported the interpretation of data in this study.

Availability of data and materials

The data reported in this paper have been deposited in the OMIX, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (<https://ngdc.cncb.ac.cn/omix>: accession no. OMIX707).

Authors' contributions

The experiment was conceived and designed by G. L., W. L., J. L. and H. W; The experiment was conducted by J. L., R. C., W. L., P. L., P. D., L. X. and G. D.; W. L., W. L. and Q. R. analyzed the data. Lab research and management supported by G. G., J. L. and H. Y.; W. L. and Q. W. wrote this paper.

Ethics Approval and Consent to Participate

The study was approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, CAAS (Permit No. LVRI-AEC-2018-001). All the procedures were conducted according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

No applicable

Competing interests

The authors declare no competing interests

References

1. Abd-Rani PA, Irwin PJ, Coleman GT, Gatne M, Traub RJ. A survey of canine tick-borne diseases in India. *Parasit Vectors*. 2011;4:141.
2. Durden LA, Merker S, Beati L. The tick fauna of Sulawesi, Indonesia (Acari: Ixodoidea: Argasidae and Ixodidae). *Exp Appl Acarol*. 2008;45:85–110.
3. Jia N, Wang J, Shi W, Du L, Ye RZ, Zhao F, et al. *Haemaphysalis longicornis*. *Trends Genet*. 2021;37:292–3.
4. Chen Z, Yang X, Bu F, Yang X, Liu J. Morphological, biological and molecular characteristics of bisexual and parthenogenetic *Haemaphysalis longicornis*. *Vet Parasitol*. 2012;189:344–52.
5. Di-Venere M, Fumagalli M, Cafiso A, De-Marco L, Epis S, Plantard O, et al. *Ixodes ricinus* and Its Endosymbiont *Mitochondria*: A Comparative Proteomic Analysis of Salivary Glands and Ovaries. *PLoS One*. 2015;10:e0138842.

6. Kim YH, Slam MS, You MJ. Proteomic screening of antigenic proteins from the hard tick, *Haemaphysalis longicornis* (Acari: Ixodidae). Korean J Parasitol. 2015;53:85–93.
7. Oleaga A, Obolo-Mvoulouga P, Manzano-Román R, Pérez-Sánchez R. A proteomic insight into the midgut proteome of *Ornithodoros moubata* females reveals novel information on blood digestion in argasid ticks. Parasit Vectors. 2017;10:366.
8. Wu WW, Wang G, Baek SJ, Shen RF. Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel-or LC-MALDI TOF/TOF. J Proteome Res. 2006;5:651–8.
9. Pierce A, Unwin RD, Evans CA, Griffiths S, Carney L, Zhang L, et al. Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases. Mol Cell Proteomics. 2008;7:853–63.
10. Craft GE, Chen A, Nairn AC. Recent advances in quantitative neuroproteomics. Methods. 2013;61:186–218.
11. Wang H, Zhang X, Wang X, Zhang B, Wang M, Yang X, et al. Comprehensive Analysis of the Global Protein Changes That Occur During Salivary Gland Degeneration in Female Ixodid Ticks *Haemaphysalis longicornis*. Front Physiol. 2019;9:1943.
12. Wang M, Hu Y, Li M, Xu Q, Zhang X, Wang X, et al. A proteomics analysis of the ovarian development in females of *Haemaphysalis longicornis*. Exp Appl Acarol. 2020;80:289–309.
13. Liu WG, Luo J, Ren QY, Qu ZQ, Lin HL, Xu XF, et al. A Novel miRNA-hlo-miR-2- Serves as a Regulatory Factor That Controls Molting Events by Targeting CPR1 in *Haemaphysalis longicornis* Nymphs. Front Microbiol. 2020;11:1098.
14. Oleaga A, Obolo-Mvoulouga P, Manzano-Román R, Pérez-Sánchez R. Midgut proteome of an argasid tick, *Ornithodoros erraticus*: a comparison between unfed and engorged females. Parasit Vectors. 2015;8:525.
15. Schwarz A, Tenzer S, Hackenberg M, Erhart J, Gerhold-Ay A, Mazur J, et al. A systems level analysis reveals transcriptomic and proteomic complexity in *Ixodes ricinus* midgut and salivary glands during early attachment and feeding. Mol Cell Proteomics. 2014;13:2725–35.
16. Heekin AM, Guerrero FD, Bendele KG, Saldivar L, Scoles GA, Dowd SE, et al. Gut transcriptome of replete adult female cattle ticks, *Rhipicephalus (Boophilus) microplus*, feeding upon a Babesia bovis-infected bovine host. Parasitol Res. 2013;112:3075–90.
17. Zha XL, Yu XB, Zhang HY, Wang H, Huang XZ, Shen YH, et al. Identification of Peritrophins and Antiviral Effect of Bm01504 against BmNPV in the Silkworm, *Bombyx mori*. Int J Mol Sci. 2020;21:7973.
18. Tellam RL, Eisemann C. Chitin is only a minor component of the peritrophic matrix from larvae of *Lucilia cuprina*. Insect Biochem Mol Biol. 2000;30:1189–201.
19. Shao L, Devenport M, Jacobs-Lorena M. The peritrophic matrix of hematophagous insects. Arch Insect Biochem Physiol. 2001;47:119–25.
20. Matsuo T, Sato M, Inoue N, Yokoyama N, Taylor D, Fujisaki K. Morphological studies on the extracellular structure of the midgut of a tick, *Haemaphysalis longicornis* (Acari: Ixodidae). Parasitol Res. 2003;90:243–8.
21. Kongsuwan K, Josh P, Zhu Y, Pearson R, Goug J, Colgrave ML. Exploring the midgut proteome of partially fed female cattle tick (*Rhipicephalus (Boophilus) microplus*). J Insect Physiol. 2010;56:212–26.
22. Rosell R, Coons LB. The role of the fat body, midgut and ovary in vitellogenin production and vitellogenesis in the female tick, *Dermacentor variabilis*. Int J Parasitol. 1992;22:341–9.
23. Boldbaatar D, Umemiya-Shirafuji R, Liao M, Tanaka T, Xuan X, Fujisaki K. Multiple vitellogenins from the *Haemaphysalis longicornis* tick are crucial for ovarian development. J Insect Physiol. 2010;56:1587–98.
24. Xavier MA, Tirloni L, Pinto AFM, Diedrich JK, Yates JR, Mulenga A. A proteomic insight into vitellogenesis during tick ovary maturation. Sci Rep. 2018;8:4698.
25. Willis JH. Structural cuticular proteins from arthropods: annotation, nomenclature, and sequence characteristics in the genomics era. Insect Biochem Mol Biol. 2010;40:189–204.
26. Gong J, Chen Y, Pu F, Sun P, He F, Zhang L, et al. Understanding Membrane Protein Drug Targets in Computational Perspective. Curr Drug Targets. 2019;20:551–64.
27. Bowman AS, Sauer JR. Tick salivary glands: function, physiology and future. Parasitology. 2004;129:67–81.
28. Šimo L, Kazimirova M, Richardson J, Bonnet SI. The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. Front Cell Infect Microbiol. 2017;7:281.
29. Ribeiro JMC, Mans BJ. TickSialoFam (TSFam): A Database That Helps to Classify Tick Salivary Proteins, a Review on Tick Salivary Protein Function and Evolution, With Considerations on the Tick Sialome Switching Phenomenon. Front Cell Infect Microbiol. 2020;10:374.

Figures

Figure 1 (A) Wayne diagram of the total protein identified by three repeated experiments on *Haemaphysalis longicornis*. R1, repeat 1; R2, repeat 2; and R3, repeat 3. (B) Distribution of the specific peptides and (C) protein coverage distribution

Figure 2

Page 17/22

Figure 3

Comparative analysis of six random screened genes (*CRK*, *flotillin*, *Mo-25*, *dystrophin*, *septin-1*, and *septin-2*) in the different developmental stages of *Haemaphysalis longicornis*.

Figure 4

Gene Ontology (GO) enrichment for the differentially expressed proteins (DEPs) ($P < 0.05$) of the different life stages of *Haemaphysalis longicornis*. (A) Unfed larva vs. egg, (B) fed larva vs. unfed larva, and (C) unfed nymph vs. fed larva. GO functional annotations in the three main categories: molecular function, cellular component, and biological process. HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph.

Figure 5

Gene Ontology (GO) enrichment for the differentially expressed proteins (DEPs) ($P < 0.05$) of the different life stages of *Haemaphysalis longicornis*. (A) Fed nymph vs. unfed nymph, (B) unfed adult vs. fed nymph, and (C) fed adult vs. unfed adult. The GO functional annotations in the three main categories: molecular function, cellular component, and biological process. HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.

Figure 6

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed proteins (DEPs) ($P < 0.05$) of the different life stages of *Haemaphysalis longicornis*. $P < 0.05$ indicates significant enrichment in the development-related pathways. The top 20 pathways are shown. (A) Unfed larva vs. Egg, (B) fed larva vs. unfed larva, and (C) unfed nymph vs. fed larva. The KEGG enrichment was measured by the Rich factor, q -value, and the number of genes enriched in this pathway. The colors and sizes of the spots represent the q -values and the number of target genes, respectively. HLEE, egg; HLUL, unfed larva; HLFL, fed larva; HLUN, unfed nymph.

Figure 7

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed proteins (DEPs) ($P < 0.05$) of the different life stages of *Haemaphysalis longicornis*. $P < 0.05$ indicates significant enrichment in the development-related pathways. The top 20 pathways are shown. (A) Fed nymph vs. unfed nymph, (B) unfed adult vs. fed nymph, and (C) fed adult vs. unfed adult. The KEGG enrichment was measured by Rich factor, q -value, and the number of genes enriched in this pathway. The colors and sizes of the spots represent the q -values and the number of target genes, respectively. HLUN, unfed nymph; HLFN, fed nymph; HLUA, unfed adult; HLFA, fed adult.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.pdf](#)