

SHORT REPORT

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The mRNA expression and enzymatic activity of three enzymes during embryonic development of the hard tick *Haemaphysalis longicornis*

Tian-Tian Zhang, Zhao-Xi Qiu, Yuan Li, Wen-Ying Wang, Meng-Meng Li, Pei Guo and Jing-Ze Liu*

Abstract

Background: Three main enzymes including cathepsin B, cathepsin D and acid phosphatase are involved in vitellin degradation, which is a major biochemical event of the embryonic development and can provide nutrients and metabolites for tick embryos. In the present study, the mRNA expression profiles and enzymatic activity of cathepsin B, cathepsin D and acid phosphatase were investigated during embryonic development in the tick *Haemaphysalis longicornis*.

Results: The results revealed that all three enzymes were expressed throughout embryonic development. Both cathepsin B and acid phosphatase transcripts were accumulated during the first four days. Cathepsin B reached its highest expression on day 5, whereas the peak expression of acid phosphatase and cathepsin D occurred on day 11. The highest activity of cathepsin B was observed on the first day of egg development, whereas cathepsin D reached its highest activity on day 13. Acid phosphatase activity increased gradually during the first five days and then remained stable until the end of egg development.

Conclusions: Three enzymes were expressed and activated in eggs, and also presented different dynamic changes with the development of embryos. The profiles of both mRNA expression and enzymatic activity of these enzymes indicate that they are controlled orderly and play multiple roles during embryonic development in ticks.

Keywords: *Haemaphysalis longicornis*, Cathepsin B, Cathepsin D, Acid phosphatase, Embryonic development

Background

Ticks are obligatory hematophagous ectoparasites of wildlife, domestic animals and humans. They are notorious vectors that can transmit various pathogens (viruses, rickettsiae, bacteria, spirochaetes and protozoans) among arthropods [1, 2]. With climate change, new tick-borne pathogens and tick-borne diseases have become severe threats to public health all over the world [3, 4]. With a high reproductive potential, the fully engorged female can lay up to several thousand eggs [5]. After engorgement, the body weight of female ticks is increased nearly

100-fold compared to unengorged ticks, and more than 50% of engorgement weight is transformed into eggs [6]. Therefore, one of the strategies to control tick-borne diseases is to explore new molecular targets for the interruption of the tick life-cycle. Embryonic development in eggs, the only non-parasitic stage, is vital in the tick life-cycle.

In ticks, vitellin (Vn) is a phosphorylated heme-lipoprotein and its degraded products provide the source of amino acids, carbohydrates, heme, and other nutrients for embryonic development and unengorged larva [7]. Regulatory mechanisms for the utilization of Vn have been implicated to rely on a variety of proteolytic enzymes in different species of insects [8–10]. Among them, three enzymes including cathepsin B [9, 11, 12], cathepsin D [13–15] and acid phosphatase [14, 16] have

*Correspondence: liujingze@hebtu.edu.cn
Hebei Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology, College of Life Sciences, Hebei Normal University, Shijiazhuang 050024, China



been extensively investigated in insects. Cathepsin B is a cysteine protease involved in a wide range of biological processes, including the degradation of regulatory proteins and digestive processes [8, 17]. As a proteolytic enzyme, cathepsin B has been reported to play an essential role in the embryonic degradation of Vn in insects [8, 9]. Cathepsin D is an aspartic protease mainly found in lysosomes. It is an intracellular digestive enzyme and is involved in the degradation or activation of proteins, hormones and other substrates [14, 16]. During the embryonic development of the triatomine *Rhodnius prolixus*, cathepsin D is an essential regulator for yolk protein degradation [16]. Acid phosphatase as an enzyme in the lysosome can catalyze the hydrolysis of orthophosphoric monoester from a wide variety of substrates [18]. It has been reported that acid phosphatase and cysteine protease cooperate to assure Vn breakdown during early embryogenesis of *Periplaneta americana* [19]; this is because acid phosphatase is able to dephosphorylate Vn, which increases its degradation by other proteolytic enzymes.

Compared to insects, the functional roles of three enzymes in Vn degradation in ticks and the regulatory mechanisms are still poorly understood. Previous studies which were only based on one species of tick, *Rhipicephalus (Boophilus) microplus*, have suggested that some enzymes may play different roles in Vn degradation *in vitro* [20–23]. However, the dynamic changes of these three enzymes during embryonic development are unknown. Meanwhile, knowledge on stage-specific proteolysis of Vn during tick embryo development and the relationship between different enzymes during this process remains scarce.

The hard tick *Haemaphysalis longicornis* is widely distributed in Australia, New Zealand, Korea, Japan and 17 provinces of China [24]. Moreover, it can transmit a large variety of pathogenic microorganisms to humans and animals, including *Theileria* spp., *Babesia gibsoni*, *Rickettsia* and *Coxiella burnetii* [25]. Recently, it was found for the first time that a large number of *H. longicornis* infested sheep in Hunterdon County, New Jersey, USA, suggesting an invasion of *H. longicornis* which presents a significant threat to human and animal health in the USA [26]. In our previous work, we reported the vitellogenesis in *H. longicornis* and its hormone regulation during ovary development [27]. Furthermore, we purified and characterized the properties of Vn from *H. longicornis*, finding that Vn from *H. longicornis* was composed of eight subunits with molecular weights of 112, 103, 80, 78, 71, 68, 62 and 52 kDa, respectively [28].

In the present study, we investigated the mRNA expression profiles and enzymatic activity of cathepsin B, D and acid phosphatase during embryonic development in *H.*

longicornis, aiming to reveal the existence and dynamic changes of three enzymes during embryonic development in tick *H. longicornis*.

Methods

Sample collection and rearing

Free-living *H. longicornis* ticks were collected on vegetation in Xiaowutai National Natural Reserve Area in China by flag dragging and reared on domestic rabbits, *Oryctolagus cuniculus*, as described by Liu et al. [29]. The rabbits were maintained at 20–25 °C with 50% RH and exposed to natural daylight cycles, and each rabbit was used only for a single infestation. After engorgement, ticks were collected and incubated for oviposition in an incubator with 75 ± 5% relative humidity (RH) and 8:16 h of a light-dark cycle (L:D) at 26 ± 1 °C. A group of eggs were collected to obtain samples during oviposition every day at the same time and kept separately in the incubator, then frozen quickly using liquid nitrogen when they reached different developmental stages and stored at -80 °C for further analysis. A control group of eggs was allowed to develop completely to determine the exact day of hatching.

RNA extraction and cDNA synthesis

Total RNA from each developmental stage (100 mg of eggs) was extracted using the AxyPrep™ Multisource Total RNA Miniprep Kit (Axygen, San Jose, USA) following the manufacturer's protocol. The RNA concentration and purity were determined using a Nano Drop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was evaluated by agarose gel electrophoresis. RNA samples were stored at -80 °C until use. The complementary DNA (cDNA) was synthesized from 4 µg of total RNA by reverse transcription reaction using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), which excluded genomic DNA contamination. The cDNA samples were stored at -20 °C until use.

Primer design

Primers were designed using the information from the GenBank sequences for different kinds of enzymes in *H. longicornis*. The genes chosen for this study are presented in Table 1. For cathepsin B, cathepsin D and acid phosphatase, primers were designed using the cathepsin B sequence (AN AB255051), aspartic protease sequence (AN EU019715) and lysosomal acid phosphatase sequence (AN HM150759) from *H. longicornis*, respectively. For control actin gene, primers were designed using the β-actin sequence (AN AY254898) from *H. longicornis*. All primers were designed with Primer Premier

Table 1 Primers for genes evaluated in the present study

Gene	Primer sequence (5'-3')	T _m (°C)	Amplicon size (bp)
Cathepsin B	F: CAACTCCTGGAACACCGAA	59.7	78
	R: GTCTTCAATGCCGCACTCA	61.0	
Cathepsin D	F: CTCGGTTCTGAATGTGCCA	60.1	165
	R: GGTGATGTTGCCAGTGTATG GTG	59.7	
Acid phosphatase	F: GAGACAGATGCCTGGAGAGC	60.8	71
	R: TCCACACTTTCTTGTCCTCCG	59.1	
β-actin	F: CGTTCCTGGGTATGGAATCG	62.5	70
	R: TCCACGTCGCACTTCATGAT	62.7	

Abbreviation: T_m, melting temperature

5 considering a theoretical optimal annealing temperature of about 60 °C, with a melting temperature (T_m) difference between the forward and reverse primers not greater than 5 °C, guanine and cytosine percentages between 40–60%, primer lengths of 18–22 bp and free of dimers and hairpins.

Amplicon sequence analysis

Positive polymerase chain reaction (PCR) products were purified and cloned into pEASY[®]-T1 Simple Cloning Vector (TransGen Biotech, Beijing, China) using pEASY[®]-T1 Simple Cloning Kit (TransGen Biotech, Beijing, China). The recombinant plasmid was used to transform the competent *Escherichia coli* DH5α cells and sent to Invitrogen (Beijing, China) for sequencing. The resulting sequences were analyzed by a BLASTn search in GenBank and by using Clustal W method in the DNANAN V6 software (Lynnon Biosoft, San Ramon, CA, USA).

Quantitative real-time PCR (qPCR)

Gene amplification was performed by qPCR in a Mx3005P qPCR system (Agilent Technologies, Santa Clara, USA) using TransStart[®] Top Green qPCR SuperMix (TransGen Biotech) following the manufacturer's instructions. The qPCR assays were conducted in 96-well polypropylene plates (Axygen, San Jose, USA) in a 20 μl reaction volume comprising 1 μl of cDNA template, 0.4 μl of each 10 μM primer (Table 1), 10 μl of 2× TransStart[®] Top Green qPCR SuperMix, 0.4 μl of Passive Reference Dye (TransGen Biotech) and 7.8 μl of H₂O. The thermal cycling program was as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The primers with high amplification specificity were verified by different peaks observed in corresponding melting curves. Each plate contained triplicate reactions for each DNA sample. Melting curves were also

traced after each assay to confirm that the fluorescence signal was retrieved from specific PCR products and to ensure the absence of primer dimers. The relative gene expression levels for each gene in each sample were calculated as Ct and transformed into relative values (RQ) by the 2^{-ΔΔC_T} method, where ΔΔC_T = (C_{T, Target} - C_{T, Actin})_{sample} - (C_{T, Target} - C_{T, Actin})_{control} [30].

Protein extraction

Eggs (1 g) were ground with a mortar and pestle in liquid nitrogen and dissolved in 20 mM sodium acetate buffer (pH 5.0). The homogenate was then centrifuged at 13,000× rpm for 10 min at 4 °C and the supernatant was transferred to a new tube for testing. The total protein concentration was determined according to Bradford [31] using bovine serum albumin (BSA) protein as the standard.

Enzymatic assays

The activity of cathepsin B and D was assessed using Activity Fluorometric Assay Kits (BioVision, Milpitas, USA) following the manufacturer's instructions. The Cathepsin B Activity Assay kit utilizes the preferred cathepsin B substrate sequence RR labeled with amino-4-trifluoromethyl coumarin (AFC). Samples that contain cathepsin B cleave the synthetic substrate RR-AFC to release free AFC. The released AFC can be quantified using a fluorometer or fluorescence plate reader at Ex/Em = 400/505 nm. The Cathepsin D Activity Assay kit utilizes the preferred cathepsin D substrate sequence GKPIILFFRLK(Dnp)-D-R-NH₂ labeled with 4-methylcoumarin-7-amide (MCA). Samples that contain cathepsin D cleave the synthetic substrate to release fluorescence, which can be quantified using a fluorometer or fluorescence plate reader at Ex/Em = 328/460 nm. The Acid Phosphatase Activity Assay kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate, which turns yellow (λ_{max} = 405 nm) when dephosphorylated by acid phosphatase.

Statistical analysis

Six biological replicates, representing different batches of separated spawning events, were performed for mRNA expression and enzymatic activity assessment. Data in all groups were analyzed using SPSS 12.0 software. The differences in mRNA expression and enzymatic activity among samples were compared by ANOVA. The differences were considered statistically significant when *P* < 0.05.

Results

Primer verification and qPCR conditions

Non-specific amplification was performed to confirm each primer pair using the T_m shown in Table 1 with the cDNA template of target genes prepared before. Amplification products were analyzed by agarose gel electrophoresis where a single bright band was observed (Fig. 1a-c). PCR amplicons were sequenced to affirm the amplicon sequences for all genes evaluated in the present study.

Cathepsin B dynamic changes during embryonic development

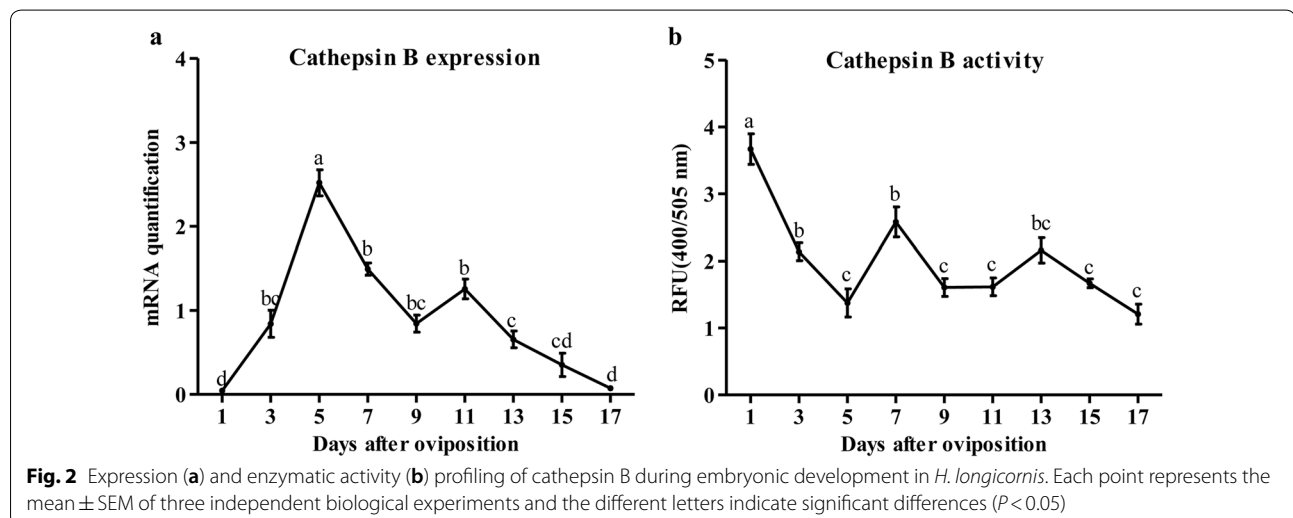
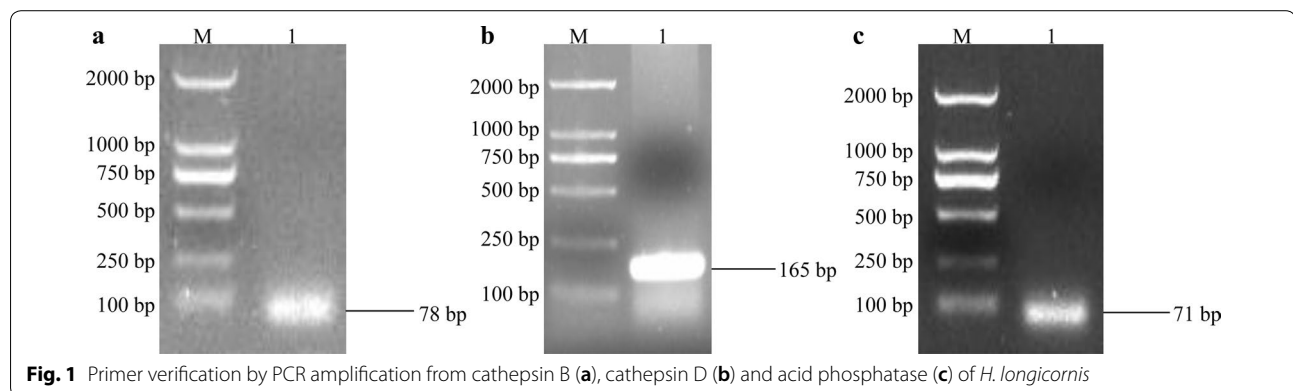
The relative expression levels of cathepsin B in the eggs from different stages of embryonic development were individually measured by quantitative real-time PCR. The results showed that cathepsin B was expressed almost throughout egg development except for the first and last day (Fig. 2a). An accumulation with significant upregulation of cathepsin B was observed in the eggs during the first five days and reached its highest expression on day

5 (ANOVA, $F_{(8,45)} = 100.02$, $P < 0.0001$). However, cathepsin B mRNA expression steadily decreased, along with the progress of embryonic development (Fig. 2a).

The enzymatic activity profile of cathepsin B is shown in Fig. 2b. A high activity level of cathepsin B was observed on the first day in comparison with later developmental stages. The activity decreased significantly on the third and fifth days and then remained relatively low until the end of egg development (ANOVA, $F_{(8,45)} = 39.66$, $P < 0.0001$) (Fig. 2b).

Cathepsin D dynamic changes during embryonic development

We analyzed cathepsin D expression profiles in different developmental stages of tick embryogenesis. At the transcriptional level, cathepsin D expression was deficient in the first nine days and obviously increased on day 11, but then rapidly and dramatically dropped (ANOVA, $F_{(8,45)} = 269.59$, $P < 0.0001$) (Fig. 3a).



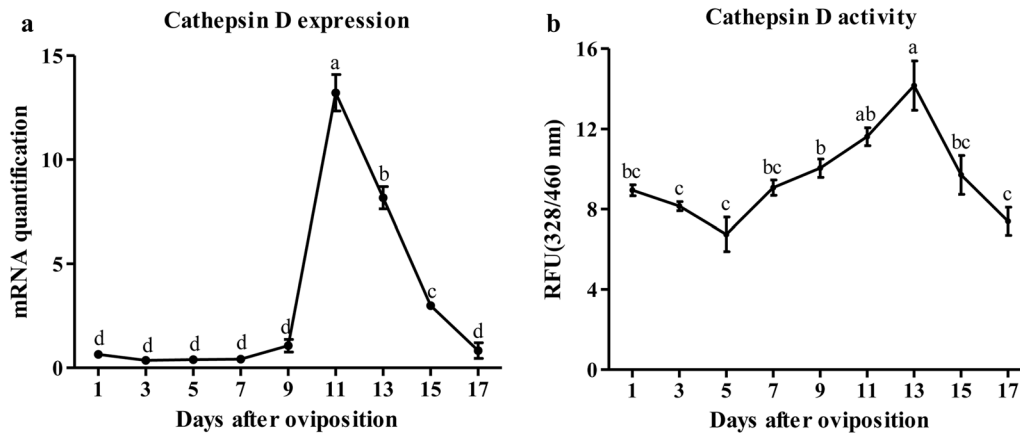


Fig. 3 Expression (a) and enzymatic activity (b) profiling of cathepsin D during embryonic development in *H. longicornis*. Each point represents the mean \pm SEM of three independent biological experiments and the different letters indicate significant differences ($P < 0.05$)

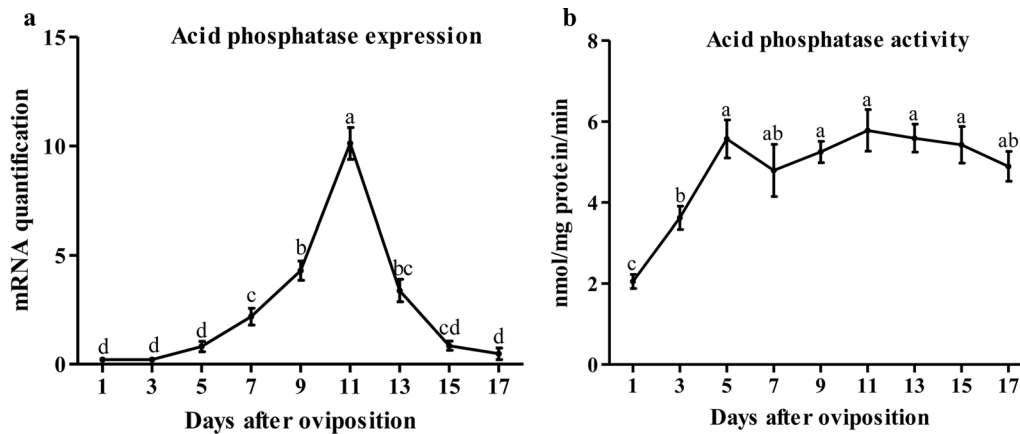


Fig. 4 Expression (a) and enzymatic activity (b) profiling of acid phosphatase during embryonic development in *H. longicornis*. Each point represents the mean \pm SEM of three independent biological experiments and the different letters indicate significant differences ($P < 0.05$)

As shown in Fig. 3b, cathepsin D activity was slowly reduced in the first three days but gradually increased during the following seven days. The highest activity of cathepsin D was observed on day 13 and then returned to the same level as in the first three days (ANOVA, $F_{(8,45)} = 21.08$, $P < 0.0001$) (Fig. 3b).

Acid phosphatase dynamic changes during embryonic development

For acid phosphatase, the relative expression analysis revealed that mRNA levels were first observed on day 5 and then accumulated with a significant increase (ANOVA, $F_{(8,45)} = 140.08$, $P < 0.0001$). It reached highest expression on day 11 and quickly reduced until the end of development (Fig. 4a).

Unlike cathepsin B and D, acid phosphatase activity increased significantly in the first five days, and then

remained stable at high levels until the end of egg development (ANOVA, $F_{(8,45)} = 17.05$, $P < 0.0001$) (Fig. 4b).

Discussion

Enzymes for protein hydrolysis in insect eggs are controlled scrupulously and orderly to ensure appropriate degradation of yolk proteins. These can be categorized into four types, namely cysteine proteases such as cathepsins B [9] and L [32], serine proteases [10], aspartic proteases [15] and acid phosphatases [14]. Among them, cathepsin B, cathepsin D and acid phosphatase have been frequently studied. In general, these enzymes are stored as an inactive form of a proenzyme in small yolk granules, but activated by the acidification of yolk granules, and then participate in digesting yolk proteins during embryonic development [33]. Analogous regulatory mechanisms in the degradation of yolk proteins are

described in many species such as *Spodoptera exigua* [15], *Dipetalogaster maxima* [14], *Blatella germanica* [34], *R. prolixus* [16], *Bombyx mori* [8], *Musca domestica* [35] and *Culex quinquefasciatus* [9].

To evaluate the dynamic changes of cathepsin B, cathepsin D and acid phosphatase during Vn degradation and embryonic development in *H. longicornis*, we observed both mRNA expression and activity of these three enzymes during egg development from a very early stage until hatching. The results showed that all three enzymes were expressed during egg development, which agrees with that observed in insects in other studies [11, 16, 19]. Both cathepsin D and acid phosphatase showed the highest mRNA expression on day 11, and cathepsin B on day 5. All three enzymes showed high activity in the eggs, but the activity patterns were not consistent with the expression profiles. The accumulation of three enzyme transcripts levels and high activity in the eggs may suggest their participation in the proteolytic process, where Vn is partially cleaved into small peptides and free amino acids [33].

Cathepsin B is a kind of proteinase in cytolysosome, which plays a crucial role in yolk utilization in oviparous animals and is able to cleave peptide bonds of their substrates [12]. In this study, cathepsin B mRNA expression significantly increased during early development (1–5 days) in the eggs but immediately decreased after that. Two noticeable decreases were observed on days 7 and 13, respectively. This expression profile is different from others such as *Helicoverpa armigera* and *Seriola lalandi*, in which cathepsin B showed the highest expression on the first day and gradually decreased during embryonic development [11, 36]. The variation tendency was, however, consistent with *M. domestica* [37]. The expression profile of cathepsin B is not uniform across different species. It is reported that increased expression of cathepsin B occurred throughout development in *Fundulus heteroclitus* [38], whereas in *Phenacoccus solenopsis*, the expression level was relatively low at the egg stage [39]. Nevertheless, the activity pattern of cathepsin B in *H. longicornis* is consistent with the results in *He. armigera*, which showed a steady decline during egg development [11].

Cathepsin D belongs to lysosomal aspartic protease, which is nearly ubiquitously expressed. This enzyme participates in several physiological processes such as protein degradation, autophagy, programmed apoptosis regulation and enzymatic precursor activation [40]. The mRNA expression and enzymatic activity of cathepsin D have been well analyzed in many insect species and a recent study showed that cathepsin D might function during metamorphosis and represent a target for insect control [15]. In this study, cathepsin D expression was

practically undetectable from day 0 to day 9, began to rise straightly on day 11, and then reduced significantly until the end of development. Consistent with this result, the trend of cathepsin D expression in *B. mori* is also first increased and then subsequently decreased [41]. The activity of this enzyme gradually accumulated during the first 13 days and significantly reduced at the end of development. This finding is similar to the activity patterns during embryogenesis in *R. prolixus* [16].

Acid phosphatases are known as hydrolases which are often associated with degradation of proteins. They usually described as lysosomal markers [14]. Our results revealed that the transcripts of acid phosphatase were extremely low during the first three days and the following trend was similar to cathepsin D, showing the highest expression on day 11. The acid phosphatase activity gradually increased from day 0 to 5, reaching a peak on day 5, and remained active until the end of egg development. This result was similar to studies in *P. americana* [19] and *R. microplus* [21], in which activity reached a peak on day 6 and 17, respectively.

The reproduction and development of ticks highly rely on Vn degradation during embryogenesis. Various proteolytic enzymes are responsible for utilization of yolk proteins during embryonic development in hematophagous arthropods and insects, including acidic cysteine proteases such as cathepsins B [9] and L [32], serine proteases [10], aspartic proteases [15] and also acid phosphatases [14]. During egg development, yolk granules are acidized, which is able to trigger the activation of proenzymes and yolk proteolysis [20]. This process has been proved in many models such as cysteine proteases in *B. germanica* and *B. mori* [34, 42], which can be activated by the acidification at embryogenesis. It has been indicated that in *P. americana*, pH acidification might be a possible mechanism for proenzymes activation during embryogenesis [19]. Moreover, acidification of yolk granules also does not occur homogeneously in invertebrate eggs. In the starfish *Pisaster ochraceus*, researchers detected differences of egg proteins in cortical granule populations [43]. Our results showed that the expression patterns of the three enzymes were different from their activity. Therefore, we think the activity of these enzymes may be controlled by the populations of acidifying yolk granules and egg proteins, which may also supervise the limited proteolysis of Vn during embryogenesis. This phenomenon was also found in ovarian tissue of *D. maxima*, where both pro-cathepsin D and cathepsin D were detected at all reproductive stages, but the highest cathepsin D activity was found at early and late atresia [14]. It is noteworthy that cathepsin B showed the highest expression on day 5, and cathepsin D as well as acid phosphatase showed the highest expression on day 11.

According to Friesen et al. [44], embryos of *Dermacentor andersoni* completed an extension of germ band around yolk on day 5, while on day 11, the differentiation of leg segments became clear and palp endites have begun to fuse. As such, we speculate that in addition to the roles in these two stages of Vn degradation, these three enzymes also participate in tissue differentiation and organ formation during embryonic development, which results in the difference between mRNA expression and enzymatic activity.

In arthropods, the interaction effect between acid phosphatase and other hydrolases on Vn degradation during embryogenesis has been mentioned. In *D. maxima*, *R. microplus* and *R. prolixus*, acid phosphatase has been proved to cooperate with aspartyl protease to degrade Vn during embryogenesis [14, 16, 21]. Furthermore, the correlation between acid phosphatase and cysteine peptidase on Vn degradation has been demonstrated in *P. americana* [19]. Conversely, although acid phosphatase and cathepsin B in *M. domestica* showed similar activity patterns during embryogenesis, the inhibition of acid phosphatase still did not affect yolk protein hydrolysis by cathepsin [37]. Unfortunately, the association among acid hydrolases is still unclear, and evidence for these processes has not yet been found in most species of ticks. In future studies, we should investigate the relationship and interaction among the three enzymes, and clarify their effect on Vn degradation and embryogenesis.

Conclusions

In this study, we investigated the mRNA expression and enzymatic activity of cathepsins B, D and acid phosphatase during embryonic development in *H. longicornis*. All three enzymes were not only expressed and activated in eggs but also presented dynamic changes with the development of embryos. Their transcription levels and activity profiles demonstrated for the first time in *H. longicornis*, that the three enzymes were existent and had different change patterns during egg development, indicating they were orderly controlled and played various roles in Vn degradation. However, further studies are required to explore the functions of these enzymes in Vn degradation during embryogenesis and their interaction. This will contribute to selecting the best potential molecular target and figuring out protective antigen for controlling tick life-cycle and disease transmission.

Abbreviations

Vn: vitellin; RH: relative humidity; L:D: light:dark photocyclus; PCR: polymerase chain reaction; qPCR: quantitative real-time PCR; RQ: relative quantity; BSA: bovine serum albumin; AFC: amino-4-trifluoromethyl coumarin; MCA: 4-methylcoumarin-7-amide; pNPP: p-nitrophenyl phosphate; Tm: melting temperature.

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Availability of data and materials

The data supporting the conclusions of this article are included in the article. Raw data used or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JZL and TTZ designed and the study and drafted the manuscript. TTZ, ZXQ and YL extracted RNA, synthesized cDNA, designed primers, performed qPCR, extracted protein, detected the activity of enzymes and analyzed data. WYW, MML and PG collected samples, participated in data collection and tick feeding. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Hebei Normal University as complying with the Animal protection law of the People's Republic of China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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