Article

Blood Digestion by Trypsin-Like Serine Protease in the Lyme Disease Vector Tick, *Ixodes Scapularis*

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ABSTRACT: *Ixodes scapularis* is the major vector of Lyme disease in the eastern United States. This species undergoes a life cycle consisting of eggs and three active stages: larva, nymph, and adult. Each active life stage takes a blood meal either for developing and molting to the next stage (larvae and nymphs) or for oviposition (adult females). This protein rich blood meal is the only food taken by *Ixodes* ticks. Most studies on blood digestion in ticks have shown that the initial stages of blood digestion are carried out by cathepsin proteases within endosomes of acidic digestive cells. However, in other hematophagous arthropods, the serine protease trypsin plays an important role in early protein degradation. In this study, we determined transcript expression of *I. scapularis* cathepsins and serine proteases, some with previously characterized roles in blood digestion. Gut pH was also determined and a trypsin-benzoyl-D, L-arginine 4-nitoanilide assay was used to measure active trypsin levels during blood digestion. Our data suggest that trypsin levels increase significantly after blood feeding and peaked in larvae, nymphs, and adults at 3, 1, and 1 days post host detachment, respectively. In addition, alkaline gut pH (8.0) conditions after *I. scapularis* blood feeding were similar to those required for trypsin activity in other arthropods suggesting these enzymes have an important and previously overlooked role in *I. scapularis* blood digestion.

Keywords: ticks; *Ixodes scapularis*; serine protease; blood digestion; trypsin

1. INTRODUCTION

Ixodes scapularis is a three host tick that requires a blood meal to complete each developmental stage, and adult females utilize blood for egg development [1]. The larvae and nymphs feed for 3-7 days whereas adult female feeding lasts for up to 10 days and consists of (i) a slow feeding period up to 5–9 days post attachment followed by (ii) rapid engorgement for 12–24 h prior to detachment from the host [2]. Rapid engorgement accounts for about two-thirds of the total blood meal. The tick gut comprises a major portion of the body and consists of a ventriculus (stomach) and several pairs of highly branched ceca extending into all regions of the body. Blood digestion putatively starts in the gut soon after ingestion and continues for several days to weeks after dropping off the host. Proteins represent about 95% of the non-water content of vertebrate blood. Consequently, hematophagous arthropods require proteases as the main enzymes in the midgut to process a blood meal [3].

A typical animal genome contains 2–4% of genes encoding for proteolytic enzymes [4]. Among these, serine proteases are the most abundant and functionally diverse group [5]. Over one third of all known proteolytic enzymes are serine proteases. The family name stems from the nucleophilic Ser in the enzyme active site. Hematophagous insects such as tsetse flies, mosquitoes, and many other insects digest the protein-rich blood meal mainly by using trypsin-like serine proteases that have a pH optimum in the alkaline range (~8.0 pH) [6]. Processing of host blood components in tick midgut, however, appears to differ greatly from that in other hematophagous arthropods. In ticks, blood

digestion is a slow process that has been shown to occur in the acidic environment of gut intracellular vesicles (endosomes), mainly by the cathepsin-like proteases [3]. In hematophagous insects, in contrast, protein digestion proceeds rapidly and takes place in the gut lumen.

Out of a total of 233 putatively active *I. scapularis* proteases thus far identified, 63 (27%) are serine proteases [3]. A multi-enzyme model for hemoglobin degradation was proposed for the European vector of Lyme disease, the castor bean tick, *I. ricinus* [7]. According to this model, the hemoglobin degradation pathway is initiated inside the acidic digestive vesicle by cysteine and aspartic endopeptidases (cathepsin L, legumain, and cathepsin D), generating large peptide fragments (8–11 kDa), followed by the action of cathepsin B and C exopeptidases, generating smaller peptides (2–7 kDa). Finally, serine carboxypeptidase (SCP, SP28 subfamily) and leucine aminopeptidase may participate in the liberation of dipeptides and free amino acids. Other studies have suggested that the final stages of hemoglobin degradation take place both in and outside of the digestive vesicles [8]. Trypsin serine proteases are active at high pH [9], in contrast to the acidic-active cathepsins. Midgut homogenates of the hard tick, *I. scapularis* (formerly *I. dammini*), were shown to lyse erythrocytes from different vertebrate bloods at an alkaline pH suggesting involvement of trypsin enzymes. Ten major blood digestive proteases (cathepsin, and aminopeptidase, and serine proteases) were proposed to be involved in blood digestion in *I. scapularis* [2]. The presence of four serine proteases on this list suggests previously unexplored roles of trypsin during *Ixodes* blood ingestion and digestion phases.

Most studies in tick blood digestion have focused on partially engorged females (up to 5 days on host, slow feeding phase), resulting in little information on the digestive profile beyond this stage. Therefore, to better understand the digestive enzyme profile of *I. scapularis*, we first tested expression of ten proteases identified previously [2] and then measured trypsin activity in unfed, partially fed, and post host detachment ticks up to 7 days (adults) and 28 days (larvae and nymphs) using benzoyl-D, L-arginine 4-nitoanilide (BApNA), a trypsin-specific substrate [10, 11]. Our results suggest that tick serine proteases are active within 24 h post host detachment (engorgement) and may play a major, previously unrecognized, role in early blood digestion in all tick life stages. Understanding the digestive profile of trypsin during blood meal digestion in *I. scapularis* improves our understanding of basic biology of ticks and may lead to new methods for tick control.

2. MATERIALS AND METHODS

Tick samples: Pathogen free *I. scapularis* were acquired from the tick rearing facility at Oklahoma State University, Stillwater, OK. Ticks were then transferred to an incubator and kept at 95% relative humidity and 20º C in our laboratory.

Blood feeding: Unfed larvae and nymphs were blood fed on mice at the University of Nevada, Reno. Both stages were allowed to detach naturally post engorgement. Once detached, the ticks were collected as soon as possible and returned to the incubator and harvested at the appropriate post blood meal intervals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Reno (IACUC # 00682). Adults were purchased from the Oklahoma University, Stillwater, OK, Tick rearing facility where adult ticks are fed on sheep.

Sample collection: Larvae and nymphs were collected at day 1, 2, 3, 7, 14, 21, and 28 days post engorgement/ host detachment. Two larvae or nymphs per sample were collected in triplicate for each time point.

Adult females were collected at 5 days post host attachment (partially fed), and at 1, 2, 3, 7, and 14 days post engorgement / host detachment. Whole guts were dissected and two guts were pooled per each triplicate sample. For unfed samples, four guts were pooled. Midguts were dissected in cold PBS buffer and rinsed with PBS to remove the blood. Once cleaned of blood, midguts were immediately transferred to either a cold 1.7 ml tube containing 200 µl of Trizol or Tris-HCl-$CaCl_2$ and stored at -80ºC until processed.

RT-PCR: Midguts were collected from adult females in Trizol reagent (Invitrogen) as described above. Samples were collected from four biological cohorts. Total RNA was extracted using Trizol reagent and a Zymo Directzol kit (Zymo Research, Irvine, California). Total RNA was DNase treated

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before using for cDNA synthesis. 1 µg DNAse-treated RNA was used for cDNA synthesis (iScript, BioRAD). For RT-PCR, 1 µl of 1:10 diluted cDNA was used as template in a 20 µl reaction. RT-PCR conditions for all four serine proteases were: Initial denaturation at 95ºC for 5 min, 95ºC for 30 sec, 55-58ºC for 30 sec (Table 1), 72°C for 30 sec, repeated for 35 cycles and final extension at 72ºC for 10 min. 10 µl of the reaction was separated by electrophoresis on a 1.2% agarose gel along with DNA ladder (Apex DNA Ladder II; Genesee) and visualized by using Amresco ethidium bromide free dye (Amresco). Primer sequences for all proteases are listed in Table 1. Tubulin was used as a housekeeping control [12].

Table 1. A list of primer pairs and the annealing temperature used for each primer pair.

Gene Name and Accession Number	Primer Pair Sequence	Annealing Temp ºC
Cath D (Aspartic) ISCW023880/ XM_002416473.1		
IscapCathDFwd	CCCTTCCGTGTGGTGTTTG	55
IscapCathDRev	AGTAGCCCTTGGTTGAGACAG	
Cath L (Cysteine) ISCW024899/ XM_002416305.1		
IscapCathLFwd	GACTTCCAGATGTACCAGGGC	55
IscapCathLRev	GAAGGATGCGGAAGTAGCCG	
Cath L (Cysteine) ISCW000076/ XM_002404428.1		
IscapCathL2Fwd	AAGTGGCCCCACTGCAACTC	55
IscapCathL2Rev	TTACCCGTAACCGCAGGAATG	
Legumain (Apartic) ISCW015983/ XM_002402043.1		
IscapLegumainFwd	CCCCTGGAGTGGTCATCAAC	55
IscapLegumainRev	TAAGTGTTTCGGAGGGCGTC	
Cath C (Cysteine) ISCW03494/ XM_002400742.1		
IscapCathCFwd	CGTTAACTACGTGTCCCCTG	57
IscapCathCRev	TAGTTGCCGACGTAATGCC	
Serine Protease 1 ISCW021184/ XM_002405400.1		
IscapSP1Fwd	AGCCTAATCAATCAAGGGCG	58
IscapSP1Rev	GACCAGTTTAGGGATGCGAG	
Serine Protease 2 ISCW006427/ XM_002435219.1		
IscapSP2Fwd	ATCCACGTTGGGAACCTTTC	58
IscapSP2Rev	CAATGGTCAAACGCCTTTCC	
Serine Protease 3 ISCW010371/ XM_002402819.1		
IscapSP3Fwd	TCTACGAGTTCCTGGGACAG	58
IscapSP3Rev	GGACCAGGGAATAATCGTCG	
Serine Protease 4 ISCW007492/ XM_002404245.1		
IscapSP4Fwd	GCTTCGTCGAAAAAGCTCAC	58
IscapSP4FRev	CAACTCTCGGCGATCTCTTC	
Leucine Aminopeptidase ISCW023735/ XM_002416067.1		
IscapLAPFwd	ACGCCCATTCTCTCACCAAG	55
IscapLAPRev	TTCGGACCCACTGCATTCTC	
Tubulin ISCW005137/ XM_002402966.1		
IscapB-TubFwd	TGAATGACCTGGTGTCCGAG	55-58
IscapB-TubRev	GCAAAGCTGTTCAAGCCTCT	

Gel images were visualized and band intensity was analyzed by densitometry using Image Lab 5.2.1 (Gel Doc EZ-Imager, BioRad). The DNA ladder was used as the standard for generating a linear regression model to determine PCR product abundance. The ratio of the tubulin control bands intensity was used to standardize values of the band from each gene at different time points.

Sample Preparation for BApNA assay: Six larvae or nymphs from each post blood meal time point were collected in triplicates (two individuals per triplicate). Samples were sonicated in 100 µl

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Tris-HCl-$CaCl_2$ buffer until completely homogenized and centrifuged at 12,000 RPM for 5 min at 4ºC. The supernatants from each individual sample were transferred to clean 1.7 ml tubes. 10 µl supernatant from each sample was added to 90 µl of Tris-HCl-$CaCl_2$ [11]. Finally, 200 µl of 4 mM BApNA was added to the samples. Samples were then placed on a shaker for 15 min, and loaded onto a 96 well plate (100 µl sample per well).

Midguts were dissected and pooled from two adults per sample. Adult midgut samples were prepared in a similar manner as described above. Trypsin (Sigma) was used to make a standard curve. Trypsin, in the presence of BApNA, cleaves p-nitroanliline off BApNA, yielding a yellow substrate that was measured at 405 nm. This is a trypsin-specific reaction that does not occur with cathepsins or chymotrypsins or other known digestive enzymes [13]

Gut lumen pH assay: Guts from unfed, partially engorged (4 days post host attachment), and engorged ticks 1-day post detachment were dissected. Blood fed guts were washed with PBS, dried and then homogenized with a plastic pestle. Unfed guts were directly homogenized. pH was measured with a universal Whatman indicator pH paper (pH range 4.0-10.0 and 1.0-11.0; Millipore Sigma).

Statistical Analysis: All experiments were replicated a minimum of three times with different biological cohorts. One-way ANOVA and Dunnet's multiple comparison were used for statistical analysis. Unfed ticks were used as a control. All data from each biological replicate was combined and significance was calculated using Graph Pad software.

3. RESULTS

Transcript expression: Transcripts of 10 proteases identified as the main proteolytic enzymes for hemoglobin degradation in *I. scapularis* [2] were examined in adult female midgut. Out of these 10, four were serine proteases (ISCW021184, ISCW006427, ISCW010371, and ISCW007492), two cathepsin L (ISCW024899 and ISCW000076), and one each of cathepsin C (ISCW03494), cathepsin D (ISCW023880), legumain (ISCW015983), and leucine aminopeptidase (ISCW023735). All sequences were confirmed by Sanger sequencing. Expression was determined at different time points: partially engorged adult females (collected 5 days post host attachment but before rapid engorgement), and at 1, 2, 7, and 14 days post host detachment (fully engorged and actively digesting blood to provision developing eggs). Out of 10 genes tested, six were expressed in unfed samples whereas four genes (ISCW023880, ISCW024899, ISCW007492, and ISCW023735) were only expressed during feeding or post blood meal (PBM) (Fig. 1 A-B). Cathepsin D expression was highest at day 1 PBM and decreased afterwards. One out of two cathepsin L paralogues (ISCW024899) expressed in all blood fed stages tested, from partially fed to 14 day PBM and the other cathepsin L (ISCW000076) expressed in unfed and partially fed females with almost no expression in fully engorged females. Legumain was expressed at low levels in unfed females, then expression increased during and after feeding. Cathepsin C also expressed at low levels in unfed females and expression was higher during and after a blood meal; peak expression was at 2 and 7 D PBM and decreased at day 14 PBM. Serine protease 1 and 2 (ISCW021184 and ISCW006427) had a similar expression pattern to one another where both expressed in unfed and partially fed females and then expression was undetectable until day 14 PBM. Serine protease 3 (ISCW010371) was faintly expressed in unfed and day 14 PBM samples. Serine protease 4 (ISCW007492) was not detected in unfed females, but expression was detected during and after blood feeding with the highest expression in 1 D PBM samples. Leucine aminopeptidase expression was not detected in unfed females and peak expression was noticed at partially engorged time point. Expression decreased afterwards and no expression was detected at day 14 PBM (Fig. 1 A-B).

Active trypsin in tick life stages: Trypsin activity increased significantly in larvae after a blood meal (Fig. 2 A). The peak trypsin activity period was 1-3 days post host detachment and levels decreased gradually afterward to nearly unfed levels at 21-28 days PBM (Fig. 2A). In our colony, larvae begin molting into nymphs within 3-4 weeks; therefore, the 28 day time-point coincides with molting.

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A

Gene	Accession ID	Timepoints: UF	PE	1D PBM	2D PBM	7D PBM	14D PBM	Size (bp)
Cathepsin D	ISCW023880							504
Cathepsin L	ISCW024899							210
Cathepsin L	ISCW000076							182
Legumain	ISCW015983							432
Cathepsin C	ISCW003494							308
Serine Protease 1	ISCW021184							382
Serine Protease 2	ISCW006427							397
Serine Protease 3	ISCW010371							512
Serine Protease 4	ISCW007492							581
Leucine Aminopeptidase	ISCW023735							714
Tubulin (Control)	ISCW005137							107

B

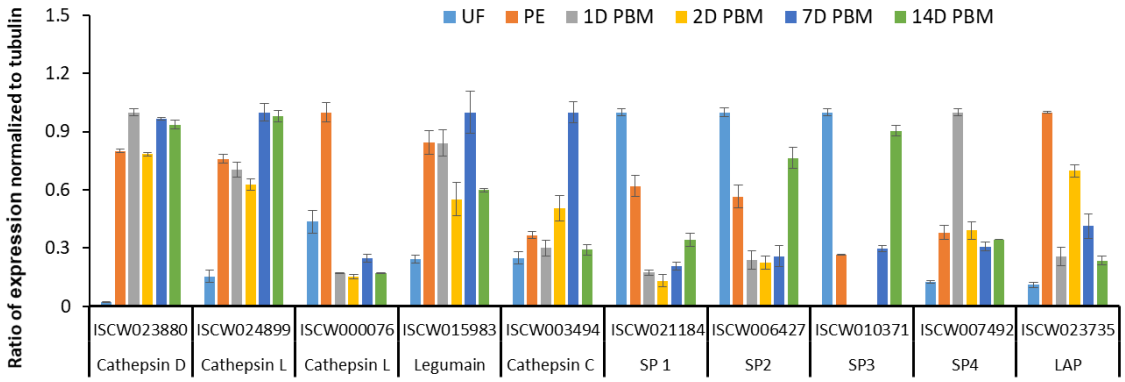

Figure 1. Transcript expression of proteases in the midgut of adult female *Ixodes scapularis*. **A:** Representative RT-PCR of ten *I. scapularis* proteases that belong to different families was carried out at different time points during blood feeding and digestion. Total RNA was extracted from a pool of 2-4 midguts at each time point and an equal amount of cDNA was used for RT-PCR. **B:** The BioRad Gel Doc EZ Images were used to determine concentrations of PCR product in each lane across the gel for each gene. The ratio of concentrations of the protease and tubulin (housekeeping gene) PCR products were calculated. After calculating the ratios, the band with highest expression in each gene was set at 1 and the values were determined accordingly and plotted to accurately depict the change in expression of each gene over time. UF= unfed female midgut, PE= partially engorged (females were pulled from the host 5 days post attachment), PBM= post blood meal (fully engorged females dropped off the host). SP= serine protease; LAP= Leucine Aminopeptidase.

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In nymphs, a similar pattern to larvae was observed. Trypsin levels increased after a blood meal and were highest at 3 days post host detachment. Subsequently, levels decreased and by 28 days trypsin levels were similar to unfed controls.

In adult midguts, no trypsin activity was detected in unfed or partially fed ticks. Trypsin activity was highest after drop off from the host (1 D PBM) and decreased gradually. By 14 D PBM, trypsin activity returned to unfed levels. Under our rearing conditions, ticks start laying eggs 7 days PBM; therefore, most blood digestion occurs during the first two weeks post host detachment.

Gut pH assay: The pH of unfed tick guts was 7.0 (neutral). Once the tick began feeding on vertebrate blood, midgut pH changed to alkaline (7.5-8.0) and remained at this pH throughout the engorgement and post detachment period.

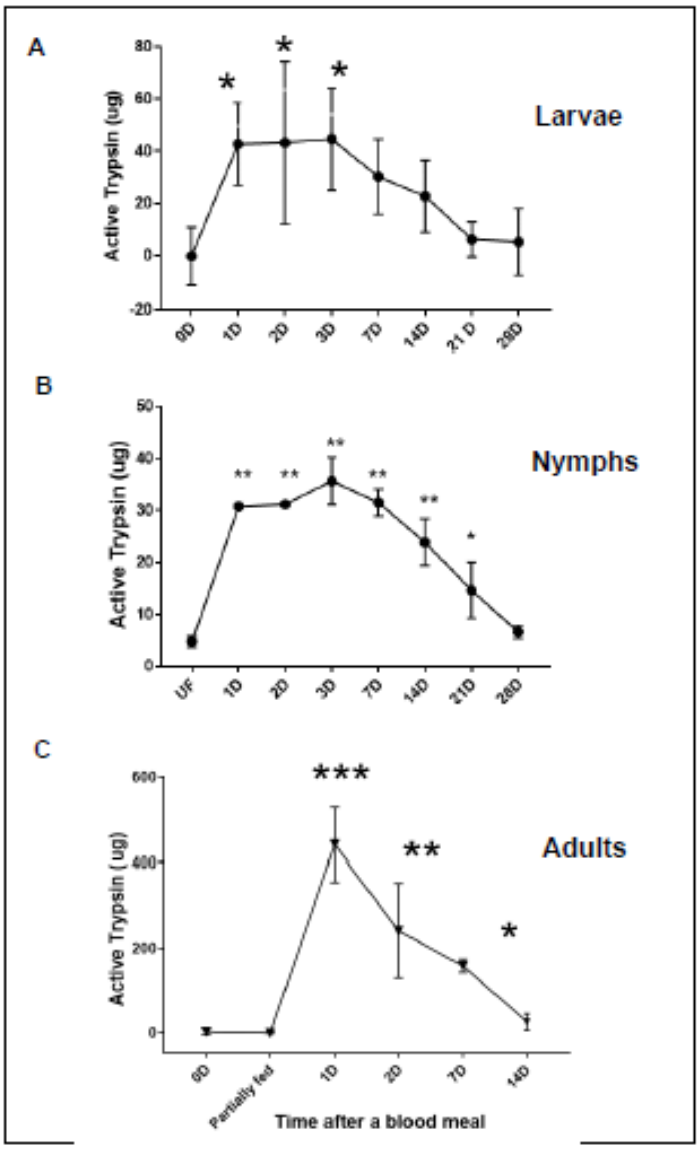

Figure 2. Trypsin activity in developmental stages and adult female. Trypsin activity was measured by the BApNA assay in unfed and blood fed ticks. **A:** Larvae and **B:** Nymphs were fed on a mouse and engorged larvae or nymphs that dropped off of the host were collected. Whole body of larvae and nymphs were homogenized for the assay. Unfed larvae and nymphs were used as a control. **C:** Adult females were fed on a sheep. Females were either pulled off the host 5 days post attachment (partially engorged) or fully engorged females that dropped off the host were collected over several days. Midguts were dissected for determination of trypsin activity. Unfed female midguts were used as a control. One-way ANOVA and Dunnet's multiple comparison were used for statistical analysis. *= 0.01; **= 0.001; ***= 0.0001.

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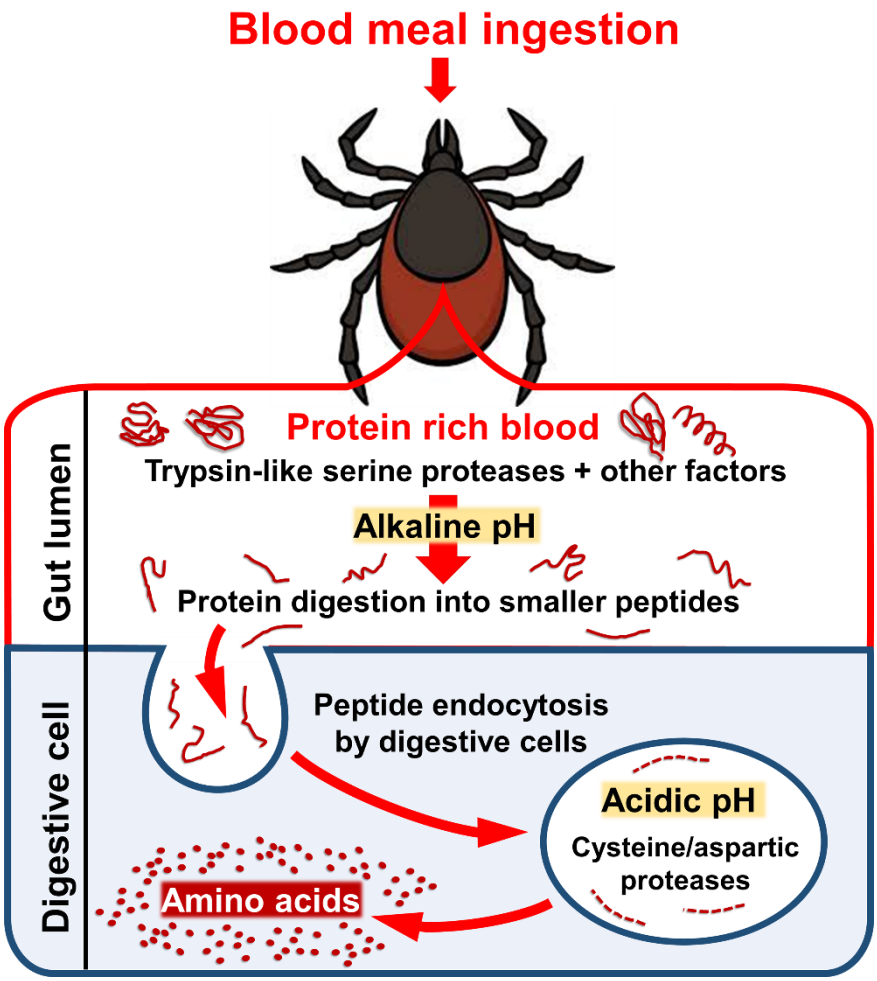

Figure 3. Proposed model of *I. scapularis* blood digestion. In this model, ingested blood proteins are first partially digested in the gut lumen where higher pH is suitable for trypsin-like enzymes. Degraded peptides are then taken into endosomes of digestive cells where low pH allows catalysis by cathepsins and other proteases that are active at acidic pH, eventually breaking the peptides into dipeptides or free amino acids.

4. DISCUSSION

In the present work, we examined an *I. scapularis* trypsin-like serine protease transcript (ISCW007492) that had increased expression during and after a blood meal and two serine proteases (ISCW021184 and ISCW006427) with high expression before a blood meal. Trypsin activity from whole body (larvae and nymphs) and midgut extracts (adult ticks) was tested by the BApNA assay in all different life stages of *I. scapularis* during blood feeding and digestion. Our trypsin assay showed that unfed ticks had no trypsin activity in gut. However, trypsin activity increases after detachment from the vertebrate host and peaks at 1-3 days post detachment in all life stages. This is in concordance with a rise in midgut lumen pH to become slightly alkaline (pH 8.0), optimal for trypsin activity.

Most previous studies have focused on the mechanism of hemoglobin degradation in ticks and suggested that the main peptidases for hemoglobin digestion are: (1) clan CA cathepsins B, C, and L; (2) clan CD asparaginyl endopeptidase (legumain); and (3) clan AA cathepsin D. Other activities detected were attributed to monopeptidases, namely a serine carboxypeptidase and a leucine metallo-aminopeptidase within gut digestive vesicles [13]. Our work suggests that protein digestion might begin in the gut lumen where the environment is more alkaline and best suited for trypsin-like enzymes. These digested peptides from the gut lumen may then be taken up by the digestive vesicles by endocytosis, as shown previously [6].

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Previous studies have also suggested hemoglobin receptor-mediated endocytosis [6, 14] occurs in the digestive vesicles. Digestive vesicles then lead to further breakdown of these peptides by creating an acidic environment suitable for cathepsin and legumain activity. Therefore, we propose a modified model of blood digestion in ticks (Fig. 3), that blood digestion occurs in both the gut lumen and digestive cells, and both trypsin-like serine proteases and cathepsins are important for degradation of blood proteins. Our results are supported by Ribeiro [15] where midgut homogenates of *I. scapularis*, (formerly *I. dammini*) lysed erythrocytes from rabbits, rats, hamsters, and guinea pigs. The midgut homogenate activity was optimal at an alkaline pH, suggestive of trypsin-like serine proteases, and this hemolytic activity was lost when homogenates were heated for 1 h at 60ºC. This activity was not detected in unfed ticks as well as ticks attached for up to 2 days to a host, and increased during the later phase of feeding. Ribeiro [15] hypothesized that this activity helped the initial process of the blood meal digestion by releasing the contents of erythrocytes for further enzymatic hydrolysis, possibly in the digestive vesicles. Our results also support this hypothesis. Two serine proteases in *Haemaphysalis longicornis* ticks were identified and characterized, and expression of both serine proteases was induced by blood feeding [16].

A remarkable property of certain insect guts is a very high luminal pH, especially in lepidopteran larvae (pH 9-12). Whereas the mosquito, *Aedes aegypti*, the midgut has an acidic pH (6.0) before a blood meal, the pH increases to an alkaline range (7.5) after a blood meal [17]. The pH of the guts of mites also strongly affects their digestive processes. For instance, gut contents of acaridid mites ranged from pH 4 to 7 [18]. Although our current method of homogenizing the gut and measuring pH suggested a rise from neutral to alkaline pH upon the consumption of a blood meal, further refinement in measurement is needed. For instance, the pH in guts of 12 species of stored product and house dust mites was determined based on the color changes of pH indicators fed to the organisms and looking at pH change microscopically. Unfortunately this is not feasible with ticks due to the dark cuticle and blood meal coloration. However, microelectrodes are frequently used to determine gut pH in insects [19-22], and we plan to utilize these in future experiments.

In this study, we correlate the serine proteases expression with trypsin activity from *I. scapularis* gut homogenates. However, direct evidence of these serine proteases as digestive proteases that can break down blood proteins remains to be confirmed. Future experiments will include the use of recombinant *I. scapularis* serine proteases for blood protein digestion assays *in vitro.* In addition, knockdown of serine protease 1, 2, and 4 using RNAi will further clarify the effect of these enzymes in *I. scapularis* blood digestion. Also, this initial exploration examined the most prominent *I. scapularis* digestive enzymes, which only included 4 out of a putative 63 serine proteases present in the genome [3]. In future studies, a more expansive screen will yield additional information on the dynamics of *I. scapularis* digestive enzymes important in blood digestion.

5. CONCLUSIONS

Most studies in ticks suggest that blood digestion occurs in the acidic environment of midgut vesicles by the cathepsin-like peptidases. In this study, we show that the trypsin-like proteases may play a significant role in initial blood protein breakdown in the alkaline gut lumen, and together both cathepsins and serine proteases lead to complete blood protein digestion. One of the major advantages of characterizing the serine proteases that regulate blood digestion is that they are generally secreted in the extra cellular environment and hence they are likely to be exposed to host antibodies [23, 24], making them suitable for anti-tick vaccine candidates.

Author Contributions: MGN and AN conceived the project and designed the experiments. JR carried out RT-PCR work. CAC and JR did the active trypsin assay. JR did densitometry and statistical analysis. MGN did gut pH assay. AN and MGN wrote the manuscript.

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Conflicts of Interest: We declare no conflict of interest.

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