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

Jeremiah Reyes[1], Cuauhtemoc Ayala-Chavez[1], Michael Pham[1], Arvind Sharma[1], Andrew Nuss[1, 2*], Monika Gulia-Nuss[1,*]

[1]Department of Biochemistry and Molecular Biology

[2]Department of Agriculture, Veterinary, and Rangeland Sciences

University of Nevada, Reno, Reno, NV, 89557, USA

*Corresponding authors; nuss@cabnr.unr.edu; mgulianuss@unr.edu

ABSTRACT

Ixodes scapularis is the major vector of Lyme disease in the eastern United States. Each active life stage (larva, nymph, and adult) 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 and therefore blood digestion is very important for tick survival. Most studies on blood digestion in ticks have shown that the initial stages of digestion are carried out by cathepsin proteases within acidic digestive cells. However, most of these studies have focused on partially engorged ticks. In other hematophagous arthropods, the serine proteases play an important role in blood protein degradation. In this study, we determined transcript expression of four *I. scapularis* serine proteases with previously characterized roles in blood digestion. RNA interference was used for functional analysis and a trypsin-benzoyl-D, L-arginine 4-

nitoanilide assay was used to measure active trypsin levels. An *in vitro* hemoglobinolytic assay was performed with or without serine protease inhibitor. Our data suggest that trypsin levels increase significantly after blood feeding and peaked in larvae, nymphs, and adults at 3, 1, and 1 day post host detachment, respectively. The knockdown of three previously identified serine proteases by RNAi negatively impacted blood intake, survival, fecundity, levels of active trypsin in the gut and resulted in lower hemoglobin degradation *in vitro*. A trypsin inhibitor, PMSF, blocked the action of trypsin in the gut extract resulting in 65% lower hemoglobin degradation. We provide evidence of the serine proteases as digestive enzymes in fully engorged, replete females. Our data also demonstrated that in addition to blood digestion, these serine proteases might have a role in blood feeding success in *I. scapularis*.

Key words: Ticks, *Ixodes scapularis*, serine protease, blood digestion, trypsin

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 before 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. Out of a total of 233 putatively active *I. scapularis* proteases thus far identified, 63 (27%) are serine proteases [3]. 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.

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 vertebrates blood at an alkaline pH suggesting the involvement of trypsin enzymes. Ten major blood digestive proteases (cathepsin, 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.

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-nitroanilide (BApNA), a trypsin-specific substrate [10-11]. Our results suggest that tick serine proteases are active within 24 h post host detachment (replete) 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 the basic biology of ticks and may lead to new methods for tick control.

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 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.

Sample collection: Larvae and nymphs were collected at day 1, 2, 3, 7, 14, 21, and 28 days post engorgement/ host detachment (repletion). 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 (replete). Whole guts were dissected and two guts were pooled per sample. Experiments were replicated with three biological cohorts. 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. Total RNA was extracted using Trizol reagent and a Zymo Directzol kit (Zymo Research, Irvine, California). Total RNA was DNase treated before using for cDNA synthesis. 1 µg DNAse-treated RNA was used for cDNA synthesis (iScript, BioRad, CA). For RT-PCR, 1 µl of 1:10 diluted cDNA was used as a 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 a final extension at 72ºC for 10 min. 10 µl of the reaction was separated by electrophoresis on a 1.2% agarose gel along with the 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].

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 band intensity was used to standardize the 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 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 at 25ºC, and loaded onto a 96 well plate (100 µl sample per well).

Midguts were dissected and pooled from two adult females per sample and 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 chymotrypsin or other known digestive enzymes [13].

RNA interference (RNAi): dsRNA was synthesized for three serine proteases: SP1 (ISCW021184), SP2 (ISCW006427), and SP4 (ISCW007492). Total RNA was extracted from unfed or one-day post host detachment tick guts using Trizol reagent and Zymo Directzol kit. DNAse treated RNA was used as the template for cDNA synthesis as described above. Primers were designed with a T7 promoter sequence on the 5' end of both forward and reverse primers (Table 1). RT-PCR conditions were the same as mentioned above in the RT-PCR section. PCR products were run on a 1.2% agarose gel. Bands were extracted from the gel using the QIAquick gel extraction kit (Qiagen) and used as a template for dsRNA synthesis using T7 Megascript kit (Invitrogen, CA). Newly synthesized dsRNA was purified using phenol-chloroform and ethanol precipitated. Sixteen unfed adult female ticks per gene were injected with 1µL of dsRNA (2µg/µL). Injections were performed with a u-200 insulin syringe on the ventral right side between the 3rd and 4th leg of the tick. Control ticks were injected with 1µl of RNase/DNase/Protease free water (11 ticks). Control and RNAi ticks were immediately placed in a holding container at 95% RH and observed for 2 h for recovery before storage. Ticks were allowed to recover for 7 days before placing on New Zealand white rabbits. Ticks were confined in capsules attached to the rabbit's back using Lamar adhesive and were allowed to feed to repletion. Capsules were made of 1.5 inch PVC tube with a Styrofoam lip attached to side going on the rabbit. Dropped off ticks were collected daily, weighed and photographed immediately after dropping off and stored in individual containers in an incubator at 20^{o}C and 95% RH. A batch of females was kept

for fecundity assessment. Females were observed daily for mortality and egg-laying. Egg mass was weighed once females stopped laying eggs.

For BApNA assay, six RNAi adult females from each of the three serine proteases were collected 1-day post drop off/repletion. Control ticks were collected at the same time. Two guts per sample were dissected (N=3) and the assay was carried out as described above.

Hemoglobin degradation assay: Midguts were dissected individually from control and RNAi females collected one-day post host detachment (hereafter post blood meal; PBM), washed, and homogenized with a pestle in 0.1 M Na-acetate, 1% CHAPS, and 2.5 mM DTT [8]. The gut extracts were centrifuged at 16000g for 10 min at 4^{o}C and filtered with a 0.22µM Polyethersulfone (PES) membrane syringe filter (Olympus). Protein concentration was measured using the BCA protein assay kit (Thermo Fisher, MA). The gut protein extracts were stored at -80^{o}C until used for assays. 0.5µg protein extract was used to digest 10µg of bovine hemoglobin in 25mM Na-citrate-phosphate (pH 7.5), 2.5mM DTT, and 25mM NaCl. Bovine trypsin (Sigma, MO) was used as a control for hemoglobin digestion. Aliquots were taken out at 0, 10, 20, and 30 min. 0.03% fluorescamine (Biotium, CA) in acetone was added to the gut extract-hemoglobin reaction to quantify the newly formed amino-terminal ends [14]. Fluorescence was measured using the Spectramax M5 microplate reader at an excitation of 370 nm and emission of 485 nm wavelengths. Measurements were performed in triplicate. For trypsin activity inhibition, gut extracts were pre-incubated with 0.1mM PMSF (Research Products International, IL) for 15 min at 37^{o}C before adding hemoglobin.

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

RESULTS:

Transcript expression: Transcripts of 10 proteases identified as the main proteolytic enzymes for hemoglobin degradation in *I. scapularis* [2] were examined in the 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 PBM (Fig. 1 A-B). Cathepsin D expression was highest at day 1 PBM and decreased afterward. One out of two cathepsin L paralogues (ISCW024899) expressed in all blood-fed stages tested, from partially fed to 14 days 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 proteases 1 and 2 (SP 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 (SP3; ISCW010371) had low expression in unfed and day 14 PBM samples. Serine protease 4 (SP4; ISCW007492) was not detected in unfed females, but the 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 afterward 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 PBM and levels decreased gradually afterward to nearly unfed levels at 21-28 days PBM (Fig. 2 A). In our colony, larvae begin molting into nymphs within 3-4 weeks; therefore, the 28 day time-point coincides with molting.

In nymphs, a similar pattern to larvae was observed. Trypsin levels increased after a blood meal and were highest at 3 days PBM. Subsequently, levels decreased and by 28 days trypsin levels were similar to unfed controls (Fig. 2B).

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 (Fig. 2C). Under our rearing

conditions, ticks start laying eggs 7 days PBM; therefore, most blood digestion occurs during the first two weeks post host detachment.

Effect of serine proteases RNAi on tick blood-feeding and physiology: We attempted to knock down all four serine proteases (Fig. 1); however, we could not get sufficient concentrations of SP3 dsRNA. Therefore, we knockdown three serine proteases (SP1, SP2, and SP4). All three serine proteases were knockdown until at least two days PBM (Fig. 3A). Serine proteases knockdown resulted in a significantly lower volume of blood ingested as indicated by the engorgement weight. Control females weighed ~200 mg whereas, SP1 knockdown weighed ~140 mg, accounting for a 29% reduction in weight. SP2 knockdown females weighed ~110 mg (45% reduction) and SP4 knockdown weighed ~78 mg (61% reduction in weight) (Fig 3 B).

Serine protease RNAi also resulted in higher mortality in ticks. About 20% of ticks died in all treatments during the recovery period post-injection. Once attached to the host, all control ticks fed to repletion whereas, two SP1 RNAi, and six each of SP2 and SP4 RNAi ticks died between day-2 and day-5 post attachment (Fig. 3C).

Serine protease knockdown ticks had reduced fecundity as indicated by the egg mass weight. The egg clutch weight in controls was ~100 mg whereas it reduced to 54 mg in SP1 and 22 mg in SP4. SP2 RNAi female did not produce any eggs (Fig. 3D). These data are very interesting; however, a small sample size (3 control females, 5 SP1 RNAi females, and one each for SP2 and SP4 RNAi), requires further validation especially for SP2 and SP4 RNAi.

There was significantly less overall active trypsin in the gut in RNAi females compared to the control at day-1 PBM (peak trypsin activity). The control ticks had ~400 mg trypsin compared to 95 (76% reduction), 240 (40% reduction), and 140 mg (65% reduction) in SP1, SP2, and SP4 RNAi females, respectively (Fig. 3E).

Hemoglobin degradation by tick gut extract *in vitro*: Bovine hemoglobin incubated with tick gut extracts at 7.5 pH resulted in free amino-terminal ends indicative of hemoglobin digestion by the gut extract. Serine protease RNAi decreased this activity further suggesting that these proteases are involved in hemoglobin degradation and therefore blood digestion. SP1 and SP2 knockdown resulted in significantly different hemolytic activity compared to the control at 30 minutes; however, there was no significant difference at 10 and 20 minutes (Fig. 4A). At 30 minutes incubation, SP1 and SP2 knockdown resulted in 29 and 25% reduction in hemoglobin breakdown activity (fluorescent activity), respectively. SP4 knockdown had the greatest effect on hemoglobin breakdown and the activity differed significantly from the control starting at 10 minutes and resulted in a 52% reduction in gut extract hemolytic activity at 30-min. incubation (Fig. 4 A).

To confirm the trypsin-like protease activity in the gut tissue extract of fully engorged tick females, we incubated gut extract from water injected ticks with a trypsin inhibitor, PMSF, prior to addition of hemoglobin. Gut extract without PMSF incubation was used as a control. Incubation with PMSF inhibited the gut extract activity by 55%. The hemoglobin degradation activity was significantly lower in PMSF incubated samples starting from 10 minutes, and it was more evident at 30 minutes as there was no increase

in activity with time as opposed to the gut sample without pre-incubation with PMSF (Fig. 4B).

DISCUSSION

In the present work, we examined *I. scapularis* midgut proteases expression including four trypsin-like serine protease transcripts. These serine proteases had a different temporal expression: two were expressed in unfed and partially engorged females, one only in unfed, and one in all blood-feeding stages tested but not in unfed guts (Fig. 1A). Since transcript abundance is not a measure of enzyme activity and higher transcript does not always result in translation, we measured active trypsin levels. Our trypsin assay showed that unfed ticks midguts did not have trypsin activity (Fig. 2C). However, trypsin activity increases after detachment from the vertebrate host and peaks at 1-3 days post detachment in all life stages (Fig. 2A-C). In *Aedes aegypti* mosquitoes, induction of trypsin biosynthesis after the blood meal is a two-phase process. The first phase of trypsin biosynthesis involves the translation of an mRNA transcript that is already there producing early trypsin. The second phase, 7 – 9 h after the blood meal, is activated by the synthesis of a new mRNA transcript that codes for late trypsin [15]. Our transcript and enzyme activity data suggest that like mosquito early trypsin, the mRNA for *I. scapularis* trypsin is already there.

Most previous studies have focused on the mechanism of hemoglobin degradation in ticks during early stages of feeding and suggested that when ticks are actively feeding on the host, 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 [16]. Previous studies have also suggested hemoglobin receptor-mediated endocytosis [6, 16] 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. However, in this study, we investigated blood digestion after repletion and off host, the stage that has not yet investigated. Our data strongly suggest that serine proteases are involved in blood digestion in the post repletion phase. Midgut extract was able to lyse hemoglobin *in vitro* and pre-incubation with trypsin inhibitor reduced this hemolysis activity (Fig. 4A, B). The knockdown of three serine proteases individually resulted in lower levels of active trypsin in BApNA assay (Fig. 3E). Serine protease knockdown also resulted in reduced hemoglobin degradation activity *in vitro* (Fig. 4A). Other studies in replete ticks have also suggested that trypsin proteases might be involved in blood digestion in replete ticks. Ribeiro [17] showed that 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. 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 latter phase of feeding. Ribeiro [17] 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. Two serine proteases in *Haemaphysalis longicornis* ticks were identified and characterized, and expression of both serine proteases was induced by blood-feeding [18]. In another study, two genes encoding trypsin-like serine proteases, *HlSP2* and *HlSP3*, in *H. longicornis* were also proposed to be involved in

blood digestion [19]. One of these *HISP* genes was further characterized and was found to be secreted in the gut lumen [20]. A previous report also demonstrated that hemolysin-like material was present in the midgut lumen of ixodid ticks [21]. Disruption of HlSP-specific mRNA by RNAi resulted in inhibition of the degradation of host erythrocyte membranes, indicating that HlSP plays a crucial role in the hemolysis in the midgut of ticks [20]. An RNAseq study comparing blood-fed and serum-fed *I. ricinus* midgut transcriptome showed that the number of genes encoding serine proteases were markedly up-regulated in the late stage of feeding [22] and a possibility of active serine proteases during the off-host stage of blood digestion was suggested. Given these data in other tick species and our results in *I. scapularis*, we propose a modified model of blood digestion in ticks (Fig. 5). We suggest that blood digestion occurs in digestive cells by cathepsins and aminopeptidases during the early digestive phase when the tick is still feeding, whereas, in replete females trypsin-like serine proteases are important for degradation of blood proteins and digestion might take place in both the gut lumen and digestive vesicles. However, the latter need to be investigated in the replete ticks.

A remarkable property of certain insect guts is a very high luminal pH, especially in lepidopteran larvae (pH 9-12). Whereas the mosquito, *Ae. 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 [18]. The pH of the guts of mites also strongly affects their digestive processes. For instance, the gut contents of acaridid mites ranged from pH 4 to 7 [24]. All the assays in this study were carried out at pH 7.5 (BApNA, Hemoglobin degradation) suggesting that these serine proteases are active at an alkaline pH and provide indirect evidence of an alkaline gut lumen environment. The *I. ricinus* midgut homogenate

activity was also optimal at an alkaline pH [17]. Another study found that a recombinant HlSP enzyme was active at pH 6.0 in *H. longicornis* [20]. We attempted to measure pH by homogenizing guts and using a universal pH paper (data not shown); however, a refined method of measurement is needed. For instance, the pH in guts of 12 species of the 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 [25-28], and we plan to utilize these in future experiments.

The knockdown of three serine proteases resulted in ingestion of lower blood volume (Fig. 3B) that correlates with lower fecundity (Fig. D). High mortality in SP2 and SP4 knockdown females post attachment (Fig. 3C) combined with reduced feeding suggest their additional roles in tick physiology that needs to be further investigated.

Here, we provide direct evidence of serine proteases as active digestive enzymes that can break down blood proteins. Future experiments will include the use of recombinant *I. scapularis* serine proteases for blood protein digestion assays *in vitro.* This initial exploration examined the most prominent *I. scapularis* digestive enzymes, which only included 3 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.

CONCLUSIONS

Most studies in ticks suggest that blood digestion occurs in the acidic environment of midgut vesicles by the cathepsin-like peptidases in partially engorged ticks. In this study, we show that the trypsin-like proteases may play a significant role in blood protein breakdown in fully engorged, replete ticks. One of the major advantages of characterizing the serine proteases that regulate blood digestion is that they are generally secreted in the extracellular environment and hence they are likely to be exposed to host antibodies [29,30], making them suitable for anti-tick vaccine candidates.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article. The midgut RNASeq datasets used during the current study for identification of serine proteases are available from the corresponding author on request.

FUNDING

This work was funded in part through an NIH R21AI128393 grant.

AUTHOR CONTRIBUTION

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 RNAi, densitometry and statistical analysis. MP, AS, and JR carried out hemoglobin degradation and trypsin inhibitor assays. AS performed statistical analysis. AN and MGN wrote the manuscript.

CONFLICT of INTEREST

We declare no conflict of interest.

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507 **Figure Legends**

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

521 **Figure 2: Trypsin activity in developmental stages and adult female.** Trypsin activity
522 was measured by the BApNA assay in unfed and blood fed ticks. **A:** Larvae and **B:**
523 Nymphs were fed on a mouse and engorged larvae or nymphs that dropped off the host
524 were collected. Whole body of larvae and nymphs were homogenized for the assay.
525 Unfed larvae and nymphs were used as a control. **C:** Adult females were fed on a rabbit.

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 and washed 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

Figure 3: Effect of serine protease knockdown on tick feeding, blood digestion, survival, and reproduction. A: Representative RT-PCR of three *I. scapularis* serine proteases. C: control ticks injected with water, RNAi: dsRNA injected ticks. SP: serine protease, SP1=ISCW021184, SP2=ISCW006427, and SP4=ISCW007492. **B:** Wet weight of ticks measured immediately after dropping off host. **C**: Percent mortality after infesting the rabbit. Dead females were counted daily until all dropped off. The percent dead ticks were calculated by combining the numbers from Day-0 (on the rabbit) until day-10 (all females dropped off). **D**: Egg clutch weight. Replete ticks were kept individually in an incubator. Number of eggs deposited each day was counted and egg clutch was weighed once females stopped laying eggs for 2 consecutive days. **E: Active trypsin levels were** measured by the BApNA assay in guts dissected from replete females, one day post host drop off. Unpaired t test with Welch`s correction was used for comparing control and a treatment (SP1, SP2 and SP4) using Graphpad Prism v8. The confidence interval was 95% and significance level was fixed @ $p<0.05$. ***P< 0.0001

Figure 4: **Hemoglobin degradation by gut extract. A**. Gut tissue was dissected from fully engorged tick females (day 1 post repletion and drop off), washed from the gut contents and protein was extracted. Bovine hemoglobin was incubated with either control

or SP1, SP2, and SP4 knockdown gut protein extracts. The relative rate of degradation of the substrate was determined using the measurement of fluorescence in a hemoglobinolytic assay. Aliquots of samples were taken over the course of 30 minutes and quantified using fluorescamine. Values are normalized to the fluorescence intensity at time 0 min and presented in absorbance units (A.U.). The error bars indicate standard deviations of the mean of triplicates. **B**. Peptidolytic activities in the gut tissue extract of fully engorged tick females was demonstrated *in vitro*. Bovine hemoglobin (Hb) was incubated with bovine trypsin or control gut sample. For serine protease inhibition assay, gut extract or bovine trypsin were pre-incubated with serine protease inhibitor PMSF before adding hemoglobin. Aliquots of samples were taken over the course of 30 minutes and quantified using fluorescamine. Values are normalized to the fluorescence intensity at time 0 min and presented in absorbance units (A.U.). The error bars indicate standard deviations of the mean of triplicates. Each treatment was compared with control for each timepoint using unpaired t test with Welch`s correction with 95% confidence interval and $p < 0.05$. *= $p<0.05$; **=$p<0.01$, ***$p<0.0001$

Figure 5: Proposed model of blood digestion in replete females. We suggest that existing model is correct for partially engorged females; however, we propose a new model for replete females. In this model, we suggest that ingested blood proteins are digested in the gut lumen where higher pH is suitable for trypsin-like enzymes. Degraded peptides might then be 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.

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Figure 1

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		Timepoints						
Gene	Accession ID	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

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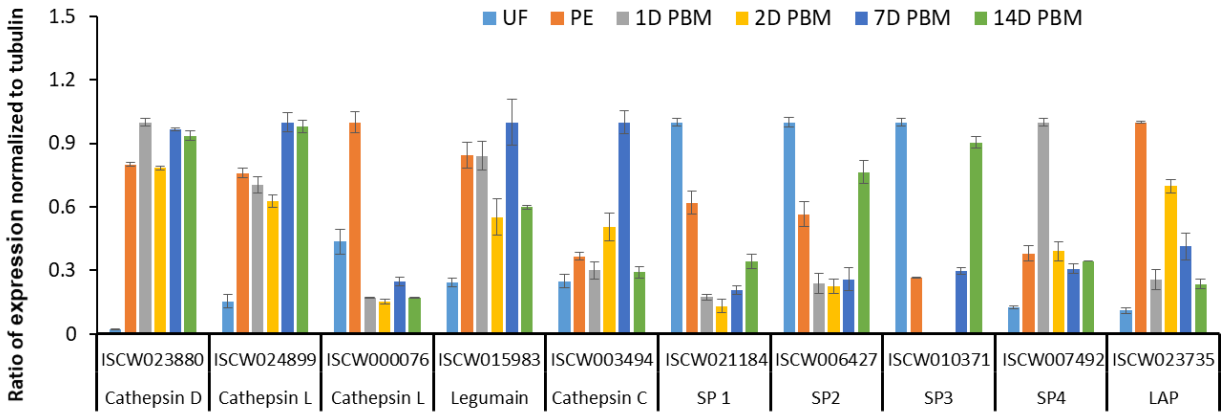

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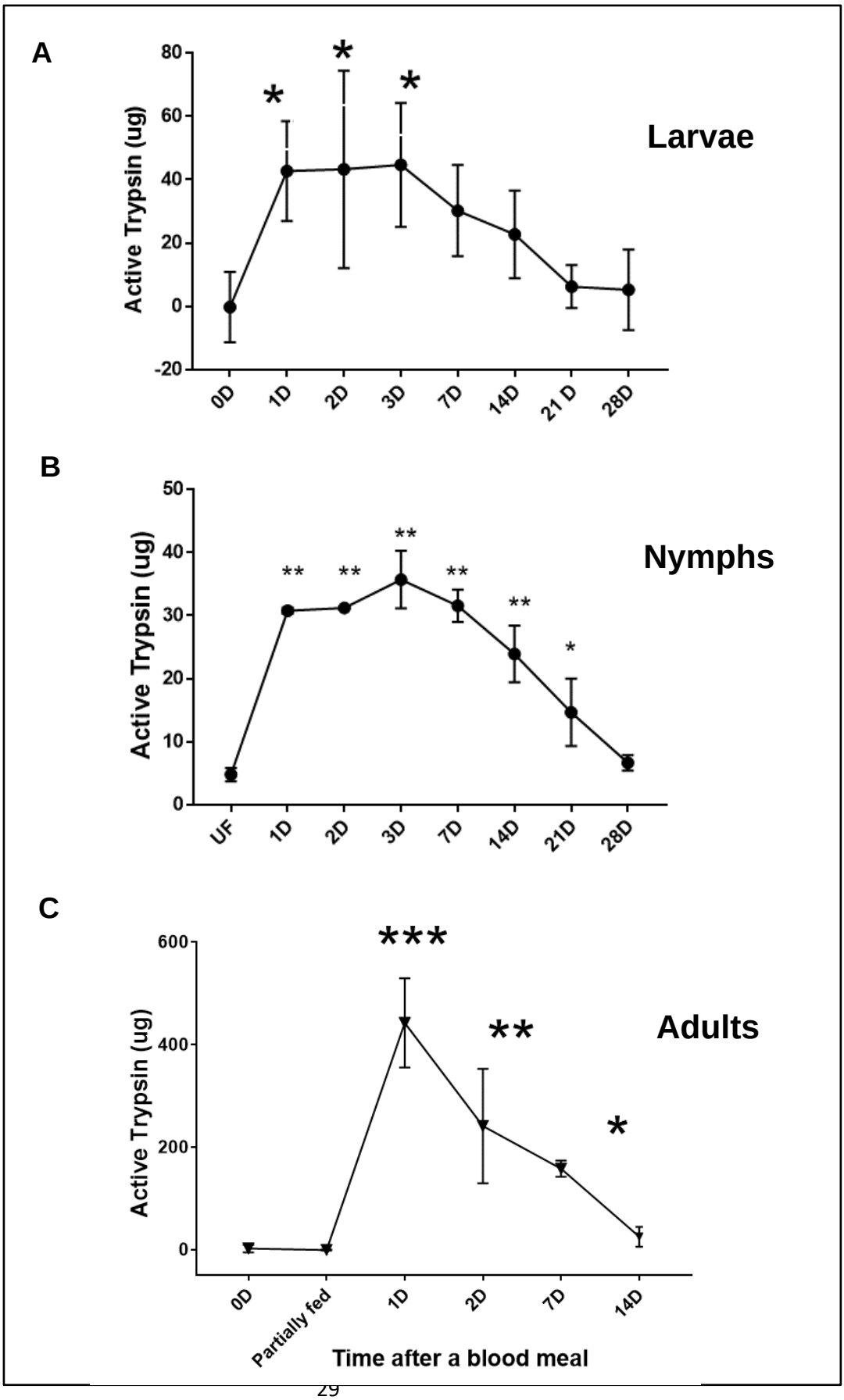
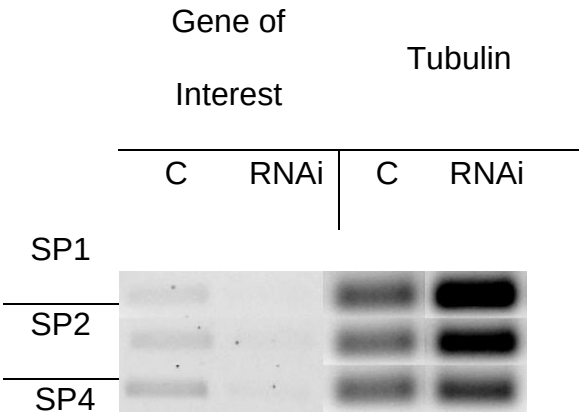
582 **Figure 2**



583 **Figure 3**

584 **A**



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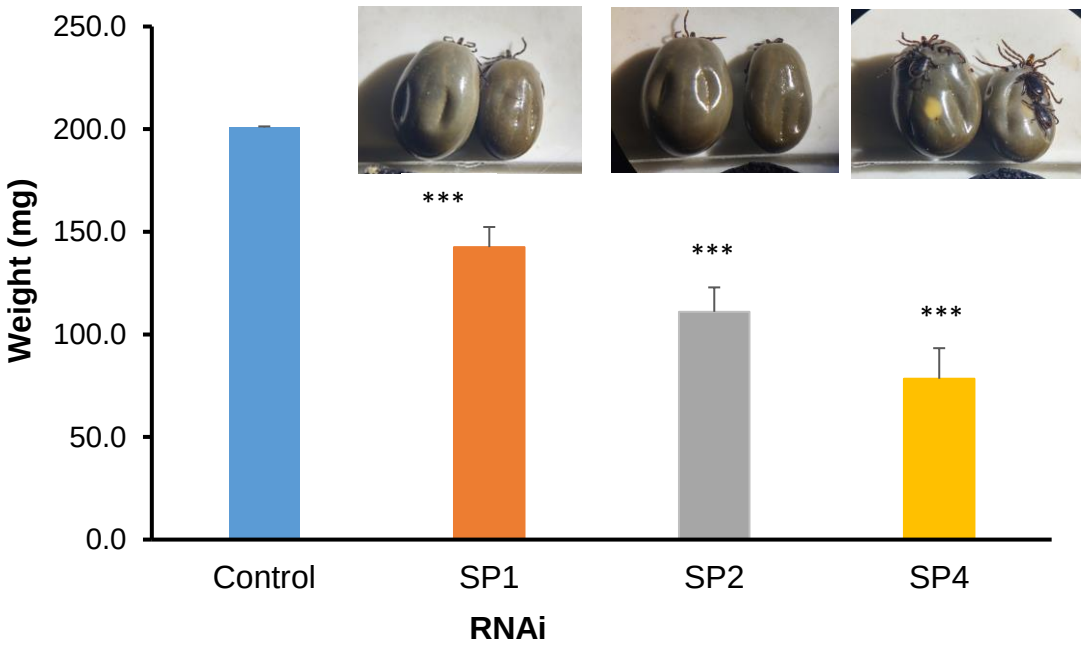
587 **B**



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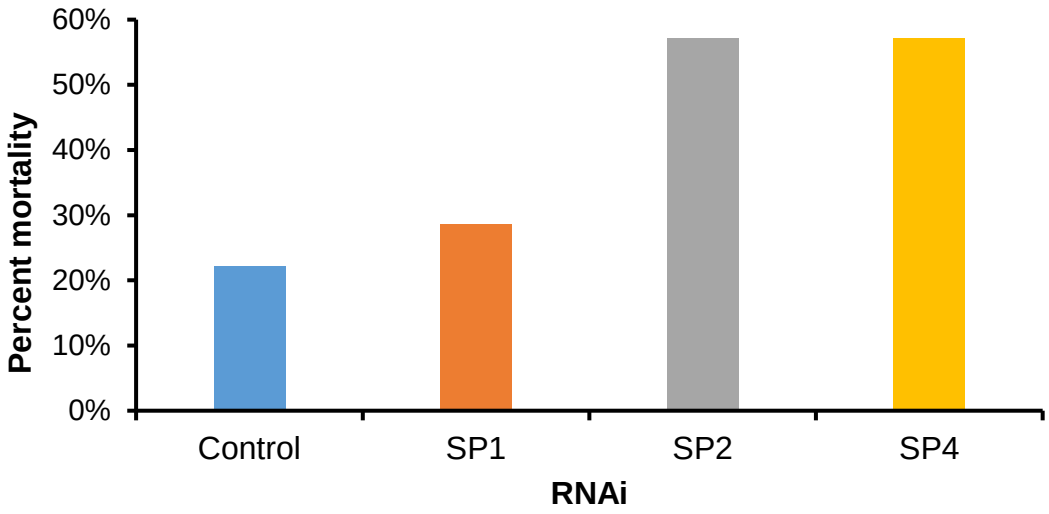
597 **C**



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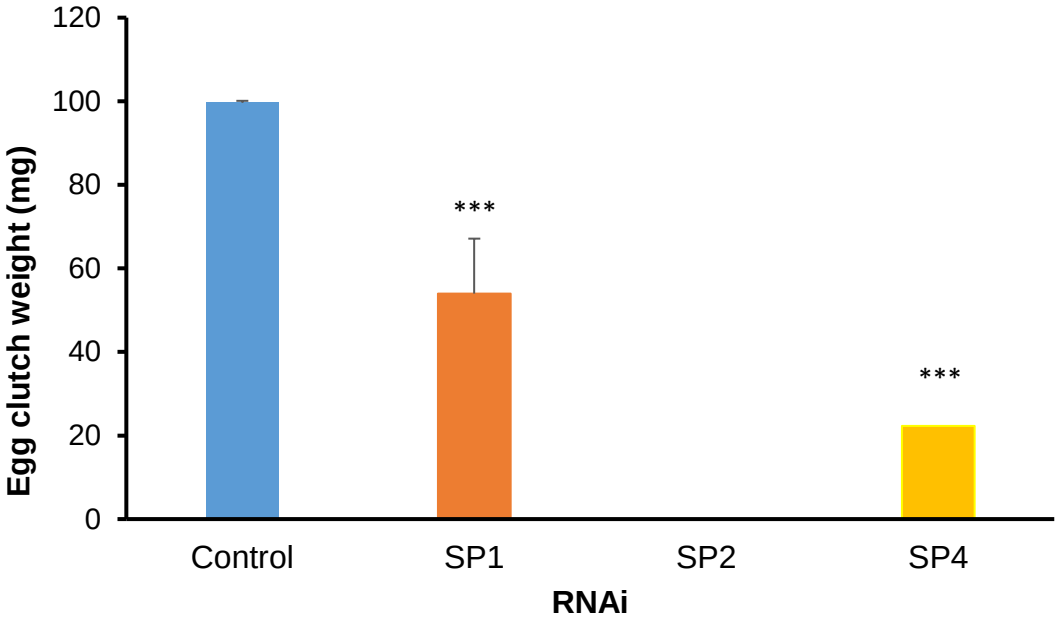
600 **D**



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603 **E**

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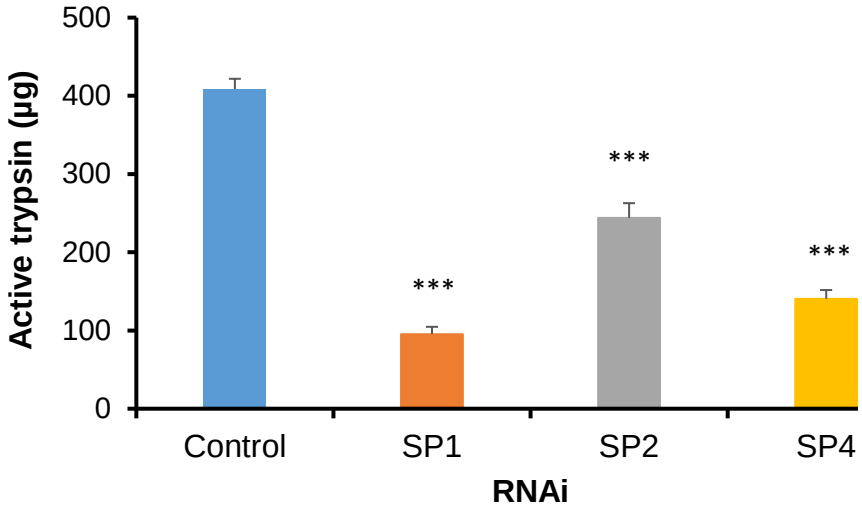

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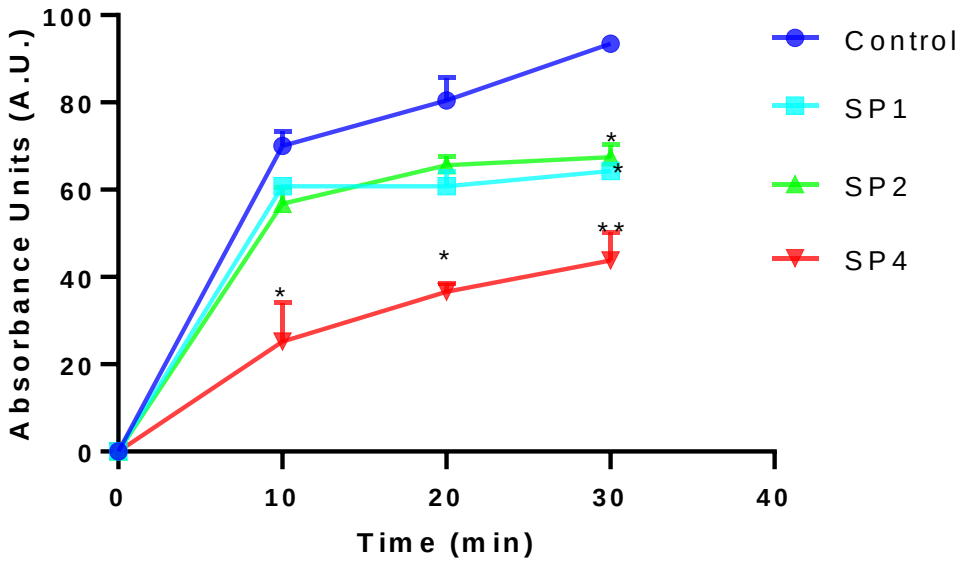
621 **Figure 4**

622 **A**



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625 **B**

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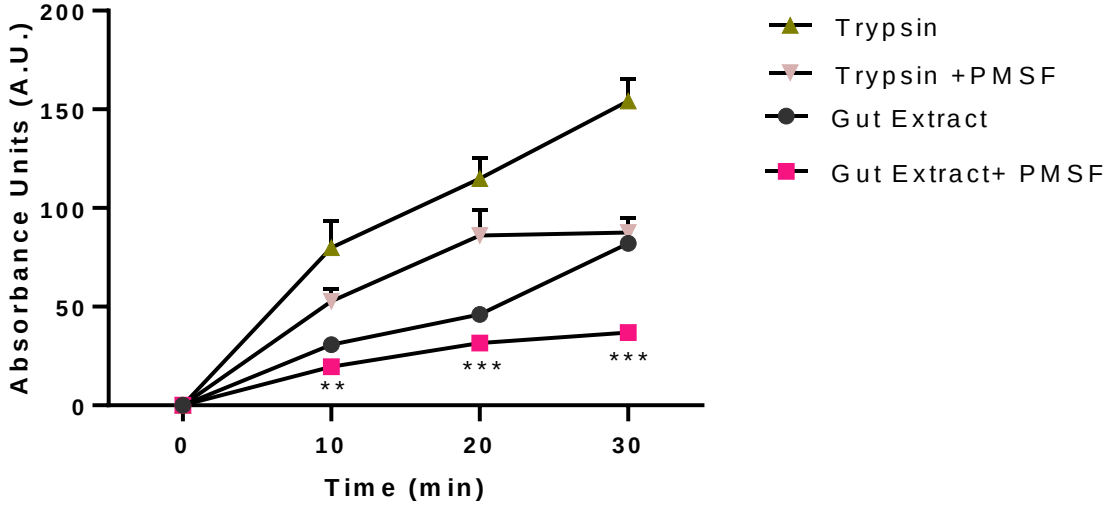

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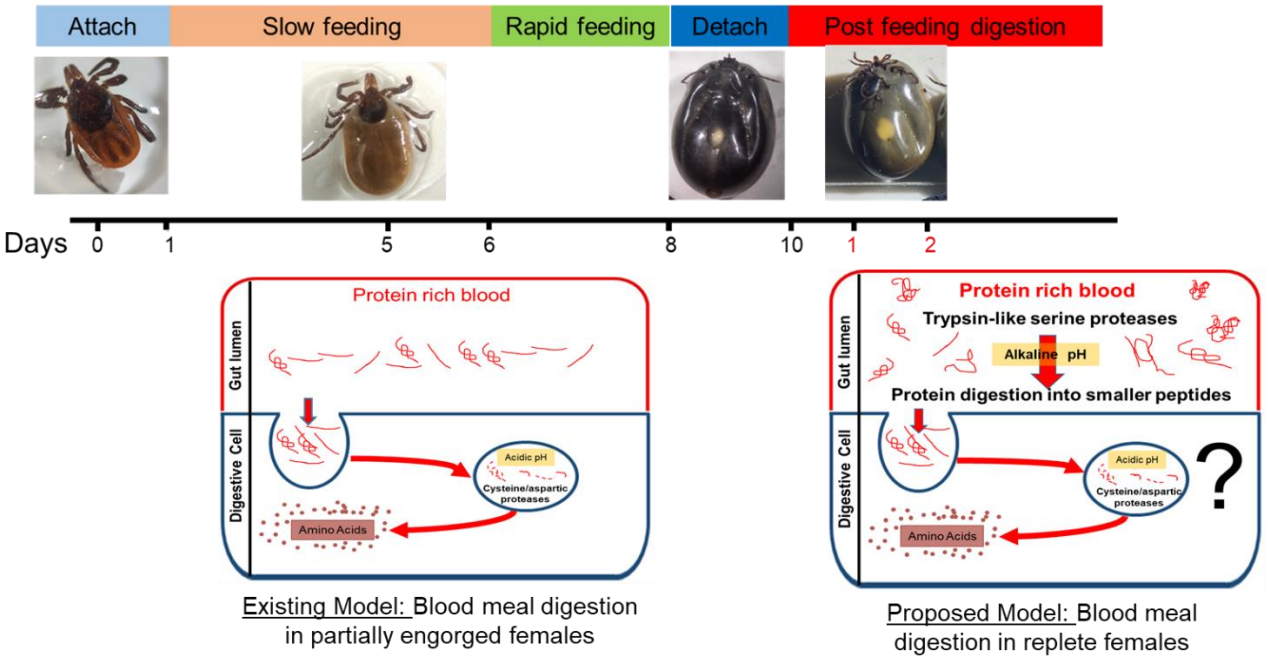
628 **Figure 5**



Existing Model: Blood meal digestion in partially engorged females

Proposed Model: Blood meal digestion in replete females

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640 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 °C
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	
T7 Serine Protease 1 ISCW021184/ XM_002405400.1		
T7IscapSP1Fwd	TAATACGACTCACTATAGGGC TTCGTCGAAAAAGCTCAC	58
T7IscapSP1Rev	TAATACGACTCACTATAGGGC AACTCTCGGCGATCTCTTC	
T7 Serine Protease 2 ISCW006427/ XM_002435219.1		
T7IscapSP2Fwd	TAATACGACTCACTATAGGGA GCCTAATCAATCAAGGGCG	58

T7IscapSP2Rev	TAATACGACTCACTATAGGGA CCAGTTTAGGGATGCGAG	
T7 Serine Protease 4 ISCW007492/ XM_002404245.1		
T7IscapSP4Fwd	TAATACGACTCACTATAGGGA TCCACGTTGGGAACCTTTC	58
T7IscapSP4FRev	TAATACGACTCACTATAGGGC AATGGTCAAACGCCTTTCC	
Tubulin ISCW005137/ XM_002402966.1		
IscapB-TubFwd	TGAATGACCTGGTGTCCGAG	55-58
IscapB-TubRev	GCAAAGCTGTTCAAGCCTCT	

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