

**Expression profiling, gene silencing and transcriptional networking of metzincin metalloproteases in the cattle tick, *Rhipicephalus (Boophilus) microplus***

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## **Abstract**

Tick proteins functioning in vital physiological processes such as blood meal uptake, digestion and reproduction are potential targets for anti-tick vaccines, since vaccination could inhibit these essential functions and ultimately affect tick survival. In this study we identified metzincin metalloproteases from *Rhipicephalus microplus* as potential vaccine candidates since they are implicated as essential to blood-cavity formation, bloodmeal digestion and reproduction in ixodid ticks. Eight transcripts encoding proteins that contain the characteristic metzincin zinc-binding motif HEXXHXXG/NXXH/D and a unique methionine containing “methionine-turn” were identified from native and in-house assembled *R. microplus* expressed sequence tag (EST) databases. These were representative of five reprotolysin-like and three astacin-like metzincin metalloproteases. Reverse transcription-PCR analysis indicated that the reprotolysins were most abundantly expressed in the salivary glands, whereas the astacins were most abundant in the midgut and ovaries. *In vivo* gene silencing was performed to assess a possible phenotype of these metalloproteases during adult female *R. microplus* blood feeding and reproduction. RNA interference (RNAi) against two of the reprotolysins and one of the astacins significantly affected the average egg weight and oviposition rate. Evidently, this reverse genetic approach enabled the evaluation of the overall vital impact of tick proteins. Finally, integrated real time-PCR studies also revealed an extensive cross organ network between the *R. microplus* metzincin transcripts, supporting the use of a combinatorial metzincin-based anti- *R. microplus* vaccine.

**Keywords** Metzincin metalloproteases, *Rhipicephalus microplus*, Expression profiling, *In vivo* RNAi

## 1. Introduction

The impact of ticks as obligate haematophagous ectoparasites and disease vectors on livestock productivity is immense (Jongejan and Uilenberg, 2004). *Rhipicephalus (Boophilus) microplus* is endemic in most subtropical and tropical regions of the world, causing annual economic losses in the hundreds of millions of dollars to cattle producers (Jongejan and Uilenberg, 2004; Estrada-Pena *et al.*, 2006). A rising concern is the continued expansion of its distribution range, especially in areas where it is accompanied by the displacement of *Rhipicephalus (B.) decoloratus* (Macleod, 1978; Mason and Norval, 1980; Tonnesen *et al.*, 2004). While *R. decoloratus* only efficiently transmits *Babesia bigemina*, *R. microplus* is a competent vector of both *B. bigemina* and *Babesia bovis*. Since the latter protozoan is more pathogenic and results in much heavier losses in susceptible livestock, the concern regarding *R. microplus* displacing *R. decolorates* is obvious. Besides the pathogens transmitted by *R. microplus* such as *Anaplasma marginale*, *Babesia* spp. and *Borrelia theileri*, heavy tick loads also cause direct harm to hosts as their feeding behavior results in blood loss, scar tissue formation and leather damage. Currently the control of *R. microplus* relies primarily on chemical acaricides. However, apart from being a pollutant to the environment, these substances augment acaricide-resistant *R. microplus* populations (Baffi *et al.*, 2007). Considering all of the above, it is clear that it is of great importance to create an alternative and more effective control strategy, to counter the negative economical impacts.

One of the most attractive alternative control approaches is to exploit the host's immunity through vaccination of livestock against ticks (Willadsen, 2004). Vaccination of livestock offers the advantages of being cost-effective, reducing environmental contamination and preventing the selection of acaricide-resistant ticks. Furthermore, by reducing tick populations and/or affecting tick vector capacity, vaccines can also indirectly prevent or reduce transmission of pathogens (de la Fuente *et al.*, 1998; , 2007). The most limiting step for the improvement and/or development of new vaccines, however, remains the identification and characterization of effective vaccine antigen targets (Jongejan *et al.*, 2007).

Based on various studies, metzincin metalloproteases have been recognized as key bioactive components in vital physiological processes of invertebrates, and therefore are considered as promising targets for control strategies against medical and economical important micro-organisms and parasites. These enzymes are multi-domain endopeptidases, which are distinguished by an extended zinc-binding consensus motif (HEXXHXXG/NXXH/D, where X represent any amino acid) in the active site and a conserved methionine-containing turn (Met-turn) underlying the active site (Bode *et al.*, 1993; Gomis-Rüth, 2009). To date, seven metzincin families have been analyzed at the structural level, including the astacin and reprotin family (reviewed by Gomis-Rüth, 2009).

Through protein degradation, protein - protein interaction modulation, extra-cellular matrix (ECM) turn-over and highly specific cleavage events, these enzymes are involved in a diverse range of essential physiological processes. Vector-borne protozoa are known to require these metalloproteases for replication and use them as virulence factors (Barrett *et*

*al.*, 2004; Gomis-Rüth, 2003.) Parasites such as hematophagous ectoparasites find metzincins essential for reproduction (Bowles *et al.*, 2007; Young *et al.*, 2000) and host infestation (Dzik, 2005; Gallego *et al.*, 2005; Francischetti *et al.*, 2003). These vital physiological implications of metzincins make them potentially attractive targets for vaccines.

Metalloproteases that form part of the array of known bioactive molecules in tick saliva have been classified as reprotin-like metalloproteases based on sequence alignment analysis (Francischetti *et al.*, 2003). To date six reprotin-like metalloprotease coding sequences have been identified in *Haemaphysalis longicornis* (Harnnoi *et al.*, 2007), five in *Ixodes ricinus* (Decrem *et al.*, 2007; , 2008) and three in *Ixodes scapularis* (Francischetti *et al.*, 2003). Based on sequence similarities, between the salivary gland metalloproteases and the hemorrhagic snake venom metalloproteases (SVMPs), and preliminary activity assays (Francischetti *et al.*, 2003) it appears that these metalloproteases contribute to the facilitation and maintenance of a fluid blood feeding cavity during long extended feeding, by performing anti-haemostatic activities such as fibrinogenolysis and fibrinolysis. Accordingly, Decrem *et al.* (2007) proposed that these salivary gland metalloproteases are adequate vaccine candidates, since an elicited antibody response would result in the inhibition of essential anti-haemostatic enzymes (Decrem *et al.*, 2007). Subsequent vaccination against these salivary gland metalloproteases did in fact affect the engorgement weight and fecundity of *I. ricinus* (with 47% and 55.4%, respectively) (Decrem *et al.*, 2007) and the mortality of *H. longicornis* (with 14.6%) (Imamura *et al.*, 2009). These reports support the notion that tick metzincin metalloproteases are promising anti-tick vaccine candidates and require further investigation to fully explore their potential.

To date, metalloproteases have not been described in any ixodid tick tissue other than the salivary glands. However, various invertebrate studies have indicated metzincin metalloproteases, including reprotolysins and astacins, in a wider variety of tissues with extensive roles in pathogen transmission (Barrett *et al.*, 2004; Gomis-Rüth, 2003), host infestation (Williamson *et al.*, 2006) and reproduction (Bowles *et al.*, 2007; Freije and Bischoff, 2006). It is therefore anticipated that similar metzincins may also occur in a wider variety of tick tissues, with similar extensive roles, contributing to the tick's successful haematophagous parasitic behavior and its success as vector of disease.

Here we report the identification of reprotolysin- and astacin-metzincin homologues from *R. microplus*, their expression profiles, phenotype assessment following *in vivo* RNAi, and evidence of a transcriptional interaction between these metzincins. This study provides a systematic approach to advance the use of metzincin metalloproteases as effective anti-*R. microplus* vaccines.

## **2. Materials and methods**

### *2.1. Experimental animals*

All animal experiments were performed at the Utrecht Centre for Tick-borne Disease (UCTD). Two 5-month-old male Holstein–Friesian calves (#7766 and #3903), which had no previous exposure to ticks, were used for tick feeding, during RNAi experiments. All tick feedings were approved by the Animal Experiments Committee (DEC) of the Faculty of Veterinary Medicine, Utrecht University (DEC No. 2008.II.07.068).

## 2.2. Ticks and tick feeding

*R. microplus* ticks (Mozambique strain) were provided by ClinVet International (Pty), Bloemfontein, South Africa and were subsequently bred and maintained at the Utrecht Centre for Tick-borne Disease (UCTD), University of Utrecht. For tick feeding, patches with inner dimensions of 60mm x 85mm were utilized. Five patches were glued to the shaved back of calf #3903 and two patches to calf #7766. Two batches of larvae (obtained from 1500 mg pooled eggs, oviposited by 25 females) were placed in 2 different patches on day 0 (patch 1) and day 1 (patch 2) on calf #7766. Approximately 400 unfed females were collected on day 16 and 200 unfed males were collected on day 16 and day 17, respectively. The collected ticks were incubated at 27°C with 95% relative humidity. On day 16 the freshly molted females were injected with prepared dsRNA or injection buffer as described below.

## 2.3. *R. microplus* reprotysin and astacin homologue identification

Basic local alignment and search tool (BLAST) searches (using the blastp and PSI-blast algorithms) were performed against the *R. microplus* nucleotide sequences available at the National Centre for Biotechnology Information (NCBI) (<http://ncbi.nlm.nih.gov>), the Bmi Gene Index (BmiGI) of expressed sequence tags (ESTs) available at the DFCI (<http://compbio.dfci.harvard.edu/tgi/>) and an in-house assembled *R. microplus* contig database. The amino acid sequence of Astacin (GenBank ID: **CAA64981.1**), the family prototype from *Astacus astacus*, and the *I. scapularis* reprotysin, MP1 (GenBank ID: **AAP22067**) (Francischetti *et al.*, 2003) were used as queries, respectively. For sequence analysis, alignments were constructed using the ClustalX program (Thompson *et al.*, 1997).

#### 2.4. Phylogenetic, topology and localization analysis

For phylogenetic analysis of the identified *R. microplus* astacin and reprotysin homologues, three available ixodid tick reprotysin-like MPs (*I. scapularis* MP1: GenBank ID: **AAP22067**); *I. ricinus* Metis 1 (GenBank ID: **AM747806**) and Metis 2 (GenBank ID: **AM747807**) and prototype astacin (GenBank ID: **CAA64981.1**) were aligned using MAFFT (v 6). Subsequent phylogenetic analysis was performed with the molecular evolution genetics analysis program (MEGA) (v 4.0) (Tamura *et al.*, 2007) utilizing neighbor-joining analysis (1000 bootstraps, substitution model: PAM Dayhoff matrix).

For localization predictions, theoretical signal peptides were determined utilizing the SignalP (v 3.0) (<http://www.cbs.dtu.dk/services/SignalP/>) computational tools. Topology analysis was performed using the TMHMM server (v 2.0) (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

#### 2.5. Expression profiling

The expression profile of each transcript within eight female tissues (haemolymph, Malpighian tubules, ovaries, fat body of fed ticks and the midgut and salivary glands of both fed and unfed ticks), seven male tissues (testis of fed ticks and the accessory glands, midgut and salivary glands of both fed and unfed ticks) and over 10 lifestages (eggs, unfed larvae, engorged larvae, unfed nymphs, engorged nymphs, unfed female, unfed male, partially fed females, fed males and females after they oviposited all eggs) was determined using reverse transcriptional (RT)-PCR. Tissue and lifestage collection procedures and RNA isolation are described elsewhere (Nijhof *et al.*, 2009; 2010). First-strand cDNA was synthesized from 1 µg total RNA using the first-strand cDNA synthesis system (Invitrogen™) and an oligo-dT primer. PCR amplification was performed using



GoTaq (Promega, USA). PCR conditions were 35 cycles of 30s at 94°C, 30s at the T<sub>m</sub> of the gene specific primers and 30s at 72°C, preceded by an initial 2 min denaturation at 95°C and followed by a final extension at 72°C for 7 min. PCR reactions were analyzed using agarose electrophoresis.

## 2.6. dsRNA synthesis

For each *R. microplus* metzincin transcript PCR-amplified cDNA templates were synthesized, utilizing gene specific primers, each with a T7 promoter sequence appended to the 5' end. Single stranded cDNA, synthesized from *R. microplus* fed mixed lifestages total RNA, served as initial template. All oligonucleotide primers used for dsRNA synthesis were synthesized by Invitrogen™ (Table 1). The region of each transcript's nucleic acid sequence which was targeted for silencing, were randomly selected and the sizes ranged from 120 - 635 bp. T7-incorporated PCR products were purified using the High Pure PCR Cleanup Micro Kit™ (Roche Diagnostics), and were used as templates to produce dsRNA using the T7 Ribomax Express RNAi system (Promega).

## 2.7. Injection of ticks with dsRNA and phenotype analysis

Injection of *R. microplus* females with dsRNA was performed as previously described in Nijhof *et al.* (2007). Freshly molted female ticks were injected with 0.5 µl dsRNA solution ( $1-2 \times 10^{12}$  molecules/µl) dissolved in injection buffer (10 mM Tris-HCl, pH 7 and 1 mM EDTA) in the following combinations: BmMP4 (gi|71726986) and BmMP5 (gi|7172684) (Group A); BmMP1 (gi|71726988), BmMP2 (gi|71726992) and BmMP3 (gi|71726990) (Group B); As51 (gi|82845051) and As70 (gi|49558770) (Group C) and AsContig (assembled from gi|8285219, gi|82845107 and gi|82843217) (Group D). Each group comprised 80 ticks. A control group (Group TE), which was injected with injection buffer

alone, was also included. Following injection the ticks were allowed to recover (for 2-5 hours at 27°C with 95% relative humidity), mortality due to injection was determined and each group (together with an excess of non-injected males) was placed in a separate patch on calf #03903. Ticks were monitored daily. Female ticks that detached upon repletion were collected, weighed and placed at 27°C with 95% relative humidity for oviposition. Several parameters including: egg weight; percentage female weight converted into egg weight; and the hatching rate were monitored and compared between dsRNA-injected and control ticks. Statistical analysis was performed using one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test with GraphPad InStat 3.10. Results were compared between the dsRNA and mock-injected ticks and P values of 0.05 or less were considered statistically significant.

#### *2.8. Gene silencing confirmation by semi-quantitative real-time PCR*

From each injected group, 15 partially fed females (with an average weight of 40 mg) were collected and dissected (giving three biological repeats, n=5). The salivary glands, midgut and ovaries were collected and total RNA was isolated from the collected tissues utilizing TRIzol (Invitrogen™ and Chomczynski, 1993). All samples were subsequently treated with DNase (Fermentas, Germany) and purified using both phenol/chloroform/isoamyl alcohol extraction and sodium acetate precipitation. cDNA was synthesized from 500 ng purified total RNA using the iScript™cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). The synthesized cDNA was diluted 1/10 times with water and served as template.

The effect of RNAi on the transcription level of each transcript was analyzed by semi-quantitative real-time PCR (semi-qPCR) using gene specific oligonucleotide primers

designed to amplify a  $\pm 100$ bp part, outside of the region that was targeted by dsRNA. All oligonucleotide primers used for real-time PCR were synthesized by Inqaba Biotec (Pretoria, South Africa) (Table 1). All real-time PCRs were performed in 10  $\mu$ l reaction volumes using the KAPA<sup>TM</sup>Syber<sup>®</sup> FAST qPCR kit (KAPABiosystems, USA), on the LightCycler<sup>®</sup> 480 (Roche Diagnostics, Germany). For each transcript a standard curve was constructed, using a dilution series of *R. microplus* mixed lifestages as template. For each primer set a reaction lacking template was included as negative control. Run-to-run differences were corrected for by an intra-calibrator being incorporated in each run. For each reaction, three technical repeats were performed. Amplification consisted of a initial activation step at 95°C for 5 minutes followed by 45 cycles of 3s at 95°C, 7s at 55°C and 4s at 72°C. Melting curve analysis, included 30s at 95°C, 30s at 55°C, followed by a continuous acquisition mode from 95°C, with a ramp rate of 0.1°C/s. Each reaction's Cq-value and melting temperature (T<sub>m</sub> of the product) were calculated with the LightCycler<sup>®</sup> 480 software and the data were analyzed with qbase<sup>PLUS</sup> software (version 1.2) (Hellemans *et al.*, 2007). All data were normalized against two reference genes, elongation factor 1-alpha (ELF1 $\alpha$ ) (GenBank ID: **EW679365**) and cyclophilin (PPIA) (GenBank ID: **CV445080**). These genes were first validated as reference genes, using qbase<sup>PLUS</sup>: their respective coefficient of variance (CV) among all tissues tested had to be less than 25% as per the acceptable limits of variation (Hellemans *et al.*, 2007). To test the significance of each transcript's percentage silencing, an unpaired t-test with unequal variances was performed. The results were compared between the dsRNA and mock-injected tissues and P-values of 0.05 or less were considered statistically significant.

To assess if non-specific silencing occurred and to investigate the differential transcriptional responses between the transcripts, the expression level of each transcript

within all the different dsRNA-injected groups (in all the relevant tissue types) was determined, using integrated semi-qPCRs. All reactions were conducted exactly as described above. Crude (non-normalized) Cq-data were exported to qbase<sup>PLUS</sup>, normalized to the two reference genes and statistically analyzed as described previously.

### **3. Results**

#### *3.1. R. microplus astacin- and reprotolysin-like homologue identification*

For reprotolysin homologue identification, *R. microplus* EST sequences with the highest similarity (E-values  $\leq \chi e^{-10}$ ) to *I. scapularis* MP1 were obtained and aligned. This resulted in the identification of five complete coding sequence (cds) translating to five putative *R. microplus* reprotolysins, namely: BmMP1 (gi|71726992), BmMP2 (gi|71726990), BmMP3 (gi|71726988), BmMP4 (gi|71726986) and BmMP5 (gi|7172684). The five reprotolysin-like *R. microplus* homologues were aligned with *I. scapularis* MP1, *I. ricinus* Metis-1 and two reprotolysin SVMPs (Fig 1).

It is evident that all five *R. microplus* sequences contain the typical zinc-binding (catalytic) domain of the reprotolysin family (HEBGHXL/FGXXHD), except for one conserve substitution in BmMP1, BmMP2, BmMP3 and BmMP5, where the first glycine was substituted with an alanine. In BmMP4 the third residue of the motif is substituted with a cysteine, instead of the usual hydrophobic amino acid. The alignment against known reprotolysins also indicates the conserved methionine adjacent to the catalytic domain (Fig 1). However, for the ixodid tick reprotolysin-like sequences a unique conserved Met-turn motif is observed: GXXMSY. None of the *R. microplus* sequences contain the conserved

RGD triplet typical of the disintegrin-domains present in some reprotolysins. However, each of the five *R. microplus* reprotolysin-like MPs possesses a cysteine-rich C-terminal domain, which is hypothesized to provide binding sites to extracellular matrix proteins (Iba *et al.*, 1999).

For astacin homologue identification, the *R. microplus* EST sequences with the highest similarity (E-values  $\leq \times e^{-10}$ ) to prototype astacin were identified and aligned. Three possible astacin-like *R. microplus* homologues were identified, namely: As51 (gi|82845051), As70 (gi|49558770) and AsContig (assembled from gi|82852194, gi|82845107 and gi|82843217) (Fig 2). All three putative tick astacin MPs contain the typical zinc-binding (catalytic) domain of the astacin family (HEBXHAAVBGFHE), except for one conserved glutamic acid to methionine substitution in As70. The conserved Met-turn is present in all three *R. microplus* astacin-like MPs, although the histidine directly adjacent to the methionine is substituted with a lysine, the structurally important tyrosine remains conserved. The typical astacin connecting segment (RXDRD), between the His-motif and the Met-turn is also present in all three *R. microplus* sequences.

### 3.2. Phylogenetic, topology and localization analysis of *R. microplus* reprotolysin and astacin homologues

Phylogenetic analysis revealed the distinct evolutionary patterns of the two different metzincin families. Except for BmMP5, which branches independently, it is evident that the reprotolysin *R. microplus* MPs, are closely related (Fig 3). As expected, the astacins grouped independently of the reprotolysins (Fig 3).

All five full length *R. microplus* reprotolysin-like sequences contained a secretory signal peptide, indicating that these proteases are most likely secreted and act extracellularly (results not shown). Topology predictions were in agreement with these findings, since no transmembrane  $\alpha$ -helices were identified for any of the reprotolysin sequences. For the three *R. microplus* astacin-like MPs the full-length coding sequences need to be determined. Therefore it can only be speculated that these *R. microplus* astacins (like all other invertebrate astacins) are not membrane associated, but most likely secreted (Sterchi *et al.*, 2008).

### 3.3. Expression profiling in *R. microplus* lifestages and tissues

From the expression profiles it is evident that the reprotolysin-like transcripts are most abundantly expressed in the salivary glands and that the astacins most abundantly in both female and male midgut and gonads (Fig 4). Both As51 and AsC were not only detected in midgut and reproductive organs but also in lower levels in the salivary glands, Malpighian tubules and accessory glands (Fig 4). Analysis of the lifestage profiles indicates that As51 is the most abundantly expressed transcript, as it was detected in high levels throughout all lifestages (Fig 5), and it was the only transcript detected in eggs.

### 3.4. *In vivo* RNAi and subsequent phenotype analysis

The five groups (consisting of 80 freshly molted *R. microplus* females each) were successfully injected, with 9% overall mortality. Tick weight after engorgement, mortality rate after placement, egg mass and oviposition efficiency (the percentage of fully fed female (ffF) weight converted into egg mass weight) were monitored for each group.

Group B and C displayed a significant phenotype, with decreased oviposited egg masses and oviposition efficiencies compared with the control group ( $P < 0.01$ ). The hatching rates of all four dsRNA-injected groups were monitored and compared to the mock-injected group but, no significant change was detected.

### *3.5. Silencing confirmation and differential transcriptional interaction analysis*

To confirm that observed phenotypes were in fact due to gene-specific silencing, the percentage silencing of each transcript within its respective dsRNA-injected group was determined, using semi-qPCRs. Expression levels of each transcript were only determined in tissues previously identified to express the transcript in question (Table 2).

The normalized transcript levels of the reprodysin-like transcripts (BmMP1, BmMP2, BmMP4 and BmMP5) (Table 3) were significantly reduced, in the salivary glands of the respective groups ( $P < 0.01$ ). For BmMP4 and BmMP5 the normalized transcript levels were reduced with 77.15% and 97.42%, respectively, in the combined BmMP4/BmMP5-dsRNA-injected group (Group A). The normalized levels of BmMP1 and BmMP2 were reduced with 64.54% and 97.36%, respectively, in the combined BmMP1/BmMP2/BmMP3-dsRNA-injected group (Group B). The normalized transcript level of As51 was significantly reduced, in both the midgut with 55.82% ( $P < 0.01$ ), the ovaries with 80.42% ( $P < 0.01$ ) and the salivary glands with 83.75% ( $P < 0.01$ ) in the combined As51/As70-dsRNA-injected group (Group C), whereas AsC was only significantly silenced within the ovaries with 62.55% ( $P < 0.01$ ) of the AsC-dsRNA-injected group (Group D), compared to the normalized levels in the mock-injected group (Table 3). Integrated real-time PCR reactions and data analysis revealed that significant non-specific

silencing took place between As51 and AsC as the normalized level of As51 was reduced with 77.79% in the midgut and 68.87% in the ovaries of Group D (Table 3).

The astacin and reprotolysin transcripts were silenced in family specific groups, to allow the identification of potential networking on transcriptional level between the five reprotolysin, the three astacins, and between two metzincin families, respectively. For this, integrated semi-qPCR studies were performed, in which the expression level of each transcript was determined within all relevant tissue types in each of the dsRNA-injected groups.

Significant up-regulation of non-silenced transcripts occurred, when one/more of the other metzincin transcript(s) were silenced (Table 3). With the silencing of the two salivary gland reprotolysins, BmMP4 and BmMP5 (Group A), the normalized levels of the other salivary gland reprotolysins, BmMP1 and BmMP2 significantly increased with 185.32% and 240.52%, ( $P < 0.01$ ), respectively. Of great interest was that the normalized level of As51 was also significantly increased with 125.44% in the midgut and 249.95% in the ovaries ( $P < 0.01$ ). The same effect was observed when the other two salivary gland reprotolysins, BmMP1 and BmMP2 (Group B), were silenced (Table 3). The normalized level of BmMP5 was significantly increased with 301.70% ( $P < 0.01$ ) in the salivary glands and the normalized level As51 with 188.13% in the midgut and 67.74% in the ovaries ( $P < 0.01$ ). Upon silencing of As51 (Group C) and AsC (Group D), a cross organ response was observed. In this case, the normalized levels of both BmMP1 and BmMP2 were significantly increased in the salivary glands of Group C with 111.44% and 191.82% ( $P < 0.01$ ) and with 257.94% and 181.60% in Group D.



## 4. Discussion

By hydrolyzing substrates such as fibrin and fibrinogen, reprotolysin metzincins are in effect capable of preventing blood clot formation and dissolving existing clots, during haematophagous feeding, by disaggregating the platelets and fibrin crosslinked matrix (Francischetti *et al.*, 2003). It has also been speculated that such metzincins may play a role in pathogen transmission, since it has been proven that *Borrelia* spirochetes up regulate the release and activation of matrix metalloprotease gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells, improving the transmission of *Borrelia burgdorferi* across extracellular matrix components (Gebbia *et al.*, 2001). Spirochete dissemination through vertebrate tissues may similarly be aided by salivary metalloproteases and other salivary anti-haemostatic agents (Maritz-Olivier *et al.*, 2007; Ribeiro, 1989), which prevent fibrin clot formation and dissolution of the fibrin clots eventually formed around the feeding cavity of ixodid ticks (Francischetti *et al.*, 2003). For this reason both Decrem *et al.* (2007) and Imamura *et al.* (2009) selected salivary gland metalloproteases from *I. ricinus* and *H. longicornis*, respectively, as novel vaccine targets. The results of both studies indicated that vaccination against these putative reprotolysin salivary gland enzymes can affect the reproductive fitness of ticks, since the female egg laying abilities were significantly affected.

Another metzincin family of interest is the astacins. Through protein degradation, protein-protein interaction modulation, ECM turn-over, extracellular coat degradation (hatching) and highly specific cleavage events such as growth factor activation, these enzymes play essential roles in diverse range of physiological mechanisms including digestion (Stöcker

*et al.*, 1993), early embryo development (dorso-ventral patterning) (Shimell *et al.*, 1991), processing of the ECM (Sterchi *et al.*, 2008) and egg hatching (Young *et al.*, 2000).

This study set out to identify reprotolysin and astacin metzincin homologues in *R. microplus* and to evaluate these enzymes as possible anti-*R. microplus* vaccine targets. Five coding sequences with significant similarity to a known reprotolysin-like ixodid tick MP, and three EST sequences with significant similarity to prototype astacin were identified. As with all members of the metzincins (Gomis-Rüth, 2009) the overall similarity and identity among the reprotolysins and astacins were low. However, analysis of the amino acid sequences (Figs 1 and 2) confirmed that the sequences contained their respective family's conserved zinc-binding his-motif as well as the conserved methionine, which clearly classified all eight *R. microplus* transcripts as metzincins. Phylogenetic analysis supports the alignment data and indicated that the five reprotolysin and three astacin *R. microplus* homologues belong to the reprotolysin and astacin metzincin families, respectively (Fig 3).

Expression profiling results indicated that the reprotolysins were most abundantly expressed in the salivary glands (Fig 4), whereas the expression of astacins was most abundant in the midgut and reproductive organs (Fig 4). The theoretical signal peptide, GPI-anchor and transmembrane  $\alpha$ -helix predictions suggest all five *R. microplus* reprotolysins to be secreted proteins. By integrating all data it can be postulated that the five reprotolysin-like MPs are secreted by the salivary glands into the saliva. Via the saliva these MPs are released into the feeding cavity, where they most probably act on ECM components, to assist in maintaining a fluid intact feeding pool (Francischetti *et al.*, 2003). For the astacin-like MPs it can be speculated, that once a blood meal is obtained, the specialized secretory cells in the midgut epithelium release these enzymes. In the midgut lumen these

astacin-like MP most probably act on imbibed ECM components. Since both As51 and AsC were detected at high levels in the reproductive organs we hypothesize that these metzincins may also be involved in the reproductive processes, oogenesis and spermatogenesis. As51 is the most abundantly expressed transcript, throughout all lifestages and is the only transcript detected in eggs. Since astacins in other invertebrates are involved in embryogenesis or egg hatching (Bond and Beynon, 1995), it can be hypothesized that this *R. microplus* astacin may have similar function. However, the exact functions and substrates of these *R. microplus* metzincin MPs remain to be determined using native- or recombinant proteins in future studies.

This work presents, to our knowledge, the first description of astacin metalloproteases in ticks and metzincin metalloproteases in tissues other than the salivary glands. Based on the data of this study and other invertebrate studies, it is evident that tick metzincins are not necessarily only involved in ECM digestion but may also be involved in other processes such as oogenesis, spermatogenesis and egg hatching.

To evaluate the vital impact of the eight *R. microplus* metzincins homologues during blood feeding and reproduction, *in vivo* RNAi was utilized. From the data (Table 2) it was evident that the silencing of the reprotin-like transcripts BmMP1, 2 and 3 (Group B) and the silencing of the astacin-like transcripts As51 and As70 (Group C) yielded the most significant phenotypes, regarding average egg weight and oviposition efficiency. Normalized transcript levels of the targeted genes (BmMP1, 2, 4, 5 and As51, AsC) were reduced by RNAi some 55 – 98% in the respective tissues. Similar degrees of silencing have been reported previously in *R. microplus* (Nijhof *et al.*, 2007). Further, real-time PCR studies and data analysis revealed that significant non-specific silencing took place

between As51 and AsC: injection of AsC dsRNA, in Group D, not only significantly reduced the normalized levels of AsC, but also resulted in a significant reduction of As51 (Table 3). Therefore it can be reasoned that the non-specific silencing of As51 in Group D also contributed to the observed phenotype of Group D. Although not found to be statistical significant, a possible *vice versa* effect cannot be excluded. Upon analysis of the alignment of the two transcripts' nucleotide sequences it is evident that there are several conserved regions (as large as 21 consecutive nucleotides), that could have attributed to the non-specific silencing.

The mechanism that impaired the ticks to successfully produce ample amounts of eggs is unknown. However, it is known that it is mandatory for adult female ixodid ticks to have successful blood meals to produce eggs (Soneshine, 1991). With the completion of feeding and digestion a hormonal signal triggers vitellogenesis, a vital process in oogenesis, which produces the yolk nutrient for the developing larvae (Soneshine, 1991). Therefore, it can be hypothesized that silencing of the reprotolysin and astacin metalloproteases (within the salivary glands and the midgut) could have indirectly impaired oogenesis by its effect on blood feeding and digestion (Lu *et al.*, 2005; Jia *et al.*, 1996; Sterchi *et al.*, 2008). Since the astacin transcripts are also present in the ovaries and are known to be involved in vital functions such as growth factor activation (Stöcker *et al.*, 1993) and embryo development (dorso-ventral patterning) (Shimell *et al.*, 1991), thus a direct effect on oogenesis cannot be excluded.

The use of integrated semi-qPCRs in this RNAi study also provided novel insight regarding gene regulation between the different members of the metzincin clan investigated. It was found that several fold up-regulation of non-silenced transcripts

occurred when other transcripts were specifically silenced (Table 3). The remarkable finding was that this was even a cross different organs (Fig 6). Upon silencing of salivary gland reprotolysins the midgut and ovary astacins were activated and *vice versa*. To fully explain these phenomena more integrated transcriptome studies are required, which will provide insight into *R. microplus* metzincin gene regulation during feeding and digestion.

Finally, it can be concluded that by means of a systematic approach we were able to identify five reprotolysin and three astacin *R. microplus* homologous and to evaluate the overall vital impact of these metzincins in *R. microplus* blood feeding and reproduction. Due to the significant phenotypes raised, current studies entail BmMP1, BmMP2 and As51 expression to evaluate them as anti-*R. microplus* protective antigens in cattle vaccination trials. As previously mentioned, vaccination with single metzincins did in fact result in some decrease of engorgement weight and ovipositioning, however, it did not have a great effect on tick mortality (Decrem *et al.*, 2007; Imamura *et al.*, 2009). Based on the differential transcriptional network observed between reprotolysins and astacins, the results support use of a combinatorial metzincin-based vaccine, which will be evaluated in a subsequent study.

### ***Acknowledgements***

Research on anti-tick vaccines is supported by the Wellcome Trust under the 'Animal Health in the Developing World' initiative through project 075799 entitled 'Adapting recombinant anti-tick vaccines to livestock in Africa'. International research was funded by the University of Pretoria's 'Postgraduate study abroad bursary program'.

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**Table 1. Characteristics of oligonucleotide primers used.**

Target name	Primer name	Primer seq 5'- 3'	Product size	Purpose
BmMP1	<b>tpBmMP1fw</b>	GCA AGA GAG ATC AAC CGA AAG		Expression profiling
BmMP1	<b>tpBmMP1rv</b>	AAA GAG AAG TTT GTC CGC AAG TA	<b>100</b>	Expression profiling
BmMP2	<b>tpBmMP2fw</b>	AAT CCG TGC GCT ATT GTT GCT AC		Expression profiling
BmMP2	<b>tpBmMP2rv</b>	GCC AAT CCA TCG TGA ATG CTA AC	<b>132</b>	Expression profiling
BmMP3	<b>tpBmMP3fw</b>	TAA GTG AAG ACA TAA CGC TGA AC		Expression profiling
BmMP3	<b>tpBmMP3rv</b>	GCT CGT GGT CGT CGT AAA	<b>144</b>	Expression profiling
BmMP4	<b>tpBmMP4fw</b>	CGG GAC ACA CTT ACG ACG GAA TA		Expression profiling
BmMP4	<b>tpBmMP4rv</b>	TCC ATG TCA GCG GTC ACG TT	<b>103</b>	Expression profiling
BmMP5	<b>tpBmMP5fw</b>	ACG GAA CGA AAT GAC ACA TAT CA		Expression profiling
BmMP5	<b>tpBmMP5rv</b>	GCA GAT CCA ACA AAG GCA TAA C	<b>128</b>	Expression profiling
As51	<b>tpAs5051fw</b>	AAC CGA CTG CTG ACG CCA TT		Expression profiling
As51	<b>tpAs5051rv</b>	CTC TGT CAA GTG ACT GCC GTC CT	<b>117</b>	Expression profiling
AsC	<b>tpAsCfw</b>	TTG AGG TGA CTG CCA TTC TTC GC		Expression profiling
AsC	<b>tpAsCrv</b>	AGA ACA GGT TGC TGA CGC CCT TC	<b>115</b>	Expression profiling
As70	<b>As70Nqfw</b>	TTG GTG TGG TGT AAG TTG ACC CT		Expression profiling
As70	<b>As70Nqrv</b>	GAA GTG ATT TGC CTG CCG TTT A	<b>116</b>	Expression profiling
BmMP1	<b>iBmMP1fw</b>	TAA TAC GAC TCA CTA TAG GGA GAT ACC AGA AGG CGA GAG GTC AAT G		dsRNA synthesis
BmMP1	<b>iBmMP1rv</b>	TAA TAC GAC TCA CTA TAG GGA GAA CAA GAA GCA GGG CAT CGT G	<b>487</b>	dsRNA synthesis
BmMP2	<b>iBmMP2fw</b>	TAA TAC GAC TCA CTA TAG GGA GAG CAC GCT TGT GGT TCA CGA A		dsRNA synthesis
BmMP2	<b>iBmMP2rv</b>	TAA TAC GAC TCA CTA TAG GGA GAC GCA TCA GGA TCG CCA TAT AGA T	<b>635</b>	dsRNA synthesis
BmMP3	<b>iBmMP3fw</b>	TAA TAC GAC TCA CTA TAG GGA GAT AAG TGA AGA CAT AAC GCT GAA C		dsRNA synthesis
BmMP3	<b>iBmMP3rv</b>	TAA TAC GAC TCA CTA TAG GGA GAG CTC GTG GTC GTC GTA AA	<b>144</b>	dsRNA synthesis
BmMP4	<b>iBmMP4fw</b>	TAA TAC GAC TCA CTA TAG GGA GAC CAC GCC ACA GGA TTG AAC		dsRNA synthesis
BmMP4	<b>iBmMP4rv</b>	TAA TAC GAC TCA CTA TAG GGA GAT ATC GTT GCC ATT TCT GTA AGG G	<b>379</b>	dsRNA synthesis
BmMP5	<b>iBmMP5fw</b>	TAA TAC GAC TCA CTA TAG GGA GAA TTG CGG AAC ATC ATT ACC ATA A		dsRNA synthesis
BmMP5	<b>iBmMP5rv</b>	TAA TAC GAC TCA CTA TAG GGA GAG GTT TCT AAG GGC ACT GTA TCG T	<b>451</b>	dsRNA synthesis
As51	<b>iAs5051fw</b>	TAA TAC GAC TCA CTA TAG GCG TGA CGG AGC CGA ATA GAC TGT		dsRNA synthesis
As51	<b>iAs5051rv</b>	TAA TAC GAC TCA CTA TAG GAA TGG CGT CAG CAG TCG GTT C	<b>610</b>	dsRNA synthesis
As70	<b>iAs8770fw</b>	TAA TAC GAC TCA CTA TAG GAC CTG CCT GCG ATT TGT TGA		dsRNA synthesis
As70	<b>iAs8770rv</b>	TAA TAC GAC TCA CTA TAG GCG CAT CCG CAG AAT ACA GCA T	<b>315</b>	dsRNA synthesis
AsC	<b>iAsContigfw</b>	TAA TAC GAC TCA CTA TAG GGA GAG TGT TTT CTG GCT CCA CGT T		dsRNA synthesis
AsC	<b>iAsContigrv</b>	TAA TAC GAC TCA CTA TAG GGA GAA TGC GGT TAC TAA GAA GGA CGA G	<b>367</b>	dsRNA synthesis
BmMP1	<b>qBmMP1fw</b>	GCA AGA GAG ATC AAC CGA AAG		Real-time PCR
BmMP1	<b>qBmMP1rv</b>	AAA GAG AAG TTT GTC CGC AAG TA	<b>100</b>	Real-time PCR
BmMP2	<b>qBmMP2fw</b>	AAT CCG TGC GCT ATT GTT GCT AC		Real-time PCR
BmMP2	<b>qBmMP2rv</b>	GCC AAT CCA TCG TGA ATG CTA AC	<b>132</b>	Real-time PCR
BmMP3	<b>qBmMP3fw</b>	TTA TGA CAG GAT CGG CTA ATC TA		Real-time PCR
BmMP3	<b>qBmMP3rv</b>	TTA TAT GAA CCA TGT ACG GCT CT	<b>120</b>	Real-time PCR
BmMP4	<b>qBmMP4fw</b>	AAC TGA CGC TGA ACC TCA GAA AG		Real-time PCR
BmMP4	<b>qBmMP4rv</b>	CCG TTG AAG AAA TGT GTC ACT TC	<b>101</b>	Real-time PCR
BmMP5	<b>qBmMP5fw</b>	ACG GAA CGA AAT GAC ACA TAT CA		Real-time PCR
BmMP5	<b>qBmMP5rv</b>	GCA GAT CCA ACA AAG GCA TAA C	<b>128</b>	Real-time PCR
As51	<b>qAs5051fw</b>	AAC CGA CTG CTG ACG CCA TT		Real-time PCR
As51	<b>qAs5051rv</b>	CTC TGT CAA GTG ACT GCC GTC CT	<b>117</b>	Real-time PCR
AsC	<b>qAsCfw</b>	TTG AGG TGA CTG CCA TTC TTC GC		Real-time PCR
AsC	<b>qAsCrv</b>	AGA ACA GGT TGC TGA CGC CCT TC	<b>115</b>	Real-time PCR
As70	<b>qAs70Nqfw</b>	TTG GTG TGG TGT AAG TTG ACC CT		Real-time PCR
As70	<b>qAs70Nqrv</b>	GAA GTG ATT TGC CTG CCG TTT A	<b>116</b>	Real-time PCR

**Table 2. Tick engorgement weight, egg mass weight and oviposition efficiency of double-stranded RNA (dsRNA)-injected *B. microplus* ticks, injected as freshly molted females. (ffF: fully fed females)**

Group	Number of ticks	Average ffF weight <sup>a</sup> (mg)	Average egg weight <sup>b</sup> (mg)	Average ffF to egg <sup>c</sup> (%)
TE	68	328±49	152±63	46%
A	68	364±71	129±54	37%
B	74	352±58	93±56*	27%**
C	79	341±73	89±72**	26%**
D	75	313±63	111±38	37%

<sup>a</sup> Ticks which completed feeding and those removed 14 days after mock- or dsRNA-injection were weighed, the average ± SD weight were calculated and compared between dsRNA and mock-injected control ticks, using the Student's t-test with unequal variance.

<sup>b</sup> The egg mass oviposited by each tick was weighed individually and compared between dsRNA and mock-injected control ticks, using one-way ANOVA and Tukey-Kramer multiple comparison test.

<sup>c</sup> The percentage fully fed female weight converted into egg mass was calculated for each tick individually and compared between dsRNA and mock-injected control ticks, using one-way ANOVA and Tukey-Kramer multiple comparison test.

(\*P<0.01, \*\*P<0.001).

**Table 3. Silencing confirmation and differential transcriptional response analysis.** The relative transcript levels in the salivary glands (SG), midgut (MG) and ovaries (O) of five partially fed females, 6 days after injected with dsRNA of BmMP4 and BmMP5 (Group A), BmMP1, BmMP2 and BmMP3 (Group B), As51 and As70 (Group C) and AsC (Group D) are represented by the calibrated normalized relative quantity (CNRQ)-values ( $\pm$ SD), normalized against ELF1- $\alpha$  and PPIA. The correlated percentage up (+) or down (-) regulation of each transcript within its dsRNA silenced group (in bold) and in the other groups are compared to its level in the control group.

		CNRQ values and percentage up or down regulation											
		BmMP1		BmMP2		BmMP4		BmMP5		As51		AsC	
Group A	SG	9.92 ( $\pm$ 1.04)	+185.32*	56.29 ( $\pm$ 7.28)	+240.52*	<b>4.95</b> <b>(<math>\pm</math>0.58)</b>	<b>-77.15*</b>	<b>4.89</b> <b>(<math>\pm</math>0.59)</b>	<b>-97.42*</b>	-	-	-	-
	MG	-	-	-	-	-	-	-	-	7.56 ( $\pm$ 0.90)	+125.44*	-	-
	O	-	-	-	-	-	-	-	-	6.01 ( $\pm$ 0.67)	+249.95*	-	-
Group B	SG	<b>1.14</b> <b>(<math>\pm</math>0.48)</b>	<b>-64.54*</b>	<b>0.43</b> <b>(<math>\pm</math>0.11)</b>	<b>-97.36*</b>	-	-	661.19 ( $\pm$ 139.15)	+301.70*	-	-	-	-
	MG	-	-	-	-	-	-	-	-	9.44 ( $\pm$ 1.04)	+188.13*	-	-
	O	-	-	-	-	-	-	-	-	2.73 ( $\pm$ 0.26)	+67.74*	-	-
Group C	SG	6.41 ( $\pm$ 0.91)	+111.44*	45.16 ( $\pm$ 7.3)	+191.82*	-	-	334.13 ( $\pm$ 59.28)	+113.04	-	-	-	-
	MG	-	-	-	-	-	-	-	-	<b>1.57</b> <b>(<math>\pm</math>0.25)</b>	<b>-55.82*</b>	0.11 ( $\pm$ 0.02)	-49.28
	O	-	-	-	-	-	-	-	-	<b>0.32</b> <b>(<math>\pm</math>0.06)</b>	<b>-80.43*</b>	0.29 ( $\pm$ 0.03)	-9.34499
Group D	SG	11.38 ( $\pm$ 1.33)	+257.94*	46.21 ( $\pm$ 6.97)	+181.60*	-	-	256.94 ( $\pm$ 35.94)	+47.38	-	-	-	-
	MG	-	-	-	-	-	-	-	-	0.71 ( $\pm$ 0.04)	-77.79*	<b>0.21</b> <b>(<math>\pm</math>0.06)</b>	<b>7.46</b>
	O	-	-	-	-	-	-	-	-	0.52 ( $\pm$ 0.11)	-68.87*	<b>0.11</b> <b>(<math>\pm</math>0.05)</b>	<b>-62.55*</b>

The normalized transcriptional level, of each transcript, was only determined within the tissue type it is known to be highly expressed in. No subsequent real-time analysis was performed for BmMP3 and As70, since both transcripts had very low expression levels. \*Denotes if the difference is significant, as determined by the student's test (\*P<0.01).

## Legends of Figures

**Fig 1. The amino acid alignment of the five reprodysin-like *R. microplus* sequences against *I. scapularis* MP1 (IsMP1: GenBank accession no. AAP22067), *I. ricinus* Metis 1 (GenBank accession no. AM747806) and two reprodysin SVMP, atrollysins.** Sequence similarity is represented by grey scaling, with black indicating 100% similarity, grey 80-90% similarity and light grey 60-70% similarity. The conserved catalytic histidine -domain is indicated by the black box and the conserved methionine with a #. The atrolysin sequences correspond to NCBI accession nos. gi|402259| (*Crotalus atrox* atrolysin B) and gi|213030| (*C. atrox* atrolysin E).

**Fig 2. Amino acid alignment of the three astacin-like *R. microplus* sequences against prototype atacin (GenBank accession no. CAA64981.1) and astacin-like MP toxin precursor of *Loxosceles intermedia* (LiMP: GenBank accession no. ABK20019.1).** Sequence similarity is represented by grey scaling, with black indicating 100% similarity, grey 80-90% similarity and light grey 60-70% similarity. The conserved catalytic histidine -domain is indicated by the black box and the conserved methionine with a #.

**Fig 3. Phylogenetic analysis of the five reprodysin-like and three astacin-like *R. microplus* MPs together with other ixodid tick MPs and astacin.** Ir refers to *I. ricinus* (Metis 1 (GenBank accession no. AM747806) and Metis 2 (GenBank accession no. AM747807), Is refers to *I. scapularis* (IsMP1: GenBank accession no. AAP22067), Bm refers to *R. microplus* (BmMP1: gi|71726988, BmMP2 gi|71726992, BmMP3: gi|71726990, BmMP4: gi|71726986, BmMP5: gi|7172684, As51: gi|82845051, As70: gi|49558770 and AsContig: assembled from gi|82852194, gi|82845107 and gi|82843217. Aa refers to *A. astacus* (astacin: GenBank accession no. CAA64981.1). Bootstrap values are shown on the lineage of the tree.

**Fig 4. Expression profiling of reprodysin- and astacin-like metzincins in *R. microplus* adult tissues.** Fig A: Female tissue: lane 1: engorged female haemolymph, lane 2: partially fed female midgut, lane 3: partially fed female malpighian tubules, lane 4: partially fed female ovaries, lane 5: partially fed female salivary glands, lane 6: unfed female midgut, lane 7: unfed female salivary glands, lane 8: partially fed female fatbody, (B: Male tissues) Lane 1: fed male ancillary glands, lane 2: fed male midgut, lane 3: fed male salivary glands, lane 4:

unfed male ancillary glands, lane 5: unfed male midgut, lane 6: unfed male salivary glands and lane 7: unfed male testis.

**Fig 5. Expression profiling of metzincins in *B.microplus* feeding lifestages.** Lane 1: eggs, lane 2: unfed larvae, lane 3: engorged larvae, lane 4: unfed nymphs, lane 5: engorged nymphs, lane 6: unfed female, lane 7: unfed male, lane 8: partially fed female, lane 9: fed male, lane 10: carcass and lane 11: no template control.

**Fig 6. Observed transcriptional responses between metzincins of the salivary gland and gut tissues.** In the first experiment (green background) midgut astacins were silenced (red arrow) resulting in the up regulation of salivary gland reprotolysins (black arrow). In a second experiment (purple background), the observation was supported by up regulation of midgut astacins (black arrow) in reprotolysin silenced (red arrow) ticks.

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IsMP1    : ---MSG---LSIKLWIVAFFSFLAEKEH---GIVYPR---MLES-----FAATGERMLKIN-DDTTLTQLQRSK-VFADDF-L : 63
Metis1   : ---MSG---LSIKLWIVAFFSFLAEKEH---GIVYPR---MLES-----FAATGERMLKIN-DDTTLTQLQRSK-VFADDF-L : 63
EmMP3    : ---MK---HSTPILFLVEVEFTWLIQAS---RVVYPR---MLES-----FADNGAKVLKIS-EDDTIALQRS-VFSEEE-L : 62
EmMP2    : MMTFLGNYSQIRADLLL LLAGVAVAGLQEN---MVVYPR---LLEE-----RGGDGMVVSIIH-DGIALALNRVVS-VTAPTLL-V : 69
EmMP4    : MVSVI-----IVIVLAIAGEAMQQP---RLVYPR---LLEE-----FSADGRMVMHLH-DCITLAIKRAS-VAAARNF-R : 60
EmMP1    : ---RVFIIILIQEALYSDAEKDV---FYVYPT---LIQE-----RSTERNLVRRLN-DKFTIALQRS-VLADKLLF : 60
EmMP5    : MKSFT---VSVACQIAVVIICRAAKLPKYR---ATVYEQ---VFDG-----E-DENTKVLKIN-DDTSLALQEPIS-VLHEDFFV : 68
gi|402259 : MIEVLL---VVICLAVFYQSSIIILESGNVNDEYVYVYPRKVTALPKGAVQPKYEDAMQYELKVNNGEPVVLHEDLNKELFSKDYSE : 83
gi|213030 : MIQVLL---VVICLAAFYQSSIIILESGNVNDEYVYVYPRKVTALPKGAVQPKYEDTMQYELKVNNGEPVVLHEDLNKELFSKDYSE : 83
                                         YP        1        r        6 6        L L        6

IsMP1    : FSTTDGNEFDYYTKAEDAER---LYHDSHMASVRVTD-DDDCVEVEGILGERLVRVPLPAMARTSNGLRPFMLYEVDF----- : 138
Metis1   : FSTTDGNEFDYYTKAEDAER---LYHDSHMASVRVTD-DDDCVEVEGILGERLVRVPLPAMARSDDGLRPFMLYEVDF----- : 138
EmMP3    : IHTHHDGEPFAIYHMNGAEMEK---NDYDRHEQMATVHVVS-QEDGLSVEGVLGHTLRIRPLEGTERSTNGLRPFMLYEVDF----- : 139
EmMP2    : VHEHENGKPIVRFYRGDDIDR---LDHEDEKQIATVMVSVVNNALHLEGGVQPRHRTSPAPEMRSEEGHVAVHVEHDEQ----- : 147
EmMP4    : VLEQEDGREVTHFFNGEDELQ---LDHEDEKQIASVHVRRRGGGVEVEGVPVPRHRTSPVHMERSEEGCLIAHMFHEIMK----- : 138
EmMP1    : VTTSDENVHLLEIDTSAIQKT---LYHDSRHRQSSVRVQQRDDAVQVEGILNDKLRIRVPEGERSMKQCILHKKIYEVNNMDEGSF : 142
EmMP5    : RTYRHGIAEHYHNVRELQKD---LYHDKKRYAAMVISEEDGAVRVEGVGANLKRIRPETAARSDDCRRLAHLENDLEEDD----- : 146
gi|402259 : THYSPDGRKTTNPSVEDHCYRGRHENDADSTASHSAC-NGLKGHFK-LQCEMYLIRPFLKLSDSEAHAVFKYENVEKED----- : 161
gi|213030 : THYSPDGRKTTNPSVEDHCYHGRHENDADSTASHSAC-NGLKGHFK-LQCEMYLIRPFLKLSDSEAHAVFKLKNVEKED----- : 161
                                         6 D    a 6        eg6 g    6 P    r    g    h    e

IsMP1    : AHENGRPHDYGST---NTSNTPVESRTGSKHSYKIPLELYPEVHLVVDSTFASAFKFDVYKVTTRFAVLTIAANLRYASFVDFPKV : 222
Metis1   : AHENGRPHDYGSP---NTTNPVERRAGGTEPQMVKIPALTYPEVHLVADSFAFAKFNFDVNVVTRVFAVLTIAANLRYESFKSPKV : 222
EmMP3    : QHDDHRRDDYGFPDNTTQALVESRLMMWVR---APRLLELNEVEVVVDFYICKALKFDEKKTARVVAIMTGSANLRYRSGVCPRI : 223
EmMP2    : PNDMFDGIVANKT---DKASLLAERQYNYQT---VPAEVTVELFVSDKIHQQRRTQTQR-LLSMQVMVA SANLRQDTSAPRI : 226
EmMP4    : ---DMHDIIVMAPIG---KGAPTVSERNYGSTQN---VPPSVTLEFEVSDRPHHQHFQTTIC-LIQYICVVA SMLRYSADTDSPRV : 215
EmMP1    : TAEETPRSSHLETNGDHSSAEPNGVNSRIERRNREFEVELHILICDQKHQSNEDKNED-LIGVMVAFMAANLRYLDIRPKV : 227
EmMP5    : SFEVYGVVEDSIGISERAARSTSGFDQTKY---NVYEMFEVEVYVCDSWFQAEVVKSMN-VTVYMTITFQVVSRYR SALRNBKV : 227
gi|402259 : EAPKMGVTDQWE---SYEPIKKASDLNLNPD---QQNLP RYIELVVADHRVFMKNSDLNIRKRVHBLVTITIGFRSLN---H : 240
gi|213030 : EAPKMGVTDQWE---SYEPIKKASDLNLNP---EH---CRYVETELVVVHGMYTKINGDSDKIRQRVHQMVIIMKESYTYMY---H : 236
                                         E 6 6 6 D        6 y    n n r y    p 6

IsMP1    : QLRTGTITMKNKPADEPYIHNVKGYEKYRNILFKEALEDENKQMK-SKPFYDTSIVLVIXTGKNNSEWVDC-KLQHWVGGYAYVGT : 306
Metis1   : QLRTGTITMKNKPADEPYIHNIRGYEQYRNILFKEILEDENTQMK-SKHFYRTAIVLVITAKNNSEWVGS-TLQSWTGGYAYVGT : 306
EmMP3    : QLRTVGTITVTKSASEEPVHVHKGVEQYRNILYGQETIKNEHEHVK-RQVYFERSDIVELLTGMSVAWEN-ALQHWVGGYAYVGG : 307
EmMP2    : KLIVVGLRVRHNAWNPEYLKGDGDKYMD---TATIINLVEHIKTRKHLYGDPEAVYIATGRDMYAMDSQ-APDTRKCLAFVAG : 306
EmMP4    : IFIVLGVTEKDE---HSPVRNGNDKXLES---ASSIEQERSYAYGKRHQGNRIVTETITGDYDYSTASCGSKSTSVLIGVYGG : 293
EmMP1    : RFLVIGTVRRK---ENPAPDNHGTTDT---TEINLRIQAYEA-EKRVPGKHFALLLVITGLLDIVKVQC-HLEKGIARAFVAA : 303
EmMP5    : YPVRRLVLELSTLQEQEKKYVYVLYNASIDG---YKSIDLKLVTVEFTRNDTYCTDMVYFVARYDMVAVYVDGSRQNSLQCYAFVGS : 309
gi|402259 : DVSTDTLQEIWS---DQDFITVQSASAKN---DINSFGEWREADLLRKRKSHDACLITAIINFEGLIIC-----RAYTSS : 306
gi|213030 : DITLQEIWS---NGDLINVQPASN---DINSFGEWRETDLRKRKSHDACLITAIINFEGLIIC-----RAYTSS : 302
                                         6 g6        6        D 1 T        g        g a5

IsMP1    : ACSEWR-VGMCEDP-PTSYGAYVFAHEFAHNLGCGHDGSGASD-WVKGHIGSADCPDDEYLMYSYKMQDERQYQFSYCCQREVRN : 389
Metis1   : ACSEWR-VGMCEDP-PTSYGAYVFAHEFAHNLGCGHDGSGANS-WVKGHIGSADCPDDEYLMYSYKMEDERQYKSPCCQREVRN : 389
EmMP3    : ACRAWR-VGMSER-VGSYGAYVFAHEFAHSLGCAHDGGSDD-WPGGHICSKLCDNLEGMMSYKFIAPNMYRFSKCCQREDITN : 390
EmMP2    : VCLELF-VALGEDN-AGSNDCMHALTTHEFAHLGAARHDGAPKTIINVPGHPGALACEFSQCYIIMSVMVVTGTNYHRSFQSVVQIRY : 390
EmMP4    : LCHEYF-VALGEDS-AGSNDCMHALLTHECGRVLGAARHDESRRVVS-WIKGDFGSMACLVKEEHEIMSVDGGVVHHHLSKQSLAQIRD : 376
EmMP1    : VCSHG-VCVADDT-ATTVMGVNVAHEFAHLGLSDDH---TSPRCPRECYLMSYVDGGLRKYRISQCSQDSIRD : 374
EmMP5    : ACSKNR-EQLGDDT-AYSVRGIRTMTHEFAHNLGCSHDTAAPGIVTAFTPDLSLQCPWEHEIIMSYYEIDIRSMQFSRCCRYDLQR : 393
gi|402259 : MGNPKRSYVIVKHSPIINLLVGVMTAHEHGHNLGMHDSKCLR-----CASIC-----IMRPGLTPGRSYEFSDDSMGYYS : 379
gi|213030 : ICDPKRSTGVVGHSEINLRVAVMTMHEHGHNLGIRHDTDSQSC-----CGYSC-----IMSPVISDEPSKYFSGSIYIQCWE : 375
                                         C    v 6 d    y g    HE H LG HD        g C    g 6Msy        fs c

IsMP1    : IYNRPEFKLRLER-YTTKTIKHS--SKLPGRMTLSNYCQRVYRYEK---GMHADETYG---VRDCRVKGTGTNT----- : 454
Metis1   : IYRRPEFKLRLER-KAKKTLR-S--SKLPGVMTSSSNYCRVYMYEK---GMHADEAYG---VKDQRVKGTTSR----- : 454
EmMP3    : IFNRPQYELRVK-NSINTGIFS--SKLPGVSSRQAYCQKIYYQYK---YVHVDKTYN---ARVCIKCYINRDG----- : 457
EmMP2    : VMRLRGPICWQVK-SQGHKVD---GYPCMLVTPLNICKYKFPREQ---SVDADTSLAV---LKRQKVRCTYSKLR-HLYGR--- : 461
EmMP4    : VVILRGLVCWQLG-NTGHYTD---GIYCMGEVAPNAYRHAEPDKQ---NVTADMESDS---MKMVMVKQYYPEYRRVCIYSSRC : 450
EmMP1    : YVRLGNSQCKRVL-TKVNYMDKH---REFRGETVGVYKYCRKRIFHYTK---GKKVKAFALKGNQDAQNCQMGCYWQGN----- : 447
EmMP5    : MWAYEYEGCLHKNNSNRFFLNWINEYYLGENLSLNRC-EMKYVQLHG---TYYLEKVID---KWYQGYIYVPEVP----- : 464
gi|402259 : FLNQYKPCCLLNKPLRIDPVS---TPVSGNELLLEAGEE----- : 414
gi|213030 : FIMNQKPCCLKKPLRTDTPVS---TPVSGNELLEAGIECDGCSLENPCYATCTCKMRPGSCAEGLCDCGRFMRKKG----- : 449
                                         C        pG        c        c        c

IsMP1    : ---NYWRITVVVGTGCGKKAAILGKCVDDIKISKDD----- : 488
Metis1   : ---MYWLLGVVGTGCGNGKAILGKCRNKIKISKD----- : 488
EmMP3    : ---DNMLITAVLGVCGSGKMCILGNTSKAFKPKSLE----- : 492
EmMP2    : -AYKFPVQVEFAPIHARCGDRTVCIIRGICGLK----- : 493
EmMP4    : FTYVTTYSLQIHAQIYMPCGGNKVICQVCGEKIVMSPQKPTTWTVADTEAKTTTPTPTTPTTATTECRDCDSTTASTT : 536
EmMP1    : ---MRTCWPTPMLERMCTLKGKTKRRCVGVGHVH----- : 478
EmMP5    : -RESSAGQWDFLFGTTCHDGYPQTHLYKRRMPGGPEKNR----- : 506
gi|402259 : ----- : -
gi|213030 : ---TVCRVSMVDRNDTCTGQSADPRNGLYG----- : 478
                                         d    c        c

```

Figure 1

```

      *          20          *          40          *          60          *          80
As51 : KALKRVHSFVTLANTCVSNHNV-SSSRASLPGHRLQ-TPEDGLVRRDGAE-TVNMGFAPLTCTAAL LMSALRAILAAPVWPHPGNQAL : 86
AsC  : ----RVYELWQRAQKIAIMHVV-----QALSVVALT-----FISSMEGRQ-----QAAP---TAKPVAQ-----WKHPGVLSL : 56
As70 : ----- : -
LiMP : -----MIKYIGVFAFLVGGFCH---DFETV ISN-----QDPIVDGM : 33
Astacin : -----MQCAVLLVLLGVVAAS-----PIIPEAARALY : 27

      *          100          *          120          *          140          *          160          *          180
As51 : ESTLLEGGDIVIP--GNSDDRTAVTEDRLWEGCKIPFVVESSLGRHTEM--TCEAMEEIESKTCVRFIPRSMERDYITIER-ESGCV : 170
AsC  : EIGGDEGGDILLP--RNRSTDRTAVTKKDELWEGCKIPFVIDAQLNRTTKK--ILKTMADTESPTCLRFVARRNCKNYVLIIR-REGCS : 140
As70 : -----KCLWEGGVIPFVTDMFFRQEGKMNHWQWMMNVEYITCLRFVRRTWEEHYIHS-EGICV : 60
LiMP : R---LVEGDMIFDD-GPLFTERNAVKYDQCLWENGHIVVEISPLRQYEQT--TREAMRTYEDNFCIKFRRTNADYVNIHV-CDRCY : 115
Astacin : YNDGMEGGDKLRAGRQPARVGAAILGDEYLLSGGVIPFVTAFAGVSGADQSA--LLSCMQEIEEKTCIREVRRTWEEHYVEITTS-ESGCV : 114
      egd          a          LWp G Ip5          6 M E TC64F R 2 Y6 I g gC

      80          *          200          *          220          *          240          *          260 #
As51 : SATGR--MRRACPVSIG-YGCLYKGTMMWHELHVAIGFVHEHSRSDRIYIVPTENAKQDAVTCHEKIKSWQNRLLTFPDMBSVMIYGS : 256
AsC  : SFVGR--QGEVQCVSLG-THCLYKGLIHELHVAIGFVHEHSRSDRDEYIVVFMENVEPENTCEHEKILAPSKNRLLTFPDMBSIMLYGS : 226
As70 : SYVGR--IGCCASISLG-RECLYEGTVVHEVHVAIGLYMHSRDRDEYLDLWKNIDPEYIDHEDRYSAEDVDLILPFDTRKSVMIYFG : 146
LiMP : SRVGKSFRCGECPEISLG-RCCTDFGTLHELHVAIGFVHEHSRDRDEYLDLWKNIDPEYIDHEDRYSAEDVDLILPFDTRKSVMIYFG : 203
Astacin : SYVGR--LSEACQVSIQANGCVYHCTIHELHVAIGFVHEHTRMDRINVTINYNQVDEPMSMTSND-IDTYSRYGELVQYYSIMFYCK : 200
      S 6G4 g Q SLg C y Gt6 HE6 Ha Gf HeH3R DRD 56 6 N F k p5d S6Mlyg

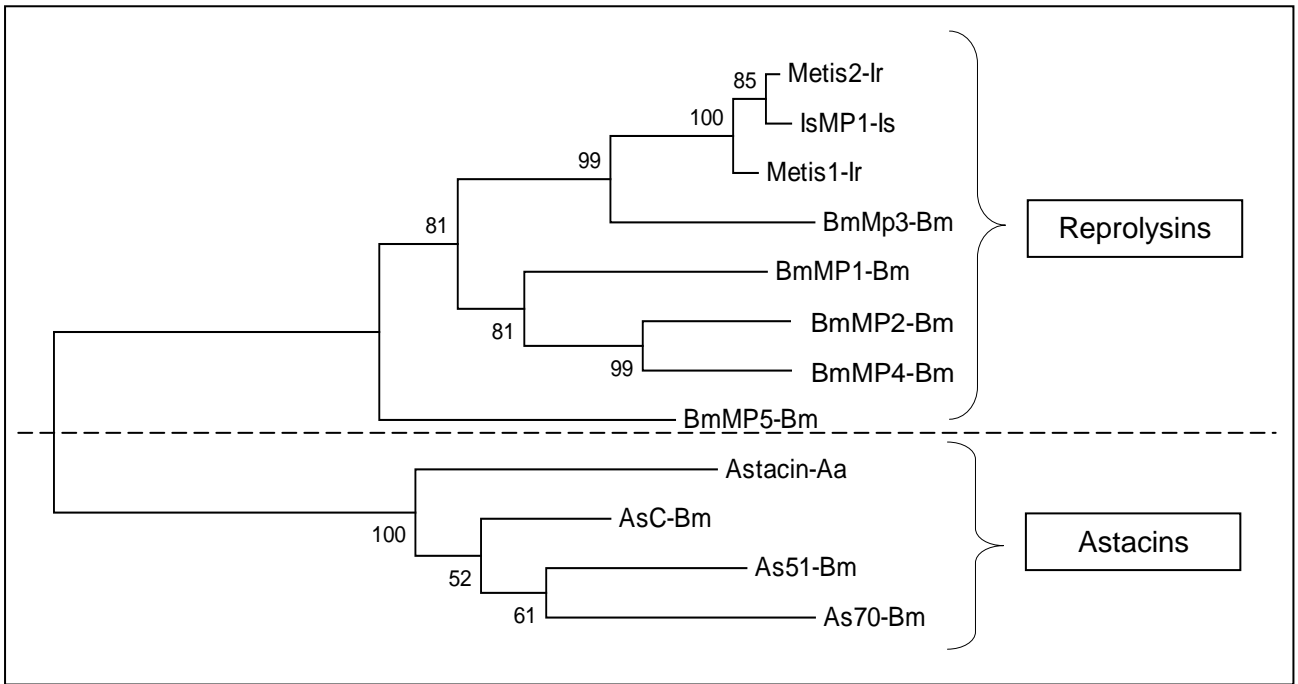
      *          280          *          300          *          320          *          340          *
As51 : HAFARAP-GILTML-AKNGSEITTEVYD----- : 281
AsC  : ETEAREP-GIVTML-AKNGSEIKDVFERPGTASVLRINLLNCKNKKK-FSIVPLYLLIYAVIQDVTLPFFHFVPSLYRRTTFVLCII : 312
As70 : DAFARAP-GILTMK-VKDGSDIPAVYNTGTISDNILISGMLNCSR---QGQLTPHQHKDCT--DMRINCFGSSS----- : 216
LiMP : YAESKDRKFRKTFEPVEFGLMKSVIQGKLSYDILVKVKNLYKCPP---VNPYPGGIRPYVNV----- : 264
Astacin : YSESIQWGVLETIVPLQNGIILITDPRAHLLQTLANQINNLNTNEC--SLRH----- : 251
      F l T6 G 6 v k d ly

      360
As51 : ----- : -
AsC  : FVMT----- : 316
As70 : ----- : -
LiMP : ----- : -
Astacin : ----- : -

```

Figure 2





**Figure 3**

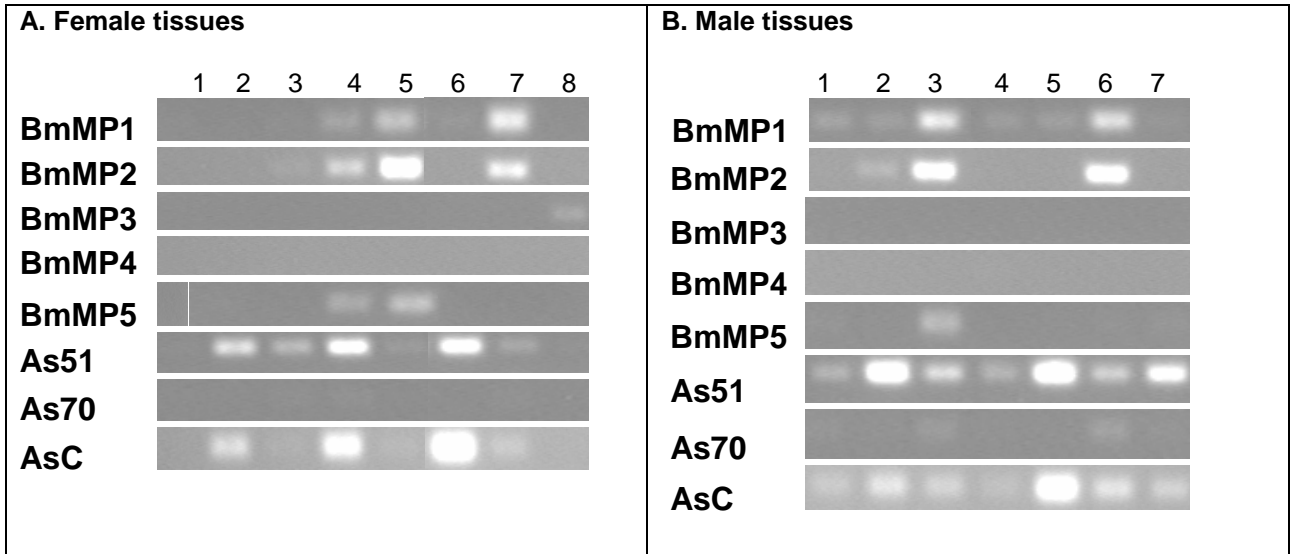


Figure 4.

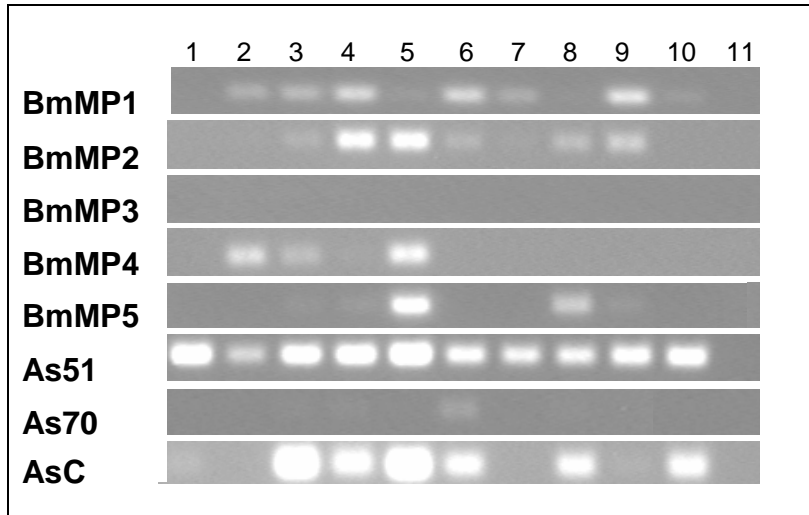


Figure 5.

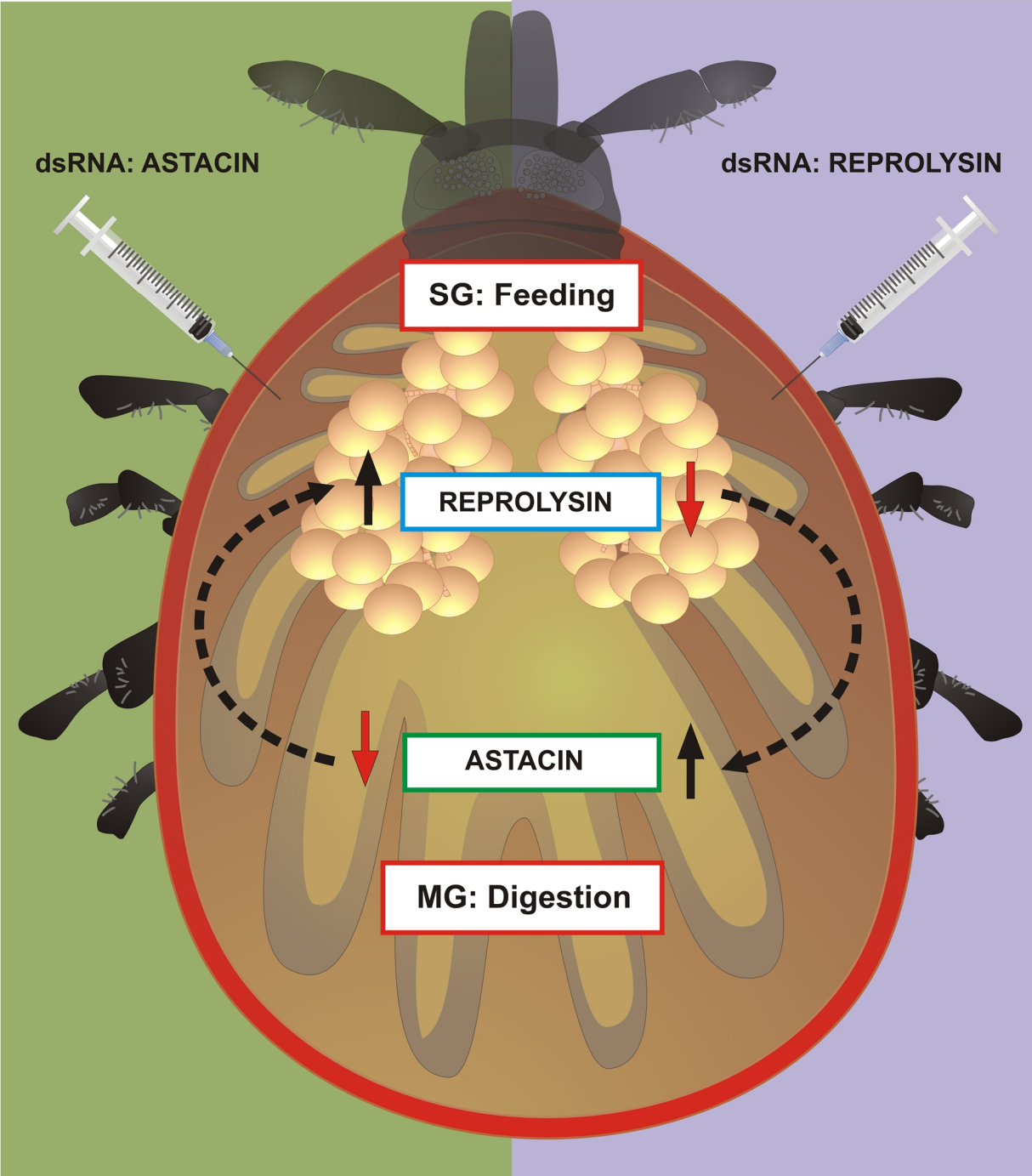


Figure 6.