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Tissue expression and the host's immunological recognition of a *Rhipicephalus microplus* paramyosin

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ABSTRACT

Rhipicephalus microplus is a parasite that causes economic losses in cattle herds, and immunological control is the most promising alternative to replace chemical control. The muscular protein paramyosin has been additionally found in non-muscle tissues and characterized as presenting activities that enable the evasion of the host's immune system in various parasites. This report investigated the recognition level of paramyosin by sera of infested bovines, its expression in tissues, organs and different life stages of *R. microplus*. ELISA analyses showed that paramyosin and salivary gland extract were recognized by infested *Bos taurus* and *B. indicus* sera. Paramyosin gene expression was evaluated in egg, larvae, adult male, and several tissues of partially- and fully-engorged females by qRT-PCR, showing the highest expression levels in fat body. These results show that *R. microplus* paramyosin is immunologically recognized during the tick infestation and together with the high transcription rate found in organs that do not present a highly developed musculature, further suggests that it may possess additional, non-muscle functions in the tick-bovine relationship.

1. Introduction

Rhipicephalus microplus is an ectoparasite that causes important economic losses in cattle herds (Jonsson, 2006; Willadsen, 2006). Conventional methods of control are based on the use of acaricides, however, their residues can cause serious impacts on the environment and contaminate meat and milk (Willadsen, 2004; de la Fuente et al., 2007). Moreover, acaricides present a high cost and their intensive use has caused the selection of resistant tick populations (Guerrero et al., 2012). Therefore, the production of a vaccine is considered one of the most promising alternative methods for tick control, which demands the identification and characterization of protective antigens.

Vaccination experiments with "concealed" antigens (which are not recognized by the host's immune system), such as Bm86 (Willadsen et al., 1989), Bm91 (Riding et al., 1994), BMA7 (McKenna et al., 1998), VTDCE (Seixas et al., 2008), BYC (Leal et al., 2006), and GST (Parizi et al., 2011), have shown to partially protect the host. When antigens were combined, this protection was increased (Willadsen et al., 1996; McKenna et al., 1998; Parizi et al., 2012), which

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indicates that antigen combinations are potentially more effective to elicit protective immune responses against tick infestations. Exposed antigens (recognized by the host's immune system) have also been investigated for host protection against ticks (Wang et al., 1998; Trimnell et al., 2002; Bishop et al., 2002).

Paramyosin (PRM) is a muscle protein found in invertebrates that was primarily isolated from large filaments of unstriated muscle of mollusks (Cohen et al., 1971) and suggested to be involved in the determination of length and stability of muscle filaments in nematodes (Mackenzie and Epstein, 1980). Beyond its structural function, it has been implicated in the modulation of the host's immune system during different parasitic infestations (Landa et al., 1993; McManus et al., 1998; Zhao et al., 2006; Valmonte et al., 2012). The PRM of parasites has been shown to inhibit the classical pathway of complement system "in vitro" (Laclette et al., 1992), and bind IgG (Loukas et al., 2001; Ferreira et al., 2002; Strube et al., 2009). Corroborating the importance of the described activities in parasite–host relationships, PRM has been suggested as a candidate antigen to compose vaccines against diseases such as schistosomiasis (Lanar et al., 1986; Zhou et al., 1999; Fonseca et al., 2004), filariasis (Nunduri and Kazura, 1989; Li et al., 1993), clonorchiasis (Wang et al., 2012) and cysticercosis (Vazquez-Talavera et al., 2001).

In this work, the recognition of paramyosin by the sera of naturally and experimentally infested bovines was evaluated and the levels of the PRM gene expression in different *R. microplus* tissues and developmental stages were measured.

2. Materials and methods

2.1. Recombinant protein expression and purification

The cDNA coding sequence of *R. microplus* PRM was previously cloned into pGEX-4T3 vector (GE-Life Sciences) to generate the recombinant paramyosin protein (rBmPRM) (Ferreira et al., 2002). Expression in the *Escherichia coli* BL21 strain and purification was followed according to Ferreira et al. (2002).

2.2. Salivary antigen preparation

Salivary antigen was obtained according to da Silva Vaz Jr et al. (1994). Briefly, partially engorged females from the Porto Alegre strain were dissected in PBS and salivary glands were separated from other organs and frozen at −70 °C. Salivary glands were macerated and sonicated (Ultrasonicator Cole Parmer, 4710, 500 W, 4 and 20% duty cycle) in a solution containing Tris/HCl 10 mM pH 8.2, 1% deoxicolate, leupeptin (8 mg/ml), pepstatin A (1 mg/ml) and TPCK (0.1 mM) and centrifuged at 32,000 × *g* for 40 min at 4 °C. The soluble fraction (supernatant) was then collected and stored at −70 °C.

2.3. Sera of naturally and experimentally infested bovines

Sera from six *Bos taurus* (Hereford) and eight *B. indicus* (Nelore) bovines from a farm in Pelotas (Brazil), within a region naturally infested with *R. microplus*, as well as the sera from non-infested *B. indicus* animals (negative controls) were kindly provided by the Departamento de Veterinária Preventiva, at the Universidade Federal de Pelotas (Brazil). Additionally, the sera from three bovines (indicated as bovines 1, 2 and 3) submitted to twelve successive experimental infestations were the same described previously by Cruz et al. (2008). Briefly, the infestation regime consisted of six initial heavy infestations with 18,000 larvae (Bagé strain) followed by six light infestations with 800 larvae. All infestations were performed once a month and along the back.

2.4. Western-blot

rBmPRM was submitted to SDS–PAGE 10% (58 μg/cm) and transferred to the nitrocellulose membrane at 70 V for 1 h at 4 °C (Dunn, 1986). Nitrocellulose strips of 4 mm were blocked for 1 h at room temperature with blocking buffer (cow non-fat dry milk 5%–PBS). Prior to the overnight incubation at 4 °C with the membrane strips, all sera were diluted 1:50 in an *E. coli* BL21 strain lysate expressing the pGEX-4T3 vector and incubated for 2 h at room temperature for removal of contaminating anti-vector and *E. coli* proteins reactive antibodies. As positive control an anti-rRmPRM hyperimmune serum (1:400) raised in bovine was used. Preparation of the *E. coli* BL21 strain lysate was performed according to Rott et al. (2000). After 3 washes with blocking buffer, the strips were incubated for 1 h with anti-bovine IgG peroxidase conjugate (Sigma), diluted 1:6000 in blocking buffer. The strips were then washed three times with PBS, and the development buffer (5 mg 3,3-diaminobenzidine in 30 ml PBS plus 150 μl H_2O_2 30% and 100 μl $CoCl_2$ 1%) was added.

2.5. ELISA

Microtitration plates were incubated overnight at 4 °C with 0.5 μg of rBmPRM or 1 μg salivary gland protein extract diluted in 50 mM carbonate/bicarbonate buffer pH 9.6 per well. Plates were washed three times with blocking buffer, blocked for 1 h with blocking buffer at 37 °C, and then incubated with bovine sera diluted 1:50 in blocking buffer for 1 h at 37 °C. Plates were washed with blocking buffer and incubated with anti-bovine IgG-peroxidase conjugate diluted 1:5000 in blocking buffer, and after, washed with PBS for three times. Then, 3.4 mg o-phenylenediamine, 5 μl H_2O_2 in 0.1 M citrate–phosphate buffer, pH 5.5 were added and incubated for 15 min in a dark room. Reaction was stopped with 12.5% H_2SO_4. The optical densities were determined at 492 nm. The analyses shown in Figs. 1 and 2 were performed in duplicates and mean and standard deviation values were used for comparison.

2.6. cDNA synthesis and qRT-PCR

Fully and partially engorged females were washed with 70% ethanol, immobilized with glue on Petri dishes and flooded in cold phosphate-buffered-saline (PBS; sodium

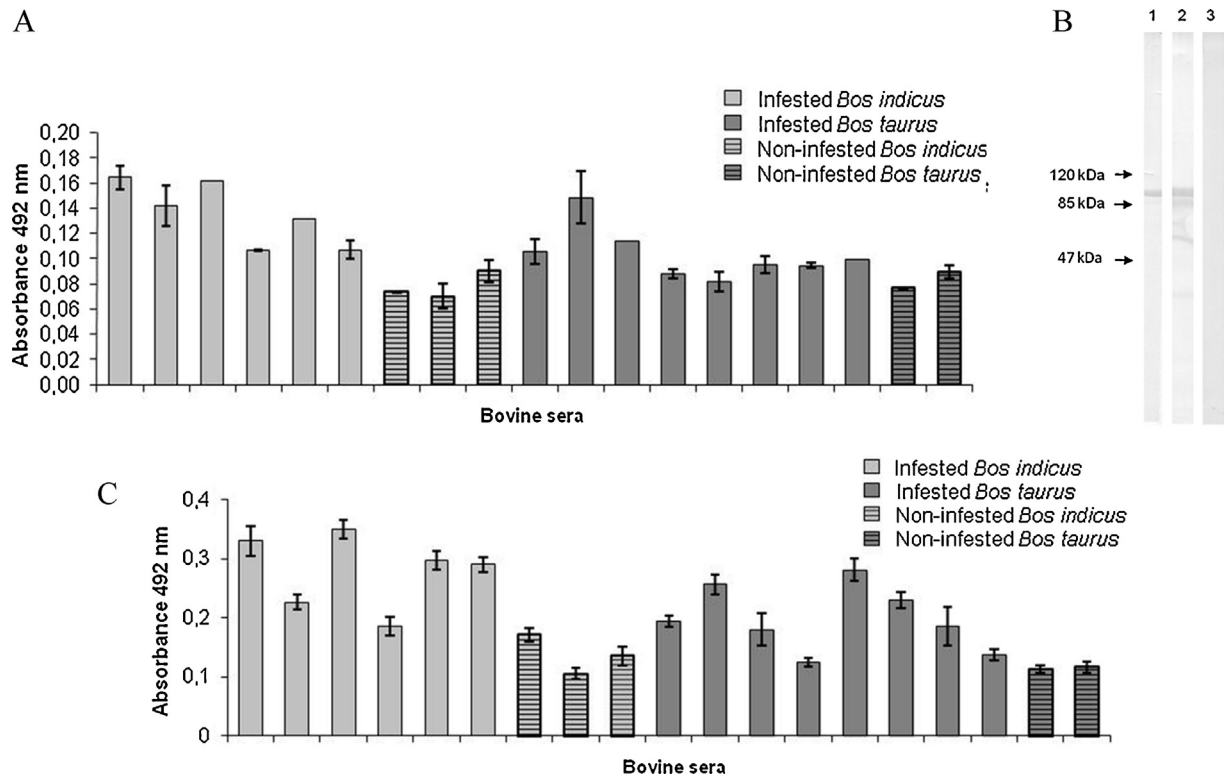

Fig. 1. Recognition of paramyosin and salivary gland extract by infested *Bos indicus* and *B. taurus* sera. (A) rBmPRM recognition by naturally infested bovine sera (individual animals are indicated by vertical bars) determined by ELISA. (B) Western-blot of rBmPRM recognition by *B. indicus*; 1, infested bovine; 2, hyperimmune anti-rBmPRM serum (positive control); 3, non-infested bovine. (C) Salivary gland recognition by bovine sera (individual animals are indicated by vertical bars) determined by ELISA. Data shown indicate the mean values of absorbance measurement from each bovine serum and error bars represent standard deviation.

phosphate (10 mM), NaCl (150 mM), pH 7.2). The dorsal cuticle was removed using a scalpel blade and gut, salivary glands, ovary and fat body were dissected with fine-tipped forceps. The fat body is spread covering the tick internal body cavity, so it was carefully removed, after all other tissues. Dissected female tissues, whole males, 5-, 10- and 15-day-old larvae and 1-, 3-, 6-, 12- and 18-day-old eggs were macerated and total RNA extracted using TRIzol (Invitrogen) according to the manufacturer's instructions.

The quantity and quality of the recovered RNA was determined spectrophotometrically at A280 nm and by the ratio A260/A280 nm, respectively. 500 ng RNA were submitted to reverse transcription, which was performed using Superscript III (Invitrogen) according to manufacturer's instructions. qRT-PCR was performed with RNAs from gut, fat body, ovary and salivary gland of partially- and fully-engorged females, 1-, 3-, 6-, 12- and 18-day-old eggs, 5-, 10- and 15-day-old larvae and adult males. The primers 5′-CCAGCGCACGATTGTTGA-3′ and 5′-CACTTGAAGGTTGCGGACTTC-3′ for BmPRM gene were used in qRT-PCR, which were designed with the Primer Express 3.0 software (Applied Biosystems, Foster City, USA) targeting the paramyosin's cDNA sequence (GenBank accession number AF479582). The samples were also analyzed with primers 5′-GGACGACCGATGGCTACCT-3′ and 5′-TGAGTTGATTGGCGCACTTCT-3′ for the 40S ribosomal protein, used as control as already described (Pohl et al., 2008). The reactions were performed using the Platinum® SYBR® Green qPCR SuperMix kit (Invitrogen), 10 pmol primers and 100 ng cDNA. The qRT-PCR reactions were performed in an Applied Biosystems Step One Plus thermocycler, and the Relative Expression Software Tool (REST) was used for data analyses (Pfaffl et al., 2002). All qPCR reactions were performed in duplicates and mean values were considered for comparison. Genomic DNA contamination was tested by performing amplification reactions using template without reverse transcription, as already described (Imamura et al., 2013).

2.7. Statistical analyses

Statistical analyses were performed using SPSS 20 (Statistical Package to Social Sciences for Windows; SPSS Inc., Chicago, IL) software by one-way ANOVA and Tukey's post-test. The values were considered different to the level of $p < 0.05$. Pearson's correlation coefficient was performed to correlate the rBmPRM and salivary gland extract ELISA recognition levels.

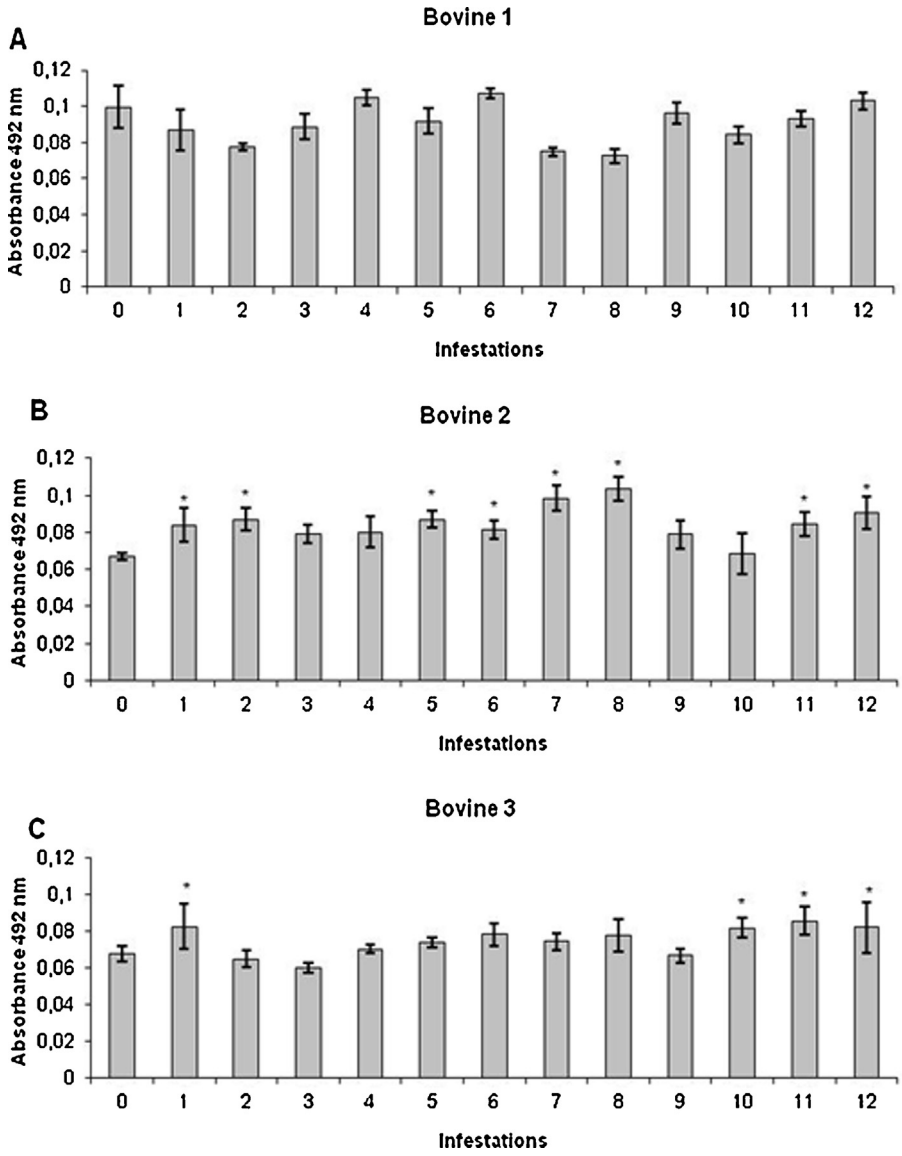

Fig. 2. Recognition of rBmPRM by sera of experimentally infested bovines *Bos taurus* 1 (A), 2 (B) and 3 (C) by ELISA. 0, sera pre-infestation; 1–12, sera post-experimental infestations. Experiments were replicated three times and data shown indicate the mean values of absorbance measurement from each serum and error bars represent standard deviation. *Statistical analysis was performed to differentiate rBmPRM recognition by infested bovines sera and non-infested by ANOVA one-way followed by Tukey's test ($p < 0.05$).

3. Results

3.1. Paramyosin is recognized by sera of naturally infested bovines

The ELISA analysis was performed to evaluate the rBmPRM recognition by sera of naturally infested *B. indicus* and *B. taurus* bovines, showing the presence of different IgG levels in the tested sera (Fig. 1A). The specificity of the antibody recognition was confirmed by Western-blot (a representative Western-blot from a positive *B. indicus* bovine serum is shown in Fig. 1B). Sera from non-infested bovines were used as negative control. In order to evaluate the overall recognition of tick antigens by the same sera, an ELISA analysis using salivary glands protein extract as antigen was performed (Fig. 1C). Accordingly, *B. indicus* and *B. taurus* sera presented different IgG levels against the tick salivary gland extract. The Pearson's correlation coefficient comparing the recognition against rBmPRM and salivary gland extract showed a $r = 0.67$ for *B. indicus* and a $r = 0.28$ for *B. taurus* sera.

3.2. Paramyosin is recognized by sera of experimentally infested bovines

The rBmPRM recognition by sera from three bovines submitted to 12 experimental successive infestations (6 heavy infestations followed by 6 light infestations) was

evaluated by ELISA (Fig. 2). Sera from one infested animal (bovine 1) showed no significant humoral response against BmPRM. The other two bovines (bovines 2 and 3) showed the presence of significant anti-BmPRM IgG in the sera obtained following different infestations, but not in all of them. Bovine 2 did not show significant anti-rBmPRM IgG levels in sera from infestations 3, 4, 9 and 10, while bovine 3 showed the presence of significant IgG levels only in sera from infestations 1, 10, 11 and 12.

3.3. Paramyosin gene expression

Fig. 3 shows a qRT-PCR analysis of the expression of the paramyosin gene (*bmprm*) within eggs, larvae, adult males and adult female organs and tissues. The highest relative *bmprm* expression levels detected in all developmental stages and tick tissues tested were observed in adult female fat body. Among the different embryonic developmental stages tested, *bmprm* expression was detected in 18-day-old eggs, while in the larval stage 5 and 10-day-old larvae showed low expression levels, with absence of expression in 15-day-old larvae. Gut showed higher *bmprm* expression in partially engorged than fully engorged females ($p = 0.004$), while similar expression levels were detected in 5- and 10-day-old larvae, ovary, salivary gland and fat body ($p = 1.00$, $p = 1.00$, $p = 1.00$ and $p = 0.982$, respectively). *bmprm* expression was also detected in adult males.

4. Discussion

A prototypical parasite concealed antigen is considered as not being able to generate an adaptative immune response under a natural infestation (Willadsen et al., 1993; Nuttall et al., 2006). In this regard, parasite muscle proteins are candidates to comprise such definition and, therefore, they may also turn into candidates to take part in a vaccine cocktail, which has initially shown to be effective against the stable fly *Stomoxys calcitrans* (Schlein and Lewis, 1976). So, *R. microplus* PRM seemed to deserve further attention, as it was shown to be present in *R. microplus* tissues that are exposed to the host's immune system (Ferreira et al., 2002) and PRMs have been shown to induce protective responses when used as immunogen against different parasite infestations (Li et al., 1993; McKenna et al., 1998; Vazquez-Talavera et al., 2001). Contrary to the initial expectations, considering that BmPRM was not identified in saliva (Ferreira et al., 2002), but consistent with the described host's immune response against PRM from other acari (Mattsson et al., 2001; Tsai et al., 2000; Lee et al., 2004), rBmPRM was recognized by sera of infested bovines, turning it from a probable concealed (Ferreira et al., 2002) into an exposed antigen.

PRM was initially described as an internal muscular protein of invertebrates, but many parasites, mainly mites and helminths, have been show to induce anti-PRM humoral immune responses in their hosts (Zhao et al., 2006; Nara et al., 2007; Ramos et al., 2003a), suggesting that the host's immune systems have direct contact with parasite PRMs. In the mites *Dermatophagoides pteronyssinus* (Tsai et al., 2005), *D. farinae* (Tsai et al., 1999) and *Blomia tropicalis* (Ramos et al., 2003b), PRM showed to represent an important allergen, and in *Schistosoma japonicum*, IgE responses to PRM were shown to predict resistance to reinfestation (Jiz et al., 2009). Anti-PRM IgG responses have been described against acari and helminths, such as *Taenia saginata* (Ferrer et al., 2003), *Trichostrongylus colubriformis* (Kiel et al., 2007), *Trichinella spiralis* (Yang et al., 2008), *B. tropicalis* (Ramos et al., 2003a), and *Sarcoptes scabiei* (Mattsson et al., 2001). The data presented herein showed that both *B. taurus* and *B. indicus* infested bovines developed IgG against rBmPRM as well as recognize salivary antigens at different levels, showing individual differences in antibody production against rBmPRM and salivary extract antigens. Serine protease inhibitor-3 (RMS-3), a salivary *R. microplus* protease inhibitor, has recently been described to be recognized by infested bovines sera, showing the development of higher IgG levels in resistant than in susceptible individuals (Rodriguez-Valle et al., 2012). In this sense, the Pearson's analysis suggests a difference in the immune response of *B. taurus* and *B. indicus* against rBmPRM and salivary gland proteins. A direct comparison between the anti-rBmPRM levels developed by the susceptible *B. taurus* and resistant *B. indicus* naturally infested bovines was not included as the individuals analyzed present different ages (around two years for *B. taurus* and over three years for *B. indicus*), and, therefore, were exposed to ticks for differing period of time (the comparison of IgG levels between *B. taurus* and *B. indicus* groups, evaluated by parametric Student's *t*-test, indicate a $p = 0.005$). Perhaps, the evaluation of a higher number of animals under controlled conditions could clarify the differences that may exist in the anti-BmPRM immune responses of resistant and susceptible cattle breeds. Additionally, on what concerns to possible cross-reacting antibodies, Valmonte et al. (2012) showed that the IgE response of ascaris-infected subjects exhibited reactivity against *B. tropicalis* paramyosin, indicating that PRM should be one of the proteins responsible for cross-reactions between house dust mites and helminthes. Therefore, although it is possible that infestations by other parasites trigger the development of IgG able to recognize BmPRM, the recognition of rBmPRM by sera from bovines experimentally infested and maintained at controlled conditions suggests that *R. microplus* infestations can induce an anti-BmPRM IgG response.

Antigens with relative mobility in SDS–PAGE consistent with BmPRM that are recognized by sera of infested and vaccinated bovines have already been described in different adult tissues and developmental stages (da Silva Vaz et al., 1994; Kimaro and Opdebeeck, 1994; Cruz et al., 2008), but unfortunately they were not characterized. Interestingly, Pruett et al. (2006) described the recognition of a 102.3 kDa antigen by the sera of *B. taurus* bovines successively infested with *R. microplus* larvae, suggesting it may be a specific marker of *R. microplus* larvae exposure. If the 102.3 kDa antigen described represents BmPRM, which presents 102 kDa, the host's immune recognition of BmPRM may initiate in the larvae stage, not necessarily depending on its presence in female adult saliva. Also, Cruz et al. (2008) analyzed tick antigen recognition by sera from 5 bovines submitted to 12 successive experimental infestations, reporting the Western-blot positive identification of molecules presenting a molecular mass

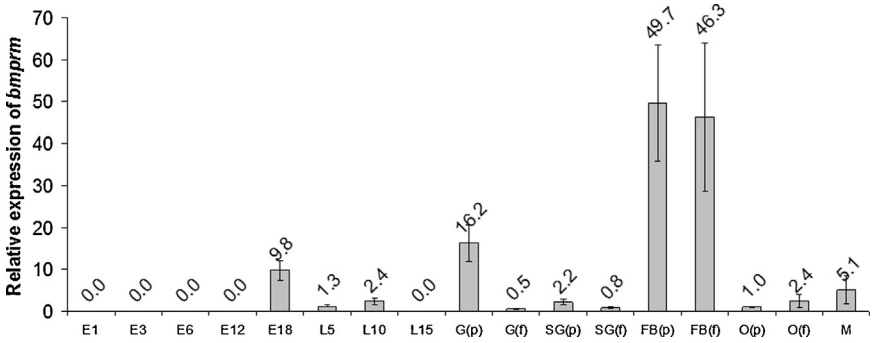

Fig. 3. Paramyosin gene expression in tick tissues and organs detected by qRT-PCR. E1, 1-day-old egg; E3, 3-day-old egg; E6, 6-day-old egg; E12, 12-day-old egg; E18, 18-day-old egg; L5, 5-day-old larva, L10, 10-day-old larva; L15, 15-day-old larva; G(p), partially-engorged female gut; G(f), fully-engorged female gut; SG(p), partially-engorged female salivary gland; SG(f), fully-engorged female salivary gland; FB(p), partially-engorged female fat body; FB(f), fully-engorged female fat body; O(p), partially-engorged female ovary; O(f), fully-engorged female ovary; M, male. Data are presented as mean values and error bars represent standard deviation.

compatible with BmPRM. Furthermore, Reck et al. (2009) showed that a pool of these sera was able to abolish anti-haemostatic activities from *R. microplus* saliva. Here, the sera from three out of the five bovines analyzed by Cruz et al. (2008) and Reck et al. (2009) were tested against rBmPRM by ELISA and two bovines showed to have developed an IgG response against BmPRM. The differences of rBmPRM recognition between bovines and after different infestations are consistent to what was described by Cruz et al. (2008). Similarly, Piper et al. (2009) reported individual variation in antigen recognition of tick extracts by IgG responses, showing distinct antibody levels between individuals of the same breed against the same tick antigens, as well as that preliminary Western-blot analysis indicated that susceptible and resistant animals produced antibodies to different tick antigens. Thus, it may be suggested that infestation levels can influence the IgG bovine response produced against BmPRM and it may vary widely among individuals.

The multifunctional nature of parasite PRMs, specially accounted by their possible immunosupressive properties, has been assigned to explain, at least partially, their distribution within the organisms. PRM was recognized in the surface of *S. mansoni* (Matsumoto et al., 1988) and *S. japonicum* (Gobert, 1998). In *Paragonimus westermani*, the protein was found in all development stages and was localized in gut and ovary (Zhao et al., 2006). In *R. microplus*, BmPRM was found in all tissues and developmental stages tested, except saliva (Ferreira et al., 2002), what was corroborated by the data of the qRT-PCR performed in this study. Indeed, *bmprm* showed to be expressed in very different levels at 18-day-old eggs, 5- and 10-day-old larvae, gut, salivary gland, fat body and ovary of partially- and fully-engorged adult females, and at adult males, but not at 1-, 3-, 6-, 12-day-old eggs and 15-day-old larvae. The *bmprm* expression found in 18-day-old eggs indicates that changes preceding hatching need higher quantities of BmPRM. The presence of BmPRM in tick tissues without a prominent musculature, such as adult female salivary gland and fat body, corroborate the indications that BmPRM plays additional roles beyond the muscle. Considering that, (i) when *R. microplus* feeds, antibodies are ingested retaining functional activity (da Silva Vaz et al., 1996), (ii) the wide distribution of BmPRM in adult tick tissues, and (iii) rBmPRM has been shown to bind immunoglobulins (Ferreira et al., 2002), the possible involvement of BmPRM in IgG clearance is reinforced, as already suggested (Ferreira et al., 2002). It has been shown that immunoglobulin binding proteins (IGBPs) of *R. appendiculatus*, *Amblyomma variegatum* and *Ixodes hexagonus* mediate the binding of IgG in gut, the transportation by the hemolymph to the salivary glands, and its secretion in the saliva (Wang and Nuttall, 1994, 1995, 1999). Indeed, the proportionally high expression of the BmPRM gene in adult female fat body indicates the high demand of the protein in the rapid ingurgitation and post-detachment tick phases. On the other hand, the higher expression of *bmprm* in the salivary glands of partially-engorged females in comparison to fully-engorged females suggests that BmPRM is not as demanded in this tissue after finishing the tick-bovine contact.

BmPRM has been shown to possess similar characteristics presented by helminthic PRMs beyond the contractile apparatus, which are considered important for parasite survival. As ticks and helminths evolved independently in their parasitic relationships, it is tempting to speculate that the non-muscle functions performed by these orthologous proteins are the result of convergent evolution. Also, helminths, mites and ticks (or at least *R. microplus*) show to induce humoral responses in their hosts, although no PRM presents a signal peptide sequence to enable their secretion. Collectively, these evidences indicate that BmPRM presents the same attributes that turned PRMs into promising targets for the development of vaccines against helminth parasites (Pearce et al., 1988; Nunduri and Kazura, 1989; Vazquez-Talavera et al., 2001; Zhang et al., 2006; Yang et al., 2010) as well as possess desirable characteristics already described to be important in immune protection development against ticks (Trimnell et al., 2002; Nuttall et al., 2006). Therefore, BmPRM represents a potential candidate to compose a cocktail vaccine against *R. microplus*.

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