QUANTITATIVE AND QUALITATIVE EVALUATION OF THE EGG PROTEINS OF *Rhipicephalus microplus* AND *Rhipicephalus sanguineus* (ACARI: IXODIDAE) DURING OVIPOSITION*

Vanessa de Almeida Raia[1+], Paulo Roberto de Carvalho Filho[2], Adriana Rayol Pedrenho[3], Walter Flausino[4] and Kátia Maria Famadas[5]

ABSTRACT. Raia V. de A., de Carvalho Filho P.R., Pedrenho A.R., Flausino W. & Famadas K.M. **Quantitative and qualitative evaluation of the egg proteins of *Rhipicephalus microplus* and *Rhipicephalus sanguineus* (Acari: Ixodidae) during oviposition.** [Avaliação quantitativa e qualitativa das proteínas dos ovos de *Rhipicephalus microplus* e *Rhipicephalus sanguineus* (Acari: Ixodidae) durante a postura]. *Revista Brasileira de Medicina Veterinária*, *34(3):177-182, 2012*. Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, BR 465 km 7, Seropédica, RJ 23890-000, Brasil. E-mail: e-mail: vanessaraiaufrrj@gmail.com

To fill in some gaps in the knowledge of the intrinsic mechanisms of the oviposition biology of *Riphicephalus microplus* and *R. sanguineus*, the protein variability in eggs according oviposition day was evaluated. Engorged females laid eggs in a controlled environment chamber (27 ± 1° C, 80 ± 5% RH, and darkness). As soon as the females began oviposition, egg samples weighing 50 mg were collected daily, placed in Eppendorf tubes and preserved at - 20ºC. The protein concentration was evaluated by the Bradford method and submitted to analyses by SDS-PAGE. After normality of the data was excluded by the Shapiro-Wilk test, the data were transformed into logarithmic values [log (X+1)] and the protein concentrations were correlated with the oviposition days using Pearson's correlation coefficient (r). During oviposition the egg protein concentrations of *R. microplus* and the days of lying were weakly positive correlated, while for *R. sanguineus* the egg protein concentrations remained constant until the last day. It cannot be inferred that the protein variation in the eggs of *R. microplus* is correlated with the oviposition day. The two tick species thus have different oviposition protein profiles, which can be use as a phenetic tool.

KEY WORDS. Tick, Posture, Electrophoresis.

RESUMO. Objetivando preencher lacunas acerca dos mecanismos intrínsecos da biologia da oviposição dos carrapatos ixodídeos, foram realizadas análises da concentração e do perfil eletroforético das proteínas dos ovos de *R. microplus* e *R. sanguineus* durante o período de postura. Fêmeas ingurgitadas de ambas as espécies efetuaram postura em estufa biológica (BOD) sob condições controladas (27 ± 1° C, 80 ± 5% UR, escotofase). Iniciada a postura, amostras de ovos foram coletadas diariamente.

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[1] Médica-veterinária, *Dr.CsVs*. Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária (IV), Universidade Federal Rural do Rio de Janeiro (UFFRRJ), BR 465 Km 7, RJ Seropédica, 23890-000, Brasil. [+]**Autor for correspondence**. E-mail: vanessaraiaufrrj@gmail.com - Capes/Faperj scholarship.

[2] Médico-veterinário, *M.CsVs*. Serviço de Vigilância Agropecuária do Porto de Santos, Superintendência Federal de Agricultura, MAPA, Rua XV de Novembro, 122, Santos, SP 11010-150, Brasil. E-mail: carvalhofilhopr@gmail.com

[3] Bióloga, *D.Sc*., Departamento de Ciências Fisiológicas, Instituto de Biologia, UFRRJ, BR 465 Km 7, Seropédica, RJ 23890-000. E-mail: apedrenho@yahoo.com.br

[4] Biólogo, *PhD*. Departamento de Parasitologia Animal (DPA), IV, UFRRJ, BR 465 Km 7, Seropédica, RJ 23890-000. E-mail: flausino@ufrrj.br - CNPq fellowship.

[5] Zootecnista. *Dr.Med.Vet.Parasitol.Vet*. DPA, IV, UFRRJ, BR 465 Km 7, Seropédica, RJ 23890-000. E-mail: kfamadas@ufrrj.br

Posteriormente, após quantificação pelo método de Bradford, foram submetidas a fracionamento em SDS-PAGE de acordo com Laemmli, 1970. Após descarte da normalidade pelo teste de Shapiro--Wilk, os valores das concentrações protéicas foram transformados logarítmicamente [log (X+1)] e correlacionados com os dias de postura através do coeficiente de correlação de Pearson (r). Durante a oviposição de *R. microplus*, a concentração de proteínas nos ovos e os dias de postura apresentaram fraca correlação positiva, em *R. sanguineus* a concentração de proteínas nos ovos permaneceu constante até o último dia. Deste modo não podemos concluir que a variação da concentração de proteínas nos ovos de *R. microplus* está relacionada aos dias de postura. Os carrapatos *R. microplus* e *R. sanguineus* possuem perfil de proteínas diferentes durante a oviposição, o que pode ser usado como característica fenética.

PALAVRAS-CHAVE**.** Carrapato, Postura, Eletroforese.

INTRODUCTION

Among ticks that attack domesticated animals, *Riphicephalus microplus* (Canestrini, 1887) and *Riphicephalus sanguineus* (Latreille, 1786) stand out (Jongejan & Uilenberg 2004). In Brazil, *R. microplus* causes economic losses to livestock breeders estimated at 2 billion dollars a year (Grisi et al. 2002). In turn, *R. sanguineus*, besides its direct parasitism (mainly of pets), is a vector of the agents causing babesiosis and ehrlichiosis and is also suspected of being a main vector of *Rickettsia rickettsii* outbreaks in urban areas (Labruna & Pereira 2001, Piranda et al. 2008, Cunha et al. 2009).

A great deal of research has been directed at controlling ticks. However, the control methods used do not always produce the expected results from a cost-benefit standpoint and ticks rapidly develop resistance to most acaricides, today the main control method (Davey et al. 2006).

Females of the main tick species lay an average of 3,000 eggs per female during several days of a gonotrophic cycle (Gallardo & Morales 1999). Since the Ixodidae are hematophagous parasites, the females need to ingest a large amount of blood from the host to serve as a source of nutrients (Sonenshine 1991) for maturation and development of the oocytes (Denardi et al. 2004).

After the blood is ingested, the hemoglobin is broken down by lysis in the midgut lumen and is absorbed by the intestinal cells, where it is slowly degraded and released into the hemolymph (Sonenshine 1991). This degradation is accomplished in the fat body, where vitellogenin is synthesized. Vitellogenin is a complex protein formed of protein sub-units (James & Oliver 1997) and is considered a heme reserve. The vitellogenin is then released into the hemolymph and selectively absorbed by the developing oocytes, where it is stored as vitellin in the yolk granules (Obenchain & Galun 1982, Sonenshine 1991, Denardi et al. 2004).

Because a large amount of vitellogenin is necessary, its release into the hemolymph and absorption by the mature oocytes does not occur simultaneously. A sufficient amount of the material needed for form the yolk granules cannot be synthesized to supply all the oocytes at the same time (Obenchain & Galun 1982, Sonenshine 1991). This can explain why the development and maturation of the oocytes in the ovary is not synchronized, so that oocytes can be found simultaneously at various developmental stages (Denardi et al. 2004).

The oviposition biology of ticks, particularly the role of abiotic factors on the number of eggs laid and hatching rate, has been well reported in the literature (Bellato & Daemon 1997). This knowledge has contributed to the formulation of tick management strategies. However, though many studies have been performed on tick reproduction physiology, there are still many important unexplained aspects of the biology of the different phases of these parasites oviposition cycle.

One of the most important physiological questions that need to be elucidated regards the intrinsic factors responsible for the lower hatching rate of the eggs in the first and last laying days. In *R. microplus*, posture lasts about fifteen days and begins to decrease larval hatchability from eggs laid on the seventh day of oviposition, reaching zero in the eggs of the last day (Bennett 1974, Hitchcock 1955). These factors result from changes in the female's metabolism during this period. This work reports the initial findings of a line of study of the physiological mechanisms that regulate the larval hatching rate of tick eggs. We assessed the relationship between the egg-laying rate and the protein concentration and composition of the eggs on different days after the start of oviposition.

MATERIAL AND METHODS

The experiment was conducted at the Laboratório de Ixodologia, Laboratório de Biologia Molecular

and Laboratório de Coccídios e Coccidioses, all part of the Departamento de Parasitologia Animal of Universidade Federal Rural do Rio de Janeiro (UFRRJ).

Engorged females of *R. microplus* and *R. sanguineus* of second and fifth generation from collection the wild respectively, were obtained from artificial infestations on cattle and rabbits, respectively. During this experimental phase, all hosts were kept in individual cages and given water and commercial feed ad libitum daily. The protocol of Ethical Principles for Animal Research of the Brazilian College of Animal Experiments was observed throughout the experiment. After collection, the female ticks were washed in a 1% hypochlorite solution, rinsed, dried on paper towel and fixed with double-sided adhesive tape in Petri dishes, which were then placed in a biological chamber for oviposition under controlled conditions (27 ± 1 ºC, 80 ± 5% RH, darkness). Eggs were obtained from a pool with 57 *R. microplus* females, with average weight of 217.9 ± 44.2 mg, and a pool with 69 *R. sanguineus* females, with average weight of 127.9 ± 30.1 mg. All females used in the study started posture on the same day, this ensure that the eggs in the sample pool gathered daily would correspond to the same laying day of each tick. During most of the laying period, daily samples of 50 mg of eggs were collected. Each sample was placed in an *Eppendorf* tube, identified and preserved in a freezer at –20 ºC. After 11 laying days there was a natural decline in the number of eggs, so it was not possible to attain the 50 mg weight threshold. Therefore, to avoid errors during the protein measurements due to the low protein concentration in the samples, we analyzed samples of eggs from first day up to the 13th oviposition day.

To measure the total protein concentrations, the eggs were defrosted at room temperature (23 ± 1 ºC), washed with phosphate buffer (0.1 M, pH 7.4) with SDS 7 mM; ground with a sterile mortar and pestle with 1ml of lysis buffer (TRIS 100 mM, containing EDTA 10 mM, 0.5% Nonidet-P 40, N--tosyl-L-lysine-chloromethylketone (TLCK) 0.1 mM and PMSF 1 mM), and then filtered through a 0.45-mm Millipore membrane. For chemical denaturation, 100µl of 5X loading buffer (TRIS 2M, pH 6.8; containing SDS 0.35 M; 50% glycerol; 25% β-mercaptoethanol and bromophenol blue) in 500µl of the filtered sample. The physical denaturation was performed at 100ºC for three minutes. Then the samples were allowed to cool at room temperature (23 ± 1ºC) and analyzed.

The proteins were quantified according to the method described by Bradford (1976), using a FEMTO spectrophotometer, to analyze 30 µg of proteins from each sample by SDS-PAGE (Laemmli 1970). After the gels were stained with Coomassie blue, the densitometric analysis was performed using a Bio-Rad densitometer and the Quantity One program.

The pre-oviposition, laying and incubation periods were observed according to Bellato & Daemon (1997). The laying rate was compared against the results of the concentration and profile of the proteins. Since the Shapiro-Wilk test showed that the data on the protein concentrations were not normally distributed, they were transformed into log (X+1) form and compared with the laying days through the Pearson correlation coefficient (r) (Ayres et al. 2005).

RESULTS AND DISCUSSION

Oviposition started two days after all of 57 females of *R. microplus* dropped off the hosts. The average laying period was 13.23 ± 5.52 days, and very few ticks were still laying eggs by the 21st day after starting. The peak occurred on the fifth day (Figure 1). The *R. sanguineus* females had a pre-oviposition period of 3.91 ± 0.70 days, an average laying period of 14.83 ± 2.62 days, and most of the ticks had stopped laying by the 19th day. The peak laying rate occurred on the fourth day after the start (Figure 1). These figures can be considered within the normal range for colonies, which were maintained at 27 ºC (Bellato & Daemon 1997), demonstrating the integrity of the colonies and assuring a normal oviposition course. The protein concentrations of the *R.*

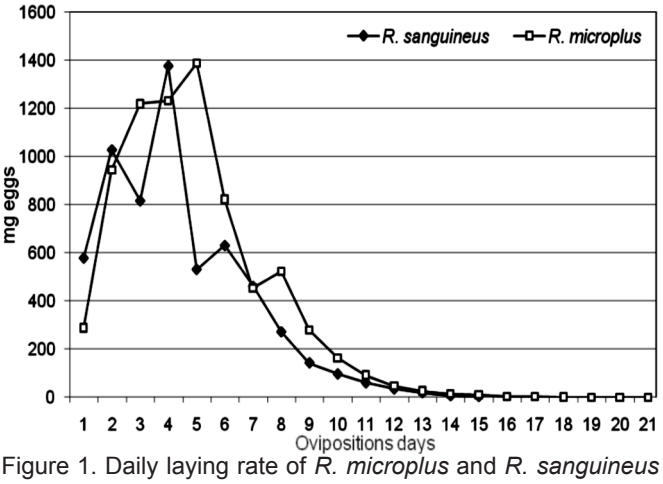

Figure 1. Daily laying rate of *R. microplus* and *R. sanguineus* females under controlled laboratory conditions (27±1 ºC, 80±5% RH, darkness).

microplus and *R. sanguineus* eggs by lying day are presented in figure 1.

In Figure 1 it can be seen that the protein concentration of the *R. microplus* eggs was constant during the initial laying period, and then increased slightly between the sixth and thirteenth days, with a positive weak correlation between the protein concentration and day (r=0.559833; p=0.003264). Figure 1 also shows that for *R. sanguineus* the protein concentration remained steady until the thirteenth day, with no correlation between the protein concentration and day of laying (r= 0.031266; p= 0.563338).

The electrophoretic fractionation by SDS-PAGE of the proteins of the *R. microplus* eggs revealed the presence of 14 protein bands, as shown in Figure 3. These bands referred to the approximate molecular weights of 226, 160, 123, 83, 74, 69, 64, 59, 56, 51, 49, 38, 36, 33 and 31 kDa and were present on all days analyzed. There were ten bands for the *R. sanguineus* eggs (Figure 4). The bands with approximate molecular weights of 215, 139, 112, 91, 81, 75, 65, 54 and 31 kDa were present during the entire period analyzed. The 38-kDa band could be seen with the naked eye during the entire period analyzed, but on the peak day (fourth day of lying) and the next three days its intensity was so low that it was not detected by the program.

There are very few studies of the hatching rate of tick larvae. Campbell & Harris (1979) suggested that the low availability of proteins and other nutrients supplied by the female and insufficient spermatozoids for fertilization are two factors responsible for the low hatching rate in the last laying days. But this explanation does not apply to the first laying days, about which some works have already found low hatching rates (Sonenshine 1991) when the females should be replete with nutrients and have sufficient spermatozoids in their receptacles.

Although it was not possible to collect 50mg of eggs on the last laying days, so we only analyzed the eggs laid up to the 13th day, the weight of the egg mass on the last two collection days (11th and 12th) was still sufficient to obtain significant measurements of the quantities of proteins. Even though the last laying days were not analyzed, we believe their inclusion would not have changed the overall profile observed.

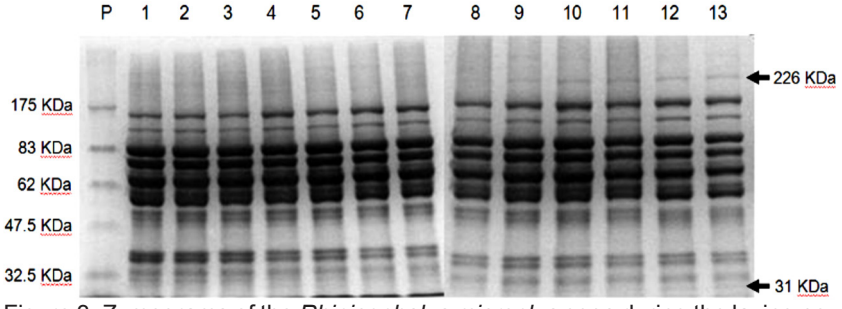

Figure 3. Zymograms of the *Rhipicephalus microplus* eggs during the laying period under controlled laboratory conditions (27±1 ºC, 80±5% RH, darkness). *P* – Protein molecular weight standard; *1 to 13* – Oviposition days; *32.5 to 175 kDa* – Protein molecular weight standard bands; *31 to 226 kDa* - Bands detected in the gel.

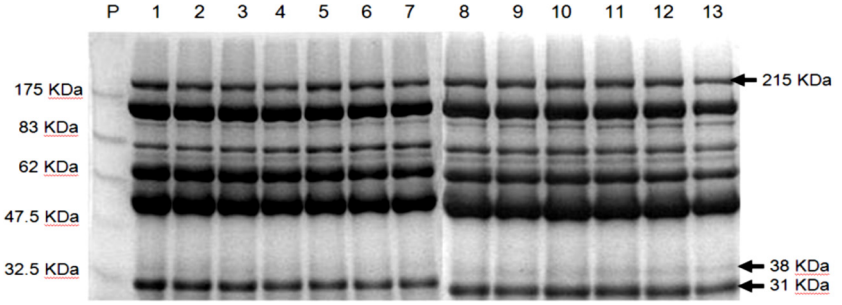

Figure 4. Zymograms of the *Rhipicephalus sanguineus* eggs during the oviposition period under controlled laboratory conditions (27±1 ºC, 80±5% RH, darkness). *P* - Protein molecular weight standard; *1 to 13* – Oviposition days; *32.5 to 175 kDa* - Protein molecular weight standard bands; *31 to 215 kDa* - Bands detected in the gel.

In *R. microplus*, the lowest protein concentration in the eggs was observed during the peak laying period, and then after the laying rate started to decline abruptly, the concentration of proteins increased and then remained stable until the 13th day. Although this result was statistically significant, we cannot say that this is the cause of decreased hatchability of eggs from the last laying days. In *R. sanguineus*, the concentration of proteins remained constant throughout the laying period, but on the peak laying day and the next three days the 38-kDa protein band was not detected by the densitometer. In this work the peak production occurred on the fourth day of oviposition. According to Bennett (1974) and Hitchcock (1955) the largest hatchability in eggs laid is in the fifth and sixth days of oviposition. These results indicate that although the total concentration of proteins did not change during the laying period, there was variation in the availability of specific proteins. Therefore, we suggest more detailed studies of these variations, with focus on the 38-kDa protein band.

The results obtained for both species were different than those found by Iwuala et al. (1981), studying *Amblyomma variegatum* and *Boophilus decoloratus*. They observed that the concentration of proteins in the eggs was higher in the initial and final oviposition phases than in the intermediate period. Therefore, they used a parabolic curve to represent the variations in the protein concentrations during oviposition. They pointed out that the protein concentrations in the eggs on the last days were not as high as in the first days, but were close to each other. Figure 2 shows that the models of the protein concentration curves obtained here are similar for the two species of the *Rhipicephalus* genus but completely different than those observed by Iwuala et al. (1981) for the species *A. variegatum* and *B. decoloratus*. Therefore, we can speculate that there is a different protein availability model during oviposition for each genus.

James & Oliver (1997) purified and partially characterized the vitellin of *I. scapularis* eggs and observed that this protein has a total weight of 480 kDa and is composed of eight sub-units with molecular weights between 48 and 145 kDa. Therefore, the protein bands between 49 and 123 kDa observed in the zymograms for *R. microplus* and the bands between 54 and 139 kDa observed in the zymograms for *R. sanguineus* could represent the vitellin sub-units.

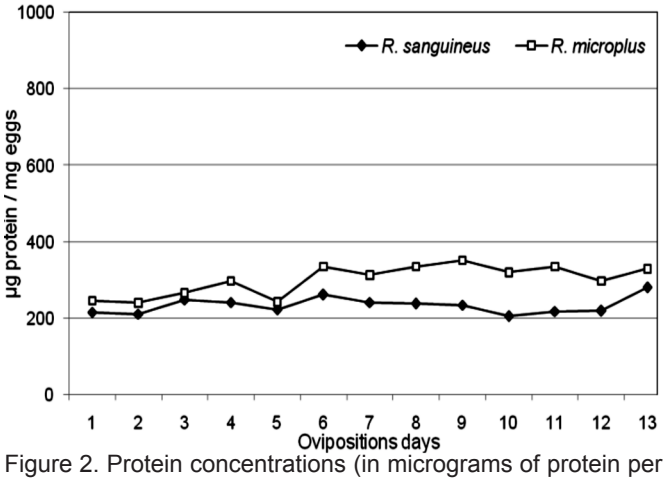

Figure 2. Protein concentrations (in micrograms of protein per milligram of egg) of *R. microplus* and *R. sanguineus* by oviposition day under controlled laboratory conditions (27±1 ºC, 80±5% RH, darkness).

In *R. sanguineus* we observed all the bands from the start of laying, with values compatible with the vitellin sub-units, except for the 91-kDa band, which was only present in the eggs from the second laying day.

Studying the degradation of proteins during the embryonic development of *R. microplus*, Logullo et al. (1998) detected the presence of the enzyme Boophilus Yolk pro-Cathepsin (BYC), which is an aspartic proteinase precursor whose function is to break down vitellin. This enzyme, when analyzed by electrophoresis, was represented by two protein bands with molecular weights of 54 and 49 kDa. Abreu et al. (2004) suggested that BYC has a molecular weight of 54 kDa, and that through acidification of the yolk granules, around the fourth day of embryogenesis, it is converted into a polypeptide with molecular weight of 47 kDa.

In this study we identified three bands in the samples of *R. microplus* eggs, with molecular weights similar to those found by Abreu et al. (2004). In their study, a 56-kDa band was present until the day analyzed, while the other two, 49 and 51 kDa, remained sharply separated until the 11th day of laying and then joined from the 12th day on. In *R. sanguineus,* we detected a protein weighing approximately 54 kDa in eggs on all the laying days studied.

The SDS-PAGE of extracts from the bodies of partially fed larvae, nymphs and adults of *R. microplus* revealed five proteins, weighing 68, 57, 50, 47 and 43 kDa (Gosh & Khan 2000). In our study, we found proteins in the eggs of *R. microplus* with molecular weights of 69, 56, 51, 49 and 38 kDa,

very near those observed by these authors. Therefore, we can speculate that these proteins play in important role in the other stages of the oviposition cycle of this species. Since they are present in eggs laid on all days of the cycle, they can be good targets for tick control studies. In *R. sanguineus*, only two protein bands had average weights (65 and 54 kDa) near those observed in *R. microplus* by Gosh & Khan (2000).

With the exception of the proteins of the vitellin structure, *R. microplus* and *R. sanguineus* have distinct protein profiles. This characteristic provides another tool for identification of these species, using recently laid eggs. We cannot attribute the low hatching rates observed in the first and last egg masses of *R. microplus* to the quantity of proteins supplied by the female. To clarify what factor or factors determine this low hatching rate, it will be necessary to evaluate the availability of other nutrients.

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