

Biological parameters of larvae, nymphs, and engorged females of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) obtained from artificial and successive infestations on *Oryctolagus cuniculus* (Linnaeus, 1758) (Lagomorpha: Ochotonidae)

[Parâmetros biológicos de larvas, ninfas e fêmeas ingurgitadas de *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) obtidas de infestações artificiais e sucessivas em *Oryctolagus cuniculus* (Lagomorpha: Ochotonidae)]

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ABSTRACT

The development of acquired resistance to *Amblyomma cajennense* ticks was evaluated. A total of 18 rabbits were uniformly distributed into three groups named GL, GN, and GA that were submitted to three consecutive infestations with, respectively, larvae, nymphs, and adults of *A. cajennense*. After the infestations, the parasitic and reproductive parameters of recovered instars were evaluated. A significant reduction of recovery rates of inoculated ticks was observed only after the third infestation of animals in groups GL and GN ($P<0.01$). The modal day for drop-off of engorged larvae and nymphs increased along the infestations. The recovered engorged nymphs presented a significant reduction of the mean body weight between the first and the third infestations ($P<0.01$). A significant reduction in the mean recovery rate of engorged females was only observed during the third infestation. However, significant reductions ($P<0.05$) in body weight, egg mass weight, and larva eclosions were observed for recovered engorged females after the second infestation onwards. The results suggest the occurrence of resistance of rabbits to *A. cajennense* infestations, being more evident against the adult stage.

Keywords: tick, Ixodidae, resistance, immunity, infestation

RESUMO

Avaliou-se o desenvolvimento de resistência adquirida frente ao parasitismo por *Amblyomma cajennense*. Para tanto, 18 coelhos foram uniformemente distribuídos em três grupos denominados GL, GN e GA e submetidos a três infestações consecutivas por, respectivamente, larvas, ninfas ou adultos de *A. cajennense*. Após a infestação, estudaram-se os parâmetros parasitários e reprodutivos dos exemplares recuperados. Apenas a partir da terceira infestação dos animais dos grupos GL e GN, pôde-se observar uma queda significativa na taxa de recuperação dos ixodídeos inoculados ($P<0,01$). O dia modal de queda das larvas e ninfas recuperadas aumentou com o decorrer das infestações. As ninfas ingurgitadas recuperadas apresentaram uma redução significativa ($P<0,01$) no peso médio corporal entre a primeira e terceira infestação. Observou-se uma queda significativa na taxa média de recuperação das fêmeas ingurgitadas somente na terceira infestação ($P<0,01$). Todavia, a partir da segunda infestação observou-se nas teleóginas recuperadas, uma redução significativa no peso corporal, peso da postura e eclodibilidade das larvas ($P<0,05$). Os dados sugerem a ocorrência de resistência em coelhos à infestação por *A. cajennense* que foi mais evidente contra o estágio adulto.

Palavras-chave: carrapato, Ixodídeo, resistência, imunidade, infestação

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INTRODUCTION

Popularly known in Brazil as “micuim”, “star tick”, or “rodoleiro” (Guimarães et al., 2001), *Amblyomma cajennense* ticks were first described in 1787, in Cayenna city, in French Guyana.

It is a three host ixodide that primarily infests horses (Borges et al., 2002). However, this tick species presents low host specificity, particularly during its immature stage of development, being also found infesting some species of reptiles, birds, and a wide variety of mammals, including man.

A. cajennense tick can be considered as one of the main links between domestic and wild animals, and man; consequently, it is a potential vector of pathogens transmitted among these hosts. This ixodide is the main vector of *Rickettsia rickettsii*, the agent of the Rocky Mountain Spotted Fever in the neotropical region (Guimarães et al., 2001).

Lopes et al. (1998), evaluating the parasitic specificity of *A. cajennense* ticks, observed that rodents and lagomorphs can be efficient hosts for the larval and nymphal stages and suggest that this fact may contribute to the maintenance and dispersion of these organisms in the environment. However, the role of these secondary hosts as tick disseminators might be significantly limited by the development of resistance of infested animals (Mulenga et al., 2000).

Acquired resistance to ixodide infestations is an unquestionable phenomenon that is well documented in the literature (Balashov, 1972; Sahibi et al., 1997; Jittapalapong et al., 2000). Ticks feeding on resistant hosts present lower fixation rate, lower weight after engorgement with relatively longer feeding periods, lower percentage of ecdysis, reduced production of eggs, and reduced viability of oviposition and larva eclosion (Sonenshine, 1993; Andreotti et al., 2002; Brossard and Wikel, 2004). Besides, as the host immune response inhibits tick feeding, there is an inhibition of its capacity to act as a vector of pathogens (Nuttal and Labuda, 2004; Valenzuela, 2004).

Some hosts fail to develop a protective immune response against ixodides (Ferreira and Silva, 1998; Castagnolli et al., 2003), demonstrating that the development of resistance is quite complex and varies within different systems in the nature.

The knowledge about the immune status of *A. cajennense* infested hosts may help the development of alternative control measures, for instance, by the use of vaccines or the selection of resistant animals. Secondly, it will help to understand the role of this ixodide as a vector of pathogens to human beings and animals.

The present study had the objective to evaluate the development of resistance to different developmental stages of *A. cajennense* inoculated in *Oryctolagus cuniculus*, using as indicators the main biological parameters observed in larvae, nymphs, and engorged females recovered after successive infestations.

MATERIALS AND METHODS

The study was carried out from March 2004 to January 2005. Before the beginning of the experiment, the pilot project (protocol 045/04) was approved by Ethic Committee for Experimentation of the University.

The ixodides were originally from a colony maintained at a laboratory of ectoparasitosis. The parasites were obtained by manual collection of engorged instars on the body of a horse (undefined breed) that had been artificially infested and kept isolated in a pen at the Departamento de Clínica e Cirurgia - Escola de Veterinária at the UFMG.

A total of 18 female New Zealand rabbits (*Oryctolagus cuniculus*), weighing approximately 2kg, were used in the experiment. These rabbits were tick naive and were originally from a commercial unit. The rabbits were kept in individual cages, placed 1m above the floor, and received water and commercial ration *ad libitum*. The animals were distributed into three groups that were identified as GL (larvae group), GN (nymph group), and GA (adult group). According to the experimental group, the rabbits received three consecutive infestations with larvae, nymphs, or adults of *A. cajennense* ticks at 15-day intervals.

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Animals in group GL received in each infestation approximately 740 larvae with ages ranging from 15 to 20 days. These larvae were obtained from aliquots of 50mg eggs/syringe. The ticks were placed alternately into the auricular cavity, starting with the right ear. In order to inhibit dispersion of ticks, a 16cm long and 7cm wide cotton bag was fixed to the basis of the ear using an adhesive glue¹ and tape, covering the whole ear, according to modifications of the technique described by Neitz et al. (1971). In addition, an adjustable hard collar was placed around the neck (Elizabethan collar) (Labruna and Leite, 1997).

Following the same methodology, the animals in group GN received in each infestation a total of 50 nymphs, approximately 20 day-old, which were placed alternately into the external auricular canal.

The rabbits in group A were infested with virgin unfed adults of *A. cajennense* ticks averaging 30 day-old. Following a modification of the technique described by Pinter et al. (2002), fur was removed from the lumbar sacral region of the experimental animals, that received a rectangular feeding chamber (7 x 8cm) fixed¹ to the shaved skin. In each infestation, 10 females and seven males were inoculated per animal inside the feeding chamber. In order to avoid the removal of the chamber and any interference of the grooming behavior of the rabbits, Elizabethan collars were used, as previously described for larva and nymph infestations.

After inoculations on the hosts, the ticks were daily examined by internal visual inspection of the feeding chambers. During the parasitic period, the feeding time of fixed ticks was recorded, as well as the number of parasites recovered from each animal, according to Sahibi et al. (1997). The detached nymphs and engorged females were sent to the laboratory, where they were individually weighed and placed into Petri dishes for incubation under BOD conditions (27°C and relative humidity of 80%). With regard to the larvae, the same procedure was applied, except the recording of body weight after engorgement.

The host resistance was based on the analysis of parasitic and reproductive parameters of recovered ticks. These included: the recovery rates of engorged ticks, the feeding period, the body weight of nymphs and engorged females, the molting rate of larva, the egg mass weight, and the larval hatchability rates. In addition, the Egg Production Index – oviposition weight/body weight of engorged female x 100 – (EPI) and the Larva Production Index – oviposition weight/body weight of engorged female x eclosion of larvae – (LPI) were calculated according to Bechara et al. (1994).

The recovery rates for each infestation within each group were compared using the chi-square test, with a significance level of 95%. The modal drop-off day for engorged larvae and nymphs were calculated, and the correlation analysis between body weight of nymphs and adults and the parasitic period was evaluated. The others parasitic and reproductive parameters were submitted to variance analysis and the means between infestations within the groups were compared using the t test (P≤0.05).

RESULTS

Data related to the ticks obtained from animals in group GL are presented in Table 1. The development of resistance against *A. cajennense* larva infestations could only be detected from the third infestation onwards. The evaluated parameters were statistically similar among the ixodides recovered from primarily infested animals in relation to those obtained in the second infestation. After the third infestation onwards, the recovery rate of engorged larvae and the correspondent modal drop-off day were considerably affected by the development of resistance in the rabbits.

The mean recovery rates of engorged larvae were 44.0%, 70.5% and 17.3%, respectively, at the first, second, and third infestations. As demonstrated by the mean recovery rate, the engorged larvae from the second infestation were significantly more efficient for feeding than those collected at the third infestation, in addition to be statistically similar to those obtained at the first infestation (P<0.05). No increase in the recovery rate between the first two infestations was observed.

¹Brascoplast®, Brascola Ltda - São Paulo, Brazil.

Table 1. Biological parameters (means±standard deviation) of *Amblyoma cajennense* engorged larvae and nymphs collected according to the stage

Parameters	Infestation		
	First	Second	Third
Larvae			
Recovery rate (%)	44.03a	70.54a	17.32b
Molting rate (%)	100.00	97.12±4.38	100.00
Nymph			
Recovery rate (%)	80.67a	81.00a	47.67b
Body weight (mg)	0.021±0.001a	0.013±0.002b	0.009±0.002c

Means in the same row followed by distinct letters are different ($P<0.05$).

The feeding or parasitic period was also influenced by the successive exposures to ticks, reflecting an increase in the modal drop-off day from the third infestation onwards. During the first and the second infestations, 43.3 and 38.5% of the larvae, respectively, detached from the animals at the fourth parasitism day. In contrast, the highest percentage of drop-off of engorged larvae in the third infestation occurred at the seventh day of feeding, when 35.3% of the parasites detached from the hosts. No significant differences ($P<0.05$) were observed between the mean molting rates for larvae recovered during the three infestations of animals in GL.

The mean recovery rates of engorged nymphs were 80.7%, 81.0%, and 47.7%, respectively, at the first, second, and third infestations. Statistical differences were not observed between the ixodides obtained from primarily infested animals and those from the animals submitted to the second challenge. At the third infestation, there was a significant decrease in the recovery rate of engorged nymphs in relation to that observed at the two previous infestations.

The modal drop-off day of engorged nymphs increased according to the parasitic exposure of the animals. Along the first infestation, 60.3% of the parasites were recovered at the fourth day of parasitism. However, at the same period during the second infestation, only 22.0% of nymphs had detached, and the highest percentage of drop-off was registered at the fifth day of feeding, when 47.7% of the inoculated specimens were recovered. In the third infestation, the modal drop-off day was the sixth

day of parasitism, when the recovery rate was 52.3%.

The mean engorged weight of recovery nymphs reduced significantly with the development of host resistance after three successive infestations (Table 1). The values observed in the first, second and third infestations were, respectively, 0.021mg, 0.013mg, and 0.009mg, with significant differences among all of them ($P<0.05$). During the three infestations, no significant correlations were observed between weight of engorged nymphs and their feeding period ($P>0.05$).

The mean recovery rates of engorged females were 33.3%, 56.7%, and 31.7%, respectively, at the first, second, and third infestations. Significant differences were observed between numbers of engorged ixodides obtained during the second infestation in relation to those obtained from primarily infested animals or animals submitted to the third parasitic challenge.

As shown in Table 2, from the second infestation onwards, the parasitic parameters were significantly reduced. The mean body weight of engorged females dropped from 783.75mg in the first infestation to 454.09mg in the subsequent exposure, with a significant difference ($P<0.05$). At the third infestation, this value increased to 529.16mg, being different from that observed for ticks obtained during the first challenge and similar to that reported for ticks from the second infestation.

Biological parameters of larvae...

Table 2. Biological and reproductive parameters (means±standard deviation) of *Amblyoma cajennense* engorged females collected from adult stage

Parameters	Infestation		
	First	Second	Third
Recovery rates (%)	33.33a	56.67b	31.67a
Body weight (mg)	783.75±172.25a	454.09±180.68b	529.16±211.20b
Feeding Period (days)	10.65±0.88a	11.14±1.06b	10.53±1.07a
Eggs mass weight (mg)	385.45±108.86a	191.71±121.16b	244.16±132.15b
Larval Hatchability rate (%)	93.50±11.52a	87.86±11.00ab	81.32±22.41b
EPI	48.62±7.58a	39.33±13.54b	42.45±13.81ab
LPI	45.93±10.03a	35.23±14.02b	36.47±14.91b

EPI: egg production index = egg mass weight (mg)/body weight (mg) x 100.

LPI: larvae production index = egg mass weight (mg)/body weight (mg) x eclosion rates (%).

Means in the same row followed by distinct letters differ significantly (P<0.05).

The mean feeding period of engorged females recovered from the second infestation increased significantly (P<0.05) in relation to the first parasitic challenge. However, from the third infestation onwards, this variable was similar to that initially observed.

With the exception of the mean larval hatchability rate, all the other reproductive variables were significantly reduced in the second infestation, although they presented an increasing tendency from the subsequent exposure onwards, with some figures similar to those observed in the first challenge.

DISCUSSION

According to Wikel (1996), the degree of host resistance to ixodid infestations can be measured by the following indicators: decrease of body weight after engorgement with consequent reduction of oviposition, increase of feeding period, and reduction of larval hatchability rate and molting rates. The inflammatory reaction in the parasite fixation site is partially responsible for resistance. More sophisticated mechanisms affect the ixodides, either locally or systemically, limiting the acquisition of food and consequently inhibiting the transmission of pathogens (Nuttall and Labuda, 2004; Valenzuela, 2004).

Tick infestations induce to a complex variety of immune responses, involving antigen presenter cells, T lymphocytes, B lymphocytes, antibodies, cytokines, complement, basophils, mastocytes, eosinophils, and a number of bioactive molecules

(Brossard and Wikel, 2004). Undoubtedly, the activation of these effector mechanisms of the immune response in a resistant host is considerably quicker and more intense (Wikel et al., 1994).

The results of the present study demonstrate that resistance to *A. cajennense* larvae in rabbits in GL became significant only after the third infestation onwards. During this period, the number of engorged larvae was significantly reduced due to the development of immune response of the challenged animals. Sanavria and Prata (1997) observed that after artificial infestations with approximately 123,840 larvae of *A. cajennense*, the rabbits presented petechias, edema, and small abscesses in the ears, at the fixation sites of larvae. These lesions were more severe after reinfestations. The present experiment used a lower number of parasites (approximately 740 larvae) and macroscopic lesions into the auricular cavity (data not shown) were only detected after the third infestation onwards and these coincided with the beginning of the development of the immune resistance. According to Kempt et al. (1976), *B. microphilus* larvae that try to feed on resistant animals become stressed and frequently die due to the several attempts to fixate on the same host.

The modal drop-off day of engorged larvae from rabbits in GL increased considerably in the third infestation. This alteration seems to be a consequence from the immune response produced by the host at the tick attachment sites, which will directly interfere within the process of tick feeding (Craig et al., 1996). Anaphylatoxins

produced by the complement activation and the histamine originated from leukocyte degranulation at the tick fixation site are responsible for the increased permeability of vascular endothelium. As a consequence, there will be local edema and serum exudation (Andreotti et al., 2002). Under these conditions, tick fixation and engorgement are seriously compromised. In the present experiment, it is possible that the reduction of larva recovery rate and the increased larva parasitic period after the third infestation occurred as a direct consequence of these phenomena. The main disadvantage for these ixodides while fixed for longer periods is that they become more vulnerable to the attack of predators as well as to the grooming behavior of the hosts.

The results obtained in the present study show that the development of immune resistance did not compromise the mean molting rate of larvae recovered from animals in group GL. According to Balashov (1972), the minimum weight required for ecdysis of semi-fed ixodides corresponds to 20% of their regular weight after engorgement. Under this perspective, probably nearly all larvae recovered in the present experiment were able to feed and to reach this minimal weight to undergo ecdysis into the next stage, even under adverse conditions of parasitism.

A. cajennense nymphal infestations on rabbits from GN determined a resistance profile similar to that observed for animals sensitized by larvae. However, the response was evaluated as intermediate when compared to that observed for larva and adult infestations.

Although the mean recovery rate of engorged nymphs was constantly high during the first two infestations, the mean weight of these nymphs was significantly reduced after the second parasitic challenge, keeping a tendency for decrease until the last infestation. Besides, the modal drop-off day of engorged nymphs increased along the successive exposures of rabbits to ticks. Considering that the first two infestations were carried out alternately into the auricular cavities, the inflammatory response that might have occurred during the second infestation did not seem to alter the capacity of nymphs to feed; however, it compromised the full engorgement of these parasites. The primary

effect of immune rejection is to 'flood' the fixation site with fluid, making it more difficult for the ticks to ingest blood (Sonenshine, 1993). Ticks engorging on resistant animals frequently present fainter coloration, suggesting a decrease of hemoglobin quantities in the ingested fluid.

During the second exposure of GA, a higher number of *A. cajennense* females were able to fixate and to engorge, in relation to those observed in the other infestations. This fact suggests an influence of possible exogenous factors on this variable. However, the mean body weight of recovered engorged females was the lowest observed among the three tick challenges, together with the egg production index (EPI) and the larva production index (LPI). Jittapalpong et al. (2000) also observed in dogs exposed to successive infestations of *Rhipicephalus sanguineus* ticks a higher rate of parasite recovery during the third infestation in association to a significant decrease of parasitic and reproductive parameters in these recovered ixodides. This fact demonstrates that the host immune response not only acts on the feeding site, but it is also possible that some defense components may cross the intestinal epithelium of ticks reaching organs and internal tissues, affecting their biological and reproductive functions. According to Wang and Nuttal (1994), the intestinal wall of ticks is permeable to the immunoglobulins produced by the hosts and once these antibodies reach the hemolymph, they can ligate to antigens associated to internal organs of these parasites. Besides, the host immune response seems to affect the physiology of ticks in a permanent manner that persists even after detachment (Sahibi et al., 1997). Vaz Jr. et al. (1996) observed that functional antibodies are present in the hemolymph of *B. microplus* engorged females for at least 48 hours after the end of the parasitic period, and that the quantity of immunoglobins present corresponds to 2% of that concentration in the serum of infested animals.

The mean recovery rate of engorged females obtained in the third infestation of the present experiment was significantly lower than that obtained during the second parasitic challenge, but it was similar to that obtained during the primary infestation. Probably the inflammatory response developed in the tick attachment sites during the experimental period was the major

mechanism responsible for the decreased number of recovered engorged females. However, there was a crescent tendency in the other parameters observed from the third infestation, with the exception of the mean period of parasitism and the mean larval hatchability rate (Table 2). It is well demonstrated in the literature that ixodides produce substances that are able to modulate the host immune response (Nuttal and Labuda, 2004). According to Wikel (1996), this modulation may act on different mechanisms of the immune system, inhibiting the complement activation, the production of antibodies during the humoral response, the proliferation of T cells, and the production of cytokines. It has been postulated that these modulation mechanisms are more evident in natural hosts with which the ixodides co-exist for longer time (Sonenshine, 1993; Mulenga et al., 2000). However, the pressure imposed by consecutive parasitic challenges on the rabbits of the present experiment, which is not present under natural *A. cajennense* infestations, may have facilitated the development of immune modulation.

Besides, there are indications that the survival of pathogens transmitted by ixodides depends on their ability to exploit the pharmacological and immunosuppressive properties of the molecules in the saliva of these parasites (Nuttal and Labuda, 2004). Ferreira and Silva (1998) observed that molecules present in the saliva of fed adult females of *R. sanguineus* favored the transmission of *Trypanosoma cruzi* by these ixodides. These finds could reinforce the importance of *A. cajennense* ticks in the transmission of *R. rickettsii* to wild reservoirs and its contribution in maintaining the Rocky Mountain Spotted Fever in endemic environments. Since the engorged females of *A. cajennense* were efficient in modulating the immune response of rabbits from the third infestation onwards, it is possible that they were also able to potencialize the transmission and the establishment of *R. rickettsii* in wild reservoirs that might have been infested by this species of ixodide.

The results from the present experiment suggest that infestation by the adult stage produce a quicker and more intense immune response, which could be observed after the second exposure onwards. On the other hand, the rabbits were more sensitive to infestations with

immature stages. Although the mean number of recovered larvae was significantly reduced after the third infestation, no effect was observed on the ecdysis rate of these recovered parasites. With regard to nymphs, the immune resistance of rabbits affected the parasite gain weight after the second exposure. However, its impact on recovery rate could only be detected at the third challenge of the animals.

Lopes et al. (1998) observed that the larval and nymphal stages of *A. cajennense* show lower parasitic specificity than the adult stages. This parasitic specificity is related to the occurrence of immune resistance during the infestations. Once the adult females feed for longer periods, releasing higher quantities of saliva at the feeding site (Balashov, 1972), it is likely that these females induce or modulate more efficiently an immune response in the host, when compared to the other stages. Whelen and Wikel (1993) reported that *Dermacentor andersoni* nymphs were less able than the adults to stimulate the production of specific antibodies in infested animals. According to Brossard and Wikel (2004), intra and inter-species variations in the saliva composition might be expected due to different antigenic stimulus in the infested animals. Besides, *A. cajennense* females introduce the proboscis deeper into the feeding sites, reaching more vascularized areas and consequently causing more damage to the host tissues. However, the intensity of the inflammatory response appears to be more dependent on the quantity of antigenic substances introduced rather than to the size and insertion of mouth parts of the ixodides (Latif et al., 1990).

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