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Original Article

Infestation of rabbits with just-molted adults of the cattle tick *Rhipicephalus microplus*: biological parameters and efficiency

Infestação de coelhos com adultos recém-mudados do carrapato-do-boi *Rhipicephalus microplus*

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Abstract

In this study, we report for the first time the successful infestation of rabbits with just-molted, unfed adults of *Rhipicephalus microplus*. Six New Zealand White rabbits were experimentally infested with 20 female and 20 male unfed adult ticks released into plastic chambers fixed on the shaved backs of each host. The attachment and feeding processes were successful. The biological characteristics of the ticks and the occurrence of adverse events in the tick-attachment area were studied. The average engorgement period was 10.7 days, and 33.3% of the engorged females completed the parasitic phase. The average weight of the recovered engorged females was 149.8 mg, with an average egg mass weight of 70.9 mg, a conversion efficiency index of 47.3%, and a hatching percentage of 88.31%. The adverse reactions found in the tick-attachment area were the usual inflammatory responses of the organism to infestation by these ectoparasites; however, it did not prevent the ticks from feeding and completing their life cycle. These data indicate that the infestation of rabbits with just-molted, unfed adult ticks could be a valuable, alternative animal model for rapid and economical evaluation of vaccine candidates and new molecules with acaricidal activity against *Rhipicephalus microplus*.

Keywords: Alternative host, life cycle, experimental infestation, animal model, feeding chambers, ectoparasite.

Resumo

Neste estudo, relata-se, pela primeira vez, o sucesso da infestação de coelhos por carrapatos adultos recém-mudados e não alimentados. Seis coelhos brancos da Nova Zelândia foram infestados artificialmente com 20 carrapatos adultos fêmeas e 20 machos adultos não alimentados no interior de câmaras de plástico fixadas no dorso de cada hospedeiro. Os processos de fixação e alimentação dos carrapatos foram bem sucedidos. Foram estudadas as características biológicas dos carrapatos e a ocorrência de eventos adversos na área de fixação do ectoparasito. O período médio de ingurgitamento foi de 10,7 dias, e 33,3% das fêmeas ingurgitadas completaram a fase parasitária. O peso médio das fêmeas ingurgitadas recuperadas foi de 149,8 mg, com peso médio da massa de ovos de 70,9 mg, índice de produção de ovos de 47,3% e porcentagem de incubação de 88,31%. Uma resposta inflamatória usual do organismo à infestação por esses ectoparasitas foi a única reação adversa encontrada na área de fixação dos carrapatos, o que não impediu que eles se alimentassem e completassem seu ciclo de vida. Esses dados apontam que a infestação de coelhos com carrapatos adultos recém-mudados e não alimentados pode ser um modelo animal alternativo e útil para a avaliação rápida e econômica de candidatos a vacinas e novas moléculas com atividade acaricida contra *Rhipicephalus microplus*.

Palavras-chave: Hospedeiro alternativo, ciclo de vida, infestação experimental, modelo animal, câmaras de alimentação, ectoparasita.

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Introduction

Rhipicephalus microplus, the most important member of the Ixodidae family from an economic point of view, is infamous for causing huge losses in livestock operations through the direct and indirect effects of feeding on host blood (Wang et al., 2019). Tropical and subtropical regions are favorable for the life cycle of *R. microplus* (Antunes et al., 2014). It is endemic to India, Asia, Africa, and the Caribbean (Estrada-Peña et al., 2006). In Cuba, *R. microplus* represents 57% of the Ixodid population (Gaínza et al., 2014). Synthetic acaricides are the main treatment of ticks infestation in the livestock, however, due to the increasing influence of acaricide resistance in ticks, the implementation of alternative strategies such as vaccines is required (Abbas et al., 2014).

R. microplus prefers to feed off cattle, although other animals can serve as occasional hosts for this species (Szabó et al., 2003). Several studies describe the infestation of *R. microplus* and aspects of its biology in alternative host species such as buffalo (Benitez et al., 2012; Obregón et al., 2010), horse (Franque et al., 2009; Rodríguez et al., 2009), llama (Aguirre et al., 2000), goat (Daemon et al., 1998; Nyangiwe & Horak, 2007), deer (Barré et al., 2002), sheep (Ma et al., 2016), dog (Franque et al., 2007), and rabbit (Amaral et al., 2012; Ma et al., 2016; Silva et al., 1996). These alternative hosts are a valuable option to accelerate the identification of novel molecules for tick control and to evaluate the efficacy of novel vaccine candidates against cattle ticks. Small laboratory animals, such as rabbits, are less expensive than cattle, and their use as surrogate hosts for tick infestation would reduce the physical space required for maintaining *R. microplus* colonies, facilitate handling and lower the maintenance costs (Amaral et al., 2012; Bonnet & Liu, 2012). Finally, the use of cattle to evaluate experimental vaccines or drugs implies the risk of compromising the meat and milk of these animals with drug residues (Senbill et al., 2018).

The infestation of rabbits with *R. microplus* larvae (Amaral et al., 2012; Ma et al., 2016; Silva et al., 1996) has been proved to be a time-consuming and low-yield procedure, not optimal for the evaluation of vaccine candidates. Infestation with larvae induces strong cutaneous hypersensitivity reactions in the attachment area of the rabbit skin, and mortality rates are high, therefore, a low percentage of the larvae manage to reach the adult stage. In this study, a variation of this procedure is proposed by infecting rabbits with just-molted, unfed adults of *R. microplus* instead of larvae. The biological parameters and the efficiency of this model are evaluated.

Materials and Methods

The experiments were approved and conducted following the guidelines of the Ethics Committee on Animal Experimentation. All procedures and samplings involving animals were carried out following the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and approved by the Ethics Committee of the Center for Genetic Engineering and Biotechnology (CIGB) (approved protocol number SG2021-4).

Ticks

The *R. microplus* specimens used in this experiment were kindly provided by the National Laboratory of Parasitology (LNP). The Cayo Coco hemoparasite-free LNP reference strain was used (Guzmán et al., 2021). Ticks were collected from clinically healthy cattle, maintained in individual boxes. The ticks on the cattle were visualized with an Optivisor DA-7 blue optical glass binocular magnifier (Donovan Optical, Inc, USA), and removed with forceps after fifteen days of infection. Just-molted adult ticks of any size were selected based on the morphological changes in the epidermis. They were identified by the remnants of the old cuticle that are still attached to the new one.

Rabbits

Six 12-week-old, female, specific pathogen-free (SPF) New Zealand White rabbits in good health conditions, weighing 2.5 kg, and naïve to ticks, were purchased from the National Center for Laboratory Animals Production (CENPALAB) and kept in the open area of CIGB animal facilities. The rabbits were kept in independent boxes throughout the experiment and fed with pelleted feed (supplied by CENPALAB) and water *ad libitum*.

Containment devices and infestation

The containment devices were constructed using cell culture plastic bottles lined with fabric and having screwcaps. Small holes were introduced in the caps using needles for facilitating oxygenation (Neitz et al., 1971; Sanavria et al., 1996). These devices were fixed on the upper back of the rabbits with a non-toxic contact cement lacking toluene.

The attachment area was previously shaved with an Electric 2000 Plus machine (Hauptner & Herberhotz GmbH & Co. KG, Germany). The shaved area was washed with 70% ethanol to remove cut hairs and sebaceous secretions that might interfere with the attachment of the devices. One device was attached to each rabbit. Twenty-four hours after attaching the devices, 20 male and 20 female just-molted, unfed adult ticks were introduced into each plastic chamber. From this moment, the animals were kept under daily observation. The engorged females detached were harvested and transferred to Petri dishes in the laboratory, where they proceeded with their non-parasitic cycle.

Non-parasitic stage

Engorged females were washed with distilled water and dried with filter paper. Subsequently, they were weighed individually and placed on Petri dishes (one tick per dish). The dishes were kept under controlled conditions of 28 ˚C temperature and 80% relative humidity with a photoperiod of 12:12 light/darkness until the end of the setting (Amaral et al., 2012). Egg masses were weighed individually and placed in glass flasks with cotton plugs (for ventilation) and kept under the same controlled conditions (humidity, temperature, and light) until hatching was completed.

The egg production index (EPI) was calculated using the formula (Bennett, 1974):

$$EPI = egg\ mass\ weight\ /\ engorged\ females\ weight\ x\ 100 \quad (1)$$

The hatching percentage was calculated by counting the larvae and the eggs under a stereomicroscope (S9-series, Leica, Germany). Three independent counts were done for each sample. A total of 300 specimens (either larvae or eggs) were counted for each replicate and added. The hatching percentage was determined by the formula:

$$Hatching\ percentage = Number\ of\ larvae\ /\ \left(Number\ of\ larvae + Number\ of\ eggs\right) x\ 100. \quad (2)$$

Histopathology of the tick attachment area

Five skin samples were collected from each rabbit after tick attachment, according to the protocol by (Szabó & Bechara, 1999). The samples were only taken from regions with partially engorged ticks and were immediately immersed in a 10% neutral buffered formalin solution for fixation. These tissues were dehydrated by immersion in increasing concentrations of ethanol, (80%, 90% and 100%) for 1 hour each. Thereafter, they were treated with xylene for 1 hour for cleaning. The tissues were removed from the cleaning agent and embedded and blocked in paraffin wax. Each sample was cut in 3-4 µm thick sections, as close as possible to the tick hypostome, using a semi-automatic vertical microtome. The sections were mounted on microscopic slides, rehydrated, and stained with hematoxylin-eosin. After fixation and staining of skin tissues the samples were embedded in Canada balsam, visualized in a DM300 transmission microscope (Leica, Germany), and photo-documented with a cyber-shot DSC-530 camera (Sony, Japan).

Statistical methods

The Kruskal-Wallis test was used to compare the reproductive parameters of the ticks collected from different groups. the differences between the individual means were determined by Dunn's multiple comparison tests. a chi-square test was used to compare the number of engorged females recovered from each rabbit. The statistical analysis was carried out using the Graph Pad Prism software, version 6.0 (Graphpad Software; La Jolla, USA).

Results

Collection and selection of ticks from cattle

Three hundred ticks attached to the epidermis were collected from cattle and sexed. Only seven of them (2.3%) had lesions in the hypostome after removal. A total of 120 females and 120 males with an intact hypostome were selected for the experiment in rabbits

General biological parameters

After 24 hours, approximately 70% of the ticks were viable and moved inside the devices in search of the best place to attach and feed. After 48 hours, a large number of ticks had already attached and were feeding.

The general biological parameters of the ticks are summarized in Table 1. The engorging period ranged from 7 to 16 days, with an average engorging period of 10.7 ± 3.5 days. The feeding process was affected by the strong hypersensitivity reactions observed in the tick-attachment area on the rabbit's skin. After the engorged females were detached, the area was reddened and inflamed. However, 33.3% of the engorged females could be recovered alive after engorgement. The engorged females weighed between 72 mg and 362 mg, with an average weight of 149.8 ± 85.8 mg, while the average weight of the egg masses was 70.9 ± 46.4 mg, and the average CEI was 47.3%. Additionally, the overall hatching percentage was 88.31%.

Table 1. General biological parameters of *R. microplus* individuals fed on rabbits.

Parameters	Mean ± STD	GM	Median	Range
Engorgement period (days)	10.7 ± 3.5	10.2	9	(7 - 16)
Recovery (%)		33.3		
Weight of engorged females (mg)	149.8 ± 85.8	131.8	106.6	(72 - 362)
Weight of egg mass (mg)	70.9 ± 46.4	60.0	51.35	(0 - 179)
CEI (%)	47.3			(0 - 52.3)
Hatching (%)	88.3			(0 - 99.4)

GM= geometric mean.

Biological parameters of ticks by engorgement day

The biological parameters of ticks per engorgement day are presented in Table 2. The collection of detached, engorged females started on day 7, and this was the day of maximal collection, reaching up to seven engorged females per rabbit. The weight of the engorged females decreased as the number of engorgement days increased. The differences in the weight of engorged females shed on day 7 and days 9, 13, and 16 were statistically significant (Figure 1A) (Kruskal-Wallis $p < 0.05$, Dunn $p < 0.05$).

Table 2. Recovery of *R. microplus* per engorgement day on rabbits.

Parameters							
Engorgement period (days)	7	8	9	13	14	15	16
Number of engorged females	10	7	5	5	4	5	4
Recovery (%)	8.3	5.8	4.3	4.3	3.3	4.2	3.3

Unlike the engorged females collected between days 7 and 13, which were green in color, the engorged females collected on days 14, 15, and 16 presented a yellow coloration and laid fewer eggs. The differences in the weight of eggs laid between days 7 and 16 were statistically significant (Figure 1B) (Kruskal-Wallis $p < 0.05$, Dunn $p < 0.05$). No differences were found in the egg production index of the engorged females collected on different engorgement days (Figure 1C) (Kruskal-Wallis $p > 0.05$), since the weight of both, the engorged females and their eggs, decreased with the increase in engorgement time.

The hatching percentage also decreased as the number of engorgement days increased. Significant differences were found in the hatching percentage between days 7 and 14 (Figure 1D) (Kruskal-Wallis $p < 0.05$, Dunn $p < 0.05$).

Biological parameters of ticks per rabbit

About six to nine engorged females were recovered per rabbit (Table 3). No significant differences were observed among the rabbits for this parameter (Chi-square, $p = 0.1860$). On day 7, two ticks were removed from

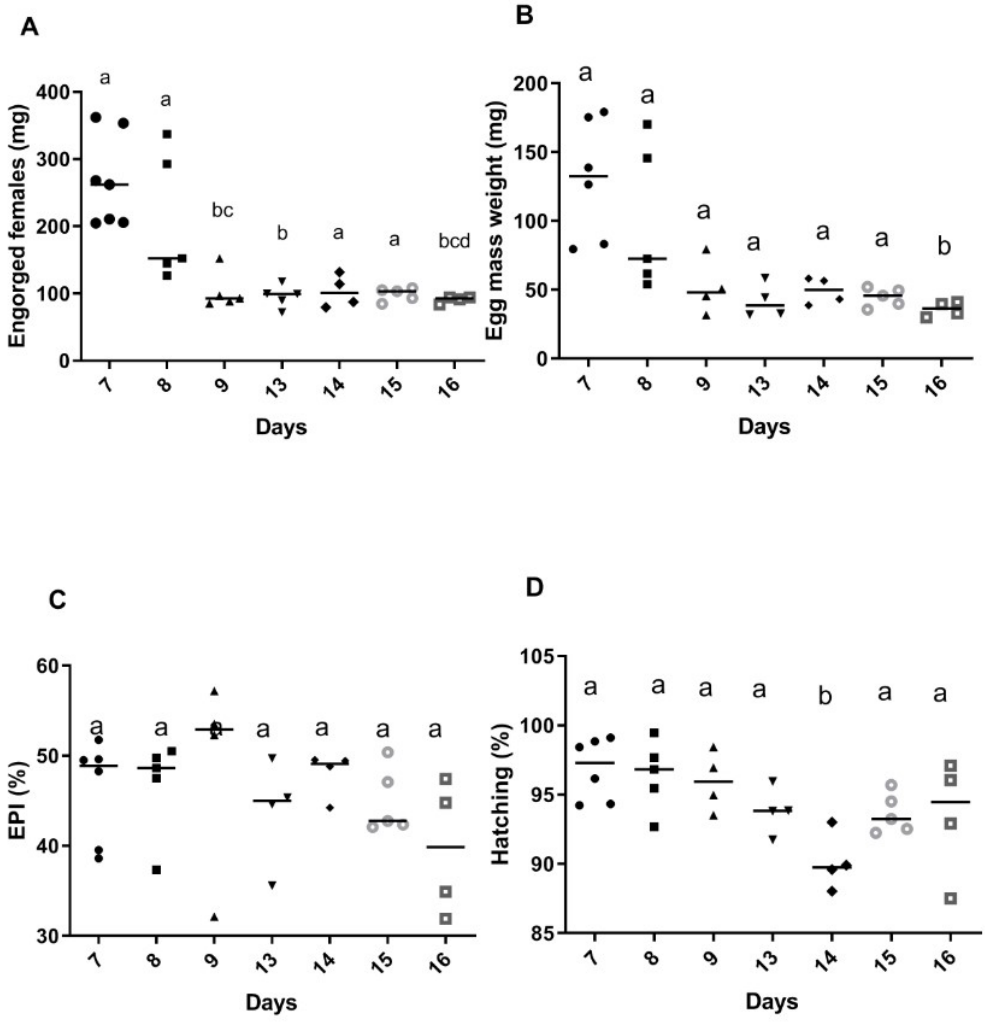

Figure 1. Biological parameters of just-molted *Rhipicephalus microplus* adults engorged on rabbits. A: average weight of engorged females per engorgement day; B: average weight of eggs masses per engorgement day; C: Egg production index per engorgement day; D: hatching percentage per engorgement day. Different letters indicate statistically significant differences among groups (Dunn test, $p < 0.05$).

Table 3. Engorged females recovery per rabbit and engorgement day.

Animal	Engorgement days							Total	Recovery (%)
	7	8	9	13	14	15	16		
1	0	3	2	0	1	3	0	9	45
2	1	0	0	0	2	1	3	7	35
3	4	0	2	3	0	0	0	9	45
4	1	1	1	2	1	1	1	8	40
5	1	0	1	0	2	0	0	6*	30
6	1	1	0	0	0	1	0	6**	30
Total	8	5	6	5	6	6	4	40	33.3
X	1.33	0.83	1	0.83	1	1	0.66	6.6	

X, mean of engorged females recovered per rabbit. The recovery was calculated as the percentage of planted adult females that were recovered at the end of the engorgement period. *2 engorged females were removed for histopathology on day 7; hence it could not be assigned to any engorgement day; **3 engorged females were removed for histopathology on day 7; hence it could not be assigned to any engorgement day.

rabbit # 5 and three from rabbit # 6, and the corresponding attachment area of the rabbit epidermis was sampled to conduct a histopathological analysis.

Adverse reactions in the tick-attachment area on the rabbits' skin

A biopsy of the tick-attachment area on the rabbit skin showed epidermal hyperplasia. Several tick-related structures could be observed, such as the cement cone (generated by the tick during the attachment process), the capitulum, which includes the mouthparts used for attachment, and the alimentary cavity (Figure 2A). The host's response to tick infestation was characterized by an inflammatory reaction with abundant eosinophils and macrophages (Figure 2B). However, this reaction did not prevent the ticks from engorging and having viable offspring.

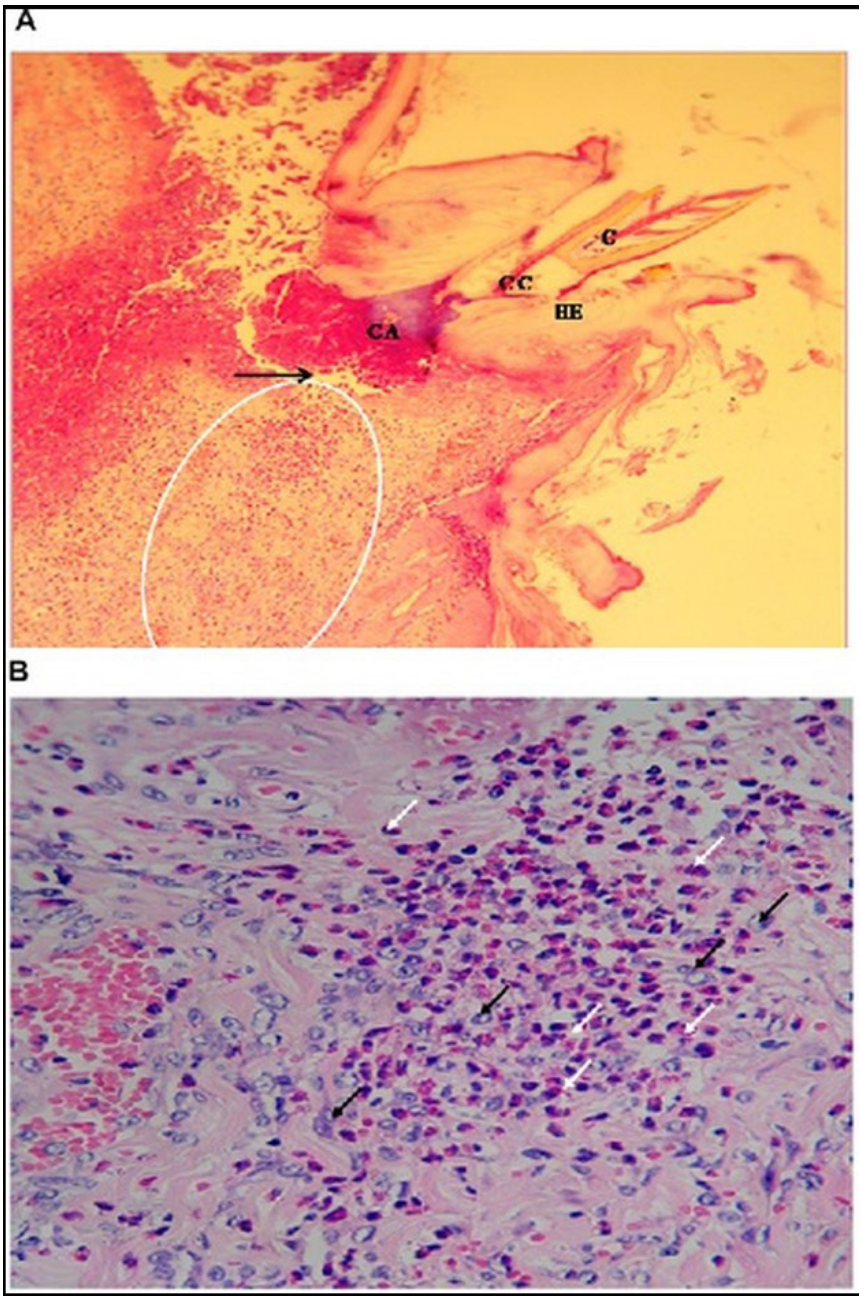

Figure 2. Histopathology of the infected rabbits' skin. A: R. *microplus* attachment site on the rabbit skin stained with hematoxylin-eosin. The arrow indicates inflammatory cell infiltration. HE: epidermal hyperplasia; CA: alimentary cavity; CC: cement cone; C: *capitulum*; ellipse: inflammatory response in the tick attachment area. B: local inflammatory response in the tick attachment area. Eosinophils (White Arrows) and Macrophages (Black Arrows).

Discussion

Studies have been conducted to analyze the attachment and feeding of *R. microplus* on alternative hosts such as horses (Franque et al., 2009), sheep (Ma et al., 2016), goats (Daemon et al., 1998; Nyangiwe & Horak, 2007), rabbits (Amaral et al., 2012; Silva et al., 1996) and dogs (Franque et al., 2007). All these species allowed the development of the parasitic cycle of these Ixodids to a certain degree, although the results were variable and depended on the host-parasite relationship. In these studies, the infestation was initiated using the *R. microplus* larvae, which seemed to be quite susceptible to the immune-inflammatory responses of these non-conventional hosts. A different approach was explored in the present study by infecting rabbits with just-molted unfed adults instead of the larvae. The unfed adults managed to attach, engorge, complete their parasitic cycle, and spontaneously detach from their host.

The overall recovery of engorged females in our experiment was 33.3%. The information on the recovery in other hosts has been variable, both in free and controlled infestations, but the general recovery has always been lower. For example, a recovery of 3.7% was reported in goats (Daemon et al., 1998); between 0.9% and 1.8% in horses (Franque et al., 2009); 0.42% in dogs (Franque et al., 2007); and 10.69% and 4.88% in rabbits infested on the ears and the back, respectively (Amaral et al., 2012). Another study carried out on bovine, sheep and rabbits, yielded recoveries of 11%, 0.47%, and 5.5%, respectively (Ma et al., 2016). It has been reported that the recovery of engorged females could be influenced by two factors: (1) the intraspecific competition between ticks for physical space, which leads to ticks maintaining a restricted area for their feeding (Silva et al., 1996), and (2) by the immune-inflammatory reaction of the host, which interferes with the blood supply to the host tissues, thereby limiting the tick's ability to acquire food (Nuttall & Labuda, 2004; Valenzuela, 2004; Cunha, 1978).

In controlled studies in bovines using containment devices with 3000 *R. microplus* larvae, the recovery of engorged females was variable and depended on the strain used, with values that fluctuated between 10% and 25%. For example, 24.03% was reported for the Camcord strain (Cuba) (Garcia-Garcia et al., 2000); 15.03% for strain A (Argentina) (Garcia-Garcia et al., 2000); 13.6% for Yerongpilly strain (Australia) (Penichet et al., 1994) and 21.3% with the Cayo Coco strain (Cuba) (Mallón et al., 2020). The recovery of engorged females in our model (33.3%) was even higher than those reported in cattle. The more logical explanation for this is that cattle are infested with larvae, therefore the ticks would have to go through three stages of development (larva, nymph, and adult), a fact that reduces the percentage of recovery, while in our model rabbits are infected with adults, which are already in the final stage of development, facilitating a higher percentage of recovery. In addition, it must be considered that the initial number of ticks used in our model to infect rabbits was low, and in cattle, the percent of recovery tend to be higher when lower number of larvae is used.

Another important parameter to be considered in these surrogate models is the duration of the engorgement period. In this study, the engorgement and copulation period of the ticks in rabbits was 10.7 days. These values are higher than those previously reported in rabbits infected with larvae: 7.03 ± 2.45 days for the dorsal region and 8.55 days for the pinna of the ears (Amaral et al., 2012). One reason that can be advanced to explain these differences is that in our model, the adult ticks initially spent some days attaching to the rabbit before beginning the engorgement phase. On the contrary, in the larvae infestation model, this is a continuous process, since nymphs are already attached, and the engorgement period begins immediately after molting.

On the other hand, the duration of the engorgement period of adult ticks in cattle was approximately eight days (Hitchcock, 1955). In a review article about the biological and ecological aspects of hard ticks, the proposed engorgement period ranked from 7 to 12 days (Echeverry & Osorio, 2016), which is very similar to the one found in the present study in rabbits.

It has been described that long engorgement periods alter the morphology of engorged females and reduce egg production (Wikel & Bergman, 1997). In this study, a yellow coloration was observed in the engorged females when the engorgement period was longer than nine days. A similar change in coloration was reported in ticks fed on the pinna of the ear in rabbits (Silva et al., 1996), contrasting with the reddish color of *R. microplus* (Garcia et al., 2019) or from chestnut to dark brown color characteristic to all *Rhipicephalus* species (Onofrio et al., 2006).

The weight of the engorged ticks is directly related to certain features of their parasitic phase. The increase in intraspecific competition can cause a decrease in the weight of the engorged female, and consequently, lower its efficiency to convert ingested food into egg mass, since the ability to produce eggs is directly related to the availability of food (Bennett, 1974). Additionally, long engorgement periods of ticks in horses were not accompanied by an increase in the engorgement of engorged females (Franque et al., 2009). Our results agreed with the previous reports, as the average weight of engorged females decreased with the number of engorgement days.

The mean weight of the engorged females in our study (149.8 mg) was higher than the ones described before in rabbits infected with larvae in the back and ear, with values of 34.43 mg and 36.30 mg, respectively (Amaral et al., 2012). A lower average weight of 38.88 mg was also described for ticks planted on the ears of rabbits (Silva et al., 1996). This lower engorgement capacity may be associated with the cumulative effects of using a non-ideal food substrate during the larval stage (Franque et al., 2009).

The weight of *R. microplus* engorged females engorged on cattle is variable. Average weights of 213 mg and 246 mg were reported in studies carried out in Australia (Hitchcock, 1955; Sutherst et al., 1973). A value of 245.5 mg was reported in Colombia (Ortíz, 1983) and 228 mg in Cuba (Vega et al., 2003). A direct comparison between the weight of engorged females from Brazil and Cuba showed comparable results of 258.7 mg and 233.9 mg (Vega et al., 2007). In summary, the average weight of the engorged females in our study was closer to the values reported for cattle than to the previous experiments of rabbit infestation with *R. microplus* larvae.

The egg production index was 47.3%, and this index was conserved throughout the entire engorging period, which indicates that the capacity for processing blood and converting it into eggs was similar for all engorged females, regardless of their weight.

On the other hand, the hatching percentage showed significant differences between days 7 and 14, with values of 96.85% and 90.13%, respectively, and average hatching of 88.31%. This hatching percentage was lower than that obtained by (Silva et al., 1996), who reported a very high hatching percentage of 96.65%. This lower value may be related to the characteristics of the strains used in both studies.

The skin lesion produced by ticks at the point of attachment, regardless of the host or the parasite species, is characterized by epidermal hyperplasia, formation of the cement cone, presence of the tick mouthparts and alimentary cavity, and a strong immune-inflammatory reaction of the host during the tick feeding process (Bechara, 2006; Engracia et al., 2017; Szabó & Bechara, 1999; Tabor et al., 2017; van der Heijden et al., 2005). All these elements were observed in this study.

In rabbits, infestation with *R. microplus* induces the production of an inflammatory cell infiltrate (with eosinophils and small macrophages) at the attachment site. The composition of the infiltrate varies depending on the relationship of the tick with its host, time, and the number of infestations (Bechara, 2006). Eosinophils have been associated with the introduction of tick saliva into the skin of a desensitized host, which causes degranulation of mast cells and basophils. This reaction possibly occurs due to enzymatic hydrolysis of the host's plasma membrane by salivary enzymes, resulting in the release of vasoactive and chemotactic factors that could contribute to the leukocyte influxes at the tick attachment sites (Tabor et al., 2017).

R. microplus is considered a very complex species that has coevolved with its host, acquiring a high degree of specificity. Therefore, from the biological point of view, using its natural host for experimental work is highly advantageous; however, it is limited by the high costs associated with the acquisition and maintenance of animals, specialized labor, and its management. In contrast, the rabbit is a very economical and practical model for tick engorgement that allows the engorgement of all stages of different tick species, making it a valuable tool for expanding investigations in search of molecules for the control of ticks.

The data generated in this study allow us to conclude that the infestation of rabbits with just-molted, unfed adults of *R. microplus* can provide some advantages over previous studies using larvae. The extended engorgement period, the higher recovery values, and the weight of the engorged females observed in this study suggest that rabbits could be a useful alternative biological model to evaluate new molecules and vaccines for the control of this ectoparasite.

Ethics declaration

The protocol was approved by the ethical committee for the use of experimental animals of the Center for Genetic Engineering and Biotechnology (CIGB); all procedures were conducted according to the established guidelines.

Conflict of interest

The authors declare that they have no competing interests.

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