UNIVERSIDADE FEDERAL DE UBERLÂNDIA

Instituto de Ciências Biomédicas Programa de Pós Graduação em Imunologia e Parasitologia Aplicadas

ESCARA NOS SÍTIOS DE FIXAÇÃO DO CARRAPATO *AMBLYOMMA OVALE*: MORFOLOGIA DAS LESÕES INFECTADAS E NÃO INFECTADAS POR *RICKETTSIA PARKERI*

ALESSANDRA CASTRO RODRIGUES

UBERLÂNDIA 05/2020

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Dissertação apresentada ao Programa de Pós-Graduação em Imunologia e Parasitologia Aplicadas da Universidade Federal de Uberlândia como requisito parcial à obtenção do título de mestre em Imunologia e Parasitologia.

Alessandra Castro Rodrigues

Prof. Dr. Matias Pablo Juan Szabó

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UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Imunologia e Parasitologia Aplicada
Av. Amazonas, s/n, Bloco 4C, Sala 4C218 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
Telefone: (34) 3225-8672 - www.imunoparasito.ufu.br - coipa@ufu.br

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Projeto de Pesquisa de vinculação:	Carrapatos e patógenos zoonóticos do Cerrado e da Mata Atlântica na interface com áreas antropizadas				

Reuniu-se, por vídeo conferência, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, assim composta: Prof. Dr. Marcelo Bahia Labruna - FMVZ/USP; Prof. Dr. Márcio Botelho de Castro -FAV/UNB; Prof. Dr. Matias Pablo Juan Szabó - HV/UFU - (Presidente) e Orientador do candidata.

Iniciando os trabalhos o presidente da mesa, Prof. Dr. Matias Pablo Juan Szabó, apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público, e concedeu a discente a palavra para a exposição do seu trabalho. A duração da apresentação da discente e o tempo de arguição e resposta foram conforme as normas do programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Matias Pablo Juan Szabo**, **Professor(a) do Magistério**

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Uberlândia, 28 de maio de 2020

Banca examinadora:

Prof. Dr. Matias Pablo Juan Szabó

(Orientador – UFU-MG)

Prof. Dr. Marcelo Bahia Labruna

(Examinador – USP-SP)

Prof. Dr. Márcio Botelho de Castro

(Examinador UNB- DF)

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Resumo

Duas Rickettsioses bem caracterizadas e transmitidas por carrapatos ocorrem no Brasil. *Rickettsia rickettsii* agente da febre maculosa, uma doença grave e com alta taxa de letalidade na região sudeste do país e *Rickettsia parkeri* cepa Mata Atlântica transmitida por carrapatos adultos de *Amblyomma ovale* causando uma doença febril não letal, mais leve e com eschar (necrose) no local da picada do carrapato. No Brasil, essa escara é considerada a principal característica clínica da infecção leve por *R. parkeri* e é utilizada para diferenciá-la da infecção grave por *R. rickettsii*. No entanto, faltam conhecimentos sobre a patogênese da escara e sua clara relação com a infecção por *R. parkeri*. Neste estudo, avaliamos macroscopicamente e histopatologicamente as lesões cutâneas de porquinhos-da-índia (*Cavia porcellus*) nos sítios de parasitismo de *A. ovale* infectados ou não infectados por *R. parkeri* cepa Mata Atlântica, bem como lesões cutâneas de Rickettsia inoculadas por agulha. As lesões também foram avaliadas em porquinhos-da-índia submetidos à primeira infestação de carrapatos (não imunizados a picada de carrapato) e naqueles sensibilizados por infestações anteriores ou infecções por *R. parkeri* (imunizados). O exame geral revelou que após o desprendimento de carrapatos infectados e não infectados e a inoculação com agulha de Rickettsia, todos os animais desenvolveram hiperemia e endurecimento da pele. No entanto, a necrose estava ausente nos animais com inoculação intradérmica de Rickettsias e era muito maior nos locais da pele onde os carrapatos se desprendiam após uma segunda ou terceira infestação de carrapatos. Esta observação mostra que a resposta imune aos carrapatos é a base da patogênese da necrose. A histopatologia revelou que vasculite e agregados intravasculares leves de fibrina eram uma característica discreta dos locais de inserção de carrapatos infectados ou não, e ausentes nos locais de inoculação por agulha da Rickettsia. No entanto, a infecção por Rickettsia aumentou e estendeu ao longo do tempo a necrose focal nos locais de fixação do carrapato, bem como o aumento do infiltrado inflamatório focal na interface derme-hipoderme. Estes resultados indicam que as escaras em cobaias expostas a carrapatos infectados por *A. ovale* são causadas pela picada de carrapatos de animais previamente sensíveis à picada de carrapatos e que a infecção concomitante por Rickettsia pode aumentar essa lesão sob avaliação microscópica. No entanto, a escara pode ser usada como sinal de picada de carrapato e, eventualmente, no local de inoculação de Rickettsia. Portanto, doenças febris com escaras ainda precisam de confirmação laboratorial da infecção por Rickettsia. Estudos adicionais são necessários para revelar a fisiopatologia da escara e morfologia relacionada em outros hospedeiros, espécies e cepas de Rickettsia.

Palavras-chave: *Rickettsia parkeri*, escara, Brasil, lesões, *Amblyomma ovale*

Abstract

Two well characterized tick-borne rickettsiosis occur in Brazil. *Rickettsia rickettsii* caused spotted-fever is a severe disease with a high case-fatality rate in the southeastern region of the country whereas *Rickettsia parkeri* strain Atlantic rainforest (ARF) infections transmitted by adult *Amblyomma ovale* ticks cause a milder non-lethal febrile disease with eschar (necrosis) at tick-bite site. In Brazil, such eschar is considered the main clinical feature of mild *R. parkeri* infection and used to differentiate it from severe *R. rickettsii* infection. However, knowledge on the pathogenesis of eschar and its clear relationship with *R. parkeri* ARF is lacking. We herein evaluated gross and histopathology of skin lesions of guinea pigs caused by *R. parkeri* ARF infected or uninfected *A. ovale* parasitism sites as well as skin lesions of needle inoculated *Rickettsia*. Lesions were also evaluated in guinea pigs undergoing first tick infestation (tick-bite naïve) and on those sensitized by previous infestations or *R. parkeri* infections (immunized). Gross examination revealed that after detachment of infected and uninfected ticks and *Rickettsia* needle inoculation all animals developed skin hyperemia and induration. Necrosis, however, was lacking from needle inoculated animals and was much larger at skin sites where ticks detached after a second or third tick infestation. This observation shows that an immune response to ticks is at the basis of necrosis pathogenesis. Histopathology revealed that vasculitis and slight intravascular fibrin aggregates were an inconspicuous feature of both infected or uninfected tick attachment sites and absent at *Rickettsia* needle inoculation sites. Nevertheless, *Rickettsia* infection enhanced and extended over time focal necrosis at tick attachment sites as well as increased focal inflammatory infiltrate at the dermis-hypodermis interface. These results indicate that eschar in guinea pigs exposed to infected *A. ovale* ticks are caused rather by tick-bite of previously tick-bite sensitized animals and that concomitant *Rickettsia* infection may increases such lesion under microscopic evaluation at least. Nonetheless eschar may be used as a tick-bite sign and eventually *Rickettsia* inoculation site. Therefore, febrile diseases with eschars still need laboratory confirmation of *Rickettsia* infection. Additional studies are needed to reveal eschar physiopathology and related morphology in other hosts and *Rickettsia* species and strains.

Key words: *Rickettsia parkeri*, eschar, Brazil, lesions*, Amblyomma ovale*

SUMÁRIO

1- INTRODUÇÃO:

Os carrapatos se destacam como artrópodes vetores pois ocupam o segundo lugar como agentes transmissores de patógenos para seres humanos e o primeiro para os animais domésticos (JONGEJAN & UILENBERG, 2004). Entre outros, estes ácaros dispõe de associações específicas com uma grande diversidade de riquetsias (PAROLA, et al., 2013; PAROLA et al., 2005; WEISBURG, et al., 1989), e atuam em muitos casos como reservatórios destes patógenos (PAROLA & RAOULT, 2001).

Rickettsioses são zoonoses causadas por bactérias intracelulares obrigatórias pertencentes ao gênero *Rickettsia* (família Rickettsiaceae; ordem Rickettsiales) e transmitidas por artrópodes como pulgas, piolhos e carrapatos (AZAD & BEARD, 1998; BLANTON & WALKER, 2017; DANTAS-TORRES, CHOMEL, OTRANTO, 2012; DUMLER, et al., 2001; GILLESPIE, et al., 2008; GILLESPIE, et al., 2009). Riquetsias patogênicas transmitidas por carrapatos pertencem ao grupo da febre maculosa e tem como principais espécies as bactérias *Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia sibirica*, *Rickettsia africae*, *Rickettsia parkeri* e *Rickettsia slovaca* dentre outras, (DAY & NEWTON, 2017; GILLESPIE, et al., 2008; VITORINO, et al., 2007). Porém, há de se considerar que além das riquetsias patogênicas, muitas não têm patogenicidade conhecida (PAROLA, et al., 2013).

Fatores ambientais e hospedeiros vertebrados determinam a presença de espécies de artrópodes e rickettsias em uma localidade. Para a transmissão das rickettsioses uma relação de mínima de equilíbrio entre a bactéria e o carrapato é necessária, assim como a alimentação deste vetor, sobretudo em hospedeiros vertebrados, estabelecendo a existência de rickettsioses distintas em cada continente do mundo (DAY & NEWTON, 2017; PAROLA, et al., 2013; RENVOISÉ & RAOULT, 2009).

Diversas espécies de riquetsias patogênicas já foram identificadas nas Américas. Na América do Norte as bactérias *Rickettsia rickettsii*, *Rickettsia parkeri* e *Rickettsia 364D*. Na América Central as espécies *Rickettsia rickettsii* e *Rickettsia africae.* Na América do Sul *Rickettsia rickettsii. Rickettsia parkeri* e *Rickettsia massiliae* são agentes etiológicos da febre maculosa (GARCÍA-GARCÍA et al., 2010; LABRUNA et al., 2011; SPOLIDORIO et al., 2010; PAROLA, et al., 2013).

No Brasil, a febre maculosa é causada pela *Rickettsia rickettsii*, espécie mais patogênica, com altas taxas de letalidade e transmitida principalmente pelas espécies de

carrapatos *Amblyomma sculptum* e *Amblyomma aureolatum* e *Rickettsia parkeri* cepa Mata Atlântica associada principalmente ao carrapato *Amblyomma ovale*, causando uma doença mais branda e autolimitante (BARBIERI et al., 2014; DE OLIVEIRA et al., 2016; KRAWCZAK et al., 2016; MEDEIROS et al., 2011; OLIVEIRA, S. V, 2017; OLIVEIRA, S. V, 2016; SILVA et al., 2011; SPOLIDORIO et al., 2010; SZABÓ, PINTER, LABRUNA, 2013).

Os sinais clínicos observados na infecção pelas duas espécies de riquetsia são inicialmente muito inespecíficos como febre, linfadenopatia, cefaleia, mialgia, mal-estar generalizado e náuseas. Em pacientes infectados com *Rickettsia parkeri,* porém, uma escara de inoculação do carrapato, caracterizada por uma lesão cutânea com centro necrótico e halo eritematoso, no local de fixação do vetor no hospedeiro é considerado um sinal clínico muito sugestivo (DE OLIVEIRA et al., 2016; FACCINI-MARTÍNEZ et al., 2014; FANG, BLANTON, WALKER, 2017; KRAWCZAK et al., 2016; SILVA et al., 2011; SPOLIDORIO et al., 2010).

Em virtude dos sinais clínicos inespecíficos e evoluções distintas da doença dependentes da espécie envolvida, se faz necessário o uso de exames complementares como PCR, isolamento em cultura, imuno-histoquímica e sorologia para a confirmação e identificação do agente etiológico (ANGERAMI, et al., 2009; FACCINI-MARTÍNEZ, et al., 2018; MINISTÉRIO DA SAÚDE, 2017). O exame sorológico é o mais utilizado para o diagnóstico, porém, devido as reações cruzadas entre as riquetsias do grupo da febre maculosa, não é possível identificar a espécie. Além disso, na fase aguda da doença não há soroconversão, o que pode interferir nas decisões de tratamento, internação do paciente e consequentemente na resolução do caso (ANGERAMI, et al., 2009; FACCINI-MARTÍNEZ, et al., 2018; MINISTÉRIO DA SAÚDE, 2017; PADDOCK, et al., 2008; PAROLA, LABRUNA, RAOULT, 2009).

Devido à semelhança dos sinais clínicos iniciais e as limitações dos exames complementares disponíveis de forma abrangente para o sistema de saúde nacional, infecções em humanos pela *Rickettsia parkeri* e *Rickettsia rickettsii* vêm sendo confundidas. Esta situação pode resultar em tratamentos equivocados ou tardios além de dificultar o estabelecimento de medidas de prevenção. Neste contexto, a escara de inoculação do carrapato, é considerado o principal sinal clínico da rickettsiose por *Rickettsia parkeri*, para o diagnóstico decisivo (ANGERAMI, et al., 2009; FACCINI-MARTÍNEZ, et al., 2018; MINISTÉRIO DA SAÚDE, 2017; PADDOCK, et al., 2008). Deve-se, porém, considerar que os aspectos morfológicos e de patogênese da escara de

inoculação são vagos e desconhecidos, respectivamente. A associação desta lesão com infecção por *Rickettsia parkeri* cepa Mata Atlântica provém de estudos envolvendo outras espécies de carrapatos e outras espécies ou cepas de riquetsias. Com a finalidade de se descrever os aspectos morfológicos das lesões cutâneas desta rickettsiose, o estudo a seguir descreveu a morfologia da lesão causada por *Rickettsia parkeri* cepa Mata Atlântica em cobaias quando transmitida pelo vetor *Amblyomma ovale* ou quando inoculada por injeção intradérmica e comparada à lesão cutânea causada pelo parasitismo do carrapato não infectado.

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2- ESCHARS AT *AMBLYOMMA OVALE* TICK ATTACHMENT SITES: MORPHOLOGY OF *RICKETTSIA PARKERI* INFECTED AND UNINFECTED LESIONS

(ESCARA NOS SÍTIOS DE FIXAÇÃO DO CARRAPATO *AMBLYOMMA OVALE*: MORFOLOGIA DAS LESÕES INFECTADAS E NÃO INFECTADAS POR *RICKETTSIA PARKERI)*

Alessandra Castro Rodrigues[a], Márcio Botelho de Castro[b], Maria Marlene Martins[a], Monize Gerardi[c], Adriane Suzin[a], João Fabio Soares[c], Felipe da Silva Krawczak[d], Marcelo Bahia Labruna[e], Matias Pablo Juan Szabó[a]*

[a]Laboratório de Ixodologia, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia, Av. Pará, 1720/Campus Umuarama-Bloco 2T, CEP 38400-902, Uberlândia, Minas Gerais, Brazil

[b]Laboratório de Patologia Veterinária, Universidade de Brasília, Av. L4 Norte, Hospital Veterinário - Campus Universitário Darcy Ribeiro, CP. 4508, Asa Norte, CEP 70910-970, Brasília, Distrito Federal, Brazil

[c]Laboratório de Protozoologia, Faculdade de Medicina Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, nº 9090, CEP 91540-000, Porto Alegre, Rio Grande do Sul, Brazil

[d]Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Campus Samambaia, Avenida Esperança, 1533, CEP: 74690-900, Goiânia, Goiás, Brazil

[e]Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva, 87, Cidade Universitária, CEP 05508-270, São Paulo, São Paulo, Brazil.

*corresponding author: szabo@ufu.br

Abstract

Two well characterized tick-borne rickettsiosis occur in Brazil. *Rickettsia rickettsii* caused spotted-fever is a severe disease with a high case-fatality rate in the southeastern region of the country whereas *Rickettsia parkeri* strain Atlantic rainforest (ARF) infections transmitted by adult *Amblyomma ovale* ticks cause a milder non-lethal febrile disease with and eschar (necrosis) at tick-bite site. In Brazil, such eschar is considered the main clinical feature of mild *R. parkeri* infection and used to differentiate it from severe *R. rickettsii* infection. However, knowledge on the pathogenesis of eschar and its clear relationship with *R. parkeri* ARF is lacking. We herein evaluated gross and histopathology of skin lesions of guinea pigs caused by *R. parkeri* ARF infected or uninfected *A. ovale* parasitism sites as well as skin lesions of needle inoculated *Rickettsia*. Lesions were also evaluated in guinea pigs undergoing first tick infestation (tick-bite naïve) and on those sensitized by previous infestations or *R. parkeri* infections (immunized). Gross examination revealed that after detachment of infected and uninfected ticks and *Rickettsia* needle inoculation all animals developed skin hyperemia and induration. Necrosis, however, was lacking from needle inoculated animals and was much larger at skin sites where ticks detached after a second or third tick infestation. This observation shows that an immune response to ticks is at the basis of necrosis pathogenesis. Histopathology revealed that vasculitis and slight intravascular fibrin aggregates were an inconspicuous feature of both infected or uninfected tick attachment sites and absent at *Rickettsia* needle inoculation sites. Nevertheless, *Rickettsia* infection enhanced and extended over time focal necrosis at tick attachment sites as well as increased focal inflammatory infiltrate at the dermis-hypodermis interface. These results indicate that eschar in guinea pigs exposed to infected *A. ovale* ticks are caused rather by tick-bite of previously tick-bite sensitized animals and that concomitant *Rickettsia* infection may increases such lesion under microscopic evaluation at least. Nonetheless eschar may be used as a tick-bite sign and eventually *Rickettsia* inoculation site. Therefore, febrile diseases with eschars still need laboratory confirmation of *Rickettsia* infection. Additional studies are needed to reveal eschar physiopathology and related morphology in other hosts and *Rickettsia* species and strains.

Key words: *Rickettsia parkeri*, eschar, Brazil, lesions*, Amblyomma ovale*

2.1- Introduction

Ticks (Acari: Ixodidae and Argasidae) transmit a diverse array of pathogens to hosts which cause a wide range of human and animal diseases, including those caused by bacteria from the order Rickettsiales (Jongejan and Uilenberg, 2004). Human tick-borne rickettsioses are caused by intracellular bacteria belonging to the spotted fever group (SFG) of the genus *Rickettsia*. This group includes over 25 formally recognized species worldwide (Parola et al., 2013). Some of these rickettsiae are now known to cause emerging human diseases even though were first recognized from their associations with different animals and their ectoparasites and only later associated with specific human diseases (Eremeeva et al., 2015).

Generally, tick transmitted SFG rickettsiae are closely related to various genetic and antigenic characteristics nonetheless include agents of severe and often fatal rickettsioses, as well as others responsible for milder, non-lethal diseases (Parola et al., 2013). Therefore, proper, and fast diagnosis is mandatory to prevent lethality mainly in the case of *Rickettsia rickettsii* infection that has a rapid and severe evolution demanding correct treatment within no later than five days of infection (Álvarez-Fernadez et al., 2017). However, serologic assay (indirect immunofluorescence antibody – IFA the reference standard for confirmation of rickettsial infection) is many times unsuitable for timely diagnosis (Biggs et al., 2016). IFA assays are insensitive in the first days when patients need medical attention and proper treatment is critical in preventing poor outcomes in the case of *Rickettsia rickettsii* infection. Furthermore, immune responses to spotted-fever group rickettsial antigens are cross-reactive and serology do not distinguish species-specific reactions. Other diagnostic methods are rather confirmative of the illness after resolution or restricted to few laboratories (Biggs et al., 2016).

Clinical diagnosis of SFG rickettsiosis is also challenging, particularly during the early stages of the illness when signs and symptoms are non-specific and similar to many other infectious diseases such as dengue, meningococcemia, measles, leptospirosis, chikungunya and Zika virus infections (Biggs et al., 2016; Álvarez Fernandez et al., 2017). Therefore, rickettsioses diagnosis relay on epidemiological history linking tick-bite in endemic regions and a nonspecific febrile illness.

Generally rickettsioses have no pathognomonic signs, although there are local, regional and systemic alterations that are suggestive such as an inoculation eschar at the tick-bite site, regional lymphadenopathy, fever and rash that may assist diagnosis

(Drexler et al., 2020; Fischer, 2018; Faccini-Martínez et al., 2014; Myers et al., 2013; Paddock et al., 2008). In fact, inoculation eschar at the infected tick-bite site has been considered a hallmark for several mild rickettsioses as well as source of *Rickettsia* DNA for diagnosis (Fischer, 2018; Myers et al., 2013; Socolovschi et al., 2012; Wang et al., 2009). Eschar (described initially as "tache noir" or black spot) is a necrotic lesion and defined as a dark, scabbed plaque overlying a shallow ulcer, typically 0.5–2 cm in diameter (Walker et al., 1988; Biggs et al., 2016). It is believed to be caused by local proliferation and spread of rickettsiae to numerous contiguous endothelial cells in the dermis, injury to vascular endothelium and occurrence of ischemic necrosis of the adjacent epidermis and dermis (Walker et al., 1988).

Until 2009, *Rickettsia rickettsii* infection was the only known tick-borne rickettsiosis to occur in Brazil. In 2010 a second agent, *Rickettsia parkeri* strain Atlantic rainforest, was shown to cause a human tick-borne spotted fever illness (Spolidorio et al., 2010) transmitted mainly by adults of *Amblyomma ovale* ticks (Szabó et al., 2013a; Faccini-Martinez et al., 2018). In the forthcoming years, by relating new laboratory confirmed cases (Silva et al., 2010; Spolidorio et al., 2010; Krawczak et al., 2016a; Sevá et al., 2019), likely cases from the past (Angerami et al., 2009) as well geographical distribution of infected vector (Moerbeck et al., 2016; Szabó et al., 2013a, Krawczak et al., 2016a; Sevá et al., 2019) it became clear that this second rickettsiosis is potentially widespread along the Brazilian Atlantic rainforest zone and underdiagnosed.

Infections with above mentioned *Rickettsia* species, as elsewhere, have quite different clinical outcomes in Brazil. Whereas *R. rickettsii* spotted-fever is a severe disease with a case-fatality rate of 55% in the southeastern region of the country (Oliveira et al., 2016) *R. parkeri* strain Atlantic rainforest infections cause a milder disease with eschar at tick-bite site but no death was attributed to it so far. At the same time the main laboratory testing for spotted fever by the Brazilian Ministry of Health consists of serological evidence of a fourfold change in immunoglobulin G (IgG)-specific antibody titers reactive to *R. rickettsii* (Oliveira et al., 2016) which does not discriminate *Rickettsia* species responsible for the illness. Furthermore, paired serum samples are taken at a minimum interval of two weeks (the first week of illness and a second 2–4 weeks later) and is rather confirmatory of the rickettsiosis but too late to institute treatment in case of severe illness. Therefore, an inoculation eschar is considered the main clinical feature of mild *R. parkeri* infection that differentiates it from severe *R. rickettsii* infection in Brazil (Faccini-Martínez et al., 2018).

However, the presence or lack of eschar must be seen with caution as sign to discriminate milder from potentially lethal rickettsioses. Not all patients with *R. parkeri* rickettsiosis exhibit eschars (Paddock et al., 2008) whereas fatal *R. rickettsii* infections may have a lesion characteristic of a rickettsial eschar (Argüello et al., 2012; Walker et al., 1981). In fact, eschar pathogenesis should be reviewed within experimental settings and considering each rickettsia and vector species as well as tick-bite exposure history of host. Levin et al. (2016) showed that in *R. rickettsii*, *R. parkeri* and *R. slovaca* infected guinea pigs, *Rickettsia* DNA could be found in skin samples from ears, away from the tick attachment site and without eschars. On the other hand, tick bite on its own may cause an eschar-like lesion according to host, tick stage and host sensitization level to the vector (Szabó et al., 1995; Szabó et al., 2006).

Ticks are known to efficiently control host´s hemostatic, inflammatory, immune, and healing mechanisms using biologically active molecules from their saliva (Hajnická et al., 2011; Kotál et al., 2015; Wikel et al., 2017). Walker et al. (1988) reasoned that tick lesions are controlled by tick pharmacologically active tick saliva components and could be not considered as eschar inducers. However, the controlling efficiency of these tick saliva molecules is greater upon their natural hosts and less so against unnatural ones (Randolph, 1979, Szabó et al., 1995; Ferreira et al., 2003; Kotál et al., 2015). For example, Lawrie et al (1999) observed that the ability of tick saliva to counteract complement activity varied according to the animal species source of serum, with specificity shown towards the most common hosts for each *Ixodes* species. Expression of immunity-mediated resistance to ticks was shown to be decreased in natural hosts whereas unnatural ones acquired a strong resistance and was expressed by a more vigorous reaction of hosts to tick-bites sometimes culminating with local necrosis (Szabó et al., 1995; Ferreira et al., 2003).

Another issue to be considered in the case of tick-borne rickettsiosis in Brazil is the tick stage that transmits the pathogen. The main vector of *R. rickettsii* in the country is the nymph of *Amblyomma sculptum* whereas adults of *A. ovale* transmit *R. parkeri* to humans (Sevá et al., 2019; Szabó et al., 2013a). In that case the size of the tick may elicit host reactions proportional to its size with different outcome in the lesion dimension. Last but not the least human reactions at tick-bite sites are rarely reported but they seem to vary and whereas some individuals lack a strong reaction, others exhibit an ulcer (necrosis) at tick attachment site (Szabó et al., 2006).

Based on the information presented above, we reason that the pathogenesis of eschars cannot be attributed solely to endothelial damage by *Rickettsia* inoculated at tick-bite site, and that host reaction to tick attachment and feeding is an important, if not the main, component of the lesion. Therefore, we herein present a morphological evaluation, gross and microscopic, of *R. parkeri* infected and non-infected *A. ovale* feeding sites on guinea pigs, during a first infestation or a third infestation and compare them to needle inoculated *Rickettsia* sites in the skin. The aim of this information is to discern host reaction to ticks from lesions caused by *R. parkeri* infection to avoid misdiagnosis of rickettsioses and other febrile diseases.

2.2- Material and Methods

2.2.1. *Rickettsia* and tick sources

Rickettsia parkeri strain Atlantic rainforest (ARF) infected and uninfected *Amblyomma ovale* ticks were obtained from laboratory colonies kept at the of Preventive Veterinary Medicine and Animal Health Department of the School of Veterinary Medicine and Animal Science of the University of São Paulo (Krawczak et al., 2016b; 2018). In one colony, close to 100% of the specimens were infected by *R. parkeri* SAR. A second colony consisted of ticks with no rickettsial infection. The infected ticks derived from naturally infected adult collected in the Atlantic rainforest within Peruíbe county, São Paulo State. *Rickettsia parkeri* ARF inoculum for intradermal injections was isolated previously in Vero cells (Szabó et al., 2013b) and consisted of approximately ~ 3,000 Vero cells infected by *Rickettsia parkeri* strain Atlantic rainforest suspended in 1 mL of sucrose-phosphate-glutamate buffer. Prior to the current study, this isolate was propagated and stored in Vero cells for a total of five passages.

2.2.2 Hosts

Guinea pigs (*Cavia porcellus*) were used as experimental hosts as indicated before (La Scola 2009). Animals were adult males and females, 60–90 days old, tick-bite naïve without any contact with acaricides or antibiotic drugs. Animals were fed with commercial pellets and water ad libitum throughout the experiment.

2.2.3 Tick infestations

Tick infestations on guinea pigs were performed inside feeding chambers (10–15 cm diameter) that were glued to the shaved back of each animal, as previously described (Szabó et al., 1995; Pinter et al., 2002). Eight couples of adult *A. ovale* ticks, 20–30 days old, were released into each feeding chamber. Feeding chambers were opened daily, position of each tick within the chamber annotated and ticks assigned to one of the experimental procedures (observation of gross skin alterations or skin biopsy at tick attachment site for histopathology).

2.2.4 Experimental protocol

Guinea pig groups were either infested with *Rickettsia*-infected or uninfected ticks or inoculated intradermally with *Rickettsia* by syringe and needle in different combinations (Figure 1 and 2). All guinea pigs were tick-bite naïve at the beginning of experiments and sequential infestations were always 30 days apart. Each experimental group had six guinea pigs at the beginning of procedures. During infestation procedures rectal temperature of animals was measured daily. Guinea pigs were considered febrile if rectal temperature was over 39,5ºC (Soares et al, 2011).

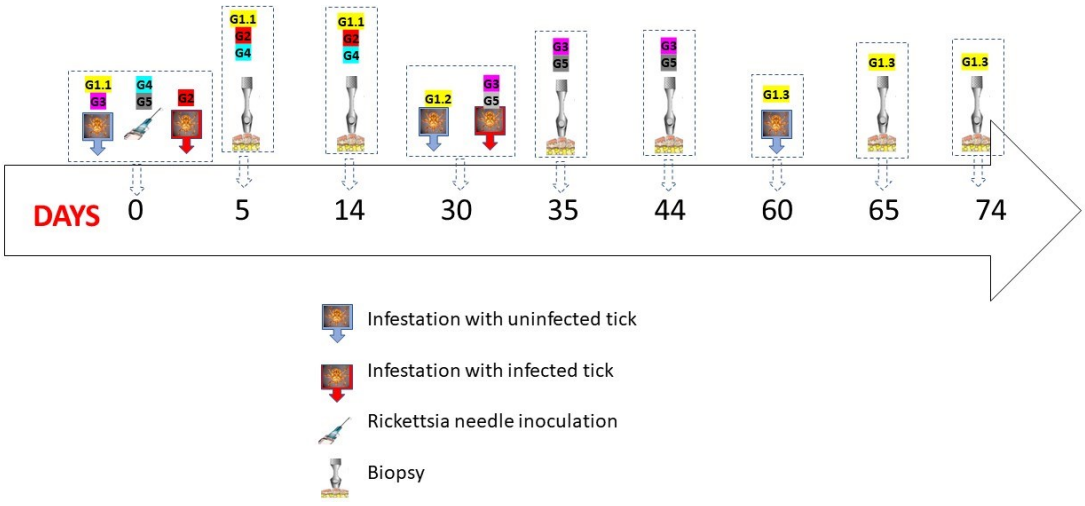


Figure.1. Sequence of procedures on each of five experimental groups to evaluate histopathology at tick bite and *Rickettsia parkeri* inoculation sites on the shaved back of guinea pigs. Procedures consisted of host infestations in feeding chambers with either *Rickettsia parkeri* strain Atlantic rainforest infected or uninfected *Amblyomma ovale* ticks or inoculation of the *Rickettsia* either intradermal or intraperitoneal. G1.1 - first infestation with uninfected *Amblyomma ovale* ticks; G1.3 - third infestation with uninfected *A. ovale* ticks; G2 - infested once with *Rickettsia parkeri* ARF strain infected *A. ovale* ticks; G3 - infested once with uninfected and followed by a second infestation with *R. parkeri* ARF infected *A.ovale* ticks; G4 - intradermally inoculated with *R. parkeri* ARF; and G5 - inoculated intraperitonially with *R. parkeri* followed by an infestation with *Rickettsia parkeri* ARF strain infected *A. ovale* ticks.

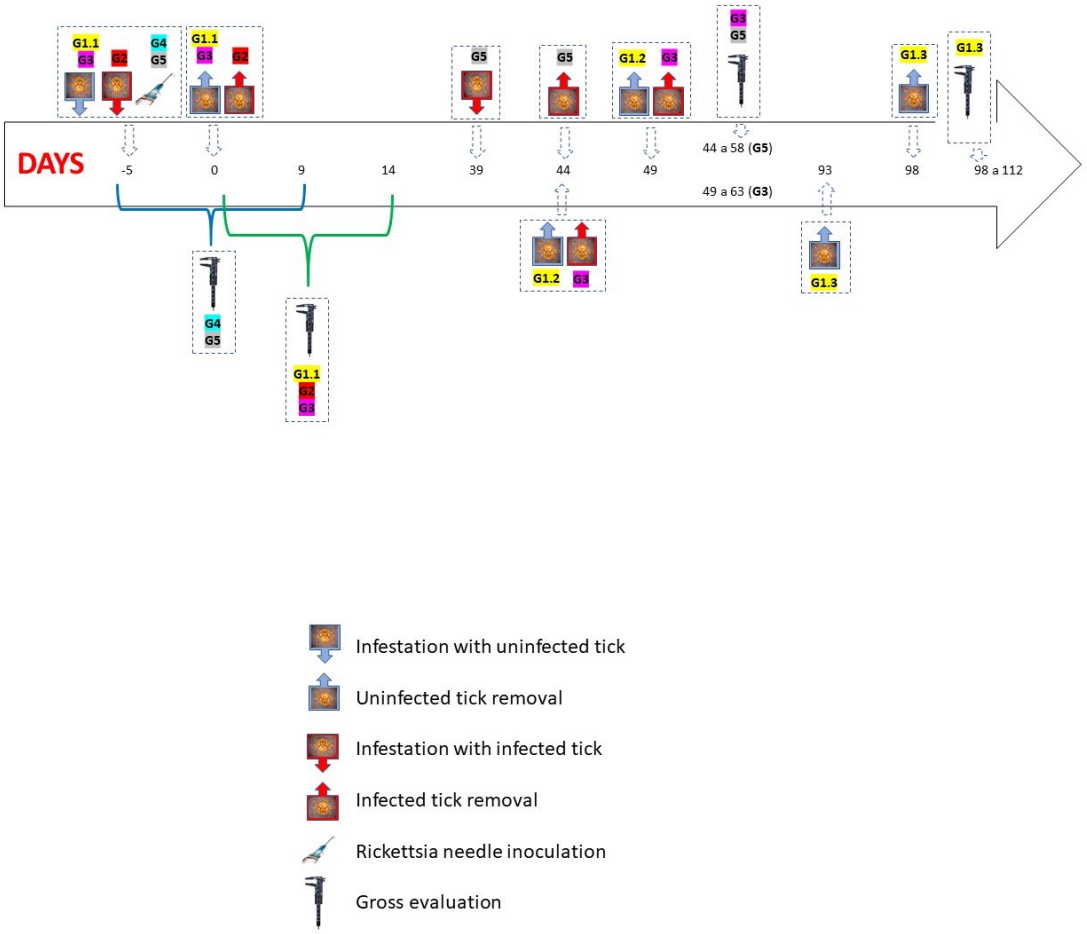


Figure.2. Sequence of procedures on each of five experimental groups for gross evaluation of tick bite and *Rickettsia parkeri* inoculation sites on the shaved back of guinea pigs. Procedures consisted of host infestations in feeding chambers with either *Rickettsia parkeri* strain Atlantic rainforest infected or uninfected *Amblyomma ovale* ticks or inoculation of the *Rickettsia* either intradermal or intraperitoneal. G1.1 - first infestation with uninfected *Amblyomma ovale* ticks; G1.3 - third infestation with uninfected *A. ovale* ticks; G2 - infested once with *Rickettsia parkeri* ARF strain infected *A. ovale* ticks; G3 - infested once with uninfected and followed by a second infestation with *R. parkeri* ARF infected *A.ovale* ticks; G4 - intradermally inoculated with *R. parkeri*

ARF; and G5 - inoculated intraperitonially with *R. parkeri* followed by an infestation with *Rickettsia parkeri* ARF strain infected *A. ovale* ticks.

Experimental group one was set up to evaluate non-immune (first infestation - G1.1) or sensitized (third infestation – G1.3) host cutaneous reactions to tick-bite and aimed analysis of tick-bite as an eschar inducing element on its own. Group two evaluated the dual effect of tick-bite and simultaneous vector inoculation of *Rickettsia* upon eschar induction without previous sensitization by the tick or *Rickettsia*. Group three evaluated the dual effect of tick-bite and simultaneous vector inoculation of *Rickettsia* upon eschar induction of a tick-bite sensitized host (second infestation) and whether host immunity to tick may interfere with *Rickettsia* transmission and eschar development. Group four evaluated the sole effect of *Rickettsia* (without tick-bite) upon eschar development. Group five evaluated the effect of *Rickettsia* sensitization on its own over a subsequent infected tick parasitism.

2.2.5 Gross evaluation of tick attachment and *Rickettsia* injected sites

For gross evaluation of tick-bite lesions, ticks were allowed to feed for five days and were thereafter gently pulled from the attachment site. These sites were marked and daily evaluation began immediately after detachment (day 0). Similarly, intradermal Rickettsia inoculation sites were evaluated daily from the moment of bacteria inoculation on (day 0). In all cases lesions were daily evaluated throughout 14 days. Such period was considered adequate for *Rickettsia* proliferation and the development of tick-bite lesions as observed before (Szabó et al., 1995; 2006; La Scola et al., 2009). Gross examination pursued necrosis (a dark, scabbed plaque and an ulcer), induration (indurated increase of skin thickness indicative of accumulation of inflammatory exudate and/or healing elements) and hyperemia (a red halo indicative of vasodilation and increased arterial blood flow). These skin parameters were measured with the aid of a Caliper and expressed in millimeters.

2.2.6 Histopathology of tick attachment and *Rickettsia* injected sites

Analysis of epidermal, dermal, and hypodermal microscopic features of tick bite lesions and at *Rickettsia* inoculation sites were performed in guinea pig skins with five and 14 days of tick attachment or five to 14 days after needle *Rickettsia* injection into

hosts. Such periods are within adult tick feeding period (Martins et al., 2012). To obtain adequate samples of tick-bite lesions, each female tick attachment site was registered daily on a map of the of each host's feeding chamber. Whenever an appropriate tick attachment period was observed, the host was anesthetized, and a biopsy was obtained of the skin with the attached tick in the center with the aid of a 5 mm diameter circular biopsy punch.

Skin biopsies were dissected and put in 10% formalin for pathological study. Sections (5 μm) were cut from the formalin-fixed, paraffin-embedded skin biopsy specimens of the cutaneous tick attachment or *Rickettsia* inoculation sites and stained with hematoxylin-eosin using routine staining methods. Serial sections were obtained from each sample and one representative section of tick attachment site chosen for analysis. Selection of the section was based on its quality and display of at least two of the following features indicative of tick attachment: 1- tick's hypostome within the dermis and/or epidermis; 2 - tick's cement cone (proteinaceous secretion that anchors the tick mouthparts into the host); 3 -feeding cavity (necrotic area surrounded by inflammatory cells); 4 – ruptured epidermis.

Each selected section was examined by light microscopy and intensity of epidermal and dermal alterations scored as follows (Gibson-Corley et al., 2013):

0 – normal; 1 – mild; 2 – moderate; 3 - severe

A sample of sections where stained with May-Grünwald Giemsa stain to differentiate polymorphonuclear cells as standardized by Szabó et al. (1999).

2.2.7 Serology for *Rickettsia*

Seroreactivity of guinea pigs to *Rickettsia* antigens was used to confirm exposure after needle inoculation or infected tick infestations with *R. parkeri*. To this end sera from all guinea pigs obtained before and 21 days after the end experimental procedures were tested by the indirect immunofluorescence assay (IFA) using crude antigens *R. parkeri* strain At24 isolate available at the Faculty of Veterinary Medicine of the University of São Paulo as described previously (Labruna et al., 2007). Briefly, starting from the 1:64 dilution sera were diluted in 2-fold increments with phosphate-buffered saline (PBS), pH 7.4 until the last reactive dilution. Slides were incubated with fluorescein isothiocyanate-labelled goat anti-guinea pig IgG (Sigma, USA). For each sample, the endpoint IgG titer reacting with *Rickettsia* antigens was determined. In each slide, a serum previously shown

to be non-reactive (negative control) and a known reactive serum (positive control) were tested at the 1:64 dilution.

2.2.8 Statistical analysis:

Gross parameters: Differences in the size (mm) of necrosis, induration, and hyperemia among the experimental groups (G1.1, G1.3, G2, G3, G4, and G5) were evaluated by Kruskal-Wallis analysis followed by Dunn's multiple comparison test. A separate analysis was done for each parameter (necrosis, induration, and hyperemia) and each day (0 to 14). Differences in the size of necrosis, induration, and hyperemia along time within groups were analyzed with Friedman test followed by Dunn's multiple comparison test (except for induration G2 that met the assumptions for an Analysis of Repeated Measures ANOVA and was followed by Tukey's multiple comparison test).

Microscopic parameters: Histopathological scoring analysis followed principles of Gibson-Corley et al. (2013). Such parameters were analyzed by either Kruskal-Wallis followed by Dunn`s test, Wilcoxon matched pairs test or Mann Whitney test. The Binomial test was used to determine relationship between *Rickettsia* infected tick-bite in the skin and vasculitis or thrombosis, in the 5^{th} or 14^{th} day of parasitism. Analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc.). Significant differences were set at $P < 0.05$.

2.2.9 Ethical statement

This study has been approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine of the University of São Paulo (protocol 3129/2013).

2.3- Results

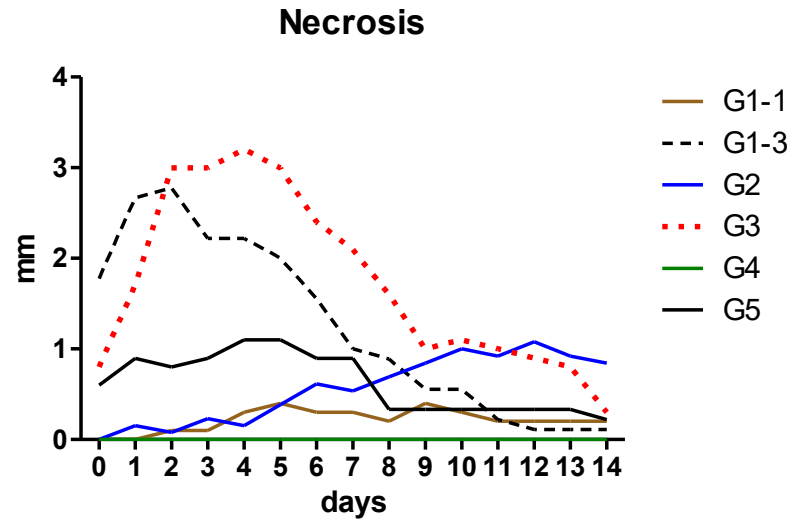
2.3.1- Gross evaluation of tick attachment and *Rickettsia* injected sites.

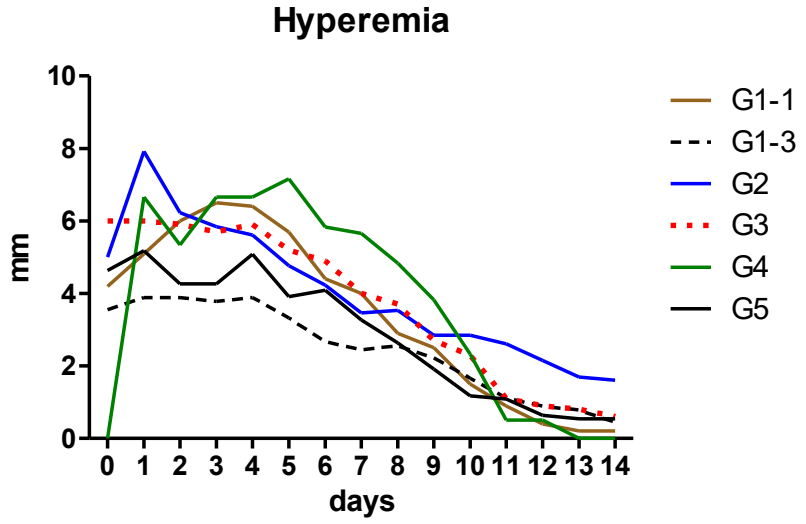
2.3.1.1 Necrosis

Necrosis was, among the three gross evaluation parameters, which displayed consistent differences among the experimental groups (Figure 1). Guinea pigs undergoing third infestation with uninfected ticks (G1.3) or second infestation with infected ticks (G3) several times exhibited significantly larger necrosis areas from day 0 (day of tick

detachment or intradermal inoculation) until day five after tick detachment in relation to other groups (Figure 2). At the same time skin necrosis size between these two groups did no differ significantly at any period ($P < 0.05$). No significant difference was observed among groups from six to the 14^{th} day post stimulus.

Significant necrosis size variation within groups and along time was observed only in guinea pigs undergoing a third or a second tick infestation (G1.3 and G3, respectively) and unrelated to *Rickettsia* infection (Friedman statistic= 80.000; DF= 14; $P<0.001$; Friedman statistic= 60.000; DF= 14; $P<0.001$; respectively). In G1.3 group, the second and fourth days` necrosis size was larger than those from days 12, 13 and 14. In the G3 group necrosis of the fourth day was larger than that of the 14th day. Group G4 did no exhibit gross necrosis at all. Since necrosis was not observed at *Rickettsia* needle inoculation sites on gross examination, three additional animals were needle injected with the double of the *Rickettsia* dose. On gross examination skin reactions of these animals were similar to those of G4 (lack of necrosis) (data not shown). Necrotic tick attachment sites and a *Rickettsia* needle inoculation site are illustrated in Figure 3.


Necrosis
mm
days
G1-1
G1-3
G2
G3
G4
G5


Hyperemia
mm
days
G1-1
G1-3
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G4
G5

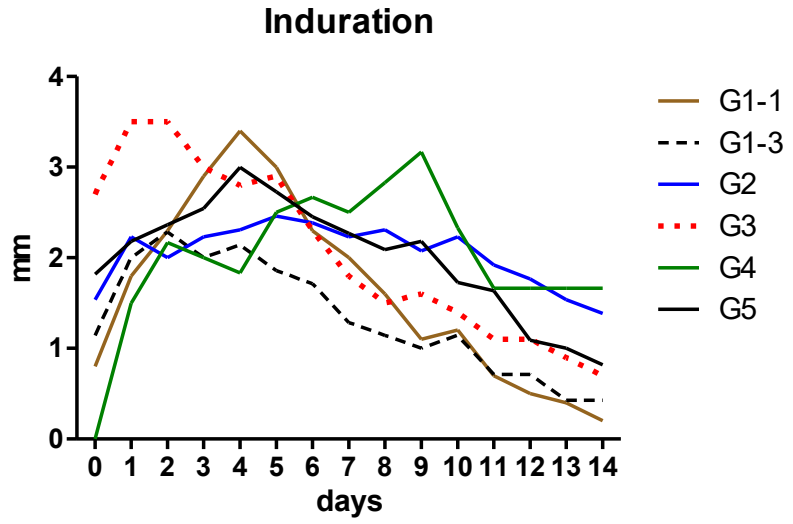
Induration
mm
days
G1-1
G1-3
G2
G3
G4
G5

Figure 1. Means (mm) of macroscopic alterations (necrosis, hyperemia, and induration) at tick attachment and/or *Rickettsia parkeri* ARF inoculation sites in the skin of guinea pigs. G1.1 - first infestation with uninfected *Amblyomma ovale* ticks; G1.3 - third infestation with uninfected *A. ovale* ticks; G2 - infested once with *Rickettsia parkeri* ARF strain infected *A. ovale* ticks; G3 - infested once with uninfected and followed by a second infestation with *R. parkeri* ARF infected *A.ovale* ticks; G4 - intradermally inoculated with *R. parkeri* ARF; and G5 - inoculated intraperitonially with *R. parkeri* followed by an infestation with *Rickettsia parkeri* ARF strain infected *A. ovale* ticks.

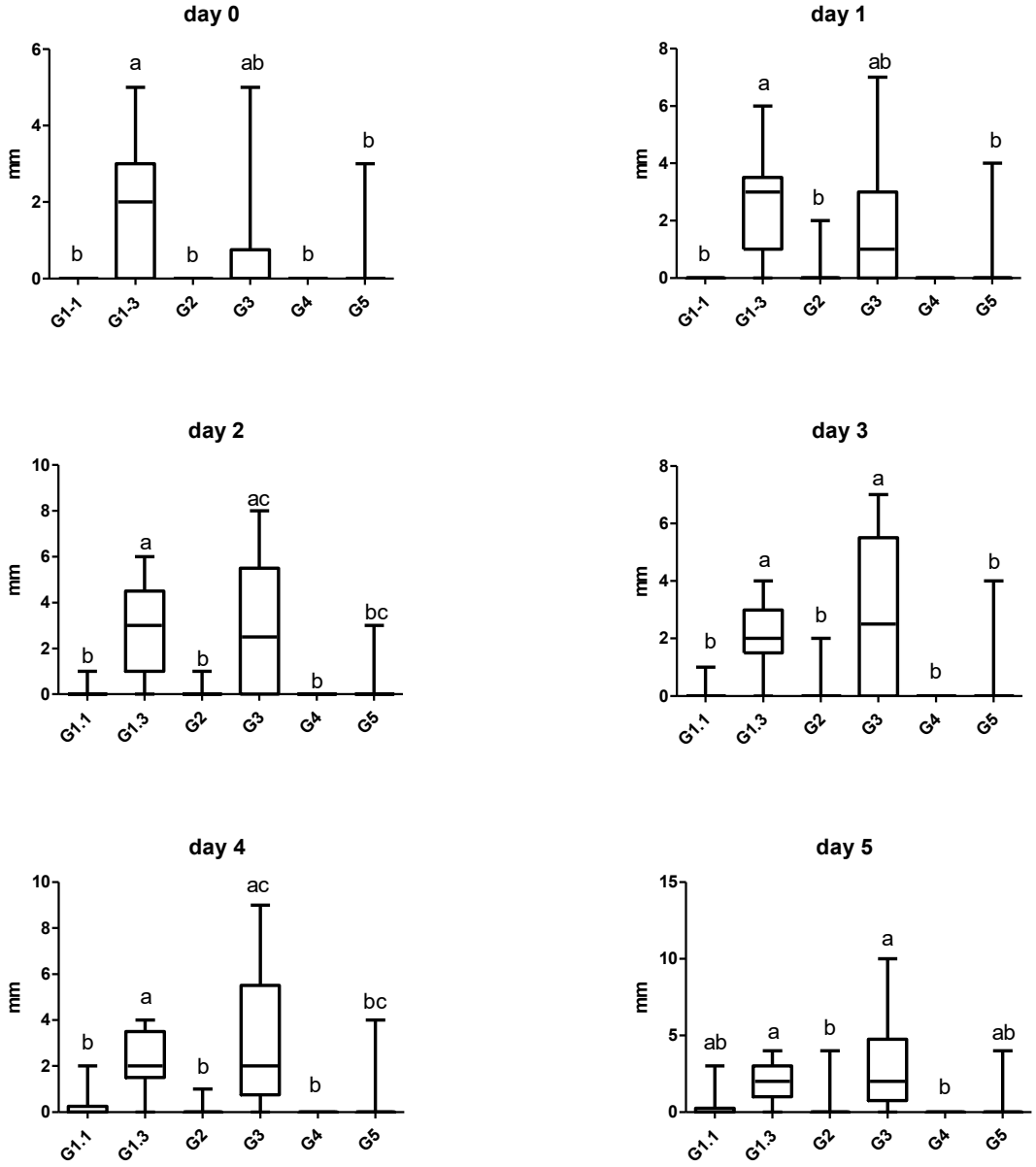

Figure 2. Significant differences in the diameter of skin necrosis at *Amblyomma ovale* tick attachment and/or *Rickettsia parkeri* ARF inoculation sites in the skin of guinea pigs at various time post stimulus and that underwent first infestation with uninfected ticks (G1.1); third infestation with uninfected ticks (G1.3); first infestation with *R. parkeri* infected ticks (G2); infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks (G3); intradermal inoculation of *R. parkeri* (G4); infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri* ARF (G5).

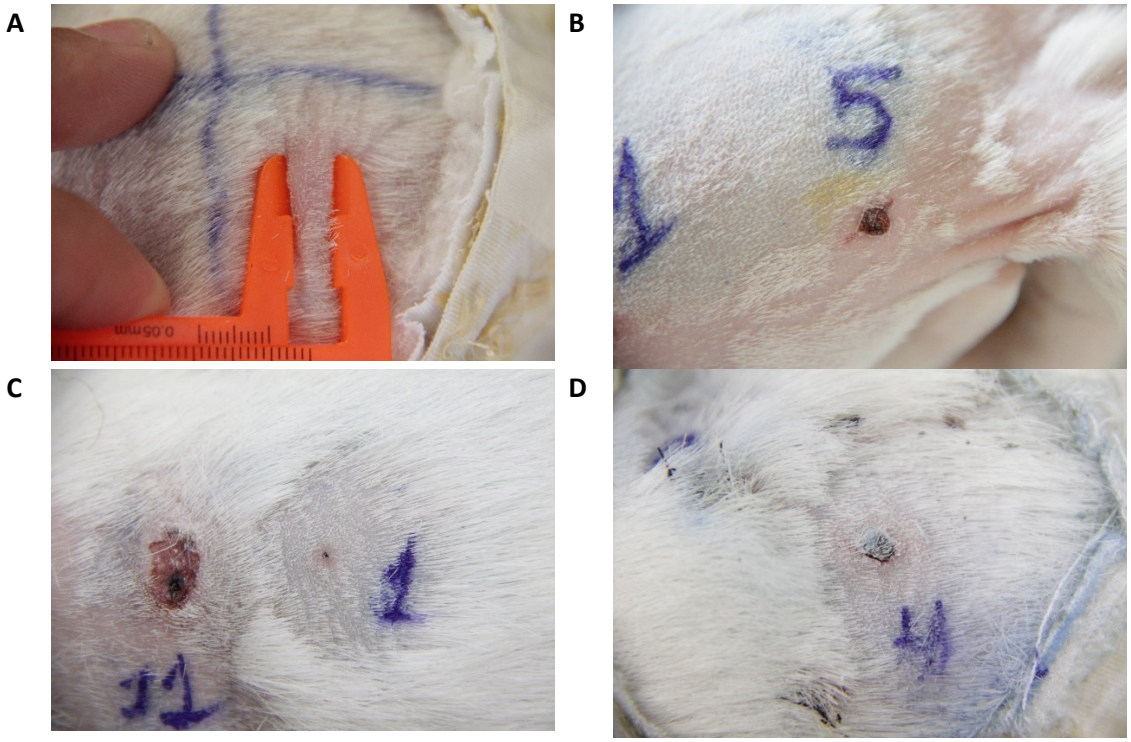

Figure 3. *Rickettsia parkeri* Atlantic rainforest strain needle inoculation and *Amblyomma ovale* tick attachment sites on the skin of guinea pigs. **A** –*R. parkeri* intradermal inoculation site; **B**- Eschar at the attachment site of an uninfected *A. ovale* tick after sensitization of the host with two previous infestations with uninfected ticks; **C**- Eschar at the attachment site of a *R. parkeri* infected *A. ovale* after sensitization with one infestation with uninfected ticks; **D** – Scab over necrotic area at the attachment site of a *R. parkeri* infected *A. ovale* after sensitization with one infestation with uninfected ticks

2.3.1.2 Hyperemia

Only minor differences in the hyperemia size were observed among the experimental groups (Figure 1). Hyperemic halo diameter was observed when ticks were detached in all groups whereas it was not observed immediately after needle *Rickettsia* inoculation into the dermis (Figure 3). Thereafter at needle *Rickettsia* inoculation sites (G4) hyperemia increased and was significantly larger until the seventh day in relation to those of the skin of guinea pigs undergoing third infestation with uninfected ticks (G1.3) (Figure 3). Significant hyperemia size variation within groups and along time was observed in all groups (data not shown). In all experimental groups, with slight variations, initial hyperemia (from 0 until 7^{th} day) was significantly larger in relation to those from the final days o observation (10^{th} – 15^{th}).

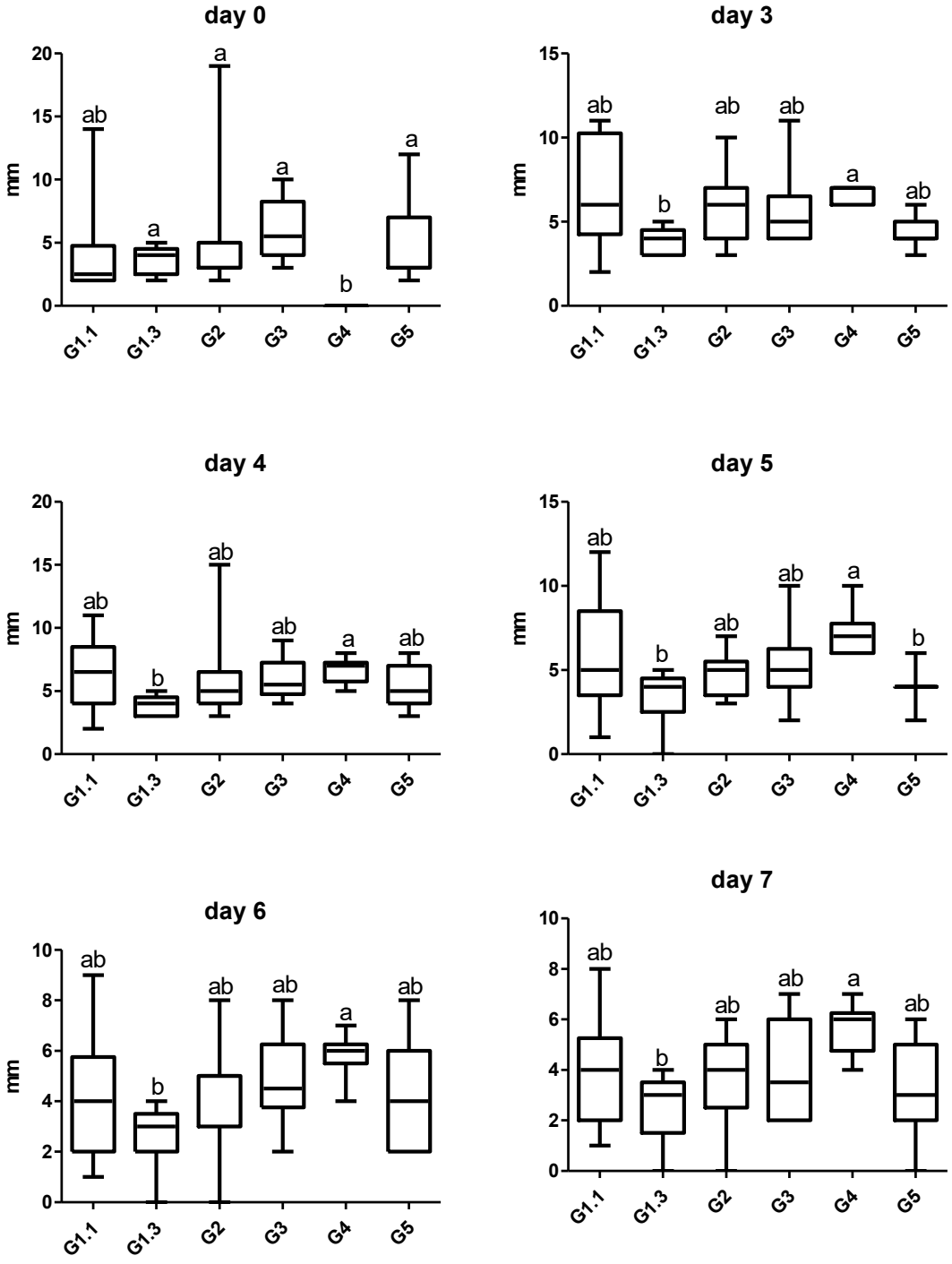

Figure 3. Significant differences in the diameter of skin hyperemia at *Amblyomma ovale* tick attachment and/or *Rickettsia parkeri* ARF inoculation sites in the skin of guinea pigs at various time post stimulus and that underwent first infestation with uninfected ticks (G1.1); third infestation with uninfected ticks (G1.3); first infestation with *R. parkeri* infected ticks (G2); infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks (G3); intradermal inoculation of *R. parkeri* (G4);

infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri* ARF (G5).

2.3.1.3 Induration

In the daily comparison among groups (Figure 4) it was observed that induration diameter on day zero was significantly wider on guinea pigs that underwent an infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks (G3) in relation to guinea pigs infested once with uninfected ticks (G1.1) or *Rickettsia* needle inoculated animals (G4). On the other hand, this last group exhibited larger induration in relation to guinea pigs infested once with uninfected ticks (G1.1) on the 14th day (G4).

Means of induration diameter followed a similar temporal pattern in all experimental groups with increase in the initial days after the end of parasitism or after needle inoculation and decrease thereafter (Figure 1). Nevertheless, such pattern varied greatly among animals within groups and attained significant values only in the guinea pig group that underwent a single infestation with uninfected ticks (G1.1). On these animals, the diameter of induration of days three and four were significantly larger in relation to days zero, and from the 11th to 14th (data not shown).

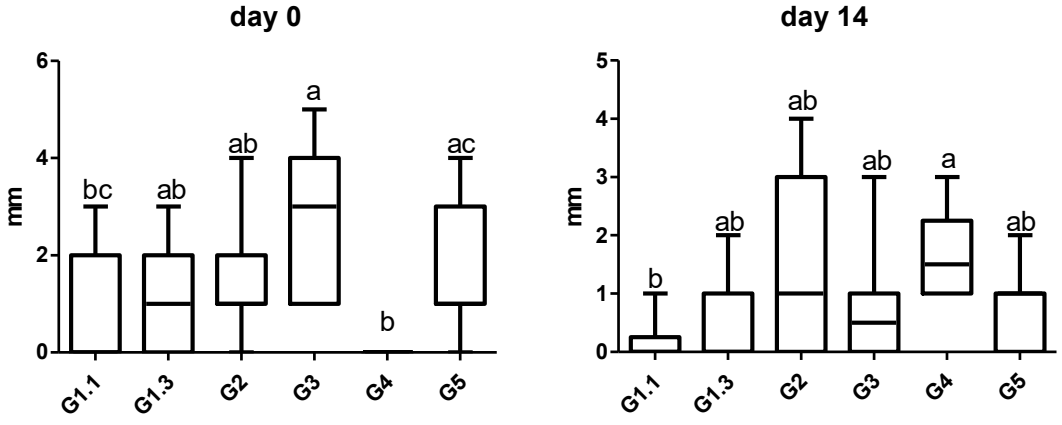

Figure 4. Significant differences in the diameter of skin induration at *Amblyomma ovale* tick attachment and/or *Rickettsia parkeri* ARF inoculation sites in the skin of guinea pigs at various time post stimulus and that underwent first infestation with uninfected ticks (G1.1); third infestation with uninfected ticks (G1.3); first infestation with *R. parkeri* infected ticks (G2); infestation with *R. parkeri* infected ticks after sensitization with one

infestation with uninfected ticks (G3); intradermal inoculation of *R. parkeri* (G4); infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri* ARF (G5).

2.3.2- Histopathology

Microscopic examination of tick-bite and *Rickettsia* inoculation sites were performed five and fourteen days after, respectively, tick attachment and *Rickettsia* needle injection. From 21,720 histopathology sections (3,620 slides with six sections each), 169 were selected for analysis.

2.3.2.1 General histopathologic features

Tick-bite lesions were characterized, irrespective of the experimental group, by a common microscopic pattern. All animals exhibited a necrotic area in the dermis beneath the tick´s attachment site (feeding cavity). Necrotic area was characterized by an approximately round cavity reaching the mid dermis, with irregular borders without a discernible tissue structure (lacking collagen and vessels) filled with cellular debris, inflammatory cells and erythrocytes. This area was surrounded by a focally intense inflammatory infiltrate. Polymorphonuclear cell infiltrates were located closer to the necrotic area whereas mononuclear infiltrates were more prominent outside surrounding the center of the lesion and deeper in the dermis. May-Grünwald Giemsa stain revealed that polymorphonuclear cells at the vicinity of the necrotic area lacked cytoplasmic granules indicating either neutrophils or other degranulated granulocytes. Cells further away but still surrounding the necrotic area, displayed eosinophilic or basophilic granules a characteristic stain and morphology of, respectively, eosinophils and basophils. In the deep dermis mononuclear infiltrate was more diffuse and constituted by macrophages and lymphocytes associated with areas of fibrous tissue (collagen). Apart from this general inflammatory cellular infiltrate at the tick´s attachment site, a perivascular infiltrate at the dermis-hypodermis interface was observed several times. This infiltrate was sometimes confluent with the general inflammatory process from the tick-bite site, but many times a separate process creating an independent focal but intense infiltrate more prominent in the skin of *Rickettsia* infected bite.

Alterations with vascular involvement included hyperemia, neovascularization, hemorrhage, and edema in the superficial dermis. Epidermal alterations were

characterized by a rupture at tick attachment site bordered by epithelium with increased thickness determined by hyperplasia (increase of the number of cell layers of the epidermis) and hyperkeratosis (increased thickness of keratin layer). Spongiosis (intercellular edema among keratinocytes) and exocytosis (inflammatory cell migration within the epithelium of skin) were also common epidermal alterations. Tick mouthparts (hypostome) could erratically be seen on the surface of epidermis or within the dermis. Laminae of a homogeneous and eosinophilic material was frequently seen around tick´s hypostome inserted into the dermis (cement cone). Inconspicuous vasculitis and small fibrin clots inside vessels were erratically seen at the dermis – hypodermis interface of the skin undergoing both infected and non-infected tick parasitism.

Except for hyperemia, *Rickettsia* needle inoculation sites (G4 experimental group) lacked features described above and were characterized by a slight dermal edema, sparse mixed inflammatory infiltrate, and mild collagenous proliferation. Three additional animals were needle injected with the double of the *Rickettsia* dose and were also evaluated in histopathology. Skin reactions of these animals were similar to those of G4 lacking necrosis, vasculitis and small intravascular fibrin aggregations (data not shown).

2.3.2.2 Necrosis

Necrosis was evident and similar in all tick infestation sites but was lacking from *R. parkeri* ARF needle inoculation sites (Figure 5). Opposite to infected, necrosis of non-infected tick-bite sites decreased significantly from the 5^{th} to 14^{th} days of tick attachment (Figure 6). Pooled data from non-infected tick bite sites (G1.1 and G1.3) compared to pooled data from infected tick-bite sites (G2, G3 and G5) showed a significantly more intense necrosis in the latter at both 5^{th} and 14^{th} days of tick attachment (Figure 7).

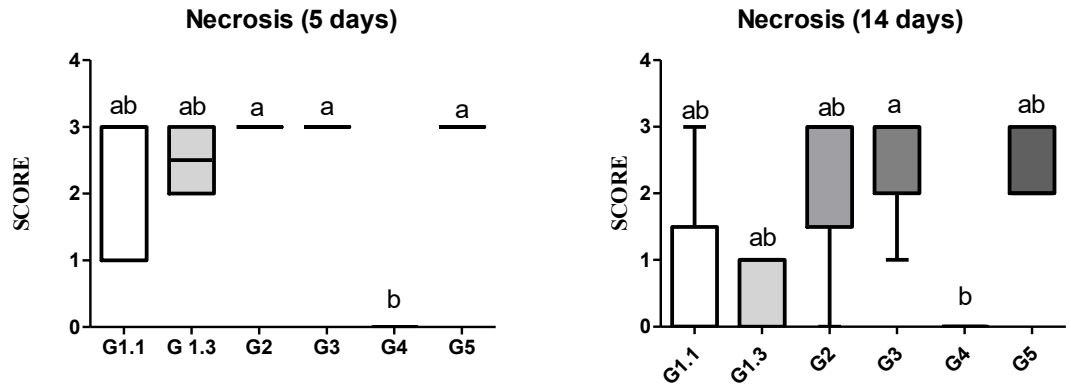

Figure 5. Necrosis at *Amblyomma ovale* tick attachment and/or *Rickettsia parkeri* strain Atlantic Rainforest inoculation sites in the skin of guinea pigs at five- and 14-days of stimulus. **G1.1-** first infestation with uninfected ticks; **G1.3-** third infestation with uninfected ticks; **G2** - first infestation with *R. parkeri* infected ticks; **G3 -** infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks; **G4-** intradermal inoculation of *R. parkeri*; **G5 -** infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri*. Different letters indicate significant differences between medians of groups (Kruskal-Wallis followed by Dunn's test; P≤0.05).

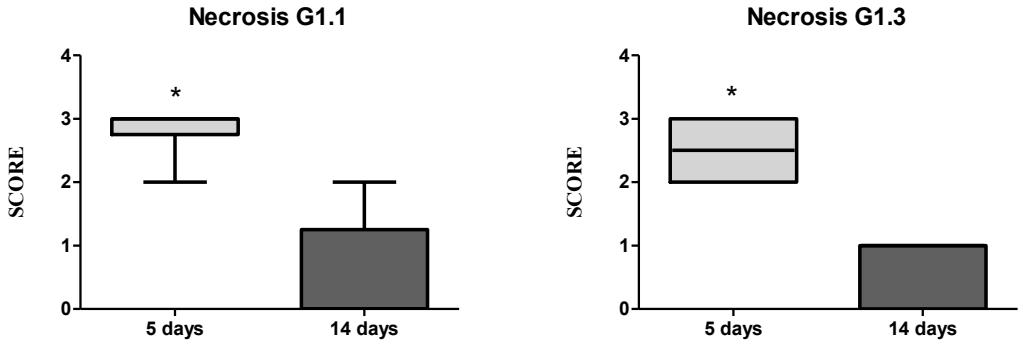

Figure 6. Necrosis intensity at *Amblyomma ovale* tick attachment sites in the skin of guinea pigs at five- and 14-days post stimulus. **G1.1-** first infestation with uninfected ticks; **G1.3-** third infestation with uninfected ticks. Asterisks indicate statistically significant differences between the two given groups (Wilcoxon matched pairs test; P≤0.05).

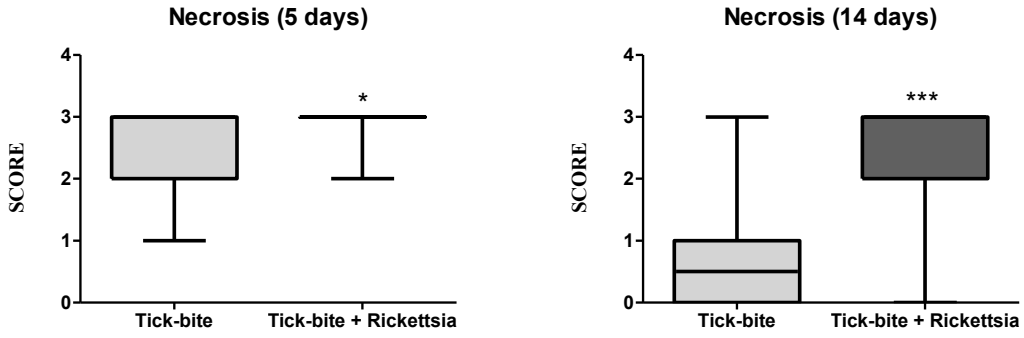

Figure 7. Necrosis intensity in the skin of guinea pigs undergoing five or 14 days of parasitism with either *Rickettsia* infected or uninfected *Amblyomma ovale* ticks. Uninfected ticks: pooled data of G1.1 and G1.3. Infected ticks: pooled data of G2, G3 and G5. Asterisks indicate statistically significant differences between the two given groups (Mann Whitney test, * P= 0.0337; *** P= 0.0004).

2.3.2.3 Inflammatory infiltrate

Inflammatory infiltrate intensity around the tick's feeding cavity in the dermis (general inflammatory infiltrate) was similar in all tick-bite sites irrespective of *Rickettsia* infection. Infiltrate was most of the time less intense in the dermis of needle inoculated *Rickettsia* (Figure 8). Inflammation intensity was similar in both days of observation (5th and 14th) within all groups (data not shown). General inflammatory infiltrate pooled data from non-infected tick bite sites (G1.1 and G1.3) compared to pooled data from infected tick-bite sites (G2, G3 and G5) showed a significantly more intense infiltrate in the latter at the 14th day of tick attachment (Figure 9).

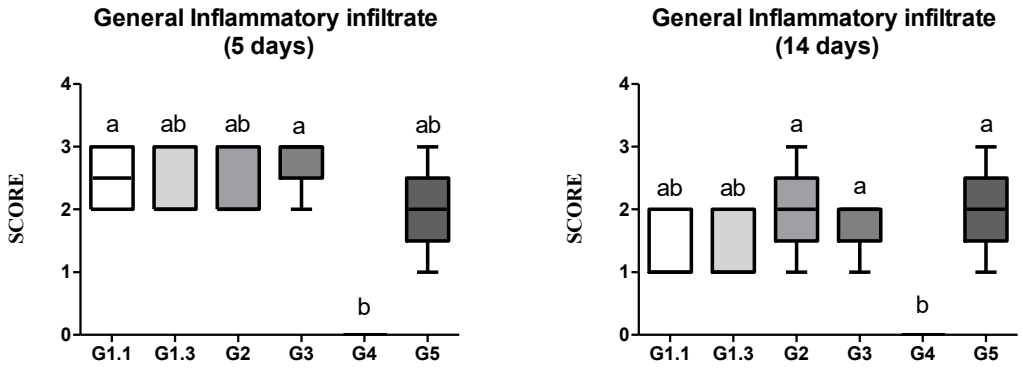

Figure 8. Inflammatory infiltrate at *Amblyomma ovale* tick attachment and/or *Rickettsia parkeri* strain Atlantic Rainforest inoculation sites in the skin of guinea pigs at five- and 14-days of stimulus. **G1.1-** first infestation with uninfected ticks; **G1.3-** third infestation with uninfected ticks; **G2** - first infestation with *R. parkeri* infected ticks; **G3 -** infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks; **G4-** intradermal inoculation of *R. parkeri*; **G5 -** infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri*. Different letters indicate significant differences between medians of groups (Kruskal-Wallis followed by Dunn's test; P≤0.05).

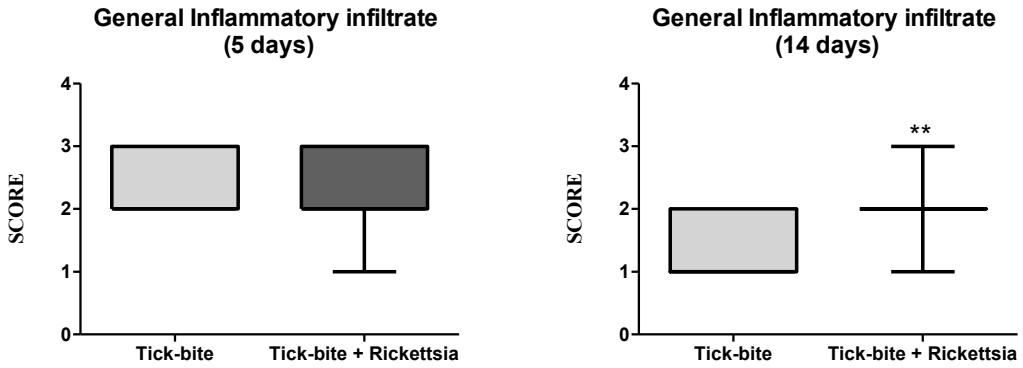

Figure 9. General inflammatory infiltrate intensity in the skin of guinea pigs undergoing five or 14 days of parasitism with either *Rickettsia* infected or uninfected *Amblyomma ovale* ticks. Uninfected ticks: pooled data of G1.1 and G1.3. Infected ticks: pooled data of G2, G3 and G5. Asterisks indicate statistically significant differences between the two given groups (Mann Whitney test, ** P= 0.0044).

2.3.2.4 Inflammatory infiltrate at the dermis - hypodermis interface

Inflammatory infiltrate at the dermis- hypodermis interface was absent in *Rickettsia* needle inoculation sites (Figure 10). A tendency for infiltrate intensification of skin parasitized by infected over uninfected ticks at this site was observed at the 14th day of parasitism and that attained a significant level between G3 (infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks) over G1.3 (third infestation with uninfected ticks) (Figure 10). Inflammation intensity at the dermis-hypodermis interface did not alter significantly from the 5th to the 14th day of parasitism or after *Rickettsia* inoculation in any of the groups (data not shown). However, comparison of pooled data (n=12) from skin bitten by uninfected ticks (G1.1 and G1.3) to pooled data (n=15) from skin bitten by infected ticks (G2, G3 and G5) showed a more intense infiltrate in the latter during the14th day of tick attachment (Figure 11).

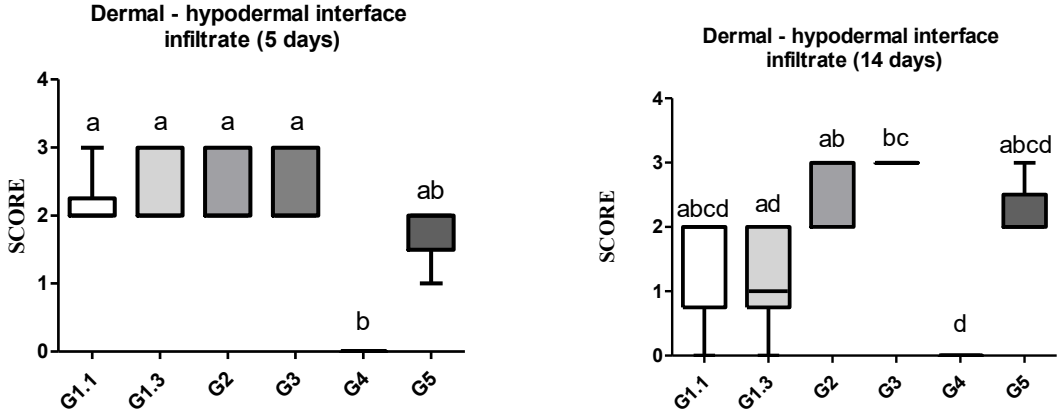

Figure 10. Inflammatory infiltrate of the dermal-hypodermal interface at *Amblyomma ovale* tick attachment and/or *Rickettsia parkeri* strain Atlantic Rainforest inoculation sites in the skin of guinea pigs at five- and 14-days of stimulus. **G1.1-** first infestation with uninfected ticks; **G1.3-** third infestation with uninfected ticks; **G2** - first infestation with *R. parkeri* infected ticks; **G3 -** infestation with *R. parkeri* infected ticks after sensitization with one infestation with uninfected ticks; **G4-** intradermal inoculation of *R. parkeri*; **G5 -** infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri*. Different letters indicate significant differences between medians of groups (Kruskal-Wallis followed by Dunn's test; P≤0.05).

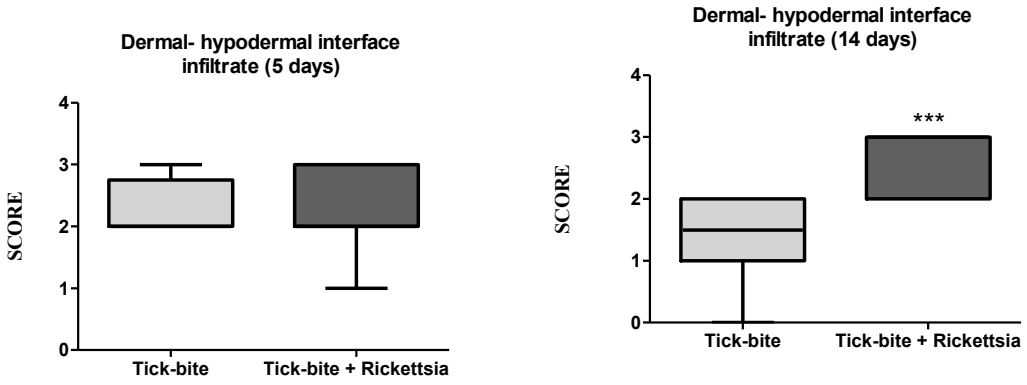

Figure 11. Inflammatory infiltrate intensity at the dermal-hypodermal interface in the skin of guinea pigs undergoing five or 14 days of parasitism with either *Rickettsia* infected or uninfected *Amblyomma ovale* ticks. Uninfected ticks: pooled data of G1.1 and G1.3. Infected ticks: pooled data of G2, G3 and G5. Asterisks indicate statistically significant differences between the two given groups (Mann Whitney test, *** P= 0.0001).

2.3.3 Vasculitis and thrombosis

An obvious thrombotic mass was absent from all analyzed sections, but small fibrin aggregates sometimes associated to a group of white cells were seen within vessel lumens. These small fibrin aggregates and inconspicuous vasculitis were erratically observed in the skin undergoing both infected and non-infected tick parasitism. Both vascular lesions were absent in the skin of guinea pigs at *R. parkeri* ARF needle inoculation sites and could not be evaluated in the center of infected or uninfected tick feeding lesions. In the latter, necrosis, and intense focal inflammatory cell infiltrate, respectively, disrupted, or overshadowed structural tissue alterations, precluding other observations. Both vasculitis and fibrin aggregates were rather located bellow the tick´s attachment site, deeper in the skin, at the dermal-hypodermal interface, rather than in the dermis at both sides of the feeding cavity.

No association was detected between *Rickettsia* infected tick-bite in the skin of guinea pigs and vasculitis in both the 5th (Z = -0.4712; P = 0.6375) and 14th (Z = 1.7225; P = 0.085) day of parasitism. On the other hand, fibrin aggregates were negatively associated with *Rickettsia* infected tick-bite of the skin on the 5th day (Z = -2.5531; P = 0.0107).

Histological features of a *R. parkeri* infected and uninfected *Amblyomma ovale* tick attachment as well as *R. parkeri* needle intradermal inoculation sites on guinea pig´s skin are illustrated in Figures 12 and Figure 13.

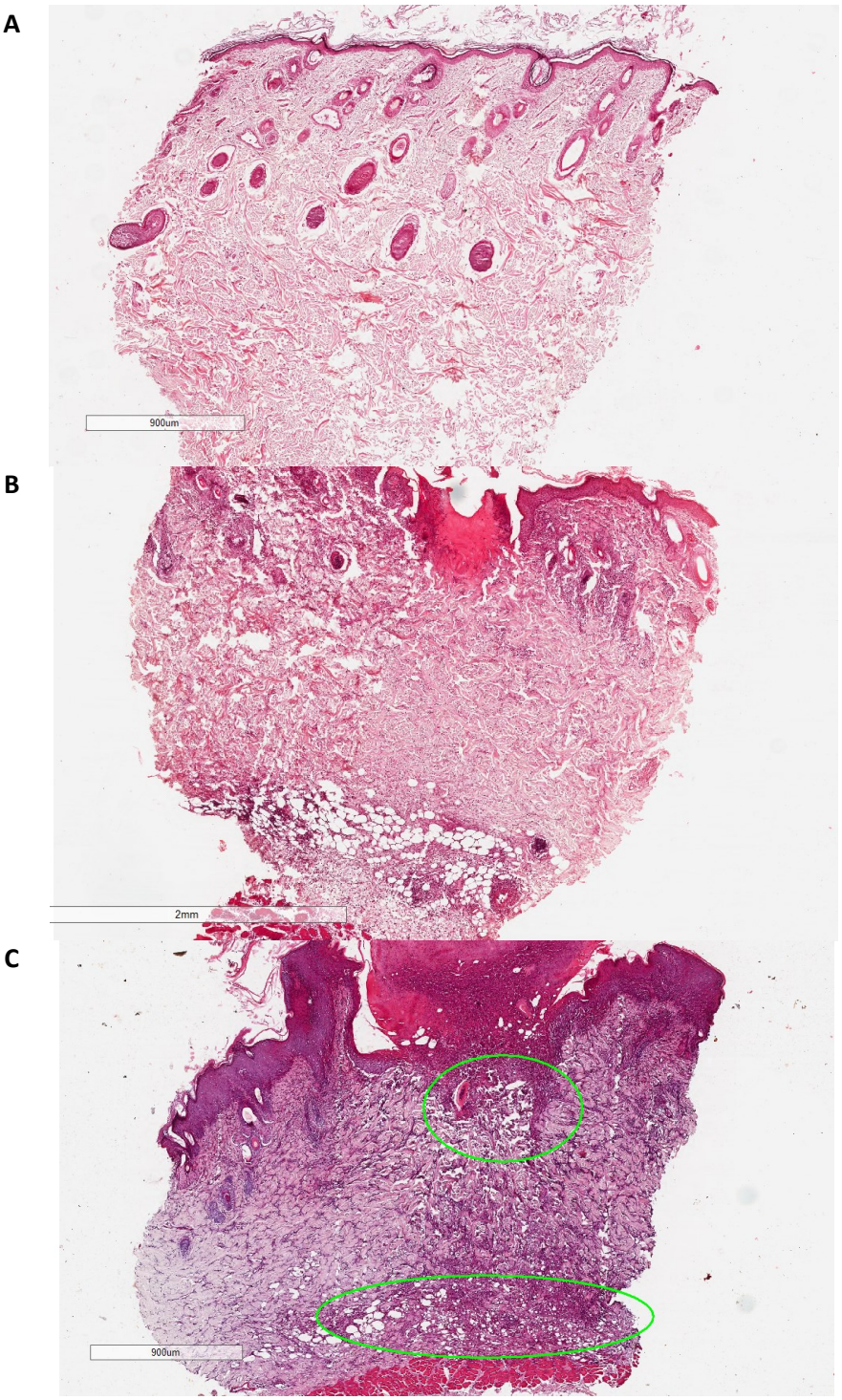

Figure 12. Histopathological features of guinea pig skin at: **A** - *Rickettsia parkeri* needle intradermal inoculation site; **B** - Attachment site of a *R. parkeri* infected *Amblyomma ovale* tick on a guinea pig undergoing fifth day of a first infestation; **C** - Attachment site

of a *R. parkeri* infected *Amblyomma ovale* tick on a guinea pig undergoing 14th day of a second tick infestation. Note de feeding cavity (small green circle on the top) and intense inflammatory infiltrate in the deep dermis/hypodermis. Hematoxylin-eosin stain.

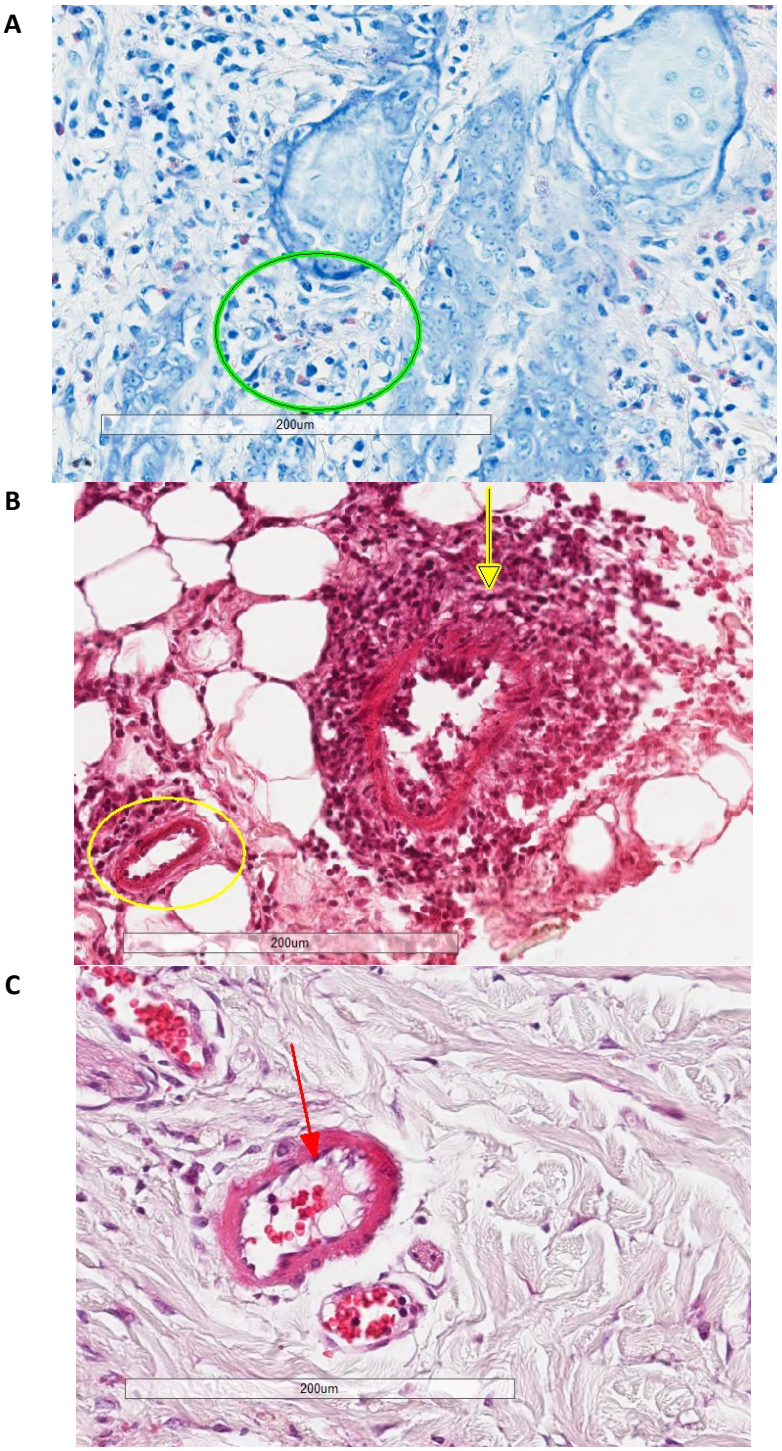

Figure 13. Histopathological features of guinea pig´s skin at the attachment site of *Amblyomma ovale* tick: **A** – undergoing fifth day of a third uninfected tick infestation. Green circle highlights eosinophils and basophils in the dermal inflammatory infiltrate (May-Grünwald Giemsa stain); **B** - Attachment site of a *R. parkeri* infected *Amblyomma*

ovale tick on a guinea pig undergoing fifth day of a first infestation. Yellow circle highlights preserved artery and arrow points deformed arteria by vasculitis. Hematoxylin-eosin stain; **C** - Attachment site on a guinea pig undergoing 14th day of a third uninfected tick infestation. Arrow indicated a small fibrin aggregate within the vessel lumen. Hematoxylin-eosin stain.

2.3.4 Serology

All animals exposed to *Rickettsia parkeri* ARF, by intradermal inoculation or infected tick-bite, developed *R. parkeri* antibody titers (Table 3). Infected tick-bite induced higher antibody titers than intradermal inoculation. Antibody titers ranged from 1:512 to 1:4096 in the case of guinea pigs that were tick infected whereas from 1:128 to 1:512 when needle inoculated. Highest titers (1024-8192) were attained after an intraperitoneal needle sensitization followed by and infected tick infestation (G5). Serum of all guinea pigs infested with only uninfected ticks or collected before stimulus with infected tick or *Rickettsia* needle inoculation did not react to *Rickettsia* antigens.

Table 3. Endpoint antibody titers by indirect immunofluorescence assay (IFA) to *Rickettsia parkeri* in guinea pigs from Groups 1 to 4 (G1- G4) at 21 days post last infestation or intradermal inoculation. G1 guinea pigs were infested three times with uninfected *Amblyomma ovale*, G2 guinea pigs were infested once with *Rickettsia parkeri* ARF strain infected ticks. G3 guinea pigs were infested once with uninfected and followed by a second infestation with *R. parkeri* ARF infected ticks and G4 guinea pigs were intradermally inoculated with *R. parkeri* ARF. G5**-** infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri*

Animal	**Experimental group**				
	G1	**G2**	**G3**	**G4**	**G5**
1	NR	4096	512	512	2048
2	NR	512	1024	512	2048
3	NR	1024	4096	256	4096
4	NR	1024	4096	256	1024
5	NR	512	2048	128	2048
6	NR	4096	512	256	8192

NR- serum nonreactive at the 1/64 dilution

2.3.5 Fever

Apart from animals from G5 group (infestation with infected ticks after sensitization with intraperitoneal inoculation of *R. parkeri*) and one animal from G3 group (guinea pigs were infested once with uninfected and followed by a second infestation with *R. parkeri* ARF infected ticks), none of the animals had fever throughout the experiments.

2.4- Discussion

All guinea pigs exposed to *R. parkeri* ARF either by tick infestation or by needle inoculation had detectable antibodies 21 days after the last experimental stimulus. As expected, seroconversion to *R. parkeri* was not observed in guinea-pigs infested with uninfected ticks and serum of hosts before experimental procedures did not react to *Rickettsia*. These results prove that an infection of hosts with *Rickettsia* occurred during their experimental exposure and that such infection had the potential to damage skin by bacterial proliferation.

Endpoint titers were high and comparable to another experimental infestation with *R. parkeri* strain ARF infected ticks (Krawczak et al., 2016b). However, titers were higher in guinea pigs that were bitten by infected ticks. One explanation for this difference is the transmission of the bacteria associated with tick saliva that may enhance *Rickettsia* proliferation by modulating local host immunity. Saliva is known to have several immunomodulatory compounds (Kotál et al., 2015) and that were shown establish an ecologically favorable niche for pathogen proliferation, a phenomenon known as saliva-activated transmission (Nuttall and Labuda 2004). However, there are other possibilities that explain lower endpoint titers. Needle injected Rickettsia dose may have been lower than that inoculated by ticks, Vero cell Rickettsia may be less virulent or intradermal inoculation was less effective than intraperitoneal route.

Gross examination revealed that after detachment of infected and uninfected ticks and *Rickettsia* needle inoculation, all animals developed skin hyperemia and induration. Both features are inflammation hallmarks and indicate, respectively, enhanced arterial blood flow as a result of vasodilation and inflammatory exudate accumulation caused by increased vascular permeability (Kumar et al., 2013).

Although hyperemia was similar in all groups, it was the prominent feature of *Rickettsia* needle inoculated sites from the first day onwards. Similarly, induration remained longer at *Rickettsia* needle inoculated sites. These observations indicate an inflammation triggered by the bacteria inoculation on its own but that was most of the time overshadowed by the inflammation induced by tick-bite.

One drawback of this study was the fact that G4 were inoculated with a R. parkeri isolate from in vitro culture, and we don´t know if this isolate decreased virulence through in vitro passages; therefore, we do not know if absence of necrosis was due decreased virulence of this isolate. Another drawback was the lack of a group inoculated only with Vero cells; therefore, we can not predict how much of the skin alterations in G4 were attributed to cell contents besides the rickettsiae. However, absence of this group does not compromise our aims, which relied primarily on the skin lesions induced by infected or uninfected ticks, and to discern how much of these lesions were induced by rickettsiae in a tick bite feeding site.

Size of necrosis was dissimilar among the various groups and, in the initial days after parasite detachment, larger in those skin that underwent a second or third tick infestation. This observation shows that an immune response to ticks is a major factor in the necrosis pathogenesis. On the other hand, *Rickettsia* by itself or an immune reaction to the bacteria, did not collaborate with necrosis under gross scrutiny. Needle skin inoculation sites of *R. parkeri* strain ARF lacked necrosis and sensitization with intraperitoneal *Rickettsia* inoculation did not increase infected tick parasitism site necrosis. Additionally, three animals were needle injected with double of the *Rickettsia* dose and still did not develop necrosis. Nonetheless it is worthwhile observing that, although did not attain significantly different values, second infestation with infected ticks provided the overall largest necrotic areas and that in the infestation with infected ticks of tick-bite naïve hosts necrosis slowly increased towards the 14th day when it was the largest.

Taken together gross observations indicate that *A. ovale* infestations cause an intense skin inflammation after tick detachment and necrosis is a prominent feature on animals that underwent a second or third infestation but not after a first one. *Rickettsia parkeri* strain ARF on its own induces inflammation, but not a visible necrosis. In this regard it was previously shown that gross lesions including necrosis are increased by immune response (second or third infestations) of unnatural hosts against ticks (Szabó et al., 1999).

Under histopathological analysis, skin lesions associated with both *R. parkeri* infected and uninfected *A. ovale* tick feeding were similar. Same lesion morphology was already described from uninfected adult *Rhipicephalus sanguineus* and *Amblyomma* spp. feeding sites in the skin of guinea pigs and capybaras, respectively (Heijden et al., 2005; Szabó et al., 1999). The center of the tick-bite lesion was characterized by a necrotic area surrounded by an inflammatory infiltrate. Necrosis located beneath tick attachment site is feature of tick and telmophage arthropod blood feeding (Kemp et al., 1982; Szabó et al., 1999; Wikel et al., 2017). The laminae of a homogeneous and eosinophilic material seen around tick´s hypostome in the dermis refer to a cement-like proteinaceous substance secreted by the tick that hardens and helps to secure attachment (Anderson and Magnarelli, 2008).

Overall, lesions under microscopic view were similar and most intense in those animals that underwent a tick bite whereas needle injection of *Rickettsia* in skin resulted in only minor inflammatory changes. Nevertheless, *Rickettsia* seemed to enhance some of the lesion parameters if inoculated into the skin by the tick. Necrosis, general inflammatory infiltrate at tick bite site and, the focal inflammatory infiltrate at the dermis-hypodermis interface were extended overtime at infected tick attachment sites in relation to those uninfected. These observations highlight important aspects of this tick-host-pathogen interface. First, *Rickettsia* inoculation by tick in relation to needle injection is more effective, a feature that was observed by the highest anti-*Rickettsia* antibody titers obtained when injected by ticks. Second, main microscopic lesions are caused by the tick-bite itself, but they can be enhanced and extended overtime by the *Rickettsia*.

Vasculitis and/or thrombus are histopathological hallmarks of many rickettsial infections and responsible for ischemic and necrotic lesions in several human and experimental rickettsiosis (La Scola, 2009; Drexler et al., 2020; Walker et al., 1981; 1988). At both *R. parkeri* infected and uninfected *A.ovale* tick feeding sites in the skin of guinea pigs these features were inconspicuous and erratic. In fact, they seemed to be related to the *A. ovale* tick feeding and not to the *Rickettsia* infection.

It must be emphasized that there were both size and temporal dissimilarities between gross observations and histopathological analyses. Necrosis on gross examination was measured with the aid of calipers and, when observed, attained from one to 10 mm in diameter whereas in histopathology the feeding´s cavity necrosis diameter was in the approximate range of 0.5 – 1.0 mm. As such, feeding cavity necrosis is a microscopic feature. In this regard, Szabó et al. (1999) described similar feeding cavity

necrosis at *R. sanguineus* attachment sites in both natural and unnatural hosts (dogs and guinea pigs, respectively) but only guinea pigs exhibited necrosis on gross examination.

In relation to temporal settings, gross examination underwent after tick detachment whereas histopathology was evaluated during tick feeding (five and 14 days). Therefore, in the latter, skin at the attachment site was under tick´s saliva modulation. Saliva of several tick species have many pharmacological properties, particularly a prominent and redundant anti-hemostatic activity that includes control of coagulation cascade and platelet aggregation (Wikel et al., 2017). Saliva also decreases several aspects of both immune and non-immune inflammatory processes by inhibiting, among others white cell chemotaxis and activation (Wikel et al., 2017). While these processes have host specific activity that explains diminished reaction to ticks and increased feeding capacity on natural hosts, they are less efficient and only partially abrogate reactions of unnatural host species (Randolph, 1979, Ferreira et al. 2003; Szabó et al. 1999). Since guinea pigs (just as humans) are not habitual adult *A. ovale* tick hosts it is possible to infer that saliva injected during parasitism will have a partial activity. Under such circumstances it is possible to propose that during feeding on guinea pigs *A. ovale*, partially inhibited host reactions including hemostatic processes. After tick detachment, and with vanishing saliva activity, host reactions were released from the incomplete pharmacological control, gained strength, and evolved to a larger necrosis visible under gross examination. Vascular damage (vasculitis) and hemostatic plugs adhered to vascular wall (thrombosis) are possible candidates in the physiopathology of skin necrosis visible on gross examination at *A. ovale* feeding sites on immune hosts. Both can cause ischemic necrosis and their only partial control (seen as the inconspicuous and erratic vasculitis and of fibrin aggregates) underscore such possibility. Neutrophil infiltrate at tick attachment sites is also capable of causing skin necrosis. These cells produce hydrolytic enzymes and reactive oxygen species but which may be partially controlled by tick saliva (Wikel et al., 2017). These processes, however, should addressed by additional research.

Taking together both gross and microscopic observations it can be affirmed that, under the experimental conditions of our work, skin eschar (necrosis or “tache noir”) was not a pathognomonic sign of *R. parkeri* strain ARF infection transmitted by adult *A. ovale* in guinea pigs. Such eschar was rather related to an immune-mediate inflammatory reaction against the tick that may be enhanced by the bacteria. The discrepancy between our observation and that of those from literature has several possible explanations. In the

case of human eschars, its origin and age are most of the time uncertain. Ticks that may have bitten the human host and transmitted *Rickettsia* are rarely recovered and therefore tick stage, species and age of lesion remain unknown, as is rare the description of previous contact with ticks of human cases (Walker et al., 1981; 1988; Drexler et al., 2020).

In relation to experimental settings like ours, but restricted to intradermal needle inoculation, La Scola et al. (2009) observed by inoculating 25 pathogenic and non-pathogenic *Rickettsia* into guinea pigs that 16 *Rickettsia* species or subspecies caused inoculation eschars in guinea pigs but in nine species or subspecies, no eschar was observed. Curiously, they observed that although belonging to the same species, *Rickettsia conorii* subsp. *conorii* and *R. conorii* subsp. *israelensis* caused eschars whereas *R. conorii* subsp. *caspia*, *R. conorii* subsp. *indica* did not. Hence this work suggests that not only *Rickettsia* species, but subspecies as well is an important feature for eschar pathogenesis. In this regard it is important to consider the multiple strains of *Rickettsia parkeri* from the New World (Nieri-Bastos et al., 2018) as basis for a wide spectrum of host reactions to infected tick-bites.

With these results we suggest that eschar in humans exposed to infected *A. ovale* ticks are caused by tick-bite of previously tick-bite sensitized human beings and eschars may occur without *Rickettsia* infection. Nonetheless eschar may be used as a tick-bite sign and *Rickettsia* inoculation site. Therefore, until additional evidence, febrile diseases related to tick-bites with or without eschars still need laboratory confirmation of *Rickettsia* infection.

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