ALEJANDRO RAMÍREZ HERNÁNDEZ

Experimental infection of Capybaras (*Hydrochoerus hydrochaeris*) with an *Amblyomma sculptum*-derived strain of *Rickettsia rickettsii*

São Paulo
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Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

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Prof. Marcelo Bahia Labruna Ph.D.

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CERTIFICADO

Certificamos que a proposta intitulada "Avaliação de capivaras (Hydrochaeris hydrochaeris) como hospedeiros amplificadores da bactéria Rickettsia rickettsii para carrapatos Amblyomma sculptum, vetor da febre maculosa brasileira ", protocolada sob o CEUA nº 4115110215 (ID 005723), sob a responsabilidade de **Marcelo Bahia Labruna** *e equipe; Alejandro Ramírez Hernández; Adriano Pinter dos Santos* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 18/10/2018.

We certify that the proposal "Evaluation of capybaras (Hydrochaeris hydrochaeris) as amplifier hosts for the bacterium Rickettsia rickettsii to Amblyomma sculptum ticks, the vector of Brazilian spotted fever ", utilizing 5 Brazilian wild species (5 males), 90 Guinea pigs (90 males), 75 Rabbits (75 males), protocol number CEUA 4115110215 (ID 005723), under the responsibility of **Marcelo Bahia Labruna** *and team; Alejandro Ramírez Hernández; Adriano Pinter dos Santos* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/18/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2015 a 12/2017 Área: Medicina Veterinária Preventiva E Saúde Animal

Origem:	Animais provenientes de campanha						
Espécie:	Espécies silvestres brasileiras	sexo:	Machos	idade:	3 a 5 meses	N:	5
Linhagem:	Não Aplica			Peso:	10 a 20 kg		
Origem:	Biotério do Departamento de Medicina Veterinária Preventiva e Saúde Animal						
Espécie:	Cobaias	sexo:	Machos	idade:	3 a 8 meses	N:	90
Linhagem:	Albina			Peso:	200 a 400 g		
Origem:	Animais provenientes de estabelecimentos comerciais						
Espécie:	Coelhos	sexo:	Machos	idade:	2 a 8 meses	N:	75
Linhagem:	Nova Zelândia Branco			Peso:	1 a 3 kg		

Registro IBAMA/Sisbio/Etc: SISBio 43259-4

Método de Captura: As capivaras serão capturadas com o uso de brete de cevação, conforme aprovado pelo SISBIO (documento em anexo) e por esta CEUA (em anexo, protocolo CEUA N 5948070314) para o projeto auxilio-pesquisa (FAPESP 2013/18046-7) referente ao presente projeto de Doutorado.

Local do experimento: Infectório Exprimental da SUCEN em Mogi Guaçu, SP

São Paulo, 18 de outubro de 2018

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretária
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

Author: RAMÍREZ HERNÁNDEZ, Alejandro

Title: **Experimental infection of Capybaras (*Hydrochoerus hydrochaeris*) with an *Amblyomma sculptum*-derived strain of *Rickettsia rickettsii***

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Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

DEDICATION

To my parents and brothers ("my tribe"), you have been the best example of discipline, dedication and enthusiasm to achieve goals and dreams in this lifepath. "Para atrás ni pa' bailar tango"…

To my beloved wife and daughter, for their limitless patience and love through this life journey.

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Firstly, I must acknowledge my advisor, professor Marcelo Bahia Labruna, for courageously accepting the guidance of this “gringo” and continuously opens doors in the prolific world of ticks and tick-borne diseases.

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Eternal gratitude to God for each life opportunity.

"There is an animal named Catíuare; abides on land and in the water…when anything alarms them they flee into the water to the bottom…are larger than a sheep, have a head in the manner of a hare, but larger, and short ears; have a stumpy tail, fairly long legs and run fast on land from one body of water to another."

H. Staden (1557)

RESUMO

RAMÍREZ HERNÁNDEZ, Alejandro. **Infecção experimental de Capivaras (*Hydrochoerus hydrochaeris*) com uma cepa de *Rickettsia rickettsii* derivada de *Amblyomma sculptum***. 2019. 74 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

A Febre Maculosa Brasileira (FMB) é reconhecida como a doença transmitida por carrapatos com maior letalidade no Brasil e outros países do hemisfério ocidental. *Rickettsia rickettsii* é seu agente etiológico e, na sua história natural, os carrapatos e mamíferos cumprem um papel essencial. No sudeste brasileiro, *Amblyomma sculptum* é o principal vetor incriminado e as capivaras têm sido reconhecidas como hospedeiros amplificadores. Estudos prévios têm comprovado que as capivaras são suscetíveis à infecção com *R. rickettsii* e apresentam uma rickettsemia prolongada que possibilita a infecção de populações de *A. sculptum* suscetíveis. Os estudos citados, usaram na infecção, uma cepa de *R. rickettsii* isolada de carrapatos *Amblyomma aureolatum* (cepa Taiaçu), sem executar novos desafios de infecção em animais imunes. No presente trabalho, apresentamos os resultados de uma infecção experimental de capivaras com uma cepa de *R. rickettsii* isolada de carrapatos *A. sculptum* (cepa Itu) identificando variáveis clínicas, hematológicas e patológicas, o período de rickettsemia e a subsequente transmissão de *R. rickettsii* a populações de carrapatos *A. sculptum* suscetíveis, com uma análise sequencial da sua competência vetorial. Adicionalmente, foram executados desafios posteriores para estudar os parâmetros citados em animais imunes ou convalescentes. Cinco capivaras, de duas áreas não endêmicas no estado de São Paulo, foram infectadas com *R. rickettsii* (cepa Itu) através de infestações com carrapatos *A. sculptum* infectados. A temperatura retal e os sinais clínicos foram avaliados por um período de acompanhamento de 30 dias e amostras de pele e sangue foram coletadas a cada dois dias, para inoculação de cobaias, extração de DNA, hematologia e reação de imunofluorescência indireta (RIFI). Adicionalmente, as capivaras também foram infestadas com carrapatos *A. sculptum* não infectados (ninfas e adultos) em duas câmaras de alimentação (uma junto com os adultos infectados e a outra separados dos adultos infectados), os quais foram posteriormente coletados, incubados e usados para infestar coelhos suscetíveis e para extração de DNA. Estes procedimentos foram repetidos através de infecções subsequentes nas capivaras. Na primoinfecção, quatro de cinco capivaras

apresentaram sinais clínicos e duas delas morreram, exibindo lesões macroscópicas vasculares durante a necropsia. Baseando-se na inoculação das cobaias, houve rickettsemia em todas as capivaras com uma duração média de 9,2 dias (intervalo: 6-20 dias). DNA de *Rickettsia* foi amplificado em amostras de sangue e pele das capivaras e algumas variáveis hematológicas (hematócrito e contagem de leucócitos) se alteraram durante a infecção. Todos os indivíduos apresentaram respostas sorológicas, mantiveram títulos de anticorpos durante o período de acompanhamento (307-555 dias) e, nos animais convalescentes, anticorpos foram detectados prévio a cada desafio ulterior. Em aquelas capivaras, não foram registrados nem sinais clínicos nem rickettsemia depois de cada um dos desafios. As amostras de carrapatos coletadas durante a primoinfecção, de todas as capivaras, amplificaram DNA de *Rickettsia* com taxas de infecção de 4,6-30% para ninfas e 5,0-100.0% para adultos. Adicionalmente, depois das infestações com estes carrapatos, os coelhos apresentaram sinais clínicos e reação sorológica. Pelo contrário, carrapatos coletados durante os desafios posteriores não amplificaram DNA de *Rickettsia* e os coelhos infestados com estes não exibiram sinais clínicos nem resposta de anticorpos. Salienta-se que um lote de carrapatos coletados da capivara 5, durante a segunda infeção, que se alimentaram junto com os adultos infectados, amplificaram DNA de *Rickettsia*, sugerindo uma provável transmissão horizontal não sistêmica. Em conclusão, no presente estudo foi corroborada a susceptibilidade das capivaras à infecção com *R. rickettsii* (cepa Itu) e a apresentação de variáveis de infecção similares quando comparada com a cepa Taiaçu. Não obstante, novas características clínicas e de infecção de carrapatos foram registradas em animais primo-infectados e imunes.

Palavras-chave: Febre Maculosa Brasileira. Febre Maculosa das Montanhas Rochosas. Carrapatos. Ixodidae. Rickettsiaceae.

ABSTRACT

RAMÍREZ HERNÁNDEZ, Alejandro. **Experimental infection of Capybaras (*Hydrochoerus hydrochaeris*) with an *Amblyomma sculptum*-derived strain of *Rickettsia rickettsii*.** 2019. 74 f. Thesis (Doctor in Sciences) – School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, 2019.

Brazilian Spotted Fever (BSF) is recognized as the most lethal tick-borne disease in Brazil and other countries in the western hemisphere. *Rickettsia rickettsii* is its etiological agent, and in its natural history, ticks and mammals perform an essential epidemiologic role. In southeastern Brazil, *Amblyomma sculptum* is the main incriminated vector and capybaras have been recognized as amplifier hosts. Previous studies have comproved that capybaras are susceptible to infection with *R. rickettsii* and that develop a rickettsemia of sufficient length to infect naïve *A. sculptum* ticks. These studies used for infection a strain of *R. rickettsii* isolated from *Amblyomma aureolatum* ticks (strain Taiaçu) without performing subsequent infection challenges in immune animals. Herein, we present the results of an experimental study infecting capybaras with a *R. rickettsii*-strain isolated from *A. sculptum* (strain Itu) identifying clinical, haematological and pathological features, rickettsemic period and subsequent transmission of *R. rickettsii* to susceptible *A. sculptum* tick populations with a sequential analysis of their vectorial competence; also, we performed subsequent infections to evaluate the mentioned variables in immune or convalescent animals. Five capybaras, from two non-endemic regions in São Paulo state, were infected with *R. rickettsii* (strain Itu) through tick infestations with infected *A. sculptum* adults. Rectal temperature and clinical signs were registered during a 30-day following period and skin and blood samples collected, each two days, for guinea pig inoculation, DNA extraction, haematology and immunofluorescence antibody test (IFA). Also, capybaras were infested with non-infected *A. sculptum* ticks (nymphs and adults) in two feeding chambers (one to feed with infected adults and another to feed separated from infected adults), which were further collected, incubated and posteriorly used for infestation of susceptible rabbits and DNA extraction. These procedures were repeated during subsequent capybara infections. During primoinfection, four out of five capybaras presented clinical signs and two died, showing vascular gross lesions at necropsy. Based on guinea pig inoculation, rickettsemia was present in all capybaras with a mean duration of 9.2 days (range: 6-12 days). *Rickettsia* DNA was amplified in blood and

skin samples from capybaras and some hematologic variables (PCV, and leucocyte count) were altered during infection. All individuals presented serological responses and maintain antibody titres during the following period (307-555 days) and, in convalescent capybaras, antibodies were detected before each subsequent infection. In those animals, no clinical signs nor rickettsemia were detected after each infection challenge. Samples of ticks collected during primoinfection of all capybaras amplified *Rickettsia* DNA with infectious rates of 4.6-30.0% and 5.0-100.0% in molted nymphs and adults, respectively. Also, after infestations with these ticks, rabbits presented clinical signs and serologic reactivity. By contrast, ticks collected during subsequent capybara infections did not amplify *Rickettsia* DNA and rabbits infested with them did not exhibited clinical signs nor antibody response. Notably, a batch of ticks collected from capybara 5, during the second infection, that fed adjacent with infected adults, amplified *Rickettsia* DNA, suggesting a probable *R*. *rickettsii* non-systemic horizontal transmission. In conclusion, in the present study it was corroborated capybara susceptibility to infection with *R*. *rickettsii* (strain Itu) and similar infection variables when comparing with strain Taiaçu. However, new clinical and tick transmission patterns were registered in first-infected and immune animals.

Keywords: Brazilian Spotted Fever. Rocky Mountain Spotted Fever. Ticks. Ixodidae. Rickettsiaceae.

SUMMARY

1 INTRODUCTION

The disease produced by the infection with *Rickettsia rickettsii*, known as Brazilian Spotted Fever (BSF), is considered the most lethal tick-borne disease in the country with high case-fatality rates (>50%) in infected patients (DE OLIVEIRA et al., 2016). A condition related, as in other countries, by the delayed case suspicion and treatment, the use of less effective antibiotics and also by the infection with high virulent strains which genetically differ between geographical regions (WALKER, 2002; EREMEEVA et al., 2003; ANGERAMI et al., 2006).

In Brazil, in spite of the recognition of the disease since 1929 (PIZA et al., 1932) it was included for compulsory notification until 2001 and obligatory notification since 2014 (DE OLIVEIRA et al., 2016). Most of cases are registered in the southeastern region, where it is considered an endemic disease. Particular ecological and epidemiological factors in this region could favour its maintenance, as the broad distribution and high environmental densities of the main recognized vector (*Amblyomma sculptum*) and high population densities of one of its primary hosts, the capybara (*Hydrochoerus hydrochaeris*) (SZABO et al., 2013). Historically, among the states from this region, São Paulo state (SP) registered the highest numbers of suspected, confirmed cases and related deaths (DEL FIOL et al., 2010; DE OLIVEIRA et al., 2016).

Ticks functions as the main reservoir of *R. rickettsii* in nature by means of transstadial maintenance and transovarian transmission mechanisms (RICKETTS, 1907). Nonetheless, due to bacterial pathogenic effects on ticks (BURGDORFER; BRINTON, 1975; NIEBYLSKI et al., 1999) and some degree of tick refractoriness to bacterial infection (SOARES et al., 2012), low natural infectious rates are commonly found. Therefore, amplifier hosts (vertebrate animals that develops rickettsemia for some days) are essential in maintaining the pathogen by its transmission to non-infected ticks feeding on them and creating new lineages of infected individuals within the population (BURGDORFER, 1988; LABRUNA, 2009).

Labruna et al. (2009) proposed five main requirements that a vertebrate species must satisfied in order to be an efficient amplifier host for *R*. *rickettsii* in a given endemic area for this bacterium. Firstly, it has to be abundant in the mentioned area; secondly, it has to be a major host for the tick vector; thirdly, it must be susceptible to the infection with *R*. *rickettsii*; fourthly, once infected with *R*. *rickettsii*, it must develop a rickettsemia of sufficient length and intensity in order to infect ticks feeding on this host; and, fifthly, it has to be a prolific species to introduce new nonimmune individuals to the population. Capybaras, in endemic BSF regions in Souheastern Brazil, fulfil the mentioned criteria, confirming its crucial role in the natural history of the disease (LABRUNA, 2013).

Experimental *R*. *rickettsii* infections have been performed in order to study susceptibility and rickettsemia in diverse animal species like birds (LUNDGREN et al., 1966), dogs (KEENAN et al., 1977; NORMENT; BURGDORFER, 1984; PIRANDA et al., 2008; LEVIN et al., 2014), lagomorphs (LUNDGREN et al., 1968; BURGDORFER et al., 1980), nonhuman primates (MOE et al., 1976; SAMMONS et al., 1976), opossums (BOZEMAN et al., 1967; HORTA et al., 2009), small mammals (BURGDORFER et al., 1966; LUNDGREN; THORPE, 1966; BOZEMAN et al., 1967), and capybaras (TRAVASSOS; VALLEJO, 1942a; SOUZA et al., 2009). In the latter, it was confirmed a rickettsemia of adequate extent and magnitude in order to infect susceptible tick populations, indicating the role of this wild rodent as amplifier host (TRAVASSOS; VALLEJO, 1942a; b; SOUZA et al., 2009).

In the mentioned previous experimental studies with capybaras, in spite of the success in developing and identifying rickettsemia and corroborating tick infection, it was registered low nymphal infectious rates, suggesting a probable refractoriness to infection in tick populations; which could be related with the use of a *R*. *rickettsii* strain derived of other tick species (strain Taiaçu from *Amblyomma aureolatum*) (SOUZA et al., 2009). Furthermore, the referred works did not evaluate if could be more than one rickettsemic period in capybaras, after subsequent bacterial challenges, which could be crucial in the BSF epidemiology. Besides, previous research has identified that as a result of an acquired immune response after primoinfection in the vertebrate host, new rickettsemic periods are absent, not contributing to the infection of new susceptible ticks (PHILIP, 1959; LUNDGREN; THORPE, 1966).

In spite of capybaras being essential in the natural history of BSF, questions remained unresolved due to its wide distribution in endemic and non-endemic localities for BSF in São Paulo state. Which factors associated with this host, the vector and/or the pathogen, could favour the circulation of *R. rickettsii* in specific area?

Herein, we present the results of an experimental study infecting capybaras with a *R. rickettsii*-strain isolated from *A. sculptum* (strain Itu) (KRAWCZAK et al., 2014) identifying clinical, haematological and pathological features, rickettsemic period and subsequent transmission of *R. rickettsii* to susceptible *A. sculptum* tick populations with a sequential analysis of their vectorial competence. Data were collected after multiple infection challenges in an extended period of evaluation. Our aim is to compare these results with those obtained in previous studies with strain Taiaçu and complement current knowledge in dynamic of *R. rickettsii* infection in this amplifier host. Results are presented in two separated papers proposed for further submission and publication

1.1 REFERENCES

ANGERAMI, R. N.; RESENDE, M. R.; FELTRIN, A. F. C.; KATZ, G.; NASCIMENTO, E. M.; STUCCHI, R. S. B.; SILVA, L. J. Brazilian Spotted Fever: a case series from an endemic area in southeastern Brazil: epidemiological aspects. **Annals of the New York Academy of Sciences**, v. 1078, n. 1, p. 170-172, 2006. DOI: 10.1196/annals.1374.030

BOZEMAN, F. M.; SHIRAL, A.; HUMPHRIES, J. W.; FULLER, H. S. Ecology of Rocky Mountain spotted fever. II. Natural infection of wild mammals and birds in Virginia and Maryland. **American Journal of Tropical Medicine and Hygiene**, v. 16, n. 1, p. 48-59, Jan. 1967. ISSN 0002-9637 (Print) http://www.ajtmh.org/docserver/fulltext/14761645/16/1/TM0160010048.pdf?expires=1549657110&id=id&accname=12715&checksum=C9DBF9343634942D476827591D8C7D0C. Viewed: Feb. 08 2019.

BURGDORFER, W. Ecological and epidemiological consideration Rocky Mountain spotted fever and scrub typhus. In: WALKER, D. (Ed.). **Biology of rickettsial diseases**. Florida: CRC, Boca Raton, 1988. v. 1, p.33-50.

BURGDORFER, W.; BRINTON, L. P. Mechanisms of transovarial infection of spotted fever Rickettsiae in ticks. **Annals of the New York Academy of Sciences**, v. 266, p. 61-72, 1975. ISSN 0077-8923 (Print) https://nyaspubs.onlinelibrary.wiley.com/doi/abs/10.1111/j.1749-6632.1975.tb35088.x Viewed Feb. 08 2019

BURGDORFER, W.; COONEY, J. C.; MAVROS, A. J.; JELLISON, W. L.; MASER, C. The role of cottontail rabbits (*Sylvilagus* spp.) in the ecology of *Rickettsia rickettsii* in the United States. **American Journal of Tropical Medicine and Hygiene**, v. 29, n. 4, p. 686-90, Jul 1980. ISSN 0002-9637 (Print). https://www.ajtmh.org/content/journals/10.4269/ajtmh.1980.29.686. Viewed: Feb. 08 2019.

BURGDORFER, W.; FRIEDHOFF, K. T.; LANCASTER, J. L., JR. Natural history of tick-borne spotted fever in the USA. Susceptibility of small mammals to virulent *Rickettsia rickettsii*. **Bulletin of the World Health Organization**, v. 35, n. 2, p. 149-53, 1966. ISSN 0042-9686 (Print). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2476118/pdf/bullwho00605-0043.pdf Viewed: Feb. 08 2019.

DE OLIVEIRA, S. V.; GUIMARAES, J. N.; RECKZIEGEL, G. C.; NEVES, B. M.; ARAUJO-VILGES, K. M.; FONSECA, L. X.; PINNA, F. V.; PEREIRA, S. V.; DE CALDAS, E. P.; GAZETA, G. S.; GURGEL-GONCALVES, R. An update on the epidemiological situation of spotted fever in Brazil. **The journal of venomous animals and toxins including tropical diseases**, v. 22, n. 1, p. 22, 2016. ISSN 1678-9180 (Linking). DOI: 10.1186/s40409-016-0077-4

DEL FIOL, F.; JUNQUEIRA, F. M.; ROCHA, M.; TOLEDO, M.; BARBERATO FILHO, S. A febre maculosa no Brasil. **Revista panamericana de salud pública**, v. 27, p.

461-6, 2010. http://www.scielosp.org/scielo.php?script=sci_arttext&pid=S1020-49892010000600008. Viewed: Feb. 08 2019.

EREMEEVA, M. E.; KLEMT, R. M.; SANTUCCI-DOMOTOR, L. A.; SILVERMAN, D. J.; DASCH, G. A. Genetic analysis of isolates of *Rickettsia rickettsii* that differ in virulence. **Annals of the New York Academy of Sciences**, v. 990, p. 717-22, Jun 2003. ISSN 0077-8923 (Print). https://nyaspubs.onlinelibrary.wiley.com/doi/full/10.1111/j.1749-6632.2003.tb07449.x Viewed: Feb. 08 2019.

HORTA, M. C.; MORAES-FILHO, J.; CASAGRANDE, R. A.; SAITO, T. B.; ROSA, S. C.; OGRZEWALSKA, M.; MATUSHIMA, E. R.; LABRUNA, M. B. Experimental infection of opossums *Didelphis aurita* by *Rickettsia rickettsii* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense*. **Vector Borne and Zoonotic Diseases**, v. 9, n. 1, p. 109-18, Feb 2009. ISSN 1557-7759. DOI:10.1089/vbz.2008.0114

KEENAN, K. P.; BUHLES, W. C., JR.; HUXSOLL, D. L.; WILLIAMS, R. G.; HILDEBRANDT, P. K. Studies on the pathogenesis of *Rickettsia rickettsii* in the dog: clinical and clinicopathologic changes of experimental infection. **American Journal of Veterinary Research**, v. 38, n. 6, p. 851-6, Jun 1977. ISSN 0002-9645 (Print)

KRAWCZAK, F. S.; NIERI-BASTOS, F. A.; NUNES, F. P.; SOARES, J. F.; MORAES-FILHO, J.; LABRUNA, M. B. Rickettsial infection in *Amblyomma cajennense* ticks and capybaras (*Hydrochoerus hydrochaeris*) in a Brazilian spotted fever-endemic area. **Parasites and Vectors**, v. 7, p. 7, 2014. ISSN 1756-3305 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3892071/pdf/1756-3305-7-7.pdf > Viewed: Feb. 08 2019.

LABRUNA, M. B. Ecology of *Rickettsia* in South America. **Annals of the New York Academy of Sciences**, v. 1166, n. 1, p. 156-166, 2009. ISSN 00778923. DOI:10.1111/j.1749-6632.2009.04516.x

LABRUNA, M. B. Brazilian spotted fever: the role of capybaras. In: MOREIRA, J.; FERRAZ, K.; HERRERA, E.; MACDONALD, D. (Ed.). **Capybara.** New York: Springer, 2013. p.371-383.

LEVIN, M. L.; KILLMASTER, L. F.; ZEMTSOVA, G. E.; RITTER, J. M.; LANGHAM, G. Clinical presentation, convalescence, and relapse of Rocky Mountain spotted fever in dogs experimentally infected via tick bite. **Plos One**, v. 9, n. 12, p. e115105, Dec. 26 2014. http://journals.plos.org/plosone/article/asset?id=10.1371/journal.pone.0115105.PDF. Viewed: Feb. 08 2019.

LUNDGREN, D. L.; NICHOLES, P. S.; THORPE, B. D. Experimental infection of lagomorphs with *Rickettsia rickettsii*. **American Journal of Tropical Medicine and Hygiene**, v. 17, n. 2, p. 213-8, Mar 1968. ISSN 0002-9637 (Print). https://www.ajtmh.org/content/journals/10.4269/ajtmh.1968.17.213. Viewed: Feb. 08 2019.

LUNDGREN, D. L.; THORPE, B. D. Infectious diseases in wild animals in Utah. VII. Experimental infection of rodents with *Rickettsia rickettsii*. **American Journal of Tropical Medicine and Hygiene**, v. 15, n. 5, p. 799-806, Sep 1966. ISSN 0002-9637 (Print).http://www.ajtmh.org/docserver/fulltext/14761645/15/5/TM0150050799.pdf?expires=1549659486&id=id&accname=12715&checksum=B4ABE33004F799C6473F1A0E1C084907. Viewed: Feb. 08 2019.

LUNDGREN, D. L.; THORPE, B. D.; HASKELL, C. D. Infectious diseases in wild animals in Utah. VI. Experimental infection of birds with *Rickettsia rickettsii*. **Journal of Bacteriology**, v. 91, n. 3, p. 963-6, Mar 1966. ISSN 0021-9193 (Print).

MOE, J. B.; RUCH, G. L.; KENYON, R. H.; BUREK, J. D.; STOOKEY, J. L. Pathology of experimental Rocky Mountain spotted fever in rhesus monkeys. **Veterinary Pathology**, v. 13, n. 1, p. 69-77, 1976. DOI:10.1177/030098587601300108

NIEBYLSKI, M.; PEACOCK, M.; SCHWAN, T. Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). **Applied and Environmental Microbiology**, v. 65, n. 2, p. 773-778, February,1999. http://aem.asm.org/content/65/2/773.full.pdf. Viewed: Feb. 08 2019

NORMENT, B. R.; BURGDORFER, W. Susceptibility and reservoir potential of the dog to spotted fever-group rickettsiae. **American Journal of Veterinary Research**, v. 45, n. 9, p. 1706-10, Sep 1984. ISSN 0002-9645 (Print). https://europepmc.org/abstract/med/6548617 Viewed: Feb. 08 2019.

PHILIP, C. B. Some epidemiological considerations in Rocky Mountain spotted fever. **Public Health Reports**, v. 74, n. 7, p. 595-600, Jul 1959. ISSN 0094-6214 (Print). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1929280/pdf/pubhealthreporig00127-0033.pdf. Viewed: Feb. 08 2019.

PIRANDA, E. M.; FACCINI, J. L.; PINTER, A.; SAITO, T. B.; PACHECO, R. C.; HAGIWARA, M. K.; LABRUNA, M. B. Experimental infection of dogs with a Brazilian strain of *Rickettsia rickettsii*: clinical and laboratory findings. **Memórias do Instituto Oswaldo Cruz**, v. 103, n. 7, p. 696-701, Nov 2008. ISSN 1678-8060 (Electronic). http://www.scielo.br/scielo.php?pid=S0074-02762008000700012&script=sci_arttext&tlng=es. Viewed: Feb. 08 2019.

PIZA, J.; MEYER, G.; GOMES, L. **Typho exanthematico de São Paulo**. São Paulo: Sociedade Impressora Paulista, 1932.

RICKETTS, H. T. Further experiments with the wood tick in relation to Rocky Mountain spotted fever. **Journal of the American Medical Association**, v. 49, n. 15, p. 1278-1281, 1907.

SAMMONS, L. S.; KENYON, R. H.; BURGER, G. T.; PEDERSEN, C. E., JR.; SPERTZEL, R. O. Changes in blood serum constituents and hematologic values in *Macaca mulatta* with Rocky Mountain spotted fever. **American Journal of Veterinary Research**, v. 37, n. 6, p. 725-30, Jun 1976. ISSN 0002-9645 (Print) https://europepmc.org/abstract/med/820224. Viewed: Feb. 08 2019.

SOARES, J. F.; SOARES, H. S.; BARBIERI, A. M.; LABRUNA, M. B. Experimental infection of the tick *Amblyomma cajennense*, Cayenne tick, with *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever. **Medical and Veterinary Entomology**, v. 26, n. 2, p. 139-51, Jun 2012. ISSN 1365-2915 (Electronic)
DOI: 10.1111/j.1365-2915.2011.00982

SOUZA, C. E.; MORAES-FILHO, J.; OGRZEWALSKA, M.; UCHOA, F. C.; HORTA, M. C.; SOUZA, S. S.; BORBA, R. C.; LABRUNA, M. B. Experimental infection of capybaras *Hydrochoerus hydrochaeris* by *Rickettsia rickettsii* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense*. **Veterinary Parasitology**, v. 161, n. 1-2, p. 116-21, Apr 6 2009. ISSN 0304-4017 (Print). https://www.sciencedirect.com/science/article/pii/S0304401708007085. Viewed: Feb. 08 2019.

SZABO, M. P.; PINTER, A.; LABRUNA, M. B. Ecology, biology and distribution of spotted-fever tick vectors in Brazil. **Frontiers in cellular and infection microbiology**, v. 3, p. 27, 2013. ISSN 2235-2988 (Electronic)
2235-2988 (Linking). DOI: 10.3389/fcimb.2013.00027

TRAVASSOS, J.; VALLEJO, A. Comportamento de alguns cavídeos (*Cavia aperea* e *Hydrochoerus capybara*) às inoculações experimentais do vírus da febre maculosa. Possibilidade desses cavídeos representarem o papel de depositários transitórios do vírus na natureza. **Memórias do Instituto Butantan**, v. 15, p. 73-86, 1942a. https://bibliotecadigital.butantan.gov.br/arquivos/87/PDF/9.pdf Viewed: Feb. 08 2019.

_____. Possibilidade de *Amblyomma cajennense* se infectar em *Hydrochoerus capybara* experimentalmente inoculado com o vírus da febre maculosa. **Memórias do Instituto Butantan**, v. 15, p. 87-90, 1942b.
https://bibliotecadigital.butantan.gov.br/arquivos/87/PDF/10.pdf

WALKER, D. *Rickettsia rickettsii*: As Virulent as Ever. **American Journal of Tropical Medicine Hygiene**, v. 66, n. 5, p. 448-449, 2002.
http://www.ajtmh.org/docserver/fulltext/14761645/66/5/12201574.pdf?expires=1549660628&id=id&accname=guest&checksum=F63F252FE6EA07C228315CA21BD052E2. Viewed Feb. 08 2019

2 OBJECTIVES

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To perform an experimental infection in capybaras with an *A*. *sculptum*-derived strain of *R*. *rickettsii* and to register clinical, infection and serological variables during the first and subsequent infections.

To perform an experimental transmission of *R*. *rickettsii* (strain Itu) to non-infected larvae and nymphs of *A*. *sculptum*, after feeding on experimental infected and subsequently challenged capybaras, and to assess the capacity of these ticks to transmit the bacterium to susceptible rabbits.

3 EXPERIMENTAL INFECTION OF CAPYBARAS (*Hydrochoerus hydrochaeris*) WITH AN *Amblyomma sculptum*-DERIVED STRAIN OF *Rickettsia rickettsii*

3.1 INTRODUCTION

Brazilian Spotted Fever (BSF) is the most lethal tick-borne disease in Brazil with increasing numbers of cases and consequent deaths. Between 2007 and 2015, 17,117 suspected cases of spotted fever (including other Spotted Fever Group *Rickettsia* species) were reported and 1,245 were confirmed in twelve Brazilian states from all regions (de Oliveira et al., 2016). Moreover, the case-fatality rates can attain values of 30% (or higher) which could be associated with low index of suspicion and misdiagnosis by health-care professionals and exposure to particular eco-epidemiological risk factors (de Oliveira et al., 2016).

Rickettsia rickettsii is the etiological agent of BSF (as known in Brazil) and of Rocky Mountain Spotted Fever, a disease registered in different American countries including United States, Canada, Mexico, Costa Rica, Panama, Colombia (known as “Tobia Fever”) and Argentina (Parola et al., 2013). This bacterium is transmitted by different tick species through the continent (*i.e*. *Dermacentor variabilis*, *Dermacentor andersoni*, *Rhipicephalus sanguineus* s.l., *Amblyomma sculptum* and *Amblyomma aureolatum*) (Parola et al., 2013). In Brazil, in the southeastern region, *A. sculptum* is the main incriminated vector, for which horses and capybaras (*Hydrochoerus hydrochaeris*) act as primary host for all parasitic stages (Labruna, 2009; Szabo et al., 2013).

Ticks function as the main reservoir of *R. rickettsii* in nature, nonetheless, due to bacterial pathogenic effects on ticks (Burgdorfer and Brinton, 1975; Niebylski et al., 1999) and some degree of tick refractoriness to bacterial infection (Soares et al., 2012), low natural infectious rates are commonly found. Therefore, amplifier hosts (vertebrate animals that develops rickettsemia for some days) are essential in maintaining the pathogen by its transmission to non-infected ticks feeding on them and creating new lineages of infected individuals within the population (Burgdorfer, 1988; Labruna, 2009). In Brazil, opossums (*Didelphis aurita*) (Horta et al., 2005) and capybaras (Travassos and Vallejo, 1942; Souza et al., 2009) have been shown to be competent

amplifier hosts of *R*. *rickettsii* for *A*. *sculptum* ticks, in contrast to horses, which could maintain high tick populations but are not competent in amplifying infection to naïve ticks (Ueno et al., 2016). Souza et al. (2009) induced infection on capybaras by the inoculation or tick exposure to strain Taiaçu of *R*. *rickettsii*, isolated from *A*. *aureolatum* ticks from Taiaçupeba (São Paulo state) (Pinter and Labruna, 2006); besides, this study followed the serological response of capybaras during 146 days post infection, without new infection challenges, which bring some interrogations about infection and serologic dynamics in a longer period of time with more than one exposure event and with a bacterium strain derived from *A*. *sculptum*, resembling natural conditions. Based on these, we present the results of an experimental infection of capybaras with an *A*. *sculptum*-derived strain of *R*. *rickettsii* including multiple challenges and the respective clinical, infection and serological monitoring.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Five capybaras were obtained from two different non-endemic areas in São Paulo state. Three animals (females) from São Paulo city (University of São Paulo -USP- campus), 2 weeks old and from the same litter; and 2 animals (one male and one female, 4 weeks old) from Pirassununga (USP campus), from two different groups. Animals were transported to the Animal Research Facility of Superintendência de Controle de Endemias (SUCEN) in Mogi Guaçu, São Paulo state. They were kept in individual boxes (3 m x 3 m) and fed daily with fresh forage, commercial guinea-pig pellets and water *ad libitum*. Occasionally, they received sugar cane and fresh corn as positive reinforcements during management. They had permanent access to a swimming pool, with daily cleaning and water replacement, for environmental enrichment. All captured animals were previously infested by *Amblyomma dubitatum* ticks under natural conditions, before starting the present study.

Laboratory guinea pigs (Hartley strain) and white New Zealand rabbits were purchased from a commercial breeder, housed in standardized laboratory cages and fed with a commercial guinea pig and rabbit pellet diet, water *ad libitum* in a room with temperature, photoperiod (12 h/12 h) and ventilation control. All guinea pigs and rabbits were tick-naïve and breed under laboratory standard sanitary conditions.

All animal procedures were authorized by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) (CEUA No. 4115110215) and procedures involving capybaras, were authorized by the Brazilian biodiversity agency SISBIO ("Sistema de Autorização e Informação em Biodiversidade"-ICMBio) (No. 43259-3).

3.2.2 Capybara infection

The study was divided into four phases (I to IV) as follows: phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5 (Figure 1).

Before primoinfection, all capybaras were clinically healthy and negative in both paired serum samples (14-day interval), tested by indirect immunofluorescence assay IFA (see below) with the antigens *R. rickettsii*, *Rickettsia parkeri*, *Rickettsia rhipicephali*, *Rickettsia bellii*, *Rickettsia typhi* and *Rickettsia felis*, as previously described (Pacheco et al., 2007; Souza et al., 2009).

For primo and subsequent infections of capybaras, two separated cotton sleeves (10-cm diameter feeding chambers) were glued on the capybara shaved dorsum, as previously described (Labruna et al., 2008; Souza et al., 2009). These chambers were labelled as chamber A (cranial position on the capybara dorsum) and chamber B (caudal position on the capybara dorsum).The minimum distance between the two chambers was 5 cm. Chamber A received 20 males and 20 females (10 males and 10 females for primoinfection of capybara 1) of *A. sculptum* derived from a colony, in the fifth generation (F5), established in the laboratory from a population adapted to capybaras, collected in Itu municipality (São Paulo) in 2012 (Krawczak et al., 2014). These adult ticks were exposed to rickettsial infection, as larvae and nymphs, through feeding on rickettsemic guinea pigs intraperitoneally inoculated with a *R. rickettsii* strain (Itu strain) that was isolated from *A. sculptum* ticks collected in the same area of origin of the progenitor ticks that generated our tick colony (Krawczak et al., 2014).

Day 0 (zero) of infection was considered as the day of infestation with infected ticks, namely, when infected unfed adults were put in the feeding chamber A. After that, and during 30 days post infection (DPI), animals were monitored daily for clinical signs and rectal temperature was registered. Also, blood samples were collected from the femoral vein for the following procedures: with anticoagulant (EDTA) for guinea pig inoculation (every 2 days), DNA extraction (every 2 days) and haematology (every 4 days); and without anticoagulant to obtain sera for IFA test (every 6 days). In addition, a skin biopsy from the abdominal region was performed with a 5 mm-punch, every 2 days. These procedures were repeated during all study phases, and in the control animal (no. 3 in phase I) the only sample collected was blood for haematology analyses. Prior to each procedure (sample collections and tick feeding chamber preparation) animals were slightly sedated with a mixture of xylazine (0.1 mg/kg) and ketamine (1 mg/kg) by the intramuscular route (IM).

In each infection phase (I to IV) a tick-naïve white New Zealand rabbit were used as control of infected ticks. At day zero of capybara infection, these animals received the same number and batch of infected ticks (adults) as the capybaras included in the respective phase. Formerly, a cotton sleeve (8-cm diameter feeding chamber) was glued on the shaved back of the rabbit as described earlier (Pinter et al., 2002). Clinical signs and rectal temperature were registered daily, between day 0 and 21 DPI, and in case of death, necropsy and organ samples (spleen) were collected and frozen. Also, serum samples were collected in a 21-day interval for IFA (described below).

3.2.3 Guinea pig inoculation

Anticoagulated blood from capybaras (1.0 ml) was inoculated into two guinea pigs, for each blood sampling, in order to identify a probable rickettsemia in the capybara, as previously described (Souza et al., 2009). For this purpose, animals were previously anaesthetised with a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg) (IM) (Gaertner et al., 2008) and a blood sample collected, by intracardiac puncture, for IFA analyses (day 0). All animals were monitored daily for clinical signs detection and rectal temperature measurement during 21 days; then, a second blood sample (day 21) was collected, as described above, and thereafter guinea pigs were euthanized with sodium pentobarbital (100 mg/kg) by intracardiac injection. A febrile guinea pig was considered

when a rectal temperature ≥ 40 ºC was registered during two or more consecutive days. Dead individuals were submitted to necropsy and samples of spleen frozen at -20 ºC.

3.2.4 Haematology tests

Non-coagulated blood samples from capybaras (0.5 ml) were used for estimation of packed cell volume (PCV), erythrocytes and leukocytes total cell count. PCV was estimated by the microhematocrit technique and red and white cells were counted in an improved Neubauer chamber with blood diluted on Gowers and Türk solutions, respectively. Subsequent procedures followed those described by Madella et al. (2006).

3.2.5 Indirect immunofluorescence assay (IFA)

Sera obtained from capybaras and guinea pigs were tested by IFA using *R. rickettsii* (strain Taiaçu) crude antigen and fluorescein isothiocyanate-labelled anti-capybara IgG (CCZ, São Paulo-SP, Brazil) or rabbit anti-guinea pig IgG (Sigma, St. Louis, USA) as previously described (Pacheco et al., 2007; Soares et al., 2012). Samples were initially tested in a 1:64 dilution (with PBS) as cut-off and those positive, further diluted in a twofold increase until reach the endpoint titre as reported earlier (Labruna et al., 2007). In each slide, previously known reactive and non-reactive serum, for both animal species, were included as positive and negative controls, respectively.

3.2.6 DNA extraction from blood and skin samples

DNA was extracted from frozen (-20 ºC) blood and skin samples using the DNeasy Blood and Tissue kit (QIAGEN Inc., Valencia, CA, USA) and following the manufacturer's protocols for purification of total DNA from animal blood and tissues. Final products were stored at -20 ºC for further amplification by polymerase chain reaction (PCR).

3.2.7 Real-time PCR from blood and skin samples

DNA obtained from blood and skin biopsies was submitted to a TaqMan real-time PCR technique to amplify a 147-bp fragment of the citrate synthase gene (*gltA*) of *Rickettsia* spp. using the primers CS-5 (forward; Guedes et. al., 2005) and CS-6 (reverse;

Labruna et. al., 2004) and an internal fluorogenic probe (6-FAM d, BHQ- 1) (Integrated DNA Technologies, San Diego, CA), in accordance with reagents and cycling conditions reported by Labruna et al. (2004). The sensitivity of the technique was determined to be 1 DNA copy of *R. rickettsii* (Labruna et al., 2004). For each reaction, a positive (DNA of *Rickettsia vini* cultivated in Vero cells) and a negative control (molecular-grade water) were included.

3.3 RESULTS

3.3.1 Capybara infection

Five capybaras were infected during four study phases. Capybara 1 was the sole animal that was re-infected three times after primoinfection (four infection challenges). Capybaras 4 and 5 had one additional infection (two infection challenges). Unexpectedly, capybaras 2 and 3 died during primoinfection, impeding further exposure.

As presented in Table 1, during primoinfection, capybaras 2, 3, 4 and 5 manifested rectal temperatures higher than 38.5 ºC with onset on 8 or 9 DPI. Outstandingly, rectal temperature reached highest values of 39.7 and 39.8 ºC in capybaras 2 and 3, respectively, which died during the rickettsemic period (see below). Also, in both individuals, rectal temperatures around 32 ºC (hypothermia) were recorded few hours prior to death. Additionally, capybaras 2 to 5 presented common clinical signs during primoinfection, such as lack of appetite, general and hindlimb weakness (supplementary video), and nasal mucous discharge. Moreover, in capybaras 2 and 3, a more severe clinical course was evident with signs like inappetence, diarrhoea, dark urine, prostration, coma, seizures and death. It is noteworthy that both individuals also presented skin manifestations such as abdominal and thoracic rash and focal purplish macules with onset at 8 DPI (Table 1 and Figure 2 A-D). The first clinical signs appeared in capybaras no. 2, 3, 5 at DPI 8 and in capybara no. 4 at DPI 9 (mean incubation period: 8,3 days).

In contrast, clinical alterations, including rectal temperature >38.5 ºC, were absent in capybara 1 during primoinfection and further challenges (phases I to IV) and in capybaras 4 and 5 during the subsequent exposure (phase IV). Also, in capybara 3, used as control during phase I, no clinical abnormality was observed (Table 1).

All control rabbits, infested with the same batch of infected ticks used for infection and challenge of capybaras, presented fever. Additionally, most of them manifested lack of appetite, auricular, preputial and scrotal vascular abnormalities (i.e. edema, erythema or necrosis), and, in the case of animals in phase I and II its condition get worst and manifested hypothermia and death (Table 1).

After death of capybaras 2 and 3 (during phase II) necropsy was practised and different macroscopic abnormalities registered. Both animals presented vascular disseminated lesions (from hyperaemia to haemorrhage) in different organs like spleen, stomach, small and large intestines, liver, lung, kidneys and adrenal glands (Figure 3 A-B and Figure 4). It was notorious a spleen enlargement in both capybaras (Figure 3 A-D) accompanied by ascites in capybara 3 (Figure 3 A). Different organs were sampled for histopathology (conserved in 10% neutral buffered formalin) and *Rickettsia* immunohistochemistry (conserved in 10% neutral buffered formalin for 24 hours and further in 70% ethanol), and are under analyses.

3.3.2 Guinea pig inoculation

Anticoagulated blood collected every two days from capybaras during infection challenges were inoculated in two guinea pigs simultaneously. During primoinfection (first challenge) of capybaras 1 to 5, fever was detected in guinea pigs inoculated with blood from capybaras at 6 to 16 DPI, being the earliest (6 DPI) in individuals inoculated with blood from capybaras 2 and 3. The proportion of febrile guinea pigs ranged from 9.4 to 61.1% with the highest values (45.0 and 61.1%) in groups inoculated with blood from capybaras 2 and 3. None of guinea pigs showed fever when inoculated with blood from capybaras 1, 4 and 5 during their subsequent challenges (Table 2).

Auricular and genital vascular signs like erythema, edema and necrosis were recorded in guinea pigs inoculated with blood from capybaras 1 to 5 during their respective primoinfection. The proportion of affected animals with these conditions ranged from 3.1 to 61.1% with the higher frequencies ($\geq$ 50.0%) in individuals inoculated with blood from capybaras 2 and 3. The mentioned vascular manifestations were absent in all animals inoculated with blood from capybaras 1, 4 and 5 during subsequent challenges (Table 2).

Guinea pig death was recorded in animals inoculated with blood from capybaras 1 to 5 at 6 to 20 DPI of their primoinfection. The frequency of dead animals ranged from 3.1 to 27.8% with the highest values in groups inoculated with blood from capybaras 2 and 3. No deaths were recorded in animals inoculated with blood from capybaras 1, 4 and 5 when they were subsequently infected (Table 2).

Anti-*R. rickettsii* IgG antibodies (≥ 1/64 dilution) were detected by IFA in guinea pigs inoculated during primoinfection of capybaras 1 to 5. The frequency of seropositive animals ranged from 9.4 to 50.0% with highest values in animals inoculated with blood from capybaras 2 and 3. Besides, guinea pigs inoculated with 6 - 18 DPI-capybara blood seroconverted with endpoints titres from 8,192 to 262,144, with higher titres in animals inoculated with blood of capybaras 2 and 3 and the lowest titres in animals inoculated with blood from capybara 4. Furthermore, none of the guinea pigs inoculated with blood from capybaras 1, 4 and 5, during their subsequent challenges, presented antibodies to *R. rickettsii* (Table 2).

3.3.3 Haematology

Haematological variables evaluated in capybaras during primoinfection and subsequent challenges were packed cell volume (PCV), erythrocyte and leucocyte count and results were compared with those from capybara 3 during phase I (infested with non-infected *A. sculptum* ticks) and reference values (Table 3, Figure 6). In general, mean PCV values during primoinfection of all infected capybaras were lower than reference. Besides, minimum PCV values of 22.2, 24.4, 25.6, 31.4 and 27.1% in capybaras 1 to 5, respectively, between 12 and 22 DPI, were registered (Figure 6). Conversely, mean values were higher during subsequent challenges in capybaras 1, 4 and 5 (except for second infection of capybara 1) and minimum values were within the reference range (Table 3).

Mean erythrocyte count in capybaras were lower than reference during primoinfection of all animals excepting no. 3 (Table 3). The lowest values were registered during 12-28 DPI in all individuals including capybara 3 (Figure 6). During subsequent challenges, mean counts of capybaras 4 and 5 were within reference values but for

capybara 1 they were slightly lower, in spite of be higher than that of primoinfection (Table 3).

Mean leucocyte count number during primoinfection was lower than reference values in capybaras 2 and 3. Besides, comparing mean counts during primoinfection and subsequent challenges in capybaras 1 and 4, lower values were evident during the first infection (Table 3). The minimum values were registered during primoinfection of capybaras 2 and 3 (phase II), capybara 1 (phase I) and capybara 5 (phase III), with 1.13, 1.38, 1.85 and 2.23 $x10^3$ cells/mm^3, respectively, at 12 DPI (Figure 6).

3.3.4 IFA

As presented in Figure 5, capybaras 1, 4 and 5 became first seroreactive to *R. rickettsii* at 16-18 DPI and remained seroreactive until the last day of sampling. In capybara 1, antibody titres were followed from 0 to 555 DPI. The first positive sample (≥ 64 titre) was registered at 16 DPI and peaked with 8,192 at 138 DPI, 18 days after the second challenge (phase II), and remained high (4,096-8,192) till 238 DPI. The lowest titre recorded was 1,024 at 388 DPI, 140 days after the third challenge. Titres at the day of the 2nd, 3rd and 4th challenges were 2,048, 2,048 and 1,024, respectively (Figure 5). For capybara 4, at 18 DPI was detected the first positive sample with a 1,024 titre. Posteriorly, they peaked in 8,192 between 46 and 83 DPI, and thereafter, the lowest detected titre was 2,048 at 189 DPI. Before the 2nd challenge (227 DPI), the titre was 4,096. For capybara 5, the first positive sample was a 2,048 titre at 18 DPI; which posteriorly reached its highest titre at 32,768 between 46 and 54 DPI. Then, titre descended to 4,096 (159 DPI), which remained till and after the second challenge. At 251 DPI, 24 days after the second challenge, capybara 5 titre decreased to 2,048. Lastly, for capybaras 2 and 3, antibodies were detected in the serum sample before death (18 and 16 DPI, respectively) with titres of 512 and 128, correspondingly (data not graphed).

3.3.5 Real-time PCR from blood and skin samples

Real-time PCR in blood samples collected during primoinfection revealed *Rickettsia* DNA in samples from capybara 2 at 14, 16 and 18 DPI, capybara 3 at 12, 14 and 16 DPI, and, capybara 5 at 12 and 14 DPI. No rickettsial DNA was detected in the blood

of capybaras 1 and 4 after primoinfection (Figure 7). In addition, *Rickettsia* DNA was detected in skin samples from all capybaras during primoinfection, as follows: capybara no. 1, DPI 16; no. 2, DPI 8, 12, 14 and 16; no. 3 DPI 10, 12 and 14; no. 4 DPI 6, 10, 12 and 14; no. 5 DPI 12, 16, 20, 22 and 26. Blood samples and skin biopsies from subsequent infections were not tested because guinea pig inoculations were negative for the presence of viable rickettsia in capybara blood at all instances (Figure 7).

3.4 DISCUSSION

Herein, we present the results of an experimental infection in capybaras with a *R. rickettsii* strain (Itu) derived from the same tick population (*A. sculptum*) used in all infestation phases. One of our main objectives, was to emulate a natural condition using infected ticks as the primary and unique way of infection for this vertebrate. Two previous published studies achieved experimental infections in this species. The earliest work published by Travassos et al. (1942), performed subcutaneous inoculations with infected blood from guinea pigs as sole way of exposition. More recently, Souza et al. (2009) executed infections by two procedures: infestations with infected *A. sculptum* nymphs (n=20) and intraperitoneal inoculation of infected organs (liver and brain) derived from guinea pigs. In the present study, we succeeded in develop primoinfection in five capybaras as evidenced by clinical and guinea pig inoculation findings.

Firstly, we confirm, in agreement with preceding studies, that capybaras are susceptible to infection with the referred strain (Itu) of *R. rickettsii*. Secondly, we did not register any clinical abnormality in control animal (capybara 3) infested with non-infected ticks during phase I of the study. While there are no reference values for normal rectal temperature of capybaras in the literature, in the present study we observed that rectal temperature of capybara 3 during infestation with noninfected ticks never exceeded 38.2 ºC. In addition, when capybaras 1, 4 and 5 were already immune (seropositive to *R. rickettsii*) and did not develop rickettsemia during subsequent challenges, their rectal temperature did not exceed 38.0 ºC (Table 1). Hence, for convenience, we adopted in the present study that rectal temperatures >38.5 ºC was considered as fever. Thus, it is noteworthy that a febrile condition was manifested by capybaras 2, 3, 4 and 5 during primoinfection, achieving registers as high as 39.8 ºC,

which differ with previous studies in which normothermic conditions were registered (Travassos and Vallejo, 1942; Souza et al., 2009). Furthermore, clinical pictures evidenced by the same febrile animals with signs like weakness, inappetence, nasal discharge, diarrhoea, nervous disorders and even death (no. 2 and 3), diverge from earlier experimental observations with absence of illness (Travassos and Vallejo, 1942; Souza et al., 2009). One plausible explanation is the infection rate of the *R. rickettsii*-infected colony of *A. sculptum* that was used for primoinfection of the capybaras. We estimated, in random samples of the same batch of ticks used for primoinfection, infection rates of approximately 30% in ticks used in phase 1 (capybara 1), and > 50% in ticks used during phases 2 and 3 (capybaras 2-5) (data not shown). Probably, these different rates reflect, in addition with the number of infected adults used for primoinfection, the contrasting results in the clinical course of capybara 1 versus capybaras 2 to 5. Higher infectious dose rates in the latter could be associated not only with severity of the fever and clinical course, but also with higher proportions of blood-inoculated guinea pigs presenting fever, vascular injuries, serological responses and even death. Moreover, *Rickettsia* DNA was only detected in blood from capybaras 2, 3 and 5. Differences in infectivity, severity of clinical course, and mortality have been previously associated with infectious dose of *Rickettsia* in dogs experimentally inoculated with incremental bacterial doses (Keenan et al., 1977). As well, Piranda et al. (2008) stated differences in severity of illness, onset of fever and rickettsemia between dogs inoculated intraperitoneally and those exposed to *R. rickettsii* via tick bite, being most severe in the latter. Piranda et al. (2008) proposed that tick-exposed dogs might have received lower infectious doses over several consecutive days during adult engorgement. Thus, capybaras infested with tick batches with higher infectious rates could finally receive higher bacterial doses during a prolonged period of tick feeding. Signs manifested in infected capybaras (no. 2 to 5) are comparable with clinical course in other *R. rickettsii* susceptible animal hosts (i.e. guinea pigs, rabbits, dogs and humans). As an example, signs like fever, anorexia, lethargy, weakness, skin rash, diarrhoea, prostration, seizures and death have been related in experimental and natural infections in dogs (Keenan et al., 1977; Piranda et al., 2008; Labruna et al., 2009; Levin et al., 2014), and are similar to those related in human cases of BSF (Angerami et al., 2009; Angerami et al., 2012). Correspondingly, gross pathological lesions related with multisystemic vascular disorders observed during necropsy of capybaras 2 and 3, concur with common findings in susceptible

hosts (Walker et al., 1980; Randall and Walker, 1984; Levin et al., 2014). Additionally, rectal temperatures and clinical signs evidenced in control rabbits infested with the same lot of ticks used for capybara infection, corroborate the infectious status of the latter in each of the study phases.

Herein, infection of guinea pigs inoculated with blood, is used as an indicative of rickettsemia in each one of the infected capybaras. Based on that, the probable rickettsemic period for each capybara was 6 days (8-14 DPI) for capybara 1, 12 days (6-18 DPI) for capybara 2, 10 days (6-16 DPI) for capybara 3, 6 days (8-14 DPI) for capybara 4, and 12 days (8-20 DPI) for capybara 5. In summary, the mean rickettsemic period for all infected animals was 9.2 days (range: 6-12 days). These results are similar to those from Souza et al. (2009), where the group of capybaras infected through tick infestation presented a continuous rickettsemic period between 6 to 15-18 DPI (total period of 9 to 12 days). Also, they are comparable with earlier registers made by Travassos et. al. (1942) where rickettsemia began at 5 DPI and lasted until 11 DPI (6 days) in capybaras exposed by intraperitoneal inoculation. In addition, similar extents (8-12 days) were registered in diverse studies with other rodent species in the United States (Lundgren and Thorpe, 1966; Burgdorfer, 1988) . By contrast, this rickettsemic period is shorter when comparing with that observed in opossums where it lasted 26 days for the species *Didelphis aurita* (Horta et al., 2009) or up to 4 weeks in the species *Didelphis virginiana* (Bozeman et al., 1967). In addition to referenced rickettsemic periods, it is noticeable that the proportion of guinea pigs with fever, vascular clinical signs and positive serum was higher when inoculated with blood from capybaras 2 and 3 in comparison with animals inoculated with blood from capybaras 4 and 5, and even more if compared with capybara 1. Besides, high endpoint titres (> 131,072) were detected in those guinea pigs inoculated with blood from capybaras 2 and 3 (Table 2). Hence, we can correlate these findings with described clinical pictures and mortality and associate them with high bacterial loads, as proposed before.

Detection of *Rickettsia* DNA in blood was accomplished in capybaras 2, 3 and 5 between 12 and 18 DPI. This detection period coincided with the worseness of clinical condition in these animals, which was more noticeable in the two formers prior to death (16 and 18 DPI for capybaras 3 and 2, respectively). Besides, the DNA detection time points are within the range of rickettsemic period, established by guinea pig

inoculation, for each capybara (see above). In the study of Souza et al. (2009), a unique sample from one capybara, intraperitoneally inoculated, yielded *Rickettsia* DNA by real-time PCR at 12 DPI. As stated before, we can infer that differences between both studies, could be related with bacterial doses circulating in those animals. Thus, it must be emphasized that DNA blood detection is not a feasible criterion to establish rickettsemic period. The intracellular nature of this bacterium with very low concentrations in blood, minimum DNA quantities obtained during extraction (200 μl of host blood), use of a very sensitive technique to detect bacterial DNA (e.g. a real-time PCR technique which detects one single copy of *R. rickettsii*) and lack of a biological 'machinery' for replication and infection development (biological model), are enough reasons to review its practical use. Furthermore, it was achieved *Rickettsia* DNA amplification in skin samples from all capybaras during primoinfection (6-26 DPI), which is similar with those results registered by Levin et al. (2016) in skin biopsies from *R. rickettsii*-infected guinea pigs (3-22 DPI). It is remarkable that in some capybaras (no. 1 and 4) DNA was detected only in skin samples, and that in other capybaras (no. 2, 3 and 5) skin samples amplified DNA earlier (no. 2 and 3) or later (no. 5) than blood specimens. Similarly, Levin et al. (2016) detected *Rickettsia* DNA in a higher proportion of ear-skin than in blood samples from *R. rickettsii*-infected guinea pigs and registered earlier or later DNA detections in skin when compared with blood specimens.

Analysing haematological parameters during primoinfection of all capybaras, it was perceptible a reduction in PCV values for all individuals, mainly during 12-22 DPI; also erythrocyte counts for all capybaras, but no. 3, were below reference values during 12-28 DPI. Souza et al. (2009) also registered this pattern in tick-infected capybaras with decrease of PCV, erythrocyte and haemoglobin during 15-21 DPI. Additionally, Keenan et al.(1977), Levin et al.(2014) and Piranda et al. (2008) also registered this haematological variation in experimentally infected dogs during the febrile period. Likewise, leucocyte counts reached the lowest values in capybaras 1, 2, 3 and 5, during primoinfection, at 12 DPI, which correlated with the febrile (for capybaras 2, 3 and 5) and rickettsemic periods (all individuals). While previous studies in capybaras did not include white blood cell count, these results are comparable with that described by Keenan et al. (1977) in experimental dogs. However, deserves attention the fact that it is most common a leukocytosis pattern in infected animals, explained mainly by predominant monocyte and granulocyte responses (monocytosis and granulocytosis)

(Levin et al., 2014). Lastly, further similar studies must attempt to perform thrombocyte count due to its variability in infected hosts (Elghetany and Walker, 1999); herein, it was not included mainly by lack of standardization in manual count and blood smear estimation for this species.

Anti-*R. rickettsii* IgG antibodies, evaluated through IFA, showed an immunological response in all capybaras. First reactive samples were detected between 16 and 18 DPI and then peaked heterogeneously (26-46 DPI) among convalescent individuals (capybaras 1, 4 and 5) and presented a variable dynamic during respective following periods (307-555 days). Maximum titres registered were 8,192 (animals 1 and 4) and 32,768 (no. 5). It is noteworthy, that capybara 1 highest titre was recorded after 2nd challenge (phase II) when death of capybaras 2 and 3 was noted, but a previous endpoint (2,048) was observed at 26 DPI (phase I). A probably strong antigenic stimulus could explain titre raise in capybara 1 after the second infection, a phenomenon not evidenced in capybaras 4 and 5. IFA results agree with those of Souza et al. (2009) in which capybaras infected through tick feeding showed the first reactive response at 12 DPI and then peaked from 21 to 30 DPI, with mean endpoint titres varying from 8,192 to 32,768, which remained till the end of the following period (146 DPI).

One main objective of the present study was to evaluate clinical, haematological, immunological and infectious variables in capybaras, after subsequent challenges with *R. rickettsii* through tick feeding. Data was collected in capybara 1 during three additional challenges at 120, 248 and 475 DPI. Besides, for capybaras 4 and 5, data was obtained for one additional challenge at 227 DPI. As stated before, none of the animals presented fever, clinical signs or haematological abnormalities during subsequent infections. Also, guinea pig inoculated with blood during these phases did not reveal fever, clinical signs or antibody responses. Moreover, through antibody follow-up period, it was noticeable the presence of titres ranging from 1,024 to 4,096, previous to each challenge, corroborating a long-lasting immune response in capybaras, even without recent antigenic stimuli (the maximum interval between challenges was 227 days). Thus, we can infer that primo infected capybaras develop a durable protective immune response that counteract further *R. rickettsii* challenges and impeded its corporal dissemination and establishment. Likewise, Travassos et. al.

(1942) observed that capybaras captured in BSF-endemic areas had a probable natural acquired immunity due to the absence of verifiable infection after challenge with *R. rickettsii*, through inoculation or tick exposure. Besides, Keenan, et al. (1977) conducted an experimental challenge of convalescent dogs with high *R. rickettsii* doses, six and twelve months after primoinfection, and found no clinical nor haematological abnormalities. In a separate publication we will evaluate this immunity condition and its association with transmission of *R. rickettsii* to non-infected *A. sculptum* ticks.

In conclusion, the present study confirms that capybaras are susceptible to the strain Itu of *R. rickettsii*, isolated from *A. sculptum* ticks. In addition, this susceptibility could be dose-dependent due to evidence of fever, illness and mortality in some of the infected animals. Rickettsemic period, hematologic and serologic patterns are similar to those reported previously by experimental capybara infection with a *R. rickettsii* strain derived from *A. aureolatum* ticks (Taiaçu). Finally, it is confirmed that infected capybaras develop a long-lasting immune response which prevent further rickettsemias.

3.5 REFERENCES

Angerami, R.N., Camara, M., Pacola, M.R., Rezende, R.C., Duarte, R.M., Nascimento, E.M., Colombo, S., Santos, F.C., Leite, R.M., Katz, G., Silva, L.J., 2012. Features of Brazilian spotted fever in two different endemic areas in Brazil. Ticks Tick Borne Dis 3, 346-348.

Angerami, R.N., da Silva, A.M., Nascimento, E.M., Colombo, S., Wada, M.Y., dos Santos, F.C., Mancini, D.M., de Oliveira, R.C., Katz, G., Martins, E.C., da Silva, L.J., 2009. Brazilian spotted fever: two faces of a same disease? A comparative study of clinical aspects between an old and a new endemic area in Brazil. Clin Microbiol Infect 15 Suppl 2, 207-208.

Arouca, M., de Miranda, L., Lopes, R., Takahira, R.K., Kohayagawa, A., Ciarlini, P., Oba, E., 2000. Valores hematológicos de capivaras (*Hydrochoerus hydrochaeris*) criadas em cativeiro no município de Botucatu, SP. Ciência Rural, Santa Maria 30, 813-817.

Bozeman, F.M., Shiral, A., Humphries, J.W., Fuller, H.S., 1967. Ecology of Rocky Mountain spotted fever. II. Natural infection of wild mammals and birds in Virginia and Maryland. Am J Trop Med Hyg 16, 48-59.

Burgdorfer, W., 1988. Ecological and epidemiological considerations of Rocky Mountain spotted fever and scrub typhus, In: Walker, D. (Ed.) Biology of Rickettsial Diseases. CRC Inc. Boca Raton, Florida, pp. 33-50.

Burgdorfer, W., Brinton, L.P., 1975. Mechanisms of transovarial infection of spotted fever Rickettsiae in ticks. Ann N Y Acad Sci 266, 61-72.

de Oliveira, S.V., Guimaraes, J.N., Reckziegel, G.C., Neves, B.M., Araujo-Vilges, K.M., Fonseca, L.X., Pinna, F.V., Pereira, S.V., de Caldas, E.P., Gazeta, G.S., Gurgel-Goncalves, R., 2016. An update on the epidemiological situation of spotted fever in Brazil. The journal of venomous animals and toxins including tropical diseases 22, 22.

Elghetany, M.T., Walker, D.H., 1999. Hemostatic changes in Rocky Mountain spotted fever and Mediterranean spotted fever. American journal of clinical pathology 112, 159-168.

Gaertner, D.J., Hallman, T.M., Hankenson, F., Batchelder, M.A., 2008. Anesthesia and Analgesia for Laboratory Rodents, In: Fish, R.E., Brown, M.J., Danneman, P.J., Karas, A.Z. (Eds.) Anaesthesia and Analgesia in Laboratory Animals. Academic Press, USA, pp. 239-297.

Guedes, E., Leite, R.C., Prata, M.C., Pacheco, R.C., Walker, D.H., Labruna, M.B., 2005. Detection of *Rickettsia rickettsii* in the tick *Amblyomma cajennense* in a new Brazilian spotted fever-endemic area in the state of Minas Gerais. Mem Inst Oswaldo Cruz 100, 841-845.

Horta, M.C., Moraes-Filho, J., Casagrande, R.A., Saito, T.B., Rosa, S.C., Ogrzewalska, M., Matushima, E.R., Labruna, M.B., 2009. Experimental infection of opossums *Didelphis aurita* by *Rickettsia rickettsii* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense*. Vector Borne Zoonotic Dis 9, 109-118.

Horta, M.C., Pinter, A., Souza, C., Joppert, A., Yai, L., Labruna, M., Schumaker, T. 2005. *Rickettsia* infection in opossums (*Didelphis* spp.) in São Paulo State, Brazil. In 4th International Conference on Rickettsiae and Rickettsial Diseases (Logroño (La Rioja), Espanha.), p. 166.

Keenan, K.P., Buhles, W.C., Jr., Huxsoll, D.L., Williams, R.G., Hildebrandt, P.K., Campbell, J.M., Stephenson, E.H., 1977. Pathogenesis of infection with *Rickettsia rickettsii* in the dog: a disease model for Rocky Mountain spotted fever. J Infect Dis 135, 911-917.

Krawczak, F.S., Nieri-Bastos, F.A., Nunes, F.P., Soares, J.F., Moraes-Filho, J., Labruna, M.B., 2014. Rickettsial infection in *Amblyomma cajennense* ticks and capybaras (*Hydrochoerus hydrochaeris*) in a Brazilian spotted fever-endemic area. Parasit Vectors 7, 7.

Labruna, M., Kamakura, O., Moraes-Filho, J., Horta, M.C., Pacheco, R., 2009. Rocky Mountain Spotted Fever in Dogs, Brazil. Emerg Infect Dis 15, 458-460.

Labruna, M.B., 2009. Ecology of Rickettsia in South America. Annals of the New York Academy of Sciences 1166, 156-166.

Labruna, M.B., Horta, M.C., Aguiar, D.M., Cavalcante, G.T., Pinter, A., Gennari, S.M., Camargo, L.M., 2007. Prevalence of *Rickettsia* infection in dogs from the urban and rural areas of Monte Negro municipality, western Amazon, Brazil. Vector Borne Zoonotic Dis 7, 249-255.

Labruna, M.B., Ogrzewalska, M., Martins, T.F., Pinter, A., Horta, M.C., 2008. Comparative susceptibility of larval stages of *Amblyomma aureolatum*, *Amblyomma cajennense*, and *Rhipicephalus sanguineus* to infection by *Rickettsia rickettsii*. J Med Entomol 45, 1156-1159.

Labruna, M.B., Whitworth, T., Horta, M.C., Bouyer, D.H., McBride, J.W., Pinter, A., Popov, V., Gennari, S.M., Walker, D.H., 2004. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. J Clin Microbiol 42, 90-98.

Levin, M.L., Snellgrove, A.N., Zemtsova, G.E., 2016. Comparative value of blood and skin samples for diagnosis of spotted fever group rickettsial infection in model animals. Ticks and Tick-Borne Diseases 7, 1029-1034.

Levin, M.L., Killmaster, L.F., Zemtsova, G.E., Ritter, J.M., Langham, G., 2014. Clinical Presentation, Convalescence, and Relapse of Rocky Mountain Spotted Fever in Dogs Experimentally Infected via Tick Bite. Plos One 9, e115105.

Lundgren, D.L., Thorpe, B.D., 1966. Infectious diseases in wild animals in Utah. VII. Experimental infection of rodents with *Rickettsia rickettsii*. Am J Trop Med Hyg 15, 799-806.

Madella, D.A., Neto, E.J.R., Felisberto, M.E., Souza, C.E.d., 2006. Valores hematológicos de capivaras (*Hydrochoerus hydrochaeris*) (Rodentia: Hydrochoeridae) de vida livre na regiao de Campinas-SP. Ciência Rural 36, 1321-1324.

Niebylski, M., Peacock, M., Schwan, T., 1999. Lethal Effect of *Rickettsia rickettsii* on Its Tick Vector (*Dermacentor andersoni*). Applied and Environmental Microbiology 65, 773-778.

Pacheco, R.C., Horta, M.C., Moraes-Filho, J., Ataliba, A.C., Pinter, A., Labruna, M.B., 2007. Rickettsial infection in capybaras (*Hydrochoerus hydrochaeris*) from Sao Paulo, Brazil: serological evidence for infection by *Rickettsia bellii* and *Rickettsia parkeri*. Biomedica 27, 364-371.

Parola, P., Paddock, C.D., Socolovschi, C., Labruna, M.B., Mediannikov, O., Kernif, T., Abdad, M.Y., Stenos, J., Bitam, I., Fournier, P.E., Raoult, D., 2013. Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev 26, 657-702.

Pinter, A., Labruna, M.B., 2006. Isolation of *Rickettsia rickettsii* and *Rickettsia bellii* in Cell Culture from the Tick *Amblyomma aureolatum* in Brazil. Annals of the New York Academy of Sciences 1078, 523-529.

Pinter, A., Labruna, M.B., Faccini, J.L., 2002. The sex ratio of *Amblyomma cajennense* (Acari: Ixodidae) with notes on the male feeding period in the laboratory. Vet Parasitol 105, 79-88.

Piranda, E.M., Faccini, J.L., Pinter, A., Saito, T.B., Pacheco, R.C., Hagiwara, M.K., Labruna, M.B., 2008. Experimental infection of dogs with a Brazilian strain of *Rickettsia rickettsii*: clinical and laboratory findings. Mem Inst Oswaldo Cruz 103, 696-701.

Randall, M.B., Walker, D.H., 1984. Rocky Mountain spotted fever. Gastrointestinal and pancreatic lesions and rickettsial infection. Archives of pathology & laboratory medicine 108, 963-967.

Soares, J.F., Soares, H.S., Barbieri, A.M., Labruna, M.B., 2012. Experimental infection of the tick *Amblyomma cajennense*, Cayenne tick, with *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever. Med Vet Entomol 26, 139-151.

Souza, C.E., Moraes-Filho, J., Ogrzewalska, M., Uchoa, F.C., Horta, M.C., Souza, S.S., Borba, R.C., Labruna, M.B., 2009. Experimental infection of capybaras *Hydrochoerus hydrochaeris* by *Rickettsia rickettsii* and evaluation of the

transmission of the infection to ticks *Amblyomma cajennense*. Vet Parasitol 161, 116-121.

Suckow, M.A., Brammer, D.W., Rush, H.G., Chrisp, C.E., 2002. Biology and Diseases of Rabbits, In: Fox, J.C., Anderson, L., Loew, F., Quimby, F. (Eds.) Laboratory Animal Medicine Academic Press, USA, pp. 329-364.

Szabo, M.P., Pinter, A., Labruna, M.B., 2013. Ecology, biology and distribution of spotted-fever tick vectors in Brazil. Front Cell Infect Microbiol 3, 27.

Travassos, J., Vallejo, A., 1942. Comportamento de alguns cavídeos (*Cavia aperea* e *Hydrochoerus capybara*) às inoculações experimentais do vírus da febre maculosa. Possibilidade desses cavídeos representarem o papel de depositários transitórios do vírus na natureza. Mem. Inst. Butantã 15, 73-86.

Ueno, T.E., Costa, F.B., Moraes-Filho, J., Agostinho, W.C., Fernandes, W.R., Labruna, M.B., 2016. Experimental infection of horses with *Rickettsia rickettsii*. Parasit Vectors 9, 499.

Van der Heijden, K.M., Szabó, M., Matushima, E.R., da Veiga, M.L., Santos, A.A., Egami, M.I., 2003. Valores hematológicos e identificação morfo-citoquímica de células sangüíneas de capivaras (*Hydrochoerus hydrochoeris*) parasitadas por carrapatos e capivaras livres de infestação. Acta Scientiarum. Animal Sciences 25, 143-150.

Walker, D.H., Crawford, C.G., Cain, B.G., 1980. Rickettsial infection of the pulmonary microcirculation: the basis for interstitial pneumonitis in Rocky Mountain spotted fever. Hum Pathol 11, 263-272.

Table 1. Clinical monitoring of capybaras (*Hydrochoerus hydrochaeris*) and control rabbits during multiple infections with *Rickettsia rickettsii* (strain Itu) via tick exposure.

	Phase							
	I		II		III		IV	
Animal No.	Rectal Temperature*	Clinical Signs ǂ	Rectal Temperature	Clinical Signs	Rectal Temperature	Clinical Signs	Rectal Temperature	Clinical Signs
1	36.7 (0.59) [35.4-37.9]	None	36.2 (0.84) [34.9-37.7]	None	36.1 (1.2) [33.4-38.0]	None	35.8 (1.05) [34.0-38.0]	None
2	-	-	36.8 (1.76) [32.0-39.7]	Abdominal and thoracic rash (8); fever (9); hindlimb weakness (11); lack of appetite (12); nasal mucous discharge (13); inappetence (14); purplish macules (18); prostration (18); coma (18); seizures (18); death (18)	+	+	+	+
3	37.1 (0.55) [35.7-38.2]	None	37.1 (1.87) [32.5-39.8]	Abdominal and thoracic rash (8); fever (9); lack of appetite (10); nasal mucous discharge (11); inappetence (14); hindlimb weakness (14); reluctance to move (15); diarrhoea (15); dark urine (15); death (16)	+	+	+	+
4	-	-	-	-	36.8 (0.84) [35.4-38.7]	Fever (9); hindlimb weakness (11); lack of appetite (11); nasal mucous discharge (12); general weakness (14)	35.8 (1.01) [34.0-37.5]	None
5	-	-	-	-	37.2 (1.13) [34.6-39.2]	Fever (8); mucous faeces (11); lack of appetite (11); general weakness (14)	36.3 (0.98) [33.9-38.0]	None

Table 1 (Cont.) Clinical monitoring of capybaras (*Hydrochoerus hydrochaeris*) and control rabbits during multiple infections with *Rickettsia rickettsii* (strain Itu) via tick exposure.

	Phase							
	I		II		III		IV	
Animal No.	Rectal Temperature*	Clinical Signs ǂ	Rectal Temperature	Clinical Signs	Rectal Temperature	Clinical Signs	Rectal Temperature	Clinical Signs
Control Rabbit	39.8 (0.99) [37.5-41.0]**	Fever (7); nasal mucous discharge (9); lack of appetite (13); dehydration (13); death (13)	39.5 (1.55) [35.0-41.1]	Fever (6); scrotal edema and erythema (8); scrotal necrosis (10); inappetence (10); preputial edema (12); ocular discharge (12); hypothermia (13); death (13)	39.6 (0.44) [39.0-40.8]	Fever (5); auricular erythema (5)	40.1 (0.75) [38.7-41.5]	Fever (8); lack of appetite (9); auricular edema and necrosis (10); scrotal erythema and edema (10); inappetence (11); scrotal necrosis (11); preputial edema (12)

Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5

* Data is shown as mean (standard deviation) [range]

** Normal range: 38.5º-39.5 ºC (Suckow et al., 2002)

ǂ Clinical sign (post-infection day of onset)

+ Dead animal

Table 2. Guinea pig (*Cavia porcellus*) inoculated with blood from capybaras (*Hydrochoerus hydrochaeris*) during multiple infections with *Rickettsia rickettsii* (strain Itu) via tick exposure.

	Febrile animals*		Auricular vascular signs**	Genital vascular signs**	Dead animals		IFA		
Capybara No.	No.	Day(s) PI†	No.	No.	No.	Day(s) PI†	Positive IFA samples	Day(s) PI	Endpoint IFA titre
	Phase I								
1	3/32 (9.4)‡	8, 10	1/32 (3.1)	3/32 (9.4)	2/32 (6.3)	10	3/32 (9.4)	8, 12, 14	8,192
	Phase II								
1	0/32 (0)	-	0/32 (0)	0/32 (0)	0/32 (0)	-	0/32 (0)	-	-
2	9/20 (45.0)	6-16	10/20 (50.0)	7/20 (35.0)	4/20 (20.0)	6, 10, 16	9/20 (45.0)	6-18	262,144
3	11/18 (61.1)	6-16	11/18 (61.1)	9/18 (50.0)	5/18 (27.8)	6, 8, 14, 16	9/18 (50.0)	6, 10-16	131,072
	Phase III								
1	0/32 (0)	-	0/32 (0)	0/32 (0)	0/32 (0)	-	0/32 (0)	-	-
4	4/32 (12,5)	8-12	3/32 (9.4)	2/32 (6.3)	1/32 (3,1)	8	4/32 (12,5)	10-14	32,768
5	6/32 (18,8)	8-16	6/32 (18.8)	5/32 (15.6)	3/32 (9,4)	8, 14, 16	7/32 (21,9)	8-20	65,536
	Phase IV								
1	0/32 (0)	-	0/32 (0)	0/32 (0)	0/32 (0)	-	0/32 (0)	-	-
4	0/32 (0)	-	0/32 (0)	0/32 (0)	0/32 (0)	-	0/32 (0)	-	-
5	0/32 (0)	-	0/32 (0)	0/32 (0)	0/32 (0)	-	0/32 (0)	-	-

Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5

* Two or more consecutive days with rectal temperature ≥ 40 ºC

** Vascular abnormalities like edema, erythema and/or necrosis

† Post-infection day in the capybara when blood was obtained and inoculated in the guinea pig

‡ Data are shown as number of affected animals/total number of animals (%)

Table 3. Hematological variables evaluated in capybaras (Hydrochoerus hydrochaeris) during multiple infections with *Rickettsia rickettsii* (strain Itu) via tick exposure. Data are presented as mean (standard deviation) [range]

	PCV (%)				Erythrocyte count (x106 cells/mm3)				Leucocyte count (x103 cells/mm3)			
	Phase				Phase				Phase			
Capybara No.	I	II	III	IV	I	II	III	IV	I	II	III	IV
1	36,8 (10,83) [22,2-50,9]	35,9 (2,15) [33,3-39,2]	38,7 (2,23) [35,0-41,4]	40,4 (1,49) [38,4-42,4]	2,56 (0,48) [1,97-3,28]	2,75 (0,31) [2,07-3,13]	2,66 (0,49) [1,80-3,36]	2,75 (0,18) [2,49-2,94]	3,46 (1,73) [1,85-6,93]	4,84 (0,75) [3,70-5,75]	4,72 (0,95) [3,28-6,38]	4,95 (0,64) [4,03-5,95]
2	-	33,8 (7,27) [24,4-40,4]	-	-	-	2,70 (0,46) [2,28-3,34]	-	-	-	1,97 (1,11) [1,13-3,23]	-	-
3	40,5 (4,46) [34,9-48,1]	34,5 (7,49) [25,6-42,6]	-	-	2,91 (0,33) [2,37-3,23]	2,95 (0,36) [2,41-3,18]	-	-	4,59 (1,08) [3,40-6,68]	2,71 (1,56) [1,38-4,53]	-	-
4	-	-	35,6 (2,32) [31,4-38,1]	40,6 (1,02) [39,2-41,6]	-	-	2,65 (0,35) [2,21-3,18]	3,05 (0,17) [2,78-3,32]	-	-	5,89 (1,28) [3,43-7,20]	7,62 (1,78) [5,48-10,55]
5	-	-	31,6 (3,34) [27,1-36,0]	40,8 (1,4) [38,3-42,4]	-	-	2,14 (0,42) [1,59-2,81]	3,05 (0,18) [2,81-3,29]	-	-	4,98 (2,06) [2,23-8,73]	4,94 (0,85) [3,80-6,28]
Reference values	46.6-51.4 (1); 43.3-52.9 (2); 38.4-42.4 (3)				3.44-3.98 (1); 2.82-3.44 (2); 4.3-4.7 (3)				3.96-6.44 (1); 7.44-15.82 (2); 3.3-7.3 (3)			

Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5.

(1) Arouca et al., 2000; (2) Van der Heijden et al., 2003; (3) Madella et al., 2006

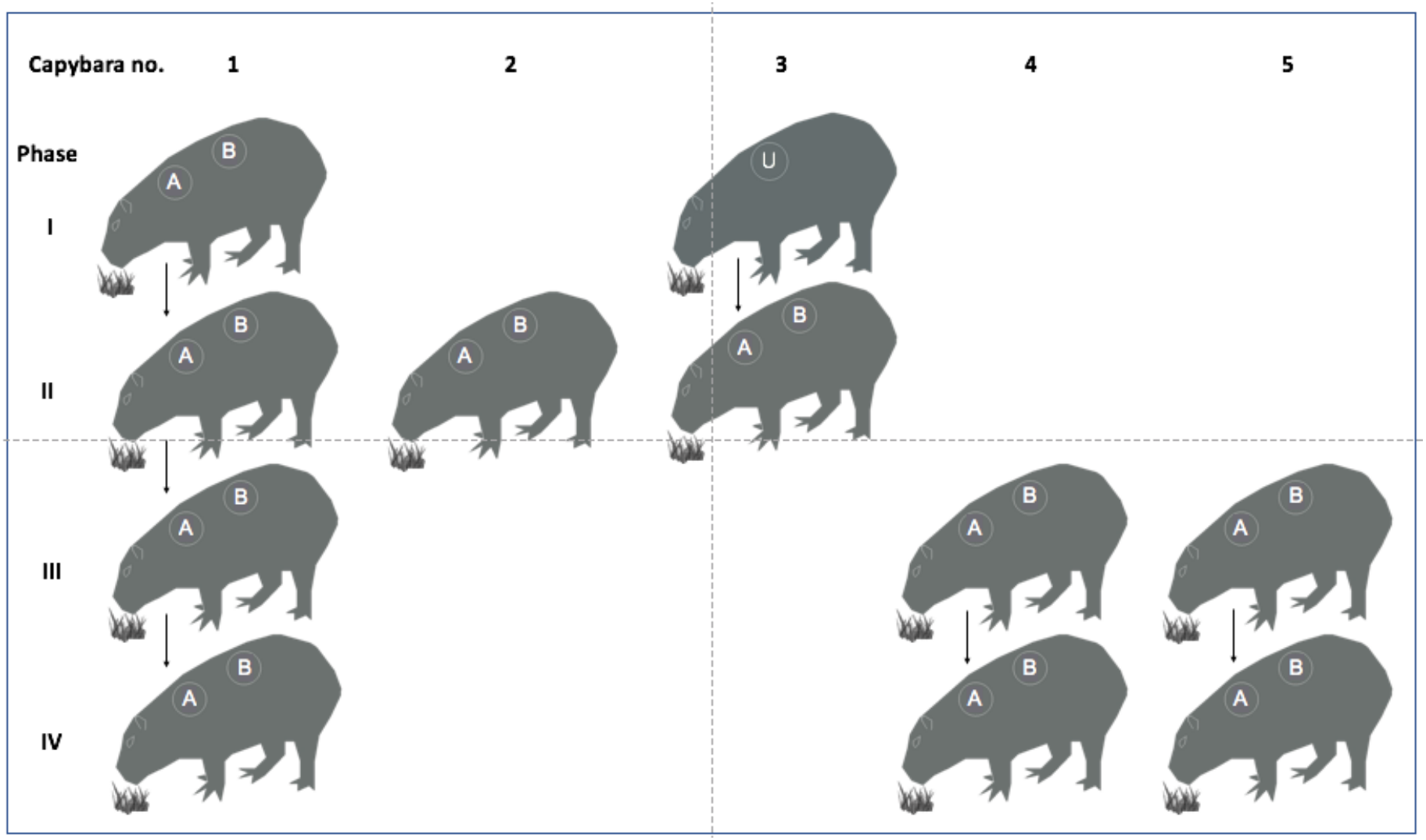


Figure 1. Scheme of the experimental infections conducted on capybaras no. 1 to 5 during the study. Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5. A. feeding chamber A; B. feeding chamber B; U. unique feeding chamber.

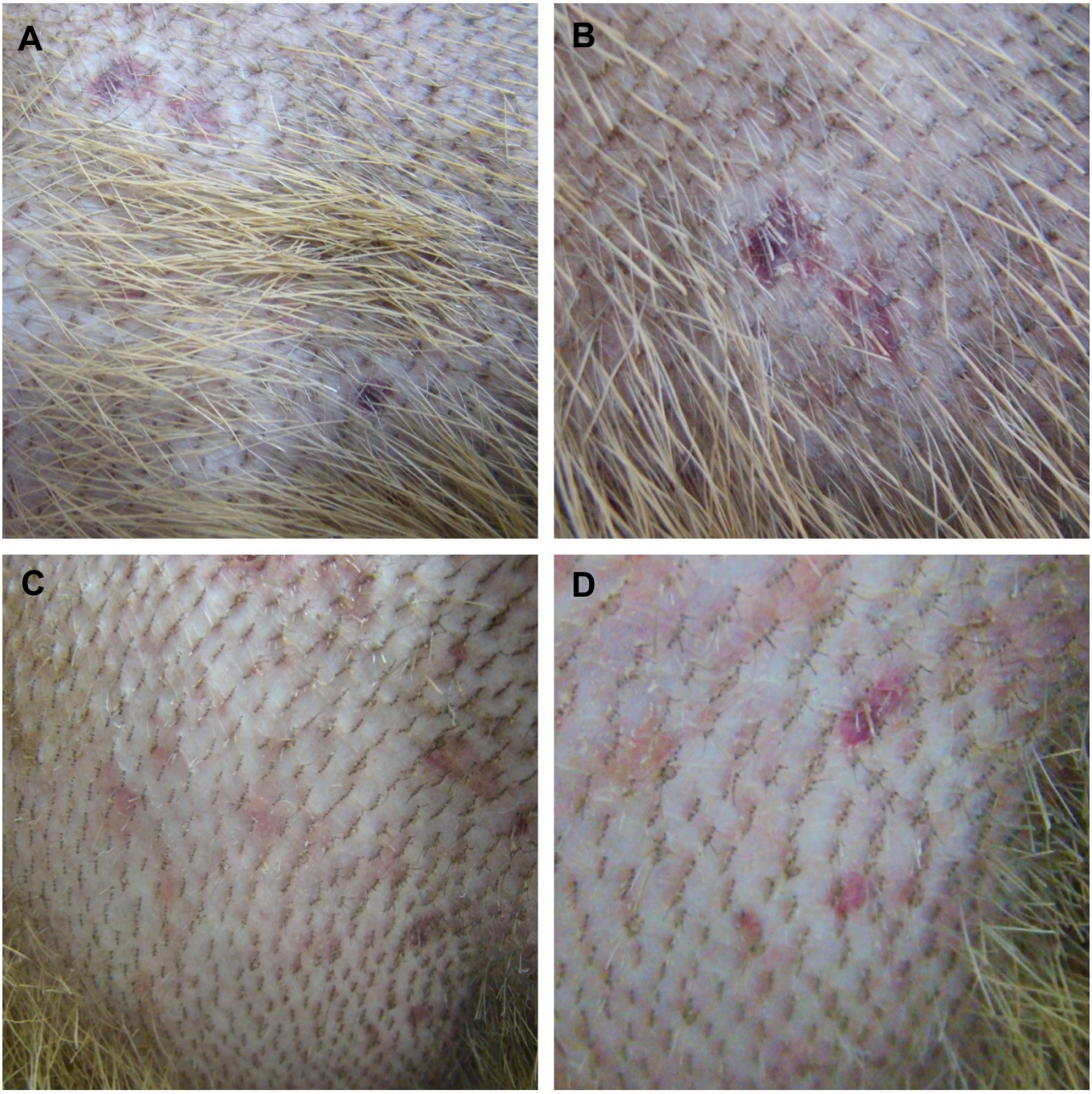


Figure 2. Skin of capybaras no. 2 and 3 during infection (phase II) with *Rickettsia rickettsii* (strain Itu) via tick exposure. A and B. Purplish macules in capybara no. 2 (18 DPI). C and D. Abdominal rash in capybara no. 3 (10 DPI).

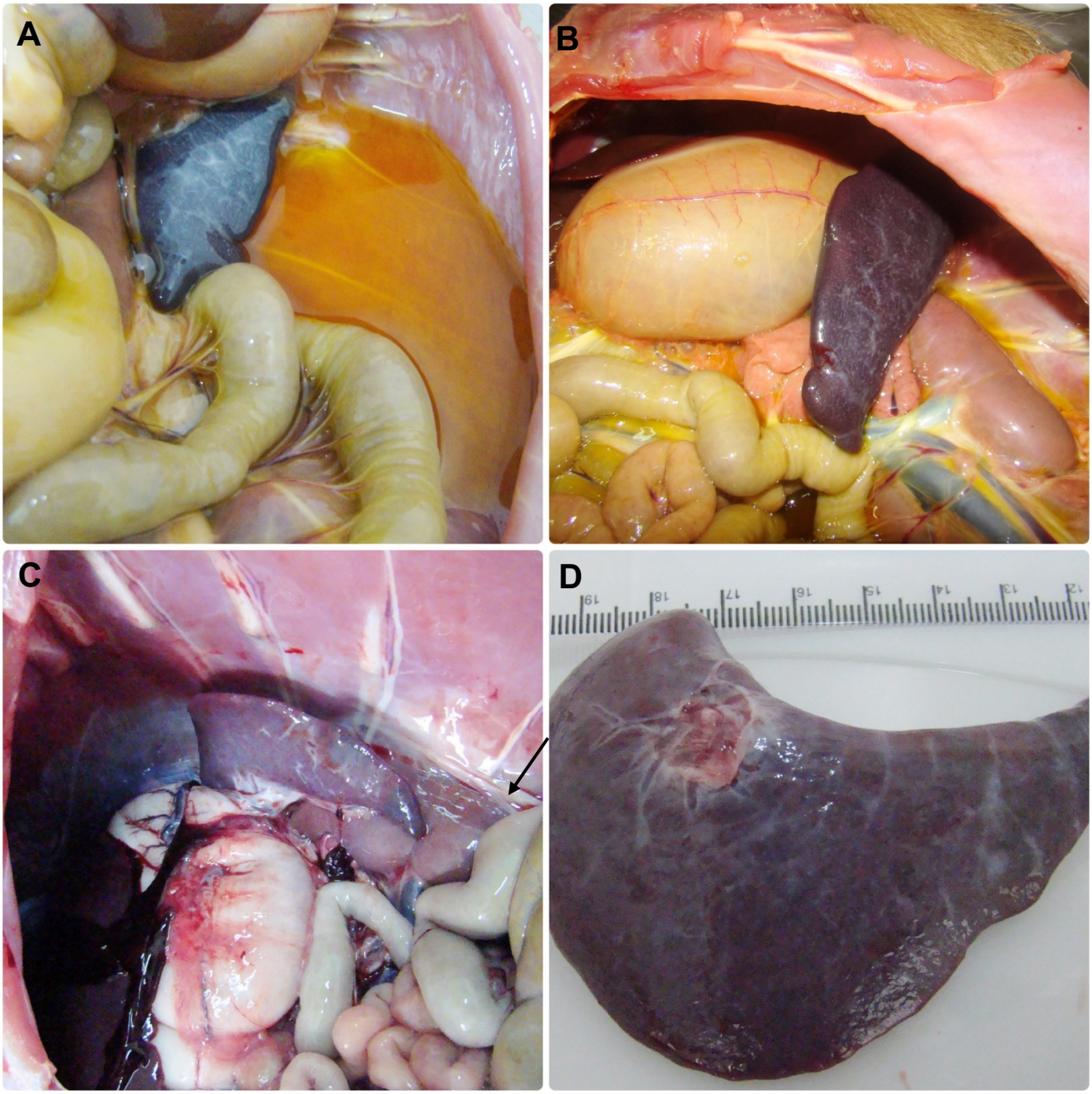


Figure 3. Abdominal cavity and spleen of capybaras no. 2 and 3 after infection (phase II) with *Rickettsia rickettsii* (strain Itu) via tick exposure. A. Abdominal cavity of capybara no. 3 with evidence of ascites and spleen enlargement (16 DPI) B. Spleen enlargement in capybara no. 3 (16 DPI). C. Spleen enlargement with apical haemorrhage (arrow) in capybara no. 2 (18 DPI) D. Enlarged spleen from capybara no. 2 (18 DPI)

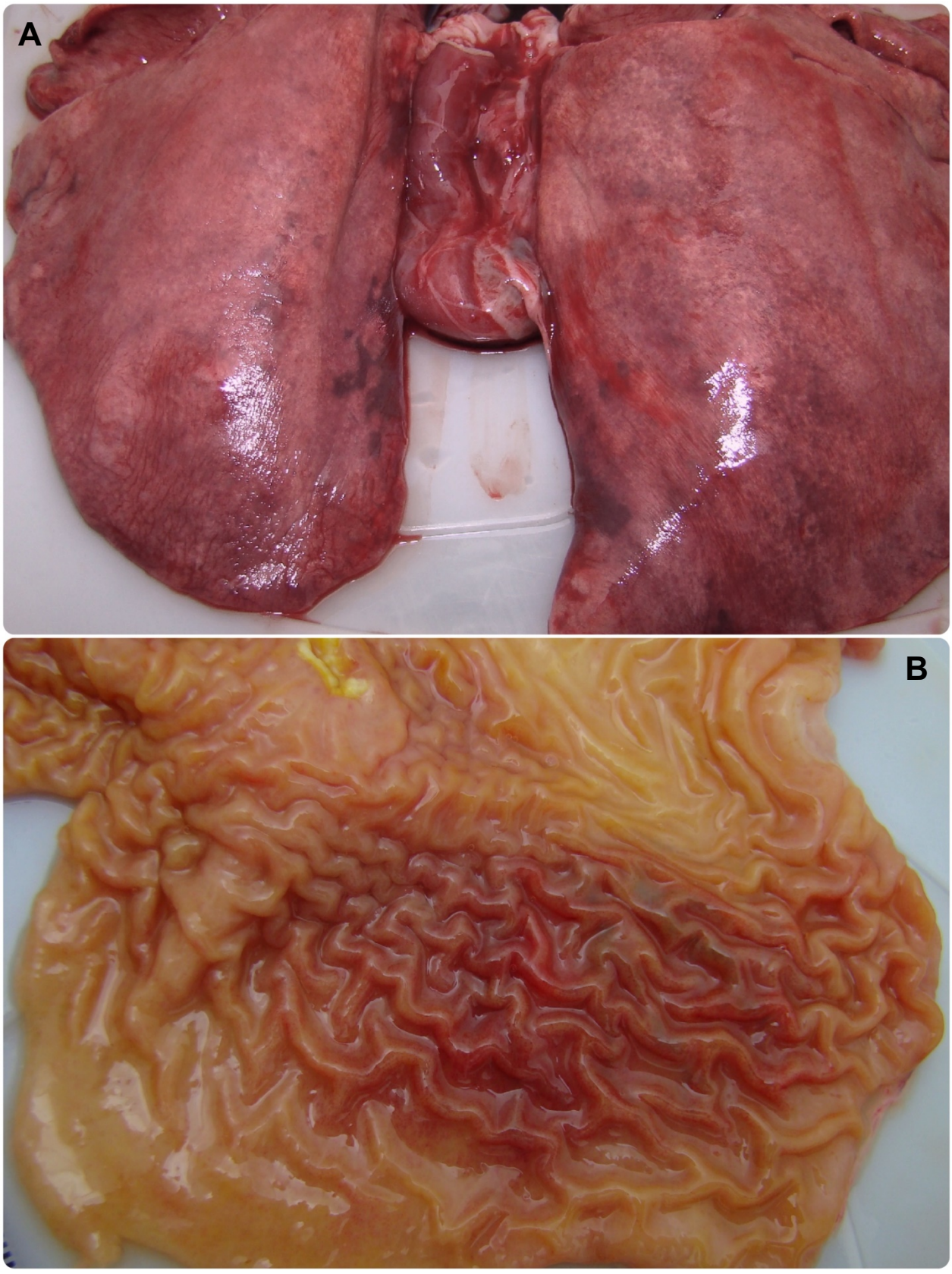


Figure 4. Lung and stomach of capybara no. 2 after infection (phase II) with *Rickettsia rickettsii* (strain Itu) via tick exposure. A. Lung of capybara no. 2 with evidence of bilateral disseminated vascular injuries (18 DPI). B. Stomach of capybara no. 2 with an extended area of haemorrhage in the mucosa (18 DPI).

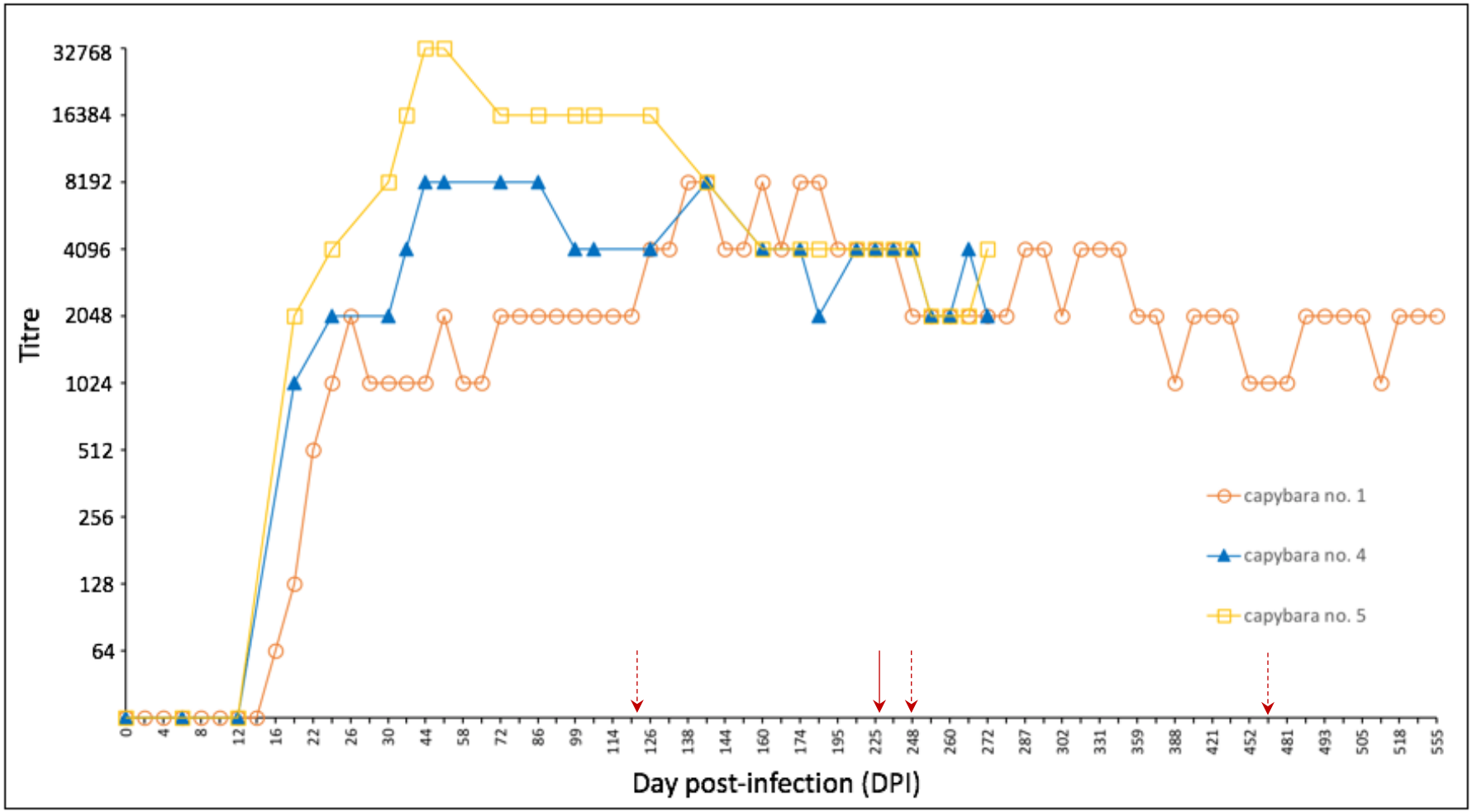


Figure 5. *Rickettsia rickettsii* antibody titres (IFA) after multiple infections with *Rickettsia rickettsii* (strain ITU) via tick exposures, in capybaras no. 1, 4 and 5. Dashed arrows indicate 2nd, 3rd and 4th challenges of capybara no. 1 at 120, 248 and 475 days after the first infection, respectively. Straight arrow indicates 2nd challenge of capybaras no. 4 and 5 at 227 days after the first infection.

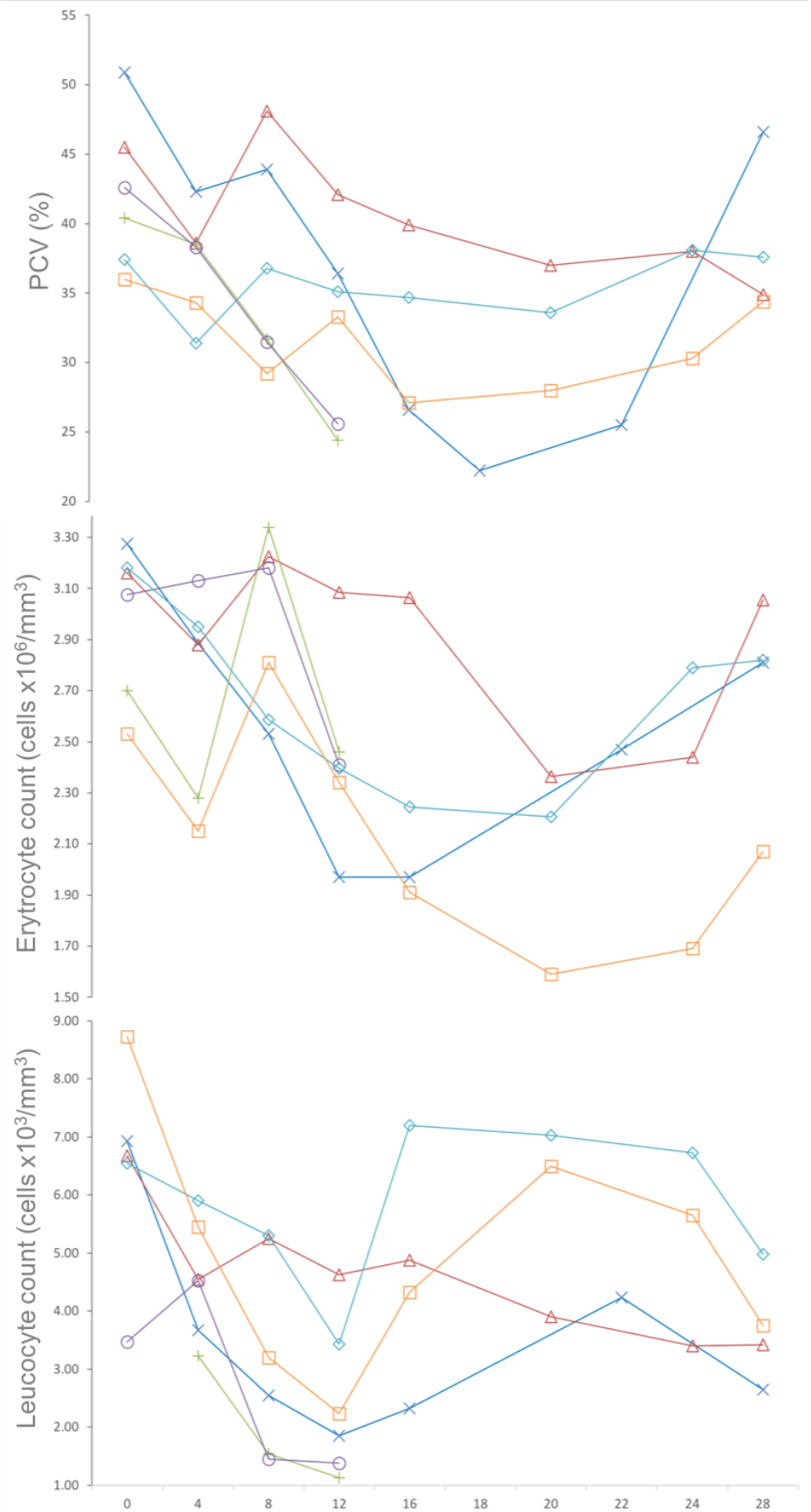


Figure 6. Haematological variables evaluated in capybaras (*Hydrochoerus hydrochaeris*) during primoinfection with *Rickettsia rickettsii* (strain Itu) via tick exposure. Capybara 1 (x); cap. 3 (control) (△); cap. 2 (+); cap. 3 (○); cap. 4 (◇); cap. 5 (□).

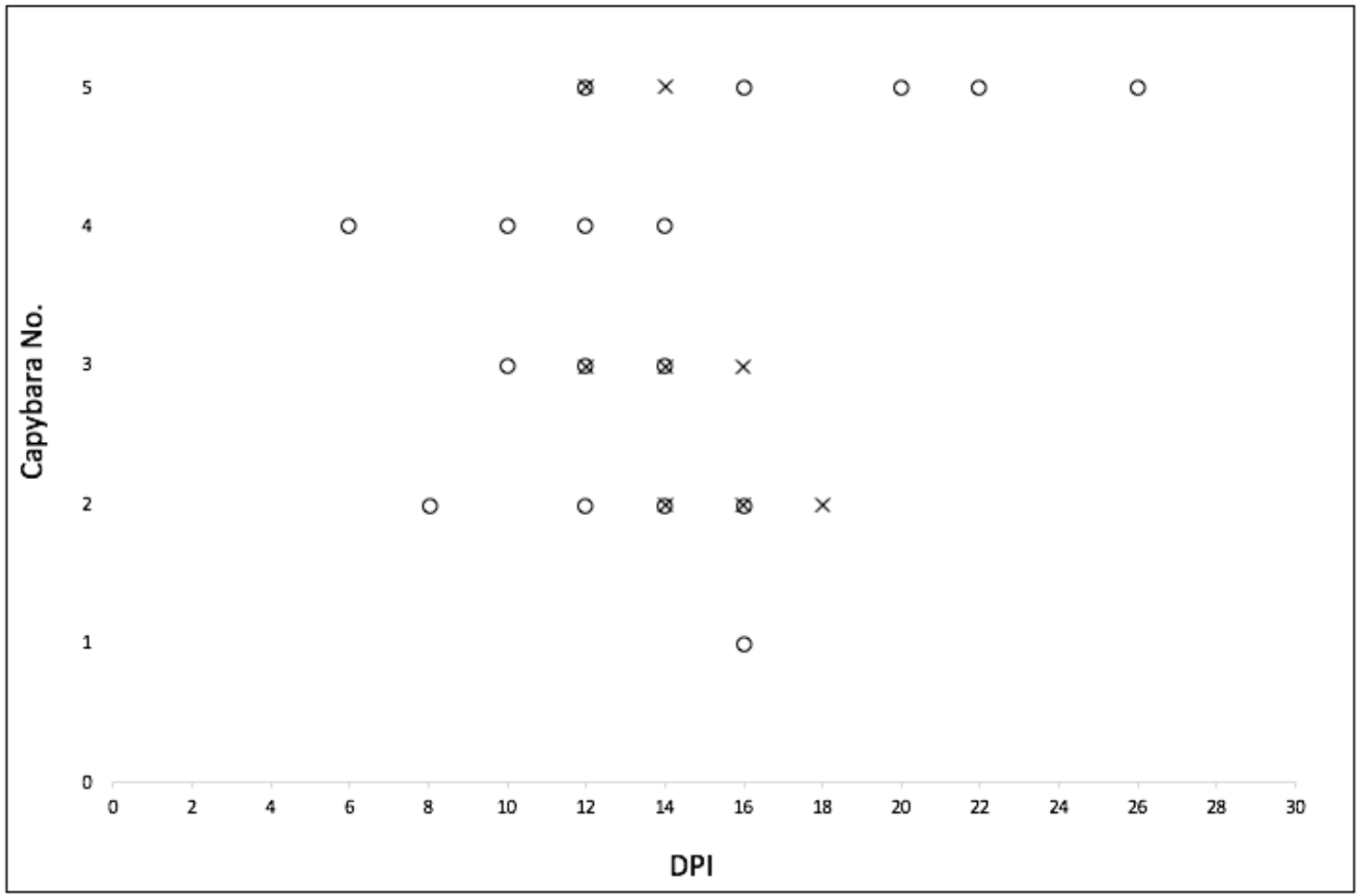


Figure 7. Molecular detection of *Rickettsia rickettsii* DNA in blood (X) and skin (O) samples in capybaras (*Hydrochoerus hydrochaeris*) during primoinfection with *R. rickettsii* (strain Itu) via tick exposure.

Supplementary video. Capybaras no. 2 and 3 during infection (phase II) with *Rickettsia rickettsii* (strain Itu) via tick exposure. Video showing hindlimb weakness in capybara no. 2 during locomotion (12 DPI).
Access link: https://www.dropbox.com/s/2ddj8jrrtgzridy/Cap_2_3_Phase%20II.m4v?dl=0

4 TRANSMISSION OF *Rickettsia rickettsii* (STRAIN ITU) TO *Amblyomma sculptum* TICKS AFTER EXPERIMENTAL INFECTION OF CAPYBARAS (*Hydrochoerus hydrochaeris*)

4.1 INTRODUCTION

Rickettsia rickettsii infection produces a disease known as Rocky Mountain Spotted Fever or Brazilian Spotted Fever (BSF), as it is recognized in Brazil. Is the most lethal tick-borne disease in the western hemisphere with high case-fatality rates (> 50% in southeastern Brazil) associated with misdiagnosis, lack of suspicion and awareness by medical services (Walker, 2002; Angerami et al., 2006; Angerami et al., 2009; de Oliveira et al., 2016).

Different tick species have been incriminated in the transmission of this bacterium in the continent; *Dermacentor variabilis* and *Dermacentor andersoni* in the United States and Canada, *Rhipicephalus sanguineus* s.l. in Southern US and north of Mexico, and members of the *Amblyomma cajennense* s.l. complex in Central and South America (Demma et al., 2006; Parola et al., 2013; Szabo et al., 2013; Tinoco-Gracia et al., 2018). Besides, in Brazil, two *Amblyomma* species have been incriminated within two different eco-epidemiological scenarios: *Amblyomma aureolatum*, in the São Paulo metropolitan area, with carnivores as main hosts for adults (Pinter and Labruna, 2006; Labruna, 2009; Szabo et al., 2013) and *Amblyomma sculptum*, in the southeastern region, where horses and capybaras (*Hydrochoerus hydrochaeris*) act as primary host for all stages (Labruna, 2009; Szabo et al., 2013). Ticks function as the main reservoir of *R. rickettsii* in nature, nonetheless, due to bacterial pathogenic effects on ticks (Burgdorfer and Brinton, 1975; Niebylski et al., 1999) and some degree of tick refractoriness to bacterial infection (Soares et al., 2012), low natural infectious rates are commonly found. Thus, a susceptible vertebrate host which develops rickettsemia of sufficient intensity and length, contribute to the infection of new lineages of ticks and the natural maintenance of the pathogen (Burgdorfer, 1988; Labruna, 2009); a role that capybaras develop in southeastern Brazil (Labruna, 2009; Labruna, 2013).

Previous experimental studies on capybaras induced infection by the inoculation or tick exposition to *R. rickettsii* strains isolated from *Amblyomma aureolatum* (Travassos

and Vallejo, 1942a; Souza et al., 2009). In the study of Souza et al. (2009), the strain Taiaçu, isolated from *A. aureolatum* collected in Taiaçupeba (São Paulo) (Pinter and Labruna, 2006), was used for the experimental infection of capybaras and transmission to susceptible *A. sculptum* nymphs (from Pedreira, São Paulo). Totally, a proportion of 20-35% adults get infected. While these rates are similar to those obtained in guinea pigs with the same bacterium strain and tick population (Labruna et al., 2008), probable higher rates could be registered with infections with a *R. rickettsii* strain isolated from *A. sculptum*, as occurred in nature. The mentioned early study evaluated infection in nymphs (and molted adults) but not in larval stage which also infests this vertebrate host (Labruna et al., 2004a). Besides, vectorial competence tests and assess of transmission in immune hosts were not performed. Hence, the aim of this paper is to describe the transmission of *R. rickettsii* (strain Itu) to non-infected larvae and nymphs of *A. sculptum*, after feeding on experimental infected and subsequently challenged capybaras, and assess the capacity of these ticks to transmit the bacterium to susceptible rabbits.

4.2 MATERIALS AND METHODS

4.2.1 Animals

As reported in a previous paper (Ramírez-Hernández et. al., not published), five capybaras obtained from two different non-endemic areas in São Paulo state were transported and infected in the Animal Research Facility of SUCEN (Superintendência de Controle de Endemias) in Mogi Guaçu (São Paulo, Brazil). All capybaras were previously infested by *Amblyomma dubitatum* ticks under natural conditions, before starting the present study. In addition, laboratory white New Zealand rabbits were purchased from a commercial breeder, housed in standardized laboratory cages and fed with a commercial rabbit pellet diet, water *ad libitum* in a room with temperature, photoperiod (12 h/12 h) and ventilation control. All were tick-naïve and bred under laboratory standard sanitary conditions.

All animal procedures were authorized by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) (CEUA No. 4115110215) and procedures involving capybaras, were

authorized by the Brazilian biodiversity agency SISBIO (“Sistema de Autorização e Informação em Biodiversidade”-ICMBio) (No. 43259-3).

4.2.2 Capybara infection

The study was divided in four phases (I to IV) as follows: Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5. All capybaras were serologically negative for *Rickettsia* (six species), in 14-day interval paired samples, before primoinfection (tested by indirect immunofluorescence assay-IFA).

As reported earlier (Ramírez-Hernández et. al., not published), for primoinfection and subsequent challenges, two separated cotton sleeves (10-cm diameter feeding chambers) were glued on the capybara shaved dorsum, as described previously (Labruna et al., 2008; Souza et al., 2009). These chambers were labelled as chamber A (cranial position on the capybara dorsum) and chamber B (caudal position on the capybara dorsum). The minimum distance between the two chambers were 5 cm. Chamber A received 20 males and 20 females of *A. sculptum* derived from a colony, in the fifth generation (F5), established in the laboratory from a population adapted to capybaras, collected in Itu municipality (São Paulo) in 2012 (Krawczak et al., 2014). These adult ticks were exposed to rickettsial infection, as larvae and nymphs, through feeding on rickettsemic guinea pigs intraperitoneally inoculated with a *R. rickettsii* strain isolated from *A. sculptum* ticks collected in the same area of origin of the progenitor ticks that generated our tick colony (Itu strain) (Krawczak et al., 2014). Day 0 (zero) of infection was considered as the day of infestation with infected ticks, namely, when infected unfed adults were put in the feeding chamber A.

4.2.3 *Amblyomma sculptum* infestations

Non-infected larvae and nymphs of *A. sculptum* ticks derived from the same progenitor colony (collected in Itu, São Paulo, Brazil), in the fifth and sixth generation (F5-F6), were used for infestation of capybaras during their experimental infection. Feeding

chambers A and B received these ticks at 0, 5, 10, 15, 20 and 26 day post-infection (DPI) of capybaras. In each infestation, approximately 300-500 larvae and 100 nymphs were used.

Both feeding chambers were opened daily to collect naturally detached engorged individuals and then transported them to the incubator (23 ± 2 ºC and 85% RH) for molting (engorged larvae and nymphs). Ticks were separated by life stage, capybara number, feeding chamber (A or B), detachment day and stage, in individual tubes.

After molting, unfed nymphs (molted from larvae) and unfed adults (molted from nymphs) were separated for DNA extraction or vectorial competence tests. For DNA extraction, tick samples were separated in five periods (I to V), related with the capybara experimental infection day, as follows: ticks detached between 0-5, 6-11, 12-17, 18-23 and 24-30 DPI; further they were individually storage in identified microtubes and frozen at -20 ºC. Likewise, ticks for vectorial competence tests were separated in three periods according to the detachment period of the previous feeding stage on capybaras (I to III): 0-10, 11-20 and 21-30 DPI and maintained in incubation until rabbit infestations. Both procedures are detailed below.

4.2.4 Rabbit infestations

In order to assess the capacity of ticks obtained from capybaras to transmit *R. rickettsii*, tick-naïve rabbits were infested with nymphs and adults that had fed as larvae and nymphs, respectively, on capybaras. Ticks were divided by capybara number (1 to 5), detachment period (I to III), stage (nymph or adult) and feeding chamber (A or B) to infest rabbits individually. Previous to infestation, rabbits were dorsally shaved and one cotton sleeve (8-cm diameter feeding chamber) glued on the skin as previously described (Pinter et al., 2002). Blood was collected at day 0 and 21 of rabbit infestation by puncture of the central ear artery (day 0) and intracardiacally (day 21). This latter sampling was performed under anaesthesia (xylazine 5 mg/kg + ketamine 35 mg/kg, intramuscularly-IM) (Flecknell, 2009). Sera were separated by centrifugation (10 min at 5.000 r.p.m.) and stored at -20 ºC for IFA test (described below).

Rabbits were daily monitored for clinical signs and rectal temperature measured during the infestation period (21 days). Febrile animals were considered when temperatures

higher than 40 ºC, for two or more consecutive days, were registered (Monteiro, 1933). Besides, in dead rabbits, a necropsy was performed and spleen samples frozen (-20 ºC) for DNA extraction. Moreover, feeding chambers were opened daily and naturally detached and engorged larvae and nymphs were collected and transported to the incubator (23 ± 2 ºC and 85% RH) for molting and oviposition, respectively. After that, random samples of ticks were preserved (-20 ºC) for DNA extraction. Finally, at day 21, after collection of the second blood sample, rabbits were euthanized with sodium pentobarbital (100 mg/kg), by intracardiac injection, under general anaesthesia (described above).

4.2.5 Indirect immunofluorescence assay (IFA)

Sera obtained from rabbits were tested by IFA using *R. rickettsii* (strain Taiaçu) crude antigen and fluorescein isothiocyanate-labelled goat anti-rabbit IgG (Sigma, St. Louis, USA). Samples were initially tested in a 1:64 dilution (with PBS) as cut-off and those positive, further diluted in a twofold increase until reach the endpoint titre as reported earlier (Labruna et al., 2007; Soares et al., 2012). In each slide, a previously known rabbit positive and negative sera were used as controls.

4.2.6 DNA extraction from tick samples

Nucleic acid (DNA) was extracted from tick samples for *Rickettsia* detection through real-time polymerase chain reaction (real-time PCR) (see below). Ticks (adults, nymphs) were processed individually. Guanidine Thiocyanate (GT) and boiling techniques were used for adult and immature stages, respectively; in both, TE buffer (10mM Tris HCl; 1mM ethylenediaminetetraacetic acid (EDTA) pH 8.0) was used for initial maceration of samples and final elution of DNA, following procedures published formerly (Horta et al., 2005; Sangioni et al., 2005; Gerardi, 2016). All extracted DNA from tick samples was conserved at -20 ºC for further PCR techniques (see below).

4.2.7 Real-time PCR

Extracted DNA from ticks (nymphs and adults), was submitted to a Taqman real-time PCR technique to amplify a 147-bp fragment of the citrate synthase gene (*gltA*) of *Rickettsia* spp. using the primers CS-5 (forward; Guedes et. al., 2005) and CS-6 (reverse; Labruna et. al., 2004b) and an internal fluorogenic probe (6-FAM d, BHQ- 1)

(Integrated DNA Technologies, San Diego, CA), in accordance with reagents and cycling conditions reported by Labruna *et al*. (2004b). The sensitivity of the technique was determined to be 1 DNA copy of *R. rickettsii* (Labruna et al., 2004b). Each reaction had a positive (DNA of *Rickettsia vini* cultivated in Vero cells) and a negative control (molecular-grade water) included.

4.3 RESULTS

4.3.1 Rabbit infestations

After molting of larvae and nymphs (to nymphs and adults, respectively), they were used for rabbit infestations with the purpose of identify *R*. *rickettsii* transmission from infected ticks. Totally, 115 animals were used in these transmission tests. As presented in Table 1, only rabbits infested with ticks collected during primoinfection of capybaras presented fever, vascular signs and anti-*R*. *rickettsii* antibodies. Contrastingly, rabbits infested with ticks collected during subsequent infections on capybaras 1, 4 and 5 did not present clinical signs or antibodies detected by IFA.

Rabbits infested with nymphs and adults collected (in the previous stage) from capybara 1 during the 1st and 2nd drop-off period (0-20 DPI), from feeding chambers A and B, presented fever. Moreover, only nymph-infested rabbits (feeding chamber B and drop-off period I and II) manifested vascular clinical signs (i.e. edema, erythema, and necrosis) in ears and genital region, and one individual died (day 15 post-infestation). The convalescent animal presented antibodies, at day 21 post-infestation, with an endpoint titre of 65,536 and an additional individual infested with nymphs (chamber A, drop-off period III) was reactive (1:64) without exhibiting clinical signs. Ticks collected in subsequent challenges (phase II, III and IV) did not generate clinical signs or serological response in infested rabbits, excepting one individual infested with adults from the 4th capybara-infection which presented three consecutive days with temperatures between 40.0 and 40.2 ºC but without exhibition of vascular signs or antibody reactivity (Table 1), refuting a possible rickettsial infection. Finally, a febrile rabbit infested with adults (chamber A, period II) did not exhibit additional signs or positive IFA serum.

Rabbits infested with nymphs and adults collected from feeding chambers A and B in the 2nd drop-off period (11-20 DPI) from capybara 2 presented fever, vascular clinical signs and antibody titres (endpoint titre: 32,768). Due to the death of this capybara, further challenges and consequent tick infestations tests were not performed (Table 1).

A high proportion (87.5 to 100.0%) of rabbits infested with nymphs and adults collected from capybara 3, in both feeding chambers, during the 1st and 2nd drop-off period, exhibited fever, vascular clinical signs and specific antibody response (endpoint titre: 65,536). As mentioned for capybara 2, further challenges and tick infestations were not performed due to the death of the animal (Table 1).

Regarding capybara 4, one rabbit infested with nymphs (2nd drop-off period, feeding chamber B) showed fever, vascular clinical signs and serologic response. An additional animal infested with nymphs (3rd drop-off period, feeding chamber A) showed antibody titres, and in both individuals the endpoint level was 16,384. Rabbits infested with ticks collected during challenge (phase IV) of this capybara did not exhibit signs or antibody response (Table 1).

Finally, 4, 3 and 6 rabbits infested with nymphs and adults collected during primoinfection of capybara 5 revealed fever, vascular signs and antibody response, respectively. Both stages were collected from feeding chambers A and B during all drop-off periods, with the exception of the 3rd period in animals with vascular lesions. The endpoint titre was 32,768 and, as mentioned for capybara 4, none of the animals infested with ticks collected during the subsequent challenge (phase IV) manifested clinical or serological reactivity (Table 1).

Some images illustrating vascular lesions observed during rabbit infestations have been included as supplementary material (supplementary Figures 1 and 2).

4.3.2 Real-time PCR

Based on data of probable rickettsemic period on capybaras, derived from guinea pig inoculation results described in a previous study (Ramírez-Hernández et al., not published), samples of ticks collected during primoinfection of all capybaras, between

6 and 17 DPI (sampling periods II and III for DNA extraction), were submitted to DNA extraction and real-time PCR amplification, as previously described. As summarized in Table 2, generally, *Rickettsia* DNA was detected in molted nymphs and adults collected during primoinfection of all capybaras and only in the challenge of capybara 5.

For capybara 1, DNA was detected only in adults (5.0-9.1%) collected from feeding-chambers A and B. In capybara 2, DNA was detected in all tick groups and the frequency of positive samples ranged from 4.6 to 9.1% in nymphs and from 35.0 to 40.0% in adults from both feeding chambers. As in the latter, DNA was detected in all tick groups from capybara 3 with proportions that ranged between 28.6-30.0% and 73.7-100.0% in nymphs and adults, respectively; conversely, only adults (10.5%) from capybara 4 (feeding chamber B) were positive. Finally, nymphs (feeding chamber B) and adults (both feeding chambers) from capybara 5 were positive with rates of 10.0% and 5.3-40.9%, respectively. In addition, it is worthy of note the presence of positive (Real-Time CT = 35) ticks (adults) collected from this capybara during the second infection in the feeding chamber A (35%), when non-infected nymphs had fed with infected adults.

4.4 DISCUSSION

The present study describes the results obtained in experimental transmission of *R. rickettsii* (strain Itu) from infected capybaras to *A. sculptum* ticks, under controlled conditions, and the subsequent evaluation of vectorial competence of these infected ticks through tick-naïve rabbit infestations. Clinical, serological and haematological findings of infected capybaras have been detailed in a separate paper (Ramírez-Hernández et al., not published). Two early studies related infection of adult *A. sculptum* ticks (reported in both as *A. cajennense*) from capybaras infected with a *R. rickettsii* strain derived from *A. aureolatum* (reported as *Amblyomma striatum* in one of these works) (Travassos and Vallejo, 1942b; Souza et al., 2009). Contrastingly with the mentioned studies, herein, we attempted to analyse not only infection on molted adults but also on molted nymphs, and, through simultaneous infestations in two feeding chambers (one with infected adults plus uninfected larvae and nymphs, and another only with uninfected larvae and nymphs), the dynamics of horizontal (systemic

and non-systemic) *R. rickettsii* transmission between ticks simultaneously feeding on a rickettsemic host, as formerly performed (Moraes-Filho et al., 2018).

Rabbits infested with ticks detached from capybara 3, used as control animal (phase I) did not exhibit clinical signs or serologic reactivity. Conversely, animals infested with molted adults and nymphs collected during primoinfection of capybaras 1 to 5, exhibited fever, clinical signs and serologic responses. Moreover, feeding periods for those ticks collected during drop-off periods I to III, in the previous phase, were within rickettsemic periods established for infected capybaras, which ranged between 6 and 20 DPI. In addition, infected rabbits were infested with molted ticks detached from both feeding chambers, demonstrating systemic horizontal transmission during host rickettsemia. Similarly, Travassos et al. (1942b) achieved infection of *A. sculptum* adults through feeding in infected capybaras between 5 to 11 DPI and checked transmission competence by infesting guinea pigs 6 days after collection; they succeeded in the animal infection and corroborated an intraestadial transmission in that tick population, without performing observations of transovarian and transestadial maintenance. By comparison, Souza et al. (2009), succeeded in infection of nymphs (*A. sculptum*) recovered between 6 and 18 DPI (within capybaras' rickettsemic period) and further examined molted adults through real-time PCR, confirming transestadial perpetuation but without performing transmission trials in susceptible hosts.

In his classical work, Ricketts (1907) postulated the two main mechanisms of tick infection with *R. rickettsii*: 1) simultaneous feeding of infected and non-infected ticks in a susceptible host (systemic horizontal transmission) and 2) transovarian passage from infected females to their progeny (vertical transmission) (Burgdorfer, 1988). As stated before, herein, it was possible to confirm *R. rickettsii* transmission in rickettsemic capybaras through simultaneous feeding of infected adults (males and females) and non-infected larvae and nymphs, in close and distant feeding sites. Rabbit infestation results obtained with ticks collected from capybaras 1, 4 and 5, during subsequent challenges (phase II to IV), evidenced an absence of *R. rickettsii* transmission due to lack of clinical signs and antibody response in those animals. These findings could be correlated with the immune status acquired by capybaras after primoinfection (detailed in a separate paper), an absence of *Rickettsia* circulation and ensuing lack of tick infection. It has been postulated that recovered or immune animals do not contribute

to the natural maintenance of *R. rickettsii* in nature (Philip, 1959; Lundgren and Thorpe, 1966) which have been corroborated in experimental studies with guinea pigs and *A. aureolatum* ticks (Moraes-Filho et al., 2018).

Additional to the systemic mechanism of transmission of *R. rickettsii* in ticks simultaneously feeding in a rickettsemic host, a non-systemic way could contribute for the pathogen acquisition when two individuals are feeding side by side, sharing saliva content, as has been confirmed for Tick-borne encephalitis virus (with *Rhipicephalus appendiculatus*) and *R. rickettsii* (with *A. aureolatum*) in guinea pigs (Labuda et al., 1993; Moraes-Filho et al., 2018). In the present study, we attempted to assess this phenomenon in immune capybaras through simultaneous infestations of infected adults with non-infected larvae and nymphs in the same feeding chamber (i.e. chamber A) and comparing it with non-infected larvae and nymphs in a separate chamber (i.e. chamber B). Results derived from rabbit infestations with molted ticks obtained from these chambers evidenced no clinical signs nor immune reactivity in animals, suggesting an absence of *R. rickettsii* transmission.

In general, *Rickettsia* DNA detection in ticks through real-time PCR confirmed the results evidenced in rabbit infestation tests. DNA was detected in molted adults and nymphs collected, from both feeding chambers, during the first infection of capybaras 1 to 5. These evidences corroborate an efficient systemic transmission between ticks during rickettsemic capybara period (primoinfection) with variable infection rates, ranging from 4.6 to 30.0% in nymphs and from 5.0 to 100.0% in adults. It is worthy to note that the highest rates were evidenced in ticks collected from capybaras 2 and 3, which could be correlated with high bacterial loads, as previously reported (Horta et al., 2009). In the earlier study of Souza et al. (2009), capybaras infected through infected-tick and intraperitoneal inoculum, were capable to infect 20-25% and 30-35% of molted adult ticks, respectively. Differences between studies could be related with initial infective dose and individual biological response in each capybara.

Rickettsia DNA amplification was absent in tick samples from capybaras 1, 4 and 5 during subsequent challenges, confirming a lack of transmission to ticks in immune or convalescent hosts. However, adults collected (as nymphs) from feeding chamber A during the challenge of capybara 5 (immune) yielded *Rickettsia* DNA. In spite of this

finding, none of the rabbits infested with these ticks presented clinical or serological reactions associated with *R. rickettsii* infection, thus confirming absence of vectorial competence. Those adults fed (as nymphs) in the same chamber with infected adults, indicating a probable non-systemic horizontal transmission between ticks. Nonetheless, the lack of pathogen transmission to susceptible rabbits ratified that solely DNA amplification is an improper criterium to inferred this tick transmission route as stated elsewhere (Moraes-Filho et al., 2018).

In conclusion, capybaras susceptible to infection with strain Itu of *R. rickettsii*, can infect *A. sculptum* larvae and nymphs (during rickettsemic period) which maintain the pathogen transstadially and are capable to transmit it to susceptible rabbits in the subsequent stage (nymphs and adults, respectively). By contrast, immune capybaras do not infect *A. sculptum* larvae and nymphs feeding on them, after subsequent *R. rickettsii* challenges. Finally, a probable non-systemic horizontal transmission route in immune hosts, that could pass bacteria between adjacent co-feeding ticks, seems to be irrelevant in the context of Brazilian Spotted Fever epidemiology. Future studies must clarify this assumption.

4.5 REFERENCES

Angerami, R.N., da Silva, A.M., Nascimento, E.M., Colombo, S., Wada, M.Y., dos Santos, F.C., Mancini, D.M., de Oliveira, R.C., Katz, G., Martins, E.C., da Silva, L.J., 2009. Brazilian spotted fever: two faces of a same disease? A comparative study of clinical aspects between an old and a new endemic area in Brazil. Clin Microbiol Infect 15 Suppl 2, 207-208.

Angerami, R.N., Resende, M.R., Feltrin, A.F.C., Katz, G., Nascimento, E.M., Stucchi, R.S.B., Silva, L.J., 2006. Brazilian Spotted Fever: A Case Series from an Endemic Area in southeastern Brazil: Epidemiological Aspects. Annals of the New York Academy of Sciences 1078, 170-172.

Burgdorfer, W., 1988. Ecological and epidemiological considerations of Rocky Mountain spotted fever and scrub typhus, In: Walker, D. (Ed.) Biology of Rickettsial Diseases. CRC Inc. Boca Raton, Florida, pp. 33-50.

Burgdorfer, W., Brinton, L.P., 1975. Mechanisms of transovarial infection of spotted fever Rickettsiae in ticks. Ann N Y Acad Sci 266, 61-72.

Burgdorfer, W., Cooney, J.C., Mavros, A.J., Jellison, W.L., Maser, C., 1980. The role of cottontail rabbits (*Sylvilagus* spp.) in the ecology of *Rickettsia rickettsii* in the United States. Am J Trop Med Hyg 29, 686-690.

Burgdorfer, W., Friedhoff, K.T., Lancaster, J.L., Jr., 1966. Natural history of tick-borne spotted fever in the USA. Susceptibility of small mammals to virulent *Rickettsia rickettsii*. Bull World Health Organ 35, 149-153.

de Oliveira, S.V., Guimaraes, J.N., Reckziegel, G.C., Neves, B.M., Araujo-Vilges, K.M., Fonseca, L.X., Pinna, F.V., Pereira, S.V., de Caldas, E.P., Gazeta, G.S., Gurgel-Goncalves, R., 2016. An update on the epidemiological situation of spotted fever in Brazil. The journal of venomous animals and toxins including tropical diseases 22, 22.

Demma, L.J., Eremeeva, M., Nicholson, W.L., Traeger, M., Blau, D., Paddock, C., Levin, M., Dasch, G., Cheek, J., Swerdlow, D., McQuiston, J., 2006. An outbreak of Rocky Mountain Spotted Fever associated with a novel tick vector, *Rhipicephalus sanguineus*, in Arizona, 2004: preliminary report. Ann N Y Acad Sci 1078, 342-343.

Flecknell, P., 2009. Laboratory Animal Anaesthesia, Third Edition. Academic Press, 300 p.

Gerardi, M., 2016. Perfil genético e susceptibilidade de diferentes populações do carrapato *Amblyomma sculptum* à infecção pelo patógeno *Rickettsia rickettsii*. Ph.D. Universidade de São Paulo, São Paulo.

Horta, M.C., Moraes-Filho, J., Casagrande, R.A., Saito, T.B., Rosa, S.C., Ogrzewalska, M., Matushima, E.R., Labruna, M.B., 2009. Experimental infection of opossums *Didelphis aurita* by *Rickettsia rickettsii* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense*. Vector Borne Zoonotic Dis 9, 109-118.

Horta, M.C., Pinter, A., Cortez, A., Soares, R.M., Gennari, S.M., Schumaker, T.T.S., Labruna, M.B., 2005. *Rickettsia felis* (Rickettsiales: Rickettsiaceae) in *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) in the State of Sao Paulo, Brazil. Arq Bras Med Vet Zootec 57, 321-325.

Krawczak, F.S., Nieri-Bastos, F.A., Nunes, F.P., Soares, J.F., Moraes-Filho, J., Labruna, M.B., 2014. Rickettsial infection in *Amblyomma cajennense* ticks and capybaras (*Hydrochoerus hydrochaeris*) in a Brazilian spotted fever-endemic area. Parasit Vectors 7, 7.

Labruna, M.B., 2009. Ecology of Rickettsia in South America. Annals of the New York Academy of Sciences 1166, 156-166.

Labruna, M.B., 2013. Brazilian spotted fever: the role of capybaras, In: Moreira, J., P.M.B. Ferraz, K., Herrera, E., Macdonald, D. (Eds.) Capybara. Springer, pp. 371-383.

Labruna, M.B., Horta, M.C., Aguiar, D.M., Cavalcante, G.T., Pinter, A., Gennari, S.M., Camargo, L.M., 2007. Prevalence of *Rickettsia* infection in dogs from the urban and rural areas of Monte Negro municipality, western Amazon, Brazil. Vector Borne Zoonotic Dis 7, 249-255.

Labruna, M.B., Ogrzewalska, M., Martins, T.F., Pinter, A., Horta, M.C., 2008. Comparative susceptibility of larval stages of *Amblyomma aureolatum*, *Amblyomma cajennense*, and *Rhipicephalus sanguineus* to infection by *Rickettsia rickettsii*. J Med Entomol 45, 1156-1159.

Labruna, M.B., Pinter, A., Teixeira, R.H.F., 2004a. Life cycle of *Amblyomma cooperi* (Acari : Ixodidae) using capybaras (*Hydrochaeris hydrochaeris*) as hosts. Experimental and Applied Acarology 32, 79-88.

Labruna, M.B., Whitworth, T., Horta, M.C., Bouyer, D.H., McBride, J.W., Pinter, A., Popov, V., Gennari, S.M., Walker, D.H., 2004b. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. J Clin Microbiol 42, 90-98.

Labuda, M., Danielova, V., Jones, L.D., Nuttall, P.A., 1993. Amplification of tick-borne encephalitis virus infection during co-feeding of ticks. Medical and Veterinary Entomology 7, 339-342.

Lundgren, D.L., Thorpe, B.D., 1966. Infectious diseases in wild animals in Utah. VII. Experimental infection of rodents with *Rickettsia rickettsii*. Am J Trop Med Hyg 15, 799-806.

Monteiro, J.L., 1933. Comportamento experimental do Coelho aos virus do "Typho Exanthemático de S. Paulo" e da febre maculosa das Montanhas Rochosas. Memórias do Instituto Butantan 8, 3-8.

Moraes-Filho, J., Costa, F.B., Gerardi, M., Soares, H.S., Labruna, M.B., 2018. *Rickettsia rickettsii* Co-feeding Transmission among *Amblyomma aureolatum* Ticks. Emerging Infectious Diseases 24, 2041-2048.

Niebylski, M., Peacock, M., Schwan, T., 1999. Lethal Effect of *Rickettsia rickettsii* on Its Tick Vector (*Dermacentor andersoni*). Applied and Environmental Microbiology 65, 773-778.

Norment, B.R., Burgdorfer, W., 1984. Susceptibility and reservoir potential of the dog to spotted fever-group rickettsiae. Am J Vet Res 45, 1706-1710.

Parola, P., Paddock, C.D., Socolovschi, C., Labruna, M.B., Mediannikov, O., Kernif, T., Abdad, M.Y., Stenos, J., Bitam, I., Fournier, P.E., Raoult, D., 2013. Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev 26, 657-702.

Philip, C.B., 1959. Some epidemiological considerations in Rocky Mountain spotted fever. Public Health Rep 74, 595-600.

Pinter, A., Labruna, M.B., 2006. Isolation of *Rickettsia rickettsii* and *Rickettsia bellii* in Cell Culture from the Tick *Amblyomma aureolatum* in Brazil. Annals of the New York Academy of Sciences 1078, 523-529.

Pinter, A., Labruna, M.B., Faccini, J.L., 2002. The sex ratio of *Amblyomma cajennense* (Acari: Ixodidae) with notes on the male feeding period in the laboratory. Vet Parasitol 105, 79-88.

Piranda, E.M., Faccini, J.L., Pinter, A., Pacheco, R.C., Cancado, P.H., Labruna, M.B., 2011. Experimental Infection of *Rhipicephalus sanguineus* Ticks with the Bacterium *Rickettsia rickettsii*, Using Experimentally Infected Dogs. Vector Borne Zoonotic Dis 11, 29-36.

Ricketts, H.T., 1907. Further experiments with the wood tick in relation to Rocky Mountain spotted fever. J. Amer. Med. Ass. 49, 1278-1281.

Sangioni, L.A., Horta, M.C., Vianna, M.C., Gennari, S.M., Soares, R.M., Galvao, M.A., Schumaker, T.T., Ferreira, F., Vidotto, O., Labruna, M.B., 2005. Rickettsial infection in animals and Brazilian spotted fever endemicity. Emerg Infect Dis 11, 265-270.

Soares, J.F., Soares, H.S., Barbieri, A.M., Labruna, M.B., 2012. Experimental infection of the tick *Amblyomma cajennense*, Cayenne tick, with *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever. Med Vet Entomol 26, 139-151.

Souza, C.E., Moraes-Filho, J., Ogrzewalska, M., Uchoa, F.C., Horta, M.C., Souza, S.S., Borba, R.C., Labruna, M.B., 2009. Experimental infection of capybaras *Hydrochoerus hydrochaeris* by *Rickettsia rickettsii* and evaluation of the

transmission of the infection to ticks *Amblyomma cajennense*. Vet Parasitol 161, 116-121.

Szabo, M.P., Pinter, A., Labruna, M.B., 2013. Ecology, biology and distribution of spotted-fever tick vectors in Brazil. Front Cell Infect Microbiol 3, 27.

Tinoco-Gracia, L., Lomeli, M.R., Hori-Oshima, S., Stephenson, N., Foley, J., 2018. Molecular Confirmation of Rocky Mountain Spotted Fever Epidemic Agent in Mexicali, Mexico. Emerg Infect Dis 24, 1723-1725.

Travassos, J., Vallejo, A., 1942a. Comportamento de alguns cavídeos (*Cavia aperea* e *Hydrochoerus capybara*) às inoculações experimentais do vírus da febre maculosa. Possibilidade desses cavídeos representarem o papel de depositários transitórios do vírus na natureza. Mem. Inst. Butantã 15, 73-86.

Travassos, J., Vallejo, A., 1942b. Possibilidade de *Amblyomma cajennense* se infectar em *Hydrochoerus capybara* experimentalmente inoculado com o vírus da febre maculosa. Mem. Inst. Butantã 15, 87-90.

Ueno, T.E., Costa, F.B., Moraes-Filho, J., Agostinho, W.C., Fernandes, W.R., Labruna, M.B., 2016. Experimental infection of horses with *Rickettsia rickettsii*. Parasit Vectors 9, 499.

Walker, D., 2002. *Rickettsia rickettsii*: As Virulent as Ever. Am J Trop Med Hyg 66, 448-449.

Table 1. New Zealand white rabbits infested with *Amblyomma sculptum* ticks collected (in the previous stage) from capybaras (*Hydrochoerus hydrochaeris*) during multiple infections with *Rickettsia rickettsii* (strain Itu) via tick exposure. Data are presented as: no. of affected animals/total no. of infested animals (%)

	Febrile animals*				Animals with vascular signs**				IFA				
Capybara No.	No.	Stage	Feeding chamber	Drop-off period	No.	Stage	Feeding chamber	Drop-off period	No. of positive samples	Stage	Feeding chamber	Drop-off period	Endpoint titer
	Phase I												
1	3/12 (25.0)	N, Ad	A, B	I, II	2/12 (16.7)†	N	B	I, II	2/12 (16.7)	N	A, B	I, III	65,536
3	0/6 (0)	-	-	-	0/6 (0)	-	-	-	0/6 (0)	-	-	-	-
	Phase II												
1	0/12 (0)	-	-	-	0/12 (0)	-	-	-	0/12 (0)	-	-	-	-
2	4/8 (50.0)	N, Ad	A, B	II	3/8 (37.5)	N, Ad	A, B	II	4/8 (50.0)	N, Ad	A, B	II	32,768
3	7/8 (87.5)	N, Ad	A, B	I, II	7/8 (87.5)	N, Ad	A, B	I, II	8/8 (100.0)	N, Ad	A, B	I, II	65,536
	Phase III												
1	0/12 (0)	-	-	-	0/12 (0)	-	-	-	0/12 (0)	-	-	-	-
4	1/12 (8.3)	N	B	II	1/12 (8.3)	N	B	II	2/12 (16.7)	N	A, B	II, III	16,384
5	4/12 (33.3)	N, Ad	A, B	I, II, III	3/12 (25.0)	N, Ad	A, B	I, II	6/12 (50.0)	N, Ad	A, B	I, II, III	32,768
	Phase IV												
1	1/11 (9.1)	Ad	B	I	0/11 (0)	-	-	-	0/11 (0)	-	-	-	-
4	0/11 (0)	-	-	-	0/11 (0)	-	-	-	0/11 (0)	-	-	-	-
5	0/11 (0)	-	-	-	0/11 (0)	-	-	-	0/11 (0)	-	-	-	-

Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5.

Stage: Nymphs (N), Adults (Ad)

Drop-off period in the previous stage during capybara infection: period I (0-10 DPI), period II (11-20 DPI), period III (21-30 DPI)

* Two or more consecutive days with rectal temperature ≥ 40 ºC

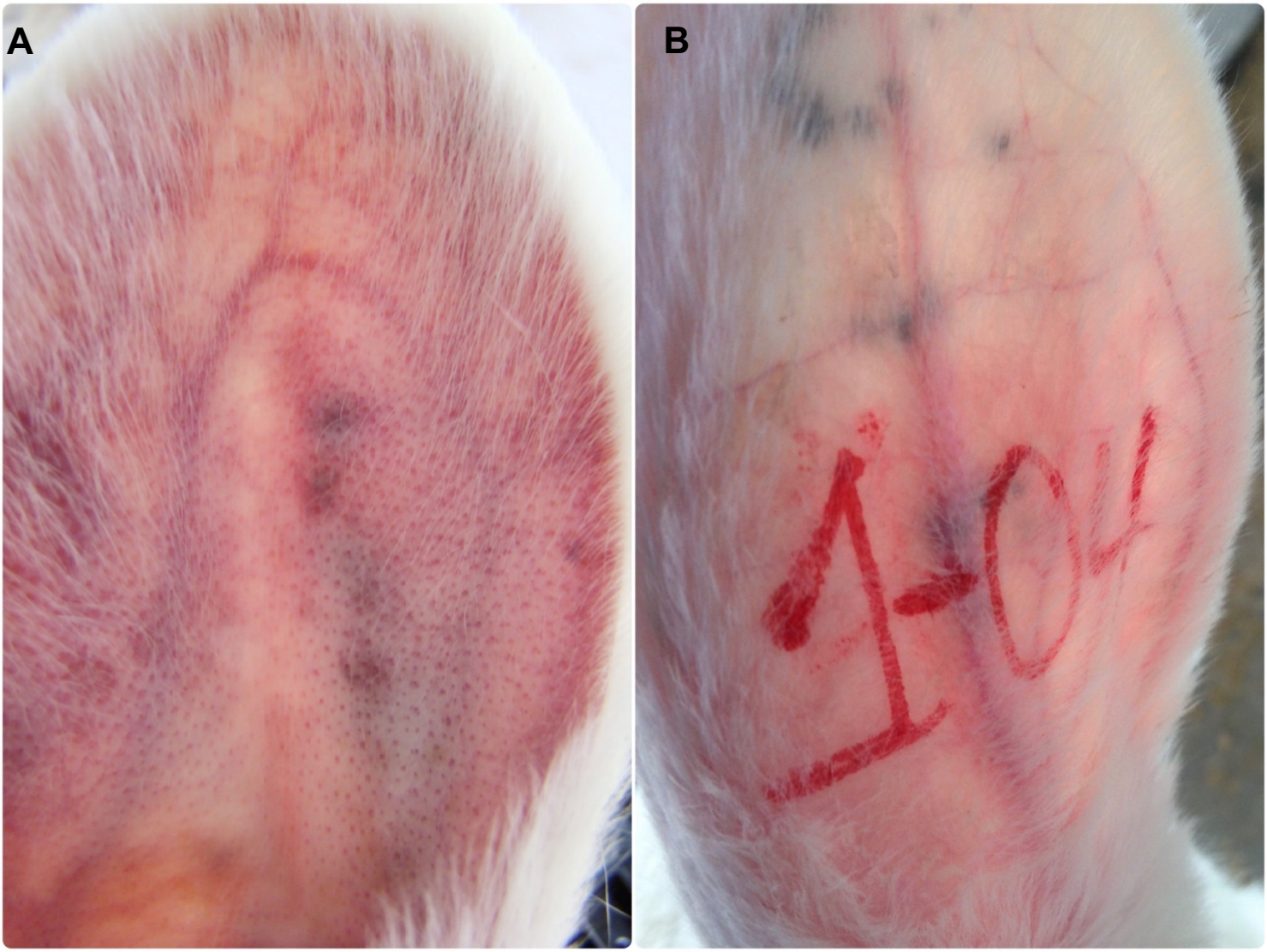
** Vascular signs like edema, erythema and necrosis

†One dead animal (stage, nymphs; feeding chamber, B; drop-off period, II)

Table 2. *Rickettsia* real-time PCR results of *Amblyomma sculptum* ticks collected (in the previous stage) from capybaras (*Hydrochoerus hydrochaeris*) during multiple infections with *Rickettsia rickettsii* (strain Itu) via tick exposure. Data are presented as no. of positive ticks/total no. of ticks sampled (%)

		Phase							
		I		II		III		IV	
		Tick stage							
Capybara No.	Feeding chamber	N	Ad	N	Ad	N	Ad	N	Ad
1	A	0/14 (0)	1/20 (5.0)	0/20 (0)	0/13 (0)	0/18 (0)	0/14 (0)	0/20 (0)	0/20 (0)
	B	0/20 (0)	2/22 (9.1)	0/21 (0)	0/20 (0)	0/15 (0)	0/20 (0)	0/12 (0)	0/19 (0)
2	A	-	-	1/22 (4.6)	8/20 (40.0)	-	-	-	-
	B	-	-	2/22 (9.1)	7/20 (35.0)	-	-	-	-
3	A	-	-	6/21 (28.6)	15/15 (100.0)	-	-	-	-
	B	-	-	6/20 (30.0)	14/19 (73.7)	-	-	-	-
4	A	-	-	-	-	0/21 (0)	0/16 (0)	0/14 (0)	0/20 (0)
	B	-	-	-	-	0/20 (0)	2/19 (10.5)	0/12 (0)	0/21 (0)
5	A	-	-	-	-	0/20 (0)	1/19 (5.3)	0/20 (0)	7/20 (35)
	B	-	-	-	-	2/20 (10.0)	9/22 (40.9)	0/8 (0)	0/20 (0)

Phase I, primoinfection of capybara no. 1 and capybara no. 3 used as no infection control; phase II, second infection of capybara no. 1 and primoinfection of capybaras no. 2 and 3; phase III, third infection of capybara no. 1 and primoinfection of capybaras no. 4 and 5; phase IV, fourth infection of capybara no. 1 and second infection of capybaras no. 4 and 5.



Supplemental Figure 1. Focal necrotic lesions in ears from rabbits infested with *Amblyomma sculptum* ticks collected (in the previous stage) from capybaras (*Hydrochoerus hydrochaeris*) during primoinfection with *Rickettsia rickettsii* (strain Itu). A. Rabbit no. 3-07 infested with molted nymphs from capybara no. 3 (feeding chamber A, drop-off period 1) B. Rabbit no. 1-04 infested with molted nymphs collected from capybara no. 1 (feeding chamber B, drop-off period 1).

Supplemental Figure 2. Genital lesions in rabbits infested with *Amblyomma sculptum* ticks collected (in the previous stage) from capybaras (*Hydrochoerus hydrochaeris*) during primoinfection with *Rickettsia rickettsii* (strain Itu). A. Scrotal edema and focal necrosis (arrow) in rabbit no. 5-03 infested with molted nymphs collected in the primoinfection of capybara no. 5 (feeding chamber A, drop-off period 2) B. Bilateral necrotic scrotal lesions in rabbit no. 5-10 infested with molted adults collected in the primoinfection of capybara no. 5 (feeding chamber B, drop-off period 2). C. Preputial edema in rabbit no. 3-12 infested with molted adults from capybara no. 3 (feeding chamber B, drop-off period 1)

5 CONCLUSIONS

Herein, it has been confirmed capybara susceptibility to infection (and the resulting disease) with the strain Itu of *R*. *rickettsii*, derived from *A*. *sculptum* ticks. In spite of a similar infection dynamic in comparison with strain Taiaçu, derived from *A*. *aureolatum*, mainly in haematological and serological parameters, duration of rickettsemic period and transmissibility to non-infected *A*. *sculptum* ticks, some biological differences have been registered. It is noticeable that capybara susceptibility could be dose-dependent, and in the case of high bacterial loads, transmitted by ticks, some clinical abnormalities could arise (e.g. fever, skin rash, general weakness, anorexia, diarrhoea) and even lead to a fatal outcome with exhibition of post-mortem disseminated vascular gross lesions. This clinical and pathological picture is comparable with those broadly described in other susceptible species (e.g. guinea pigs, rabbits, dogs and humans). Besides, the mean rickettsemic period, calculated by results of guinea pig inoculation, was 9.2 days (range: 6-12 days), period in which non-infected *A*. *sculptum* ticks (larva and nymphs) feeding simultaneously with infected ticks (adults) acquired *R*. *rickettsii* and maintain it through transestadial transmission, corroborating an efficient systemic horizontal transmission route between adjacent and non-adjacent co-feeding ticks. New-infected nymphs and adults (infected in the previous stage) could transmit viable *R*. *rickettsii* to susceptible rabbits, which exhibit typical clinical signs and serological reactivity. Herein, it also has been registered higher tick infection rates, in comparison with those previously reported with strain Taiaçu, probably associated with elevated circulating *R*. *rickettsii* loads in blood of infected capybaras. Finally, here it has been corroborated that immune or convalescent capybaras did not develop new rickettsemic periods after subsequent infection challenges, as was previously hypothesized, and consequently, there is an absence of *R*. *rickettsii* transmission to non-infected ticks feeding on them. Nonetheless, a probable non-systemic horizontal transmission route between adjacent co-feeding ticks, sharing saliva content, could pass bacteria; but, due to absence of vectorial competence of this new-infected co-feeding ticks, we can infer that it is of minimum relevance for *R*. *rickettsii* preservation in natural conditions.