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Experimental infection with *Rickettsia rickettsii* in an *Amblyomma dubitatum* tick colony, naturally infected by *Rickettsia bellii*

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ABSTRACT

Amblyomma dubitatum engorged females, naturally infected by *Rickettsia bellii*, were used to establish a laboratory colony. Larvae, nymphs, and adults were exposed to two strains of *Rickettsia rickettsii* by feeding on needle-inoculated guinea pigs, and thereafter reared on uninfected guinea pigs. After acquisition feeding, engorged larvae and nymphs molted to nymphs and adults, respectively, which were shown to be infected (confirming transstadial perpetuation), and were able to transmit both strains of *R. rickettsii* to uninfected animals, as demonstrated by clinical, serological, and molecular analyses. However, the larval, nymphal, and adult stages of *A. dubitatum* showed to be only partially susceptible to *R. rickettsii* infection, since in all cases, only part of the ticks became infected by this agent, after being exposed to rickettsemic animals. While transovarial transmission of *R. rickettsii* was inefficient in the *A. dubitatum* engorged females of the present study, 100% of these females passed *R. bellii* transovarially. Because it has been reported that a primary infection by a *Rickettsia* species would preclude transovarial transmission of a second *Rickettsia* species, it is likely that the ineffectiveness of *A. dubitatum* to perpetuate *R. rickettsii* by transovarial transmission was related to its primary infection by *R. bellii*; however, it could also be related to unknown factors inherent to *A. dubitatum.* The relevance of *A. dubitatum* as a natural vector of *R. rickettsii* to humans or animals is discussed.

Introduction

The genus *Rickettsia* comprises Gram-negative, coco-bacilli bacteria that multiply exclusively within eukaryotic cells. Many *Rickettsia* species cause diseases in humans and animals, to which they are vectored by lice, fleas, ticks, or mites (Merhej and Raoult, 2011). Traditionally, *Rickettsia* species have been classified into two groups: the typhus group (TG), containing *Rickettsia prowazekii* and *Rickettsia typhi*, which are transmitted by lice and fleas, respectively, and the spotted-fever group (SFG), containing >20 species, most of which have tick vectors (Parola et al., 2005). Other rickettsiae, such as *Rickettsia bellii*, the most common *Rickettsia* species reported in ticks from the New World, have shown antigenic and genetic particularities that preclude their inclusion in either the TG or SFG (Parola et al., 2013).

The bacterium *Rickettsia rickettsii* is the etiological agent of Rocky Mountain spotted fever (RMSF), the most severe spotted fever of the world, with confirmed occurrence in Canada, United States, Mexico, Costa Rica, Panama, Colombia, Argentina, and Brazil (Parola et al., 2013). In Brazil, the disease is usually referred to as Brazilian spotted fever, with current case fatality rate of 20–40% (Del Fiol et al., 2010). Different tick species have been implicated as vectors of *R. rickettsii* for humans. Whereas, the ticks *Dermacentor andersoni* and *Dermacentor variabilis* are the main vectors in the United States, ticks of the *Amblyomma cajennense* complex are the most common vectors in Central and South America, including Brazil (Parola et al., 2013). Other tick species have been incriminated as vectors in some particular areas, such as *Rhipicephalus sanguineus* in northern Mexico and Arizona (Demma et al., 2005; Eremeeva et al., 2011), *Amblyomma americanum* in North Carolina (Breitschwerdt et al., 2011), and *Amblyomma aureolatum* is the metropolitan area of São Paulo, southeastern Brazil (Ogrzewalska

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et al., 2012). In Costa Rica, a high virulent strain of *R. rickettsii* was isolated from the rabbit tick *Haemaphysalis leporispalustris*, which usually does not bite humans (Fuentes et al., 1985; Hun et al., 2008). As such, *H. leporispalustris* has been treated as a potential enzootic vector of *R. rickettsii* in nature (Fuentes et al., 1985).

The capybara tick, *Amblyomma dubitatum*, is widely distributed in Argentina, Brazil, Paraguay and Uruguay, where capybaras (*Hydrochoerus hydrochaeris*) serve as the main host for both immature (larvae, nymphs) and adult stages of this tick species (Nava et al., 2010). Recent studies in Brazil have reported different *A. dubitatum* populations to be infected by distinct *Rickettsia* species. Labruna et al. (2004) found that 40 and 7.5% of an *A. dubitatum* population in the state of São Paulo were infected by *R. bellii* and a *Rickettsia parkeri*-like agent (strain Cooperi), respectively. A *Rickettsia tamurae*-like agent (strain Pampulha) was found infecting 70.6–100% of the ticks from two *A. dubitatum* populations in the states of Minas Gerais and Rio de Janeiro (Almeida et al., 2011; Spolidorio et al., 2012). Finally, one study in the state of São Paulo showed that among 16 populations of *A. dubitatum*, 10 (62.5%) were infected by *R. bellii*, with infection rates varying from 6.1 to 44.9% (Pacheco et al., 2009).

Many RMSF-endemic areas in southeastern Brazil are characterized by the presence of overgrown populations of capybaras that sustain sympatric populations of *A. cajennense* and *A. dubitatum* (Perez et al., 2008; Brites-Neto et al., 2013; Krawczak et al., 2014). Because *A. cajennense* is a natural vector of *R. rickettsii* in these areas, and the capybaras are considered an efficient source of infected blood meal for *A. cajennense* ticks (Souza et al., 2009), natural contact of *A. dubitatum* with *R. rickettsii*-infected hosts is likely to occur in these areas. However, infection of *A. dubitatum* with *R. rickettsii* has never been reported. Therefore, the present study performed experimental infection of *A. dubitatum* ticks with two different strains of *R. rickettsii* from Brazil, in order to evaluate the vector competence of the tick for the agent of RMSF, and the maintenance of the bacterium by transstadial and transovarial perpetuation within the tick population. The whole study was performed with a tick colony originated from engorged females that were naturally infected by *R. bellii.*

Materials and methods

Ticks

Ten engorged females of *A. dubitatum* were collected from a naturally infested capybara in Jambeiro Municipality (23°15′16″S, 45°41′37″W), state of São Paulo, southeastern Brazil. Females were taken to the laboratory and held in an incubator set at 27 °C, 85% RH, and scotophase, where they laid eggs, from which hatched larvae were used to start the present study. In order to check the rickettsial infection status of the engorged females (tested at the end of oviposition) and their offspring, they were tested by two PCR protocols, one specific for *R. bellii*, and one specific for the SFG rickettsiae (protocols described below). Throughout the present study, tick's free-living stages were always held in a single incubator under the same conditions cited above.

R. rickettsii strains

Experimental infections were performed in parallel with two strains (Itu and Taiaçu) of *R. rickettsii.* Strain Itu was originally isolated from an *A. cajennense* tick pool from Itu (23°16′S, 47°19′W), state of São Paulo through the inoculation of guinea pigs with infected tick homogenate and subsequently adaptation of the strain to Vero cell culture (Krawczak et al., 2014). Strain Taiaçu was originally isolated from an *A. aureolatum* tick from Mogi das Cruzes (23°38′S, 46°11′W), state of São Paulo through the inoculation of guinea pigs with infected tick homogenate and subsequently adaptation of the strain to Vero cell culture (Pinter and Labruna, 2006). Only the guinea pig lineages of both strains (never in vitro cultured) were used in the present study. For this purpose, guinea pig infected organs were thawed at room temperature, crushed in a mortar with brain–heart infusion (BHI), and the resultant homogenate was used to inoculate guinea pigs intraperitoneally, as described below. All procedures described below were done in parallel with strains Itu and Taiaçu.

Animals

Guinea pigs (*Cavia porcellus*) used in the study were adult males and females, 60–90 days old. All animals were obtained from an animal room with no history of tick infestation or tick-borne disease or any contact with acaricides or antibiotic drugs. During the experiment, animals were fed with commercial pellets and water *ad libitum*. Care was taken to assure that animals were not having any contact with antibiotic or acaricide during the experiment. This study was previously approved by the Ethical Committee on Animal Research of the Federal Rural University of Rio de Janeiro (protocol number 376/2013).

Tick infestations

Tick infestations on guinea pigs were performed inside white cotton sleeves (10–15 cm diameter) that were glued to the shaved back of each animal, as previously described (Pinter et al., 2002; Labruna et al., 2011). Ticks (larvae, nymphs, or adults 20–30 days old) were released into the sleeve. Larval infestations consisted of approximately 1000–2500 larvae per guinea pig; nymphal infestations consisted of 250 nymphs per guinea pig, and adult infestations consisted of 5–15 couples per guinea pig. The sleeves were opened daily, and the detached engorged ticks were removed, and immediately taken to the incubator where molting (for engorged larvae and nymphs) or oviposition (for engorged females) was observed daily. Engorged females were individually weighed in an electronic balance (precision of 0.001 g) at the detachment day.

Experimental protocol

The following procedures with ticks exposed to *R. rickettsii* were done in parallel and independently for strains Itu and Taiaçu. Two guinea pigs were each inoculated intraperitoneally with a *R. rickettsii* strain. The four inoculated animals (2 per rickettsial strain) as well as five other uninfected control guinea pigs were each infested with *A. dubitatum* larvae derived from the field-collected engorged females. Engorged larvae recovered in guinea pig infestations were used to form two independent experimental groups: group GL, consisting of ticks exposed to *R. rickettsii* during the larval stage; and the control group (CG), consisting of larvae not exposed to *R. rickettsii.* Engorged larvae recovered in these infestations were held in the incubator for molting. The resultant nymphs were used to infest new guinea pigs as follows: GL nymphs (previously exposed to *R. rickettsii* during the larval stage) were allowed to infest two uninfected guinea pigs; part of the CG nymphs were used to infest six uninfected guinea pigs (keeping the status of *R. rickettsii*-free control group), and another part of CG nymphs were used to infest two guinea pigs inoculated intraperitoneally with a *R. rickettsii* strain. These last nymphs formed the group GN, consisting of ticks exposed to *R. rickettsii* during the nymphal stage. Engorged nymphs recovered in these infestations were held in the incubator for molting. The resultant adults were used to infest guinea pigs as follows: GL adults (previously exposed to *R. rickettsii* during the larval stage) were allowed to infest three uninfected guinea pigs; GN adults

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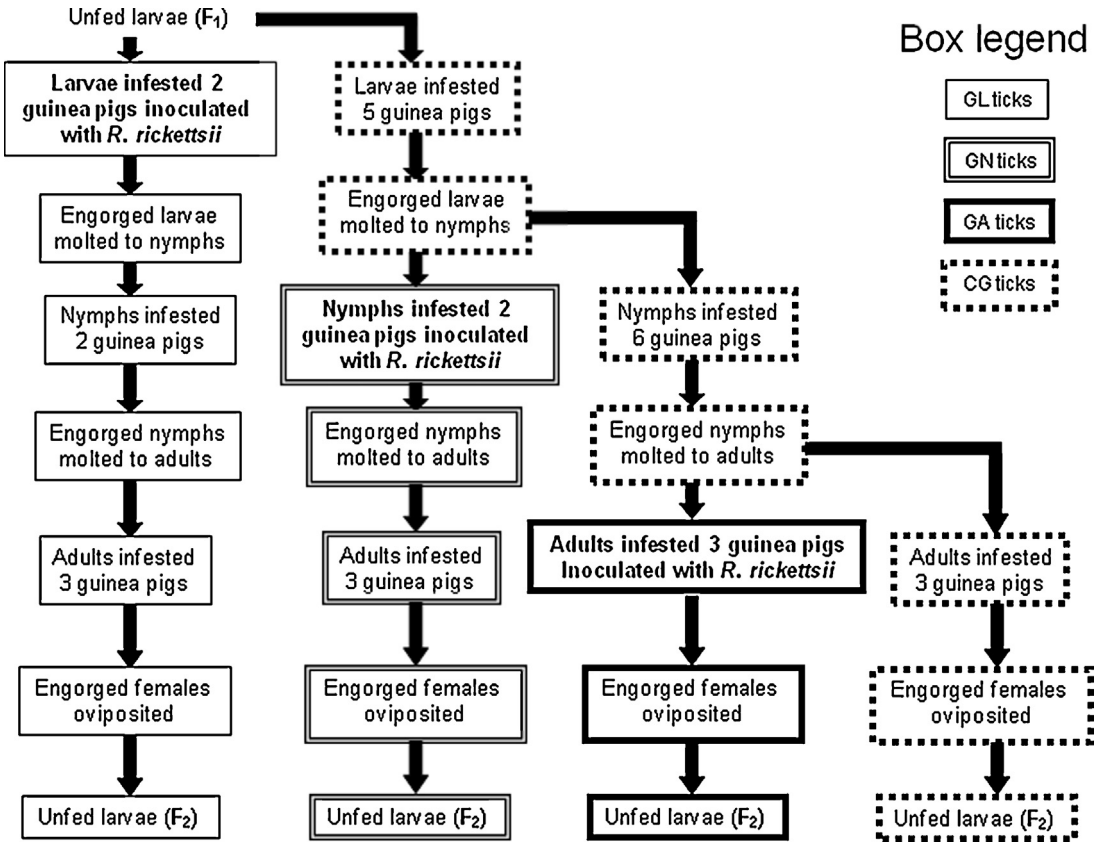

Fig. 1. Diagram illustrating experimental procedures with *Amblyomma dubitatum* ticks from F_1 larvae to F_2 larvae. GL, GN, and GA ticks were exposed to *Rickettsia rickettsii*-infected animals during the F_1 larval, nymphal, and adult stages, respectively, and thereafter they were reared through the F_2 larval stage on susceptible guinea pigs. CG ticks were the control group, never exposed to *R. rickettsii*. All procedures in this figure were done in parallel for two *R. rickettsii* strains (Taiaçu and Itu).

(previously exposed to *R. rickettsii* during the nymphal stage) were allowed to infest three uninfected guinea pigs; part of the CG adults were used to infest three uninfected guinea pigs (keeping the status of *R. rickettsii*-free control group), and another part of CG adults were used to infest three guinea pigs inoculated intraperitoneally with a *R. rickettsii* strain. These last adults formed the group GA, consisting of ticks exposed to *R. rickettsii* during the adult stage. Recovered engorged females representing the four experimental groups (GL, GN, GA, CG) were taken to the incubator, where egg oviposition and larval eclosion was observed. These procedures are summarized in Fig. 1.

Larval and nymphal infestations on guinea pigs inoculated intraperitoneally with *R. rickettsii* were performed at the same day of rickettsial inoculation. Because previous experiments (unpublished data) showed that *A. dubitatum* adult ticks can take up to 2 weeks to attach and start engorgement on guinea pigs, rickettsial inoculation was done only 14 days after placement of adult ticks inside the cotton sleeves. All infested animals had their rectal temperature measured from 0 to 21 days after infestation (DAI). For guinea pigs infested with adult ticks, which sometimes stayed on host for longer than 21 days, rectal temperature was measured until the detachment of the last engorged tick. Guinea pigs were considered febrile when rectal temperatures were >39.5 °C (Monteiro, 1931). The occurrence of scrotal or vagina injury (edema, congestion, necrosis), typical of *R. rickettsii* acute infection in guinea pigs (Monteiro, 1931) were annotated.

In all infestations, approximately 1.5 mL of blood was taken via cardiac puncture of each guinea pig under anesthesia 21 DAI. Samples were centrifuged (1500 *g*, 10 min) to obtain sera, which were tested for the presence of antibodies reactive to *R. rickettsii* and to *R. bellii* as described below. If a guinea pig died before 21 DAI, it was subjected to necropsy and a blood sample was collected directly from the heart's right ventricle. In addition, a lung sample was collected and evaluated by PCR for rickettsial infection, using the protocol described below.

Rickettsial detection by PCR assay

Attempts to detect rickettsial DNA in ticks were performed on unfed nymphs and adults, engorged females at the end of oviposition, and egg pools containing 20–40 eggs/pool. DNA extraction of ticks was performed by using guanidine thiocyanate, as previously described (Sangioni et al., 2005). This DNA extraction protocol has been shown in our laboratory to be 100% efficient for *R. rickettsii*-infected ticks (Labruna et al., 2008, 2011). Random samples of ticks were processed individually. Guinea pig lung fragments were submitted to DNA extraction using the DNeasy tissue Kit (Qiagen, Chatsworth, CA), according to the manufacturer's protocol for isolation of DNA from animal tissues. Blank tubes were always included in the DNA extraction procedures, as negative controls of this step.

All DNA samples were tested by two PCR protocols, one specific for SFG rickettsiae (here designated as the "SFG-PCR"), and one specific for *R. bellii* (here designated as the "*R. bellii*-PCR"). The SFG-PCR consisted of primers Rr190.70p (5′-ATG GCG AAT ATT TCT CCA AAA-3′) and Rr190.701R (5′-GTTCCGTTAATGGCAGCATCT3′) targeting a ≈630-bp fragment of the rickettsial 190-kDa outer membrane protein gene (*ompA*), as previously described (Eremeeva et al., 2006). For the *R. bellii*-PCR, primers (forward: 5′-ATCCTGATTTGCTGAATTTTTT-3′; reverse: 5′-TGCAATACCAGTACTGACG-3′) were designed to be specific for a 338-bp fragment of the *R. bellii* citrate synthase gene (*gltA*), as previously described (Szabó et al., 2013). Random samples (3 ticks)

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of PCR products from both PCR assays were DNA sequenced as previously described (Labruna et al., 2004), in order to verify the *Rickettsia* species.

Indirect immunofluorescence assay (IFA)

Guinea pig blood serum samples were individually tested by the indirect immunofluorescence assay (IFA) using crude antigens derived from *R. rickettsii* strain Taiaçu, and *R. bellii* strain Mogi, as previously described (Labruna et al., 2007a). Each serum was diluted in two-fold increments with PBS from 1:64 to the end-point titer (Labruna et al., 2007a). A commercial fluorescein isothiocyanate-labeled rabbit anti-guinea pig IgG (Sigma Diagnostics) was used. In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested at 1:64 dilution.

Statistics

The proportions of *R. rickettsii*-infected ticks between tick stages or between ticks exposed to different *R. rickettsii* strains, and the proportions of engorged females that successfully oviposited were compared by the chi-square test. Engorged female mean weight values were compared between groups by the Student's *t*-test. Analyses were performed using the program Minitab® Release 16. Significant differences were considered for $P < 0.05$.

Results

All 10 *A. dubitatum* engorged females that were used to start this study, and their respective offspring were shown by PCR to be naturally infected by *R. bellii;* i.e., they were all positive by the *R. bellii*-PCR, and negative by the SFG-PCR. Since we failed to find *R. bellii*-free ticks to start the experimental infections, we decided to follow on the study even knowing that the ticks were infected by *R. bellii.*

Irrespective of the strain, all guinea pigs ($n = 14$) inoculated with *R. rickettsii* had fever which started 3–5 days after inoculation and lasted for 2–7 days (Table 1). Except for one animal (guinea pig number T-2), all inoculated guinea pigs died of severe rickettsiosis between 7 and 11 days after inoculation. Their lung was PCR positive for SFG-rickettsiae, and some of them already presented high antibody endpoint titers to *R. rickettsii*. Since tick feeding period overlapped the febrile period (considered to be the rickettsemic period) in all inoculated animals, all ticks feeding on inoculated animals were exposed to *R. rickettsii*. Infection status (rectal temperature, serology, or lung PCR) of *R. rickettsii*-inoculated guinea pigs, and of those infested with ticks previously exposed to *R. rickettsii*-inoculated hosts are shown in Table 1.

A portion of GL unfed nymphs and adults, and GN unfed adults were shown to be PCR-positive for SFG rickettsiae, with infection rates varying from 23.3 to 50.0% for ticks exposed to strain Itu, and 5.3 to 24.2% for ticks exposed to strain Taiaçu (Table 2). These results indicate that only part of GL and GN ticks were able to maintain the rickettsial infection to subsequent tick stages by transstadial passage, after being exposed to *R. rickettsii* through feeding on inoculated guinea pigs. With a single exception, infection rates by SFG rickettsiae were statistically similar ($P > 0.05$) among nymphs and adults exposed to the same *R. rickettsii* strain. On the other hand, exposure to *R. rickettsii* strain Itu resulted in significantly higher ($P < 0.05$) infection rates by SFG rickettsiae when compared to exposure to *R. rickettsii* strain Taiaçu (Table 2).

Our results showed that after being exposed to *R. rickettsii* during the larval (GL ticks) and nymphal (GN ticks) stages, the subsequent stages were capable to transmit *R. rickettsii* to susceptible animals

Table 1
Clinical and serological data of guinea pigs experimentally infected with *Rickettsia rickettsii* (strain Itu or Taiaçu). GL, GN, and GA tick groups consisted of *Amblyomma dubitatum* F_1 larvae, nymphs, and adults, respectively, that were initially fed on guinea pigs inoculated intraperitoneally (IP) with *R. rickettsii* (strain Itu or Taiaçu). Thereafter, GL F_1 nymphs and adults, and GN F_1 adults were fed on uninfected guinea pigs.

Tick group	Strain Itu							Strain Taiaçu						
	Guinea pig no.	Infection route	Fever period (DAI)	Scrotal or vaginal injury (DAI)	Death (DAI)	Endpoint titer to *R. rickettsii*[+]	Endpoint titer to *R. bellii*[*]	Guinea pig no.	Infection route	Fever period (DAI)	Scrotal or vaginal injury (DAI)	Death (DAI)	Enpoint titer to *R. rickettsii*[+]	Endpoint titer to *R. bellii*[*]
GL	I-1	IP	3–9	8	11	2048	64	T-1	IP	3–8	8	10	2048	<64
	I-2	IP	3–7	7	9	1024	<64	T-2	IP	3–11	11	No	32768	256
	I-3	F_1 nymphs	8–12	16	No	16384	4096	T-3	F_1 nymphs	6–13	12	No	8192	256
	I-4	F_1 nymphs	7–11	11	14	1024	<64	T-4	F_1 nymphs	7–13	12	15	512	<64
	I-5	F_1 adults	8–13	13	19	8192	2048	T-5	F_1 adults	No	No	No	<64	<64
	I-6	F_1 adults	9–12	13	17	2048	<64	T-6	F_1 adults	10–12	13	13	128	<64
	I-7	F_1 adults	14–17	18	18[*]	<64	<64	T-7	F_1 adults	No	No	No	<64	<64
GN	I-8	IP	4–8	9	10	256	64	T-8	IP	4–9	9	11	512	64
	I-9	IP	5–7	No	8[*]	<64	<64	T-9	IP	4–8	10	11	256	128
	I-10	F_1 adults	10–13	No	No	16384	16384	T-10	F_1 adults	18–22	22	26	128	<64
	I-11	F_1 adults	12–14	15	17[*]	<64	<64	T-11	F_1 adults	8–11	13	No	1024	512
	I-12	F_1 adults	11	No	No	512	<64	T-12	F_1 adults	10–12	No	No	8192	8192
GA	I-13	IP	3–4	No	7[*]	NS	NS	T-13	IP	4–8	No	11	1024	128
	I-14	IP	4–7	No	9[*]	NS	NS	T-14	IP	4–8	8	10	64	<64
	I-15	IP	4–6	No	8[*]	<64	<64	T-15	IP	4–8	No	11	64	<64

DAI: day after IP inoculation with *R. rickettsii* or infestation with *R. rickettsii*-infected ticks.
NS: no serum sample could be collected from this animal.
+: PCR performed on DNA extracted from lung fragment yielded amplification of a portion of the rickettsial *ompA* gene.
[*] Guinea pig blood was collected for serology at the day it died, or 21 DAI.

Table 2
Proportion of *Amblyomma dubitatum* unfed ticks infected by spotted fever group (SFG) rickettsiae, as determined by a SFG-specific PCR protocol targeting a portion of the rickettsial *ompA* gene on ticks after molting. GL and GN tick groups consisted of *A. dubitatum* larvae and nymphs, respectively, that were initially exposed to feed on guinea pigs inoculated intraperitoneally (IP) with *R. rickettsii* (strain Itu or Taiaçu). Thereafter, GL nymphs were exposed to uninfected guinea pigs in order to obtain unfed adults. CG ticks (control group) consisted of *A. dubitatum* never exposed to *R. rickettsii*.

Tick group	Tick stage	No. of PCR-positive ticks/No. of tested ticks (%)*	
		Strain Itu	Strain Taiaçu
GL	Nymphs	14/60 (23.3)[a, A]	5/60 (8.3)[a, B]
GL	Adults	20/44 (45.5)[b, A]	2/38 (5.3)[a, B]
GN	Adults	17/34 (50.0)[b, A]	8/33 (24.2)[a, B]
CG	Nymphs	0/44 (0)	
CG	Adults	0/22 (0)	

* Proportion values followed by different letters in the same row (lowercase letters) or line (capital letters) are significantly different ($P < 0.05$).

(Table 1). However, while all 8 guinea pigs (I-3 to I-7; I-10 to I-12) infested with strain Itu-exposed ticks (GL nymphs and adults, and GN adults) became infected by *R. rickettsii* (confirmed by IFA and/or PCR of lung samples, besides clinical signs), at least 2 guinea pigs (T-5 and T-7) infested with strain Taiaçu-exposed ticks did not become infected by *R. rickettsii*, since they remained asymptomatic and seronegative to *R. rickettsii* (Table 1).

Generally, engorged females exposed to strain Itu tended to achieve higher engorgement weights than engorged females exposed to strain Taiaçu, although their differences were not statistically significant ($P > 0.05$). On the other hand, engorged females of the CG group, never exposed to *R. rickettsii*, had the highest engorgement weights, which were significantly higher ($P < 0.05$) than the weights of engorged females exposed to either Itu or Taiaçu strain (Table 3).

All engorged females were tested by the SFG-PCR at the end of oviposition, in order to verify if they were infected by SFG rickettsiae. Overall, 37.0 to 83.3% of the engorged females of the tick groups GL, GN, and GA were infected by SFG rickettsiae at the end of oviposition, or at 30 days after detachment from host (for females that did not oviposit). Again, strain Itu tended to generate higher infection rates than strain Taiaçu (Table 3). Egg pools oviposited by engorged females were tested by PCR for rickettsial infection. From 19 females infected with strain Itu, and 38 infected with strain Taiaçu, all egg pools were negative by the SFG rickettsiae PCR, except for a single GN engorged female previously exposed to strain Taiaçu. This result indicates that transovarial transmission of *R. rickettsii* did not occur, except in a single female.

Throughout the study, no animal or tick was shown to be infected by SFG rickettsiae in the CG group, since all PCR performed on CG ticks were negative for the rickettsial *ompA* gene, and all guinea pigs infested with CG ticks remained asymptomatic and seronegative at 21 DAI. These results were expected because CG ticks composed the *R. rickettsii*-free control group that was reared in parallel to the infected groups in the present study.

All tick DNA samples that were tested by the SFG-PCR were also tested by the *R. bellii*-PCR, which amplified *R. bellii* DNA in 100% of the samples from GL, GN, GA, and CG tick groups. This result indicates that 100% of the *A. dubitatum* ticks, regardless of having acquired infection by *R. rickettsii*, remained infected by *R. bellii* through the study. All guinea pigs infected with CG ticks (infected solely by *R. bellii*) were seronegative for *R. bellii* at 21 DAI, while guinea pigs exposed to *R. rickettsii*-infected tick groups (GL, GN, GA) had endpoint titers to *R. bellii* generally much lower than the endpoint titers elicited to *R. rickettsii* (Table 1). All guinea pig lung DNA samples were negative by the *R. bellii*-PCR.

Table 3
Engorgement weight, oviposition success, and infection by spotted fever group (SFG) rickettsiae (determined by a SFG-specific PCR) in *Amblyomma dubitatum* engorged females. GL, GN, and GA groups consisted of engorged females that were initially exposed to *Rickettsia rickettsii* during the larval, nymphal, and adult stages, respectively.

Tick group	Engorged female weight (mg)*		No. of PCR-positive females+/No. of recovered engorged females (%)		No. of females that oviposited/No. of PCR-positive females (%)+		No. of females that oviposited/No. of PCR-negative females (%)+	
	Strain ITU	Strain Taiaçu	Strain ITU	Strain Taiaçu	Strain ITU	Strain Taiaçu	Strain ITU	Strain Taiaçu
GL	223.8 ± 119.7 (74–372)	213.2 ± 122.1 (28–423)	3/5	10/27	1/3	4/10	2/2	7/17
GN	229.0 ± 103.9 (68–396)	231.4 ± 133.0 (29–505)	8/14	18/28	3/8	3/18	4/6	5/10
GA	214.2 ± 107.9 (111–462)	153.2 ± 133.3 (20–464)	8/19	10/12	5/8	2/10	9/11	0/2
Total	221.0 ± 105.2 (68–462)a	209.8 ± 129.8 (20–505)a	19/38 (50.0)	38/67 (56.7)	9/19 (47.4)	9/38 (23.7)	15/19 (78.9)	12/29 (41.4)
CG	292.7 ± 129.4 (77–552)b		0/22 (0)		Not available		17/22 (77.3)	

+Engorged females were tested by PCR targeting a portion of the rickettsial *ompA* gene at the end of oviposition or at the 30th day after detachment from the host.
* Values are presented as mean ± standard error (range); mean values followed by different letters are significantly different ($P < 0.05$).

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PCR products from 3 unfed ticks (2 GL adults infected with *R. rickettsii* strain Itu, and 1 GL adult infected with *R. rickettsii* strain Taiaçu) were DNA-sequenced. The products of the *R. bellii*-PCR were all identical to a corresponding sequence of the *gltA* gene of *R. bellii* in GenBank (accession number JQ906786). The products of the SFG-PCR were all identical to a corresponding sequence of the *ompA* gene of *R. rickettsii* in GenBank (CP003305).

Discussion

The present study shows that larvae, nymphs, and adults of *A. dubitatum* were less susceptible to *R. rickettsii* infection since in all cases only part of the ticks (5.3–50%) maintained the infection through transstadial perpetuation, after being exposed to rickettsemic animals. These results are similar to a previous study that performed experimental infection of *A. cajennense* ticks with strain Taiaçu, which infected 0–62.2% of the exposed larval and nymphal ticks upon feeding on rickettsemic guinea pigs (Soares et al., 2012). On the other hand, these results contrast to previous studies that showed strain Taiaçu to infect 80–100% of the *A. aureolatum* and *R. sanguineus* ticks exposed to rickettsemic guinea pigs (Labruna et al., 2008, 2011). Indeed, an important difference between those and our studies is that neither the *A. cajennense* nor the *A. aureolatum* colony of the previous studies was infected with *R. bellii.* Herein, all *A. dubitatum* ticks were originally infected by *R. bellii*, and even under these circumstances, some of them acquired and maintained the *R. rickettsii* infection through transstadial perpetuation. Natural infection by two or even three *Rickettsia* species in a single tick specimen has been reported in the literature, including *R. bellii* and a *R. parkeri*-like agent infecting an *Amblyomma ovale* specimen (Szabó et al., 2013), *R. rickettsii* and *Rickettsia amblyommii* infecting an *A. americanum* specimen (Berrada et al., 2011), *R. bellii* and *Rickettsia rhipicephali* infecting a *D. variabilis* specimen (Wikswo et al., 2008), and a triple infection by *R. bellii*, *Rickettsia montanensis* and *R. rickettsii* in a *D. variabilis* specimen (Carmichael and Fuerst, 2010).

While 100% of the *A. dubitatum* ticks of the present study were infected by *R. bellii*, no guinea pig from the CG group became seropositive to *R. bellii*, while no *R. bellii* DNA was detected in lung samples from the GL, GN, and GA guinea pigs that died during the febrile period. These results suggest that *R. bellii* was not tick-transmitted to CG guinea pigs. Guinea pigs exposed to ticks infected by both *R. rickettsii* and *R. bellii* generally elicited endpoint titers much higher to *R. rickettsii* than to *R. bellii*, indicating that reactions to the later agent were a result of cross-reactions with anti-*R. rickettsii* antibodies. In fact, because *R. rickettsii* and *R. bellii* are phylogenetically distant species, experimental infection of animals (including guinea pigs) with *R. rickettsii* is usually characterized by > four-fold higher endpoint titers to *R. rickettsii* than to *R. bellii* (Piranda et al., 2008; Horta et al., 2010; Pacheco et al., 2011).

Among the *A. dubitatum* engorged females that oviposited in the present study, all had their egg mass positive by the *R. bellii*-PCR, indicating efficient transovarial transmission of this rickettsia, just as observed in the original females collected from capybaras and used to start the present study. Transovarial transmission of *R. bellii* to 100% of the tick offspring has been previously observed in *Ixodes loricatus* (Horta et al., 2006). On the other hand, efficient transovarial transmission of *R. rickettsii* in *A. dubitatum* ticks was not observed, since none of the females infected with strain Itu laid infected eggs, and among the 38 females infected with strain Taiaçu, only one (2.63%) had an egg mass positive by the SFG-PCR. This result contrasts to the ones reported by Soares et al. (2012), who observed transovarial transmission of strain Taiaçu in 6–43% of the *R. rickettsii*-infected *A. cajennense* females, and to Labruna et al. (2011), who observed transovarial transmission of strain Taiaçu in 100% of the *R. rickettsii*-infected *A. aureolatum* females. Because it has been reported that a primary infection by a *Rickettsia* species would preclude transovarial transmission of a second *Rickettsia* species (Burgdorfer, 1988; Macaluso et al., 2002), it is likely that the ineffectiveness of *A. dubitatum* to perpetuate *R. rickettsii* by transovarial transmission was related to its primary infection by *R. bellii*; however, it could also be related to unknown factors inherent to *A. dubitatum.*

Engorged females of *A. dubitatum*, infected by either strain Itu or strain Taiaçu, had significantly lower engorged weights than *R. rickettsii*-free engorged females. Similar results were reported for *A. aureolatum* and *A. cajennense*, since *R. rickettsii*-infected engorged females of both species had significantly lower engorged weights, when compared with conspecific uninfected females (Labruna et al., 2011; Soares et al., 2012). Indeed, lower engorged weights also affected the proportions of engorged females that oviposited, which were 47.4 and 23.7% for *A. dubitatum* females infected with strains Itu and Taiaçu, respectively, and 78.9 and 41.4% for the respective *R. rickettsii*-free female counterparts (Table 3).

Significantly, more ticks sustained a *R. rickettsii* infection when exposed to strain Itu than to strain Taiaçu. It is noteworthy that strain Itu was isolated from *A. cajennense* ticks collected in an area of the state of São Paulo that had capybaras and *A. dubitatum* (Krawczak et al., 2014). It is possible that this strain has been passed through *A. dubitatum* ticks under natural conditions; therefore, strain Itu would be more adapted to this tick species than strain Taiaçu. This later strain was isolated from *A. aureolatum* in an area that had neither capybaras nor *A. dubitatum* (Pinter and Labruna, 2006), and was previously shown to be highly pathogenic for guinea pigs, which usually infected 100% of the *A. aureolatum* ticks that fed on then during rickettsemia (Labruna et al., 2008, 2011). A possible higher capacity of strain Itu to compete against *R. bellii* in tick tissues could also have affected our results, although *A. aureolatum* populations are found infected by both *Rickettsia* species under natural conditions (Pinter and Labruna, 2006; Ogrzewalska et al., 2012).

A recent study showed that capybaras are efficient amplifier hosts of *R. rickettsii* for *A. cajennense* ticks (Souza et al., 2009). Indeed, the reemergence of RMSF in many areas of southeastern Brazil has been attributed to the overgrown population of capybaras during the last few decades (Labruna, 2009). In these RMSF-endemic areas, capybaras sustain populations of both *A. cajennense* and *A. dubitatum* ticks, albeit the former species usually outnumbers the later (Perez et al., 2008; Brites-Neto et al., 2013; Krawczak et al., 2014). Although human infestations by *A. dubitatum* has been recorded (Labruna et al., 2007b), it is not a common finding. In contrast, *A. cajennense* is the most common human-biting tick in southeastern Brazil (Guglielmone et al., 2006). Therefore, even though the present study showed that *A. dubitatum* ticks can acquire, maintain by transstadial perpetuation, and transmit *R. rickettsii* to a susceptible host, the relevance of *A. dubitatum* as a vector of *R. rickettsii* to humans should be minimal, or overshadowed by *A. cajennense.*

On the other hand, *A. dubitatum* could play an important role as an enzootic vector of *R. rickettsii* between capybaras. However, it is possible that this tick species alone is not able to sustain a *R. rickettsii* infection through consecutive generations, due to the ineffectiveness of transovarial transmission elicited by a primary infection by another *Rickettsia* species, or because of unknown intrinsic factors, yet to be evaluated.

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